The Cryopreservation of Pacific Oyster (Crassostrea gigas) Gametes
Introduction

The aquaculture, or farm fishery, industry in British Columbia is a rapidly expanding sector important to the provincial economy. In 2002, an estimated C$287 million in provincial revenue was contributed by the aquaculture sector in British Columbia, with C$29 million contributed through the sale of farmed shellfish and 53% of that figure as farmed oysters (BC Government Statistics). The oyster species with the most promising world market is *Crassostrea gigas*, better known as the Pacific or Japanese oyster. The market, as of 1996, was an estimated US$3.23 billion (FAO), making it one of the most profitable species to farm, and the market continues to expand. In British Columbia, however, the colder coastal waters makes the natural habitat in which these oysters will spawn, or reproduce, a very limited region (Kruzynski, 2004). The use of hatchery fertilization, including cryopreservation of gametes, has provided a way around the natural constraints on the oyster’s breeding habits, and thus greatly contributed to the aquaculture sector expansion currently underway in British Columbia.

Cryopreservation is the preservation of cells or tissues by freezing them at extremely low temperatures. This is an extremely advantageous means of supplying continuous gametes and embryos, no matter the spawning season, in aquaculture practices. There are many advantages to cryopreservation, including the avoidance of costs of otherwise having to manage a broodstock, and the role that it plays in selective breeding, where it can aid in providing greater control over parental combinations (Tervit et al., 2005). Also, as noted by He et al. (2004), the cryopreservation of tetraploid sperm can allow for the crossing between tetraploids and diploids, resulting in triploid oysters, a
commercially preferable oyster due to the reduced gonadal development, and thus, improved meat quality and growth obtained.

While an extremely useful procedure, the freezing and thawing that accompanies cryopreservation can be potentially damaging to the cell, even leading to the formation of ice crystals that can cause cell death (Harvey & Ashwood-Smith, 1982). A means of improving cryopreservation is the addition of cryoprotectants, which can act to prevent the damage associated with dehydration or ice crystal formation (Ieropoli et al., 2004). Though these cryoprotectants can have such a positive effect on the cryopreservation of cells, there is also a downside to their use. Depending on the cryoprotectant used, the concentration used, and the time that the cells are exposed to it, a toxic effect can be seen. Therefore, a balance must be found between the positive, protective effects of cryoprotectants, and the negative, toxic effects that can also be seen (Chao et al., 1997).

Due to all of the considerations to be made when cryopreserving cells, this paper focuses on the cryopreservation of C. gigas spermatozoa and oocytes separately, and the different factors that must be taken into account to do so as effectively as possible.

Cryopreservation of Spermatozoa

Although cryopreservation of male C. gigas gametes has been used for a number of years, a widespread and comprehensive procedure that optimizes recovery of spermatozoa that produce viable offspring has not been verified, and thus C. gigas spermatozoa cryopreservation has not become available on a commercial scale. A majority of the complications come from the fact that no universal cryoprotectant has been found, and those that are used based on their efficacy in other species show some
variation in optimal concentration with regards to *C. gigas*. These findings are most likely due to the fact that both the type and concentration of a cryoprotectant have been shown to be species-specific (Gwo, 1994).

Among the cryoprotectants shown to have a desirable combination of low toxicity and a sufficient protectant ability are dimethylsulfoxide (DMSO/Me$_2$SO), ethylene glycol (EG), 1-2 propylene glycol (PG) (Ieropoli et al., 2004) and methanol (MeOH) (Dong et al., 2005). Ieropoli et al. (2004) demonstrated that DMSO/Me$_2$SO, EG, and PG had similar effects on preservation of spermatozoa samples, and each showed only slightly toxic effects once the concentrations rose above 10% in the sample, while Dong et al. (2005) showed similar results with the use of DMSO/Me$_2$SO, they also demonstrated that the use of MeOH at a 6% concentration produced a high post-thaw motility rate (70%) and a fertilization rate that was 98%. Together, these results demonstrate a wide range of cryoprotectants that will produce desirable results when used at acceptable concentrations, as well as a possible front runner in the quest for an ideal cryoprotectant in the form of MeOH.

