

## Post-cooling damage to *Piaractus mesopotamicus* embryos at different stages of development

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**ABSTRACT** - The present study investigated the effects of 6h- and 10h-storage at -8°C on the quality and hatch rate of *Piaractus mesopotamicus* embryos at 4 stages of development. Embryos were exposed to a cryoprotectant solution, cooled down at a rate of 1°C.min<sup>-1</sup> to -8°C, and stored at this temperature for 6h and 10h, respectively. For control treatment, viable embryos at the 4 developmental stages studied, were selected and taken immediately to the incubator, without going through cooling. The results were evaluated using a multivariate statistical technique (factor analysis). Damage was characterized according to the following variables: uniformity, adhesion, symmetry, margins, and inclusion. Two factors that best explained the variance of each parameter were defined. The control group had the highest hatch rates, and a weak relationship with embryo damage. Although treatments involving 6h and 10h cooling exhibited lower hatch rates and a higher association to damage. The information obtained in this study is useful in promoting improved cryopreservation techniques for fish embryos, indicating the probable conditions under which certain injuries are more frequent.

Keywords: cryopreservation, embryogenesis, multivariate analysis, South American fish

## Danos causados a embriões de *Piaractus mesopotamicus*, em diferentes estádios de desenvolvimento, após técnica de resfriamento

**RESUMO** - O presente estudo investigou o efeito da estocagem de embriões de *Piaractus mesopotamicus* em quatro diferentes estádios de desenvolvimento, durante 6 e 10 horas a -8°C na qualidade e taxa de eclosão. Os embriões foram expostos à solução crioprotetora e passaram por curva de resfriamento de 1°C.min<sup>-1</sup> até atingir -8°C, onde foram mantidos por 6 e 10 horas, respectivamente. Para o tratamento controle, embriões viáveis nos quatro estádios de desenvolvimento estudados, foram selecionados e levados a incubadoras, sem passar por resfriamento. Os resultados foram avaliados usando estatística multivariada (análise de fatores). Os danos causados pelo resfriamento foram caracterizados de acordo com as variáveis: uniformidade, adesão, simetria e bordas das células, além de inserção no vitelo. Foram definidos dois fatores que conseguiram reter maior variância contida nos dados. O grupo controle apresentou alta taxa de eclosão e baixa relação aos danos verificados nos embriões. Enquanto os tratamentos com 6 e 10 horas após resfriamento tiveram taxas de eclosão mais baixas e alta associação aos danos. Os resultados encontrados são importantes, pois indicam condições prováveis em que ocorrem lesões durante o processo de resfriamento das células, contribuindo assim para o aperfeiçoamento da técnica de criopreservação de embriões.

Palavras-chave: análise multivariada, criopreservação, embriogênese, peixe sulamericano

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## INTRODUCTION

A number of factors can compromise the reproduction and artificial propagation of fish of economic interest (Romagosa 2008). Therefore, gamete cryopreservation is crucial to ensure the formation of semen and embryo banks for endangered species and to develop genetically variable farmed fish.

Cryopreservation techniques aim to maintain cell metabolism in a quiescent state, thereby prolonging cell and tissue conservation (Neves et al. 2014). In recent years, efforts from the fishing industry, in particular, have rapidly enhanced the technical capacity of preserving eggs from fish and aquatic invertebrates (Carolsfeld 2003). Special attention has been devoted to the cryopreservation of fish embryos, but the results obtained remain inconsistent (Zhang et al. 2007). However, in addition to sensitivity to cold (Zhang; Rawson 1995), one of the main problems of cryopreservation is the low permeability of embryo membranes to water and cryoprotectants (Hagedorn et al. 1997, Lopes et al. 2011). Embryo susceptibility to exogenous factors imposed by cryopreservation depends on the stage of development (Ahammad et al. 2003), i.e., early-stage embryos are more vulnerable to cryopreservation, but become more resistant as cleavage advances and cell size decreases, producing a more suitable surface-volume ratio (Calvi; Maisse 1998).

