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The effects of cryoprotectants on chilled pirapitinga (*Piaractus brachypomus*) embryos at various ontogenetic stages

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Abstract. Cryopreservation has not been successfully used to preserve fish embryos, although chilling techniques have been used with good results. The aim of this study was to chill *Piaractus brachypomus* embryos at different stages of development in some cryoprotectants and for various periods of chilling. Embryos at the following ontogenetic stages were used: blastoderm – 1.2 hours post-fertilization (hpf); epiboly – 5 hpf; blastopore closure – 8 hpf; and appearance of optic vesicle – 13 hpf. One hundred embryos were selected from each of the four stages and chilled in methanol, methylglycol or dimethylsulfoxide (DMSO) for 6, 8, 10 or 12 hours, at 2ºC. The total number of treatments was 4 stages x 3 cryoprotectants x 4 periods of chilling. The highest percentage of normal and live larvae (30.6%) was observed when embryos were chilled at 13-hpf in methanol for 6 hours. In general, larvae chilled at a more developed stages (8 and 13 hpf), in methanol and for shorter periods could survive chilling and develop normally, compared to the other treatments. Therefore, *P. brachypomus* embryos at the optical vesicle appearance stage (13 hpf) should be chilled in a solution containing 17.5% glucose and 10% methanol for up to eight six at 2°C.

Key words: Aquaculture, South American fish, embryonic stage, Larval survival

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Introduction

Cryopreservation techniques have been successfully used since the 1970s to preserve the embryos of several species of mammals (DOBRINSKY, 2002) and marine invertebrates, including shellfish (CHAO et al.1997), sea urchins (ASAHINA & TAKAHASHI, 1979) and polychaetes (OLIVE & WANG, 1997). Several studies on the cryopreservation of zebrafish embryos have also been performed (LIU et al.1998; HAGEDORN et al.2004). With the rapid growth of marine aquaculture, several attempts to cryopreserve other types of fish embryos have been carried out in recent years, most notably for species with large commercial value, such as *Paralichthys olivaceus* (CHEN & TIAN, 2005; ZHANG & RAWSON, 2003), *Scophthalmus maximus* (CABRITA et al.2003; ROBLES et al.2003), *Sparus aurata* (BEIRAO et al.2006; CABRITA et al.2006) and *Pagrus major* (DING et al.2007). These

efforts, however, have yielded relatively low hatching and larval survival rates. Although efficient cryopreservation techniques for fish embryos are still lacking, chilling techniques are already being used. Recent studies have shown that *Piaractus mesopotamicus* embryos can be stored at low temperatures (-8ºC) without significantly decreasing the hatching rates (LOPES et al.2011; LOPES et al.2012; STREIT Jr et al.2007).

The aforementioned chilling techniques require embryos to be placed in a cryoprotectant solution and stored at low temperatures. Studies concerning stage-dependent chilling sensitivity of fish embryos showed that post-gastrulation stage is the least sensitive to chilling injury (DINNYES et al.1998; HAGEDORN et al.1997; LIU et al. 1993). It was found that 24-h stage of Common carp (*C. carpio* L.) embryos were stored at 4 to -2 ºC in a medium containing 2.0 M methanol and 0.1 M trehalose for a period of 14 days (AHAMMAD et al.2002). Recent studies have evaluated both chilling protocols and cryoprotectant toxicity in *Prochilodus lineatus* (COSTA *et al.*2012). However, literature on the embryo chilling of freshwater tropical fish species remains scarce.

Pirapitinga (*Piaractus brachypomus*) is native to the Amazon and Araguaia-Tocantins basins, and it can grow to 80 cm in length and weigh up to 20 kg. This fish has scales and feeds primarily on fruits and aquatic plants, although it will also eat smaller fish. *P. brachypomus* is a very important commercial species, both as an ornamental fish and for human consumption (VAL & VAL-ALMEIDA, 1995).

Studies on the characterization and cryopreservation of *P. brachypomus* sperm were conducted by

Nascimento et al. (2010), but their embryo-chilling protocols are currently unavailable.

The aim of this study was to chill *Piaractus brachypomus* embryos at 2ºC for various periods of chilling. Because the post-gastrulation stage is critical for low temperature storage the embryos were stored in a glucose solution combined with cryoprotectants.

Materials and Methods

Embryo collection

This experiment was conducted between December 2011 and April 2012 at the Aquaculture Research Center (Centro de Pesquisas em Aquicultura – CPAq) of DNOCS in Pentecoste, Ceará, which is located at 03º45'00"S and 039º10'24"W and is 82 km from the state capital Fortaleza.

Fish were maintained in irtrearing ponds with a surface area of 350 m^2. Twenty males and 16 females of *P. brachypomum* with an average age of >3 years were tagged with chips to avoid data loss.

