Review

Biobanking of a Marine Invertebrate Model Organism: The Sea Urchin

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Abstract: The sea urchin has long been used as an invertebrate model organism in developmental biology, membrane transport and sperm oocyte interactions, and for the assessment of marine pollution. This review explores the effects of cryopreservation and biobanking in the biology and development of sea urchins, all the way from germaplasm through to juveniles. This review will provide an integral view of the process and all that is known so far about the biology of cryopreserved sea urchins, as well as provide an insight on the applications of the biobanking of these model organisms.

1. Introduction

Sea urchins can be found from shallow shores to deep waters. There are more than 700 known species widespread within the five oceans [1]. Some sea urchins are a key group of herbivores due to their grazing control of the macro algal cover, playing an important role in the functioning, dynamics and structure of benthic assemblages [2], while others present a omnivorous feeding behavior [3]. Sea urchins are well-known as models for studying larvae biology and development; they have been used as a model organism to study reproduction and early cell differentiation processes, sperm oocyte interactions, and efflux transport and apoptosis [3–6].

Sea urchins have also been extensively used in ecotoxicology. Current strategies of water quality assessment integrate the chemical analysis with biological parameters to evaluate the effects of pollution on living resources [7,8] Due to the sensitivity of gametes and early stages of development to pollutants present in seawater—even at very low concentrations—sea urchin sperm and embryo-larval bioassays have been routinely used for water quality assessment for decades [7,9,10].

Most sea urchins are edible. There are few toxic species; there are venomous species in the Indo-Pacific including the Red Sea (*Diadema setosum*, *Toxopneustes pileolus*, *Tripneustes gratilla*), in the Caribbean (*Diadema antillarum*), and in the tropical and subtropical Atlantic coasts of South America and Africa (*Tripneustes ventricosus*). Despite these few being toxic, not many sea urchin species are consumed. There is evidence that those known to be edible have been collected from the oceans as early as 2400 B.C. [3]. With modern times, sea urchin fisheries have flourished around the world, and most of them still remain quite traditional. With the actual means of transportation, distribution and the increasing value or sea urchin roe, the rate of extraction is leading to the overfishing of many populations [11]. Due to the concern about the collapse of natural populations [3], there has been active research in the following fields: echinoculture or land-based culture [12]; larval and juvenile feeding [13,14]; artificial feed [15]; larval rearing systems [16]; gonad enhancement [17–19]; and wild stock enhancement [20].

Sea urchins are being collected from the sea, consumed as food, used for bioassays and kept in research facilities all over the world. Cryopreservation can be a very powerful biotechnological tool to enhance the use of sea urchins as a model species for research, enhance the use of embryo-larval bioassays, preserve genetic resources against extinction and increase the culinary interest in sea urchin roe. The development of cryopreservation protocols for different sea urchin cell types will allow the establishment of biobanks, providing year-round access to high quality material, regardless of the reproductive season, the oceanic, meteorological and environmental conditions that can be a source of uncertainty and variability [21] Access to cryopreserved embryos will allow using these model organisms in laboratories all around the world, regardless of their facilities or their proximity to a natural population of sea urchins. It will be a very useful tool for aquaculture production, fisheries conservation and wild stock enhancement [10], allowing spat supply all year round without the need of conditioning broodstock for out of season reproduction—which is expensive, time consuming and often unfruitful [22]. It will also provide flexibility for selective breeding programs [22,23] by allowing crossings of individuals from different reproductive seasons. Although gametes and/or embryo cryopreservation protocols have been successfully developed for many valuable species as sturgeons [24], salmonid fishes [25] due to their aquaculture interest, or marine invertebrates as oysters, mussels or abalone [22,26,27], arthropods, like the marine shrimps [28–30], little research has been carried out regarding sea urchin cryopreservation(example of development on Figure 1).

Gametes and embryo cryopreservation protocols and cryopreservation effects have been studied and published for 11 sea urchin species, the most common of which are *Hemicentrotus pulcherrimus*, *Strongylocentrotus nudus* and *Stongylocentrotus intermedius* by Asahina and Takahashi [31,32], Naidenko *et al.* [33] and Naidenko and Koltsova [34], *Tetrapigus niger* by Barros *et al.* [35,36], *Evechinus chloroticus* by Adams and Hessian [37] and Adams *et al.* [38] and *Paracentrotus lividus* embryos by Bellas and Paredes [39] and Paredes and Bellas [10].

