Toxicity of cryoprotectants on *Prochilodus lineatus* (Valenciennes, 1837) (curimba) embryos in an experimental incubator (Characiformes: Prochilodontidae)

Daniella A. J. Paula[1], Mônica R. F. Machado[2], Luis D. S. Murgas[3], Denílson Rabelo[4], Márcio G. Zangerônimo[3], Rafael V. R. Neto[5], Estefânia S. Andrade[3] and Eliane A. Andrade[3]

This paper investigated the effect of cryoprotectant substances on *Prochilodus lineatus* embryos in an experimental incubator. The prospective study applied combinations of polyvinyl alcohol, hydroxyethyl cellulose, gelatin and fetal bovine serum with dimethyl sulfoxide and ethylene glycol in a new experimental incubator. The morphology of embryos, larval viability and the efficiency of experimental incubators in maintaining the quality of embryos were evaluated. This study demonstrates the efficient association between hydroxyethylcellulose and dimethyl sulfoxide as greater viability ($p<0.05$) was found for embryos ($72.9 \pm 23.9\%$). It should also be noted the permeation of cryoprotectants in embryos through the changes found in chorion diameter, embryo diameter and embryo volume comparing the treatments versus control group (water) ($p<0.05$), this results can help in future cryopreservation protocols. Although the temperature and oxygenation differed between the usual and experimental incubators ($p<0.05$), the results showed a high fertilization rate ($79.6 \pm 13.2\%$) for experimental incubators ($p<0.05$) which is sufficient for the maintenance of embryos in a cryoprotective environment and effectively allows experimentation for long periods with cryoprotectant substances. Cryopreservation of fish embryos has not been accomplished yet and new approaches are required for understanding the permeability of teleost embryos, especially in Brazilian native species.

Este trabalho avaliou o efeito de substâncias crioprotetoras sobre embriões de *Prochilodus lineatus* em uma incubadora experimental. O estudo aplicou combinações de álcool polivinílico, hidroxietilcelulose, gelatina e soro fetal bovino com dimetilsulfóxido e etilenoglicol em uma nova incubadora experimental. Foram avaliadas a morfologia dos embriões, a viabilidade larval e a eficiência das incubadoras experimentais na manutenção da qualidade dos embriões. Este estudo demonstra a associação eficiente entre hidroxietilcelulose e dimetilsulfóxido pela maior viabilidade ($p<0,05$) encontrada para os embriões ($72,9 \pm 23,9\%$). Deve-se notar também a permeação dos crioprotetores nos embriões através das alterações encontradas no diâmetro córion, diâmetro do embrião e no volume do embrião comparando os tratamentos ao grupo controle (água) ($p<0,05$), estes resultados podem ajudar em futuros protocolos de criopreservação. Embora a temperatura e a oxigenação diferiram entre as incubadoras comuns e as experimentais ($p<0,05$), os resultados mostraram elevada taxa de fertilização ($79,6 \pm 13,2\%$) para incubadoras experimentais ($p<0,05$), o que é suficiente para a manutenção de embriões em ambiente crioprotetor e permite efetivamente a experimentação por longos períodos com substâncias crioprotetoras. A criopreservação de embriões de peixes ainda não foi realizada e novas abordagens são necessárias para a compreensão da permeabilidade dos embriões de teleósteos, especialmente em espécies nativas brasileiras.

Key words: Fertilization, Freshwater fish, Morphology, Reproduction.

[1]Universidade Federal de Lavras, Departamento de Zootecnia, Campus Universitário, 37200-000 Lavras, MG, Brazil. daniufla2002@yahoo.com.br (DAJP)
[2]Pontifícia Universidade Católica de Goiás, Av. Universitária, 7460-5010 Goiânia, GO, Brazil. monicavet_2@hotmail.com (MRFM)
[3]Universidade Federal de Lavras, Departamento de Medicina Veterinária, Campus Universitário, Centro, 37200-000 Lavras, MG, Brazil. lsmurgas@ufla.br (LDSM), zangeronimo@ufla.br (MGZ), esandrade@bol.com.br (ESA), eandrade70@yahoo.com.br (EAA)
[4]Universidade Federal de Goiás, Instituto de Química, IQII, 74001-970 Goiânia, GO, Brazil. rabelo.denilson@gmail.com (DR)
[5]Universidade Estadual Paulista, Campus Experimental de Registro, Nelson Brihi Badur, 11900-000 Registro, SP, Brazil. rafaneto2003@yahoo.com.br (RVRN)

Introduction

Piracema fish comprise a significant proportion of the Brazilian fish fauna and their reproduction occurs once a year. They would not only benefit from programs to recover rivers, but also programmes for the reproduction of these species in captivity by artificial propagation and reintroduction (Murgas *et al.,* 1997; Viveiros *et al.,* 2010a). Cryopreservation of embryos is one alternative for the conservation of fish species as this technique allows maintenance of cells in liquid nitrogen for long periods and preserves genetic material.