P. brachypomum viable embryos were collected from a pool of eggs originated from eight breeding pairs. Females were induced with two intramuscular injections of 0.5 and 5.0 mg-kg crude carp pituitary extract (cPE; Danúbio Aquacultura, Blumenau, Santa Catarina), within a 12-hour interval. Males were induced with a single dose of cPE at 1.0 mg kg^{-1}. The gametes were released after 280 hours-degree, and the recently fertilized eggs were transferred to 18-L conical incubators in a flow-through system. Embryo development was monitored until larval hatching, and the water temperature was maintained at 28±1°C.

Control Embryos

The control group was used to determine the influence of egg-selection and manipulation at the different ontogenetic stages. The control group

was composed of four subgroups (n = 100 viable embryos), each representing one of the following stages (LOPES et al., 2011): Control 1: blastoderm – 1.2 hours post-fertilization (hpf) (~64 cells); Control 2: epiboly – 5 hpf (25% epiboly); Control 3: blastopore closure – 8 hpf (90% epiboly); and Control 4: appearance of optic vesicle – 13 hpf. (Figure 1). Control embryos were incubated without the addition of cryoprotectants or chilling in 3-L incubators in a flow-through system until larval hatching (approximately 18 hpf). (Figure 1).

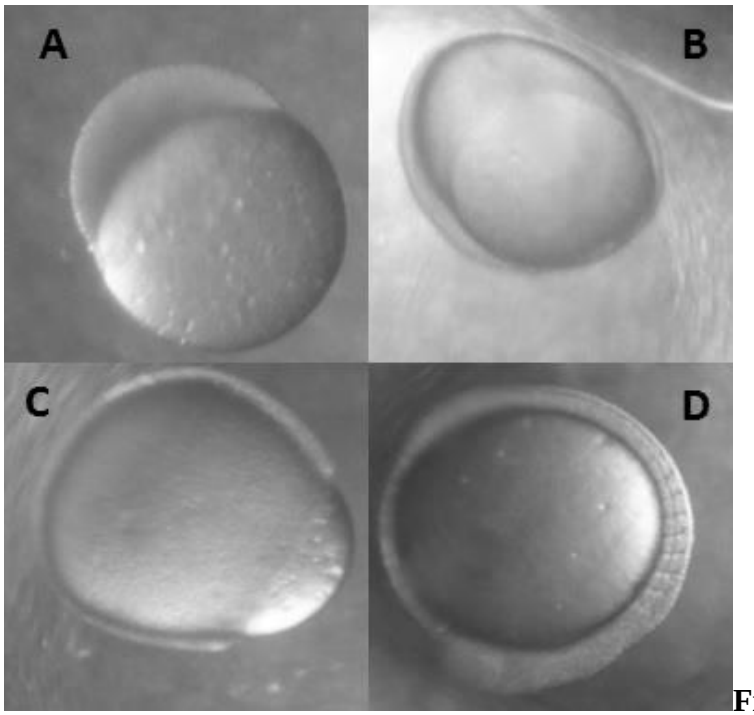

Figure 1

Figure 1 Embryonic stages of *Piaractus brachypomus*. a) Blastoderm stage (~1,2h post fertilization). b) Epiboly stage (~5 h post fertilization). c) Blastopore closure stage (~8h post fertilization). d) The appearance-of-optic-vesicle stage (~13h post fertilization).

Chilled Embryos

Viable embryos (n = 100 viable embryos/treatment) collected at those four stages (1.2, 5, 8 and 13 hpf) were placed in a Vacutainer® tube containing 17.5% glucose (FORNARI et al.2011)

and one of the following cryoprotectants (10%): methanol, methylglycol or dimethylsulfoxide (DMSO).

Then, tubes were transferred to a polystyrene box containing ice under constant temperature control and chilled at a rate of approximately 1ºC per minute (LOPES et al. 2012) from 20°C down to 2ºC for 6, 8, 10 or 12 hours. The total number of treatments was 4 stages x 3 cryoprotectants x 4 periods of chilling. The tubes were then removed from the chilling device, incubated for 5 minutes at room-temperature water and the embryos were transferred to 3-L flow-through incubators until embryonic development were completed (Figure. 2).