Low temperatures are known to damage cells and tissues, and the resistance of fish eggs and embryos to cooling varies according to the techniques applied. For instance, Morris and Watson (1984) showed that different procedures, such as (a) direct rapid cooling with thermal shock, and (b) slow cooling achieved with indirect, long-time exposure to cold, cause different embryonic damage.

The first cryopreservation protocols applied to embryos from native South American fish were developed by Ninhaus-Silveira et al. (2009) for *Prochilodus lineatus* and by Streit Jr. et al. (2007) for *Piaractus mesopotamicus*. *P. mesopotamicus* embryos have been recently tested to develop cooling protocols with different cryoprotectants (Streit Jr et al. 2007, Digmayer 2010, Fornari et al. 2011, Fornari et al. 2012, Neves et al. 2014) and diverse embryo stages (Lopes et al. 2011). However, many aspects, such as identifying the type of damage and its cause must be studied to establish efficient cryopreservation protocols. As such, the present study investigated the damage caused to *P. mesopotamicus* embryos at 4 different stages of development from storage at -8°C for 6h and 10h.

## MATERIALS AND METHODS

### *Embryo production*

Embryos were obtained from broodstock *P. mesopotamicus* aged four years and kept in fish farming ponds (0.4 kg fish.m<sup>-2</sup>). Six couples of reproducers were hormonally induced using carp pituitary extract in two doses (0.5 and 5.0 mg.kg<sup>-1</sup>), with an interval of 12 hours for the females, and 1.0 mg.kg<sup>-1</sup> (single dose) for the males (intramuscularly). After 275h<sup>0</sup>C, gametes releases were performed (extruded), with posterior fertilization. An egg pool was transferred to conical incubators (7L) with continuous water flow for embryonic development.

### *Embryo cooling*

Viable embryos were selected from the incubators at four development stages: blastoderme (BD) at the 64 cell stage, 1.4 hours post-fertilization (hpf); 25% epiboly movement (EP), 5.2 hpf; 90% epiboly movement and blastophore closure (BL), 8 hpf; formation of the embryonic optic vesicle (OP), 13.3 hpf. The embryos were held in Vacutainer® tubes, to which a cryoprotectant solution containing 10% methanol and 0.5M sucrose was added. The tubes were then passed through a series of ice-filled coolers, producing a cooling curve as described by Lopes et al. (2012), with temperature decreasing at  $1^{\circ}\text{C}\cdot\text{min}^{-1}$ , in order to control the decline in temperature during the cooling curve, six coolers containing ice were used (each cooler was kept at a constant temperature: 20, 10 and  $0^{\circ}\text{C}$ , respectively), monitored with thermometers, until reaching  $-8\pm 2^{\circ}\text{C}$  in a freezer, where they were stored for 6h and 10h. For control purposes, viable embryos at the four developmental stages studied were selected and taken immediately to the incubator, where they remained until hatching.

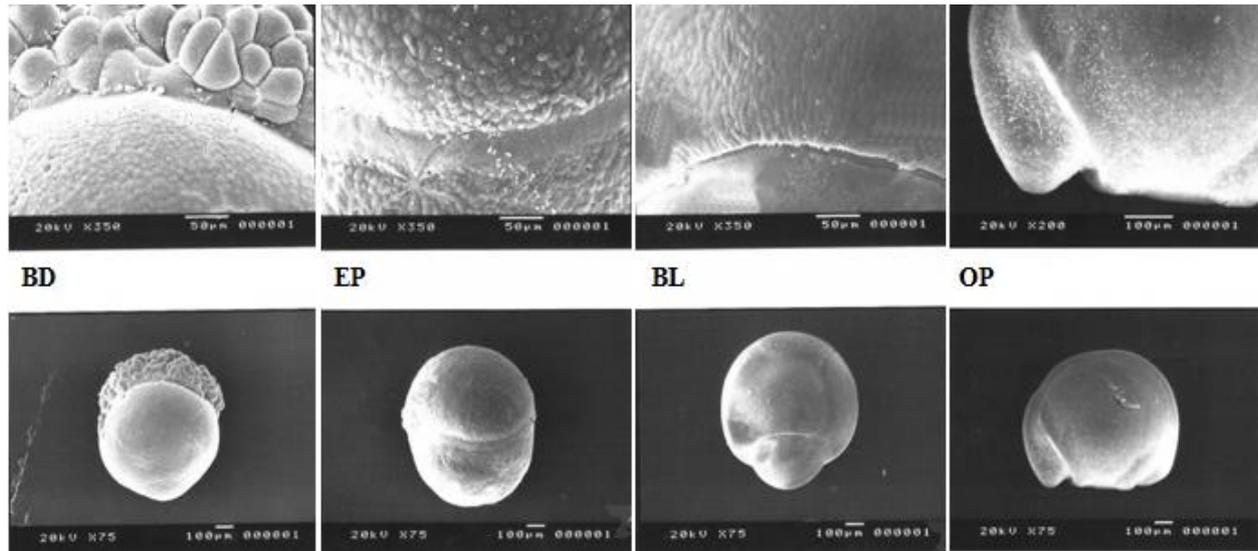
Hatch rates (percentage values for total larvae) were recorded for the eight treatment combinations (four embryo stages and two cooling periods) and a control group (viable embryos at the four stages of development). The 100 embryos from each treatment were kept in 3L incubators with continuous flow (six repetitions).

