Cryopreservation of shellfish sperm, eggs and embryos

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ABSTRACT

Cryopreservation of gametes and embryos plays a key role in the advanced areas of animal production. It will also play a central role in future aquaculture. The techniques to cryopreserve gametes are well understood in domestic animals such as sheep and cattle. In contrast, research on shellfish cryopreservation is scarce. A major limitation of previous shellfish work is that fertilisation or short-term survival has been taken as the measure of success, which has not been validated in terms of successful development and growth of larvae. This manuscript briefly describes the approach to the problem by this research team and highlights some of the similarities and differences between the procedures used with shellfish and domestic animal gametes.

Keywords: shellfish; cryopreservation; sperm; eggs; embryos; diluents; fertility.

INTRODUCTION

NZ has three significant shellfish aquaculture industries: Greenshell mussel (*Perna canaliculus*), Pacific oyster (*Crassostrea gigas*) and paua (*Haliotis iris*). Each of these industries has great potential for growth and innovation. For example, hatchery-based selective breeding could provide improved Greenshell™ mussel lines, a Pacific oyster that is distinguished from the commodity produced elsewhere, and the development of a paua suited for pearl production thus contributing to a whole new industry which is currently in its infancy. Comparable goals have long ago been reached in domestic animal breeding. Cryopreservation of gametes and embryos plays a key role in the advanced areas of animal production. It will also play a central role in future aquaculture.

The storage of frozen sperm, eggs and embryos will bring several major benefits to the aquaculture industry, such as:- reduced cost of brood stock conditioning; higher efficiency of resource use (overcoming seasonal limitations); reduced cost of spat production; selective breeding; maintenance of threatened genetic strains; as well as having benefits for shellfish aquaculture research (e.g., removal of seasonal constraints).

The techniques to cryopreserve gametes are well established in domestic animals such as sheep and cattle. In contrast, research on shellfish cryopreservation is relatively new. A major limitation of previous shellfish work is that fertilisation or short-term survival has been taken as the measure of success, which has not been validated in terms of successful development and growth of larvae.

Cryopreservation of sperm has been achieved overseas with variable results for Pacific oysters and related species (Zell *et al*., 1979; Bougrier & Rabenomanana, 1986; Yankson & Moyse, 1991) and for abalone [= paua], (Matsunaga *et al.*, 1983; Tsai & Chao, 1994). These studies suggest that sperm cryopreservation is feasible but the true viability of the resultant larvae has not been tested by rearing them beyond settlement (the transition from the swimming larval form to the attached adult form). All attempts to cryopreserve unfertilised eggs or 2-8 cell embryos of the Pacific oyster have been unsuccessful (Renard, 1991; Grout *et al*., 1992; Gwo, 1995). Late embryonic stages of Pacific oysters, and the Blue mussel (*Mytilus galloprovincialis*) are less vulnerable to cryoprotectants than early embryos (Toledo *et al*., 1989; Grout *et al*., 1992; Chao *et al*., 1994), and encouraging results have been obtained with D-stage oyster larvae [the second larval stage that has a D-shaped shell, from 1 day after fertilisation until several days old] (Grout *et al*., 1992).

This manuscript will briefly describe the approach to the problem taken by this research team and will highlight some of the similarities and differences between the procedures used with shellfish and domestic animal gametes. For the purpose of illustration, results obtained with the Pacific oyster are presented.

MATERIALS AND METHODS

Location: Work was conducted at the Glenhaven Aquaculture Centre, Nelson

Animals: Sexually mature Pacific oysters were obtained from commercial farms, during the reproductive season.

Gamete collection; Sperm and eggs were obtained primarily by physical stripping of the gonad after opening the shell. In some cases spawning was induced by changes in water temperature and addition of inactivated sperm.

Gamete handling: Sperm was held on ice (around 0^{o}C) in a concentrated form, while eggs were held in seawater at ambient temperature (about 20^{o}C).

Cryo-protectants: A range of different cryo-protectants (CPAs) at various concentrations was investigated for both sperm and eggs. Dimethyl sulfoxide (DMSO), glycerol, ethylene glycol, propylene glycol and methanol were those most commonly used.

