Cryopreservation of Embryos and Oocytes of South American Fish Species

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1. Introduction

The importance of animal genetic resources for wildlife maintenance as well as farming production has become more and more evident in recent years. Fish stocks are globally threatened mainly due to overfishing and environmental pollution. Cryopreservation of aquatic germplasm brings the possibility of preserving the genome of endangered species, increasing the representation of genetically valuable animals for farming purposes and avoiding genetic losses through diseases and catastrophes.

In fish, successful cryopreservation of semen from many species has been well documented and cryopreserved semen has been used for reproduction of many wild and farmed species. Attempts to cryopreserve fish embryos have been conducted over the past three decades, nevertheless successful cryopreservation protocol for long-term storage still remains elusive.

In this chapter we cover the cryobiology applications on assisted reproduction for fish farming, focused on embryo and oocyte cryopreservation. Our research group has been working in this area for more than 10 years and the chapter shows the main results that we have achieved from researches with fish embryos cryopreservation. The barriers that have been identified as a hamper for successful cryopreservation and the sensitiveness of tropical fish embryos to low temperature exposure are detailed here.

Recently, researches have reported that the use of oocytes may offer some advantages when compared to fish embryos, improving the chances of a successful cryopreservation. On this point, the chapter presents the most recent achievements in oocytes cryopreservation from South American fish, new trends and future works on this area.

2. Brazilian aquaculture growth and researches in cryobiology area

The history of Brazilian aquaculture dates back to 1930's when the first scientific studies related to fish farming were carried out by Rodolpho von Ihering [1], however, the farming activities had bit higher impulses from the 1970's by creation of research center facilities like CEPTA (Center for Research and Training in Aquaculture) and the insertion of academic activities related to aquaculture at universities. From the mid 90's the entry of some private companies boosted the sector, and from the 2000's aquaculture started to represent an important activity of the Brazilian agribusiness.

Fish farming is the sector of the Brazilian animal production industry which has displayed the highest growth in recent years, reaching 544,490 t in 2011, representing an increase of 38.1% compared to its production in 2010 [2]. From the total fish farmed in 2011, about 46% (250,046 t) of the production is from South American species [2].

The growth in fish production has called attention of numerous companies on planning the fish farming development, and biotechnology area has contributed decisively to this. Germplasm cryopreservation brings the possibility of preserving specific species or strains of particular interests, increasing the representation of genetically valuable animals, extending the reproductive life of a particular animal, and avoiding genetic losses through disease, catastrophe, or transfer between locations [3]. In fish farming, the successful cryopreservation of gametes, eggs, and embryos will offer new commercial possibilities, allowing the unlimited production of fry and potentially more robust and better conditioned fish as required [4]. Germplasm cryopreservation also provides a secure method for preserving the genomes of endangered species, having an important role in conservation and preservation of genetic biodiversity of aquatic resources [5].

Research on cryopreservation of gametes and embryos from South American species focused initially on those fish with commercial interest, and more recently has also focused on species which have environmental importance. The first record published in the literature regarding semen cryopreservation from South American species was in 1984 [6] using *Prochilodus scrofa* = *Prochilodus lineatus* and *Salminus maxillosus*. Afterwards, papers started to be published involving other species such as: *Rhamdia hilarii* [7]; *Leporinus silvestrii* [8]; *Piaractus mesopotamicus* [9, 10]. In the 2000's studies on semen from South American species took different lines of research, not only investigating other species such as *Leporinus macrocephalus* [11], *Brycon cephalus* [12], *Brycon orbygnianus* [13], *Colossoma brachypomus* [14], but also testing different protocols for semen cryopreservation [15, 16], and the fertilizing capability after thawing [17, 18].

In the 2000's researches on cryopreservation of fish embryos began in Brazil, with the first tow theses performed on the subject in the country [19, 20] and having the first papers published in 2007 (*Piaractus mesopotamicus*) [21] and in

2008 (*Prochilodus lineatus*) [22]. Currently, three research groups have been working on fish embryo cryopreservation research: *Aquam* Group (Federal University of Rio Grande do Sul - UFRGS), PeixeGen Group (Maringá State University - UEM) and the Cryopreservation of Gametes and Embryos of Neotropical Fish Group (Universidade Estadual Paulista Júlio de Mesquita Filho - UNESP). However, other groups have started activities in this research area, as NAQUA Group (Federal University of Lavras) and certainly new scientific papers related to this area will begin to be published in the next years.