The curimba (*Prochilodus lineatus*) is a migratory native species of South America (Viveiros *et al.,* 2009) used in commercial polycultures and for fishing subsistence or commercialization (Maduenho & Martinez, 2008). Furthermore, it is used as a model in research in the reproductive biotechnology of Brazilian fish (Viveiros *et al.,* 2010b), thanks to its high prolificacy and ease of management. As a migratory species, curimba needs to swim upriver to spawn and this cycle has been drastically affected (Paula *et al.*, 2012). Cryopreservation is a biotechnology for its conservation and a tool for assisted reproduction on a large scale.

However, this technique does not constitute an effective protocol, mainly because of the difficulties in developing substances that penetrate the chorionic sac and protect the embryo from formation of intracellular ice crystals during the procedure (Kusuda *et al.*, 2002; Ninhaus-Silveira *et al.*, 2009). Although Hagedorn *et al.,* (1996) observed in images by magnetic resonance that methanol permeated the entire zebrafish embryo within 15 min and propylene glycol and dimethyl sulfoxide exhibited little or no penetration over 2.5 h and considered that the yolk syncytial layer may be selectively permeable to specific charges or conformations.

Various cryoprotectant solutions have been used in different protocols with different results. For zebrafish (*Brachydanio rerio*), 2M methanol proved to be more effective and less toxic (Zhang *et al.*, 1993). For curimba, 1.5 M of dimethyl sulfoxide and propylene glycol proved to be less toxic, promoting the highest percentage of survival (78.95%) after toxicity assays, but no embryos survived after cryopreservation (Ninhaus-Silveira *et al.,* 2009).

Sucrose and methanol were tested in cryopreservation of pacu (*Piaractus mesopotamicus*), solutions containing 8.5%, 17%, 25.5% and 34% of sucrose associated with 9% methanol determined a reduced level of injury on morphological structures of fish embryos, although no embryo survived after freezing (Fornari *et al.,* 2011). The use of 10% methanol associated with 0.5M sucrose resulted in 49% of larvae survival, when cooling of embryos was performed after blastopore closure (Lopes *et al.,* 2011).

Substances such as polyvinyl alcohol (Naitana *et al.,* 1997; Wowk *et al.,* 2000; Checura & Seidel 2007) and fetal bovine serum (Vajta *et al.,* 1999) have been successfully tested in freezing embryos of mice, sheep and cattle.

According Nowshari & Brem (2000) polyvinyl alcohol appears to be a suitable replacement for biologically derived macromolecules like fetal bovine serum and eliminates the chance of contamination with infectious agents; it can be substituted for fetal bovine serum in medium for freezing mouse embryos. In addition, substances such as hydroxyethyl cellulose and gelatin have not been tested on embryos of any species, and present the characteristics of cryogels with complex structures, having porous compartments which originate from gelation of concentrated aqueous solution (Shapiro & Shapiro, 1999).

If fish embryos have multi-compartmentalized structures the exchange of water/cryoprotectant is difficult and during their early stages they are more permeable (Martínez-Páramo *et al.,* 2008; 2009). The exposure time to cryoprotectant on the protocols, the fresh embryos are typically exposed for only few minutes, at ambient temperature, after blastopore closure (8 h post-fertilization) and cooled (Lopes *et al.,* 2011). New models of incubators could allow the maintenance of embryos in a cryoprotective environment, combined with a suitable environment for embryo development.

Thus the objective of the study was to investigate the effect of cryoprotectant substances on curimba embryos in an experimental incubator, allowing a longer period of exposure to the external cryoprotectants polyvinyl alcohol, hydroxyethyl cellulose, gelatin and fetal bovine serum in association with the internal cryoprotectant ethylene glycol or dimethyl sulfoxide.

Material and Methods

Animals and incubation. The experiment took place from December 2010 to February 2011 at the Estação Ambiental de Itutinga da Companhia Energética de Minas Gerais (Itutinga Environmental Station of the Companhia Energética de Minas Gerais) (EAI-CEMIG), in the city of Itutinga, Minas Gerais, Brazil. Six pairs of wild curimba (mean weight females: 1191 ± 496 g and mean weight males: 675 ± 296 g), kept in inland hatcheries in the station for a maximum of three years, were used. They were ready to reproduce, and were selected by abdominal massage to check the release of sperm in males and evaluation of the urogenital papillae in females (prominent with pinkish or reddish coloration and genital orifice slightly open). During the breeding season the females have a bulging belly and are removed from water for visualization of the papillae. If the papillae are reddish in color the fish is selected for application of hormones.

The specimens were sent to masonry aquariums and received hormonal application of crude carp pituitary extract (Argent Chemical Laboratory, Redmond, WA, USA). The initial and final doses of hormone given were 0.5 and 5 mg/kg of fish and 0.4 and 4 mg/kg of fish, for females and males respectively. The application of the hormone was done intramuscularly, with a syringe of 1 mL and near the

base of the dorsal fin, at intervals of 12 h (Murgas *et al.,* 1997; Ninhaus-Silveira *et al.,* 2006).