### *Embryo morphology*

Five embryos from each treatment combination (four stages, two cooling periods and control, with six repetitions each), a total of 360 embryos, were fixed and stored in 2.5% glutaraldehyde solution in 0.1M cacodylate buffer, pH 7.2, until morphological evaluation.

After mechanical removal of the chorion, embryos were kept in a fixing solution (4% glutaraldehyde) and dehydrated in a series of increasing ethyl alcohol concentrations (10, 30, 50, 70, 80, 90%), for 10 min in each phase. The embryos were also passed through three 100% ethyl alcohol baths and dried in a  $\text{CO}_2$  critical point dryer (BAL-TEC, model CPD 030). Embryo fragments were mounted on metal stubs and coated with gold palladium alloy in a Denton Desk II coating unit (Deltion Vacuum).

The samples were examined and electron micrographs taken in a JEOL scanning electron microscope (JSM-5410). Embryo images from the different treatments and stages of development (Figure 1) were evaluated to characterize damage according to the following variables: uniformity (unequal cell size), adhesion (intercellular adhesion), symmetry (asymmetrical cell positioning), margins (poorly defined cell margins) and inclusion (cell inclusion in the yolk) (Shields et al. 1997). Damage was evaluated on a quantitative scale from 1 (abnormal) to 3 (normal).



**Figure 1.** Scanning microscopy of *P. mesopotamicus* embryo at four stages of development: blastoderm (BD), 25% epiboly (EP), blastopore closure (BL) and optic vesicle formation (OP)

#### Data analysis

The study was arranged in a 4x2 completely randomized factorial design (four embryo stages, two cooling periods) and one control treatment, with six repetitions per treatment. Hatch rates were compared using ANOVA followed by the Tukey test for multiple comparisons. Tests were performed at a significance level of 0.05 using the SAS package (2002).