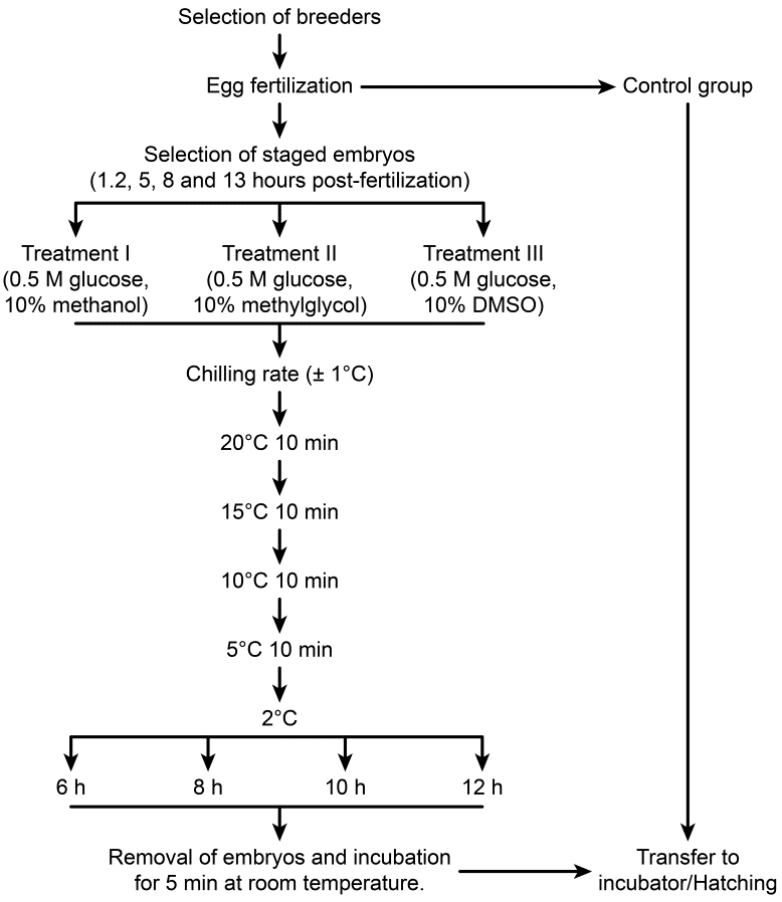

Figure 2. A flowchart showing the *P. brachypomum* embryo-chilling procedure for four embryonic stages and four storage times. Adapted from LOPES et al. (2011).

Post-hatching evaluation

After approximately 18 hpf, hatched larvae were removed from each incubator and the percentage of live and normal developed larvae was estimated for each of the 48 treatments.

Values are expressed as mean ± standard deviation (SD). When data did not fit the normal distribution, an arcsin transformation was performed. Data were tested for significant differences using ANOVA, followed by the Tukey HSD test, when applicable. The level of significance for all statistical tests was set at 0.05. All statistical analyses were performed using the R software package.

Results

Control Embryos

The percentage of live and normal larvae developed from embryos submitted to selection and manipulation at the four ontogenetic stages, but without cryoprotectants or chilling (control embryos) are shown in Table 1.

Table 1 The percentages of live control *P. brachypomum* larvae (showing complete embryonic development) that developed from embryos chosen at 1.2, 5, 8 and 13 hours post-fertilization (hpf).

Embryonic Stage	**Live Larvae (%)**
blastoderm – 1.2 hpf	78.2 ± 1.0
epiboly – 5 hpf	83.3 ± 1.0
blastopore closure – 8 hpf	83.4 ± 1.0
appearance of optic vesicle – 13 hpf	87.0 ± 1.0

Each mean±standard error of the mean value represents three replicates consisting of 100 embryos each.

Chilled Embryos

In general, the larval survival rate for methanol-chilled embryos was significantly higher (31%) than the rate of methylglycol (17%) and DMSO-chilled embryos (19%). No significant differences ($p>0.05$) were observed between the cryoprotectant groups for the 5- and 8-hpf stages; however, embryos chilled at the 13-hpf stage showed significantly higher larval survival rate (31%) compared with those treated at the other stages, regardless of cryoprotectants. Lower larval survival rates were observed for methylglycol-chilled embryos at the epiboly stage (5 hpf) (12%) and for DMSO-chilled embryos at the blastoderm stage (1.2 hpf) (5 %) (Figure 3).

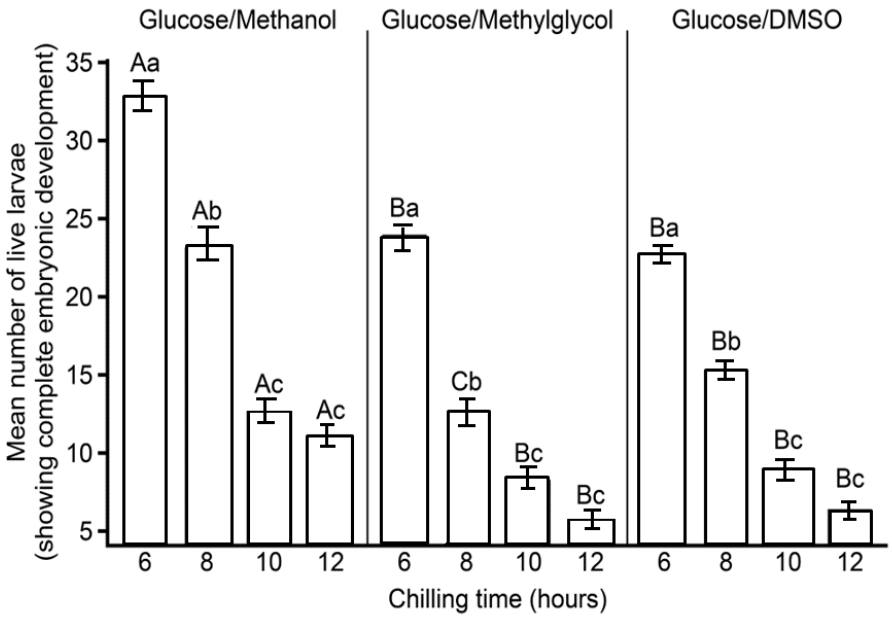

Figure 3

Figure 3 The percentage of live and normal *P. brachypomus* larvae originated from embryos at different ontogenic stages (from 1.2 to 13 hours post-fertilization) and chilled in glucose combined with methanol, methylglycol or DMSO for 6 to 12 hours (pooled data). Uppercase letters represent differences between cryoprotectants for the same stage and lowercase letters represent differences between stages within the same cryoprotectant. ANOVA and Tukey's test.