Diluents: A range of diluents from seawater through specific salt solutions to polysaccharides in distilled water was tested. Dilution ratios (gamete: diluent) and rates of addition and removal of the CPA in diluent were also investigated.

Freezing methods: Sperm, eggs and embryos were frozen using liquid nitrogen vapour in programmable freezers, for controlled rate freezing. Vitrification systems that involved rapid cooling directly into liquid nitrogen were investigated for eggs and embryos.

Fertilisation Assay: Because fertility is an important end point and this was as easy to measure as any other sperm characteristic a miniaturised fertilisation assay was

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developed. This comprised the comparison of the fertilisation rates of samples of the frozen/thawed sperm at several concentrations and used a sample of fresh sperm, again at several concentrations, as a control. Duplicate fertilisation assays were conducted on each thawed straw and results presented are means of the duplicates. Data presented are not corrected for fresh sperm fertility. Assays employed multi-well tissue culture plates with a standard volume of seawater and number of eggs per well. The assays were performed on the bench in atmospheric conditions at room temperature (about 20^{o}C) and development was halted by the addition of fixative after a set period of time to expedite the reading of the results. The wells were examined under a microscope and the proportion of eggs having cleaved was used as the fertilisation value. These values were then plotted against sperm concentration for the comparison of the different treatments. The better treatments were eventually tested in large-scale assays using about a million eggs and the resultant embryos reared through to spat following standard hatchery procedures.

Evaluation of eggs and embryos: Eggs and embryos were microscopically examined immediately post-thaw and following CPA removal for membrane integrity and cytoplasmic normality. In some cases, eggs were subjected to fertilisation assays and embryos were returned to seawater tanks to assess developmental ability.

RESULTS

Gamete production

Male: A large, ripe male will produce around 7 ml of semen at a concentration of 2.0 x10^{10} sperm per ml (total of 10^{12} sperm per animal).

Female: A large ripe female can provide 10-15 ml of stripped gonad tissue yielding 50 to 100 million eggs.

Sperm Freezing

A series of multi factorial designed experiments was performed to examine a range of factors that influence the post thaw survival and fertility of sperm.

Effect of CPA: A series of trials was performed testing a range of CPAs at different concentrations; both for their toxicity to sperm and post thaw survival of sperm. Overall, DMSO at an in-straw concentration of 5% was the most effective CPA for sperm (Figure 1).

Effect of Diluent: A number of different diluents have been described as giving survival after freezing and three of these were compared (Figure 2). A polysaccharide, at high concentration in distilled water, was most successful under our conditions.

Effect of males: Some differences between batches of males were seen in the post thaw fertility (Figure 3).

Effect of dilution rate: Sperm to diluent ratios of 1:1 to 1:20 were examined. Optimal dilution ratio was around 1:10 (Figure 4).

Rate of sperm dilution pre freezing and post thaw: Moderate increases in fertility were seen when the diluent and CPA mixture was added to sperm in a stepwise manner and also when thawed sperm was placed into seawater by stepwise dilution (data not shown).

Effect of pre-freeze holding time: Sperm were held on ice for periods of up to 4 hours, between collection and processing, without any marked deleterious effect on post thaw fertility (data not shown).

Effect of equilibration temperature: Sperm were held in the diluent - CPA mixture at different temperatures (0^{o}C v 10^{o}C v 20^{o}C) for periods of 10 to 30 minutes. There were no significant differences between treatments (data not shown).

Effect of freezing rate: Freezing rates of -1^{o}C/min, -5^{o}C/min, -20^{o}C/min and -50^{o}C/min were evaluated with the better results being obtained at the faster freezing rates. However, there were interactions between freezing rate, dilution rate and diluent concentration (Figure 5).

Effect of thaw rates: No differences were found between the standard thaw (15 sec at 20^{o}C) and rapid thawing (2 sec at 75^{o}C) (data not shown).

Effect of straw size: Straws of 0.25ml, 0.5ml and 2.5ml were tested and at the faster freezing rates there were no straw size effects.

