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Survival of *Macrobrachium amazonicum* embryos submitted to cooling

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Summary

Cooling techniques have several applications for reproduction in aquaculture. However, few studies have sought to create protocols for cooling and cryopreservation of *Macrobrachium amazonicum* embryos. Thus, the objective of this work was to verify the survival of *M. amazonicum* embryos and the correlation between embryonic volume and mortality of *M. amazonicum* embryos after cooling. Embryo pools were collected from three females and divided into two treatment groups: dimethyl sulfoxide (DMSO) 3% and ethylene glycol (EG) 0.5%, both of them associated with 2 M sucrose. Positive and negative control groups consisted of seawater 10%. Aliquots of 10 µg of embryos were placed in Falcon® tubes containing a cryoprotectant solution and submitted directly to the test temperature of 2°C for 2 and 6 h of cooling. Further analysis of survival and embryonic volume were performed under a stereoscopic microscope. Data were subjected to analysis of variance (ANOVA), and means were compared using the Tukey test at 5%. The highest embryonic survival rate was observed after the shortest storage time for both the DMSO 3% and the 0.5% EG groups, with survival rates of 84.8 ± 3.9 and 79.7 ± 2.8%, respectively. There was a reduction in survival after 24 h, with the DMSO 3% group presenting a survival rate of 71.7 ± 6.6%, and the EG 0.5% group, 66 ± 6.9%. Survival showed a statistically significant difference when compared with the positive controls after 2 h and 24 h of cooling, with 99 ± 0.5% and 95.8 ± 1.5% survival rates, respectively. There was no significant statistical difference in the embryonic volume, but it was possible to observe a change in the appearance of the embryos, from a translucent coloration to an opaque white or brownish coloration, after 24 h in incubators. Thus, it can be concluded that survival is inversely proportional to storage time and that, although there was no change in the embryonic volume after cooling, a change in the appearance of embryos could be observed.

Keywords: Amazonian prawn, Cryopreservation, Cryoprotectants, Protocol

Introduction

Macrobrachium amazonicum, also known as cinnamon prawn, is a freshwater species with a high potential for aquaculture. Among the main characteristics of this animal, it is possible to highlight the rapid growth, the resistance, the rusticity, the easy reproduction and the development in captivity (Maciel & Valenti, 2009).

The techniques of cooling and cryopreservation are often used in studies on mammals of zootechnical interest. However, in aquatic invertebrates, specifically shrimps, most of these studies focus on spermatophore, researches conducted with embryos are still

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incipient. In animal reproduction, this technique can be used for the preservation of gametes, transport of genetic material and preservation of endangered breeding lines (Diwan *et al*., 2010). In general, several factors must be considered for the success of cooling and cryopreservation protocols, such as species, stage of embryonic development, concentration of cryoprotectants, extender composition, equilibrium and rehydration time, rate of cooling and heating (Tsai & Lin, 2012).

The development of cooling and cryopreservation protocols for aquatic invertebrates presupposes knowledge of the embryonic development of the species and the mechanisms that involve cryopreservation, as well as overcoming the barrier imposed by the inborn sensitivity of these animals to low temperatures. The alternative for overcoming problems caused by low temperatures is the use of internal and external cryoprotectants, which aim to reduce cryoinjury caused by cooling processes (Castro *et al*., 2011).

With respect to aquatic invertebrates, further studies need to be conducted on cooling and cryopreservation of spermatophores, embryos and larvae. In this context, studies have been carried out on several species such as oysters (Liu & Li, 2008; Suquet *et al*., 2014), crabs (Huang *et al*., 2013) and shrimps (Alfaro *et al*., 2001; Bambozzi *et al*., 2014; Ferreira *et al*., 2015). For the mussel *M. galloprovincialis*, Wang *et al*. (2011) highlights the use of 5% DMSO associated with trehalose 0.2 M for the cryopreservation of the larvae of this species, with a larval survival rate above 50%. Regarding the penaeid shrimp (*Trachypenaeus byrdi*), Alfaro *et al*. (2001) found the nauplius stage to be more resistant to cooling and exposure to cryoprotectants than advanced embryonic stages. As to the ornamental shrimp *Stenopus hispidus*, Lin *et al*. (2013) verified that the concentration of 1 M of methanol was efficient at protecting embryos of this species, having observed an increased tolerance to cooling as development occurs. As regards the freshwater prawn *M. amazonicum*, Ferreira *et al*. (2015) described methanol as the least toxic CPA among the cryoprotectants tested [DMSO and ethylene glycol (EG)].