Figure 1. Development schedule: *P. lividus* at 20 °C from cryopreserved blastula embryos all the way to settlement (**a**–**c**) and juveniles (**d**). Larvae achieve competence to settle after 21 days in incubation. Competent larvae were transferred to previously-conditioned petri dishes for settlement for 18 days. (d.p.s: days post settlement). All the details of the larval rearing can be found at Paredes *et al.* [40].

2. Principles of Cryopreservation

Cryobiology is a well-developed and worldwide-recognized technique for achieving long-term storage of biological material at low temperatures. Cryopreservation consists of freezing, banking and thawing living organisms, cells or tissues in the presence of cryoprotecting agents (CPAs) [41]. Many chemicals have been identified as having cryoprotective action, for example, dimethyl sulfoxide (Me_2SO), propylene glycol (PG), ethylene glycol (EG), glycerol (GLY), methanol (MetOH), sucrose (SUC) and other sugars or polyvinylpyrrolidone (PVP). It is critical to achieve a compromise between the ability of the CPA to protect the cells against cryopreservation, and the CPA toxicity [39,42,43].

When cells are exposed to a solution containing a CPA, the differences in the osmotic pressure of the solution inside and outside the cell will cause an initial diffusion of water from the cell to equilibrate the osmotic pressures [44] At the same time, the higher concentration of the CPA in the external solution will cause the diffusion of the CPA into the cell. The process will stop once the

concentration of the CPA equalize outside and inside the cell. At that point, the volume in equilibrium will be slightly larger than the initial due to the presence of CPA inside the cell [44].

The freezing point of the external solution will depend on the solution media and the CPAs present. Once the cooling starts the external media will freeze before the cells and water inside will be under its thermodynamic freezing point. Water below its freezing point is defined as supercooled and has a higher vapor pressure at a given temperature than the ice outside the cell [41,45]. As long as this difference in potential remains, water will slowly leave the cell and freeze externally, and the cell will dehydrate. Concentration of solutes inside the cell will increase and the potential reduced to reach the thermodynamic equilibrium, after which the cell will freeze without the formation of internal ice crystals, which will be lethal. Once successfully cryopreserved, the cells or embryos need to be transferred to liquid nitrogen for conservation.

Banking in liquid nitrogen (−196 °C) will ensure the conservation of cryopreserved cells as long as necessary [46,47]. At this low temperature, no chemical reactions take place and cellular metabolism is on hold. Once cells are stored in liquid nitrogen, their viability would only be affected either by background radiations, which at normal level will take 2000 years to become a hazard to stored cells [48], or human mishandling [49].

The fundamental steps in the cryopreservation process are:

1. Selection of the right CPA: It is necessary to know or study the toxic effects and toxicity thresholds of the different chemical compounds in order to select the right CPA or combinations of CPAs to be used in cryopreservation, as well as the temperature of addition and equilibration.
2. The addition and removal of CPAs: Factors such as membrane permeability, temperature, and osmotic excursions effects need to be taken into account.
3. Selecting the volume of the sample: The volume of the sample will affect the cooling and thawing rates and will also determine which programmable freezer to use and the liquid nitrogen storage needs.
4. Choosing/designing the right cryopreservation protocol: Determining the cooling rate/s, the temperature of seeding, the thawing rate and the ending point of the cooling are important key points.
5. Correct storage without temperature fluctuations: Samples should be continuously stored below their glass transition temperature; temperature excursions out of this range will result in recrystallization of the ice, a process in which large ice crystals grow at the expense of smaller ones, causing leading to reduced cell viability or even cell destruction.

3. Biobanking of Model Organisms

The idea of what a biobank is can be easily understood, although there are some different definitions and disagreements among scientists on the specifics [50]. Many scientific, medical, national and international agencies often refer to the biobanking of human biological material or genetic information. However, the idea of biobanking can and has been applied to animal biological material and genetic information. Biobanking for our purposes can be defined in a simple way: as a long-term biorepository for storage of biological material at low temperatures.

A model organism can be defined as a non-human species that has been extensively studied to understand different biological phenomena with the expectation that knowledge made in the model will provide insight into other organisms. There are several desired characteristics on a model organism: Ease of year-round breeding in the laboratory is one of the most important ones and sometimes quite complicated or expensive to achieve. Often characteristics such as short life cycle, well developed techniques for genetic manipulation, and simple living requirements are valuable when choosing a model organism to work with [51,52].

