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First steps towards *Echinometra lucunter* embryo cryopreservation

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ABSTRACT

We have studied the sensitivity to cryoprotecting agents of different embryos of the local sea urchin, *Echinometra lucunter* which is the species used for embryo-larval bioassays in Brazil. We have located significant differences between both species sensitivity to cryoprotecting agents; while for *P. lividus* propylene glycol was the less toxic compound for most development stages, whereas for *E. lucunter* is was the most toxic and the least toxic was Dimethyl sulfoxide. There is a significant difference between development stages as well; in the case of *P. lividus*, the blastula embryo was the most resistant to the cryoprotecting agents, meanwhile for *E. lucunter,* it was the fertilized oocyte. This is a very promising result, a really early embryo that is not extremely sensitive to Me_2SO. Our next aim is to develop a cryopreservation protocol for *E. lucunter* early embryos and test them in an embryo-larval bioassay.

1. Introduction

Echinometra lucunter is a tropical sea urchin with great ecological importance and which populates the Brazilian coast, particularly in the south and southeast in high densities [18].

Sea urchins have been extensively used in ecotoxicology assays in the last decades. Current strategies of water quality assessment integrate the chemical analysis with biological parameters to evaluate the effects of pollution on living resources [8–10,12]. Due to the sensitivity of the early stages of development to pollutants present in seawater-even at very low concentrations-sea urchin embryo-larval bioassays have been routinely used for water quality assessment for decades.

Toxicity bioassays consist on the exposure of a representative amount of organisms to a range of chemical concentrations or field samples and to register/quantify the effects (the endpoint can be any quantifiable response that can be related to the chemical dose or exposure) over a fixed period of time [17,30]. Sea urchins present numerous advantages for ecotoxicological studies due to their abundance, wide distribution, easy recollection and low laboratory maintenance [15]. Sea urchin larvae were firstly proposed as water quality indicator in the early 1950's by Wilson [29].

The application of these biological techniques to environmental monitoring presents several advantages: bioassays integrate the effects of all substances present in a complex mixture [16], including interactions among substances, they provide predictions and early warning of environmental impacts, serving as diagnostic tools [28]. Cause-effect relationships can be examined by dose response experiments with individual substances or with water or sediment dilution series [16], a great number of response variables can be examined over different exposure times, effects on different biological organization levels can be predicted [16] and results can be used to develop water quality criteria [30].

Methodological advances in cryopreservation procedures for marine organisms [31] may be a solution to overcoming the constraint of the reproductive seasonality in embryo-larval bioassays and provide biological material available throughout the year [19,20].

The design of a cryopreservation protocol for the cryopreservation and cryobanking of gametes and embryos to be used for marine quality assessment could ensure the accessibility to high quality reproductive material all year round, as an option to conditioning adults for out of season reproduction, which is a very time consuming and expensive process [24]. In addition to that, the dependence from field collection is highly inconvenient (weather issues, punctual pollution events add too many degrees of uncertainty to the reliable supply) and it has an impact on the local populations.

The aim of this work is to study the sensitivity of *Echinometra luchunter* to cryoprotecting agents as a first step to develop a cryopreservation protocol for sea urchin cells with ecotoxicological applications.

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2. Material and methods

2.1. Biological material

Mature sea urchins, *E. lucunter*, were collected in the Curva da Baleia beach (Jacaraípe, Serra, Espírito Santo – Brazil) during the natural spawning season. Animals were transported to the laboratory in a portable icebox and maintained in aquaria with running natural sea water until the experiments. Sea urchins were fed with the green algae *Ulva* sp.

Gametes were obtained by injecting 1,5 -3 mL of KCl (0,5 M) in the oral area [1]. At least three males and three females were used in each experiment. Mature oocytes were transferred to a measuring cylinder with 100 mL of ASW and their quality was checked under microscope. Only batches of mature eggs that were spherical and undamaged were used for the experiments. Sperm mobility was checked under microscope and the sperm solution was stored at 4 °C until use. The sperm was diluted in ASW (1:49) and 1–2 mL of the sperm solution was added to the eggs solution for fertilization. After 30 min, the solution containing eggs and sperm was checked under microscope to make sure that all the eggs were fertilized (by observation of the elevation of the fertilization membrane).

