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Membrane permeability characteristics and osmotic tolerance limits of sea urchin (*Evechinus chloroticus*) eggs☆

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Abstract

Development of effective cryopreservation protocols relies on knowledge of the fundamental cryobiological characteristics for a particular cell type. These characteristics include osmotic behaviour, membrane permeability characteristics, and osmotic tolerance limits. Here, we report on measures of these characteristics for unfertilised and fertilised eggs of the sea urchin (*Evechinus chloroticus*). In NaCl solutions of varying osmolalities, sea urchin eggs behaved as ideal linear osmometers. The osmotically inactive volume (v_b) was similar for unfertilised and fertilised eggs, 0.367 ± 0.008 (mean $\pm$ SE) and 0.303 ± 0.007, respectively. Estimates of water solubility (L_p) and solute permeability (P_s) and their respective activation energies (E_a) for unfertilised and fertilised eggs were determined following exposure to cryoprotectant (CPA) solutions at different temperatures. Irrespective of treatment, fertilised eggs had higher values of L_p and P_s. The presence of a CPA decreased L_p. Among CPAs, solute permeability was highest for propylene glycol followed by dimethyl sulphoxide and then ethylene glycol. Measures of osmotic tolerance limits of the eggs revealed unfertilised eggs were able to tolerate volumetric changes of −20% and +30% of their equilibrium volume; fertilised eggs were able to tolerate changes ±30%. Using membrane permeability data and osmotic tolerance limits, we established effective methods for loading and unloading CPAs from the eggs. The results of this study establish cryobiological characteristics for *E. chloroticus* eggs of use for developing an effective cryopreservation protocol. The approach we outline can be readily adapted for determining cryobiological characteristics of other species and cell types, as an aid to successful cryopreservation.

Keywords: Sea urchin; Eggs; Membrane permeability characteristics; Osmotic tolerance limits; Cryopreservation

Sea urchins are used as a model system in several areas of research including developmental biology and embryology [10,11], marine pollution [8,17,38], and aquaculture and fisheries [15,21]. The ability to cryopreserve both sperm and eggs

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would be of practical benefit to these areas of research. It would provide a reliable source of gametes for experiments and juvenile production outside the natural breeding season, as well as enabling total control over parental input [5,6,46].

Successful cryopreservation of oocytes from mice and cattle has been reported and, while still at an experimental stage, initial reports concerned with the cryopreservation of human oocytes are promising [1,37,41,53]. However, post-thaw survival, fertilisation rates, and development rates have often been low or highly variable. Existing protocols for these species require refining and improvement before oocyte cryopreservation can become a routine procedure like sperm and embryo cryopreservation. Attempts to cryopreserve unfertilised sea urchin eggs have so far been unsuccessful [2,3]. However, for fertilised eggs, 10% survival has been reported following freezing and thawing for eggs of the sea urchin, *Strongylocentrotus intermedius* [3]. Although this is relatively low, it indicates that effective cryopreservation of sea urchin eggs is feasible.

A key to successful cryopreservation has been the use of controlled slow cooling and vitrification as methods of freezing. These methods generally require the presence of a permeating cryoprotective agent (CPA). Most cells are more permeable to water than to CPAs [12,40]. Therefore, they initially shrink when a CPA is added and then swell to a new equilibrium volume as the CPA permeates. Likewise, when a CPA is removed from cells, the cells swell initially and then shrink back to isotonic volume as the CPA diffuses out. The volumetric changes caused by the addition and removal of CPAs can be injurious if they exceed the cells' osmotic tolerance limits. In practice, these changes can be minimised by adding and removing the CPA in steps or removing the CPA in the presence of an osmotic buffer such as sucrose.

If the fundamental cryobiological characteristics of a particular cell are known, they can be used to formulate optimal methods for loading and unloading CPAs [12,22,27,39,47]. The characteristics include: surface area to volume ratio, osmotically inactive volume (v_b), water permeability (L_p), solute (CPA) permeability (P_s), effect of temperature on L_p and P_s (their activation energies (E_a)), and osmotic tolerance limits of the cell. Knowledge of these characteristics may also be used to predict the rates of water loss in cells frozen at different cooling rates [7,32,33].

This study describes the determination of the membrane permeability characteristics and osmotic tolerance limits of unfertilised and fertilised eggs of the sea urchin, *Evechinus chloroticus*. In addition, optimised methods for loading and unloading common CPAs from these eggs are given. The results presented in this study will be of practical benefit to future studies developing methods to cryopreserve these eggs.

Materials and methods

Reagents

Reagents used in the study were of analar grade or higher. Dimethyl sulphoxide (Me_2SO) was obtained from BDH supplies (Poole, England). Ethylene glycol (EG) and propylene glycol (PG) were obtained from Sigma Chemicals (St. Louis, MO). Cryoprotectant solutions were prepared in artificial seawater, pH 8.2, so that the molal concentration of salts were the same as seawater in all solutions [16].

Sea urchin collection, maintenance, and spawning

Mature sea urchins (*E. chloroticus*) were collected by SCUBA divers from the Marlborough Sounds and Doubtful Sound, New Zealand during the natural spawning season (October to March). Sea urchins were maintained in the laboratory in running seawater at ambient temperature and fed *Macrocystis pyrifera* in continuous supply.