Frozen versus fresh sperm: The comparisons of fertility for fresh and frozen sperm in the earlier trials showed the need for a 10^{3} fold increase in numbers of frozen sperm to achieve comparable fertility. However, increases in fertility through changes to diluent concentration, dilution rate and rates of freezing indicate that a multiplication factor of about 10^{2} is currently required (see Figure 4).

Large-scale fertilisation. Large-scale fertilisations of selected batches of eggs (fertilised with either the fresh or frozen sperm from the same males) were successfully reared through to beyond settlement. Compared to fresh sperm, frozen sperm gave lower fertilisation and much greater variation among families. However, development through to ~10 mm spat was at least as high for the larvae resulting from the frozen sperm as the fresh sperm.

Eggs and Embryos:

Eggs. In initial experiments the immediate post-thaw membrane integrity of eggs was generally high but the majority of eggs lysed or exhibited blebbing of the egg membrane upon dilution of the CPA.

Observations:

1. Good quality eggs must be selected for freezing.
2. The presence of macromolecules during freezing and dilution may improve survival.
3. Stepwise addition and removal of CPA improves survival.

Improvements to cryopreservation methods have increased the proportions of eggs that maintained their membrane and morphological integrity. However when these eggs were subjected to fertilisation assays rates of fertilisation were low (Table 1).

Similar observations have been made for the limited vitrification studies; membrane and morphological integrity

TABLE 1: Representative results of morphological normality and fertilisation of Pacific oyster eggs following cryopreservation: effects of rate of CPA addition and removal.

Cryoprotectant		Percentage of Eggs		
		Normal		Fertilised
Addition	Removal	Immediate Post-thaw	Following dilution	
Fast	Fast	70	50	2 to 10%*
	Stepwise	75	75	10 to 25%*
Stepwise	Fast	80	50	0 to 2%
	Stepwise	90	75	0 to 1%

* **Range from fertilisations with 10^{4} to 10^{6} sperm per ml**

FIGURE 1: Effect of DMSO concentration (◆=5% DMSO, □=10% DMSO, Δ =15% DMSO) on the fertilisation curves of frozen Pacific oyster sperm.

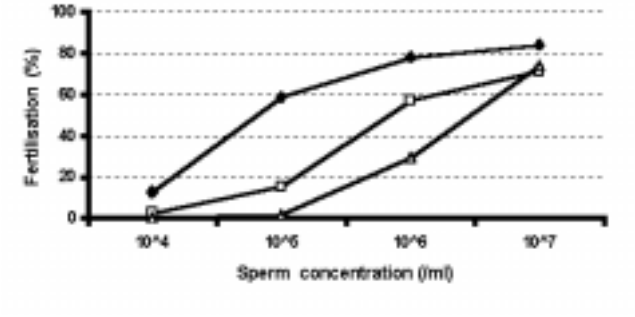


FIGURE 2: Effect of diluent type (◆=polysaccharide, □=complex salt solution,Δ =seawater) on the fertilisation curves of frozen Pacific oyster sperm.

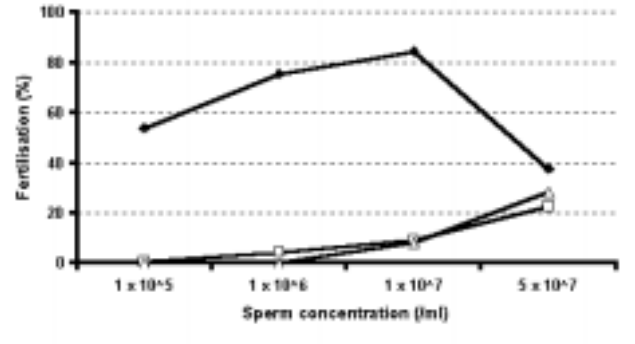


FIGURE 3: Effect of different batches of sperm (◆=Males A, □=Male B, Δ=Males C) on the fertilisation curves of frozen Pacific oyster sperm.