2.2. Toxicity tests

Toxicity tests were undertaken on the fertilized egg (30 min post-fertilization) and blastula (6 h post-fertilization). We used in this experiment 0.04 M Trehalose (TRE) as non-permeant CPA in all the solutions and for the permeant CPAs: Dimethyl sulfoxide (Me_2SO), Propylene glycol (PG) and Ethylene glycol (EG) [3,11,23].

Cryoprotecting agents were prepared in ASW and the concentrations selected and methodology were similar to those reported by Ref. [23] for *P. lividus*. Concentrations tested, addition methods and exposure times are summarized in (Table 1).

Total time of equilibration with the CPAs never exceeded 15 min. After the CPA removal, the embryos were rinsed carefully with ASW to remove the excess of CPA by using a 60 μm mesh, then transferred to experimental vials with 4,5 mL of ASW and incubated for 36–42 h (26 °C), until the 4-arm-pluteus stage was reached in controls (n = 5 per treatment).

2.3. Statistical analysis

A one-way ANOVA test (SPSS v.15.0) followed by a Dunnet post-hoc test was used to determine both the most suitable addition methodology and the toxicity parameters NOEC (No Observed Effective Concentration) and LOEC (Lowest Observed Effective Concentration).

3. Results

There are significant differences between the effects of CPA exposure of the two development stages studied, being the fertilized egg the stage that showed more resistance to CPAs, presenting higher NOEC levels (Table 2). Exposure to our three selected CPAs (Me_2SO, EG and PG always in combination with 0.04 M TRE) had different effects depending of the methodology of exposure (Table 1, Fig. 1), the best results have been obtained when using the methodology of equimolar stepwise addition which yields a total equilibration time of 15 min and stepwise removal by FSW addition (see normal larvae parameters, Fig. 2).

Table 1
Addition/dilution methodology for CPAs and total equilibration time at room temperature (20 °C).

Treatment	Addition Methodology	Total equilibration time (min)	Removal Methodology
1	15 equimolar steps of 1 min [24]	15	12 equimolar steps of 1 min [24]
2	1 step (5 min of exposure)	5	1 step
3	1 step (15 min of exposure)	15	1 step
4	3 equimolar steps	5	3 steps

Table 2
NOEC/LOEC levels calculated for the most successful Addition/removal methodology and for each CPA, data compared with published data for the same cell types with another species of sea urchin, *P. lividus*.

		E. lucunter		*P. lividus* [23]	
		Treatment 1		Treatment 1	
		15-equimolar step		15-equimolar step	
		NOEC	LOEC	NOEC	LOEC
Fertilized oocyte	Me_2SO	1.5 M	2 M	1 M	1.5 M
	EG	0.5 M	1 M	1 M	1.5 M
	PG	>1.02 M		0.68 M	1.36 M
Blastula	Me_2SO	< 1 M	1 M	1.5 M	2 M
	EG	<0.5 M	0.5 M	1 M	1.5 M
	PG	<0.34 M	0.34 M	1.36 M	2.04 M

The no observed effect concentrations (NOEC) and lowest observed effect concentrations (LOEC) were calculated for the 15 equimolar stepwise addition (treatment 1) that in general showed the best results regarding the percentage of normal larvae development (Table 2). Results were compared with the NOEC/LOEC data available from Ref. [23] for *P. lividus*, having found important differences between the sensitivity to CPA's between different species for each one of the cell types (Table 2).

NOEC levels for Me_2SO exposure of the fertilized egg is 1.5 M in the case of *E. lucunter* but it is lower (1 M) for *P. lividus*. In the case of PG *E. lucunter* shows a similar pattern, NOEC is > 1.02 M but a lower results was obtained for *P. lividus,* with a NOEC of 0.68 M. In the case of EG, NOEC is 0.5 M but *P. lividus* showed a higher resistance with a NOEC of 1 M.