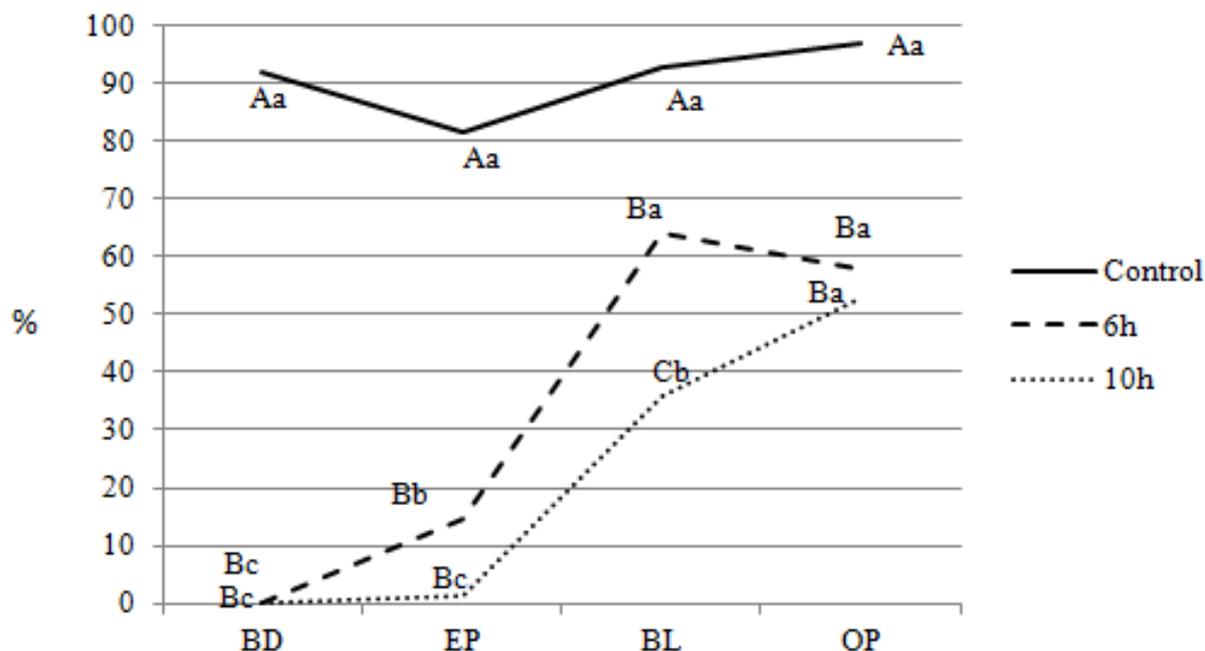
Embryo damage was analyzed through exploratory factor analysis (Thrustone 1931). This method evaluates the nature of the intervariable relationship by rotating the factor axes, using StatSoft (2010).

All experimental protocol was approved by the institutional Animal Care and Use Committee (Process n° 008004/08).

## RESULTS

No interaction effects between embryo development stages and cooling time were detected. Embryos subjected to the different treatments had lower hatch rates than those from the control treatment ( $P < 0.05$ ).

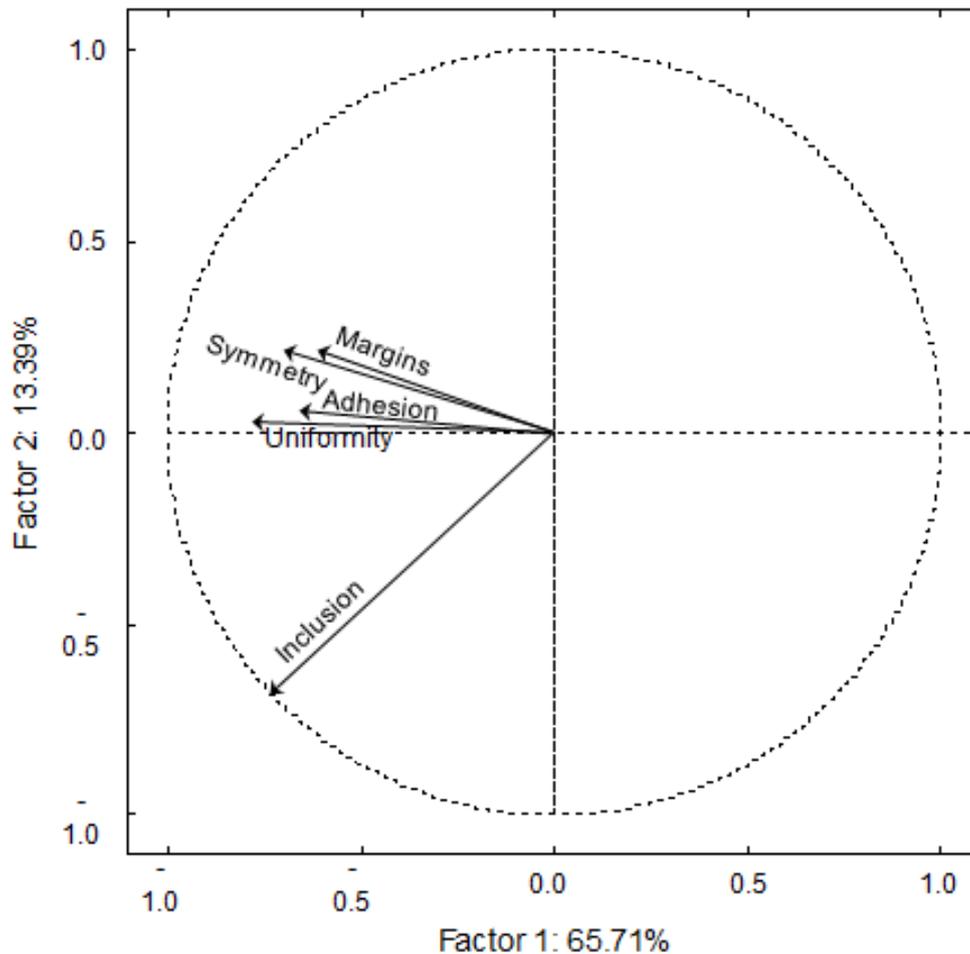
For treatments with 6h cooling, embryos at BL and OP stages had higher hatch rates than at BD and EP stages. For 10h cooling, however, only embryos at the OP stage had more than a 50% hatch rate (Figure 2).