Spawning was induced by injecting 1.5–3 ml of 0.5 M KCl through the peristomal membrane into the coelom of each urchin [48]. Spawning typically started within 1 min of injection. Females were inverted over a beaker containing seawater and left to release eggs. "Dry" sperm were aspirated from the surface of each male. Gametes from each individual were maintained separately.

Eggs were fertilised by adding fresh sperm at a ratio of 100–1000 sperm/egg. Experiments with

fertilised eggs were initiated 15 min after sperm were added to the eggs.

Osmotic behaviour

A key assumption of membrane transport models is that cells follow the Boyle–van't Hoff relationship and respond to changes in osmolality as ideal osmometers [18,20,22,54]. That is, they show a linear relationship between equilibrium cell volume and the reciprocal of extracellular osmolality. To confirm that sea urchin eggs meet this assumption, the equilibrium volume of unfertilised and fertilised eggs was measured following exposure to anisoosmotic NaCl solutions. These solutions were prepared by diluting a 2-M NaCl solution with Milli-Q water to obtain osmolalities in the range 0.6–2.0 Osm. The osmolality of each solution was calculated from its molarity as described [52]. The osmolality of seawater was determined using a vapour pressure osmometer (Wescor model 5500, Wescor, Logan, UT).

Fifty microlitres of packed eggs (approximately 10^5 eggs/ml) was added to a petri dish containing 25 ml NaCl test solution. After the eggs had equilibrated (15–90 min dependent on test solution), the petri dish was placed over an inverted microscope (Olympus IMT-2, Ina, Japan). Images of individual eggs were recorded on a video recorder (Panasonic model AG-6730, Matsushita Electric Industrial, Osaka, Japan) connected to a video camera on the microscope (Panasonic model WV-CL 350/A, Matsushita Electric Industrial, Osaka, Japan). Recorded images were digitised and analysed using NIH Image (v 1.61, US National Institutes of Health, USA). The maximum and minimum cross-sectional diameters of each egg were measured and depending on the shape, volume was calculated using the formula for either a sphere or a prolate spheroid. For each test solution, 50 eggs from each of five females were analysed. Egg volume was normalised to the average volume of an egg in seawater. The normalised volumes were plotted according to the Boyle–van't Hoff relationship (normalised volume versus 1/osmolality) and a regression line was fitted to assess linearity. The fraction of the egg volume considered to be osmotically inactive (v_b) was determined from the plot by extrapolating the concentration of the extracellular solution to an infinite osmolality.

Determination of water and solute permeability of sea urchin eggs (L_p and P_s)

The volume changes of eggs exposed to different CPA solutions were measured. Three CPAs (Me_2SO, EG, and PG) were evaluated in artificial seawater at 5, 10, 15, and 20 °C. As a comparison, the volume changes of eggs exposed to 2.0 Osm NaCl were also recorded to determine water permeability in the absence of permeating solutes.

Approximately 20 μl of packed eggs was added to a temperature-controlled chamber (Heat Controller 1.0, Campus Electronics, Dunedin, New Zealand) that was pre-filled with 2 ml CPA solution. The volumetric change of each egg was recorded over a 20-min period using the microscope and video analysis system described. Throughout the experiment, the temperature of the CPA solution was maintained within 1 °C of the test temperature. For each treatment, an average of 20 eggs was analysed from five females. To alleviate any impact of egg age, five different females were used for each CPA across the entire experiment.

The experimental data were fitted to a two-parameter model to determine the water (L_p) and solute permeability (P_s) of unfertilised and fertilised sea urchin eggs [9,22]. In the model, water flux is expressed as

$$\frac{dV_w}{dt} = -L_p ART(M^e - M^i), \tag{1}$$

where V_w is the volume of water inside the cell, L_p is the water permeability, A is the surface area of the cell, R is the universal gas constant (1.987×10^{-3} kcal/K/mol), T is the absolute temperature, and M^e and M^i are the external and internal osmolality, respectively. The surface area of the plasma membrane was assumed to be smooth and related to the initial cell volume (V_0) by

$$A = 4.836 V_0^{2/3} \tag{2}$$

(*Note.* The error in using this formula (which assumes the eggs are spherical) for eggs that are

prolate spheroids rather than the prolate spheroid formula which takes into account eccentricity was estimated to be less than 1% (data not shown)). Solute flux is expressed as

$$\frac{dN_s}{dt} = P_s A(a_s^e - a_s^i), \tag{3}$$

where N_s is the number of moles of permeating solute inside the cell, P_s is the solute permeability, and a_s^e and a_s^i are the external and internal activity of the permeating solute, respectively.

To fit the experimental data to the two-parameter model and derive best estimates of L_p and P_s, least-squares curve fitting was carried out with the aid of Berkeley Madonna 8.0 software (University of California, Berkeley, CA). Integration was performed using fourth-order Runge–Kutta integration. This was done for each individual egg. Only eggs that were considered approximately round or prolate were analysed.