Twenty-four hours after the initial dose, the urogenital papillae were cleaned and dried with paper towels to prevent contamination and activation of gametes with water, urine or faeces. Then hand massage of the coelomic wall was performed gently in cranial-caudal orientation to promote the release of gametes (spawning 214 ± 66.61 g; sperm: 2.83 ± 1.72 mL).

The fertilization of gametes was performed with the addition of water from the tank itself. The amount 6 ± 1 g of embryos from each curimba couple were weighed and then taken to incubators containing the experimental treatments in section "Effect of cryoprotectant" where they remained for 8 h.

The experimental incubators were made of polyvinyl chloride tubes (PVC) (6 cm in diameter, 10 cm high and with 400 mL capacity) and these were arranged on a wooden support compatible with the pipe size. The incubators were supplied with oxygen through a tank aeration sponge, centered on the PVC pipe and connected to oxygen inhalers (Aquarium Air Pump, Power$_{TM}$ 100) through hoses suitable for aquariums (Fig. 1). The incubator system was a closed system without recirculation of water but with constant aeration, thus maintaining the concentration of cryoprotective agents and was compared the funnel incubator.

Fig 1. Representative image of the experimental incubators.

The Núcleo de Inovação Tecnológica, NINTEC/ UFLA, conducted prior searches of the national banks and international patents to validate the uniqueness of the technology entitled 'Sistema de Incubação Experimental para Embriões de Peixe' ('Experimental System for Incubation of Fish Embryos') and subsequently applied for a patent under the provisions of Law 9.279 of 14 May 1996, which describes the rights and obligations relating to industrial property, the technology attended the necessary prerequisites for patenting. The technology was deposited at INPI (National Institute of Industrial Property), on 12 July 2013, as nº BR 10 2013 017858 6, and will remain there

for the requisite 18 months. A copy of the filed process was archived at NINTEC/UFLA for purposes of evidence.

The temperature and oxygen in the experimental incubators were measured every hour for 10 h. Type funnel incubators (200 L) are commonly used in commercial fish farms and were also checked and used as control. They are made of glass fibre, tubular, in support tripod type, with constant circulation of water at 26.98 ± 1.05 °C during experiment.

The fertilization rate of both types of incubator was measured approximately 8 h after fertilization of gametes in terms of the relationship between the number of good embryos and overall embryos counted in a sample (200 embryos), multiplied by 100. Good embryos constituted transparent embryos with development in 90% of epibolia phase. Opaque embryos could not permit to identify their phase, consequently they were deemed dead and were not considered any further.

Subsequent to exposure time (8 h), the embryos were transferred to commonly used 1L incubators (Felizardo *et al.,* 2010) in a masonry tank with constant water circulation and oxygenation to complete their development. After hatching, the larvae were evaluated in a stereomicroscope, and the total number of viable larvae was counted (i.e. those which had hatched and were not deformed).

Effect of cryoprotectant. Four substances, polyvinyl alcohol (PVA), hydroxyethyl cellulose (HEC), gelatin (GEL) in concentrations of 0.5% and 1% and fetal bovine serum (BSF) in concentrations of 4% and 8% were tested and combined with dimethyl sulfoxide (DMSO) and ethylene glycol (EG), both in concentrations of 3%. The substances tested in this study have the potential to be external cryoprotectant by its degradability and form gels. The solutions were added to a medium (160 mL) to develop embryos, E3, composed of 8.6 g NaCl, 0.38 g KCl, 1.46 g of $CaCl_2$ and 2.45 g $MgSO_4$ (Di Prinzio *et al.,* 2010; Haffter *et al.,* 1996). An additional treatment containing only water from the recirculation system was used as control to compare the solutions that were prepared 12 h prior for component stabilization.

Morphometric analysis. During the eight hours while the embryos were kept in cryoprotectants images were captured of three embryos per treatment in 15, 30 min and 60 min. These images were evaluated with the public domain software ImageJ (Schneider *et al.,* 2012). The medium chorion diameter (CD), the medium embryo diameter (ED) and the medium volume in μm were used to measure morphology. The axes were used to determine the volume of all embryos with a prolate spheroid formula ($V=4/3 \pi ab^2$), whereby a and b were the major and minor semi-axes (Hagedorn *et al.,* 1998).

Statistics. The experimental randomized block design (RBD) was used with repeated treatments and six blocks.

The treatments were arranged in a 4x2x2+1 factorial structure (substances, concentrations of substances, internal cryoprotectants and control). To evaluate the effect of the incubators a completely randomized design was adopted with two treatments, experimental and funnel type incubators, with eight replicates.