In methanol-chilled embryos, the percentage of live and normal larvae significantly decreased as the storage time increased, from 33% live and normal larvae after 6 hours of chilling, compared to 12 % after 12 hours. Larvae survival rate were not significantly different ($p>0.05$) between methylglycol- or DMSO-chilled embryos after 6 and 12 hour of storage. However, methylglycol-chilled embryos led to a significantly low survival rate (6%) compared with embryos chilled in the other cryoprotectants (12 %) after 8 hours of storage (Figure 4).

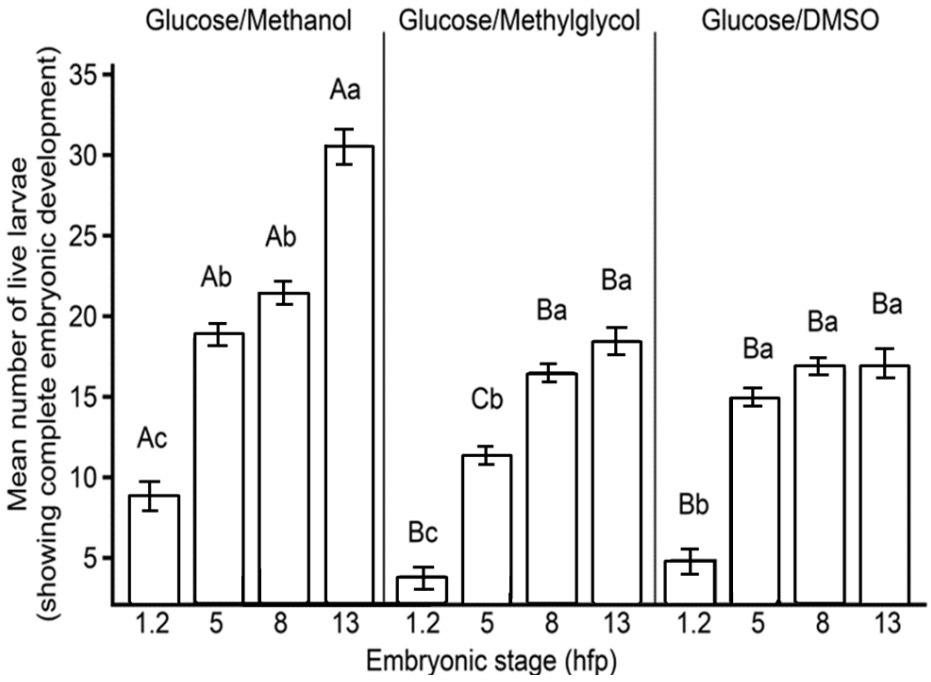

Figure 4 The percentage of live and normal *P. brachypomus* larvae originated from embryos at different ontogenic stages (pooled data) and chilled in glucose combined with methanol, methylglycol or DMSO for 6 to 12 hours post-fertilization (hpf). Uppercase letters represent differences between cryoprotectants for the same period of chilling and lowercase letters represent differences between periods of chilling within the same cryoprotectant. ANOVA and Tukey's test.

Larvae survival always decreased as the period of chilling increased, regardless of ontogenic stage or cryoprotectant (Figure 4). Embryos chilled at the blastoderm stage (1.2 hpf), live larvae were observed after up to 8 hours of chilling at 2ºC, with significantly higher larval survival rates for methanol-chilled embryos (17%); however, few live larvae were observed after 10 hours of chilling for any of the three cryoprotectants (Figure 5A) .

For embryos chilled at the epiboly stage (5 hpf), the larval survival rates decreased as the chilling times increased, but live larvae were still observed after 12 hours of chilling for all three treatments except in the blatoderm stage (1,2 hpf) (Figure 5B). For embryos chilled at the blastopore-closure stage (8 hpf), the larval survival rate for methanol-chilled embryos was significantly different (19%) from those of the other cryoprotectants after 6 hours of chilling; however, no significant differences ($p>0.05$) were observed between the treatment groups after 8, 10 or 12 hours of chilling.

The mean numbers of live larvae were not significantly different ($p>0.05$) between the glucose-methylglycol and the glucose-DMSO treatment groups for any of the chilling times (Figure 5C).

For embryos chilled at the appearance-of-optic-vesicle stage (13 hpf), the methanol-chilled embryos had significant higher in the number of live larvae after 6 (47%) or 8 hours (40%) of chilling compared with the other treatments (30%); survival rates decreased for the two longest storage times (Figure 5D).

