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Cryoprotectant agents and cooling effect on embryos of *Macrobrachium amazonicum*

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Summary

There are few reports of cryopreservation and injuries in *Macrobrachium amazonicum* embryos. Thus, the aim of this study was to analyze the effects of cryoprotectants agents and cooling on stage VIII of this species. Fertilized eggs from ovigerous females were removed from the incubation chamber, then placed in 10 ml Falcon tubes with a cryoprotectant solution and saline-free calcium solution. Thus, the embryos underwent a cooling curve of 1°C per min until reaching 5°C, and then were stored for 2 h. The tubes containing the embryos were washed to remove the cryoprotectant, acclimated for 5 min and then transferred to 50 ml incubators. At the end of the 24-h period, living embryos from each tube were counted and tabulated. A pool of embryos was fixed with 4% formaldehyde and then subjected to histology using 3-mm thick sections and stained with haematoxylin/eosin. Another pool was used for biometric analysis in which length, width and volume were analyzed. The cryoprotectants agents used were: dimethylsulfoxide (DMSO), methyl alcohol, ethylene glycol at 1, 5 and 10% and sucrose (0.5 M). Variance analysis was performed followed by Tukey's honest significant difference (HSD) test at 5% significance level. DMSO cryoprotectant affected embryo survival the least with rates of 71.8, 36.2 and 0% for concentrations of 1, 5 and 10%, respectively. Ethylene glycol caused 100% mortality at all the concentrations used. It was not possible to observe the interference of cooling and cryoprotectants on embryonic structures in this study.

Keywords: Amazon river prawn, Chilling, Cooling protectors, Fertilized eggs, Morphology

Introduction

Macrobrachium amazonicum, a native species from Brazil, has gained momentum in the economy and culture of freshwater prawns because grows easily, is resistant to disease, is unaggressive, grows quickly and has an agreeable taste (Lobão & Rojas, 1991; Moraes-Riodades & Valenti, 2001).

Macrobrachium amazonicum females carry their eggs from fertilization to hatching in an abdominal incubation chamber. This process lasts 14 days and is divided into eight embryonic stages: I, fertilized egg; II, cleavage; III, blastula/gastrula; IV, germinal disc; V, embryonised nauplius; VI, post-nauplius; VII, protozoa; and VIII, zoea (Vigoya, 2012).

Studies on the cryopreservation of embryos are gaining prominence in order to secure commercial production and preserve natural stocks of species targeted for cultivation. Embryo cryopreservation is also important because it facilitates the spread of animal genetic material on a large scale and favours embryo storage for prolonged periods with reduced

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loss of development capacity after defrosting (Kaidi *et al*., 1999). This fact is supported by research suggesting that, overall, crustacean embryos can be tolerant to freezing (Baust & Lawrence, 1977; Sansone *et al*., 2005).

Several steps are required to cryopreserve gametes and embryos and small mistakes in those steps may irreversibly damage the biological material (Tiersch & Green, 2011). Therefore, the process needs to be understood to prevent possible damage.

Among the extensive research done on *M. amazonicum*, almost nothing is known about its cooling process, as well as the effect of cryoprotectants agents on their embryonic stages. During preservation at low temperatures, low-molecular-weight substances are incorporated with the aim to protect biological structures from damage by lowering the temperature and providing a favourable environment for survival of the stored cell (Purdy, 2006). Cryoprotectants are classified into permeable and non-permeable cryoprotectants. They enter the cell and form hydrogen bonds with intracellular water molecules, lowering the freezing temperature and avoiding ice crystal formation (Vajta & Nagy, 2006). The non-permeable cryoprotectants remain in the extracellular medium, removing free water and promoting its removal from extracellular spaces by osmosis (Vajta & Nagy, 2006; Pereira & Marques, 2008). The permeability of these cryoprotectants is associated with embryo species (Friedler *et al*., 1988), embryonic development (Leiboo, 1986), surface to embryo volume ratio (Hapletoft, 1984), cryoprotectant agent (Schneider & Mazur, 1986) and exposure temperature (Trounson, 1986).

In contrast, there are two reasons to explain flaws in the action of cryoprotectants: toxicity, which limits the concentration at which it can be used and reduces the effectiveness of cryoprotective action; and the choice of cryoprotectant agent, which can have a direct effect on cryoinjury, for example by changing the polarity of the extracellular medium and causing membrane damage (Landim-Alvarenga, 1995).