Activation energies (E_a) for L_p and P_s

For determination of the activation energies (E_a) for L_p and P_s, an Arrhenius relationship was assumed. Namely,

$$P = P_0 \exp\left(-\frac{E_a}{RT}\right), \tag{4}$$

where P is the membrane permeability parameter of interest and P_0 is a constant determined by fitting the experimental data. Arrhenius' plots of the natural logarithm of L_p and P_s against the reciprocal of absolute temperature were used to obtain values of E_a from the equation

$$E_a = -R \cdot \text{slope}, \tag{5}$$

where R is the universal gas constant.

Determination of osmotic tolerance limits

The osmotic tolerance limits of unfertilised and fertilised eggs were determined by assessing the development of eggs following their equilibration with anisoosmotic NaCl solutions. Test solutions were prepared by diluting a 3-M NaCl stock solution with Milli-Q water to obtain osmolalities in the range 0.4–5.2 Osm [52]. Approximately 100 µl of seawater containing eggs was added to a small beaker containing 10 ml of test solution at 15 °C. After a period of equilibration (10–160 min dependent on test solution), the eggs were returned to seawater and their subsequent development up to the blastula stage was assessed. The Boyle–van't Hoff relationship and the measured inactive volumes, v_b, were used to calculate the expected equilibrium volume of the eggs in each solution. Comparing these volumes with the percentage of eggs successfully developing to the blastula stage allowed a determination of the volume changes that the eggs were able to tolerate. The experiment was carried out on eggs collected from five individual females.

Modelling loading and unloading of CPA from eggs

The values determined for the osmotically inactive volume (v_b), water permeability (L_p), solute permeability (P_s), and activation energies for L_p and P_s(E_a) were used in combination with knowledge of the volume changes that the eggs could tolerate to formulate optimal protocols for loading and unloading CPAs from the eggs.

Statistical analysis

Statistical analysis was performed using Minitab 13.31 software (Minitab, State College, PA). Regression analysis was used to assess the relationship between osmolality and normalised egg volume. For the experiment investigating the effect of fertilisation, CPA, and temperature on water and solute permeability, treatment means were compared using ANOVA with animal included as a nested factor. The data were normalised by natural log transformation before being analysed and residuals were checked to ensure that the assumptions of heterogeneity and normality were not violated. When appropriate, the Tukey test was used to identify significant differences between treatment groups. The Kramer modification was made when treatment groups of unequal sample size were compared [23,55].

Results

Osmotic behaviour

Sea urchin eggs followed the Boyle–van't Hoff relationship and behaved as ideal linear osmometers over the range 0.6–2.0 Osm (Fig. 1; $R^2 = 0.812$ for unfertilised eggs and 0.846 for fertilised eggs). The osmolality of seawater was determined to be 0.996 ± 0.002 Osm (mean ± SE). The osmotically inactive volume (v_b) was found by extrapolating the concentration of the extracellular solution to infinite osmolality. This gave a value of 0.367 ± 0.008 (mean ± SE) for unfertilised eggs and 0.303 ± 0.007 for fertilised eggs.

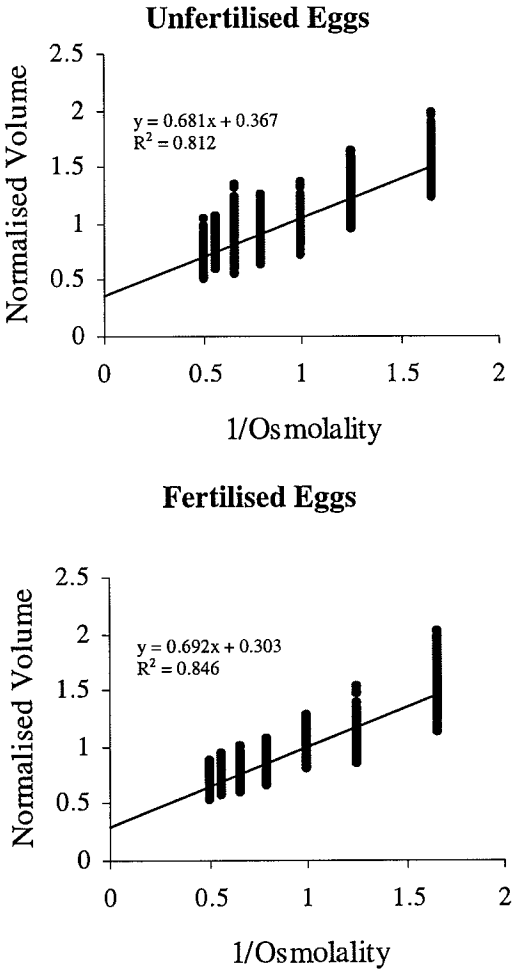


Fig. 1. Boyle–van't Hoff plots for unfertilised (upper panel) and fertilised (lower panel) sea urchin eggs. The data points represent the normalised volume (mean ± SE) of eggs equilibrated in NaCl solutions ranging from 0.6 to 2 Osm and in seawater (0.996 Osm). For each osmolality tested, a total of 250 eggs was measured from five females.