The design of cryopreservation protocols for the germaplasm model organism is the way to overcome the logistic issues of obtaining and maintaining those organisms—lowering the costly effort

and reducing the time dedicated [53]. There are different model organisms. At one end, we have the classical models, which have most of their biological processes well studied. At the other end are the emerging model organisms, which might be well studied for particular biological phenomena but have attracted less interest, and therefore their biological processes are in general less known. Developing a cryopreservation protocol for the latter is far more challenging.

The most commonly used model organisms is a quite restrictive list; on one side, it makes sense that scientists all over the world use the same model organisms, since data will be easily to compare, but this list should not be exclude emerging or specific model organisms. The most used model organisms representing different animal taxa are: mice (whose germaplasm and embryos have multiple cryopreservation protocols published like, [54]), drosophila [55], zebrafish (whose cryopreservation protocol is under study but the cryopreservation of fish oocytes or embryos hasn't been achieved yet) or, in the case of marine invertebrates, oysters, e.g., [22,56]. Cryopreservation of these model organisms in genome banks is extremely useful for biological research and the biomedical community [57].

3.1. Cryopreservation of Sea Urchin Gametes, Embyros and Larvae–Historical Landmark Events

Within marine invertebrate cryopreservation published research, mollusks and echinoids together constitute 72% of the research effort. Mollusks represent most of the work published, being oysters the best studied due to their global economic importance. In particular, *Crassostrea gigas* represents 64% of the total work published on oysters, followed by *Crassostrea virginica* with 12% [58].

Echinoids have attracted much less interest; sea urchins alone represent 28% of the publications on marine invertebrate cryopreservation. All of the published work that has been done covers the 11 different species listed here: *Hemicentrotus pulcherrimus*, *Strongylocentrotus nudus*, *Strongylocentrotus intermedius*, *Anthiocidaris crassispina*, *Loxechinus albus*, *Tetrapigus niger*, *Strongylocentrotus droebachiensis*, *Evechinus chloroticus*, *Paracentrotus lividus*, *Pseudocentrotus depressus*, *Urechis unicintus*. Regarding the cell types, work published on sea urchin cryopreservation, 41.2% are papers about sperm cryopreservation, 29.4% are on larvae, 17.6% embryo, and only 11.76% regarding oocyte cryopreservation (Table 1).

Table 1. List of cryopreservation studies published on Sea urchins sperm, oocytes, embryos and larvae from 1960 to 2015. Survival assessment results shown are the highest ones reported in each study. Cryoprotecting agents (CPAs) acronyms: Me_2SO: Dimethyl sulfoxide, EG: Ethylene glycol, PG: Propylene glycol, MetOH: Methanol, Gly: Glycine, G: Glycerol, AceT: Acetamide, SUC: Sucrose, TRE: Trehalose, PVP: Polyvinylpyrrolidone.

Species	Cell	Paper	Cryopreservation Protocol	Survival Assessment
Hemicentrotus pulcherrimus	Embryos	Asahina and Takahashi [35,36]	Me_2SO 1.5 M. Cooling rates 10–40 °C· min^{-1}. Thawing in air 15 °C· min^{-1}	10% Development to larvae
	Larvae	Asahina and Takahashi [31]	Me_2SO 1 M. Cooling rates 10–40 °C· min^{-1}. Thawing in air 15 °C· min^{-1}	90% Survival
Strongylocentrotus nudus	Larvae	Asahina and Takahashi [59]	EG 1.5 M. Cooling rate 10 °C· min^{-1}.Thawing rate 7 °C· min^{-1}	90% Active swimming

