CRYOPRESERVATION OF AQUATIC GAMETES AND EMBRYOS: RECENT ADVANCES AND APPLICATIONS

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Summary

Although successful cryopreservation of fish eggs and embryos is still elusive, spermbanks which are currently feasible can play a crucial role in aquaculture and conservation management (McAndrew *et al.*, 1993). Heterogenous results and cryopreservation of small volumes of milt, however, are likely to prevent uptake of this technology. The possible consequences of previously neglected variables such as milt collection techniques and precooling storage, handling and cooling conditions for consistent cryosuccess are therefore considered here together with possible causes of cryoinjury. Recent developments in field and large scale cryopreservation techniques and their applications in aquaculture and conservation are considered.

To increase the likelihood of successful cryopreservation of fish eggs and embryos, concerted research effort is required to identify and address fundamental cryobiological constraints.

Introduction

Following cryopreservation of Atlantic herring (*Clupea harengus*) testes (Blaxter, 1953), the feasibility of successfully cryopreserving spermatozoa has been demonstrated in over 200 fish species (Stein cited by Billard *et al.*, 1995a) notably for the salmonids, carps, tilapias. Although a number of different protocols, even for the same species are advocated in the literature (see reviews by Leung and Jamieson, 1991; McAndrew *et al.*, 1993) the components of cryopreservation are the same. Freshly collected semen is diluted with a balanced salt solution containing either 7-10% dimethyl sulphoxide (Me_2SO), glycerol or methanol, cooled as pellets on dry ice or more commonly in straws over liquid nitrogen (LN), stored in LN and rapidly warmed in a 20-40°C water bath prior to inseminating eggs. The interpretation of published results, however, is fraught with difficulties. Often components of cryopreservation such as, prefreezing milt quality and storage conditions, packaging, cooling, warming, insemination and protocol evaluation vary considerably between and within studies (Billard *et al.*, 1995a; Rana, 1995a&b). This together with the empirical and trial and error approach has led to variable results (Fig. 1).

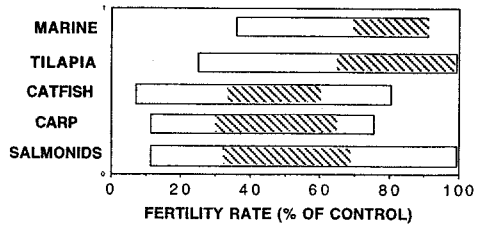

Fig.1 Range of viability of post-thawed spermatozoa. Shaded area = majority data.

Successful cryopreservation of aquatic eggs and embryos is limited to a few groups of invertebrates, notably the Pacific oyster (*Crassostrea gigas*). Although low permeability and surface:volume ratio and high internal water are suggested the fundamental reasons for cryofailure of fish eggs and embryos remains unclear. In this presentation recent information on precooling cryoprotectant toxicity, permeability and osmotically active water will be discussed.

Components of spermatozoa cryopreservation

Collection technique and gamete quality

Sperm fitness and in particular the inorganic and organic seminal plasma composition shows high intra and interspecies variation (Leung and Jamieson, 1991; Billard *et al.*,1995a; Rana,1995a&b).

To date semen quality analysis is based on milt samples expressed by abdominal pressure but unavoidable urine contamination can dilute milt by as much as 80% (Rana, 1995a) and may result in false intra-individual variation.

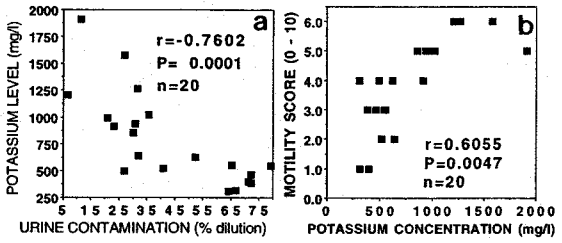

Fig 2. Effect of (a) urine contamination on potassium levels and (b) milt potassium levels on motility.

In Atlantic salmon, *Salmo salar*, the wide range (220-300mOsm/kg) in milt osmolality (Munkittrick and Moccia, 1987) was probably due to urine contamination. The osmolality of catheterised salmon milt is highly homogenous (300-330 mOsm/kg vs 180-290 mOsm/Kg; Rana, 1995a). The heterogeneity in urine contamination (Fig 2a) and K^+ of contaminated milt may also reduce the proportion of active spermatozoa (Fig 2b).