3. Main Difficulties in Cryopreserving Fish Embryos

Fish, reptiles, birds and amphibian embryos contain a considerable amount of yolk and represent a complex multicompartmentalized biological class, whose cryopreservation has constantly failed [22]. Rall [23] pointed out five features that would hinder the cryopreservation of oocytes and embryos from teleosteans:

- Cells of great size and a large final size of the embryos: In general, fish embryos are much larger than most mammalian embryos. Compared to a human zygote, which has about 100 µm in diameter, a fish zygote (> 1 mm in diameter) comes to be a thousand times larger. This large size of fish embryos results in a low surface / volume ratio and lower membrane permeability to water and cryoprotectant solutions.

- Distinct osmotic properties for each compartment of the embryo: the complex membrane structures hamper considerably the transport between each other;

- Chorion: the low permeability of this membrane envelope makes it as almost impassable;

- High sensitivity at low temperatures: fish embryos are too sensitive to sub-zero temperatures exposure.

Within this context, several studies have been carried out in order to overcome the pointed difficulties: Microinjection of cryoprotectants directly into the cytoplasm [24], use of negative pressure to increase permeability to the cryoprotectant agents (CPAs) [25] chorion removal [26]; microinjection of anti-freeze proteins [27], even the application of hydrostatic pressure [25, 28-31]. Nevertheless, all these attempts have failed. Some researchers state that precise knowledge of embryo-permeability would be one of the keys for designing and implementing a successful cryopreservation protocol.

4. Results Achieved According to the Cryopreservation Technique Employed

The initial focus of the earlier studies on cryopreservation of South American fish embryos was to assess the embryo sensitivity to different CPAs and exposure times, as well as the best stage of embryonic development to be used. As there was no reference to undergo cryogenic experiments with embryos of South American fish species, the initial studies were based on researches using *Cyprinus carpio* [29] and *Danio rerio* [28].

In early tests it was identified that for embryos of South American species, the intermediate stages of development, post-morula 8 hours post fertilization (hpf) were less susceptible to injuries caused by CPAs [20]. Methanol, dimethyl sulfoxide (DMSO) and ethylene glycol as permeating and sucrose as non-permeating CPAs led to better results, then started to be used to compose protocols of further researches [19, 20].

4.1. Chilling:

To the South American species, designing a practical and efficient chilling protocol was the main objective attempted by the research teams. Unlike freezing technique where specific cooling rates can be achieved by using liquid nitrogen (LN), and once frozen in liquid nitrogen samples can be stored for extended periods of time; the chilling technique consists of exposing cryoprotected fish embryos to subzero temperatures (normally using a refrigerator) followed by short storage periods [32, 33]. This method can be very useful for hatchery management since it allows synchronizing the development of embryos collected from different spawning events and may optimizes the use of hatchery facilities, especially in remote areas where facilities are very limited.

We chilled *Piaractus mesopotamicus* embryos using a chilling solution composed by 0.5 M (17.1%) sucrose and 2.81 M (9%) methanol for six hours at -8 °C, which resulted in a hatching rate of 69.2% [21]. The same protocol was used for other South American fish embryos, achieving good results as for *Brycon orbinyanus* with hatching rate above 62% [34], *Rhinelepis aspera* upper 50% [35], for *Salminus brasiliensis* with hatching rate of 67.06 % [36], and for *Colossoma macropomum* achieving a hatching rate of 77.6% [36].

Increasing the exposure time of embryos to chilling was a study carried out by Digmayer [37] with *Piaractus mesopotamicus*. The author achieved 26.04% of cold preservation for 24 hours at -8 ° C embryos using a cryoprotectant solution containing 0.25 M sucrose associated with 2.2M methanol. However, in this study the author increased the sucrose concentration from 0.5 to 0.73 M and methanol concentration was also increased from 2.81 to 3.12 M. There was reported some hatching rate in 24 hours exposure, but not after 36 hours.

Lopes et al. [38] using the protocol set by Digmayer [37] assessed the chilling of *Piaractus mesopotamicus* embryos at four stages of development and confirmed to be the post- morula stage (8 hpf) as the best one in terms of

hatching rate.

