Progress, challenges and perspectives on fish gamete cryopreservation: A mini-review

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Abstract

Protocols for the cryopreservation of fish gametes have been developed for many different fish species, in special, freshwater salmonids and cyprinids. Methods for sperm freezing have progressed during the last decades due to the increasing number of potential applications: aquaculture (genetic improvement programs, broodstock management, helping with species having reproductive problems), biotechnology studies using model fish species (preservation of transgenic or mutant lines), cryobanking of genetic resources from endangered species, etc.

This mini-review tries to give an overview of the present situation of this area of research, identifying the main challenges and perspectives, redirecting the reader to more in-depth reviews and papers.

Keywords

Sperm; oocyte; aquaculture; cryobanking; endangered species; biotechnology

Highlights

-Freezing protocols have been developed for many fish species

-Gamete cryopreservation has applications in aquaculture, biotechnology or cryobanking

-The lack of standardization limits the industrial use of fish gamete cryopreservation

-PGCs, spermatogonia or somatic cells are alternatives for fish genome preservation

-The improvement of techniques for sperm quality evaluation is required

1. Progress

1.1. Applications of fish gamete cryopreservation

Cryopreservation of fish gametes has evolved during the last decades due to the increasing number of potential applications. The most evident is its use for aquaculture purposes, allowing the improvement of broodstock management at hatcheries (for example, modifying the offspring production season), preserving the genetically selected strains resulting from genetic improvement programs, or helping with species having reproductive problems as lack of synchronization in the gamete production of male and females (as in the case of the European eel, *Anguilla anguilla*; Asturiano et al., 2004) or with those having a low sperm production (as in the case of F1 Senegalese sole, *Solea senegalensis*; Cabrita et al., 2006).

Another potential application is the preservation of genetic material from individuals of natural populations of fish species in the initial phases of the domestication process and genetic modifications. This can assist in maintaining the original wild genotypes for the recovery of genes in the future (becoming a phenotypic backup), contrarily with happened for example in the case of the domestication process of bovine cattle (Vandeputte, 2011). Other possible conservation-related uses include the storage of genetic resources of the increasing number of fish in the lists of endangered species, allowing cryobanking for biodiversity (Van Der Walt et al., 1993; Martínez-Páramo et al., 2009, 2016), or in the case of fish species recently attracting the interest of cryobiologists and aquaculturists, mainly in South America and Asia (Viveiros and Godinho, 2009).

Moreover, the increasing use of aquatic models such as zebrafish in studies of biotechnology, toxicology or pharmacology, requires the use of transgenic lines, knockout and mutant strains that need adequate storage (Kollár et al., 2015; Tiersch et

al., 2011).

1.2. Cryopreservation of fish sperm

Fish genome cryobanking has been attempted using different cell types (see section 2.3.; Labbé et al., 2013). However, spermatozoa have been the objective of most of the studies, making sperm cryopreservation the most established and commercialized technique. The choice of this type of cell is because it is easy to collect in most of the fish species, has a simple cellular structure and a small size and high chilling resistance, making these cells easy to preserve in many fish species. Moreover, reconstruction of individuals can be done by normal fertilization (or androgenesis), but it allows the preservation of only male germplasm.

Some previous publications have reviewed fish sperm cryopreservation subject (Suquet et al., 2000; Cabrita et al., 2009a; Kopeika and Kopeika, 2008; Tiersch and Green, 2011; Figueroa et al., 2014). The Table 1 summarizes studies on cryopreservation of sperm from fish species published during the last 15 years, including the cryoprotectants used (and their concentrations) and the best results obtained in each case in terms of post-thaw motility, cell viability and fertilization rates.

2. Challenges

2.1. When biodiversity means problems

With 25,000 to 30,000 species, fish are the largest group of vertebrates, displaying an extreme biodiversity (Near et al., 2013). This biodiversity is evident in the significant differences found in gamete (spermatozoa) morphology and biology (Mattei, 1991). During the cryopreservation steps of cooling, freezing and thawing, some biophysical and chemical processes such as osmotic changes, dehydration and

rehydration, cell volume changes, ice crystals formation, cryoprotectants toxicity, etc., occur and cells (gametes or others) are more or less sensitive to these changes being species-specific (Cabrita et al., 2014). Thus, cryopreservation protocols must be adapted to find species-specific compromises, and the increasing numbers of studies describing methods to cryopreserve sperm in many species, evidences this diversity (Cabrita et al., 2009a).