In this context, the cooling process and cryopreservation represent a viable alternative for preserving embryos, and also for reproduction on a commercial scale. Thus, the cooling process becomes a tool that is directly linked to the independence of natural reproductive periods and conservation of natural populations. Therefore, the aim of this study was to evaluate the effects of cooling and cryoprotectant agents (dimethylsulfoxide, methyl alcohol, ethylene glycol and sucrose) on *M. amazonicum* embryos.

Materials and Methods

Selection of animals

The animals used in this experiment were collected in Colosso Lake, located in Fortaleza, Ceará State, Brazil (03°77′89.10″S; 038°47′18.68″W), during the months of June, July and August 2015.

The prawn were caught with the aid of tailored polyethylene terephthalate (PET) bottle traps. Then the animals were transported in coolers to the Laboratory of Prawn Farming (LACAR) located at the Institute of Biomedical Sciences of the Ceará State University in Fortaleza, Ceará State.

Females were stored in a tank containing 25 ppm formaldehyde solution for 30 min for disinfection. Females were maintained in 500-litre fibreglass tanks, under aeration, at a temperature of 29°C and a 12 h:12 h photoperiod. Feed was provided twice a day, at 8 a.m. and 5 p.m. with commercial feed for shrimp in 35% crude protein. The tank was cleaned daily by siphoning before the first meal to remove food debris and faeces. Temperature, pH, ammonia and nitrite levels were measured daily each morning. The tanks were covered with screens to prevent animals escaping.

Cooling embryos of Amazon river prawn *Macrobrachium amazonicum*

Every day females in the final stage of embryonic development were selected. Their eggs were removed and subjected to analysis in a stereomicroscope to determine the embryo stage. Only embryos at stage VIII were used. The characterization of stage VIII was performed according to a study by Vigoya (2012). After the stage was determined, the embryos were removed from the female's incubation chamber with fine-point tweezers. Embryos were then were weighed (0.01 g) and placed in 10 ml Falcon tubes, to which had already been added the cryoprotectant (ACP) and saline-free calcium solution. The Falcon tubes were placed in ice coolers, in which the temperature was monitored constantly, such that embryos underwent a cooling curve of 1°C per min down to 5°C, and then were stored for 2 h. The tubes were removed from the cooler and the embryos were washed 10 times with tap water to remove the ACP and acclimated for 5 min in water at room temperature and then transferred to 50 ml incubators with aeration. After the end of the 24-h period, living embryos from each tube were counted and tabulated. Embryo homeowners that did not show any movement of internal organs were considered to be dead; those that showed that movement of the internal organs was present were considered to be alive.

The treatments were: control (calcium-free saline); T1, 1% DMSO + 0.5 M sucrose; T2, 5% DMSO + 0.5 M

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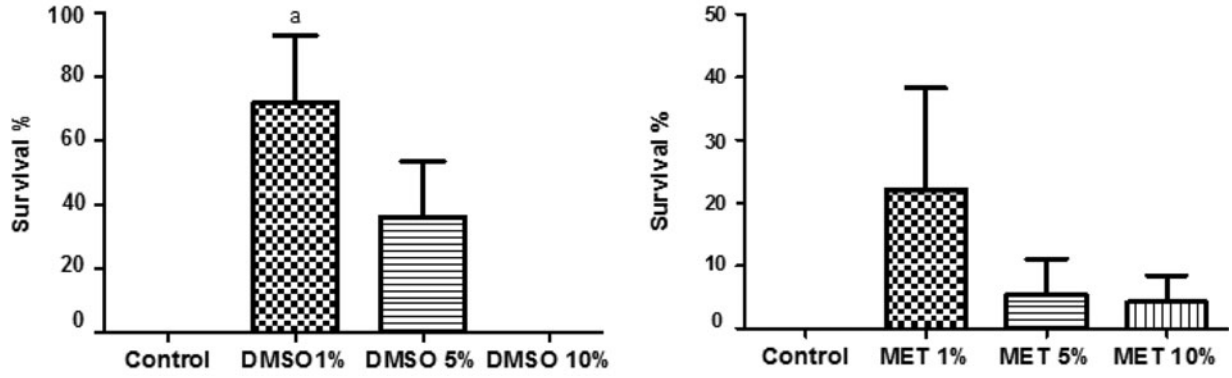

Figure 1 Embryo survival after cooling at different concentrations of dimethylsulfoxide (%), and methyl alcohol (%). Tukey's honest significant difference (HSD) test at 0.05%.

sucrose; T3, 10% DMSO + 0.5 M sucrose; T4, methyl alcohol 1% + 0.5 M sucrose; T5, methyl alcohol 5% + 0.5 M sucrose; T6, methyl alcohol 10% + 0.5 M sucrose; T7, ethylene glycol 1% + 0.5 M sucrose; T8, ethylene glycol 5% + 0.5 M sucrose; and T9, ethylene glycol 10% + 0.5 M sucrose.

