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Cryobiology of cephalopod (*Illex coindetii*) spermatophores ☆

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ABSTRACT

Cephalopod culture is expected to increase in the near future and sperm cryopreservation would be a valuable tool to guarantee sperm availability throughout the year and to improve artificial insemination programs. We have studied the tolerance of spermatophores from the oceanic squid *Illex coindetii* to several cryoprotectants, in two toxicity experiments and a cryopreservation test. Five permeating cryoprotectants were tested: Dimethyl sulfoxide (Me2SO), methanol, glycerol, propylene glycol and ethylene glycol. In the first experiment, spermatophores were exposed to the five cryoprotectants at 5% (v/v) and 15% (v/v) at 4 °C for 5 min. In the second experiment, spermatophores were exposed to the cryoprotectants at 15% using different exposure times: 5, 15 and 30 min. In a third experiment, we tested two cryopreservation protocols: LN_2 vapor or −80 °C freezer, using a 15% cryoprotectant and 15 or 30 min of exposure. Viability and mitochondrial activity were assessed using Mitotracker deep red, YOPRO1 and Hoechst 33342, by flow cytometry. Spermatozoa in this species remain viable after cryoprotectant exposure but their quality decreased considerably after cryopreservation, only 5–10% of spermatozoa being motile. Flow cytometry demonstrated that Me2SO may be the most appropriate cryoprotectant for *I. coindetii* spermatozoa, and shows a first approach on cephalopod sperm cryopreservation, opening new possibilities for the research and culture of this group of molluscs.

Introduction

Cephalopods are a group of marine molluscs of great importance not only for the seafood industry, but also for the biomedical, pharmaceutical and cosmetic industries. They are mainly obtained by fishing, whereas their aquaculture production is still very limited: 3,652,632 tons by fishing versus 10 tons by aquaculture during 2010 [10]. Nevertheless, cephalopod aquaculture production is expected to increase in the near future attending to recent scientific advances [8,23,35,37]. The expansion of cephalopod breeding will require advances in reproductive management and the development of reproduction technologies.

In particular, sperm cryopreservation is a technique that allows spermatozoa to be stored indefinitely. Among the many advantages for breeding programs, it allows sperm availability throughout the year (especially important in seasonal species, or when gamete production differs among sexes or is unpredictable), and a more efficient management of fertilization and selection programs [40]. Specifically, artificial insemination programs would benefit from sperm cryopreservation, improving the culture of oceanic squid species by using *in vitro* fertilization [38,41]. These technologies could also be used for the preservation of endangered cephalopod species such as *Nautilus pompilius* [9].

In cephalopods, spermatozoa can be collected either from mature males or from copulated females. Males produce spermatophores that are stored in the spermatophoric organ (Needham's sac). Copulated females have ejaculated spermatophores called spermatangia anchored to their bodies. Spermatozoa from spermatophores and spermatangia have similar fertilizing capacity and fertilization rates [24].

Studies have been carried out on spermatozoon structure and morphological characteristics in several species of cephalopods [5,7,13,14,16,17,25,27], spermatophore physics, morphology and physiology [2,33], and spermatangium characteristics [19–21,32]. Nevertheless, very little information on spermatophores or spermatangium is available [22]. As far as we know, the only precedent on cephalopod spermatophore refrigeration was reported by Naud & Havehand [31] for the cuttlefish *Sepia apama* and no published information exists on sperm, spermatophore or spermatangium cryobiology for any cephalopod species.

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In this study, we used the broadtail shortfin squid *Illex coindetii* (Vérany, 1839), which is a medium-sized oceanic species widely-distributed on both sides of the Atlantic Ocean and in the Mediterranean Sea. This species belongs to the Ommasthephidae family ("flying squid"), which includes many species worldwide, including many of great commercial importance (half of the world's squid captures correspond to one species of this family, *Dosidicus gigas*) *I. coindetii* is captured throughout the year, mostly from bottom and pelagic trawls, and has a high commercial interest [35]. *I. coindetii* males produce a mean of 465 spermatophores, with lengths ranging from 11 to 38 mm, the length being proportional to male size [15,18]. Copulated females have a mean of 484 spermatangia (13 mm mean length), attached in 1 to 6 bulbs on the internal mantle cavity, at the base of the gills [18]. Images of a spermatophore and spermatangia of *I. coindetii* are shown in Fig. 1, while further information can be found in Nigmatullin et al., 2003 [33].

