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Short communication

Freezing of viable embryos and larvae of marine shrimp, *Penaeus semisulcatus* de Haan

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Although sperm cryopreservation has been carried out successfully in a number of commercially important aquatic species, particularly in some teleost fish (see review of Rana in Muir & Roberts 1993) and also shellfish (Subramoniam 1993), the technology is still not at the stage of advanced commercial application that is seen in domestic mammals. Cryopreservation of eggs and embryos, of aquatic animals however, is a virgin field in cryobiology and has not yet received any appreciable amount of attention. The first successful attempt at the cryopreservation of embryos of sea urchin was reported by Asahina & Takahashi (1978). Later Zell (1978) and Erdahl & Graham (1980) have reported preliminary attempts to freeze the eggs of rainbow trout. Studies have been carried out to cryopreserve the embryos of Japanese medaka *Oryzias letipes* Schlegel (Aril, Namai, Gomi & Nakazawa 1987), rainbow trout *Oncorhynchus mykiss* Walbaum (Nilsson & Cloud 1993) and zebra fish *Brachydanio rerio* Weber & de Beaufort (Zhang, Rawson & Morris 1993). In recent years some attempts have also been made to cryopreserve the embryos and nauplii of shrimp *Penaeus indicus* H Milne Edwards (Subramoniam & Newton 1993; Subramoniam 1994; Simon, Dumont, Cuende, Diter & Aquacop 1994). From a review of the literature it appears that studies on the cryopreservation of embryos and larvae of fish and shellfish is still in a rudimentary phase and much remains to be done. The present study was carried out with a view to developing a method for the successful freezing of viable embryos and larvae of marine shrimp, using the experimental protocol now described.

Mature green tiger prawns, *Penaeus semisulcatus* de Haan were collected from Gulf of Mannar and brought to the Central Marine Fisheries Research Institute's hatchery at Mandapam Camp, Tamilnadu, South India. After acclimation to hatchery conditions they were made to spawn in the laboratory either naturally or by means of the eyestalk ablation technique. After spawning, the eggs were collected and their developmental stages monitored carefully. Once the eggs entered the advanced embryonic stage (8–10 h after spawning) they were used for cryopreservation studies. Experimental trials were also made simultaneously to cryopreserve freshly hatched larvae (nauplii). The cryoprotectants used for freezing the embryos and nauplii were dimethylsulfoxide (DMSO; S.d. Fine Chemicals Ltd, Bombay), glycerol and mixtures of DMSO and glycerol. The extender used to dilute the cryoprotectants was standard physiological saline. Its composition is given in Table 1. Different concentrations of the cryoprotectants DMSO and glycerol (5, 10, 15, 20, 25 and 30%) were prepared in physiological saline solution and tested for their efficacy in preserving the viable embryos and larvae at low freezing temperature. There was no significant change in the pH of the cryoprotectants after diluting

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with physiological saline solution. The embryos and nauplii at the required stage were collected by filtering them through nylon bolting silk (400 mesh) and approximately 200 embryos per 100 ml vial were placed in different grades (5–30%) of DMSO, glycerol and a mixture (1 : 1) of DMSO and glycerol. The embryos and nauplii were transferred to the 100 ml plastic vials as quickly as possible. The different grades of cryoprotectants were pre-chilled at 5 °C before use. After transferring the embryos and nauplii into the different grades of cryoprotectants, all the vials were immediately transferred to a programmable cryostat (American Opticals, USA) held at –10 °C. The overall cooling rate was adjusted to produce a temperature drop of –1 °C per 5 min. The viability of the cryopreserved embryos and nauplii was monitored at regular intervals of 1, 2, 4 and 6 h. At each interval frozen embryos and nauplii were thawed to room temperature, washed throughly in physiological saline solution and then transferred in glass troughs (10 litre capacity) containing aerated sea water

Table 1 Composition of extender used. The pH of extender solution 7.5

Chemical ingredients	(gm %)
NaCl	0.650
KCl	0.014
$CaCl_2$	0.012
$MgCl_2$	0.005
$NaHCO_3$	0.020
$KHCO_3$	0.001
Glucose	0.100

Table 2 Hatching rate (%) of embryos (freeze–thawed) of shrimp *P.semisulcatus* frozen (–10 °C) in different cryoprotectants. When Dimethylsulfoxide (DMSO) and glycerol were used independently, the hatching rate of embryos was nil at the concentrations 5–30%. Values are given as means ± SD

	Concentrations (%) of cryoprotectants used						
	5	10	15	20	25	30	Duration of cryopreservation (h)
DMSO + Glycerol	55 ± 3	50 ± 2	50 ± 2	40 ± 2	–	–	1
	40 ± 2	50 ± 3	50 ± 3	50 ± 2	–	–	2
	40 ± 2	50 ± 3	50 ± 2	50 ± 2	–	–	4
	40 ± 1	50 ± 2	50 ± 3	50 ± 3	–	–	6