Therefore, the present work aimed to verify the survival rates of *M. amazonicum* embryos submitted to low temperatures. In addition, this work verified the correlation between embryonic volume and mortality after cooling.

Materials and methods

Sample collection location

The animals used in this experiment were collected in Sapiranga Lake, located in Fortaleza, Ceará, Brazil (03°47′54,79″S; 038°27′28,73″W).

Capturing and sorting the animals to obtain embryos

The prawns were captured with trawls and transported in thermal boxes for sorting at the Laboratory of Prawn Farming (LACAR), of the Higher Institute of Biomedical Sciences (ISCB), of the State University of Ceará (UECE), located in Fortaleza, Ceará.

For this experiment, healthy ovigerous females were separated at the final stage of embryonic development.

Experimental design

Embryos at the final stage of development were removed from the ventral cavity of 15 females of *M. amazonicum*, cooled to 2°C, and stored for 2 or 6 h.

To study the effects of cooling on the survival of embryos of *M. amazonicum*, two treatment groups received internal cryoprotectants: 3% dimethyl sulfoxide (DMSO) and 0.5% EG. Both were associated with sucrose at 2 M concentration, totaling two cryoprotectant solutions: 3% DMSO + 2 M sucrose; and 0.5% EG + 2 M sucrose. Positive (not cooling) and negative (cooling) groups were used, both consisting of seawater at 10‰.

For the cooling procedure, the CPA solutions were put into Falcon® conical tubes, diluted with 10‰ seawater, with a final volume of 10 ml, in a proportion of 7:2:1 (10‰ seawater: external cryoprotectant: internal cryoprotectant). The treatment and control groups were duplicated and repeated five times. Each pool of embryos consisted of a group of three females. Thereby, 10 μg of embryos were added to each Falcon® tube containing cryoprotectant solutions. The tubes were removed from room temperature (25.7 ± 0.7°C) and placed directly in a thermal box filled with ice at controlled temperature. In the positive control treatment group, the embryos were not cooled and were stored for up to 24 h (Fig. 1).

After 2 h or 6 h of storage, the tubes containing the embryos were removed from the thermal boxes, acclimated for 5 min in water at room temperature (25°C), and washed in tap water for removal of the cryoprotectant solutions and subsequent survival analysis under stereomicroscope LEICA DFC 295. After the first survival analysis, the embryos were placed in 50 ml incubators. After 24 h, a new survival analysis was performed under stereomicroscope.

To verify embryonic volume, random samples were photographed at three distinct moments: before the start of the experiment, immediately after cooling, and after 24 h in the incubators. Thus, the major (length) and the smaller (width) axes were measured under a LEICA DFC 295 stereomicroscope, coupled with a photomicrography equipment in the LAS V3.6 program. According to Odinetz-Collart & Rabelo (1996), the following formula was used for the

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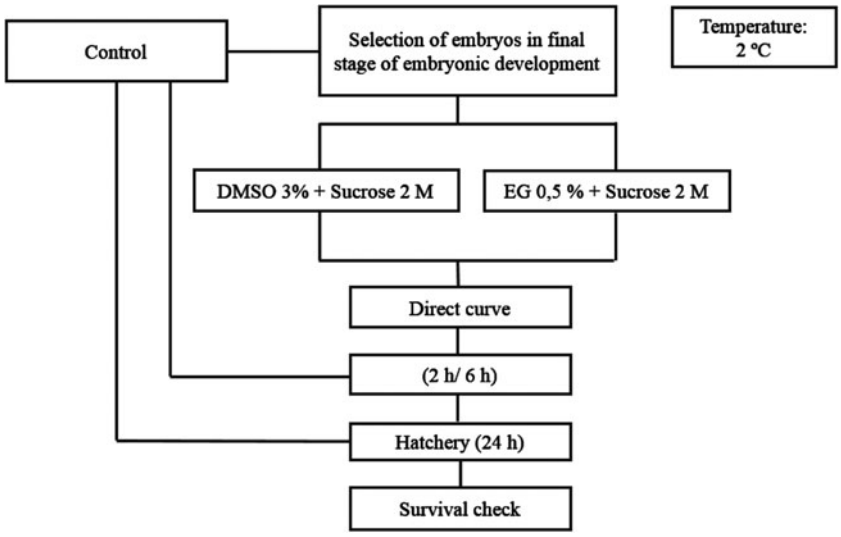

Figure 1 Experimental design flowchart.