Table 1. *Cont.*

Species	Cell	Paper	Cryopreservation Protocol	Survival Assessment
Strongylocentrotus intermedius	Embryos	Asahina and Takahashi [36,59]	EG 1.5 M. Cooling rate 10 °C· min^{-1}. Thawing rate 7 °C· min^{-1}	90% Active swimming
		Gakhova *et al.* [60]	Me$_2$SO 1–1.5 M. Cooling rate 6–8 °C· min^{-1}. Thawing in water bath 19 °C	⩾90% survival
		Naidenko *et al.* [33]	Me$_2$SO 1.5 M. Acording to Gakhova *et al.* (1988) *	0.1%–0.2% Development to 2nd generation
		Naidenko *et al.* [34]	Me$_2$SO 1 M + 1 mg· mL^{-1} Antioxidant. Acording to Gakhova *et al.* (1988) *	60% swimming post-thaw, 1% develop.
		Odintsova *et al.* [61]	0.84 M Me$_2$SO + 40 mM TRE + 0.15% (*w/v*) Antiox. Cooling rate 7 °C· min^{-1}. Thaw in water bath 10–15 °C	40% survival
	Larvae	Asahina and Takahashi [59]	EG 1.5 M. Cooling rate 10 °C· min^{-1}. Thawing rate 7 °C· min^{-1}	90% Development
		Naidenko *et al.* [34]	Me$_2$SO 1 M + 1 mg· mL^{-1} Antiox., According to Gakhova *et al.* (1988) *	20% Active Swimming
Anthocidaris crassispina	Sperm	Wu *et al.* [62]	According to H. Kurokura *et al.* (1989) *	10% Motility
Loxechinus albus	Larvae	Barros *et al.* [35]	Me$_2$SO 1 M. Two step cooling rate 3:10 °C· min^{-1}. Thawing in water bath 15 °C 30 s	77% Survival after 24 h., 55% Survival after 21 d
Tetrapigus niger	Sperm	Barros *et al.* [35,36]	Me$_2$SO 1.2 M. Two step cooling rate 6:25 °C· min^{-1}. Thawing in water bath 17 °C 12 s	96% Fertilization, 56% Development after 24 h
Strongylocentrotus droebachiensis	Sperm	Dunn and McLachlan [63]	1.7 M Me$_2$SO. Cooling rate 5 °C· min^{-1}. Thawing at room temperature 45 min.	Motility score 4 of 10
Evechinus chloroticus	Sperm	Adams *et al.* [37]	0.35–1.06 M Me$_2$SO. Cooling rate 50 °C· min^{-1}. Thawing in water bath 15 °C 30 s	85% Fertilization
	Larvae	Adams *et al.* [38]	Me$_2$SO 1.5 M. Cooling rate 2.5 °C· min^{-1}. Thawing in water bath 15 °C 30 s	91% Motility
Paracentrotus lividus	Sperm	Fabbrocinni *et al.* [64]	1 M Me$_2$SO. Cooling rate 20 °C· min^{-1}. Thawing rate 15 °C· min^{-1}	90% motility, 50% Normal Larvae
	Oocytes	Paredes and Bellas [43]	Me$_2$SO NOEC:0.5 M, EG NOEC:1 M, PG NOEC: 0.68 M	Toxicity tests of CPAs
	Embryos	Bellas and Paredes [39], Paredes and Bellas [10,21]	Me$_2$SO 1.5 M + 0.04 M TRE. 1 °C· min^{-1}. Thawing in water bath 18 °C	50%–80% Normal larvae after 96 h. 25% settlement
Pseudocentrotus depressus	Sperm	Kurokura *et al.* [65]	1.4 M Me$_2$SO. Cooling rate 6 °C· min^{-1}. Thawing rate 10–15 °C· min^{-1}	13%–33% Fertilization

The first study published in sea urchin cryopreservation dates back to 1961 by Asahina [66] (Table 1) using unfertilized *S. nudus* oocytes to study intracellular freezing, when oocytes were held at −16 °C without using any cryoprotective additive. Six years later, Asahina [67] published another paper on freezing injury on sea urchin oocytes. In both, he studied the mechanisms of freezing injury caused by formation of ice crystals inside the cell or in the extracellular solution, establishing that *S. nudus* cells were quite sturdy, resisting exposure to relatively low temperatures (−10 to −16 °C), but on a short period of time (less than 6 h); when exposed to low temperatures for longer periods, though many oocytes looked structurally intact, once fertilized, all of them failed to develop.

Lanan [68], working on oyster sperm, was the first to publish in the field of marine invertebrate cryopreservation. In 1973, Dunn and McLachlan [59] (Table 1) cryopreserved the sperm of

S. droebachiensis with 1.5 M Me$_2$SO. In their research, Dunn and McLachan [59] reported that after cryopreservation sperm's motility was compromised. However, by increasing the sperm-oocyte ratio, they could achieve 95% fertilization.

