Notas Científicas

Viability of dourado embryos cooled in different cryoprotectant solutions

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Abstract – The objective of this work was to evaluate the effect of different cryoprotectants on the viability of dourado (*Salminus brasiliensis*) embryos. Ten cryoprotectant solutions were tested. For each solution, 300 embryos were selected at the closing of the blastopore stage, and 300 more embryos were used as a negative control. After cooling (-8°C for 6 hours), the embryos were rehydrated directly in the incubator until hatching. The best result is obtained with the cryoprotectant solution containing 9% methanol associated with 17% sucrose, resulting in a larvae hatching rate of 67.06%.

Index terms: *Salminus brasiliensis*, cryopreservation, fish embryo, embryo preservation, larvae eclosion.

Viabilidade de embriões de dourado refrigerados em diferentes soluções crioprotetoras

Resumo – O objetivo deste trabalho foi avaliar o efeito de diferentes crioprotetores na viabilidade de embriões de dourado (*Salminus brasiliensis*). Foram testadas dez soluções crioprotetoras. Para cada solução, 300 embriões foram selecionados na fase do fechamento do blastóporo, e mais 300 embriões foram utilizados como controle negativo. Após o resfriamento (-8°C por 6 horas), os embriões foram reidratados diretamente na incubadora até a eclosão. O melhor resultado é obtido com solução crioprotetora com 9% de metanol associado a 17% de sacarose, o que resultou em taxa de eclosão de larvas de 67,06%.

Termos para indexação: *Salminus brasiliensis*, criopreservação, embrião de peixe, preservação de embriões, eclosão de larvas.

Cryopreservation of fish embryos has been studied for several years (Neves et al., 2012), but this biotechnology is still not controlled. Despite that, efforts during the last decade have resulted in some progress, mainly on cooling techniques (Fornari et al., 2011).

For mammals, the embryo freezing technology is successfully used and has brought great advances in animal production (Kuleshova & Lopata, 2002). However, for fish embryos, the viability of these techniques depends on characteristics such as cold sensitivity, cell membrane permeability, and amount of vitellus (Valdez Junior et al., 2005). According to Fornari et al. (2012), these traits can vary among species. The most studied methods to overcome those challenges are freezing (through cooling curves), vitrification (ultrafast cooling), and cooling itself, which consists on preserving the embryos for a short period, to prolong their development (Fornari et al., 2010).

The preservation of fish embryos through cooling requires safe transportation of embryos from remote facilities until incubation, the optimization of incubators, lab facilities, and transportation logistics in order to avoid large volumes of water and oxygen from affecting the synchronization of embryos in different developmental stages and to prevent pathogen transmission to other environments (Valdez Junior at al.,

2005). Moreover, the development of this technology may contribute to understanding embryo resistance to low temperatures and cryoprotectant efficiency (Zhang et al., 2003), which will allow to determine the best moment to submit the embryos to freezing, or "seeding", and to preserve them in a quiescence state. This knowledge is important for advances in fish production as well as for the preservation of endangered species (Diwan et al., 2010; Fornari et al., 2010).

In Brazil, fish production has increased over the last few years, and some native species have been attracting the attention of Brazilian fish farming. The dourado (*Salminus brasiliensis*) is one of the species with great economical potential. This species reproduces from October to January, does not show parental care, has a fish-based diet, and can migrate over distances of up to 1,000 km (Weingartner & Zaniboni Filho, 2005).

The technique of cooling fish embryos has showed good results when associated to intracellular (dimethyl sulfoxide, ethylene glycol, and methanol) and extracellular (sucrose and glucose) cryoprotectants, being those substances indispensable to preserve embryos bellow 0°C (Zhang et al., 2001; Ahammad et al., 2003; Fornari et al., 2011). For some Neotropical species, such as pacu (*Piaractus mesopotamicus*) (Streit Junior et al., 2007) and black catfish (*Rhinelepis aspera*) (Fornari et al., 2011), the best results were obtained with sucrose associated with methanol during cooling to -8°C. Zhang et al. (2003) suggest cooling of zebra fish (*Danio rerio*) embryos with 0.5 mol L^{-1} solutions in methanol, whereas Ahammad et al. (2003) recommend methanol associated with sucrose for cooling common carp (*Cyprinus carpio*) embryos. Some studies suggest cooling of fish embryos at the blastopore closing stage, when they are more resistant to cold (Lopes et al., 2011).