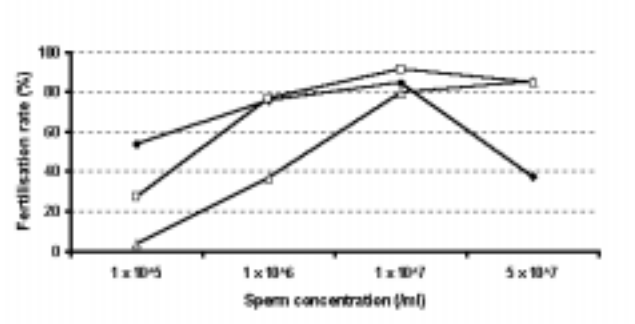


FIGURE 4: Effect of sperm: dilution ratio (◆=1:1, □=1:10, Δ =1:20, O = fresh sperm controls) on the fertilisation curves of frozen oyster sperm. (Note the 10 to 10^2-fold difference in fertility between fresh and frozen sperm.

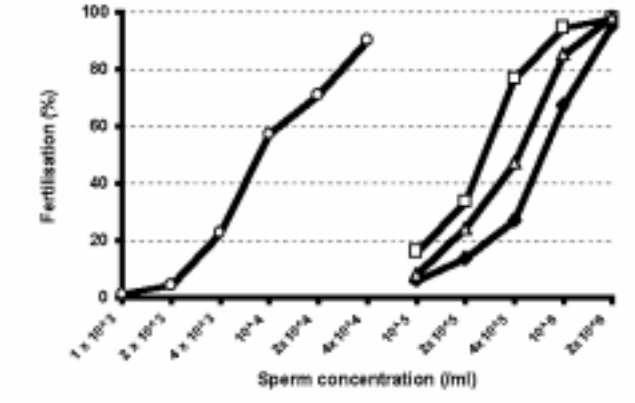


FIGURE 5: Interaction between diluent concentration (solid lines 0.7M v dotted lines 0.45M polysaccharide), freezing rate (□ -5°C /min or Δ -50°C/min) and sperm to diluent ratio (closed symbols 1:1 or open symbols 1:10).

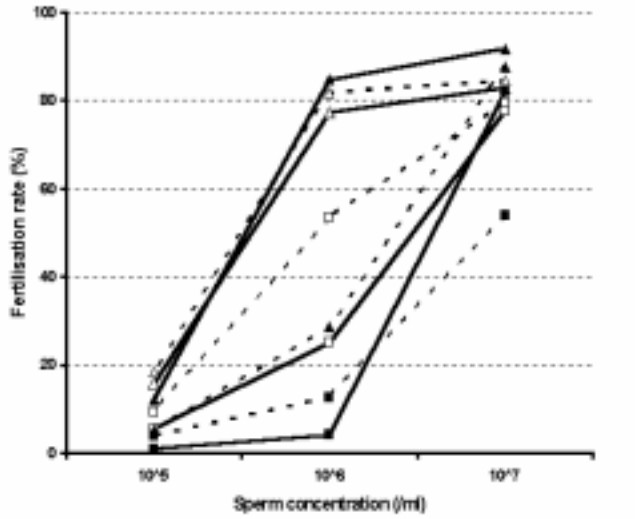
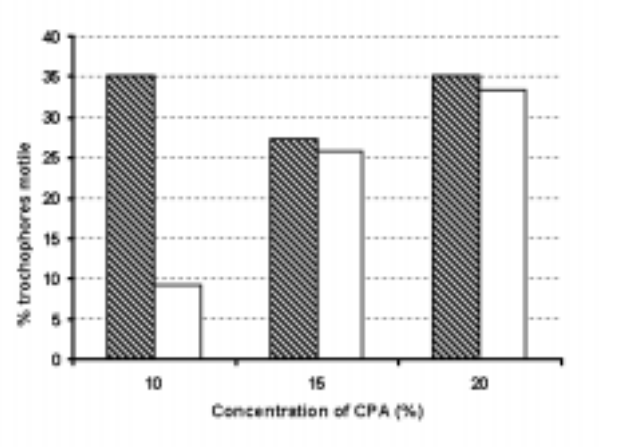


FIGURE 6: The effect of cryoprotectant concentration and its rate of addition on the post-thaw motility of Pacific oyster trochophores (hatched area = stepwise, open =single step).



can be improved to acceptable level but few eggs fertilise.