Such variability is likely to be further confounded by intra-male variation in protein and osmolality of urine (Rana, 1995a). The overall, resultant variability in milt may reduce sperm quality and alter cooling properties of the milt in an unpredictable and random manner. In simulated trials the post-thaw motility score (0-10) of Atlantic salmon milt containing 0-25% urine was reduced from 6 to 4 (Rana, unpublished data).

Duration of precooling storage

Although it is widely recommended that spermatozoa be cryopreserved immediately after collection this may not always be practical under hatchery situations. Detrimental effects of precooling storage, however, are unclear and results contradictory. In rainbow trout (*Oncorhynchus mykiss*) a 6 delay in cooling chilled

milt reduced post-thaw fertility rates from 74 to 52% (Schmidt-Baulain and Holtz, 1989). Simarily, in Atlantic halibut (*Hippoglossus hippoglossus*), motility was reduced from 80 to 5% within 7h of collection. In contrast, Baynes and Scott (1987) report higher post-thaw fertility following 26h storage at 0°C. The reasons for the variability are unclear. In carp (*Cyprinus carpio*), 8-10h storage at 4°C resulted in a decline in intracellular ATP and morphological changes (Billard *et al.*, 1995b). Recent evidence supports the view that spermatozoa activated prior to cooling retain their potential to be successfully cryopreserved. In *Oreochromis niloticus*, storage of milt at 4°C for up to 6 days had no significant effect on post-thaw fertilization rate of eggs (Rana *et al.*, 1990). In Atlantic salmon, up to 40-50% of spermatozoa diluted in modified Cortlands (Truscott, *et al.*, 1964) containing 5% methanol and held at -4°C for up to 24 days prior to cryopreservation were motile but the extent of recovery was dependent on dilution ratio (Fig. 3).

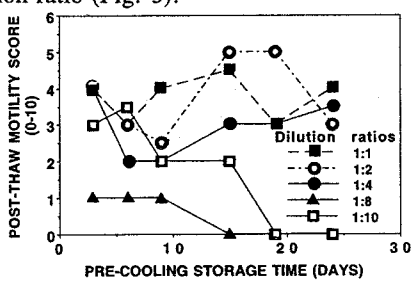

Fig.3 Precooling storage time on post-thaw motility.

Cryodiluents and dilution ratio

Undiluted gametes cannot survive the traumas of cooling and warming. Complex diluents (see reviews by Scott and Baynes, 1980; Leung and Jamieson, 1991; McAndrew *et al.*, 1993) used to dilute semen offer no advantage over simple osmotically balanced solutions such as those containing 0.3-0.6M sucrose and 10% Me_2SO (Holtz *et al.*, 1991) or glycerol (Piironen, 1993). Whilst simple extenders are equally successful it must be emphasised that the results do not suggest any improvement in the efficacy of protocols rather they confirm our limited knowledge of the cryobiological processes associated with spermatozoa cryopreservation. Studies on rainbow trout by Holtz *et al.*(1991) using a 0.6M sucrose cryodiluent reported maximum fertility rate of 87% using pellet technique. These levels of post-thaw fertility rates, however, are achieved using up to 100 times more milt than normal (Billard, *et al.*, 1995a) with up to 200 eggs. Whilst this level of efficacy may be regarded as acceptable for the salmonids which produce copious volumes of milt (Holtz *et al.*, 1991) it is wholly inadequate in, for example, *Haplochromid* spp. which produce maximum milt volumes of 5-10μl.

Milt is usually diluted up to ten fold prior to cooling. Sperm density, however, exhibits high seasonal and intra-individual differences (Leung and Jamieson, 1991). Consequently, sperm density can vary by as much as 300% for any given dilution ratio. High cell densities may also result in compression damage. During cooling water freezes and cells can be compressed in residual water channels. In red blood cells the density of cells within these channels influences post-thaw viability of cells (Mazur and Cole, 1985). Therefore, in fish sperm cryopreservation standardised cell density should dictate the dilution ratio.