A few years later, Asahina and Takahashi [32,57] published a paper on the sperm and embryo cryopreservation of *H. pulcherrimus*, *S. intermedius* and *S. nudus* using 1.5 M Me$_2$SO (Table 1). They observed the same effect of reduced post-thaw sperm motility as Dunn and McLachan [59] reported and the resulting fertilization rate was 75%. Those fertilized embryos were incubated and developed normally to pluteus larval stage. Cryopreserved embryos, on the contrary, survived but showed a higher degree of abnormalities and developmental delay that had fatal results within 3 days post thawing.

In the 90's, Naidenko *et al.* [33,34] evaluated the viability of embryos and larvae of *S. intermedius* cryopreserved in 1.5 M Me$_2$SO alone and in combination with the antioxidant pigment Echinochrome-A isolated from sea urchins (Table 1). For the first time, a complete larval rearing of the cryopreserved embryos was carried out, and, despite reporting that larvae post-thaw presented severe abnormalities, they found that a symbolic 0.1%–0.25% of embryos (cryopreserved at blastula stage) metamorphosed into juveniles. Pluteus larvae endured cryopreservation better than earlier embryos, and the larvae acquired a higher than normal pigmentation when cryopreserved using a combination of Me$_2$SOand antioxidant pigments with a 100% survival after 4 days incubation.

In 2003, Adams *et al.* [69] studied for the first time membrane permeability characteristics and osmotic tolerance limits and their application in cryopreservation of oocytes of the sea urchin *E. chloroticus* (Table 2). In 2004, this research group published extensive work on the effects of cryopreservation on sperm cells. They concluded that lower concentrations of Me$_2$SO (0.35–1.06 M) were more effective in preserving the fertilization ability, whereas higher concentrations preserved mitochondrial function and membrane integrity, items that were crucial for sperm motility and sperm-oocyte recognition. A cryopreservation study with sea urchin larvae from *E. chloroticus* [38] pointed out that four-arm pluteus larvae could be reared to settlement, achieving a 6% survival after 30 days incubation, despite the fact that many larvae showed a high degree of abnormalities. This paper has reported the highest survival of sea urchin embryo or larvae to date.

Table 2. Permeability parameters for sea urchin oocytes. Selected data from [69–72].

Species	CPA	T (°C)	Lp (μm/min/atm)	Ps (10^{-3} cm/min)
Evechinus chloroticus	Water	20	0.191 ± 0.001	
		10	0.097 ± 0.007	
	Me$_2$SO 1.5 M	20	0.169 ± 0.009	0.295 ± 0.022
		10	0.072 ± 0.004	0.071 ± 0.009
	EG 1.5 M	20	0.167 ± 0.010	0.261 ± 0.037
		10	0.057 ± 0.003	0.055 ± 0.006
	PG 1.5 M	20	0.153 ± 0.008	0.526 ± 0.042
		10	0.070 ± 0.004	0.066 ± 0.006
Arbacia punctulata	Water	21	0.107	
		12	0.043	
	EG 0.5 M	20.3	n/a	$0.32 \pm$ n/a
		10.2	n/a	$0.09 \pm$ n/a

Paredes and Bellas [43] working with sea urchin *P. lividus* studied the toxicity of cryoprotective agents for oocytes, fertilized oocytes and early embryos (Figure 2). Using EG, PG, Me$_2$SO, TRE and PVP, they calculated the toxic thresholds (see Section 2, compounds with cryoprotective action). On the basis of the NOEC (No Observed Effect Concentration) and LOEC (Lowest Observed Effect Concentration) calculated levels, EG was the least toxic CPA. However, in cryopreservation tests, Me$_2$SO resulted into highest survivals post-freeze. This was in accordance with all the previously published papers.

Paredes and Bellas [10,21]) established a protocol for blastula cryopreservation using 1.5 M Me$_2$SO with 40 mM TRE obtaining larvae that developed more slowly than unfrozen controls within the first 48 h, after which the larvae grew, achieving, on average, 50% of the size of the unfrozen controls after 96 h (Figure 2, Table 1). The percentage of abnormalities during larval development is minimal and, once incubated, to juveniles. After 20 days post-thaw, 25% of the larvae settled.