Water permeability (L_p) and solute permeability (P_s) of sea urchin eggs

When sea urchin eggs were exposed to CPA solution their volume decreased rapidly during the first few minutes of exposure. After reaching a minimum, their volume gradually increased again over several minutes. On occasion the volume did not increase and this was interpreted as membrane damage. The permeability of the eggs to water (L_p) and solute (P_s) was determined by least-squares fitting a curve to the experimental shrink–swell data using the two-parameter model described [22]. A representative example is shown in Fig. 2. The values of L_p and P_s derived for each CPA and temperature are summarised in Table 1 for both unfertilised and fertilised eggs.

The value of L_p varied significantly between unfertilised and fertilised eggs and among the CPA types and temperatures evaluated ($p < 0.001$). Interactions among the three factors were also highly significant ($p < 0.001$). Overall, the value of L_p for unfertilised eggs was approximately half that for fertilised eggs. The value of L_p was also affected by the presence of a CPA (Tukey test, $p \leqslant 0.05$). In

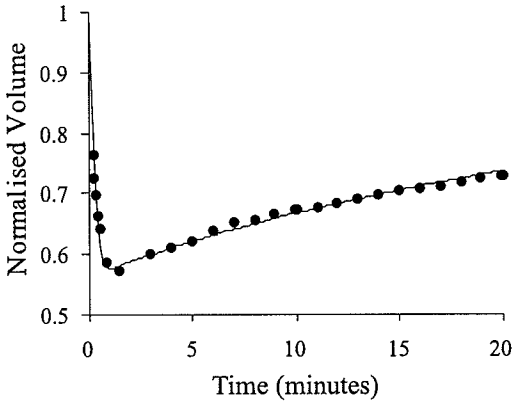


Fig. 2. Volumetric response of an individual fertilised sea urchin egg exposed to 1.5 M Me_2SO in artificial seawater at 15 °C. The data points represent observed volume changes. The solid line represents fitted volume changes.

Table 1
Values for L_p and P_s for sea urchin eggs exposed to 1.5 M CPA in artificial seawater at various temperatures

CPA	Temperature (°C)	n	L_p (μm/min/atm)	P_s (10^{-3} cm/min)
Unfertilised eggs				
Dimethyl sulphoxide	5	24	0.062 (0.003)	0.030 (0.007)
	10	28	0.072 (0.004)	0.071 (0.009)
	15	25	0.117 (0.005)	0.137 (0.013)
	20	18	0.168 (0.009)	0.295 (0.022)
Ethylene glycol	5	25	0.046 (0.004)	0.027 (0.003)
	10	20	0.057 (0.003)	0.055 (0.006)
	15	16	0.094 (0.007)	0.121 (0.018)
	20	18	0.167 (0.010)	0.261 (0.037)
Propylene glycol	5	17	0.048 (0.012)	0.030 (0.005)
	10	20	0.070 (0.004)	0.066 (0.006)
	15	17	0.110 (0.006)	0.212 (0.019)
	20	23	0.153 (0.008)	0.526 (0.042)
No CPA	5	18	0.057 (0.002)	
	10	17	0.097 (0.007)	
	15	22	0.113 (0.008)	
	20	16	0.191 (0.001)	
Fertilised eggs				
Dimethyl sulphoxide	5	15	0.104 (0.005)	0.125 (0.016)
	10	22	0.145 (0.006)	0.184 (0.015)
	15	13	0.150 (0.009)	0.367 (0.039)
	20	8	0.223 (0.025)	1.001 (0.106)
Ethylene glycol	5	25	0.096 (0.004)	0.060 (0.008)
	10	26	0.147 (0.005)	0.182 (0.024)
	15	12	0.203 (0.009)	0.392 (0.035)
	20	21	0.250 (0.012)	0.726 (0.059)
Propylene glycol	5	19	0.113 (0.022)	0.067 (0.005)
	10	25	0.150 (0.006)	0.202 (0.017)
	15	20	0.208 (0.011)	0.515 (0.033)
	20	16	0.371 (0.014)	0.868 (0.059)
No CPA	5	21	0.142 (0.004)	
	10	21	0.232 (0.011)	
	15	15	0.431 (0.020)	
	20	22	0.646 (0.023)	

Data presented are means (and SE).

general, L_p decreased in the presence of a CPA. The decrease was significant for fertilised eggs in all of the CPAs evaluated but for unfertilised eggs, the decrease was only significant for eggs in EG (Tukey test, $p \leqslant 0.05$).

Solute permeability varied significantly between unfertilised and fertilised eggs and was affected by CPA type and temperature ($p \leqslant 0.05$). However, there was also a significant three-way interaction between fertilisation, CPA type, and temperature ($p = 0.005$). Overall the value of P_s was lower for unfertilised eggs than for fertilised eggs. Among the CPAs evaluated, P_s was highest for PG followed by Me_2SO and then EG (Tukey test, $p \leqslant 0.05$).