At the 8-hpf stage, we observed no statistically significant differences ($p>0.05$) in the larval survival rates of embryos chilled in methylglycol or DMSO for any of the four periods of chilling (Figure 5).

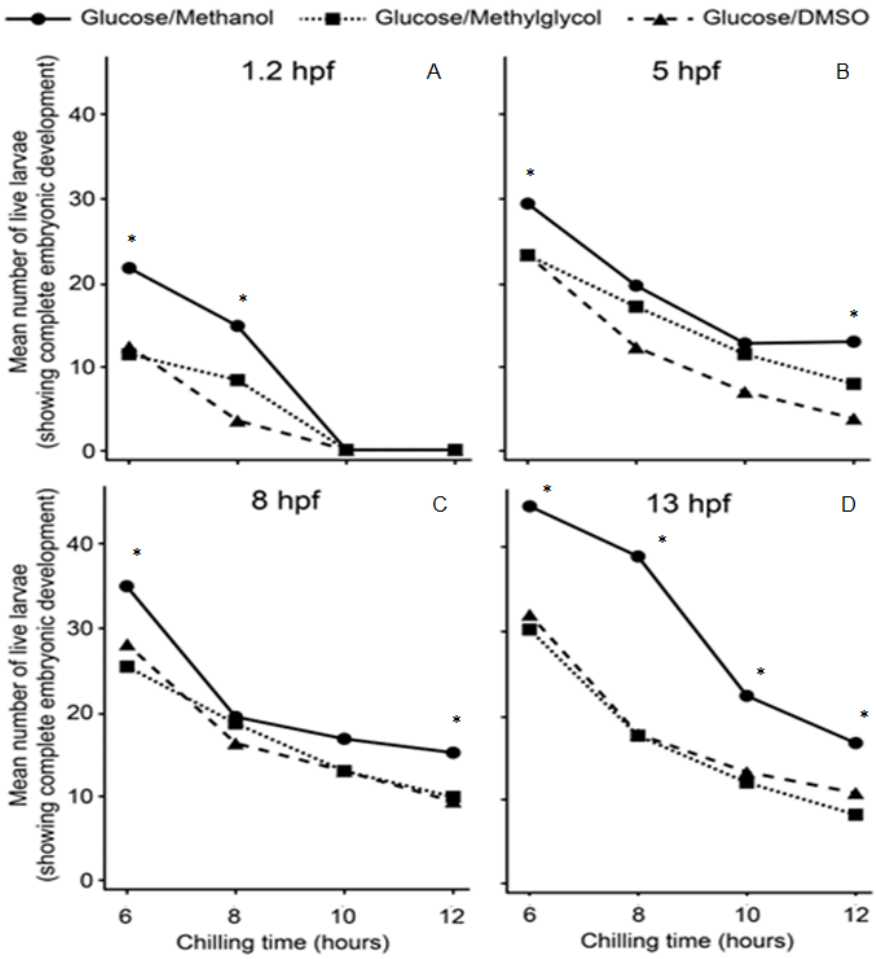

Figure 5 The mean of live and normal *P. brachypomus* larvae originated from embryos at different ontogenic stages and chilled in glucose combined with methanol, methylglycol or DMSO for 6 to 12 hours. a) Blastoderm stage (~1,2h post fertilization). b) Epiboly stage (~5 h post fertilization). c) Blastopore closure stage (~8h post fertilization). d) The appearance-of-optic-vesicle stage (~13h post fertilization).

Discussion

In this study, it was observed the best results with respect to the number of completely developed live larvae following shorter storage times and treatment at progressively later embryonic stages, suggesting that the storage time has a strong influence on the hatching success of chilled *P. brachypomus* embryos. Differential sensitivity to chilling at different developmental stages has been reported

for the embryos of many fish species, including rainbow trout (HAGA, 1982; MADDOCK, 1974), fathead minnows (BEGOVAC & WALLACE, 1989), carp (JAOUL & ROUBAUD, 1982; ROUBAUD et al.1985), zebrafish (HAGEDORN et al. 1997; ZHANG & RAWSON, 1995) and goldfish (LIU et al. 1993). The majority of these studies show that post-gastrulation embryos are less sensitive to cold-induced damage. This is consistent with our findings, and indeed, it was observed the best results when *P. brachypomus* embryos were chilled at an even more advanced developmental stage, namely, after the appearance of the optic vesicle (13 hpf). This phenomenon is likely due to the fact that early-stage embryos are more susceptible to cytotoxicity (BART, 2000), as their metabolic regulatory pathways are not well developed at these stages, making them unable to compensate for the toxicity of the cryoprotectants (LAHNSTEINER, 2008).

Of the cryoprotectants tested, methanol resulted in higher larval survival rates compared with glucose-methylglycol or glucose-DMSO. Furthermore, the chilling of *P. mesopotamicus* embryos with methylglycol (STREIT Jr. et al. 2007) led to lower hatching rates. It is possible that methylglycol may significantly interfere with embryonic metabolism, leading to cellular breakdown and, consequently, embryonic death.