P. lividus showed higher resistance to CPA exposure in the blastula stage. In the case of *E. lucunter,* it is in fact the fertilized egg the most tolerant cell type to these chemical compounds, showing lover NOECs for every CPA tested (Table 2).

4. Discussion

It has been proved that the correct selection of both the most suitable CPA type and concentration is one key step for a successful cryopreservation of marine invertebrates [2,23]. In the case of early-development stages of marine invertebrates, this factor is even more crucial due to the high sensitivity of those cells to chemicals present in the water; a quality that makes them an optimal model organisms to be used in the evaluation of water quality [8,16].

The determination of the optimal CPA is species and development stage specific [4,14,23,27] our findings reinforce this finding. The behaviour after exposure to different CPAs of different cell types from our local sea urchin species point towards significantly different results from the same development stages from a different sea urchin species. Therefore, the differences in sensitivity to the cryoprotecting agents would determine no only the optimal compound (optimal from the low toxicity point of view, which might not be offering the maximum cryoprotection as determined by Refs. [11,23]) and the optimal concentration to use, but it will also point towards the most resistant cell types which would be the target for developing the cryopreservation protocol.

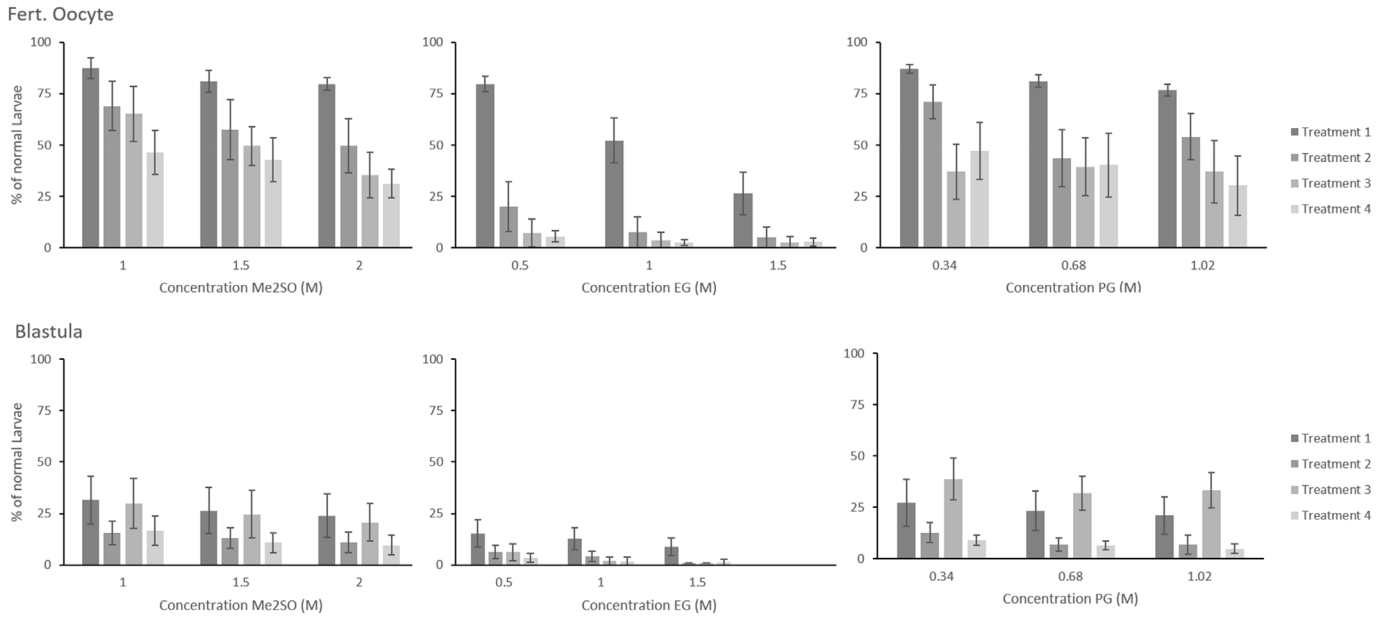

Fig. 1. Relative percentage of development to normal 4-arm pluteus larvae after exposure and removal of CPA's compared to controls. All permeant CPA's were combined with 0.04 M TRE. The four exposure treatments are explained in detail in Table 1. Average (n = 5) ± std error.