**Figure 2.** Hatch rate (total larvae) of *P. mesopotamicus* embryos at blastoderm (BD), 25% epiboly (EP), blastopore closure (BL) and optic vesicle formation (OP) stages, under control treatment or subjected to cooling for 6h and 10h. \*Different uppercase letters within a same embryo stage and lowercase letters within a same treatment differ statistically (Tukey test,  $P < 0.05$ )

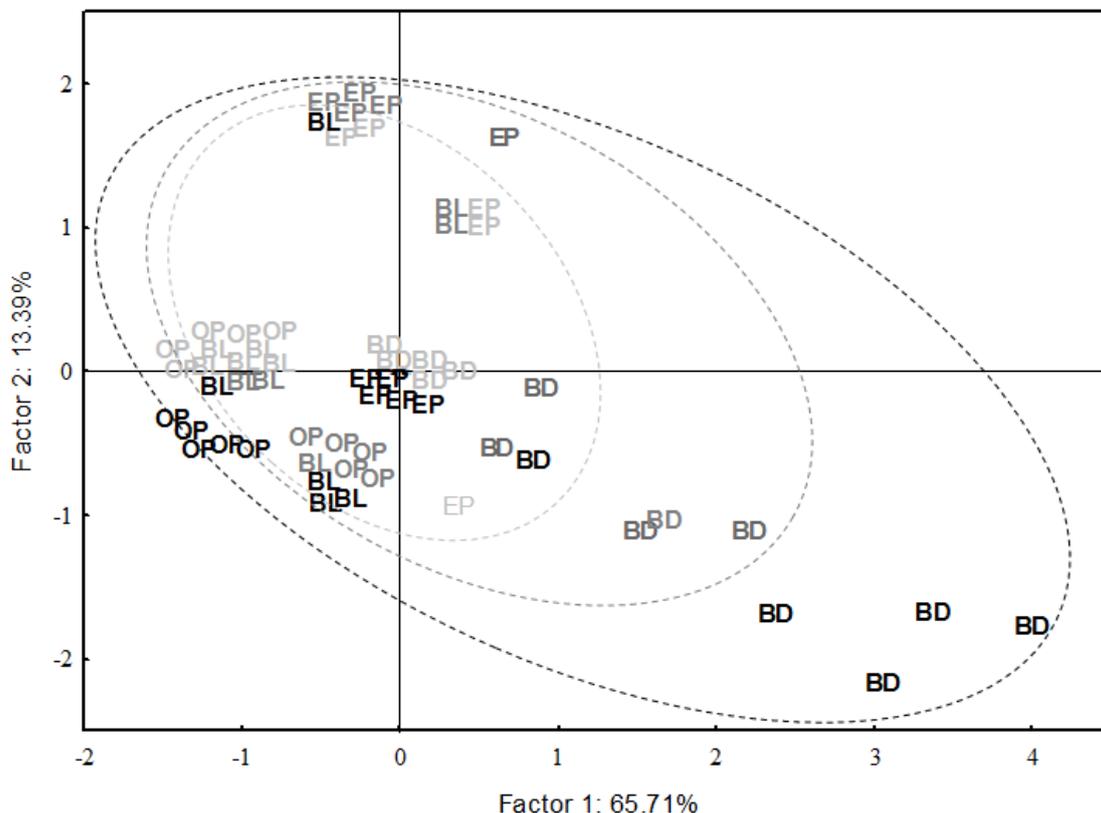
According to Thurstone (1931), the sum of the eigenvalues in factor analyses was assumed to be 10. Therefore, for the dataset analyzed, the eigenvalue of 3.29 obtained for Factor 1 was significantly higher than the other eigenvalues and explained 65.71% of the proportion of variance. Factor 2 had an eigenvalue of 0.669 and explained 13.39% of the variance. Assuming Kaiser's criterion, since Factor 1 and 2 together account for 79.10% of total variance, the analysis must consider only two factors. The other individual eigenvalues were non-significant since each one explained less than 10% of the variance.