Activation energies for L_p and P_s were determined from the slopes of the Arrhenius plots of $\ln(L_p)$ or $\ln(P_s)$ against 1000/T (Figs. 3 and 4). The value of E_a for L_p ranged from 11.4 to 14.5 kcal/mol

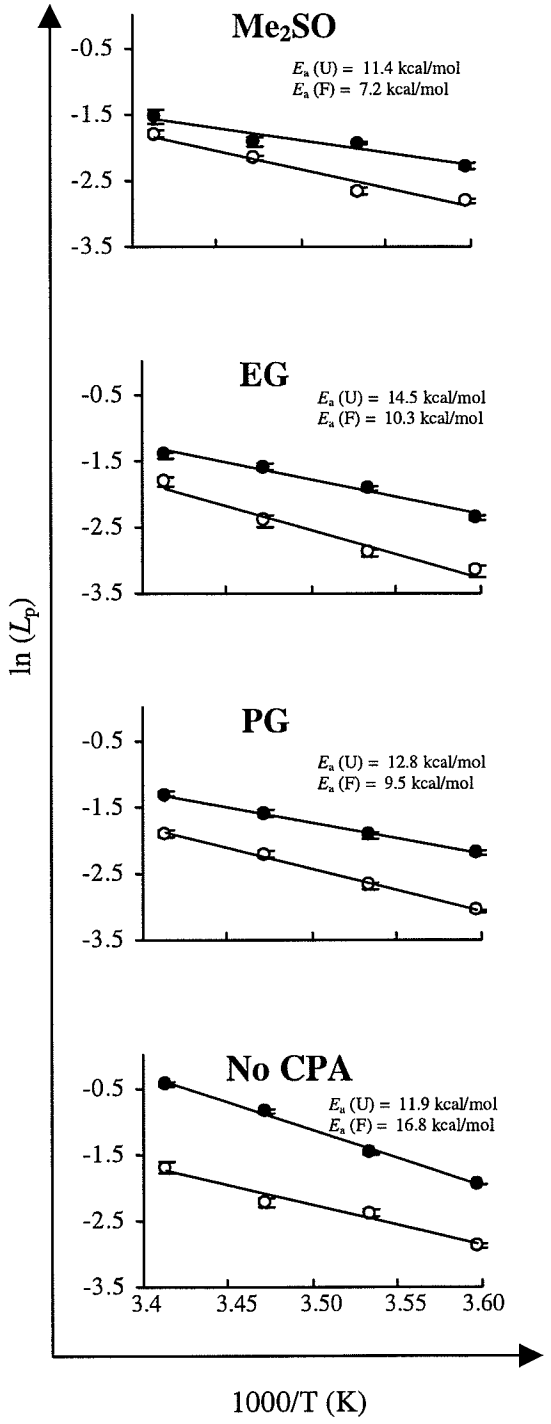


Fig. 3. Arrhenius plots of water permeability (L_p) for unfertilised (U; open symbols) and fertilised (F; closed symbols) sea urchin eggs in the presence or absence of different CPAs. Three CPAs were evaluated: Me_2SO, EG, and PG. E_a is the activation energy for L_p. Data shown are means ± SE.

for unfertilised eggs and from 7.2 to 16.8 kcal/mol for fertilised eggs. For P_s, the value of E_a ranged from 24.2 to 33.1 kcal/mol for unfertilised eggs and from 23.2 to 29.2 kcal/mol for fertilised eggs.

Osmotic tolerance limits for sea urchin eggs

More than 85% of unfertilised eggs, equilibrated in 0.73–1.57 Osm NaCl solutions were able to develop successfully to the blastula stage when

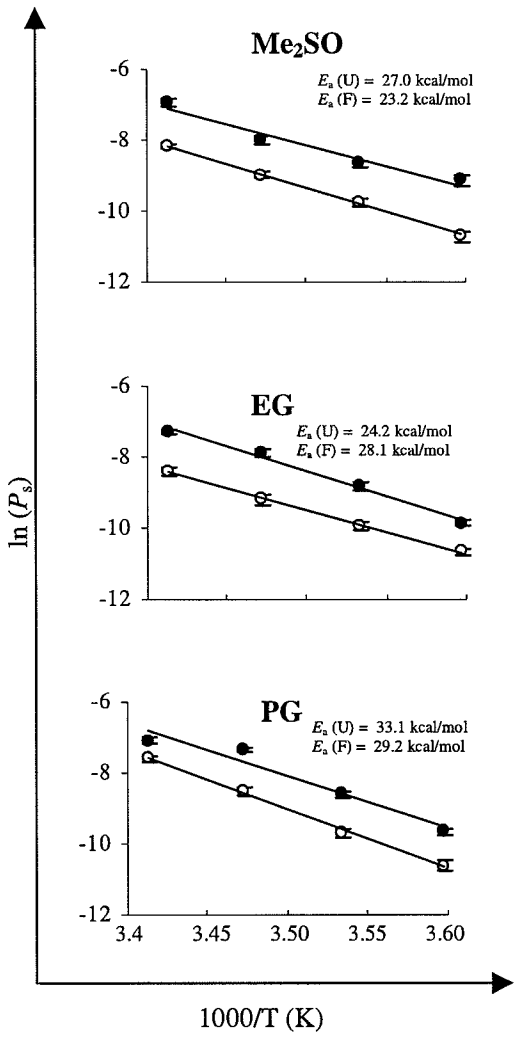


Fig. 4. Arrhenius plots of solute permeability (P_s) for unfertilised (U; open symbols) and fertilised (F; closed symbols) sea urchin eggs for three CPAs: Me_2SO (top), EG (middle), and PG (bottom). E_a is the activation energy for P_s. Data shown are means ± SE.