It is well known that the use of cryoprotectants is required to ensure proper cell protection during the cryopreservation process. However, these agents can be toxic to spermatozoa, and the evaluation of such effects should be carefully studied before designing any cryopreservation protocol [36]. Moreover, the equilibration time in a solution with cryoprotectants should be long enough to allow the cryoprotectant to interact with the cells while minimizing toxic effects. Permeating cryoprotectants exert their protective effects by entering the cell, and require some time to permeate the plasma membrane and equilibrate with the external concentration, depending on its chemical structure and temperature. Furthermore, the cell must also undergo osmotic changes while the cryoprotectant enters and equilibrates [26], and recover from them. Moreover, the cryopreservation protocol (cooling and thawing rate, cooling and freezing method, container size and shape, etc.), critically affects post-thawing sperm viability, and it could interact with the effects of the cryoprotectant, either in a positive or detrimental way.

In this study, we carried out several toxicity experiments in an attempt to collect basic data on the tolerance of from *I. coindetii* spermatozoa to several permeating cryoprotectants, including a preliminary cryopreservation trial to investigate post-thawing sperm quality after using selected cryoprotectant protocols. In this species, the manipulation of spermatophores is easier than free spermatozoa, therefore we used whole spermathophores as the experimental units for the toxicity and cryopreservation trials, rather than free spermatozoa.

Materials and methods

Animals and samples

Mature individuals of the broadtail shortfin squid, *I. coindetii*, were captured by the local bottom-trawl fleet in the Mediterranean near Barcelona, Spain, (April–September, 2010). Whole squid were transported in ice to the laboratory. Spermatophores were collected by dissecting the spermatophoric organ (Needham'sac) of mature males measuring from 107 mm to 163 mm (mantle length) and weighing from 48 g to 158 g. Spermatangia were collected from copulated females measuring from 152 mm to 198 mm (mantle length) and weighing from 100 g to 196 g. Bulbs of spermatangia were dissected from the females using scissors, placed on a 1-mm mesh and vigorously flushed with seawater to remove organic debris from the surface. Three to four hours after squid collection at sea, spermatophores and spermatangia were individually placed in 5 ml plastic containers, covered with 0.2-μm filtered sea water (FSW) and stored at 4 °C for 12–14 h before being sent to the University of León at the same temperature. Samples were processed approximately 48 h after squid collection at sea.

Scanning electron microscopy of Illex spermatozoa

Spermatangia from copulated females were placed in a Petri dish and cut into small portions (<2 mm length) using scissors. To promote sperm activation, the chopped sperm mass from groups of 3–4 bulbs of spermatangia was added to a glass container with 10 ml of FSW and gently shaken. The milky solution was filtered through a 100-μm mesh to remove spermatangia capsule debris. Cover glasses were submerged in the milky solution to be impregnated by sperm, then fixed in 2.5% glutaraldehyde in FSW for 15 h, washed in FSW, followed by dehydration in an increasing concentration (v/v) of ethanol (20%, 30% and 50%) and stored in ethanol 70% at 4 °C. At the beginning of the SEM preparation, the samples were again dehydrated in an increasing concentration of ethanol (80%, 90%, and 95%) until saturated in absolute ethanol. Each ethanol bath lasted 10 min. After complete dehydration in the ethanol series, the samples were dried to the critical point in a Bal-Tec CPD 030 Drier using CO_2 as the transition liquid. After the drying stage, the samples were mounted on stubs with double-sided conductive sticky tape to orientate them in the preferred position. The mounted samples were sputter coated with gold–palladium in a Polaron Sputter Coater SC500 and then observed using a scanning electron Hitachi S3500N microscope with working voltages of 5 kV. Measurements of spermatozoa were obtained using the Image-Pro Plus 5.0 image analyser.

Experimental design

Experiment 1: toxicity study comparing different concentrations of cryoprotectants

Five permeating cryoprotectants were used for toxicity studies: Me2SO, methanol, glycerol, propylene glycol and ethylene glycol. Spermatophores were exposed to each cryoprotectant (5% and 15% (v/v) in FSW) at 4 °C. Incubation in FSW without cryoprotectant

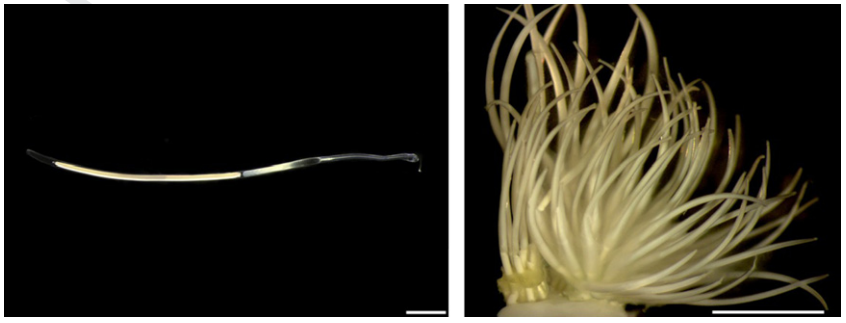

Fig. 1. *Illex coindetii* spermatophores from the spermatophoric organ (Needham's sac) of a mature male (left), and a bulb of spermatangia from a copulated female (right). Scale bar: 2 mm.