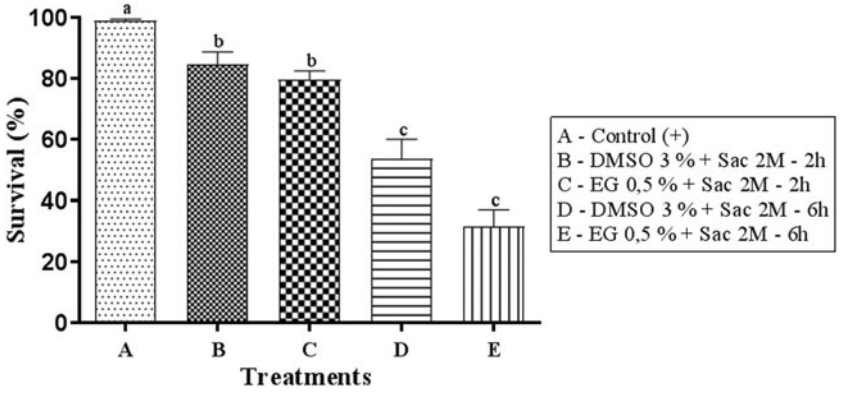

Figure 2 Survival of embryos of *M. amazonicum* in the final stage submitted to treatment with DMSO and EG for 2 and 6 h of cooling. [a–c]Different letters indicate a significant difference between the results.

calculation of the embryonic volume: $V = \pi.a.b^2/6$, where 'a' is the length and 'b' is the width.

Statistical analysis

Values were expressed as mean ± standard error of the mean. The analysis of variance (ANOVA) was used to verify the occurrence of significant differences between the cooling results, followed by the Tukey test with a significance level of 5% in GraphPad Prism software 7.

Results

Embryonic survival

Embryonic survival after 2 h of cooling in the presence of 3% DMSO and 0.5% EG was 84.8 ± 3.9 and 79.7 ± 2.8%, respectively. After 6 h of cooling, the 3% DMSO group presented a survival rate of 53.5 ± 6.5%, while the 0.5% EG group presented a survival rate of 31.6 ± 5.5%, neither with significant statistical difference ($P > 0.05$). However, the groups exposed to cooling for 2 or 6 h presented a significant statistical difference when compared to the positive control group, which reached a survival rate of 99 ± 0.5% ($P \leq 0.05$) (Fig. 2).

After 24 h of incubation, the embryos cooled for 2 h in the presence of 3% DMSO and 0.5% EG presented survival rates of 71.7 ± 6.6 and 66 ± 6.9%, respectively, without significant statistical difference ($P > 0.05$). Embryos cooled for 6 h in the presence of 3% DMSO and 0.5% EG presented survival rates of 18.9 ± 4.5 and 17 ± 3.5%, respectively, after 24 h of incubation, without significant statistical difference ($P > 0.05$). Again, after 24 h in incubators, the groups submitted to cooling for 2 or 6 h showed a significant statistical difference when compared with the positive control ($P \leq 0.05$), which presented a 95.8 ± 1.5% embryonic survival rate (Fig. 3).

The negative control groups cooled for 2 or 6 h presented survival rates of 20.9 ± 4.4% and 0%, re-

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Table 1 Volume of *M. amazonicum* embryos submitted to cooling for 2 h and 6 h

	Control (+) (mm^3)	Control (−) (mm^3)	DMSO 3% Sac 2 M (mm^3)	EG 0.5 % Sac 2 M (mm^3)
Pre-experiment	0.56 ± 0.01	0.66 ± 0.22	0.66 ± 0.22	0.64 ± 0.22
Post 2 h cooling	0.66 ± 0.02	0.64 ± 0.22	0.64 ± 0.22	0.63 ± 0.22
Post 24 h incubation	0.63 ± 0.03	0.66 ± 0.23	0.67 ± 0.23	0.68 ± 0.23

Table 2 Volume of *M. amazonicum* embryos submitted to cooling for 2 and 6 h, after 24 h of incubation.