Biometric embryos

After cooling, eggs and embryos were photographed on a LEICA DFC 295 stereoscopic microscope and their images were analyzed to determine biometric indices and measurements using the LEICA LASV3.6 program. The following biometric variables were analyzed: major axis equal in length; minor axis equal width and the corresponding volume $V = \pi.a.b^2/6$, in which a is the length of the egg, and b is the width of the egg.

Histology of eggs and embryos

After cooling, eggs and embryos were fixed in 4% formaldehyde solution for 24 h. Subsequently the samples were transferred to a 70% alcohol solution and kept at room temperature. Then they went through a dehydration process consisting of baths of ascending alcohol solutions, in the following order: 80% alcohol, 90% alcohol, and absolute alcohol. Each bath lasted 1 h. After the alcohol baths, samples were soaked in xylene to facilitate the paraffin infiltration. Immediately afterwards, the material was placed in moulds and embedded in paraffin.

When cold, the material was taken to the microtome and 3-mm thick sections were cut. The sections were stained with haematoxylin/eosin.

Histological sections were analyzed under a LEICA DM 2000 microscope at the Laboratory of Prawn Farming, Ceará State University.

3.5 Statistical analysis

Variance analysis followed by Tukey's HSD test at a 0.05% significance level was used to verify the occurrence of significant differences between results. Data were analyzed with the program GraphPad Prism® version 5.01.

Results

Survival

Analysis of 1, 5 and 10% DMSO concentration groups showed that survival rates were 71.8, 36.2 and 0% respectively, with a significant difference in the 1% DMSO group compared with the control group and the 10% DMSO group ($P \leq 0.05$). Analysis of 1, 5 and 10% methyl alcohol groups showed that embryo survival was 22, 0.5 and 0.4% respectively, and there was no significant difference between groups ($P > 0.05$). Analysis of the effect of ethylene glycol showed that embryos died at all three concentrations used (Fig. 1).

Egg biometrics

Stage VIII eggs were elliptical, with length always greater than width. The lengths of eggs subjected to 10% DMSO were significantly different that of other groups ($P < 0.0001$), with the lowest average length (1.20 mm) for this treatment. For egg width, no significant difference ($P > 0.0001$) between control treatment and 5% DMSO treatment and also between 1% DMSO and 10% DMSO treatment was observed. Smaller widths were observed for the 1% DMSO and 10% DMSO treatment groups, with values of 0.93 mm and 0.94 mm respectively. For volume, significant differences for treatment groups with