The results obtained for percentage viable larvae were subjected to nonparametric statistical analysis by the computer package SAS (1999) and, in the case of significant effect, the Kruskal-Wallis test was applied at 5% significance level. ANOVA was conducted for the variables temperature, oxygen and fertilization rate in the incubators and, in the case of significant effect, the means were subjected to the Scott-Knott test. ANOVA was also conducted for morphometric analysis and, in the case of significant effect, the means were subjected to the Dunnett test.

Results

The rate of fertilization did not differ between the incubators (p>0.05). Temperature in the funnel-type incubators was higher (p<0.05) than in experimental incubators and dissolved oxygen in the funnel-type incubators was higher (p<0.05) than in the experimental incubators during the time of evaluation (Table 1).

Table 1. Average temperature (°C), average oxygen (mg/L), average fertilization rate (%) and standard deviation in the experimental and funnel type incubators used in the experiment with *Prochilodus lineatus* embryos (n=6). [a]Averages followed by different letters in the line differ, according to the Scott-Knott test (p<0.05).

Time (Hours)	Temperature (C)[a]		Oxygen (mg/L)[a]	
	Funnel type	Experimental	Funnel type	Experimental
5:30	25.2±0.7a	22.6±2.6b	7.8±0.5a	7.1±0.1b
6:30	24.7±1.1a	23.3±2.1b	8.3±0.8a	7.5±0.2b
7:30	25.3±0.7a	21.7±3.2b	10.7±2.5a	8.8±1.1b
8:30	25.2±0.8a	23.3±2.1b	8.4±0.9a	7.3±0.1b
9:30	26.3±0.0a	24.7±1.1b	8.1±0.7a	7.3±0.1b
10:30	26.5±0.1a	25.5±0.6b	7.7±0.4a	6.2±0.6b
12:30	27.3±0.6a	25.8±0.4b	8.8±1.1a	6.2±0.6b
13:30	26.2±0.0a	26.0±0.2b	8.1±0.6a	6.7±0.2b
14:30	27.3±0.6a	26.4±0.1b	8.1±0.7a	6.2±0.6b
15:30	27.3±0.6a	26.6±0.1b	8.0±0.6a	6.4±0.5b

Fertilization rate (%) NS	
Funnel type	Experimental
88.1±15.8	79.6±13.2

The treatments consisting of 3% DMSO combined with 0.5 and 1.0 % PVA, 0.5% GEL and 1.0% HEC had a higher percentage (p<0.05) of viable larvae in relation to the use of EG for the same combinations, but the HEC 1.0% was better when combined with 3% DMSO. It was observed that during the embryos exposure to HEC they were dehydrated without breaking the membrane. The use of DMSO combined with HEC 1.0% showed similar results (p>0.05) for the percentage of viable larvae compared with the control. On the other hand, it was observed that the other treatments were lower (p<0.05) than for the control group.

When DMSO was used as internal cryoprotectant, the highest (p<0.05) percentage of viable larvae were obtained with combinations of 1.0% HEC compared with the combinations of 0.5% HEC, 1.0% gelatin, BSF 4% and 8% and 0.5% PVA. No differences were observed between the other treatments. With EG, the combination with 1.0% gelatin presented a higher (p<0.05) percentage of viable larvae in relation to BSF 8% and 0.5% PVA. However, no significant differences (p>0.05) were observed between the other treatments (Table 2).

Table 2. Average percentage (%) and standard deviation of viable larvae of *Prochilodus lineatus* subjected to different cryoprotectants. [a,b] Averages followed by different capital letters in the line and small letters in the columns differ, according to the Krustal-Wallis test (p<0.05). PVA: polyvinyl alcohol; GEL: gelatin; HEC: hydroxyethylcellulose; BSF: fetal bovine serum. [1] Differs from control, according to the Krustal-Wallis test (p<0.05).

Solution	Dose	Internal Cryoprotectant	
		DMSO	Ethylene glycol
PVA	0.5%	34.4±29.0 Abc	8.4±18.4 Bbc
	1.0%	52.7±24.0 Aab	11.2±13.1 Babc
Gelatin	0.5%	54.4±37.9 Aab	16.1±25.2 Babc
	1.0%	40.4±26.4 b	39.2±31.8 a
Hydroxyethyl Cellulose	0.5%	46.3±38.9 b	25.4±29.5 ab
	1.0%	72.9[1]±23.9 Aa	29.4±33.7 Bab
BSF	4%	16.1±19.3 c	13.2±32.4 abc
	8%	13.4±21.9 c	0.0 c
Control		83.3 ± 16.59	