returned to seawater and fertilised. In contrast, the percentage of eggs developing to the blastula stage at lower and higher osmolalities was markedly lower. The corresponding volume changes at 0.73 and 1.57 Osm were calculated from the Boyle–van't Hoff relationship to be +30% and −20% of the equilibrium volume (see Fig. 1). For fertilised eggs, equilibration with solutions less than 0.69 Osm or greater than 1.74 Osm caused a marked decrease in the percentage of eggs developing to the blastula stage after return to seawater. The corresponding volume changes for these osmolalities for fertilised eggs were calculated to be ±30% of the equilibrium volume.

Modelling CPA loading and unloading

Using the limits of volume change tolerated by the eggs, it was possible to model methods for loading and unloading CPAs. Fig. 6 shows the optimised methods for unfertilised eggs and fertilised eggs for each CPA. The methods minimise the volume changes to within the upper and lower limits tolerated by the eggs. For loading unfertilised eggs, CPAs must be added in two fixed molarity steps 20 min apart to avoid injury. For fertilised eggs, however, the CPAs can be loaded in a single step. For unloading CPAs from unfertilised eggs, Me_2SO and EG should be unloaded in three fixed molarity steps 30 min apart. Propylene glycol can be unloaded in two fixed molarity steps since it is more permeable. For fertilised eggs, all three CPAs should be unloaded in three fixed molarity steps 10 min apart. The number of steps and the time interval between steps can be reduced during unloading by removing CPAs in the presence of an osmotic buffer such as sucrose. Fig. 7 shows the volumetric changes of unfertilised eggs during unloading of DMSO with and without sucrose. In the presence of sucrose, DMSO can be unloaded in two fixed molarity steps 20 min apart instead of three steps 30 min apart.

Discussion

Membrane permeability characteristics and osmotic tolerance limits can vary among cell types and among species for the same cell type [14]. In this study, the membrane permeability characteristics and osmotic tolerance limits of unfertilised and fertilised eggs of the sea urchin, *E. chloroticus*, were investigated in order to develop optimised methods for loading and unloading CPAs. This information will aid in the development of an effective protocol for cryopreserving eggs of *E. chloroticus*. Knowledge of the membrane permeability characteristics will also enable water loss during freezing to be modelled.

The assumption that sea urchins eggs behave as ideal osmometers and respond to changes in os-

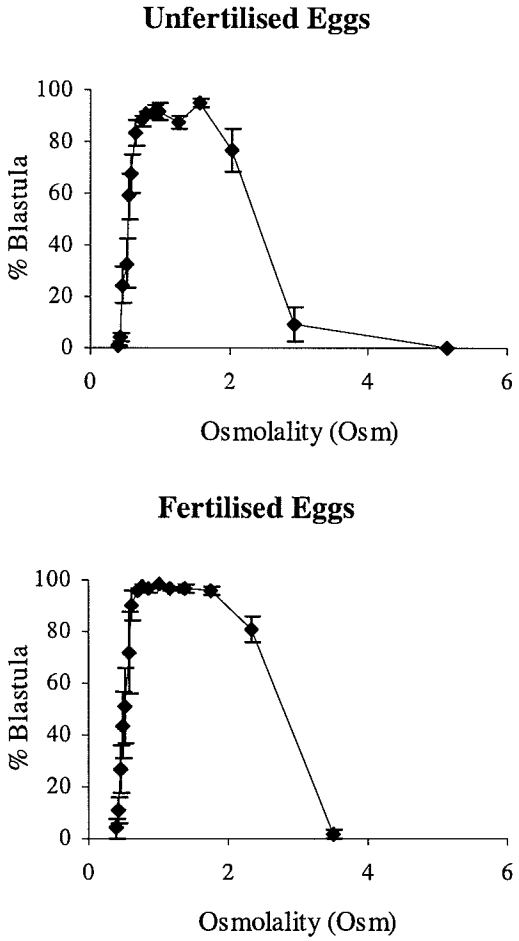
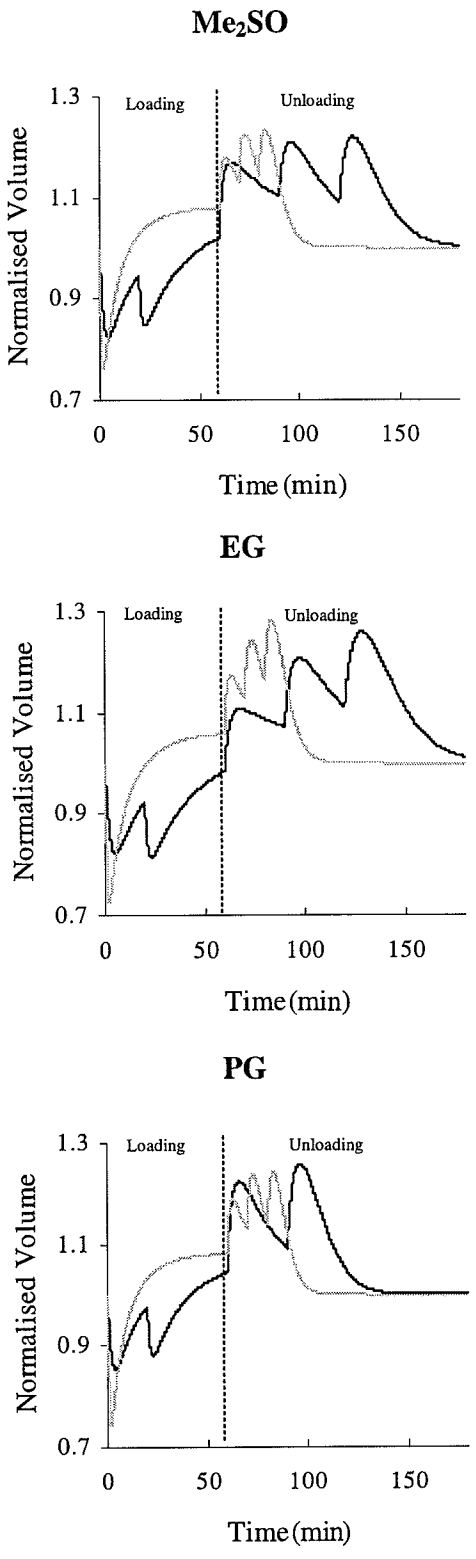