	Control (+) (mm^3)	Control (−) (mm^3)	DMSO 3% Sac 2 M (mm^3)	EG 0.5 % Sac 2 M (mm^3)
Pre-experiment	0.56 ± 0.01	0.65 ± 0.16	0.70 ± 0.23	0.67 ± 0.23
Post 2 h cooling	0.62 ± 0.02	0.67 ± 0.20	0.69 ± 0.23	0.61 ± 0.17
Post 24 h incubation	0.63 ± 0.03	0.68 ± 0.23	0.72 ± 0.23	0.66 ± 0.22

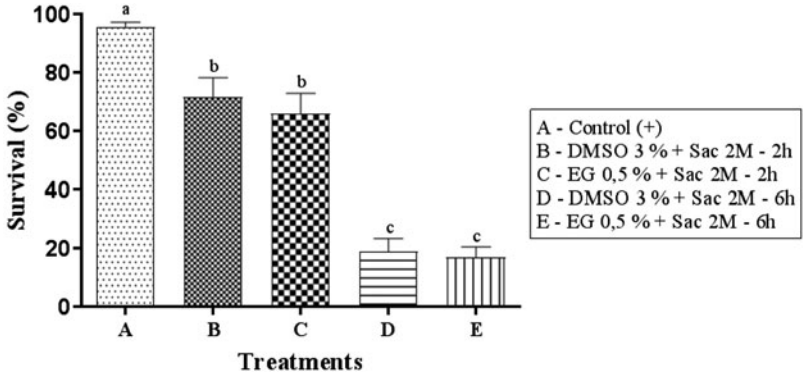

Figure 3 Survival of embryos of *M. amazonicum* in the final stage after 24 h incubation. [a−c]Different letters indicate a significant difference between the results.

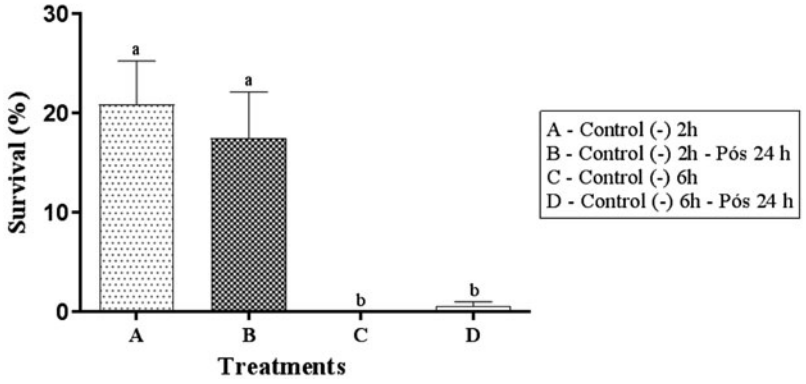

Figure 4 Survival of embryos of *M. amazonicum* in the final stage submitted to control treatment after 2 and 6 h of cooling and after 24 h incubation. Different letters indicate a significant difference between the results.

spectively, showing a statistically significant difference ($P \leq 0.05$). After 24 h of incubation, there was a reduction in the survival rates of the negative control groups, with the survival rate of the negative control cooled for 2 h reducing to 17.5 ± 4.6%, and that of the negative control cooled for 6 h reducing to 0.6 ± 0.4%, showing a statistically significant difference ($P \leq 0.05$) (Fig. 4).

Embryonic volume

Regarding embryonic volume, there was no significant statistical difference ($p > 0.05$) between the treatment groups cooled for 2 and 6 h, neither in the pre-experiment, nor after 2 or 6 h of cooling, or after 24 h of incubation, as seen in Tables 1 and 2. However, it was possible to observe that the embryos' coloration changed from a translucent coloration in the