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was used as a control. After 5 min, the spermatophores were passed to two dilutions (1/2 and 1/4) with FSW, and finally placed in pure FSW. Each washing step lasted 2 min. After the third washing step, the spermatophores were placed on a glass slide and dissected using fine forceps. The viability and mitochondrial activity of the sperm mass was evaluated immediately by flow cytometry. Spermatophores from six males were used in this experiment.

Experiment 2: toxicity study comparing different exposure times to cryoprotectants

The same five permeating cryoprotectants were used (Me2SO, methanol, glycerol, propylene glycol and ethylene glycol) at 15% (v/v). Spermatophores were exposed to the cryoprotectant at 4 °C for 3 times: 5, 15 and 30 min. Incubation in FSW without cryoprotectant was used as a control. The spermatophores were then washed in two progressive dilutions of FSW (1/2 and 1/4), and finally placed in pure FSW. Each washing step lasted 2 min. After the third washing step, the spermatophores were assessed as in Experiment 1. Spermatophores from ten males were used in this experiment.

Experiment 3: cryopreservation of spermatophores (liquid nitrogen vapor vs. cryopreservation at −80 °C)

Spermatophores were loaded into cryovials containing 1 mL of FSW with each of the five permeating cryoprotectants (Me2SO, methanol, glycerol, propylene glycol and ethylene glycol) at 15% (v/v). Two trials were performed. In the first one, the spermatophores were exposed to the cryoprotectant at 4 °C for 15 min or 30 min, and then frozen using LN_2 vapors, as described later. In the second one, the spermatophores were exposed to the cryoprotectant at 4 °C for 15 min. The samples were then frozen using either: (1) LN_2 vapors or (2) cryopreservation at −80 °C. In the first method (LN_2), cryovials were placed 1 cm above LN_2 inside a closed styrofoam box. After 30 min, the cryovials were immersed in LN_2 and stored in cryoboxes in Dewar tanks containing LN_2. The freezing rate was −15 °C/min from 4 °C to −20 °C and −51 °C/min from −20 °C to 100 °C (determined in previous studies) [4]. In the second method (freezing at −80 °C), cryovials were put in a cryo-freeze container (Nalgene, Denmark), which was placed in a −80 °C freezer providing a −1 °C/min cooling rate (manufacturer's specifications). Samples frozen at −80 °C were stored at that temperature.

Thawing was carried out after one week. The cryovials were immersed in a 30 °C water bath for 2 min 20 s. After thawing, the spermatophores were washed and dissected as described for experiments 1 and 2. Viability and mitochondrial activity of the sperm mass were assessed immediately. Spermatophores from eight males were used in this experiment. For freezing in LN_2 vapor after 30 min of exposure to cryoprotectant and for freezing at −80 °C, experiment was performed in triplicate.

Evaluation of sperm viability and mitochondrial activity

Sperm viability (plasma membrane integrity) and mitochondrial activity were evaluated using fluorescent probes and flow cytometry. After extracting the sperm mass, it was diluted with 50 µl FSW added on the slide. Twenty-five microliters were added to 300 µl of FSW in a polypropylene tube, with 100 nM Mitotracker deep red (MT), 100 nM YOPRO1 and 5 µM Hoechst 33342 (H342). The MT and YOPRO1 were purchased from Invitrogen (Barcelona, Spain) and the H342 from Sigma (Madrid, Spain). Our group has successfully tested these stains in fish (unpublished data) and mammalian spermatozoa [28]. Mitotracker deep red (MT) accumulates into mitochondria with high membrane potential, thus discriminating cells with active mitochondria. YOPRO1 can penetrate cells with increased membrane permeability, intercalating into DNA and thus staining the nuclei of these cells. Hoechst 33342 (H342) is known to permeate the membranes of mammalian spermatozoa, staining the nuclei of all spermatozoa [30]. However, in the present study we found that this dye could not enter squid spermatozoa with intact membranes (YOPRO1–) and only stained part of the YOPRO1+ subpopulation. Therefore, it seems that H342 stains squid spermatozoa when membranes are damaged. YOPRO1+/H342– spermatozoa were considered membrane-intact, but with increased permeability, whereas YOPRO1+/H342+ spermatozoa were considered membrane-damaged.

