

## NEW APPROACHES TO THE CRYOPRESERVATION OF FISH OOCYTES AND EMBRYOS

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### Keywords

Cryopreservation, fish, embryos, oocytes, conservation

### Summary

The dramatic decline in fish populations necessitates urgent action to enable gametes and embryo cryopreservation as an aid to conservation. However, high yolk content and low membrane permeabilities have frustrated their successful cryopreservation, by limiting water removal and cryoprotectant penetration. Research at Luton is exploring new approaches to overcoming these barriers to their cryopreservation.

The collapse of both fresh water and marine fish populations is causing great concern, and there is an increasing loss of fish species from many habitats. Retention of the genetic resource and the opportunity to ensure species survival and possible restocking depends on our ability to conserve relevant material. Ideally such a conservation programme would involve the cryobanking of eggs, sperm and early embryos. Whilst the cryopreservation of fish sperm has, in the main, been relatively successful, fish oocytes and embryos have not been successfully cryopreserved. There appear to be two main barriers to their cryopreservation, (i) low membrane permeability, makes the removal of water from the material and the penetration by cryoprotective agents difficult; and (ii) the large yolk mass of the oocyte and early embryo represents a compartment that is particularly difficult to reduce water activity. Both these features result in ice crystal formation during the freezing process. In addition the oocytes and embryos are prone to chilling injury unrelated to ice crystal damage.

Research on fish embryo cryopreservation at Luton is currently focused on the three new approaches: (i) permeabilisation of embryo membranes, through media modification and ultra-sound treatment, (ii) direct modification of the yolk mass by micro-manipulation, and (iii) the use of impedance spectroscopy for rapid assessment of embryo membrane permeability. The approach to fish oocyte cryopreservation has been directed at optimizing low toxicity cryoprotectant mixtures, and their use in protecting oocytes at a range of sub-zero temperatures. Both the embryo and oocyte programmes are considering both slow cooling and rapid cooling (vitrification) protocols as the freezing method.

In the study on the effect of external medium composition on membrane permeability of both intact and dechorionated zebrafish (*Danio rerio*) embryos, different concentrations of Hanks Balanced Salt Solution (HBSS) with varying levels of calcium ions. The results showed external medium composition to have no effect on membrane permeability at early developmental stages. However, at later stages of development, embryos spawned into 30% HBSS were less permeable than embryos in system water, irrespective of calcium concentration. A decrease in permeability was observed with dechorionated embryos in 30% HBSS, however the effect was not significant. These findings suggest that the composition of the external medium affects the permeability of the chorion or, more likely, diffusion through the perivitelline space between the vitelline membrane and the chorion.

In ultrasound studies, embryos at 8-16 cell and 50% epiboly stages (in embryo medium) were treated in an ultrasound bath for 5 and 15min at 22°C with a range of frequencies (24, 48, 240, 480, 1000, 10000 and 50000KHz) and voltage (50, 75, 100, 125, 150 and 175V) combinations. Earlier stage embryos were found to be more sensitive to ultrasound treatment and significant differences were found when embryos were treated for different time periods. The study also showed that the higher frequencies do not have significant effect on embryo survival.

To study the impedance change of zebrafish embryos during cryoprotectant exposure, 3, 6 or 9 embryos at 50% epiboly stage were loaded into the holding well in embryo medium, which was then removed and replaced by 0.5, 1.0, 2.0 or 3M methanol or DMSO. The impedance change of the embryos in cryoprotectant solutions were monitored over 30min at room temperature immediately after embryos were exposed to cryoprotectants at a frequency range of 10 Hz – 10<sup>6</sup> Hz. The impedance change of the untreated embryos in embryo medium was used as control. Results from this study identified the optimum frequency range to be 10<sup>2</sup> - 10<sup>3</sup> Hz that provided good sensitivity and reproducibility. Significant impedance changes were detected after embryos were exposed to different concentrations of cryoprotectants. The relationships between change of impedance and cryoprotectant penetration are currently being established.

In addition to protocol design for successful cryopreservation of oocytes and embryos, studies have been undertaken on the impact of cryopreservation on genetic integrity. Mitochondrial DNA from fish embryonic cells showed an increased frequency of base pair mutation following cryo-treatment.

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