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Brief communication

The potential for cryopreserving larvae of the sea urchin, *Evechinus chloroticus* ☆

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Abstract

Larvae of the sea urchin, *Evechinus chloroticus*, at varying stages of development, were assessed for their potential to survive cryopreservation. Ethylene glycol (EG) and dimethyl sulphoxide (Me_2SO), at concentrations of 1–2 M, were evaluated as cryoprotectants (CPAs) in freezing regimes initially based on methods established for freezing larvae of other sea urchin species. Subsequent work varied cooling rate, holding temperature, holding time, and plunge temperature. Ethylene glycol was less toxic to larvae than Me_2SO. However, no larvae survived freezing and thawing in EG. Larvae frozen in Me_2SO at the gastrula stage and 4-armed pluteus stage regained motility post-thawing. The most successful freezing regime cooled straws containing larvae in 1.5 M Me_2SO from 0 to −35 °C at 2.5 °C min^{-1}, held at −35 °C for 5 min, then plunged straws into liquid nitrogen. Motility was high 2–4 h post-thawing using this regime but decreased markedly within 24 h. Some 4-armed pluteus larvae that survived beyond this time developed through to metamorphosis and settled. Different Me_2SO concentrations and supplementary trehalose did not improve long-term survival. Large variation in post-thaw survival was observed among batches of larvae produced from different females.

Keywords: Freezing; Sea urchin; Marine invertebrate; Larvae; Pluteus; *Evechinus chloroticus*

Marine invertebrates are becoming increasingly used in laboratory experiments, bioassays, aquaculture, and fisheries gene banking. However, their use is associated with logistical problems that include seasonal constraints in access to ripe animals; conditioning broodstock for experiments and juvenile production out of season; and genetic heterogeneity between experiments which adds variation to experimental results. The use of cryopreserved larvae is one route to overcoming these problems and providing a continual year-round source of material.

Techniques for cryopreserving larvae have been investigated previously for several marine invertebrates (see [23] for review). Development of cryopreserved larvae through to metamorphosis, settlement and beyond has been reported in only a few studies [12,16,18]. In sea urchins, a range of larval stages have been evaluated and successful cryopreservation has mainly been achieved with the blastula and

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pluteus stages, although some studies also report post-thaw survival at the gastrula and prism stages [3–7,12,16,17].

This study evaluated the feasibility of cryopreserving larvae of the New Zealand sea urchin, *Evechinus chloroticus*. Preliminary experiments investigated the toxicity of two CPAs, dimethyl sulphoxide (Me_2SO) and ethylene glycol (EG), which had been used in previous studies to cryopreserve larvae of other sea urchin species. Freezing and thawing methods that were successful for these other species were used as the basis for initial attempts at cryopreservation. Subsequent experiments examined the effect of different cooling rates, holding temperatures, holding times, and ice seeding. The effect of adding the extracellular CPA trehalose was also evaluated. Larvae produced using eggs from different females were assessed to see if they differed in their ability to survive cryopreservation. Finally, the ability of larvae that survived cryopreservation to develop to later stages and reach settlement was also studied.

Sea urchin maintenance, spawning, and fertilization were carried out as described previously [1]. Resulting larvae were maintained at a density of 10–20 mL^{-1} in culture jars containing 4 L of 1 μm-filtered seawater (FSW). The cultures were stirred continuously by suspended paddles that moved backward and forward over the cultures every few seconds. Approximately half of the FSW in the cultures was replaced every 2–3 days. Swimming larvae were selected for experiments by removing the stirring paddles, decanting the larvae at the surface and pouring onto a mesh sieve. The larvae were then resuspended at a density of ~800–3000 mL^{-1} and experiments were started within 5 min to minimize any effects of holding the larvae at such high concentrations.

The toxicity of dimethyl sulphoxide (Me_2SO) (BDH Supplies, Poole, England) and ethylene glycol (EG) (Sigma Chemicals, St. Louis, MO) was first evaluated on two larval stages, gastrula and 4-armed pluteus. Larvae were exposed to 1, 1.5 or 2 M CPA at 15 °C for up to 30 min. Different methods of adding and removing the CPAs were tested to try to reduce perceived osmotic shock to larvae. The effect of adding and removing Me_2SO on ice rather than at 15 °C to reduce toxicity was also evaluated. The ability of larvae to recover motility following CPA exposure and removal was used to assess toxicity.

In general, larvae stopped swimming within seconds of being exposed to CPA. The larvae started moving again approximately 1–2 h after CPA removal, and motility was assessed at this time. Many larvae remained on the bottom of the tissue culture well rather than swimming in the water column or at the surface as was observed with the untreated (control) larvae. Different batches of larvae responded variably to Me_2SO and EG (Table 1). Overall, recovery of motility was high following exposure to 1 or 1.5 M Me_2SO but decreased following exposure to 2 M Me_2SO for 30 min. Exposing the larvae to Me_2SO on ice, rather than at 15 °C, did not reduce this effect. The effect of EG was dependent on the stage of development. A notable decrease in motility was observed in gastrula stage larvae following exposure to 2 M EG. In contrast, the motility of 4-armed pluteus larvae remained high in all concentrations of EG.

Table 1
Effect of cryoprotectant (CPA) exposure on motility of sea urchin larvae

Larval Stage	CPA	CPA concentration (M)	Percentage of motile larvae after CPA addition and removal
Gastrula	Me_2SO	1	87–92
		1.5	80–94
		2	46–83
	EG	1	93–95
		1.5	93–94
		2	16–47
4-Armed pluteus	Me_2SO	1	78–93
		1.5	83–100
		2	21–100
	EG	1	97–99
		1.5	91–98
		2	93–95

Larvae were exposed to indicated concentrations of CPA for 30 min and then removed gradually over 30 min in 15 fixed molarity steps at 1 min intervals or by continuous dropwise addition. Data presented represent two experiments carried out on batches of larvae from pooled sperm and eggs. Control larvae were >95% motile in all batches. Experiments were conducted at 15 °C.