After 5 min at ambient temperature, the sample was analyzed by flow cytometry (CyAn ADP, Beckman Coulter. Fullerton, CA, USA), carrying out a multicolor experiment. H342 was excited by a violet laser (405 nm) and the emission collected using a 450/50 nm filter; YOPRO1 was excited by a blue laser (488 nm) and the emission collected using a 530/40 nm filter; Mitotracker deep red was excited by a red laser (635 nm) and the emission collected using a 665/20 nm filter. Events were first plotted in a forward scatter vs. sideward scatter plot, and a gate was defined around the cloud of events corresponding to spermatozoa (validated using H342+ events, unequivocally identified with spermatozoa). Only events falling in that gate were considered as spermatozoa for fluorescence analysis. The fluorochrome combination allowed us to distinguish four subpopulations of spermatozoa: H342–/YOPRO1– were considered membrane-intact (viable) spermatozoa; H342–/YOPRO1+ were considered spermatozoa with increased membrane permeability; H342–/YOPRO1–/MT+ were considered viable spermatozoa with intact membranes that also had active mitochondria, and were expressed as the ratio of YOPRO1–/MT+ within YOPRO1– (membrane intact) spermatozoa; H342+ spermatozoa were considered non-viable (damaged membranes). Ten thousand events were read per sample.

Evaluation of sperm motility

On some occasions (this could not be performed systematically), sperm motility was subjectively checked after activating the spermatozoa with FSW. Motility was observed at room temperature using a Nikon E800 equipped with a 10× objective and phase contrast optics.

Statistical analysis

The statistical analyses were performed using the R statistical environment [6]. Viability and mitochondrial activity (as a % of each sperm subpopulation) were analyzed using linear mixed-effects models, with cryoprotectant, concentration, exposure time or cryopreservation method as fixed effects (depending on the experiment), and using the male as the grouping factor for the random part of the model. When required pairwise comparisons, were carried out using Tukey correction. Results are shown as mean ± -SEM, unless otherwise specified.

Results

Scanning electron microscopy of Illex spermatozoa

Observations from scanning electronic microscopy showed that *I. coindetii* spermatozoa have a cylindrical-shaped head and two tails (Fig. 2). Thirteen spermatozoa were measured, presenting mean ± SD values. The whole head region (acrosome + nucleus + nuclear appendage or mitochondrial spur) measured 6.83 ± 1.39 µm long (n = 13). The acrosome is a tronco-conical structure 0.43 ± 0.17 µm long positioned at the apex of the nucleus. The nucleus is elongated (5.12 ± 1.25 µm long, 1.39 ± 0.08 µm wide), being broadest at three-quarters of its length in correspondence with the

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annular atria (the insertion of the tails into the nucleus-spur border), a funnel-like structure which the long flagella projects. A cone-shaped appendage (1.41 ± 0.60 μm long) projects behind the posterior part of the nucleus. The tails were 53.22 ± 11.50 μm long and 0.24 ± 0.05 μm wide (n = 22). The structure became more filamentous in the distal part of the tail. The total length of the spermatozoon was 58.49 ± 4.40 μm (n = 22).

Flow cytometry analyses of fresh spermatoza

The flow cytometry analyses yielded defined populations according to previous studies in other species, showing high repeatability [30]. Flow cytometry assays of the untreated spermatophores showed that a large proportion of spermatozoa were membrane-intact (57.2 ± 6.1%), with a minor population of spermatozoa showing increased membrane permeability (18.5 ± 2.1%). Almost all spermatozoa with intact membranes also had active mitochondria (95.0 ± 0.7% of YOPRO1 – spermatozoa).