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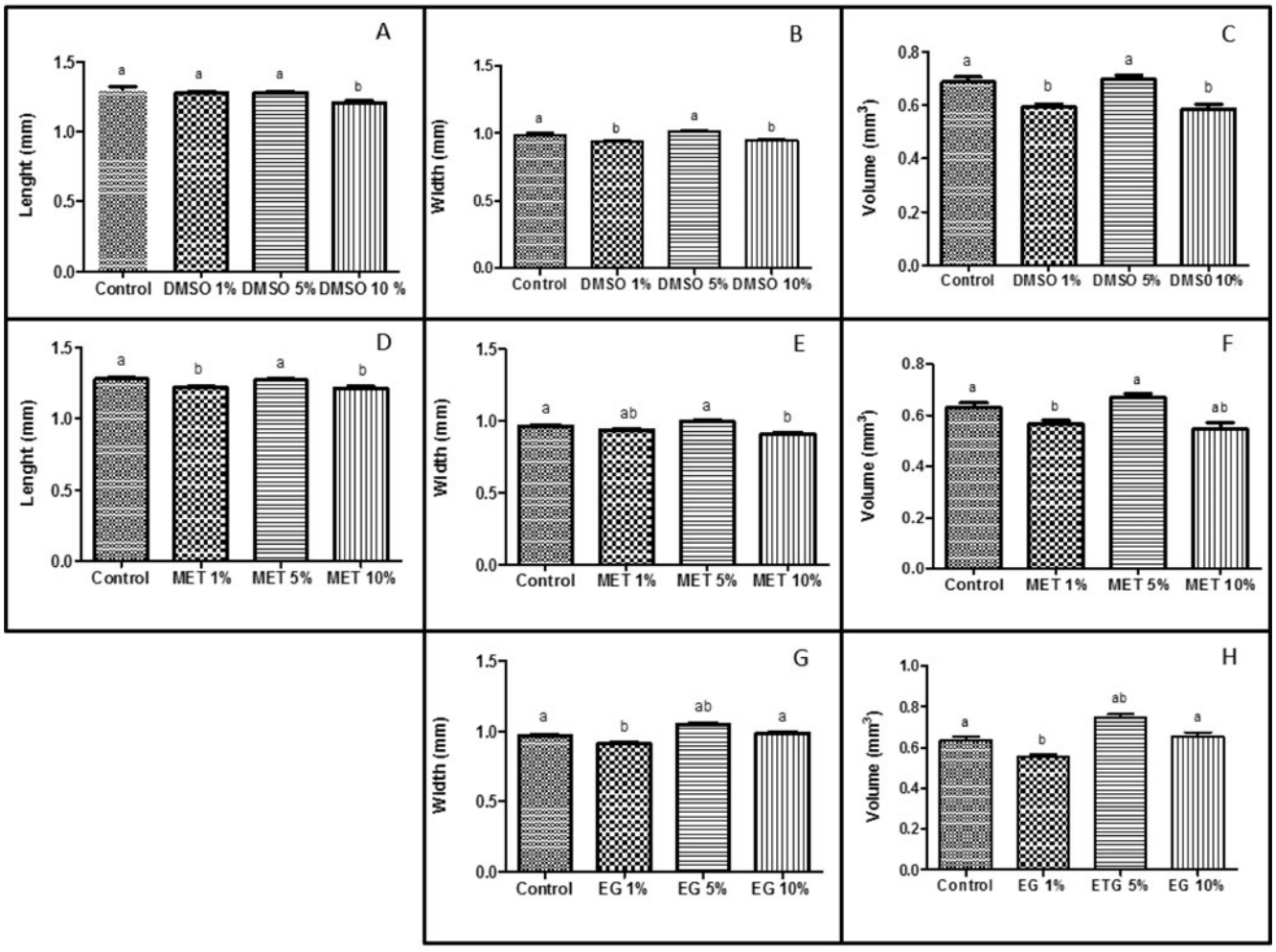

Figure 2 Length, width and volume at different concentrations of dimethylsulfoxide (%), methyl alcohol (%) and ethylene glycol of *M. amazonicum* embryos. (*A*) Length DMSO. (*B*) Width DMSO. (*C*) Volume DMSO. (*D*) Length methyl alcohol. (*E*) Width methyl alcohol. (*F*) Volume methyl alcohol. (*G*) Length ethylene glycol. (*H*) Volume ethylene glycol. [a,b,c,d]Averages with no common letters in columns differ from each other by Tukey test at $P < 0.0001$.

5, 1, and 10% DMSO ($P < 0.0001$) were observed, but volume was not significantly different from the control treatment ($P > 0.0001$). Following treatment with 10% DMSO, minor changes in length and volume were observed, compared with other DMSO concentrations used (Fig. 2).

Significant statistical difference was observed in the length of cooled eggs following treatment with 5% methyl alcohol compared with 1% and 10% methyl alcohol ($P < 0.0005$); longer lengths were observed in the control treatment (1.27 mm) and 5% methyl alcohol (1.27 mm) groups. Statistically significant difference was observed between the 10% methyl alcohol treatment and the control treatment and the 5% treatment ($P < 0.0001$) groups, with averages of 0.90 mm, 0.96 mm and 0.99 mm, respectively. A considerable difference in egg volumes for the control treatment group compared with 1% methyl alcohol and 10% methyl alcohol groups was observed and for 5% methyl alcohol treatment compared with 1% methyl alcohol treatment, and 10% treatment ($P < 0.0001$) groups. Cold eggs treated with 10% methyl alcohol had lengths, widths and volumes (Fig. 2).

There was no statistically significant difference in the length of colds eggs in the three ethylene glycol concentration groups. There was no significant statistical difference between treatments in the width and volume of eggs ($P < 0.0001$), except for the control treatment with 10% ethylene glycol. Egg measurements were lower in 1% concentration (0.90 mm long and 0.63 mm wide) and higher in 5% concentration ethylene glycol (1.05 mm long and 0.65 mm wide) groups. Treatments with cryoprotectants at 5% showed similarity with the control treatment in terms of volume (Fig. 2).