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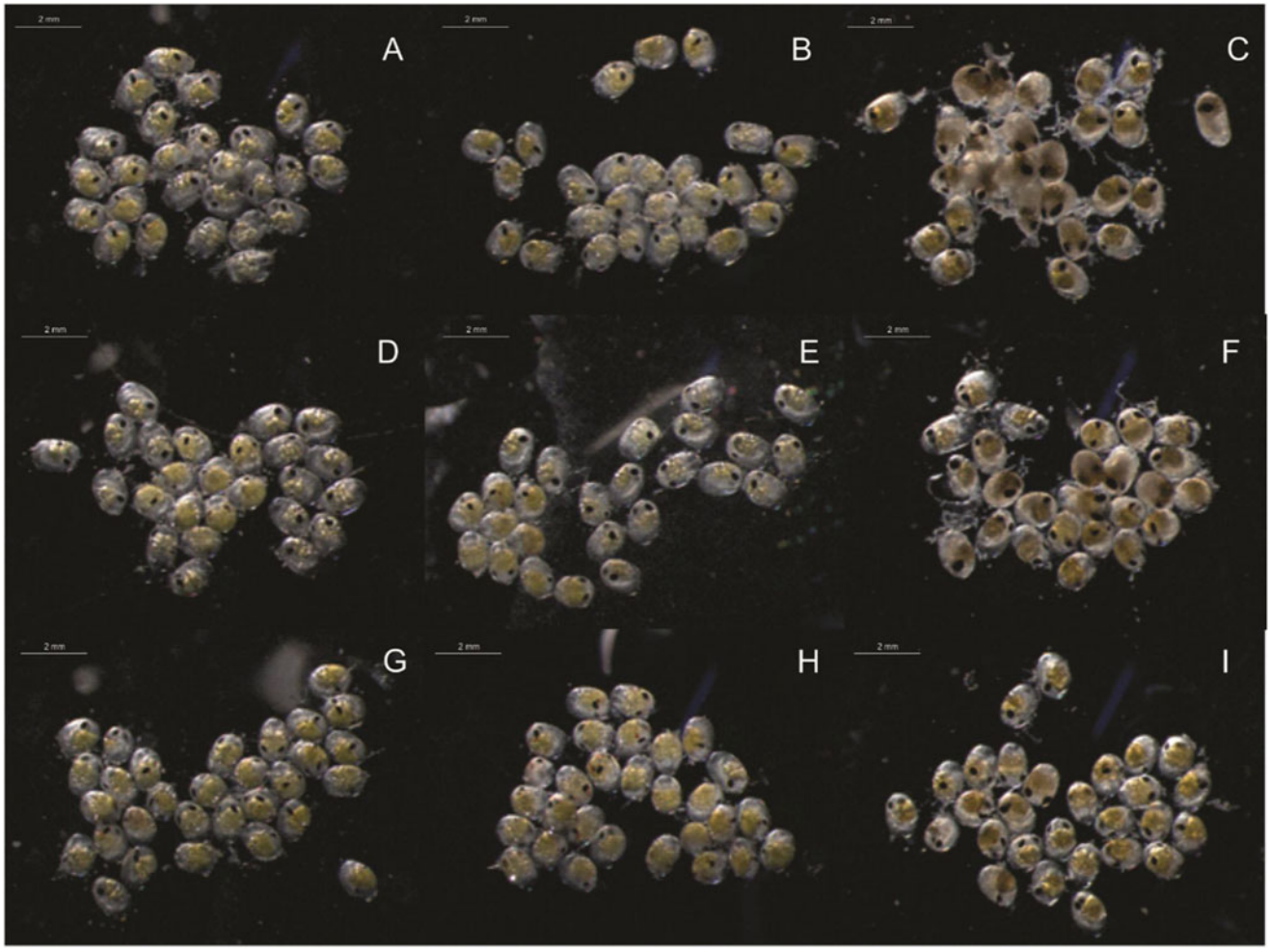

Figure 5 Appearance of embryos of *M. amazonicum* during a 2 h cooling treatment. (*A*) Negative control: pre-experiment. (*B*) Negative control: after 2 h cooling. (*C*) Negative control: after 24 h incubation. (*D*) DMSO 3% Sac 2 M: pre-experiment. (*E*) DMSO 3% Sac 2 M: after 2 h cooling. (*F*) DMSO 3% Sac 2 M: after 24 h incubation. (*G*) EG 0.5% Sac 2 M: pre-experiment. (*H*) EG 0.5% Sac 2 M: after 2h cooling. (*I*) EG 0.5% Sac 2 M: after 24 h incubation.

pre-experiment to an opaque white or brownish coloration in some embryos at the end of the experiment, after 24 h in the incubators (Figs 5 and 6).

Discussion

The present study emphasizes the importance of the type of cryoprotectant used, the stage of embryonic development, time of exposure to the cryoprotectant, and shows the influence of cooling on *M. amazonicum* prawn embryos.