As Table 1 reports, the three most successful cryopreservation protocols for sperm cryopreservation are those reported by: Barros *et al.* [35,36] for *T. niger* equilibrating the sperm for 15 min. at 20 °C with 1.2 M Me$_2$SO using 0.25 mL straws, the cooling rate used was 6 °C· min^{-1} to −20 °C and 25 °C· min^{-1} to −150 °C, and the straws were then plunged into liquid nitrogen. Thawing was performed by immersion in a water bath at 17 °C for 12 s. Obtaining 96% fertilization rate and 56% development of the fertilized embryos after 24 h. Adams *et al.* [37] obtained 85% fertilization using cryopreserved sperm of *E. chloroticus* when using lower concentrations of CPA, incubated between 28 and 41 min at 0 °C prior to cooling at 50 °C· min^{-1}, and thawing was done by immersion in a water bath at 15 °C for 30 s. Fabrocinni *et al.* [64] obtained cryopreserved *P. lividus* sperm presenting 90% motility and 50% normal larvae development after 48 h using 1 M Me$_2$SO + 0.04 M TRE, with a cooling rate of 20 °C· min^{-1}, and thawing was performed by immersion in a water bath of 30 °C for 2 s.
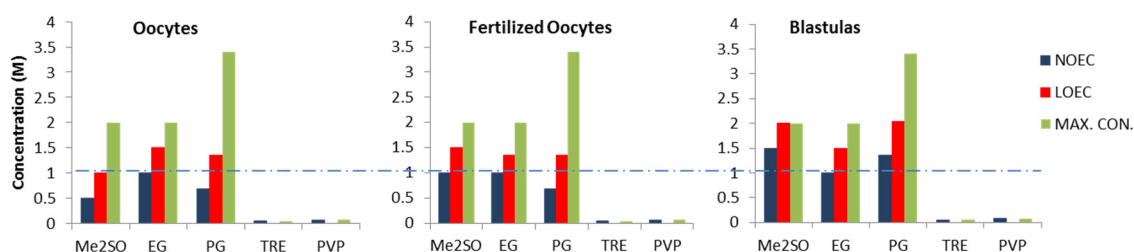

Figure 2. NOEC, LOEC and Highest tested concentration (Max. Con.) For three cell types for *P. lividus* (Data from [39,43]). **Blue** line at 1 M represents on average the most used concentration in literature. Results obtained by the evaluation of normal shape and development of embryos for 48 h exposed to increasing dose of these chemical compounds. Common abnormalities produced from this exposure can range from complete inhibition of development, abnormal shape or swimming behavior to abnormal growth. There are no protocols described for sea urchin oocyte cryopreservation, although there are some studies about membrane permeability and sensitivity to cryoprotecting agents.

The most successful embryo cryopreservation protocol for sea urchins has been reported by Paredes and Bellas [10,21] using 8-h-old *P. lividus* blastulas, with a 15 min stepwise addition of 1.5 M Me$_2$SO plus 40 mM TRE at 20 °C, using 2 mL cryovials and a cooling rate of 1 °C· min^{-1} to −80 °C (with seeding for 2 min at −12 °C). Thawing was performed by immersion in a 18 °C water bath until the ice melted and cryoprotecting agents were removed stepwise in 12 min. This protocol yielded 80%–50% normal larvae after 96 h, and those larvae were at least 50% the size of the unfrozen controls and produced a settlement to juveniles of 25% in comparison to controls.

Barros *et al.* [35] published the most successful protocol for sea urchin larvae cryopreservation so far, using the sea urchin *L. albus* with 1 M Me$_2$SO (equilibration time of 20 min at 0 °C). With a two-step cooling rate of 3 °C· min^{-1} to −10 °C and 10 °C· min^{-1} to −150 °C. Thawing in a water bath at 15 °C for 30 s resulted in 77% survival after 24 h and 55% survival after 21 days.