2.2. Lack of standardization: a problem to compare results and to arrive to the industry

The main objective has always been maintaining a high sperm fertilizing ability after thawing. However, the difficulties in obtaining reproducible results using sperm cryopreserved using the published methods have limited the use of cryopreserved sperm in production.

Recent scientific discussion have evidenced the need of standardization in different aspects of this area of research, as definition of basic concepts (extenders, cryoprotectant concentrations, dilution ratios), work protocols (sperm concentration determination, sperm cryopreservation methods, equilibration time, handling of straws, polystyrene box or controlled-rate freezer, type of vials, thawing systems, calculation of fertilization and hatching rates, osmolality measurements, sperm quality evaluation, etc) or even reporting of results (Rosenthal et al., 2010; Horváth et al., 2012a).

Although some recent efforts have been made in this regard (Benson et al., 2013; Gallego et al., 2012, 2013; Kása et al., 2014, 2015; Vílchez et al., 2014), new efforts must be made for a complete description and standardization of protocols for sperm cryopreservation, including a very wide area of topics: determination or estimation of sperm motility, substances used for activation of sperm, details of dilution of sperm with extender and cryoprotectants (new ones as the antifreeze proteins, AFPs, or better

combinations of classic ones), use of straws (sealed or unsealed), cooling of samples (dry ice *vs*. liquid nitrogen, styrofoam box *vs*. programmable freezer), methods of calculating fertilization and hatching results.

Regardless of the very high number of publications on this topic, few of the published methods have been adapted to aquaculture practice. There can be several reasons for this failure of application; however, one of them is beyond doubt the lack of standardization not only in methodologies but also in reporting them correctly. The difficulties in interpretation and replication of methods lead to a disappointment and ultimately rejection by the aquaculture industry. We also need to understand that in most fish species sperm is not a limiting factor during induced spawning and, moreover, individual selection is not as advanced in fish as it is in terrestrial livestock.

2.3. Alternative cells

Fish genome cryobanking has been attempted using different cell types: spermatozoa, oocytes, spermatogonia and primordial germ cells (PGCs), as well as somatic cells, blastomeres and embryos (Labbé et al., 2013).

The cryopreservation of fish oocytes has severe limitations because of their large cell volume, the presence of a chorion, the low permeability to cryoprotectants, and a high chilling sensitivity. Different studies have been carried out in zebrafish, as well as other marine and freshwater species, including cryoprotectant toxicity, chilling sensitivity, membrane permeability and cryopreservation (cooling rates, vitrification) of oocytes at different stages of development or ovarian fragments (Zhang et al., 2007; Godoy et al., 2013; Streit Jr. et al., 2014; Marques et al., 2015; reviewed by Martínez-Páramo et al., 2016). However, development of protocols for *in vitro* maturation of ovarian follicles after cryopreservation is required for the use of cryopreserved oocytes (Seki et al., 2008,

2011; Tsai et al., 2010). Thus, oocyte cryopreservation is still in its experimental phase and far from aquaculture applications.

The preservation of spermatogonia and primordial germ cell guarantees the full individual genome. These cells have been cryopreserved successfully in several fish species (Yoshizaki et al., 2011; Robles et al., in press). However, its use requires the development of specific biotechnological tools, such as transplantation.

Fish embryo cryopreservation could be perfect for the establishment and management of genetic selection programs in fish farms. However, they have low membrane permeability, low surface-to-volume ratio, large size, high yolk content and high chilling sensitivity (Hagedorn and Kleinhans, 2000), which is the primary reason for the very limited number of preliminary positive results (Chen and Tian, 2005; Martínez-Páramo et al., 2008; Robles et al., 2005).