Table 3 Revival rate (%) of nauplii (freeze–thawed) of shrimp *P.semisulcatus* frozen (–10 °C) in different cryoprotectants. When DMSO alone was used as a cryoprotectant the revival rate of nauplii was found to be nil at concentrations 5–30%. Values are given as means ± SD

	Concentrations (%) of cryoprotectants used						
	5	10	15	20	25	30	Duration of cryopreservation (h)
Glycerol	10 ± 1	–	–	–	–	–	1
DMSO + Glycerol	55 ± 3	55 ± 3	50 ± 2	50 ± 2	–	–	
Glycerol	5 ± 1	–	–	–	–	–	2
DMSO + Glycerol	55 ± 2	55 ± 3	50 ± 3	50 ± 2	–	–	
Glycerol	5 ± 1	–	–	–	–	–	4
DMSO + Glycerol	55 ± 2	55 ± 3	50 ± 2	50 ± 2	–	–	
Glycerol	5 ± 1	–	–	–	–	–	6
DMSO + Glycerol	40 ± 1	50 ± 1	50 ± 2	50 ± 2	–	–	

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(salinity 35%) to study their viability. The viability of the embryos was determined by allowing them to hatch out successfully as nauplii. In the case of the cryopreserved nauplii, viability was demonstrated by reviving their activity on thawing. All experiments were repeated three times and the results obtained are pooled and given in Tables 2 and 3.

It was found that when DMSO and glycerol were used independently as cryoprotectants, the viability of the freeze–thawed embryos and nauplii in all the grades was nil as the embryos did not hatch out and the nauplii were dead. However, 40–50% of the embryos preserved in concentrations of 5–20% of the mixture of DMSO and glycerol, hatched out successfully to nauplii after 1–6 h of preservation. In the higher concentrations of cryoprotectant, i.e. 25–30%, however, the embryos did not show any signs of hatching.

The nauplii preserved in the different grades of DMSO (5–30%) did not revive at all. Those preserved in glycerol, however, did show a limited capacity for revival, although it was only 5–10% in the 5% concentration of glycerol after 1 and 2 h of preservation. No nauplii survived in the remaining grades of glycerol. In the mixture (DMSO + glycerol) the revival rate of freeze–thawed nauplii was 50% or more up to 6 h in the 5–20% grades. However, the revival rate was nil in the 25 and 30% concentrations. The growth in the case of both the nauplii of the freeze–thawed embryos and the revived frozen nauplii was monitored until they grew to the size of juveniles and was found to be normal when compared with their control counterparts.

Limited success has been claimed in the cryopreservation of viable eggs/embryos and larvae of finfish and shellfish. The reason given is that in higher animals, due to the large size of the eggs and embryos, there will be interference in the penetration of the cryoprotectants and thus prevention of uniform cooling during the cryopreservation process. Sometimes the large yolk present in the eggs and embryos tends to develop crystals while freezing and damage the egg structures (Seymour 1994). In shrimps, although the size of the eggs and embryos is small, the eggs naturally absorb water soon after their release in order to get swollen and activated for fertilization. After fertilization, a strong hatching envelope (protective extra-cellular matrix) forms around the egg (Clark & Griffin 1993). The presence of water and the thick protective envelope surrounding the eggs makes the freezing of viable eggs and embryos problematic (Simon *et al.* 1994). Subramoniam & Newton (1993) were the first to successfully preserve nauplii of *P. indicus*. They held them at −30 °C using liquid nitrogen and reported survival of 82% for nauplii frozen to −30 °C and 63% at −40 °C. They further reported that the toxicity response of the various cryoprotectants (glycerol, ethylene glycol, methanol, DMSO, formamide) to nauplii was similar but while cryopreserving the embryos using the cryoprotectants detailed above they found that glycerol was more toxic to the morulae stage embryos. They could not achieve any success with regard to the cryopreservation of embryos.

Zhang *et al.* (1993), while working on cryopreservation of zebra fish embryos, studied the toxicity response of different cryoprotectants such as methanol, DMSO, glycerol, ethenediol and sucrose on different stages of embryonic development. They found that DMSO and ethenediol are more toxic to fish embryos of heart beat stage. They obtained the best embryo survival rate at −10 °C and the least was at −30 °C. Ice formation within the egg was found to be the main factor affecting the survival of the embryos. In the present study, when DMSO and glycerol were used independently they proved to be more toxic. Similarly, higher concentrations of the cryoprotectants (25 and 30%) were found to be unsuitable for cryopreservation at −10 °C. Further investigation is warranted to refine the technique, once the exact nature of the damage to the embryos and nauplii by various factors during cryopreservation is known.

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