3.2. Cryopreservation and Cryoinjuries

There is general an agreement that membranes are often damaged during the freezing process [56,73,74], and sea urchins are probably not an exception. Although there is research published on sea urchin embryos reporting positive results after freezing, such as an initial 90% survival reported with *H. pulchehimus*, *S. intermedius* and *S. nudus* embryos, the resulting larvae suffered severe abnormalities, and development was impaired after only three days [31,32,59,75]. Cryopreserved

embryos were able to be reared to juveniles for *S. intermedius* [33,34,61] and, despite suffering some abnormalities, 0.1%–0.2% survived to settlement. An encouraging cryopreservation study was published by Adams *et al.* [38] on *E. chloroticus* four-arm Pluteus larvae, although many larvae showed membrane damage they obtained 6% post-thaw larvae settlement (30 days of larval rearing), which was a significant improvement of the previous protocols published. If the cryopreservation protocol does not work, the cells that survive—if any—will present lethal abnormalities that will impair development and cause death within a few hours or days.

The cryopreservation protocol described for *P. lividus* embryos [10,39] yields a good blastula survival (over 50% after cryopreservation), less than 25% abnormalities, but interestingly cryopreserved embryos showed a delay in development when compared with fresh controls. Cryopreserved embryos grow only 20% of the control size in the first 48 h, mostly remaining as blastulas or gastrulas, but, from that point on, larvae start to develop and grow faster. The percentage of larval growth, compared to controls at 96 h, was 50% growth on average, but with significant variability during the reproductive season (the highest reported was over 80% of control size).

This phenomenon of growth delay during the first hours post-cryopreservation has also been seen in other marine embryos or larvae as mussels or oysters [58,76]. However, the question of what this means remains unanswered. It is not clear what is occurring within the first hours of embryo incubation post-cryopreservation. During the first hours post-cryopreservation and thawing, several processes might be happening synchronously causing the delay of growth. First, it might be due to the slow activation of metabolism following the exposure to cold temperatures, since, when cells are stored in cryogenic temperatures, all metabolic reactions are on hold, and it seems reasonable to suggest that reactivating the cellular mechanisms might take time since metabolic reactions are temperature-dependent. Secondly, there might be an activation of the membrane-restoration mechanisms present in sea urchins [77] because it is well-known that the membrane fluidity and integrity might be reduced due to the cryopreservation process; this has been detected in human sperm and mouse oocytes [78,79]. It is also well-known that there is an active production of Reactive Oxygen Species (ROS) post-cryopreservation for sperm cells of several species [80]. Therefore, the development delay might be related to using the vitellogenic energy remaining to activate mechanisms to deal with ROS instead of growth [78–80]. These processes are very likely quite general to cells during the freeze-thawing process but have not been studied in marine organisms.

When incubated for longer periods, larvae that survived cryopreservation [21] will successfully grow (though a little slower) and reach the four-arm pluteus stage after 96 h of incubation (when controls reach this development stage after 48 h). When a complete larval rearing with cryopreserved embryos was carried out, it resulted in 29% survival by day 20 and a settlement percentage of 7% by day 18 post-competence (71% survival and 25% settlement in comparison to fresh controls) [10]. This protocol for sea urchin embryos is the only one published that produces high viable outcome with embryos that can grow into larvae and metamorphose and settle into juveniles.

3.3. Sea Urchin Cell and Membrane Characteristics

During cryopreservation cells undergo dehydration and osmotic volume excursions, and some cells are more sensitive than others. The volumetric changes caused by the addition and removal of the cryoprotecting agents and the freezing process itself can be damaging if they exceed the osmotic tolerance limits. In order to formulate optimal methods of loading and unloading cryoprotecting agents to minimize damage, there are some cell characteristics that need to be known: Surface area to volume ratio, water permeability (Lp), solute permeability (Ps), their activation energies (Ea) and the osmotically inactive volume of the cell (Vb). These parameters are related as follows:

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

$$dN_s/dt \ = \ Ps \, A \, (a_s^e - a_s^i) \tag{2}$$

$$V \ = \ V_w \ + \ V_b \tag{3}$$

These are water permeability and solute permeability equations for modeling water and solute movements through membranes, where V represents the cell volume, V_w is the water volume inside of the cell, *t* is time, A is the surface area of the cell, R is the universal gas constant, T is the absolute temperature, M^e and M^i are the external and internal osmolality. Ns is the number of osmoles of permeating solutes, and a^e_s and a^i_s are the external and internal activity of the permeating solutes.