The cryopreservation of somatic (diploid) or embryonic cells (including PGCs) is an alternative to the cryobanking of gametes. They can be a good source of diploid genome to reconstruct fish (reviewed by Labbé et al., 2013). Moreover, they can easily be collected (e.g.: fins clips that regenerate easily). However, the use of these cells means to develop a series of complex and specific techniques as cell culture, nuclear transfer or the transplantation of the thawed cells into recipient fish (of the same or related species) for individual restoration (Siripattarapravat et al., 2011; Chenais et al., 2014), that must be explored in different fish species (reviewed by Martínez-Páramo et al., in press).

2.4. Vitrification

This technique tries to prevent the negative effects of crystallization happening in the conventional cryopreservation methods mixing cryoprotectants at very high concentration and using very high freezing rates, getting the solidification of external

and internal media into an amorphous/glassy state without formation of harmful ice crystals (Fahy et al., 1984).

First applications of vitrification to the cryopreservation of sperm of different species have been published (channel catfish, *Ictalurus punctatus;* Cuevas-Uribe et al., 2011a; green swordtail, *Xiphophorus hellerii*; Cuevas-Uribe et al., 2011b; rainbow trout, *Oncorhynchus mykiss*; Figueroa et al., 2013; Atlantic salmon, *Salmo salar*; Figueroa et al., 2015; Tambaqui, *Colossoma macropomum*; Varela Jr. et al., 2015; Eurasian perch, *Perca fluviatilis* and European eel, *Anguilla anguilla*; Kása et al., in press). However, concentrated cryoprotective solutions are toxic for cells and very high freezing rates can be difficult to achieve with large samples, limiting the applicability of the vitrification to low sperm volumes (2-4 µl; using cryoloops or cryotops). Thus, it does not seem to be able to replace conventional freezing used in most fish species. A real practical application is just feasible in small model species as zebrafish due to the low volumes of sperm that they produce.

Vitrification presents a viable alternative to freezing for the cryopreservation of various teleost tissue types. Vitrification has been tested on somatic cells such as caudal fin explants (Cardona-Costa et al., 2006) as well as testicular cells (Bono-Mestre et al., 2009), embryo blastomeres (Cardona-Costa et al., 2007), oocytes (Guan et al., 2010) and ovarian tissue (Streit et al., 2015). In case of testicular tissue, vitrification was found to be more effective than conventional freezing in terms of cell survival (Bono-Mestre et al., 2009). The efficiency of testicular tissue vitrification and its potential for transplantation and production of germ-line chimeras has been recognized in avian and mammalian species (Liu et al., 2013; Gouk et al., 2011), thus opening the possibilities of its application in teleost species, as well.

213 *2.5. Evaluation of gamete quality*

214 Fast and accurate techniques for the evaluation of the quality of fish gametes are
215 needed both for the selection of sperm samples and for the establishment of sperm
216 cryopreservation programs by companies. Many different techniques have been
217 developed for gamete quality evaluation (Cosson et al., 2008; Figueroa et al., 2016;
218 Pérez et al., 2009; Sørensen et al., 2013; Valdebenito et al., 2015) including sperm
219 volume, color and density, spermatozoa motility and morphometry parameters (CASA
220 and ASMA software; reviewed by Mylonas et al., in press), and seminal plasma
221 composition (Pérez et al., 2003; Lahnsteiner, 2009).

222 However, new techniques require new approaches and parameters to evaluate the
223 freezing-thawing processes, and to provide in-depth information on the effects of these
224 processes undergoing during freezing-thawing that reduce sperm quality (Bobe and
225 Labbé, 2010; Cabrita et al., 2009b; Martínez-Páramo et al., in press; Mylonas et al., in
226 press). For example, the cryopreservation process can induce different types of damage
227 to the spermatozoa, such as DNA fragmentation (Bungum et al., 2011; Cabrita et al.,
228 2005b, 2014; Chohan et al., 2006; Pérez-Cerezales et al., 2010; Riesco et al., 2011), and
229 changes in the protein profile of spermatozoa (Zilli and Vilella, 2012) as well as
230 increases on the production of reactive oxygen species (ROS) inducing alterations at
231 DNA level (Aitken and Baker, 2006; Martínez-Páramo et al., 2012; Thomson et al.,
232 2009). Other techniques provide information about specific damage to certain genes and
233 mRNA (Cartón-García et al., 2013; Guerra et al., 2013) and potential epigenetic
234 damages (Labbé et al., submitted). Specifically, in cryopreserved PGCs, different
235 methylation patterns were found in several genes (e.g *vasa*) (Riesco and Robles, 2013).