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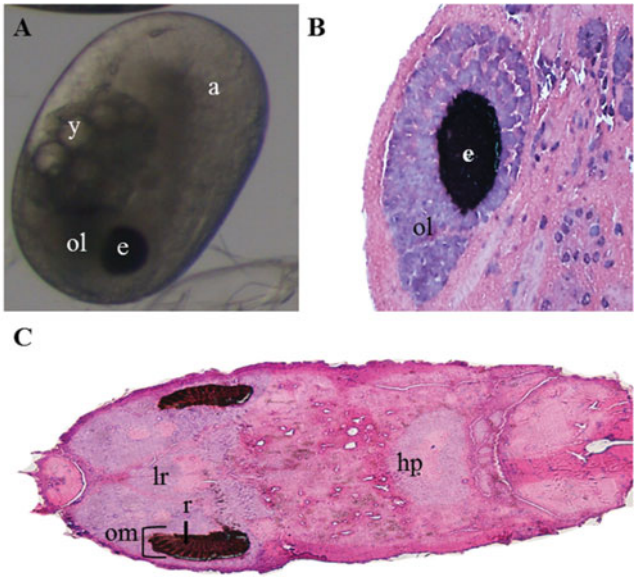

Figure 3 Photomicrography of *M. amazonicum* eggs at stage VIII. (*A*) Photograph of the embryo (×40 magnification). (*B*) Transverse view of the eye (×40 magnification). (C) Upper transverse view (×20 magnification). ab, abdomen; lr, lymph node region; hp, hepatopancreas; om, ommatidium; r, rhabdom; ol, optic lobe; e, eye; y, yolk. Haematoxylin/eosin staining.

Embryo histology

In this study, no interference of cooling and cryoprotectants on the embryonic structure of *M. amazonicum* was observed, with no changes in the cell clusters of structures present at stage VIII, such as optic lobe and eyes, ventral nerve cord, brain nodes, oesophagus, mid intestine, later intestine and telson. The yolk is located in the dorsal part of the embryo and in the eighth and last stage the amount of the yolk is small because it has been consumed by the embryo for the formation of structures during embryonic development; yolk granules serve as a nutritional reserve for the larva. The musculature is evident and developed at this stage, especially in the abdomen. The animal occupies practically all the space of the egg and folds over itself, leaving it next to the anterior and posterior regions. The ocular structure appears circular. There were no changes in embryo structure during treatments, independent of survival (Fig. 3).

Discussion

The survival of embryos cooled with ethylene glycol was similar to survival observed by Ferreira *et al.* (2015), which demonstrated the high toxicity of this cryoprotectant for this species at 5 and 10% concentrations. Ethylene glycol (1,2-ethanediol) is a low-molecular-weight cryoprotectant (62.07 g/mol), which has low toxicity and greater penetration capacity in cells. In recent studies, ethylene has shown excellent results for bovine (Sommerfeld & Niemann, 1999; Martinez *et al.*, 2002; Mucci *et al.*, 2006), swine (Berthelot *et al.*, 2007; Cuello *et al.*, 2007), goat (Guignot *et al.*, 2006) and sheep (Garcia-Garcia *et al.*, 2006; Bettencourt *et al.*, 2009) embryo cryopreservation. Bedore (1999) and Leibo (2008) state that there are differences in the efficiency of cryoprotectants for the various species.

While working with DMSO and pirancajuba (*Brycon orbignyaus*) sperm, Galo *et al.* (2011) observed a decrease in normal gametes and in increase in secondary injuries after cooling from 62.20 to 54.60% and 8.5 to 15%, respectively. Streit-Junior *et al.* (2009) obtained similar results with *Piaractus mesopotamicus* sperm using 10% DMSO, which caused a large increase in the amount of primary pathologies.

In embryos, the formation of ice crystals, the toxicity of the cryoprotectant solution and osmotic shock are factors that cause most damage in the cooling process. In addition, temperatures above 0°C are known as the danger zone, because they damage cell membrane lipids, causing structural and functional changes in the membrane (Zeron *et al.*, 1999; Vajta & Kuwayama, 2006; Yavin & Arav, 2007).

Vajta & Nagy (2006) postulate that temperatures between 15 and −5°C are related to damage of microtubules and cytoplasmic lipid droplets, which prejudices subsequent meiotic divisions. Injuries to microtubules are considered reversible, while damage to lipid drops contribute to the death of cryopreserved embryos.