The water and solute permeability of sea urchin cells have been studied by McCutcheon and Lucké [70,71], Stewart and Jacobs [72], Leitch [81], Lucké *et al.* [82], and Adams *et al.* [69]. It has been proved that sea urchin *Evechinus chloroticus* oocytes [69] follow the Boyle-van't Hoff relationship, which states that volume varies linearly as a function of the reciprocal of the osmolality. Sea urchin oocytes behave as ideal osmometers within the range of 0.6–2.0 Osm. This idea of sea urchin oocyte membranes having a perfect semipermeable membrane had also been pointed out by McClutcheon *et al.* [70] when studying the permeability of *Arbacia punctulata* oocytes. The permeability of a cell to water will vary with temperature, but will also depend on the membrane composition and method of transport (transport through channels or membrane diffusion); therefore, it may vary among cells types. The same is true for the solute permeability (Ps) (Table 2).

Oocyte volume varies widely among sea urchin species. *Evechinus chloroticus* oocytes have a volume of 7.57×10^{-6} cm^3, 36.7%, 30.3% of which is osmotically inactive volume (V_b) for oocytes and fertilized oocytes, respectively (Adams *et al.*, 2003). *Paracentrotus lividus* oocytes volume is 3.94×10^{-6} cm^3 and, according to Leitch [81], 22.6% is their osmotically inactive volume. Similarly, *Strogylocentrotus purpuratus* oocytes have a volume of 2.60×10^{-7} cm^3 with a 24.5% osmotically inactive volume [81] *Strongylocentrotus franciscanus* oocytes are slightly higher, with a volume of 11.6×10^{-7} cm^3, but their osmotically inactive volume is lower, 18% [81].

There are almost no data available regarding all those parameters listed in Section 3.3 for oocytes, neither for other types of cells, such as sperm, embryos or larvae, nor for the remaining sea urchins species insofar as the author is aware. Therefore, until these factors are studied, we can only assume that those parameters could be extrapolated to other sea urchin species and similar cells for modeling volumetric responses with the appropriate caution about the interpretation of the results.

4. Conclusions

Sea urchins are being collected, used and kept in research facilities all over the world. However, sea urchins are not just useful as research model organisms; there is a real interest in sea urchin aquaculture [12–16], population enhancement for fisheries conservation [20], ecological conservation [83], and marine pollution assessment [7,8].

Sea urchins are a very useful model organism in that they are easy to obtain from nature, easy to work with, very fertile, and their embryos and larvae are easy to culture. On the other hand, due to their marked reproductive seasonality, it is impossible to collect mature sea urchins year-round, and maintaining a stock of mature sea urchins all year is very difficult and expensive when possible. The establishment of biobanks in those facilities where sea urchins are used as model organisms would help overcome the constraint of year-round biological material supply. However, it should be noted that sea urchin biobanks could also be of potential interest for the industries of aquaculture, fishery conservation and environmental monitoring.

It is therefore remarkable that, despite some of the pioneering work in cryopreservation that was done with marine invertebrates such as oysters [68] and sea urchins [31] there has been little research carried out in sea urchin cryopreservation and biobanking thereafter. Only in recent years have researchers re-discovered the usefulness of its cryopreservation and increased their interest in biobanking for different purposes [10,21,37–40,43,61,64].

Currently, sea urchin sperm can be cryopreserved with 90% motility and 95% fertilization [64] on *P. lividus*; on the other hand, results of sea urchin oocyte cryopreservation results are far less optimistic [37,43]. Oocytes are chill and CPA-sensitive. As an alternative, there has been an intensive

research interest for embryo and larvae cryopreservation with positive results [33,38,39]. Embryos and larvae are the most tolerant to cryopreservation, with oocytes being the most sensitive. Still, all the knowledge available is restricted to the 11 species hitherto cryopreserved. In all cases, the most efficient conditions for embryo or larvae cryopreservation are slow cooling rates (less than 7 $°C\cdot min^{-1}$), the seeding of the samples, and Me_2SO as cryoprotecting agent (concentrations ranging from 1 to 1.5 M). There is a lack of knowledge of the membrane permeability characteristics of most of the sea urchins species, though some similar results could be expected when applying protocols already developed to other species. Without the knowledge about cell characteristics and sensitivity, this information can only be used as a preliminary approach, as it has been extensively proved that cryopreservation protocols are species and cell-specific. An intense research effort is needed towards widening the knowledge of sea urchin membrane parameters, modeling the addition and dilution of cryoprotecting agents, studies of membrane composition, and long-term viability of cryopreserved cells in order to increase the number of species that are available to biobank and use when necessary.

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