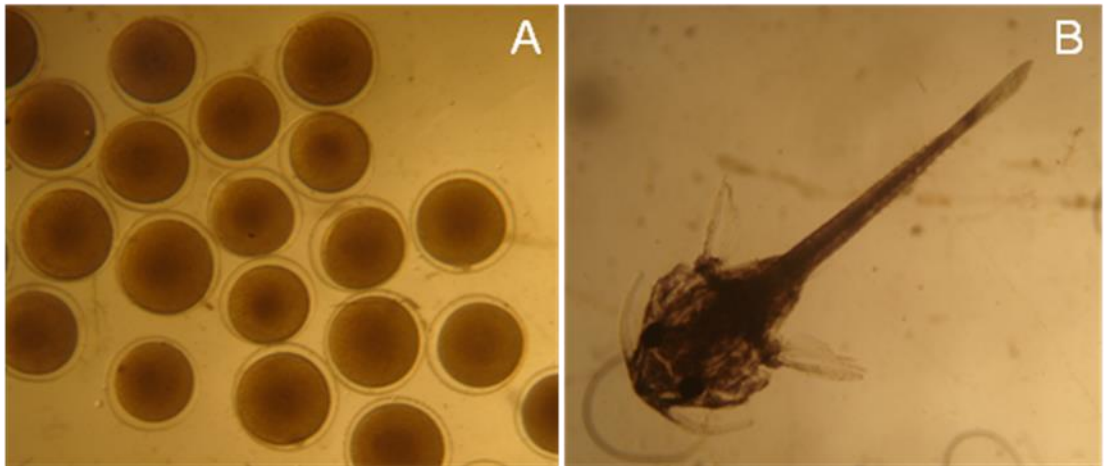

Figure 1. (A) *Rhinelepis aspera* embryos at blastoporous closing stage (75% epiboly movement) 9 h post-fertilization and (B): newly hatched larva of *Rhinelepis aspera* after exposure to a cooling protocol carried out by Fornari et al. [35]. Digital images (7.2 megapixels) obtained by using a stereomicroscope. A: ×30 magnification. B: ×10 magnification.

The exposure time (3, 6, 9, 12, 18 and 24 hours) was also tested to embryos of another South American species, *Prochilodus lineatus,* using the standard chilling protocol (0.5 M sucrose and 2.81 M methanol) employed by Streit Jr. et al. [21]. In this study it was possible to estimate that at every hour under chilling the hatching rate decreased by 6.58% and after 12 hours none larvae hatched.

The embryos of *Piaractus mesopotamicus* were exposed by Fornari et al. [33] to -8 °C for 6, 12, and 24 hours, using a fixed methanol concentration of 2.81 M combined with different sucrose concentrations (0.25, 0.50, 0.75 and 1 M). There was no influence between the combinations of cryoprotectants on the hatching rate up to 12 hours chilling, except for the treatment combining 2.81 M methanol with 0.99 M sucrose, in which no larvae hatched after this exposure time.

The combination of permeating and non-permeating cryoprotectants can be a research line that may produces good results. Working with *Brycon orbignyanus* Paes [39] exposed the post-morula stage embryos to a chilling solution composed by 0.5 M sucrose and methanol (2.81, 1.4 and 0.94 M), ethylene glycol (1.45, 0.72 and 0.48 M), and DMSO (1.15, 0.57 and 0.38 M) at tow controlled temperatures (0.0 and 8.0 °C) for 6, 10, 24, 72 and 168 hours. According to the author, the best result was observed when combining 1.4 M methanol and 0.57 M DMSO associated with 0.5 M sucrose, independent of the tested temperature. According to Milliorini [39] *Brycon orbignyanus* embryos are less sensitive to chilling than *Salminus brasiliensis* embryos. This is another important aspect, the range of sensitivity from some South American fish species when exposed to low temperatures.

Results obtained from these researches on embryos cryopreservation by chilling have great importance and can help in developing a successful freezing protocol which would allow preserving embryos for longer periods.

4.2. Freezing:

The knowledge on the stage of development which South American fish embryos are less sensitive to cryoprotectants and low temperature exposure was the starting point to initiate the studies on embryos freezing in Brazil. The choice by intermediate stages of development, morula and post-morula (≈8hpf) was based on information reported by Gwo et al. [41] when working with red drum (*Sciaenopus ocellatus*), found that embryos at morula stage showed higher resistance to chilling than the eight-cell stage (embryos at earlier stages) and gastrula (embryos at later stages). Ninhaus-Silveira et al. [22] reported that *Prochilodus lineatus* embryos at morula stage were more resistant than those which had between 4 and 6 somites. This information corroborated to the observation made by Streit Jr. [20] pointing 8hpf as the embryonic stage that best survived to exposure to different cryoprotectants.

Pre-cooling in cryopreservation of cell is essential for the success of the process. When the cooling is sufficiently slow, cells are able to lose water rapidly by osmosis, tolerating dehydration and maintaining the equilibrium of the water chemical potential of the intracellular and extracellular solutions [42]. However, the author shows that even then the exposure of cells to cryoprotectant solution causes an osmotic stress, which depending on the concentration, time and temperature of exposure to the solution can lead the cell to collapse. On the other hand, if cooling is too fast, the

chemical potential of water and extracellular solution decreases faster than the intracellular water, resulting in remaining intracellular water that eventually will form intracellular ice crystals, which are often lethal to cells [3].