Figure 3 shows intervariable and interfactor correlations. Embryo uniformity, adhesion, symmetry and margins are closer to Factor 1, this holds most of the original values analyzed, whereas inclusion is closer to Factor 2. This indicates that the variable inclusion was not as significant as the others in evaluating embryo damage resulting from experimental conditions.

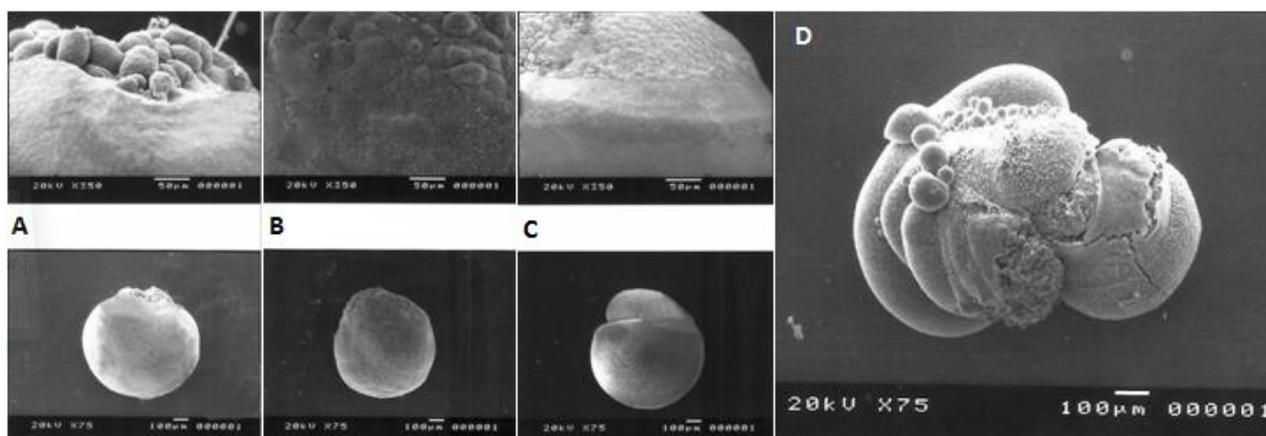


**Figure 3.** Accumulated eigenvalues for Factor 1 and Factor 2 according to the variables uniformity (unequal cell size), adhesion (intercellular adhesion), symmetry (asymmetrical cell positioning), margins (poorly defined cell margins) and inclusion (cell inclusion in the yolk) of *P. mesopotamicus* embryos at 4 stages of development and 3 cooling conditions (6h, 10h or not cooled)

Treatment distribution in relation to the Factors is shown in Figure 4. The control group had the most divergent characteristics, occupying a substantial area in the plot (largest circle), which was distant from the Factor 1 axis, particularly for embryos at BD stage. This is likely because BD embryos from the control group were not exposed to low-temperature storage and had higher hatch rates than the other treatments. Treatments cooled for 6h (T6; smallest circle) and 10h (T10; intermediate-sized circle), however, were closer to Factor 1 and Factor 2 axes, indicating a significant relationship with embryo damage and lower hatch rates compared to the control treatment. Damages did not differ between embryo development stages, even when hatch rates were below 15% (stages BD and EP) (Figure 5).