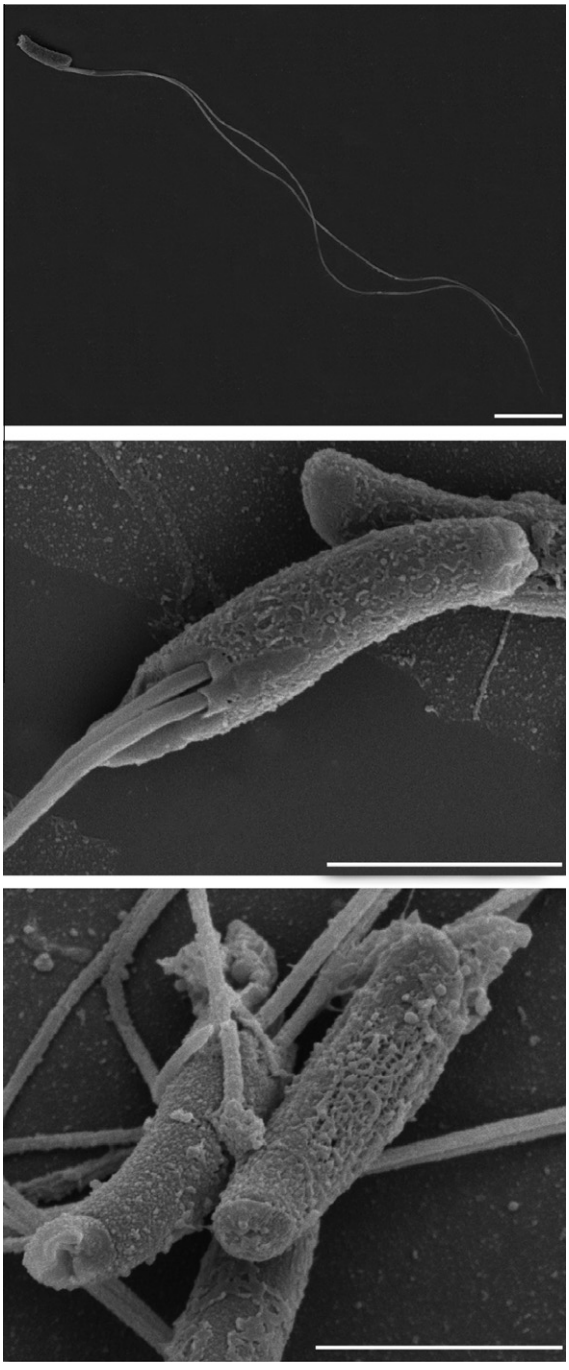

Fig. 2. Scanning electron microscope images of *Illex coindetii* spermatozoa obtained from spermatangia, showing the whole spermatozoon with two tails (top), the head region with the annular atria (center) and acrosomal region (bottom). Scale bar: 3 μm.

Results of the toxicity assays

In Experiment 1, toxicity assays comparing 5% and 15% of the five cryoprotectants showed minor differences among cryoprotectants or concentrations (Fig. 3). Only the concentration, as a main effect, significantly affected the proportion of spermatozoa with intact membranes (P < 0.001) and damaged membranes (P = 0.011), but neither the cryoprotectant type as a main effect nor the interaction of concentration × cryoprotectant type were significant. Subjecting the spermatophores to 5% cryoprotectant resulted in a slight decrease in membrane integrity (intact membrane: 48.9 ± 3.6%; damaged membrane: 26.2 ± 3.4%), which was significant in comparison with the control (intact membrane: 57.2 ± 6.1%, P < 0.001; damaged membrane: 24.3 ± 5.6%, P = 0.018), or with 15% (intact membrane: 54.9 ± 3.1%, P < 0.001; damaged membrane: 24.0 ± 2.5%, P = 0.003). The effect of the concentration was also significant for the proportion of spermatozoa with increased membrane permeability and with active mitochondria, but in this case, we detected a significant interaction between the concentration and the cryoprotectant. Therefore, we performed individual comparisons among treatments. A comparison within each cryoprotectant showed that the proportion of spermatozoa with intact membranes was higher for 5% methanol (31.5 ± 6.2%) and 5% ethylene glycol (28.2 ± 5.4%) than for the control (18.5 ± 2.1%) and 15% ethylene glycol (13.7 ± 3.1%), respectively, with no other significant differences found. The proportion of spermatozoa with active mitochondria (within membrane-intact spermatoza) was lower in 5% methanol (86.0 ± 3.5%), 15% glycerol (90.8 ± 2.5%) and 15% Me2SO (87.4 ± 3.2%) in comparison with comparing with the control. Similarly, 15% Me2SO was significantly lower than the control and 5% Me2SO (96.1 ± 0.3% overall). Nevertheless, we have to consider the higher viability of samples frozen with 15% Me2SO, and therefore when considering the absolute proportions of spermatozoa with active mitochondria the differences were not significant (5%: 48.2 ± 3.4%; 15%: 42.0 ± 2.2%; P > 0.05). Taking into account the results of Experiment 1, there were few differences between the two concentrations. Therefore we selected the 15% concentration for carrying out Experiment 2. The time-dependent variation of the membrane and mitochondrial status for each cryoprotectant are shown in the Fig. 4. There were few differences among cryoprotectants, and membrane status was not significantly affected by exposure time (although there was a trend towards increasing membrane damage with time). However, glycerol yielded a lower proportion of spermatozoa with intact membranes (P < 0.05 comparing with Me2SO or ethylene glycol) and a higher proportion of spermatozoa with damaged membranes (P < 0.05 comparing with ethylene glycol at 5 min; P < 0.01 comparing with ethylene glycol and methanol; P < 0.001 comparing with ME2SO at 15 min). At 30 min, glycerol yielded the highest proportion of membrane damage (40.5 ± 9.7%), although differences were not significant due to an increase in within-replicate variability at that time. Nevertheless, models showed an interaction of cryoprotectant and time for glycerol, indicating a significant decrease in sperm viability with time when using this cryoprotectant. Both membrane permeability and mitochondrial activity were significantly affected by the incubation time, whereas the cryoprotectant type did not show significant effects. Thus, the population of spermatozoa with increased membrane permeability grew from 21.1 ± 1.6% at 5 min to 34.7 ± 2.3% at 30 min (P = 0.021). Likewise,