Fig. 5. Percentage (mean ± SE) of unfertilised (upper panel) and fertilised (lower panel) sea urchin eggs developing to the blastula stage following equilibration in NaCl solutions of varying osmolalities. Data represent the response of eggs from five individual females. Unfertilised eggs were fertilised by adding sperm after the eggs were returned to seawater.



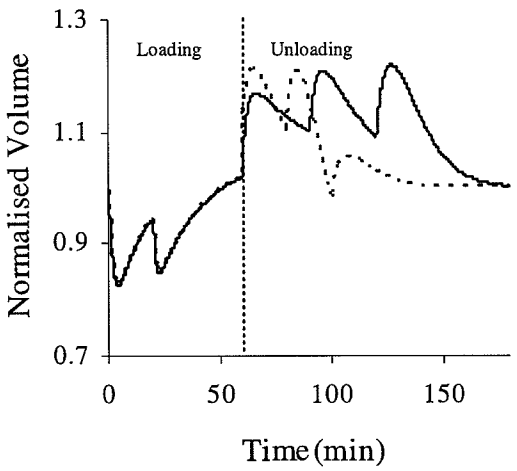


Fig. 7. Predicted volumetric response of unfertilised eggs during loading of Me_2SO and then unloading either with (dotted line) or without (solid line) sucrose at 15 °C. The eggs are loaded in two fixed molarity steps, 20 min apart, then unloaded either in three fixed molarity steps 30 min apart or in the presence of sucrose, in two fixed molarity steps 20 min apart. Eggs unloaded in the presence of sucrose are exposed to 0.15 M sucrose in the first unloading step and 0.3 M sucrose in the second step.

molality according to the Boyle–van't Hoff relationship was validated for the range 0.6–2.0 Osm. The osmotically inactive volume (v_b), determined by extrapolating the data to infinite osmolality, was similar for unfertilised and fertilised eggs, 0.367 ± 0.008 (mean $\pm$ SE) and 0.303 ± 0.007, respectively. These values are within the range, 0.11–0.54, determined for eggs of other sea urchin and marine invertebrate species [24–26,31,34,35,45]. The range of values is large, even when only the values for different sea urchin species are considered (0.11–0.46).

Fig. 6. Predicted volumetric response of unfertilised (black line) and fertilised (grey line) sea urchin eggs during loading and unloading of Me_2SO, EG, and PG in fixed molarity steps at various time intervals at 15 °C. The steps minimise the volumetric changes that the eggs undergo to within the tolerated limits. For unfertilised eggs, modelling predicts that the CPAs should be added in two fixed molarity steps that are 20 min apart to avoid excessive shrinking. For unloading, Me_2SO and EG should be unloaded in three fixed molarity steps 30 min apart. For PG, only two steps are required. For fertilised eggs, the CPAs can be added in one step but should be unloaded in three steps 10 min apart to avoid excessive swelling.

The values of L_p and P_s and their respective E_a determine the amount of shrinkage and swelling that a cell will undergo during CPA loading and unloading. For a given P_s and temperature, a higher L_p will result in a greater amount of shrinkage and swelling. Conversely, for a given L_p and temperature, a higher P_s value will result in a smaller amount of shrinkage and swelling. A high P_s will also reduce the time required for a CPA to permeate or leave a cell.

The values of L_p determined in the present study were within the range of values determined for other sea urchin and marine invertebrate species [24–31,35,45,49]. Water permeability was found to increase following fertilisation and in fact, when averaged across all other factors, the value of L_p for fertilised eggs was approximately double the value for unfertilised eggs. Water permeability was reduced in the presence of a CPA. Other studies on eggs and other cell types have reported similar changes in L_p in the presence of CPAs [13,14,36,42,50]. It is interesting that, in this study, the effect was only significant for fertilised eggs and for unfertilised eggs in EG. It is possible that the presence of a CPA has less effect on low L_p values than on higher values. Certainly in fertilised eggs, the effect was more obvious at higher temperatures where the values of L_p were also higher.