Cooling method and rates

Although cooling rate is the most critical phase in cryopreservation (Grout and Morris, 1987) it is the least standardised variable in fish sperm cryopreservation studies.

The cooling rate and its reproducibility varies with the cooling method. In insulated boxes, dewar necks and pellets, the pre- and post freezing phases of the cooling rates are driven by the difference between ambient and straw temperature and therefore the cooling rates between these phases can vary by as much as 500%. Consequently, cooling rates in straws within and between runs can be highly variable (Table 1). Such variation can be minimised by cooling milt in controlled rate coolers or specially designed heat sinks (see below).

Table 1 Mean cooling rates within straws

	Insulation Box[1]		Dewar[2]	
	PrF[3]	PoF[3]	PrF[3]	PoF[3]
°C/min	34	8.1	16.1	17.8
Range	25-41	5-13	6-36	6 - 2 7
CV(%)	14	35	44	34
No. straws	12	12	28	28

[1]Straws positioned 8cm above LN surface, [2]Goblet placed 2cm below the neck of dewar, [3] Prf and PoF = pre- and post freezing cooling rates, respectively.

Evaluation of protocols

To date there is no convincing correlation of sperm fitness and fertility rate. Recent studies on rainbow trout (Gallant and McNiven, 1991) show that neither motility ratings, LDH leakage nor proportion of permeablised cells have any significant bearing on the fertility rate of eggs (Fig. 4). One possible reason may be that like motility rating samples for evaluation are not taken from the site of fertilization and consequently bear little relation to fertilization.

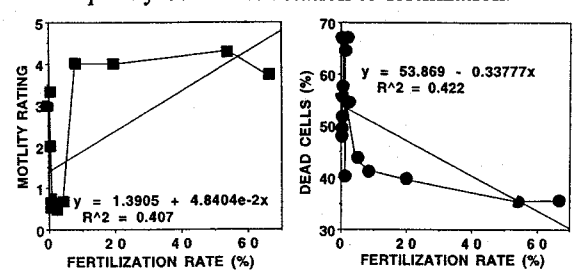

Fig. 4. Relationship between a) permeabilised cells and b) motility and fertility. *Data adapted from Gallant and McNiven, 1991.*

Possible causes and nature of cryoinjury

Despite advances to date at best only 30-50% of spermatozoa retain their post-thaw motility and we have little insight into when the damage occurs, the exact causes of damage and its significance for the outcome of cryopreservation. Recent studies suggest cells are fatally damaged along the entire

cryopreservation process. A substantial proportion of cells are damaged prior to cooling (Lawrence, 1992, Lahnsteiner *et al.*, 1992, Linhart *et al.*, 1993, Lawrence and Rana, in press). Fluorometric assessment of *O. niloticus* sperm membrane integrity during key phases of cryopreservation revealed that up to 35% of cells are permeabilised (ie. damaged) following 30min equilibration in a 10% methanol cryodiluent prior to cryopreservation and a further 26% during the cooling/warming stages (Fig. 5).

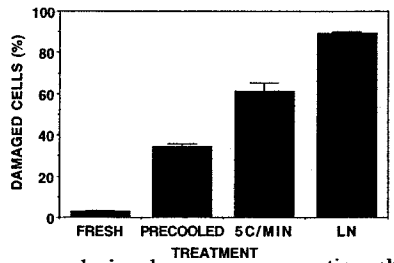

Fig 5. Damage during key cryopreservation phases.

Such damage is also observed as lower post-dilution motility following 5-20 min equilibration and this may be further reduced at higher cryoadditive concentrations (Linhart *et al.*, 1993).

Field applications

The use of the pellet technique for field cryopreservation, though convenient, is impractical for rational long term genetic resource banking. The shelf life of dry-ice is short, particularly in the tropics, and perhaps more importantly frozen samples cannot be sealed to prevent possible cross contamination of diseases in storage vessels. In addition, the thawing of large numbers of pellets is equally problematic. The fusing of pellets during thawing reduces the warming rates within aggregated pellets in an unpredictable manner.

To overcome these short comings for the tilapias a fixed rate portable cooler (FRPC) has recently been developed to generate reproducible linear cooling rates and can be used in the field for up to two weeks. Typical results from field cryopreservation trials are shown in Table 2.