Embryos. All cryopreservation studies have utilised the trochophore larval stage.

Observations:

1. Motility of trochophores can be maintained even when they are exposed to cryoprotectants. The extent and vigour of motility is, however, dependent upon both cryoprotectant type and its rate of addition.
2. Similar to that observed for eggs, immediate post-thaw morphology is good but deteriorates during CPA removal.
3. CPA concentration and rate of its addition affect post-thaw motility (Figure 6).
4. Most post-thaw trochophores are morphologically abnormal (Table 2).
5. Few of the post-thaw trochophores continued normal development.
6. Vitrification trials have resulted in no motile trochophores post-warming although the incidence of morphological abnormality was reduced.

TABLE 2: Effects of freezing and thawing rates on the motility and morphological normality of Pacific oyster trochophores after thawing.

		Estimated Percentage of Trochophores			
Freezing rate	**Thawing rate**	**Immediate post-thaw**		**After CPA dilution**	
		Motile	**Damaged**	**Motile**	**Damaged**
Fast	Fast	50	5	60	80
	Slow	20	5	20	90
Slow	Fast	20	5	40	90
	Slow	10	5	10	90

DISCUSSION

The major differences in the approach to studies on the cryopreservation of shellfish sperm and that on domestic animals is that associated with the evaluation of the samples post thaw. With domestic animal semen the evaluation is based on sperm motility and viability as indirect markers of fertility. This is due to the difficulties (cost and logistical) of obtaining fertility information on each semen treatment investigated. These constraints even apply to the use of IVF to provide an indirect indication of fertility. The very large number of eggs obtained and the rapid development of the fertilised embryos of shellfish at ambient temperatures enable a very rapid, relevant and cheap direct measure of fertility to be obtained.

Apart from semen collection, the semen handling methods, types of CPAs and freezing procedures are very similar to those used for domestic animals. The choice of diluents differs basically because of the difference in requirements at the site of fertilisation. In domestic animals the need for packaging semen to fertilise the female's single egg has led to highly diluted sperm being suspended in material capable of maintaining their motility and viability. For shellfish however, the requirement is for packaging of the large numbers of sperm (large volumes and high concentrations) required to perform bulk fertilisation of millions of eggs.

With shellfish we have observed a very rapid loss of sperm motility upon dilution to low concentrations, while the motility of sperm held in a concentrated form is maintained for several days.

While the sperm freezing research with oysters has now produced a procedure that provides repeatable good post thaw fertility (capable of bulk fertilisation and subsequent development of normal spat) its application to other shellfish species has not been automatic. Modification of a number of factors has been necessary to obtain successful results, and protocols require further development.

Our preliminary studies have shown that oyster eggs and embryos can, to a degree, survive exposure to cryoprotectant solutions and cooling to low temperatures. In addition there are a number of differences between mammalian and shellfish eggs and embryos in handling and assessment.

Hundreds of thousands of shellfish eggs and embryos are available for use in any one batch (in the studies reported here these were used at concentrations of between 500,000 and 1 million/ml), which is vastly different to cases with mammalian eggs or embryos. Thus shellfish eggs and embryos are processed in a manner similar to processing semen and assessments of survival are performed on a "per straw" basis rather than an individual embryo basis. It may well be that survival rates around 20-30% in samples containing many thousands of embryos are adequate.

Our studies with eggs and embryos of other shellfish species have also identified differences and similarities in their responses to cryopreservation procedures. Although our modifications to the cryopreservation procedure for oyster eggs resulted in an increased number of post-thaw eggs with apparently normal morphology, fertilisation rates of these eggs remained low. This highlights the need for more fundamental studies of membrane permeability to cryoprotectants, of water transport kinetics and of the physiological events during fertilisation in these species.

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