In this study, there was no satisfatory result when chilling *P. brachypomus* embryos in DMSO. In contrast, methanol and DMSO were less toxic for red drum (*Sciaenops ocellatus*) embryos compared to glycerol, methylglycol, sucrose and sea salt (ROBERTSON et al. 1988). However, for other embryos, such as turbot (*Scophthalmus maximus*), DMSO

appears to be less toxic than methanol or methylglycol (CABRITA et al. 2003). Furthermore, the toxicity of specific cryoprotectants may be affected by the embryonic stage at the time of treatment. DMSO was less toxic than methanol during the morula and segmentation stages in carp (*C. carpio*) embryos, whereas methanol was less toxic than DMSO after the initiation of heart beating (DINNYÉS et al. 1998).

Methanol penetrates cells significantly faster than DMSO or glycerol in salmonid embryos (HARVEY & ASHWOOD-SMITH, 1982). The superior penetrative ability of methanol is primarily due to its low molecular weight, which is one of the most desirable characteristics for an embryonic cryoprotectant. These findings are consistent with those of Zhang et al. (1993), who observed that methanol rapidly penetrates the chorion of zebrafish embryos. Additionally higher hatching rates were observed in zebrafish embryos treated with methanol compared with embryos treated with DMSO or methylglycol (ZHANG et al.1993). In more recent studies on *Piaractus mesopotamicus* and *Prochilodus lineatus* embryos, better results were obtained using combinations of methanol and sucrose prior to embryo storage at negative temperatures (LOPES et al.2011; LOPES et al.2012; COSTA et al.2012).

Of the cryoprotectants used in this study, DMSO and methyl glycol have similar molecular masses (~78 and 76 g-mol, respectively), whereas the molecular mass of methanol is less than half of those values (32.04 g-mol). Therefore, in the context of embryonic chilling, differences between DMSO and methylglycol are unlikely to be due to differences in molecular size. However, due to its small size, methanol is significantly more permeant, and its effects should be tested at other concentrations to better

understand its cryoprotective properties (XIAO et al.2008).

Therefore, *P. brachypomus* embryos should be chilled at the optic vesicle appearance stage (13-hpf) in a solution containing 17.5% glucose and 10% methanol for up to 6 hours at 2°C. This is the first report on the successful chilling of *P. brachypomus* embryos. Despite of these promising results, many variables remain to be addressed in future studies, such as factors that affect chilling damage, embryonic permeability and osmoregulation, as well as the effectiveness of other cryoprotectants.

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References

AHAMMAD M.M.; BHATTACHARYYA D.; JANA B.B . The hatching of common carp (*Cyprinus carpio* L.) embryos in response to exposure to different concentrations of cryoprotectant at low temperatures. **Cryobiology,** v.44, p. 114-121, 2002. doi: 10.1016/S0011-2240(02)00012-3

AHAMMAD M.M.; BHATTACHARYYA D.; JANA B.B. Stage-dependent hatching responses of rohu (*Labeo rohita*) embryos to different concentrations of cryoprotectants and temperatures. **Cryobiology**, v.46, p. 1-16, 2003. doi: 10.1016/S0011-2240(02)00138-4

ASAHINA E.; TAKAHASHI, T. Cryopreservation of sea urchin embryos and sperm. **Development, Growth and Differentiation**, v. 21, p. 423-430. 1979. doi: 10.1111/j.1440-169X.1979.00423.x

BART, A. New approaches in cryopreservation of fish embryos, In: CRYOPRESERVATION IN AQUATIC SPECIES. World Aquaculture Society, Baton Rouge, 2000. p.179-187.

BEGOVAC P.C.; WALLACE R.C. Major vitelline envelope proteins in piperfish oocytes originate within the follicle and are associated with the Z3 layer. **Journal of Experimental Zoology**. v.251, p. 56–73. 1989. doi: 10.1002/jez.1402510108

BEIRÃO, J.; ROBLES, V.; HERRAEZ, M.P.; SARASQUETE, C.; DINIS, M.T.; CABRITA, E. Cryoprotectant microinjection toxicity and chilling sensitivity in gilthead seabream (*Sparus aurata*) embryos. **Aquaculture**, v.261, p. 897-903, 2006. doi: 10.1016/j.aquaculture.2006.07.039

CABRITA, E.; ROBLES, V.; CHEREGUINI, O.; WALLACE, J.C.; HERRÁEZ, M.P. Effect of different cryoprotectants and vitrificant solutions on the hatching rate of turbot embryos (*Scophthalmus maximus*). **Cryobiology**, v.47, p. 204-213, 2003. doi: 10.1016/j.cryobiol.2003.10.001

CABRITA E.; ROBLES V.; WALLACE J.C.; SARASQUETE M.C.; HERRÁEZ M.P. Preliminary studies on the cryopreservation of gilthead seabream (*Sparus aurata*) embryos. **Aquaculture**, v.251, p.245–255. 2006. doi: 10.1016/j.aquaculture.2005.04.077.