The objective of this work was to evaluate the effect of different cryoprotectants on the viability of dourado embryos.

The experiment was carried out at the hydrology and aquaculture station of Duke Energy Brasil, in the city of Salto Grande, SP, Brazil, during the reproductive season of migratory fishes in 2008.

To obtain embryos, ten females and ten males with an average of 4 to 2 kg, respectively, were selected according to external reproductive characteristics: females with a soft and arched abdomen, and a reddish urogenital papilla; and males releasing semen with a gentle abdominal pressure. Fishes were hormonally inducted for spawning with carp pituitary extract. After spawning, embryos were placed in conic incubators (7 L).

After blastopore closing, about 6 hours after fertilization, 3,000 embryos were selected and divided into groups of 100 embryos. Embryos were collected using Pasteur pipettes, immersed in a Petri dish with water, and subjected to ten solutions with different cryoprotectants: sucrose and glucose (extracellular), and methanol, dimethyl sulfoxide, and ethylene glycol (intracellular) (Table 1). Cryoprotectant concentrations were based on the cooling protocol suggested by Streit Junior et al. (2007) for pacu. Solutions (6 mL) and eggs were placed in glass tubes. Three groups of 100 embryos were directly transferred to the incubator as a negative control group, without cryoprotectants, in a solution containing only water.

The slow cooling method was used, according to Ahammad et al. (2003). Tubes containing embryos in solutions were placed in water at 15ºC during 10 min, then transferred into water at 5°C for an additional 10 min, and placed in a refrigerator at -8°C for 6 hours. For rehydration, tubes were kept closed and placed in incubators. After 2 min, tubes were opened and embryos were released into water at environmental temperature (27±0.8°C) until eclosion. When eclosion was detected, larvae and failed eggs were removed to determine the eclosion rate.

A random design with ten treatments (solutions) and three replicates was used. Data were submitted to the chi-square test, at 5% probability, using the Table procedure of the SAS software.

Table 1. Composition of cryoprotectant solutions used on the cooling process of dourado (*Salminus brasiliensis*) embryos.

Solution	Cryoprotectants (%)					Water
	Sucrose	Glucose	Methanol	DMSO[(1)]	EG[(2)]	(%)
1	17.1	-	9	-	-	73.9
2	17.1	-	-	9	-	73.9
3	8.5	8.5	9	-	-	73.9
4	8.5	8.5	-	9	-	73.9
5	17.1	-	-	-	9	73.9
6	8.5	8.5	-	-	9	73.9
7	17.1	-	4.5	4.5	-	73.9
8	17.1	-	4.5	-	4.5	73.9
9	17.1	-	-	4.5	4.5	73.9
10	-	-	-	-	-	100

[(1)]Dimethyl sulfoxide. [(2)]Ethylene glycol.

Embryos not subjected to cooling or cryoprotectants showed a hatching rate of 79.01%, indicating good viability of the selected embryos. The embryos cooled without cryoprotectants also had a very low level of outbreak, only 0.5% (Table 2), a result of the cell damage caused by cold. These changes are related to structural and functional damages to the layer of phospholipids on the cell membrane when exposed to low temperatures (Farkas et al., 2001).

However, the use of cryoprotectants was still efficient. The combination of sucrose and methanol proved to be the best combination tested, with hatching rate of 67.06%. A solution containing a mixture of sucrose, glucose, and methanol was also effective, allowing the appearance of 55.75% of larvae. Other combinations of cryoprotectants do not appear to be effective against cold damage to cells, with very low rates of hatching or no hatching at all (Table 2).