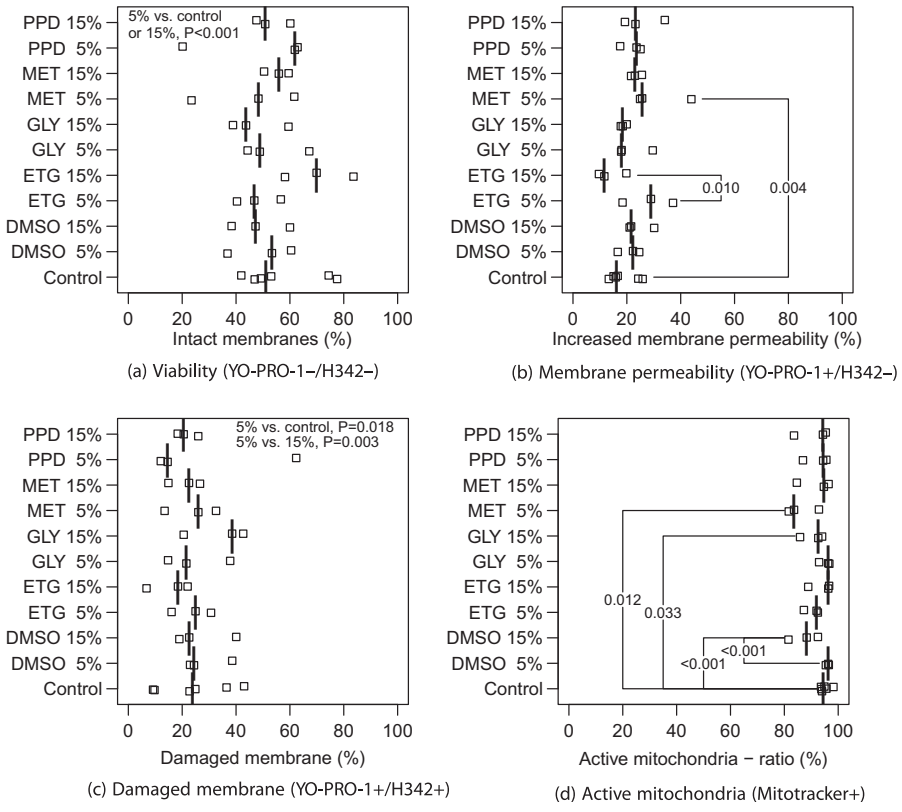

Fig. 3. Spermatozoa plasma membrane status (intact, increased permeability and damaged) and mitochondrial status after subjecting the *Illex coindetii* spermatophores to 5% or 15% of different permeating cryoprotectants (ETG: ethylene glycol, GLY: glycerol, MET: methanol, PPD: 1,2-propanediol) for 5 min, or to medium without cryoprotectant (Control). Medians in each treatment are indicated by vertical lines. When only significant main effects were detected, they are indicated in the inset text. If interactions between cryoprotectant type and concentration were significant, we carried out a pairwise comparison among treatments. In this case, lines join significantly different treatments (*P* value showed). Active mitochondria ratio is the proportion of spermatozoa with active mitochondria within the intact membrane population.

almost all spermatozoa with intact membranes showed active mitochondria at 5 min (91.0 ± 1.3%), this proportion lowering to 43.5 ± 5.7% at 30 min ($P = 0.007$).

Results of the cryopreservation trials

In Experiment 3, we tested the suitability of the cryoprotectants for freezing the spermatophores. Despite the good membrane and mitochondrial status of the spermatozoa in previous experiments, quality decreased considerably after cryopreservation using LN_2 vapors, with membrane integrity and mitochondrial status dropping almost to 0% in most treatments. Only 15% Me2SO, either with 15 or 30 min of incubation before freezing, showed some ability to preserve membrane integrity [5 ± 1.5% vs. 1.5 ± 0.5% (pooled results from the other cryoprotectants); $P < 0.001$]. Me2SO also achieved a higher percentage of spermatozoa maintaining active mitochondria (9.4 ± 1.6% vs. 4.4 ± 0.7%; $P < 0.01$) and a lower percentage of membrane-damaged spermatozoa (85.6 ± 2.8% vs. 94.1 ± 1.1%; $P < 0.001$). Using a freezer (−80 °C) for freezing the cryovials resulted in less than 1% viable spermatozoa in all treatments. Motility was checked in several samples, and only those frozen with Me2SO and with LN_2 vapors maintained a low proportion of spermatozoa capable to swim after freezing-thawing and activation (5–10% of motile spermatozoa).

Moreover, freezing/thawing seems to damage the spermatophore. We could observe signs of wear on the spermatophore, and spontaneous spermatophoric reaction when washing the thawed spermatophores. Nevertheless, these observations seemed unrelated to the quality of spermatozoa (data not shown).