Lopes et al. [43] using the protocol set by Digmayer [37] tested three cooling curves for *Piaractus mesopotamicus* embryos at post-morula stage (8hpf) at -8 °C and concluded that 1 °C/s is better than 0.5°C/s and 35 °C/s. This result corroborates the observations made by Zhang et al. [30] with embryos of the model species *Danio rerio* and testing the effect of slow (1 °C/min), intermediate (30 °C/min) and fast (≈300 °C/min) cooling curves, using methanol as cryoprotectant.

Likewise studies with other fish species, successful results for long-term storage of South American fish embryos is still remains elusive. However, researches have been focused on trying to understand the extent of injuries in embryonic tissue during the freezing process. In the freezing protocol tested for *Prochilodus lineatus* by Ninhaus-Silveira et al. [22], it was possible to observe some evidences of the problems caused by freezing. Although authors have found intact blastoderm, after scanning microscopy analysis they verified nuclei with disorganized chromatin and cracks in the plasma membrane, which compromised the microvilli, causing embryo death.

Piaractus mesopotamicus were exposed by Neves et al. [44] to six freezing protocols: 0.1 M sucrose plus ethylene glycol (1.2, 1.61 and 2.09 M) or methanol (2.18, 3.12 and 4.06 M). After thawing the chorion was observed to be broken and missing blastoderm in all treatments, which was crucial to the embryos death.

4.3. Vitrification:

Vitrification was first developed by Rall and Fahy [45] to overcome the problems of intracellular ice formation and has the advantage of minimize the procedure time and eliminate the need for a programmed freezing machine. Vitrification is the solidification of a liquid brought about not by crystallization but by an extreme elevation in viscosity during ultra-rapid cooling rates [4].

Vitrification of *Colossoma macropomum* embryos (≈8hpf) was carried out by Fornari [46] using methanol, DMSO, ethylene glycol and glycerol at concentrations of 10, 20 and 30%. Although hatching did not happen, when using scanning electron microscopy (SEM) (Figure 2), it was noted that the embryos exposed to methanol treatments had the chorion and some cellular structures preserved.

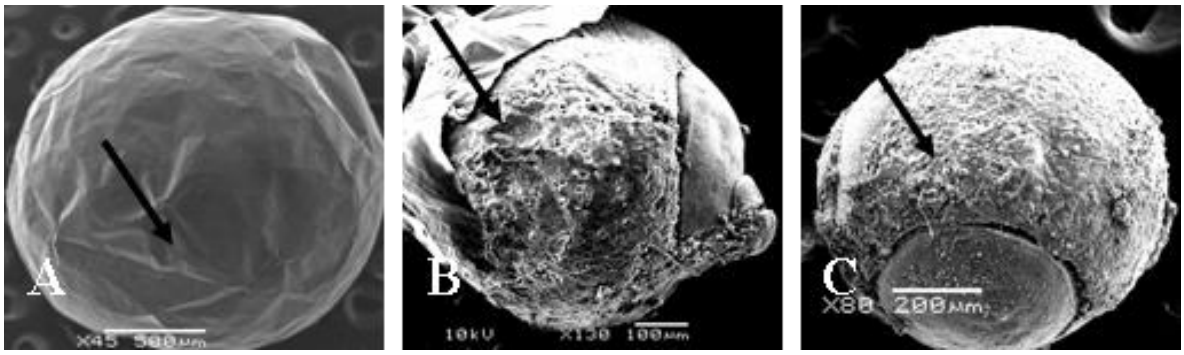

Figure 2. Scanning electron micrograph of *Colossoma macropomum* embryos after exposure to a vitrification protocol carried out by Fornari [46]. A: embryo displaying intact chorion; B: embryo with manually removed chorion, displaying damaged blastoderm; C: embryo at blastoporous closing stage displaying cell injuries.

5. Cryopreservation of Fish Oocytes

Sensitivity of fish oocytes and embryos to low temperatures exposure is specific for each species and for each cell types [30, 31]. The assessment of damages caused by cryopreservation is of great importance as it can be used to characterize the intensity of cell damage, which can incapacitate the cells to overcome such injuries and eventually lose their viability [47].

The difficulties in cryopreserving fish embryos, previously reported in this chapter are mitigated in oocytes due to its smaller size and less sensitivity to low temperature and CPAs exposure, besides the absence of fully formed chorion, which makes them more permeable to water and solutes. Fish oocytes have some advantages over other species, especially at early stages of development [4, 48, 49].