The medium chorion diameter (CD) subjected to 15 minutes of cryoprotectants decreased (p<0.05) for DMSO PVA 1.0%, EG PVA 1.0%, DMSO PVA 0.5%, EG GEL 1.0%, EG HEC 1%, EG HEC 0.5%, DMSO BSF 8%, EG BSF 8%, DMSO BSF 4% and EG BSF 4% compared with

the control. The CD subjected to 30 min of cryoprotectants decreased ($p<0.05$) compared with the control for DMSO PVA 0.5%, DMSO BSF 8%, EG BSF 8% and EG BSF 4% and increased ($p<0.05$) compared with the control for all other treatments except for EG GEL 1.0%, DMSO GEL 0.5%, EG GEL 0.5% and DMSO BSF 4%, which did not present significant difference ($p>0.05$). The CD subjected to 60 min of cryoprotectants decreased ($p<0.05$) compared with the control for DMSO PVA 1.0%, EG PVA 1.0%, DMSO PVA 0.5%, EG BSF 8%, DMSO BSF 4% and EG BSF 4% and increased ($p<0.05$) compared with the control for all other treatments except for EG GEL 1.0% and DMSO BSF 8%, which did not present significant difference ($p>0.05$) compared with the control (Table 3).

Table 3. Average chorion diameter (CD) (µm) and standard deviation of *Prochilodus lineatus* embryos subjected to different cryoprotectants for different duration (up to 60 min). DMSO: dimethylsulfoxide; EG: ethylene glycol; PVA: polyvinyl alcohol; GEL: gelatin; HEC: hydroxyethylcellulose; BSF: fetal bovine serum; 8: 8%; 4: 4%. [a]*Differs from control, according to the Dunnett test ($p<0.05$); CV = coefficient of variation.

Treatment	15 min	30 min	60 min
WATER	4031.30±37.01	2078.00±61.50	3555.30±101.10
DMSO PVA 1.0%	3057.30±37.44*	3022.66±58.50*	3039.00±63.37*
EG PVA 1.0%	3058.00±48.49*	3346.00±21.00*	3134.70±10.06*
DMSO PVA 0.5%	3306.00±96.06*	1651.66±29.02*	3117.70±21.59*
EG PVA 0.5%	4118,70±72,14	2295,33±72,14*	4094,70±39,20*
DMSO GEL 1.0%	3913.30±48,08	2363.66±59.24*	3963.70±85.00*
EG GEL 1.0%	3590.70±42.50*	2137.33±24.68	3510.30±44.41
DMSO GEL 0.5%	4020.70±101.00	2127.33±72.23	3623.00±45.39*
EG GEL 0.5%	4041.30±69.00	2095.33±156.01	3775.00±110.72*
DMSO HEC 1.0%	4107.30±48.05	2206.66±69.06*	3766.00±101.37*
EG HEC 1.0%	3708.30±95.51*	2271.33±48.34*	3684.30±17.95*
DMSO HEC 0.5%	3934.70±53.37	2229.33±8.32*	3751.30±51.01*
EG HEC 0.5%	3809.00±164.50*	2375.33±164.50*	4074.70±13.79*
DMSO BSF 8%	3568.00±265.51*	1947.33±67.68*	3523.00±49.66
EG BSF 8%	3621.70±95.51*	1927.33±60.04*	3413.70±153.95*
DMSO BSF 4%	3712.00±16.09*	2066.33±21.03	3406.30±160.12*
EG BSF 4%	3724.00±47.50*	1953.33±13.31*	3400.00±54.36*
CV (%)	2.76	2.70	2.23

The average embryo diameter (ED) subjected to 15 min of cryoprotectants increased ($p<0.05$) compared with the control for all treatments except for DMSO PVA 1.0%, EG PVA 1.0%, DMSO PVA 0.5%, EG GEL 1.0%, EG HEC 0.5%, EG BSF

4%. The ED subjected to 30 min of cryoprotectants increased ($p<0.05$) compared with the control for DMSO PVA 1.0%, EG PVA 1.0%, DMSO GEL 1% and DMSO HEC 0.5%. The embryo diameter (ED) subjected to 60 min of cryoprotectants decreased ($p<0.05$) compared with the control for DMSO PVA 0.5%, EG PVA 0.5%, DMSO GEL 1.0%, EG GEL 1.0%, EG HEC 0.5%, EG HEC 1.0%, DMSO BSF 8%, DMSO BSF 4% and EG BSF 4% and increased ($p<0.05$) compared with the control for EG BSF 8% (Table 4).

Table 4. Average embryo diameter (ED) (µm) and standard deviation of *Prochilodus lineatus* embryos subjected to different cryoprotectants for different duration (up to 60 min). DMSO: dimethylsulfoxide; EG: ethylene glycol; PVA: polyvinyl alcohol; GEL: gelatin; HEC: hydroxyethylcellulose; BSF: fetal bovine serum. [a]*Differs from control, according to the Dunnett test ($p<0.05$); CV = coefficient of variation.