The concentrations of DMSO and EG used in the present study were defined based on a previous work (Ferreira *et al.*, 2015). In the current study, it was possible to observe that both cryoprotectants, 3% DMSO and 0.5% EG, associated with 2 M sucrose, provided a high survival rate of *M. amazonicum* embryos submitted to 2°C for 2 h or 6 h. However, embryos submitted to the treatment with DMSO showed greater resistance to low temperatures. In penaeid shrimps, Alfaro *et al.* (2001) observed that *Trachypenaeus byrdi* and *Penaeus stylirostris* nauplii were more resistant than embryos, when exposed to DMSO. Different from that observed by Subramoniam & Arun (1999) in nauplii of *P. monodon*, which were exposed to 2.8 M of DMSO for 20 min, showing a mortality rate above 50%. Although the mechanism used by cryoprotectants to protect cells against the effects of cooling has not been fully understood, there are studies on marine decapod crustaceans that highlight the amount of lipid present in embryos as a fundamental factor for the success of cryopreservation (Alfaro *et al.*, 2001; Lin *et al.*, 2013)

In general, cryopreservation of eggs, embryos and larvae of fish and invertebrates have limiting factors. Some of these factors may be the considerable size

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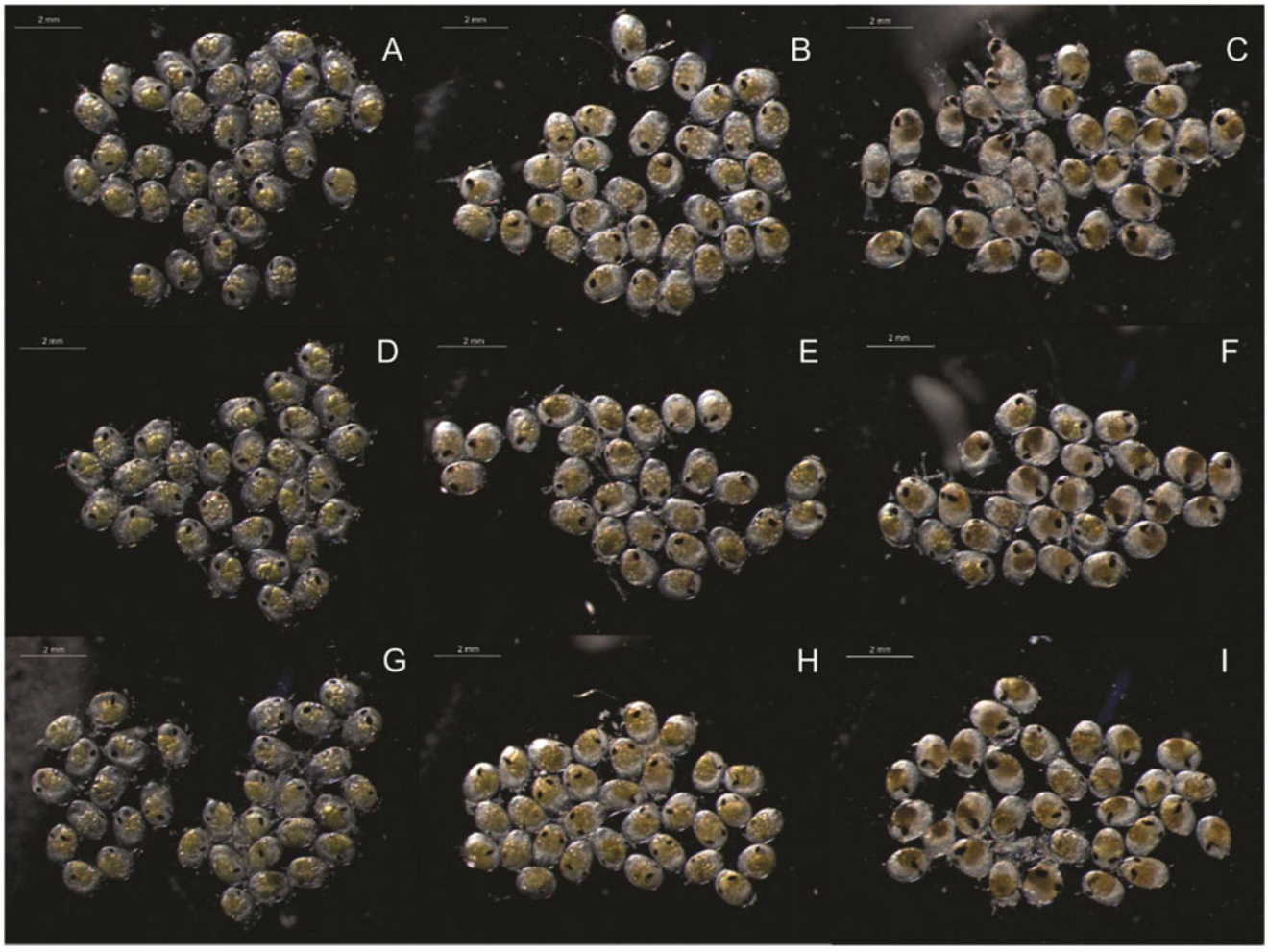