Table 2 Viability of *O. niloticus* spermatozoa cryopreserved in the FRPC

Country	n	Eggs	Fert.(%)	spm.eggx10^6
Sri Lanka	5	110-200	71.4 (5.1)	0.47(0.4)
Mexico	12	235-240	91.2 (3.0)	0.29 (0.01)

Large scale cryopreservation of semen

Reports on the use of large (4.5-5ml) straws and pellets for practical cryopreservation are limited. The The viability of post-thawed rainbow trout milt diluted (1:3) in 5.4% glucose and 10% egg yolk containing 10% Me_2SO and cooled on dry ice in 4.5ml straws average 84% (1.1 x10^7sperm/egg) using 700 eggs (Wheeler and Thorgaard, 1991). For Atlantic salmon, milt diluted in modified Cortlands containing 10% methanol and cooled linearly at 50°C/min could fertilize around 3500 unhardened eggs (Table 2). Under controlled cooling conditions the sperm:egg ratio could be reduced from 4.2 to 1.7 x10^6 without incurring any significant loss in fertility rate. At 1:8, 1500 eggs were fertilized (Table 3). In catfish (*Silurus glanis*) 6500 eggs were successfully fertilized with 5ml pellets (Linhart *et al.*, 1993).

Table 3 Mean post-thaw viability[1] of salmon milt packaged in 5ml straws at varying dilution ratios.

M:D[2]	No.eggs used	Fert. rate[1]	sperm:egg x10^6
Fresh	5134(460)	92(2.8)[a]	0.35
1:1	5867(283)	61(1.9)[b]	4.2
1:2	6143(814)	59(3.5)[b]	2.7
1:4	5911(191)	59(0.6)[b]	1.7
1:8	5039(631)	31(4.0)[c]	1.1

[1] as mean [%(SEM); n=2] eyed eggs. [2] M:D= milt: extender. Means with different superscripts are significantly ($P<0.05$) different (analysis on arc-sine transformed data).

Cryopreservation of aquatic eggs and embryos

Bivalves

Among the invertebrates, bivalves are currently the most studied group. Pacific oyster embryos have been successfully cryopreserved by cooling between 0.5-2°C/min (Rana *et al.*, 1992; Lin *et al.*, 1993; Chao et al., 1993) and by vitrification (Lin *et al.*, 1993) and to date post-thaw success has been largely quantified as post-thaw ciliary movement. Post-thaw success using vitrification is low (<1%) but higher recoveries (20-50%) are reported following slow cooling (Rana *et al.*, 1992; Chao *et al.*, 1993). The ability of embryos to survive cryopreservation is reported to vary with size of eggs, embryonic stage, cryoprotectant type and concentration and cooling rate (Rana *et al.*, 1992; Gwo, 1995). Cryopreservation of unfertilized eggs and 2-8 cell stage embryos were unsuccessful and the trochophore and D-larvae were demonstrably the most tolerant to cryopreservation.

Cryobiological progress in fish eggs and embryos

Although a few reports claim successful recovery of fish embryos from LN (Zhang *et al.*, 1989; Leung and Jamieson, 1991) there is to date no reproducible cryopreservation protocols. Review of recent data suggest that while freezing contributes to fatality there is a gradual loss in viability during the entire cooling protocol (Lin *et al.*, 1993; Zhang *et al.*, 1993; Adam, 1995). Studies by Adam (1995) suggest that between 20-80% mortality may occur prior to freezing due to cryoprotectant toxicity and cold shock damage and therefore the importance of prefreezing damage may be underestimated.

Cryoprotectant-induced injury during the long equilibration and prefreezing phase can be significant. The causes of such damage remain unclear. In rosy barbs (*Puntius conchonius*) and zebra fish (*Brachydanio rerio*) embryos, high cryoprotectant concentration significantly reduced the enzymatic activity of LDH and G-6-PDH. This decrease which was highest for the blastula stage, was attributed to the rupturing of the embryo caused by hydrostatic pressure within the perivitelline space and subsequent denaturation of the leached enzymes by the cryoprotectant (Adam *et al.*, 1995).