**Figure 4.** Treatment distribution in the factor analysis. The experimental treatments combined the use of pacu fish embryos at four stages of development and cooling for 6h (light gray) or 10h (dark gray) at  $-8^{\circ}\text{C}$ ; control treatments (black) were not cooled. Embryo stages were blastoderm (BD), 25% epiboly (EP), blastopore closure (BL), and optic vesicle formation (OP)



**Figure 5.** Damage to *P. mesopotamicus* embryos: A. Blastoderm (BD) stage after 6h storage; B. blastoderm (BD) stage after 10h storage; C. Epiboly (EP) stages after 6h storage; and D. Epiboly (EP) stages after 10h storage, at  $-8^{\circ}\text{C}$ , respectively

## DISCUSSION

The main goal of cooling protocols is to prolong embryo life by storing it at low temperatures, thereby achieving a survival rate close to that obtained for non-cooled embryos.

Under normal conditions, *P. mesopotamicus* larvae hatch between 15h at 30°C and 18h at 27°C (Urbinati; Gonçalves 2010). Therefore, storing viable *P. mesopotamicus* embryos for more than 10h (more than half the ordinary incubation time) produced encouraging hatch rates of over 50%. However, this was obtained only for embryos at the two most advanced stages of development: blastopore closure (BL) and optic vesicle formation (OP). According to Calvi and Maise (1998), reduced cell size after cleavage of more developed embryos is decisive in improving cryopreservation of fish embryo, corroborating observations obtained in the present study for *P. mesopotamicus*.

Evaluating and determining the nature of damage caused to embryos by exposure to low temperatures is important in developing cell protection protocols. However, *P. mesopotamicus* embryos exhibited a similar damage pattern, irrespective of their stage of development and cooling time (6h to 10h). However, different types of damage were observed, the most common related to embryo uniformity, cell adhesion, symmetry and margin irregularity, corroborating Shields et al. (1997) study on the relationship between embryonic cell abnormalities (blastoderm stage) and fish embryo survival.

Cryopreservation can severely compromise embryo organization because ice crystals are formed at around -15°C even in the presence of cryoprotectants (Hagedorn et al. 2004), damaging cell membranes (Dobrinsky 2002). Cryopreservation can also cause ultrastructural modifications in *P. mesopotamicus* embryos, as observed by Neves et al. (2014) in embryos thawed after storage below -33°C.

Although the conditions used in the present study (use of cryoprotectant solution and temperatures above -8°C) were not suitable for crystal formation, the consequences of exposure to negative temperatures, characterizing embryo damage from cooling, can be clearly observed at the end of storage time. Promising results, about 50% of hatching rate to more advanced stages of development has been observed in embryos cooling pacu temperature when used around -8°C (Lopes et al. 2011). Likewise, in this study were found hatching rates above 50%, at stages BL and OP with 6h and 6 and 10h storage at -8°C, respectively. However, provided that conditions for no formation of ice crystals (presence of cryoprotectant solution at nontoxic concentrations and storage temperature of at least -8°C), these hatching rates were lower than controls for all stages studied (above 80%). Thus, the consequences of exposure to freezing temperatures can be clearly observed after the end of storage period, featuring turn on injuries caused by cooling of embryos.

## CONCLUSION

*P. mesopotamicus* embryos can be stored at -8°C for up to 10h, provided that they are at advanced stages of development, such as blastopore closure (BL) up to 6h, and optic vesicle formation (OP) up to 10h. The present study could not determine which combination between stage of development and cooling period had the greatest affect on embryos, using the procedure of electron microscopy for morphological evaluation. However, other techniques, such as transmission electron microscopy, analysis of expression of genes related to embryonic development, can be employed to assist in this evaluation and to refine further results. The

information obtained in present study can support future studies and contribute to developing cooling protocols that avoid embryo damage from low temperatures and cryopreservation assays.

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