In general, the hatching rate was higher when only methanol was used as the intracellular cryoprotectant than when ethylene glycol and dimethyl sulfoxide were used alone or in combination. Furthermore, when methanol was combined with dimethyl sulfoxide, the results were better than when no methanol was included in the solution. The efficiency of methanol as an intracellular cryoprotectant is well documented in other species. Pacu embryos were kept for 6 hours at -8°C in a solution of 10% methanol in a study carried out by Streit Junior et al. (2007). Ahammad et al. (2003) observed a hatching rate of 48% in rohu (*Labeu rohita*) embryos cooled to -4°C in 9.6% methanol solution. The effectiveness of cold methanol to prevent cell damage was also observed by Zhang et al. (2003) when evaluating zebra fish embryos. The authors attributed this to methanol protection capacity, which maintained the physical-chemical integrity of the phospholipid layer in the embryo cell membrane.

When the embryos were cooled in ethanol-particle solution containing ethylene glycol combined with dimethyl sulfoxide, the hatching rate was very low, respectively, 6.04 and 4.93%. However, when only dimethyl sulfoxide or ethylene glycol was present in the solution no larvae emergence was observed, which may indicate the toxicity of these substances to the embryo. Streit Junior et al. (2007) also reported no larvae hatching from chilled pacu embryos using ethylene glycol as an intracellular cryoprotectant.

In this experiment, the addition of glucose to methanol/sucrose solution decreased the hatching rate significantly (Table 2). Streit Junior et al. (2007) also found a decrease in the hatching rate of pacu when glucose was combined in a methanol/sucrose solution. Ahammad et al. (1998) preserved carp embryos at -6°C using a solution of methanol/sucrose and observed good results for Indian major carp (*Cirrhinus mrigala*), but not for other species, which was attributed to genetic effects.

The best efficiency of sucrose, compared with glucose, as an extracellular cryoprotectant can be explained by the complexity of sugar. An oligosaccharide would have an additional protection compared to simple sugars, promoting better dewatering of embryos and preventing the formation of ice crystals within the cell (Lopes et al., 2011). Zhang et al. (2001) found that sucrose contributed to the stability of the cell membrane, especially in reducing the mechanical deformation caused by the entry and exit of water from intracellular cryoprotectants. Fornari et al. (2012) also described the effectiveness of sucrose to maintain osmotic pressure, despite the water flow and the use of internal cryoprotectants.

The results obtained in the present study with solutions of methanol/sucrose are similar to those found for other fish species, such as zebra fish (Zhang et al., 2001), carp (Ahammad et al., 1998), pacu (Streit Junior et al., 2007), and black catfish (Fornari et al., 2011).

Table 2. Eclosion rate of dourado (*Salminus brasiliensis*) larvae from embryos cooled at 8°C for 6 hours in different cryoprotectant solutions[(1)].

Cryoprotectant solution	Eclosion rate (%)	Standard error
1) 17.1% Suc + 9.0% Met	67.06a	2.60
2) 17.1% Suc + 9.0% DMSO	1.35d	2.34
3) 8.5% Suc + 8.5% Glu + 9.0% Met	55.75b	5.71
4) 8.5% Suc + 8.5% Glu + 9.0% DMSO	0	0
5) 17.1% Suc + 9.0% EG	0	0
6) 8.5% Suc + 8.5% Glu + 9.0% EG	0	0
7) 17.0% Suc + 4.5% Met + 4.5% DMSO	6.04c	4.46
8) 17.0% Suc + 4.5% Met + 4.5% EG	4.93c	8.55
9) 17.0% Suc + 4.5% DMSO + 4.5% EG	0	0
10) 100% water	0.50d	0.87

[(1)]Means followed by equal letters, in the columns, do not differ by the chi-square test, at 5% probability. Values are means of three replicates. Suc, sucrose; Glu, glucose; Met, methanol; DMSO, dimethyl sulfoxide; EG, ethylene glycol.

It is possible to preserve viable embryos of dourado (*Salminus brasiliensis*) for 6 hours at -8°C in cryoprotectant solution. The solution combining methanol (9%) with sucrose (17%) was an effective cryoprotectant for this species. The evaluated cryoprotectants have potential for future studies of fish embryo cryopreservation.

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