Fish eggs and embryos are prone to cold shock damage and by 0°C up to 70% of embryos can be fatally damaged depending on cooling rate, cryoprotectant type and concentration and embryonic stage. In most studies DMSO offers the best pre freezing cryoprotection. Subzero cooling is equally traumatic and by -20 to -40°C the viability of the most resistant stage, the heart beat stage, is reduced to 10-20% (Lin et. al., 1993; Zhang et al., 1993; Adam, 1995.

To improve the low water permeability of rosy barb and zebra fish embryos (0.003-0.008μmsec^{-1} at 20°C) the feasibility of electroporation has been investigated (Adam, 1995).

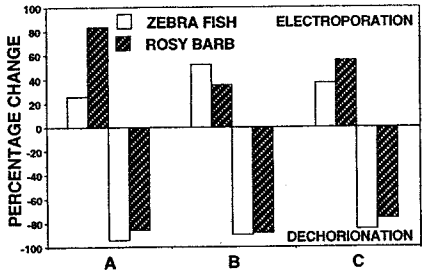

Fig.6 Effect of electroporation and dechorionation on water permeability of (A) cleavage, (B) epiboly and (C) closure of blastopore embryos.

In intact rosy barb embryos at the cleavage, epiboly and closure of blastopore stages electroporation increased permeability coefficients by 83, 34 and 56%, respectively (Adam, 1995). Much of this increase, however, may be due to improved chorion permeability. The permeability of dechorionated embryos was reduced by as much as 80% (Fig. 6) suggesting that most exchange occurs between the perivitelline space and exterior.

The removal of intracellular water is regarded as crucial for cryosuccess. Recent studies by Hagedorn *et al.* (1993) on 8h old zebra fish embryos reported a volume reduction of 8-13%. This relatively small reduction in volume probably reflects the embryonic stage used and that whole egg diameters rather than actual changes in yolk diameters were measured.

Nuclear magnetic resonance (NMR) estimates of intracellular water in epiboly and closure of the blastopore embryos suggest that 22-24% of the water could be removed (Adam, 1995). In precleavage rosy barb embryos, however, up to 36% of the water from the yolk mass (Rana *et al.*, 1995c) and around 50% from the whole egg can be removed (Fig. 7).

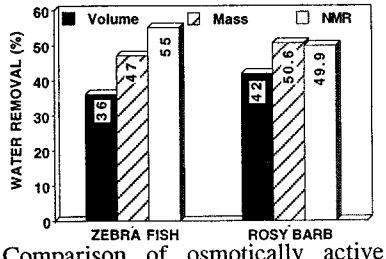

Fig. 7 Comparison of osmotically active water removed from precleavage embryos by three techniques. (*data adapted from Adam, 1995*)

The osmotically non-active volumes do not deviate markedly from other recently cryopreserved biological systems such as *Drosophila* (Steponkus *et al.*, 1990).

The requirement of adequate dehydration for cryopreservation may not be universal. *Artemia* cysts undergo natural dehydration and have a hydraulic conductivity (0.24 μmmin^{-1}.atm^{-1}) similar to mammalian embryos (0.27-1.27 μmmin^{-1}.atm^{-1}). By allowing them to hydrate in water for a fixed period of time, varying levels of internal water can be simulated and cysts cryopreserved. Moreover, the cysts can be decapsulated to improve permeability. These studies demonstrate that despite containing up to 60% water, post-thaw decapsulated and normal cysts could hatch into normal nauplii (Fig. 8).

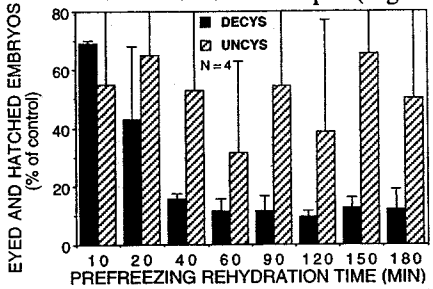

Fig. 8 Post-thaw viability of hydrated *Artemia* cysts. DECYS = decapsulated, UNCYS = encysted.

The cooling process can be observed using a cryo-microscope and internal freezing detected by flashing or blackening of the embryo. Cryomicro- scopic observations show that fully hydrated *Artemia* cysts do not show any evidence of internal freezing. Elucidation of the mechanisms for *Artemia* may provide an insight into methods for improving fish egg and embryo cryopreservation.

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