236 The improvement of these techniques is allowing the development of better
237 cryopreservation methods, although an evident lack of standardization can compromise

238 the comparison of results between different laboratories and further applications (see
239 section 2.2.).
240
241 **3. Perspectives**
242 *3.1. Cryopreservation and industry*
243 In mammals (both cattle and humans) cryopreservation means an important
244 commercial business, while this is yet to happen in aquatic species. Trying to solve the
245 lack of commercial-scale know-how for scaling-up to industry and practical
246 aquaculture, national or supranational specialized centers should be created to improve
247 the standardization (definitions, methodologies, reporting), and offer quality assessment
248 and cryobanking services (linked to genetic programs, endangered species management,
249 or preservation of special samples). Unfortunately, this activity is currently carried out
250 primarily by research centers that concentrate on conservation-related issues (O'Reilly
251 and Doyle, 2007; Streit et al., 2013) and not by commercial companies that use
252 cryopreserved sperm in their genetic improvement programs.
253 Commercial application of fish sperm cryopreservation is hindered by several factors.
254 Sperm cryopreservation came to the aid of dairy cattle farming exactly when the
255 industry needed it in the 1950-ies. Due to a continuously increasing selection pressure
256 and the development of artificial insemination (AI) techniques, the individual value of
257 dairy bulls increased significantly (Chandler and Godke, 2011). Cryopreservation
258 solved a very stressing problem: how to use the sperm of a few valuable bulls to
259 simultaneously fertilize the oocytes of several thousand cows on various continents.
260 However, all this was a result of a very long domestication process. Aquaculture on the
261 other hand is still in its infancy: higher yields can still be achieved using technologies
262 that do not require genetic improvement (e.g. formulation of feeds, stocking rates,

263 vaccination, etc.). Thus, sperm cryopreservation will be achieved commercially in some
264 species where the protocols are better developed, the aquaculture industry has
265 demanded such tools and there are economic interests. For other species, sperm
266 cryopreservation will be used occasionally or in niche areas for solving acute problems.
267 Nevertheless, aquaculture is also a very rapidly developing industry. During the last
268 years the development of genetic improvement programs has happened and there is a
269 growing offer of cryobanking services for several salmonids and other species
270 (reviewed by Martínez-Páramo et al., in press). Several companies that supply the
271 cryopreservation industry have a line of products specifically for fish, such as extenders
272 and activating solutions and there is at least one company based in Norway that offers
273 commercial cryopreservation services to the aquaculture industry, exclusively. All this
274 means that cryopreservation in aquatic species is slowly gaining momentum along with
275 the development of aquaculture, especially in the salmon industry where marker assisted
276 selection has already been introduced and QTL (Quantitative Trait Loci) mapping is
277 becoming increasingly important (Yue, 2014; Tsai et al., 2015).
278
279 *3.2. Development of alternative techniques*
280 The techniques dedicated to preserving oocytes, embryos or larvae could be a key area
281 of research, although much effort has been made with little success. An alternative or
282 complementary way is to improve emerging biotechnological techniques, as the use of
283 PGCs, spermatogonia or alternative diploid cell sources for genome preservation and
284 transplantation (Labbé et al., 2013; Chenais et al., 2014). However, these techniques
285 will need improvement in several technical aspects (reviewed by Martínez-Páramo et
286 al., in press) such as cell isolation, identification, labelling, transplantation, nuclear
287 transference or genome inactivation of the recipient. Moreover, better and simpler

methods are required for DNA integrity evaluation, types of cryodamage (induced by freezing, thawing or cryoprotectants) on the chromatin and cellular structures and their epigenetics consequences, or regarding the production and effects of reactive oxygen species, to guarantee the sperm viability after every step of these processes and the embryo survival (Chenais et al., 2014). And basic research is required on aspects such as cell reprogramming, to regenerate fish producing gametes from somatic cells after transplantation, or germ cell pluripotency (Martínez-Páramo et al., in press; Robles et al., in press).