In 1963, Mazur demonstrated that the output speed of intracellular water is related to the increased concentration of extracellular solutes, determining osmotic equilibrium. Thus, the freezing rate determines the degree of cell shrinkage and the presence or absence of crystals (Mazur, 1984).

Woods *et al.* (2004) discussed the thawing process with emphasis on the effect of cell rehydration and removal of cryoprotectant on cell viability post-cooling, showing that this is also important for the survival of cryopreserved material. During cooling, from freezing to thawing there is flow of water and cryoprotectant agents across the cell membrane, thus there is change in cell volume, and consequently its structure. Therefore, any failure in the process can modify the quantity of liquid in the egg, which could explain the smaller volumes found in this study.

However, the use of 5% DMSO in the cryopreservation of *Macrobrachium rosenbergii* shrimp sperm was successful, showing a viability rate of 64.8% (Goldberg *et al.*, 2000). Going back to the literature,

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it has been seen that higher DMSO concentrations decreased the survival of the studied structures, results that support our findings. For cryopreservation of *Litopenaeus vannameii* sperm cells with cryoprotectants 10% DMSO and 10% ethylene glycol for 0, 30, 60 and 90 days, Uberti (2012) obtained mortality rates between 23.17 and 82.11% for DMSO and 29.94 to 83.72% for ethylene glycol.

The literature reports widespread use of DMSO as cryoprotectant in concentrations ranging from 1 to 32%. However, due to its high water solubility, as a downside, DMSO presented the ability to cause changes in the cell membrane, which damages cells and makes it impracticable for use. Even at very low concentrations, DMSO can be toxic in some biological systems, especially when these are held for long periods at temperatures above 5°C (Hubálek, 2003).

Preston & Coman (1998) reported that methyl alcohol was a less toxic cryoprotectant to embryos at 1 and 2 M concentrations, both at the initial stages and at the final stages of *Penaeus esculentus*, when compared with ethylene glycol and DMSO.

Evaluation of the toxicity of various cryoprotectants in *Penaeus monodon* embryos showed that the use of 10% DMSO for 10 min resulted in a better embryo survival rate, followed by ethylene glycol at 5% for 10 min and finally methyl alcohol, which caused the death of all embryos. Unlike our study, here, methyl alcohol showed better results when compared with ethylene glycol (Vuthiphandchai *et al.*, 2005).

When studying the feasibility of *P. monodon* spermatophores with cryoprotectants DMSO, ethylene glycol and methyl alcohol, Vuthiphandchai *et al.* (2007) found that 5 and 10% DMSO achieved the best results of 81.7 and 80% respectively. In our studies, DMSO showed similar results. The differences in terms of embryo sensitivity to various cryoprotectants in conducted research showed the diversity of species tolerance to cryoprotectant agents.

Their concentrations were found to decrease viability rates, which may be related to a combination of internal and external agents, showing that these should be used together to improve the results. Pereira & Marques (2008) considered that using cryoprotectants this way is a factor that should be considered to increase the efficiency of embryo protection in the cooling process and to decrease intracellular ice crystals.

Histological and biometric analyses in our study did not find any differences in embryonic structures between treatments in which there was survival and treatments in which there was no survival, indicating that factors related to low survival of treatment with ethylene glycol are not factors that affect the embryo cell skeletons and may be on a smaller scale such as damage to membranes, cell organelles, the cytoskeleton and the nucleus.

Major development of the abdominal muscles occurred, which reveals the importance of this region at the time of embryo break out, because egg membrane ruptures in this area (Vigoya, 2012). The small amount of yolk found in our study coincides with the results of Yao *et al.* (2006), establishing the need for external energy resource independence soon after hatching and ensuring the successful first moult. Vigoya (2012) clarifies embryonic development in the early stages, showing differentiation in the anterior and posterior regions, as well as longitudinal growth at the V stage, and explaining why length vales always larger than width values.

Consequently, histological analysis carried out in this study concluded that different cryoprotectants and different concentrations used here did not produce changes in the embryonic structures, which differed from the expected findings, and showed that injuries are caused at small levels, possibly intracellularly.

Therefore, the conclusion is that methyl alcohol and DMSO were the cryoprotectant that show the best results for survival of embryos, mainly at 1% concentration. The embryos did not change in cell clusters, indicating injuries at the cellular level. The results of this research showed the need to test new associations and new concentrations of cryoprotectants to achieve more effective responses for reducing injuries in this species during the cooling process.

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