Treatment	15 min	30 min	60 min
WATER	1186.00±37.98	694.33±7.93	1381.70±29.02
DMSO PVA 1.0%	1218.00±22.60	1163.00±35.11*	1348.70±31.34
EG PVA 1.0%	1215.00±29.73	1153.33±15.30*	1308.30±25.50
DMSO PVA 0.5%	1176.70±5.56	669.66±11.50	1230.30±76.03*
EG PVA 0.5%	1284.00±37,87*	724.66±5.50	1197.30±68.79*
DMSO GEL 1.0%	1329.30±18.08*	759.66±28.51*	1232.70±32.41*
EG GEL 1.0%	1194.00±25.57	692.00±61.58	1156.00±100.80*
DMSO GEL 0.5%	1267.70±11.59*	693.67±29.02	1365.30±174.13
EG GEL 0.5%	1285.70±51.59*	701.66±34.00	1361.00±97.68
DMSO HEC 1.0%	1248.70±9.86*	709.33±61.13	1320.00±5.68
EG HEC 1.0%	1238.70±7.76*	685.33±36.29	1047.30±35.57*
DMSO HEC 0.5%	1283.70±22.74*	819.33±3.51*	1311.70±53.50
EG HEC 0.5%	1169.70±37.00	698.33±44.50	1201.00±158.52*
DMSO BSF 8%	1242.00±67.06*	673.00±52.31	1263.00±59.13*
EG BSF 8%	1253.70±15.37*	653.33±9.07	1524.70±65.04*
DMSO BSF 4%	1244.30±66.02*	669.66±18.02	1252.70±77.13*
EG BSF 4%	1172.00±38.43	674.00±23.45	1175.30±63.51*
CV (%)	2.93	4.40	6.34

The medium volume after 15 min of cryoprotectants was different ($p<0.05$) from that of the control for all treatments except for EG GEL 1.0%, DMSO GEL 0.5% and EG GEL 0.5%. The medium volume after 30 min of cryoprotectants was different ($p<0.05$) from that of the control for all treatments except for EG GEL 0.5%. The medium volume after 60 min of cryoprotectants was different ($p<0.05$) from that of the control for all treatments except for EG GEL 1.0%, DMSO GEL 0.5% and DMSO BSF 8% (Table 5).

Table 5. Average volume embryo (mm^3) and standard deviation of *Prochilodus lineatus* embryos subjected to different cryoprotectants for different duration (up to 60 min). DMSO: dimethylsulfoxide; EG: ethylene glycol; PVA: polyvinyl alcohol; GEL: gelatin; HEC: hydroxyethylcellulose; BSF: fetal bovine serum. [a]*Differs from control, according to the Dunnett test (p<0.05); CV = coefficient of variation.

Treatment	15 min	30 min	60 min
WATER	1122.35 ± 6.45	82 ± 69.82	644.85 ± 95.70
DMSO PVA 1.0%	349.6 ± 14.99*	354.9 ± 29.60*	322.1 ± 24.08*
EG PVA 1.0%	355.51 ± 16.51*	535.89± 22.55*	350.31 ± 8.38*
DMSO PVA 0.5%	465.16 ± 2.76*	30.60 ± 48.45*	351.31 ± 48.19*
EG PVA 0.5%	1104.32 ± 9.47*	100.72 ± 30.08*	1112.33 ± 70.93*
DMSO GEL 1.0%	1008.61 ± 10.28*	119.31 ± 78.73*	974.78 ± 102.10*
EG GEL 1.0%	793.54 ± 4.63*	86.56 ± 23.20	618.18 ± 42.99
DMSO GEL 0.5%	1092.90 ± 11.79*	93.13 ± 7.27	691.50 ± 28.93
EG GEL 0.5%	1183.48 ± 25.06	87.51 ± 2.48	815.84 ± 114.40*
DMSO HEC 1.0%	1077.32 ± 42.52*	108.31 ± 10.96*	807.80 ± 95.17*
EG HEC 1.0%	771.87 ± 57.66*	109.16 ± 5.56*	741.96 ± 40.07*
DMSO HEC 0.5%	926.41 ± 35.50*	108.57 ± 8.07*	814.09 ± 35.50*
EG HID 0.5%	846.63 ± 137.54*	118.70 ± 2.87*	1029.91 ± 24.94*
DMSO BSF 8%	641.65 ± 164.61*	62.35 ± 10.56*	633.16 ± 16.71
EG BSF 8%	645.39 ± 91.97*	54.20 ± 8.14*	507.68 ± 109.31*
DMSO BSF 4%	832.34 ± 53.27*	70.65 ± 5.05*	544.20 ± 90.64*
EG BSF 4%	802.30 ± 41.38*	65.74 ± 0.76*	521.39 ± 10.10*
CV (%)	9.70	8.40	9.90

Discussion

Fertilization success is probably one of the earliest estimators that can be used for accurately estimation of egg quality and it is the most integrative estimator of sperm quality. Indeed, the ability to fertilize or be fertilized is one of the key components of gamete quality. Recording of fertilization rates is relatively easy in some species, thanks to the transparency of their eggs (Bobe & Labbé, 2010). Our results show high fertilization rates in both incubator types and can be considered satisfactory in a system of artificial reproduction, as in the case of funnel-type incubators, and an experimental system, as in the case of experimental incubators. The experimental incubators are advantageous because they allow normal development of embryos in solutions with fixed concentrations and require little space.