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766 **Table 1.** Studies on sperm cryopreservation of fish species (published from year 2000 on) indicating the cryoprotectant(s) used in each case and
767 giving (when mentioned) the best results on post-thaw motility (% motile cells), viability (% live cells) and fertilization rate (%).

Species	**Post-thaw**			**Cryoprotectants****	**Reference**
	Motility (% or arbitrary scale*)	**Viability (%)**	**Fertilization (%)**		
Acipenser baerii	-	-	6.0-29.6	10% Methanol	Glogowski et al., 2002
(Siberian sturgeon)	51	40	20-35	10% Methanol	Judycka et al., 2015
Acipenser brevirostrum	13	-	19-39	10% Methanol	Horváth et al., 2005
(Shortnose sturgeon)	15	5	39-40	5% Methanol	Horváth et al., 2008
Acipenser ruthenus (Sterlet)	33.5	-	9.5-32.7	7.5% Methanol	Lahnsteiner et al., 2004
Acipenser sturio (European sturgeon)	60-70	-	34	10% Methanol	Urbányi et al., 2004
Anguilla anguilla	36.6	-	-	10% DMSO	Asturiano et al., 2004
(European eel)	36	-	-	10% Methanol	Müller et al., 2004
	47	-	-	10% DMSO	Szabó et al., 2005
	-	63.2	-	10% Methanol	Marco-Jiménez et al., 2006
	51.9	58.26	-	10% DMSO	Asturiano et al., 2007
	~18-22	~30-35	-	10% DMSO	Garzón et al., 2008
	38	-	-	10% DMSO	Peñaranda et al., 2009
	-	-	33	10% DMSO	Asturiano et al., in press
Anguilla japonica (Japanese eel)	37-46.6	-	-	10% DMSO	Tanaka et al., 2002
Barbus grypus (Shabout)	41.3	-	36.1	10% DMSO	Doğu, 2012
Brycon insignis (Tiete tetra)	76-88	51-69	-	10% Methyl glycol	Viveiros et al., 2011
Brycon nattereri (Pirapitinga)	45-72	-	-	10% Methyl glycol	Oliveira et al., 2007
Brycon opalinus	18-88	10-80	-	10% Methyl glycol	Viveiros et al., 2012

(Pirapitinga-do-sul)					
Brycon orbignyanus (Piracanjuba)	26-66 28-63 42	- - -	- - -	10% Methyl glycol 10% Methyl glycol 10% Methyl glycol	Maria et al., 2006 López et al., 2015 Viveiros et al., 2015
Clarias gariepinus (African catfish)	13.7-44 70.7	- 52.1	82.2-86.7 -	10% DMSO 8% DMSO	Horváth and Urbányi, 2000 Rurangwa et al., 2001
Coregonus lavaretus (Whitefish)	27.5	-	-	10% Methanol	Ciereszko et al., 2013
Cyprinus carpio (Common carp)	69	-	56	10% DMSO	Linhart et al., 2000
Danio rerio (Zebrafish)	35 <20 ~60	- 40 -	13 - 15	8% Methanol 20%, DMF 8% Methanol	Yang et al., 2007 Asturiano et al., 2015 Kollár et al., 2015
Dicentrarchus labrax (European sea bass)	- 35.3	~35-48 69.9	- -	10% DMSO 10% DMSO	Cabrita et al., 2011 Martínez-Páramo et al., 2012a
Epinephelus marginatus (Dusky grouper)	36.8	22.5	65.1	10% DMSO	Cabrita et al., 2009c
Gadus morhua (Atlantic cod)	13-64 ~55 ~38-58 44.2	- - - -	9-69 - - 52.4	10% DMSO 10% PG 10% PG 10% 1,2 propanediol	DeGraaf and Berlinsky, 2004 Rideout et al., 2004 Butts et al., 2010 Butts et al., 2011
Hucho hucho (European huchen)	45	-	87-88	9% Methanol	Nynca et al., 2015a
Hippoglossus hippoglossus (Atlantic halibut)	80	-	97	10% DMSO 10% DMA 10% methanol	Babiak et al., 2008
Hypophthalmichthys molitrix (Silver carp)	30	-	-	16% DMSO	Dzuba and Kopeika, 2002
Lateolabrax maculatus (Spotted sea bass)	50-75	-	-	5-10% DMSO	Gwo, 2010
Maccullochella peelii (Murray cod)	51	63	11	10% Methanol	Daly et al., 2008