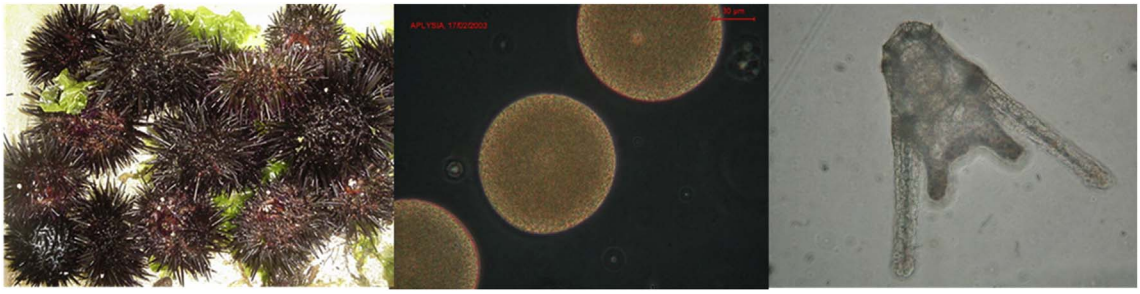

Fig. 2. *Echinometra lucunter* adults, *E. lucunter* oocytes (average size 82.9 ± 0.98 μm by Ref. [13] - 98.1 ± 14.0 μm by Ref. [6]), very distinctive shape of a normal 4 arm pluteus larvae, average size after 36–42 h is 307.8 ± 22.3 μm by Ref. [6].

From our results and previously published research on sea urchin different cell types, the sensitivity to CPAs varies widely along development. Both [3] and [23] pointed out that despite EG being the less toxic compound to sea urchin cells (fertilized oocyte, blastula and gastrula), no survivals have been obtained when cryopreserving the cells using EG as a cryoprotecting agent, both obtained survivals after choosing Me_2SO instead, despite they have described it previously described as the most toxic CPA. This points out that it is a very important to balance the toxicity and the cryoprotection offered by the CPA. With the exception of some pioneer work published by Ref. [5] with EG as cryoprotectant, all the embryo/larvae cryopreservation published results point out that Me_2SO will be the most suitable cryoprotecting agent (Refs. [21,22] with *Strongylocentrotus Intermedius*, Ref. [7] with *Loxechinus albus,* Ref. [3] with *Evechinus chloroticus* or Ref. [23] with *Paracentrotus lividus*). Our research shows in this line great promise due to the low toxicity to Me_2SO showed by the fertilized oocyte.

In addition, some cells are very sensitive to osmotic changes and therefore we have explored several addition/removal methods. After testing 4 different methodologies the best results have been obtained when using the methodology published by Ref. [23] of equimolar stepwise addition which yields a total equilibration time of 15 min.

Previous experiments with larvae from similar molluscs proved that using similar organisms and cell types [25,26] one cryopreservation protocol for one species could be potentially used as step-one to develop the protocol for another species. Once selected the optimal CPA (with the optimized balance between toxicity and cryoprotection) and if there are not major differences in membrane permeability, cell type or cell size; the optimal cooling rate could still be extrapolated from one species to another [25,26].

To conclude, this research provides for the first time information about the sensitivity to cryoprotecting agents for *Echinometra lucunter* cells as a first step to stablish a cryopreservation protocol to cryopreserve early embryos for water quality assessment purposes. Future studies will focus on the development and evaluation of a cryopreservation protocol, short and long term survival.

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