The fact that temperature in the funnel-type incubators was higher than in experimental incubators may be attributed to the conditions in which the funnel-type incubators are used, as they receive water from a tank with a heating system and constant renewal, which keeps the temperature constant. However, the experimental incubators had no exchange of water or heating system and were controlled by room temperature. The lower temperature oscillation leads to a lower frequency of heat shocks, which can help the overall performance of the incubation of eggs and larvae (Johnston & Vieira, 1996).

The time difference for embryonic development may be directly related to incubation temperature (Saka *et al.,* 2004). This was observed for curimba embryos in average temperatures of 24°C and 28°C, where hatching occurred 22 and 14 h after fertilization, respectively (Ninhaus *et al.*, 2006). As the temperatures were very close to the comfort range, our larvae took less time to hatch, but the incubation maintained the viability of the larvae, which was observed in satisfactory development rates.

According to Rodrigues-Galdino *et al.,* (2009) water temperature is a major determining factor in fish development. It is always advisable to use the optimal temperature for the species. Studies show that acceptable temperature for the incubation of eggs of tropical fish varies from 22°C to 31°C, and the best temperature is 27°C (Johnston & Vieira, 1996). All temperatures in both incubators were around this comfort range for most of the evaluated period.

The dissolved oxygen in the funnel-type incubators was higher than in the experimental incubators during the time of evaluation, justified by the conditions in which the funnel-type incubators are used, as they have constant water renewal and oxygenation. However, this difference in oxygen concentration did not affect embryo viability. Studies show that oxygen consumption by the eggs is very low in the early stages of development, and accelerates gradually (Strecker *et al.,* 2011). The oxygen demand of the developing embryo increases dramatically with temperature, i.e., dissolved oxygen should not be separated from other factors synchronous with temperature, pH, and altitude, among others (Barroso & Santos, 2006).

The DMSO is a widely used cryoprotectant during the cooling processes, both for the cryopreservation/ vitrification of semen and embryos and this cryoprotectant has as advantage the rapid permeation into the membrane (Hagedorn *et al.,* 1997a, b, 1998; Zhang & Rawson 1996). In our study the DMSO showed lower toxicity during incubation of the embryo for longer periods compared with ethylene glycol, being the cryoprotectant suggested for these conditions. However, the concentration and the cryoprotectant entering the yolk/YSL region should be widely studied. Ultra structural experiments by Hagedorn *et al.,* (1998) suggested that cryodamage led to the death of the embryos in part because of insufficient cryoprotectant entering the yolk/YSL region.

The fact that the internal cryoprotectant 3% EG showed a lower percentage of viable larvae in relation to

the 3% DMSO, combined with 0.5 and 1.0% PVA, 1.0% HEC and 0.5% GEL, can be related to the detrimental effect of the cryoprotectant, because it may interfere with the metabolism of the embryo to cause cell disruption and consequently death (Streit Jr *et al.,* 2007). These results are in agreement with those obtained by Hagedorn *et al.,* (1997b), who observed that the exposure of three-somite embryos to 1.5 M glycerol or ethylene glycol was deleterious for zebrafish, whereas ethylene glycol caused the blastoderm to dissociate from the yolk within 60 minutes.

The fact that HEC determines higher rates of embryo viability shows that this polymer could be a future means of cryopreservation, mainly because it has the viscosity of the various products that are used and a low freezing temperature, according to the manufacturer. From the results of the present work, we can assume that this substance was less toxic to the embryos. Our work is probably the first to describe the use of this substance during the embryonic development in this species. The HEC is a natural polysaccharide with chemical modifications and the beneficial effect of this compound is probably owed to its properties of non-toxicity, biocompatibility and biodegradability (Peng & Chen, 2010). Dehydration was observed for the embryos of EG HEC 1.0% and EG HEC 0.5% and did not observe to other treatments during the first 15 min, which may be because of the action of internal cryoprotectants, and after this period, the embryos were hydrated in all treatments after 30 and 60 min of exposure. However, hydration can be a barrier to cryopreservation whereas dehydration is expected to prevent formation of intracellular ice.

On the other hand, the ED of the embryos exposed to certain combinations of HEC increased during the first 15 min. Similar results were observed by Lahnsteiner (2008) for zebrafish in that the diameter of the yolk increased after 5 min exposure to DMSO and sucrose solutions. Lahnsteiner concluded that this process was coupled with the shrinkage of the perivitelline space and speculated that during shrinkage the egg lost its tension, enabling the yolk to expand, and during further incubation the further increase in the width of the perivitelline space and in the egg tension could have led to recompression of the yolk.