Variability in cooling rates have also proven to be difficult to unify into a standard practice. The key is to find a rate which allows for fast cooling to temperatures needed to store the spermatozoa in liquid nitrogen, within a time frame that ensures the majority of the gametes do not perish (lyse) due to excessive osmotic pressure within the cell from the formation of intracellular ice fragments (IIF) (He et al., 2004). Dong et al. (2005) have shown that a cooling rate of 5°C min$^{-1}$ to a temperature of -30°C, followed by a secondary cooling at a rate of 45°C min$^{-1}$ to -80°C, produced spermatozoa that upon thawing had properties most similar to fresh semen, and produced the highest rate of
fertilization. After reaching the -80°C temperature point, the samples could be immersed in liquid nitrogen for transportation or long term storage. The discovery that a two-stage cooling process produces more viable gametes opens a host of possibilities, as with better preservation and storage techniques comes the ability to ship greater quantities further distances and ensure viability at the destination, all points that will lead to a stronger aquaculture product.

Further research is needed to elaborate the most efficient methods for all stages of cryopreservation of spermatozoa in *C. gigas*, as a unified approach will make improving the techniques easier as well as ensuring that the market is satisfied with the product and return they receive from the process.

**Cryopreservation of Oocytes**

In contrast to the relatively low numbers of oocytes necessary for cryopreservation to be successful in human in vitro fertilization procedures, up to hundreds of thousands are needed in aquaculture. While previous oocyte freezing protocols have been severely limited to a handful experiments (e.g., Naidenko, 1997), a new study by Tervit et al. (2005) introduces a protocol that has been found to successfully cryopreserve *C. gigas* oocytes. A variety of different combinations and concentrations of cryoprotectants were studied, as well as the cooling rates of oocyte samples, and following thawing, the percentage of oocytes fertilized was analyzed. The proportion of thawed oocytes that were able to be fertilized and develop normally to eyed larvae was the measure of successful cryopreservation. It was found that EG (10%) and DMSO/Me₂SO (15%) were the most effective cryoprotectants. As well, the use of Milli-
Q water instead of seawater as a base medium improved fertilization. It is suggested by the authors that this may be due to reduced exposure to high Na\(^+\) concentrations, and thus reduced osmotic stress on the oocytes. The most favorable cooling rate was found to be 0.3 °C min\(^{-1}\).

A different study by Nascimento et al. (2005) on oyster gametes and embryos aimed to determine the best cryoprotectant to use based on differing toxicities of three common cryoprotectants. Exposure for 10, 20, or 30 minutes to differing concentrations of DMSO/Me\(_2\)SO, PG, or MeOH was tested to determine the concentration at which chronic effects were first seen. No significant differences were seen in the exposure to DMSO/Me\(_2\)SO on the toxicity to the gametes. In the case of PG, however, while there was an increase in toxicity with increased exposure for spermatozoa, no such increase in toxicity was seen for oocytes. This suggests that PG may be a suitable cryoprotectant for the preservation of oyster oocytes.

Based on these two relatively new studies, the optimal base mediums and cooling rates from holding temperatures can be concluded. Additionally, after studying the effects of various cryoprotectants, it appears that EG or DMSO/Me\(_2\)SO may be the most useful. Though it was found that PG did not have an increase in toxic effects in the study by Nascimento et al. (2005), Tervit et al. (2005), found that PG was less effective than other cryoprotectants studied. It should also be noted that MeOH was found to be the least effective, and most toxic for oocytes, suggesting that MeOH is not a suitable cryoprotectant to use.
Conclusion

With the variation in the conditions required for cryopreservation between spermatozoa and oocytes, it is easy to see why forming a single, unified cryopreservation procedure is so elusive. While certain conditions prove to be toxic to one, the other gamete has been shown to thrive, thus complicating the pursuit to find a commercially viable avenue for cryogenically processed gametes of *C. gigas*. With this species being such an important component of the burgeoning aquaculture industry in British Columbia, a province hard-hit by a downturn in its primary sectors such as forestry, it is vital to conduct further research. A full understanding of the most effective methods of preserving gametes cryogenically will allow this industry to grow to its full potential, and allow it to reap the benefits of such an important technology.
References


Chao NH, Lin TT, Chen YJ, Hsu HW, Liao IC. Cryopreservation of late embryos and early larvae in the oyster and hard clam. Aquaculture 1997; 155:31-44.