Solute permeability has been reported for eggs of only two other marine invertebrate species, the sea urchin, *Arbacia punctulata* and the abalone, *Haliotis diversicolor* [27,49]. The values of P_s determined in the present study were considerably lower than values determined for *A. punctulata* eggs in EG ($2.46 \times 10^{-3} \pm 0.50 \times 10^{-3}$ cm/min for unfertilised eggs and $5.99 \times 10^{-3} \pm 1.42 \times 10^{-3}$ cm/min for fertilised eggs; temperature not given) [49] but similar to those determined for unfertilised eggs of *H. diversicolor* in PG ($0.084 \times 10^{-3} \pm 0.017 \times 10^{-3}$ cm/min at 10 °C and $0.154 \times 10^{-3} \pm 0.035 \times 10^{-3}$ cm/min at 20 °C) [27]. As for water permeability, solute permeability was also found to increase following fertilisation. When averaged across all other factors, the value of P_s for the sea urchins increased approximately 1.5- to 2.5-fold. The value of P_s varied significantly between CPAs. Overall, PG was the most permeable CPA, followed by Me_2SO and then EG. PG is therefore more favourable as a CPA for these eggs since the higher solute permeability permits shorter exposure times for loading and unloading and thus minimises toxic effects resulting from long exposure.

Similar increases in L_p and P_s have been reported in eggs of *A. punctulata* following fertilisation [49]. Studies examining the ultrastructure of sea urchin eggs have shown that the egg plasma membrane is composed of thousands of microvilli [4,44,45]. The actual surface area of the eggs is therefore much higher than the values estimated in this study from the eggs' initial volumes assuming a smooth surface. Indeed, it has been estimated for eggs of other sea urchins that the actual surface area is approximately two times larger than the area calculated for a spherical surface of the same diameter [43,44]. This implies that the values of L_p and P_s are actually lower than estimated here. Although this will impact on the values of L_p and P_s, the fitted response is still close to the experimental data when modelled since the lower value for surface area has been used in both instances. The values of L_p and P_s determined in this study are therefore "effective" values rather than true membrane values. However, they enable the response of the cells to be modelled, which was the main purpose of this study. Surface area has also been shown, in studies on other sea urchin eggs, to increase following fertilisation as the microvilli elongate during development [43]. For eggs of the sea urchin, *Strongylocentrotus droebachiensis*, the increase in surface area between unfertilised and fertilised eggs is approximately 2.7-fold. This increase in surface area may explain the difference in the values of L_p and P_s between unfertilised and fertilised eggs that were observed and that have been reported. Consistent with the observed increase in P_s for sea urchins, fertilisation-induced increases have also been reported for fertilised murine oocytes [19].

The values of E_a for L_p calculated in this study were all greater than 7 kcal/mol with most values greater than 10 kcal/mol. It has been suggested that an E_a less than 6 kcal/mol implies transport through channels whereas an E_a greater than 10 kcal/mol implies transport through channel independent diffusion [51]. Although there is a

recognised need for caution in interpretation, it is likely that in sea urchin eggs, water is being transported through channel-independent diffusion such as passive bilayer diffusion.

The osmotic tolerance limits of the eggs were determined to assist in the development of optimised methods for loading and unloading CPAs from the eggs. The lower and upper limits were determined to be 0.8 and 1.3 for unfertilised eggs and 0.7 and 1.3 for fertilised eggs at 15 °C. The difference in the lower volume limit tolerated by the eggs is unclear.

The small difference in the Boyle–van't Hoff relationship between the eggs may mean that unfertilised eggs were exposed to higher NaCl osmolalities than fertilised eggs for a given volume change. Certainly, the range of osmolalities tolerated by the eggs was similar (see Fig. 5). It is known that membranes may be damaged and become leaky at high ionic concentrations. However, we were unable to confirm this as a cause or whether there was a true difference in lower volume limit. Preliminary attempts to determine osmotic limits using sucrose as a non-ionic solute, instead of NaCl, were unsuccessful because the eggs floated in the sucrose solutions. Therefore, for modelling CPA loading and unloading, it was decided that the volume limits would be kept to within those determined using the NaCl solutions.

Using modelling, it was possible to design optimised methods for loading and unloading CPAs from the eggs. Such methods ensure that any volume change the eggs undergo is restricted within tolerable limits and minimise the amount of time that eggs are exposed to CPA. If an osmotic buffer, such as sucrose, is present during unloading, the exposure time is further reduced.

The membrane permeability characteristics determined may also be used to determine rates of water loss in eggs during freezing at different cooling rates. In combination with further empirical studies, the results of the work presented here will aid in the development of an effective protocol to cryopreserve *E. chloroticus* eggs. The study reconfirms that cryobiological characteristics of a cell can vary among species and cell types and must be determined for each new species/cell type that a protocol is being developed for. However, the approach taken to in this study is readily adaptable to other species.

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