Discussion

This is the first attempt to explore conditions for spermatophore cryopreservation in Cephalopoda. The interest of this study lies in the fact that some species within this class could be cultured in the near future, and thus the availability of cryopreservation methods for long-term sperm storage would be a valuable tool for farms and to maintain diversity in farmed stocks, or to select and breed desirable traits. Whereas no information is available for cephalopods, there are reports of successful cryopreservation of spermatozoa and spermatophores in other marine invertebrates. As an example, spermatophores and spermatozoa have been cryopreserved in the mud crab (*Scylla serrata*) [3], in the black tiger shrimp (*Penaeus monodon*) [34,39] and in the giant freshwater prawn (*Macrobrachium rosenbergii*) [1]. For practical reasons, we have only used whole spermatophores or spermatangia, rather than the free sperm mass. The possibility of cryopreserving whole spermatophores offers several advantages, since manipulation is easier and the spermatophores are structures whose function is to protect spermatozoa from stress, and to maintain them in a quiescent state. However, since spermatophores isolate spermatozoa from the environment, they might interfere with the cryopreservation process (delaying slowing cryoprotectant equilibration, for instance). We did not perform a comparison by using free spermatozoa in the three experiments, so this issue remains unsolved for now.

This study also presents a working flow cytometry protocol for analyzing squid spermatozoa, which allows the membrane and mitochondrial status of squid spermatozoa to be assessed (adapted from published protocols in other species [30]). Interestingly,

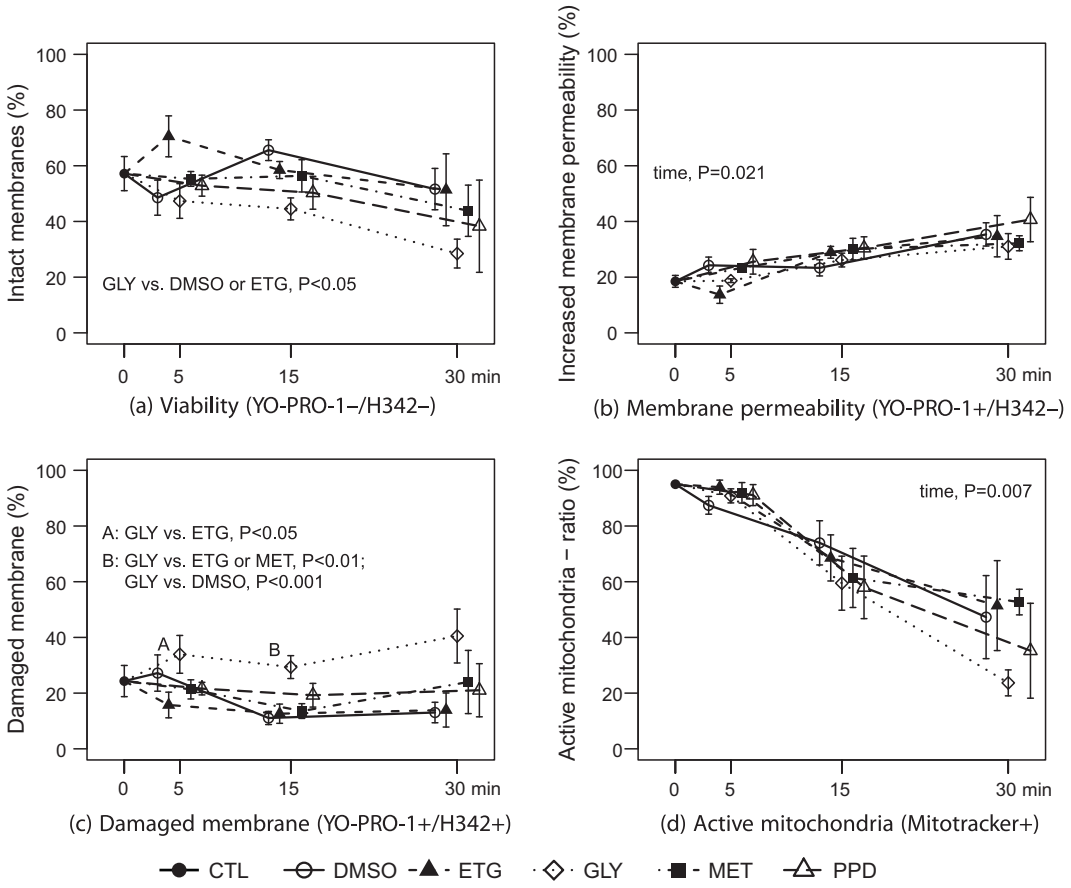

Fig. 4. Time dynamics of membrane and mitochondrial status of *Illex coindetii* spermatozoa (mean ± SEM at 5, 15 and 30 min), after submitting the spermatophores to 15% of the cryoprotectants Me2SO, ethylene glycol (ETG), glycerol (GLY), methanol (MET) or 1,2-propanediol (PPD). When only significant main effects were detected, they are indicated in the inset text. If interactions between cryoprotectant type and incubation time were significant, we carried out a pairwise comparison among treatments within each time. In this case, different letters indicate incubation times when treatments were significantly different (differences detailed in the inset text). Active mitochondria ratio is the proportion of spermatozoa with active mitochondria within the intact membrane population.