Recently, Digmayer [50] assessed the sensitivity of *Colossoma macropomum* oocytes by exposing to cryo-solutions containing 1.6 M methanol and two concentrations (0.25 and 0.50 M) of non-permeating cryoprotectants (glucose , sucrose , trehalose and fructose) using a slow cooling protocol (1 °C/min until seeding) following storage in

liquid nitrogen. After SEM analyses, in the protocol consisting of 1.6 M methanol and 0.25 M sucrose it could be observed that oocytes maintained intact some morphological structures, like the micropyle.

Digmayer [50] also worked with *Colossoma brachypomus* oocytes at room temperature (28.4 ºC) combining methanol (1.6 and 3.1 M) and DMSO (0.7 and 1,3 M) with 0.25 M sucrose diluted in 50% L-15 medium or Hanks' solution. In this study, the least toxic combination of CPAs was 1.6 M methanol with 0.25 M sucrose diluted in Hanks' solution. Morphological structure of *Colossoma brachypomum* oocytes was evaluated by Digmayer [50] after exposure to 3.1 M methanol associated with sucrose (0.45, 0.63, 0.81 and 0.99 M) in Hanks' solution. It was noted that about 44 and 40% of the oocyte structure was preserved (well defined blastoderm, intact chorion, acidophilus and uniform yolk) when sucrose was used at 0.45 and 0.99 M, respectively.

Results obtained from South American fish oocytes studies are promising, however several points should be further clarified in order to help in achieving a successful cryopreservation protocol.

6. Germplasm Banking and Genetic Improvement Programs in Fish Farming

In Brazil the use of gene banks starts driving to a professionalism level and an example serves very well to illustrate this situation. The use of a germplasm bank was crucial to the operation of the first genetic improvement program for native fish species implanted in the country. The important increase of aquaculture led the Brazilian government to create a national program for research and development of aquaculture, named AQUABRASIL. Among the AQUABRASIL's research areas, the genetic improvement program (GIP) for two South American native species tambaqui (*Colossoma macropomum*) and cachara (*Pseudoplatystoma reticulatum*) has been considered as great importance. It is important to highlight that this genetic improvement program was developed based on a similar program carried out by the World Fish Center (Malaysia) and implemented for tilapia in Brazil in 2005.

In the first four years of the GIP the genes supplier populations were identified and the families were formed, for later fingerlings production. The semen bank enabled the required mobility, since the distances between base populations were higher than 2000 km. Transporting breeders for a long distance would be costly and risky, requiring a logistics that would involve land and air transportation, beyond too time to get the program's deployment and also the possible loss of these specimens. It was much easier to transport cryopreserved semen in liquid nitrogen dewars.

The protocols used for semen freezing were: YEAR 1 - Cryopreservation protocol suggested by Carolsfeld et al. [15] for Characidae: DMSO + glucose + egg yolk; and for Siluridae: powder milk + methanol. Egg yolk and powder milk were thereafter replaced by BTS (Beltsville Thawing Solution®). YEAR 2 and 3 - Cryopreservation protocol from studies carried out by Murgas et al. [51] using semen of South American species; YEAR 4 - Cryopreservation protocol employed only to composition of *C. macropomum* families, replacing DMSO by dimethylformamide (DMF), based on studies performed by Varela Júnior et al. [52].

Currently the GIP is still being developed and germplasm bank acts as a reservoir of genetic material for an eventual replacement or change in focus of the desired genetic trait.

7. New Trends and Future Works in the Area.

Future studies in the area should try to find solutions that allow the cryopreservation of fish embryos. The path for future researches with South American species unfailingly passes through a better understanding of the injuries caused to the cell when exposed to CPAs and sub-zero temperatures in different protocols (slow, rapid or ultra-rapid cryopreservation). Advances in devices development as well as biochemical and molecular techniques can help in elucidating some questions and allow being closer to a successful cryopreservation.

A new research line aims to cryopreserve fish ovarian tissue instead of oocyte or embryo and seems to generate encouraging results. Recently, Godoy et al. [4] using *Danio rerio* as a model observed that ≈60% of the membranes of stage III ovarian follicles in tissue fragments remained intact after vitrification. This research line is currently under investigation by our research group involving South American species.

Another point that we should pay greater attention is related to genetic and behavioral changes induced in the larvae, juveniles and even in adult life after being exposed to cryo-solutions. Studies on this subject may lead to changes in cryopreservation procedures, including other animal species.

8. Acknowledgements

We thank Boa Esperança Fish Farm (Rondônia - Brazil), Buriti Fish Farm and the "Delicious Fish" Farm (Mato Grosso - Brazil) for the partnership in research.

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