CHAO, N.H.; LIN, T.T.; CHEN, Y.J.; HSU H.W.; ILIAO, C. Cryopreservation of late embryos and early larvae in the oyster and hard clam. **Aquaculture**, v. 155, p.31-44, 1997. doi: 10.1016/S0044-8486(97)00107-5

CHEN, S.L.; TIAN, Y.S. Cryopreservation of flounder (*Paralichthys olivaceus*) embryos by vitrification. **Theriogenology**, v. 63, p. 1207-1219, 2005. doi: 10.1016/j.theriogenology.2004.06.007

COSTA, R.S.; VELARDI, J.M.C.; SENHORINI, J.A.; VERÍSSIMO-SILVEIRA, R.; SILVEIRA, A.N. Resfriamento de embriões de peixes neotropicais In: AQUACIÊNCIA, Palmas-TO, 2012.

DING, F.H.; XIAO, Z.Z.; LI, J. Preliminary studies on the vitrification of red sea bream (*Pagrus major*) embryos. **Theriogenology**, v. 68, p. 702-708, 2007. doi: 10.1016/j.theriogenology.2007.05.064

DINNYÉS, A.B.; URBÁNYI, B.; BARANYAI, I. Chilling sensitivity of carp (*Cyprinus carpio*) embryos at different developmental stages in the presence or absence of cryoprotectants: work in progress. **Theriogenology,** v. 50, p.1-13, 1998. doi: 10.1016/S0093-691X(98)00108-3

DOBRINSKY, J.R. Advancements in cryopreservation of domestic animal embryos, **Theriogenology**, v. 57, p.285-302, 2002. doi: 10.1016/S0093-691X(01)00672-0

HAGA, Y. On the subzero temperature preservation of fertilized eggs of rainbow trout. **Nippon Suisan Gakkaishi,** v.48, p. 1569–1572. 1982. doi:10.2331/suisan.48.1569

FORNARI, D.C.; RIBEIRO, R.P.; STREIT, D.P.; VARGAS, L.; BARRERO, N.M.L.; MORAES G.V. Freezing injuries in the embryos of *Piaractus mesopotamicus.* **Zygote,** v.19, p.345-350. 2011. doi: 10.1017/S0967199410000432

HAGEDORN, M,; KLEINHANS, F.W.; FREITAS R.; LIU J.; HSU E.W.; WILDT D.E.; RALL W.F. Water distribution and permeability of zebrafish embryos, *Brachydanio rerio*. . **Journal of Experimental Zoology.** v. 278, p. 356–371.1997. doi: 10.1002/(SICI)1097-010X

HAGEDORN, M.; PETERSON, A.; MAZUR, P.; KLEINHANS, F.W. High ice nucleation temperature of

zebrafish embryos: slow-cooling is not an option. **Cryobiology,** v.49, p. 181–189.2004. doi: 10.1016/j.cryobiol.2004.07.001

HARVEY, B. Cooling of embryonic cells, isolated blastoderms, and intact embryos of the zebra fish *Brachydanio rerio* to minus -196°C. **Cryobiology** v.20, p. 440–447.1983. doi: 10.1016/0011-2240(83)90034-2

HARVEY, B.; ASHWOOD-SMITH, M.J. Cryoprotectant penetration and supercooling in the eggs of Salmonid fish. **Cryobiology** v.19, p. 29–40. 1982. doi: 10.1016/0011-2240(82)90122-5

HOLT, W.V. Basic aspects of frozen storage of semen. **Animal Reproduction Science**, v.62, p. 3–22. 2000.doi: 10.1016/S0378-4320(00)00152-4

JAOUL, A. ; ROUBAUD, P. Resistance de l'oeuf de carp commune (*Cyprinus carpio* L. Cyprinidae) a des chocs thermiques chauds ou froids. **Canadien Journal of Zoology**, v. 60, p. 3409–3419.1982.doi: 10.1139/z82-431

LAHNSTEINER, F. The effect of internal and external cryoprotectants on zebrafish (*Danio rerio*) embryos. **Theriogenology,** v.69, p.384–396. 2008. doi: 10.1016/j.theriogenology.2007.10.007

LEIBO, S.P. Sources of variation in cryopreservation. In: Cryopreservation in aquatic species. (ed by Tiersch T.R., Mazik P.M..), pp. 75–83. 2000. World Aquaculture Society, Baton Rouge.