Although the PVA did not show satisfactory results compared with other treatments, this substance may play an important role as an external cryoprotectant, mainly because of its degradability and non-toxic properties (Batyrbekov *et al.,* 1998). The use of cryogels has shown increased viability in smooth muscle cells, when combined with DMSO (Vrana *et al.,* 2011), and there are studies that prove the reduction in ice crystal formation, derived from freezing (Holt, 2003). Our results also show the beneficial effects of these cryoprotectants for freezing, explained by CD and combinations of cryoprotectants internal PVA induced loss of water in the embryo at 15 min (except

EG PVA 0.5%), and this behaviour continued throughout the 30 and 60 min exposure, including the EG PVA 0.5% treatment. Evaluating the ED some of the embryos exposed to combinations of PVA, increased at 15 and 30 min and only two treatments led to dehydration at 60 min (DMSO PVA 0.5% and EG PVA 0.5%). PVA is a biomaterial with potential for biomedical applications and the design of new nanostructured devices; it is water soluble, biocompatible and has physical properties (Paradossi *et al.,* 2003) such as a rubbery nature and a high degree of swelling in water (Nikolaos & Stauffer, 1991)

Gelatin associated with EG resulted in higher rates of viable larvae compared with 8% BSF. Gelatin is a composite of essential and non-essential amino acids that may have contributed to the nutrition of the embryos without being highly toxic. Furthermore, gelatin is used as a delivery vehicle for the controlled release of bioactive molecules (Young *et al.,* 2005) and used for pharmaceutical and medical applicatons because of its biodegrability (Tabata & Ikada, 1998).

The results for CD and ED with gelatin were very variable, but we observed compensation between two measures. Similar results were observed in zebrafish by Lahnsteiner (2008) in that the further increase in the width of the perivitelline space and in the egg tension could have led to recompression of the yolk. BSF is a compound of bovine blood proteins and may have been toxic to curimba embryos, which has not been observed for bovine embryos as it accounts for an increase in their growth (Lonergan *et al.,* 1999).

Zhang & Rawson (1996) measured the volume in the outer chorion to determine the cryoprotectant permeation of intact six-somite and 27h embryos. Importantly, after 30 minutes of exposure, all embryos shrank, except those which received EG GEL 0.5% treatment in our study. Hagerdorn *et al.,* (1998) also observed for zebrafish that embryos shrank over time and did not re-expand while immersed in DMSO or propylene glycol for long periods (i.e., 60 min). Our results show that in the presence of DMSO or EG there is membrane water permeability and this is important to measure, in addition the exposure time, mainly in future protocols for cryopreservation. According to Hagedorn *et al.,* (1997a, b) the efficacy of cryoprotectant permeation and equilibration is crucial for successful cryopreservation procedures.

BSF in association with EG may have caused a change in the homeostasis of embryos, and some authors report a low potential for detoxification of regulatory pathways in early ontogenetic stages since in these phases the embryos are still undeveloped and unable to compensate for the toxic effect of cryoprotectants (Lahnsteiner, 2008). It was observed during this study that use of 4% and 8% fetal bovine serum led to severe dehydration of the embryos, which we believe was deleterious.

BSA and FCS (fetal calf serum or BSF) are commonly used in freezing solutions at 4 mg/mL or 10% respectively

in bovine embryos, and according to Gordon (1994) are widely used in the freezing medium as extracellular cryoprotectants. The study by Pugh *et al.,* (1998) compared the addition of fetal bovine serum and fetal serum albumin (BSA) in a culture medium of bovine embryos and observed that the survival of late morulae/early blastocysts following freezing was improved in the presence of additional BSA but not BSF in vitro. Dehydration of the embryo before freezing would be an interesting approach in the cryopreservation process, thereby avoiding the formation of intracellular ice.

Our results for CD and ED show that BSF matched with internal cryoprotectants dehydrates the embryos during the majority of the exposure time and forces the water out of the embryo and consequently its dehydration. Thus, the use of fetal bovine serum with internal cryoprotectants before freeze drying may cause this result but during a short exposure because for 8h the damage curimba embryos had low survival rates.

The unsuccessful cryopreservation of fish embryos may be related to the lower permeability of the embryonic membranes (Hagedorn *et al.,* 1997b). Thus, embryos exposed to low concentrations of internal cryoprotectant (3% would be least toxic), throughout the period of embryonic development from fertilization to 8 h approximately, when blastopore closure occurs (Botta *et al.,* 2010), might allow for greater input of cryoprotectant in all embryonic compartments.

The combinations of tested cryoprotectants maintained the viability of curimba embryos except for the combination of ethylene glycol and 8% BSF. The use of combined DMSO and 1.0% HEC is suggested as a potential solution for embryo cryopreservation since toxicity is lower. It should also be noted the permeation of cryoprotectants in embryos through the changes in chorion diameter, embryo diameter and embryo volume, this results can help in future cryopreservation protocols. The use of a reduced model of experimental incubators is an alternative for studying the toxic effect of cryopreservation solutions on embryo development for long periods and for maintaining embryos viability.

Acknowledgements

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