Melanogrammus aeglefinus (Haddock)	11-53 ~58	- -	0.33-53 ~85	10% DMSO 10% PG	DeGraaf and Berlinsky, 2004 Rideout et al., 2004
Misgurnus anguillicaudatus (Loach)	72	-	28-32	15% Methanol	Yasui et al., 2009
Morone saxatilis (Striped bass)	45 10	- 56.5	54 -	7.5% DMSO+75mM glycine	Woods III et al., 2009 Frankel et al., 2013
Mugil soiuy (Marine haarder)	90	-	-	16% DMSO	Dzuba and Kopeika, 2002
Oncorhynchus mykiss (Rainbow trout)	- 1-29 43-58 49.9 -	- - 60.3-77.3 - -	94.4 8-65 72.8-84 84.4 ~30-40	10% DMA 7% DMSO 7% DMSO 9% Methanol 10% Methanol	Babiak et al., 2002 Robles et al., 2003 Cabrita et al., 2001 Ciereszko et al., 2014 Horváth et al., 2015a
Pagrus major (Red seabream)	64.8	-	>90	15% DMSO	Liu et al., 2007
Paralichthys orbignyanus (Flounder)	2.5*	43	78	10% DMSO	Lanes et al., 2008
Perca fluviatilis (Eurasian perch)	43-90	-	-	10% Methanol	Bernáth et al., 2015
Polyodon spathula (Paddlefish)	85 -	- -	80 48	10% Methanol 5% Methanol	Horváth et al., 2006 Horváth et al., 2010
Prochilodus lineatus (Curimbatá)	75 73	- -	- -	10% Methyl glycol 10% Methyl glycol	Viveiros et al., 2010 Viveiros et al., 2015
Salmo marmoratus (Marble trout)	11-15	-	15-60	10% Methanol	Horváth et al., 2015a
Salmo salar (Atlantic salmon)	8.2	-	58.7	10% Methanol	Dziewulska et al., 2011
Salmo trutta m. fario (Brown trout)	53-56 73.8	- -	29-42 >90%	10% Methanol 7.5% Methanol	Horváth et al., 2015a Nynca et al., 2014
Salvelinus fontinalis (Brook trout)	56.8	-	36.5	7.5% Methanol	Nynca et al., 2015b
Scaphyrinchus albus	57-70	-	79-85	10% Methanol	Horváth et al., 2005

(Pallid sturgeon)					
Scophthalmus maximus (Turbot)	75	-	68	10% DMSO	Suquet et al., 2009
Sparus aurata (Gilthead seabream)	~65 58.3-70 ~30-55 68	- 22.7-86.9 ~45-60 87	- 75.6 - -	5% DMSO 5% DMSO 5% DMSO 5% DMSO	Fabbrocini et al., 2000 Cabrita et al., 2005 Cabrita et al., 2011 Gallego et al., 2012
Tinca tinca (Tench)	-	-	85	10% Methanol	Lujić et al., in press
Tor tambroides (Malaysian Mahseer)	54.9-69.4	-	13.4-36.8	10% DMSO	Chew et al., 2010
Tor douronensis (Malaysian Mahseer)	74	-	-	10% DMSO	Chew et al., 2010
Thymallus thymallus (Grayling)	- - ~30 68	- - - -	51.1 59-60 ~60 ~50	10% Methanol 10% Methanol 10% Methanol 9% Methanol	Horváth et al., 2012b Horváth et al., 2015a Horváth et al., 2015b Nynca et al., 2015a

*** Dimethyl-acetamide (DMA); Dimethyl-formamide (DMF); Dimethyl-sulfoxide (DMSO); Propylene glycol (PG)*