Hoechst 33342, which stains the whole sperm population in other species [28], could not permeate the membrane of squid spermatozoa (we cannot exclude the presence of membrane transporters causing Hoechst 33342 excursion), therefore identifying a disrupted-membrane sperm subpopulation.

Our results show that untreated spermatophores, despite being refrigerated for two days, had a high proportion of membrane intact spermatozoa (57.2%, SD: 15.0). These data suggest that spermatophores can be successfully transported in FSW at 4 °C and conserved for up to 48 h at least before being processed, although further experiments are necessary. Experiments in crustaceans have showed that refrigerated spermatophores can maintain high sperm viability for weeks and even a month and Naud & Havehand [31] reported that in spermatophores of the cuttlefish *Sepia apama* stored at 4 °C motility was still observed in resuspendedsperm after two months.

One of the most important steps in designing a cryopreservation protocol is the selection of a proper cryoprotectant combination as well as the optimum concentrations and exposure times [11]. Although cryoprotectants prevent cell damage during freezing/thawing, they are usually toxic [36]. In our study we have found that, when applied to spermatophores/a, cryoprotectant exposure exerts few negative effects on *Illex* spermatozoa. We have to take into account that both the addition and removal of permeating cryoprotectants subject the spermatophore and the spermatozoa to fast osmotic shocks [12], which could affect the protective role of the former and the post-thawing viability of the latter.

It could be argued that the spermatophores/a may hamper the entrance of cryoprotectants, therefore limiting their toxic effects on spermatozoa. However, in our time exposure experiment we used an equilibration time of up to 30 min, which is fairly long. Indeed, we detected some negative effects on membrane permeability and mitochondria with time, which could possibly be related (since the mitochondrial function would affect membrane permeability [29]). Interestingly, glycerol caused a higher increase in membrane damage, but no differences were observed in mitochondrial status or membrane permeability in comparison with the other cryoprotectants.

Our results suggest that *Illex* spermatozoa might be resistant to osmotic insults or to other toxic effects of the cryoprotectants. However, we must be aware of the limitations of our experiments. Moreover, we still have to determine the role of the spermatophore as a barrier for cryoprotectants. Replicating our experiments in released spermatozoa (more complex, though, due to the different procedures for washing free cells) would allow us to estimate the "real" resistance of squid spermatozoa to cryoprotectants.

Nevertheless, the results we obtained could be useful for designing future experiments. The toxicity results alone highlight glycerol as the least suitable option. They also indicate that cryoprotectant concentrations around 5% would be less effective (at least when the spermatozoa are kept within the spermatophore) suggesting that a successful protocol should use higher concentrations, while combining this higher concentration with a relatively short exposure time (better results when using 15% and 5 min).

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Unfortunately, our cryopreservation trial was not useful for confirming our previous findings, due to the extremely low viability obtained after thawing. Nevertheless, this experiment suggests that Me2SO would be the most promising cryoprotectant of those tested in this work. As previously stated, although there are practical reasons for cryopreserving whole spermatophores (easy manipulation), future experiments might be aimed not only at improving the cryopreservation protocol, but also at freezing free spermatozoa. The use of a cryoprotectant could not only be more effective, but it would also enable new approaches such as using straws instead of cryovials to be tried out, allowing faster and more uniform cooling of the sample. In fact, a shortcoming of our experimental design was that we only assayed one thawing speed (30 °C for 2 min 20 s). We chose this thawing protocol because it is described in the bibliography together with a variety of freezing protocols. However, it is true that the thawing protocol should be determined by the freezing protocol (especially regarding the speed of heat exchange). Therefore, we cannot discard that our post-thawing results are associated to the use of an inappropriate thawing protocol.

In conclusion, this study provides the first data on the toxic effects of cryoprotectants used in cephalopod spermatozoa, including a very preliminary cryopreservation trial. *Illex* spermatozoa displayed few signs of toxicity when exposed to cryoprotectants, but the attempts at cryopreservation were unsuccessful. The toxicity results showed that glycerol might be inadequate for *Illex* spermatozoa, whereas Me2SO seemed to be the most adequate, especially at 15% and after an exposure treatment of 5 min. We have also presented a method to assess membrane and mitochondrial status in cephalopod spermatozoa by using flow cytometry, which was fast and effective. Although this study was limited and our results must be considered with caution, it might help to design future experiments aimed at achieving sperm cryopreservation in Cephalopoda. Freezing free spermatozoa instead of the whole spermatophore should be attempted in order to better understand the cryobiology of squid sperm, although the convenience of freezing whole spermatophora remains.

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