Figure 6 Appearance of embryos of *M. amazonicum* during a 6 h cooling treatment. (*A*) Negative control: pre-experiment. (*B*) Negative control: after 2 h cooling. (*C*) Negative control: after 24 h incubation. (*D*) DMSO 3% Sac 2 M: pre-experiment. (*E*) DMSO 3% Sac 2 M: after 2 h cooling. (*F*) DMSO 3% Sac 2 M: after 24 h incubation. (*G*) EG 0.5% Sac 2 M: pre-experiment. (*H*) EG 0.5% Sac 2 M: after 2 h cooling. (*I*) EG 0.5% Sac 2 M: after 24 h incubation.

of eggs and embryos, the embryonic stage and the amount of yolk, which may interfere in the penetration of cryoprotectants, leading to the formation of ice crystals that cause damage to the structures (Seymour, 1994). In addition, Zhang & Rawson (1996) point out that the early stages have a large amount of yolk, which is one of the main obstacles to cryopreservation.

In decapod crustaceans, eggs are small but have a large amount of yolk with phospholipids rich in polyunsaturated fatty acids (PUFAs) (Harrison, 1990; Ravid *et al*., 1999). In crustaceans, these phospholipids contain low levels of omega-6 (arachidonic acid 20: 4 omega-6) and high levels of omega-3 (eicosapentaenoic acid 20: 5 omega-3 and docosahexaenoic acid 22: 6 omega-3) (Chapelle, 1986; Harrison, 1990; Alfaro *et al*., 2001; Lin *et al*., 2013). Regarding marine animals, it is believed that high levels of these fatty acids are related to the lipid melting point, since the omega-3 structure allows for a higher degree of instauration than the structures of omega-6 or omega-9. Therefore, phospholipids from crustaceans allow for a greater flexibility of the membrane structure at low temperatures (Alfaro *et al*., 2001; Lin *et al*., 2013).

In animal cells, injuries caused by cooling are probably due to the phase transition in membranes, when the lipids are supposed to change from a liquid to a gel phase between 0°C and 20°C, with loss of fluidity (Wada *et al*., 1990; Hays *et al*., 2001). According to Mazur *et al*. (1992), injury caused by cooling increases with time of exposure to critical temperatures, a feature observed in the present work, with an increased mortality of *M. amazonicum* embryos cooled to 2°C for up to 6 h.

The positive control group of the present study showed healthy embryos with a translucent outer membrane, which enabled the observation of the movement of the internal structures of the embryo and, thus, the survival count. The negative control

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group showed a high number of embryos with opaque and whitish coloration. This characteristic is present in embryos after cooling and was observed by Fornari *et al*. (2014), although in cooled embryos of piracanjuba (*Brycon orbignyanus*). According to the author, such a characteristic is used as evidence of ice crystal formation, which causes embryonic mortality.

Regarding embryonic volume, according to Miliorini (2012), the use of cryoprotectants in fish embryos, besides avoiding crystallization, has been suggested as a way to provide a favorable osmotic environment. Thus, inappropriate concentrations would promote an increase in the osmotic concentration within the embryo and the cells, with consequent inflow of water. As the embryo and cells are already enlarged by crystallization, the membranes may suffer fractures at various points. For the embryos of *M. amazonicum* in the present study, there was no difference in the embryonic volume between the 2 h or 6 h of storage in low temperature and after 24 h in incubators, when compared with the positive control group. This fact may highlight the absence of an osmolality-related problem, despite the occurrence of mortality due to the formation of ice crystals.

It has been concluded that, in both treatment groups (DMSO and EG), the survival rate decreased as a result of the time of exposure to cooling and not as a result of the cryoprotectant tested. Although there was no change in the embryonic volume as a result of the time of exposure to cooling, a change in the translucent appearance of the embryos has been observed. Consequently, further studies are needed to verify the potential of *M. amazonicum* for cooling and cryopreservation.

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Conflict of interest

The authors declare there are no conflicts of interest.

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