LIU K.; CHOU T.; LIN H. Cryosurvival of goldfish embryos after subzero freezing. **Aquatic Living Resources,** v.6, p. 145–153. 1993.doi: 10.1051/alr:1993007

LIU, X.H.; ZHANG, T.; RAWSON, D.M. Feasibility of vitrification of zebrafish embryos using methanol. **Cryoletters,** v.19, p. 309–318. 1998.doi: 10.1006/cryo.1996.0001

LOPES, T.S.; ROMAGOSA, E.; STREIT, Jr D.P.; RIBEIRO, R.P.; DIGMAYER, M Cooling of pacu (*Piaractus mesopotamicus*) embryos at various stages of development for 6 or 10 hours. **Theriogenology,** v.75, p.570–576. 2011.. doi: 10.1016/j.theriogenology.2010.09.030

LOPES, T.S; STREIT, Jr. D.P.; FORNARI, D.C.; de OLIVEIRA, D.; RIBEIRO, R.P.; ROMAGOSA, E. Chilling curves for *Piaractus mesopotamicus* (Holmberg, 1887) embryos stored at -8°C. **Zygote,** v.21, p. 345 – 350. 2012.doi: 10.1017/S0967199412000020

MADDOCK, B.G. A technique to prolong the incubation period of brown trout ova. **Progressive Fish-Culturist,** v.36, p 219–222. 1974.doi: 10.1577/1548-8659(1974)36[219:ATTPTI]2.0.CO;2

NASCIMENTO, A.F.; MARIA, A.N,; PESSOA, N.O.; CARVALHO, M,.A.M.; VIVEIROS, A.T. Out-of-season sperm cryopreserved in different media of the Amazonian freshwater fish pirapitinga (*Piaractus brachypomus*). **Animal Reproduction Science,** v.118, p.324–329. 2010. doi: 10.1016/j.anireprosci.2009.07.002

OLIVE, P.J.W.; WANG, W.B. Cryopreservation of *Nereis virens* (polychaeta Annelida) larvae: the mechanism of cryopreservation of a differentiated metazoan. **Cryobiology,** v.34, p. 284–294.1997. doi: 10.1006/cryo.1997.2006

ROBERTSON, S.M.; LAWRENCE, A.L.; NEILL, W.; ARNOLD, C.R.; McCARTHY, G Toxicity of the cryoprotectants glycerol, dimethyl sulfoxide, ethylene glycol, methanol, sucrose, and sea salt solutions to the embryos of red drum. **Progressive Fish-Culturist,** v.50, p. 148–154. 1988. doi: 10.1577/1548-8640(1988)050<0148:TOTCGD>2.3.CO;

ROBLES, V.; CABRITA, E.; REAL, M.; ÁLVAREZ, R.; HERRÁEZ, M.P. Vitrification of turbot embryos: preliminary assays. **Cryobiology,** v.47, p 30–39.2003. doi: 10.1016/S0011-2240(03)00066-X

ROUBAUD, P. ; CHAILLOU, C. ; SJAFEI, D. Variations cycliques de la tolerance a un thermique froid appliqué au cours de la segmentation de l'embryon de la carpe commune (*Cyprinus carpio* L.). **Canadien Journal of Zoology**,v.63,p. 657–663. 1985. doi: 10.1139/z85-094

VAL, A.L.; VAL-ALMEIDA, V.M.F. Fishes of the Amazon and their environment: physiological and biochemical aspects. Springer Heidelberg. 1995. cap.1, p.39-63.

STREIT Jr. D.P.; DIGMAYER M.; RIBEIRO R.P.; SIROL R.N.; MORAES G.V.; GALO J.M. Embriões de pacu submetidos a diferentes protocolos de resfriamento [Pacu embryos submitted to different cooling protocols]. **Pesquisa Agropecuária Brasileira,** v.42, p. 1199–1202. 2007. doi: 10.1590/S0100-204X2007000800018

XIAO, Z.Z.; ZHANG, L.L.; XU, X.Z.; LIU, Q.H.; LI, J.; MA, D.Y.; XU, S.H.; XUE, Y.P.; XUE, Q.Z. Effect of cryoprotectants on hatching rate of red seabream (*Pagrus major*) embryos. **Theriogenolog,** v.70, p. 1086–1092. 2008. doi: 10.1016/j.theriogenology.2008.06.028

ZHANG, T.; LIU, X.Z.; RAWSON, D.M. Effects of methanol and developmental arrest on chilling injury in zebrafish (*Danio rerio*) embryo. **Theriogenology,** v.59 p. 1545–1556. 2003. doi: 10.1016/S0093-691X(02)01199-8

ZHANG, T.; RAWSON, D.M .Studies on chilling sensitivity of zebrafish (*Brachydanio rerio*) embryos. **Cryobiology,** v.32, p. 239–246. 1995. doi :10.1006/cryo.1995.1023

ZHANG, T.; RAWSON, D.M.; MORRIS, G.J. Cryopreservation of pre-hatch embryos of zebrafish (*Brachydanio rerio*) embryos**. Aquatic Living Resources,** v.6, p. 145–153. 1993.doi: 10.1051/alr:1993014

ZHANG, Y.Z.; ZHANG, S.C.; LIU, X.Z.; XU, Y.J.; HU, J.; XU, Y.Y.; LI, J.; CHEN, S.L. Toxicity and protective efficiency of cryoprotectants to flounder (*Paralichthys olivaceus*) embryos. **Theriogenology,** v.63, p.763–773. 2005. doi: 10.1016/j.theriogenology.2004.04.011