Manuscript
Click here to view linked References

1 **CRYOBIOLOGY OF CEPHALOPOD *(Illex coindetti)* SPERMATOPHORES**

2 Vanesa Robles[1,2*#], Felipe Martínez-Pastor[1,2*], Giuliano Petroni[3] , Marta F. Riesco[1,2],
3 Anna Bozzano[3], Roger Villanueva[3]
4 *Authors have equally contributed to the work
5 [1] Department of Molecular Biology, Faculty of Biology, University of León, 24071,
6 Spain
7 [2] INDEGSAL, University of León, 24071, Spain
8 [3] Institut de Ciències del Mar (CSIC), Passeig Maritim de la Barceloneta 37-49, 08003-
9 Barcelona, Spain
10 [#]corresponding author: Vanesa Robles, Tel. (+34) 987291000 ext 5678 email
11 v.robles@unileon.es
12
13
14

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% to 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.

Keywords: Mollusca, spermatophore, spermatangia, spermatozoa, cryopreservation, flow cytometry.

39 **1. Introduction**

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

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

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

62 Studies have been carried out on spermatozoon structure and morphological
63 characteristics in several species of cephalopods [5,7,13,14,16,17,25,27], spermatophore
64 physics, morphology and physiology [2,33], and spermatangium characteristics
65 [19,20,21,32]. Nevertheless, very little information on spermatophores or
66 spermatangium is available [22]. As far as we know, the only precedent on cephalopod

67 spermatophore refrigeration was reported by Naud & Havehand [31] for the cuttlefish
68 *Sepia apama* and no published information exists on sperm, spermatophore or
69 spermatangium cryobiology for any cephalopod species.

70 In this study, we used the broadtail shortfin squid *Illex coindetii* (Vérany, 1839),
71 which is a medium-sized oceanic species widely-distributed on both sides of the
72 Atlantic Ocean and in the Mediterranean Sea. This species belongs to the
73 Ommasthephidae family ("flying squid"), which includes many species worldwide,
74 including many of great commercial importance (half of the world's squid captures
75 correspond to one species of this family, *Dosidicus gigas*) *Illex coindetii* is captured
76 throughout the year, mostly from bottom and pelagic trawls, and has a high commercial
77 interest [35]. *I. coindetii* males produce a mean of 465 spermatophores, with lengths
78 ranging from 11 to 38 mm, the length being proportional to male size [15,18].
79 Copulated females have a mean of 484 spermatangia (13 mm mean length), attached in
80 1 to 6 bulbs on the internal mantle cavity, at the base of the gills [18]. Images of a
81 spermatophore and spermatangia of *Illex coindetii* are shown in Figure 1, while further
82 information can be found in Nigmatullin et al., 2003 [33].

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

95 and it could interact with the effects of the cryoprotectant, either in a positive or
96 detrimental way.

97 In this study, we carried out several toxicity experiments in an attempt to collect
98 basic data on the tolerance of from *I. coindetii* spermatozoa to several permeating
99 cryoprotectants, including a preliminary cryopreservation trial to investigate post-
100 thawing sperm quality after using selected cryoprotectant protocols. In this species, the
101 manipulation of spermatophores is easier than free spermatozoa, therefore we used
102 whole spermathophores as the experimental units for the toxicity and cryopreservation
103 trials, rather than free spermatozoa.
104

105 **2. Materials and Methods**

106 ***2.1 Animals and samples***

107 Mature individuals of the broadtail shortfin squid, *Illex coindetii*, were captured
108 by the local bottom-trawl fleet in the Mediterranean near Barcelona, Spain, (April to
109 September, 2010). Whole squid were transported in ice to the laboratory.
110 Spermatophores were collected by dissecting the spermatophoric organ (Needham'sac)
111 of mature males measuring from 107 mm to 163 mm (mantle length) and weighing
112 from 48 g to 158 g. Spermatangia were collected from copulated females measuring
113 from 152 mm to 198 mm (mantle length) and weighing from 100 g to 196 g. Bulbs of
114 spermatangia were dissected from the females using scissors, placed on a 1-mm mesh
115 and vigorously flushed with seawater to remove organic debris from the surface. Three
116 to four hours after squid collection at sea, spermatophores and spermatangia were
117 individually placed in 5 ml plastic containers, covered with 0.2-µm filtered sea water
118 (FSW) and stored at 4 °C for 12-14 h before being sent to the University of León at the
119 same temperature. Samples were processed approximately 48 h after squid collection at
120 sea.
121

122 ***2.2 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.

2.3 Experimental design

2.3.1 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

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

154

155 *2.3.2 Experiment 2: Toxicity study comparing different exposure times to*
156 *cryoprotectants*

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

165

166 *2.3.3 Experiment 3: Cryopreservation of spermatophores (liquid nitrogen vapor vs.*
167 *cryopreservation at -80 °C)*

168 Spermatophores were loaded into cryovials containing 1 mL of FSW with each
169 of the five permeating cryoprotectants (Me2SO, methanol, glycerol, propylene glycol
170 and ethylene glycol) at 15% (v/v). Two trials were performed. In the first one, the
171 spermatophores were exposed to the cryoprotectant at 4 °C for 15 min or 30 min, and
172 then frozen using LN_2 vapors, as described later. In the second one, the spermatophores
173 were exposed to the cryoprotectant at 4 °C for 15 min. The samples were then frozen
174 using either: (1) LN_2 vapors or (2) cryopreservation at -80 °C. In the first method (LN_2),
175 cryovials were placed 1 cm above LN_2 inside a closed styrofoam box. After 30 min, the

176 cryovials were immersed in LN_2 and stored in cryoboxes in Dewar tanks containing
177 LN_2. The freezing rate was -15 °C/min from 4 °C to -20 °C and -51 °C/min from -20 °C
178 to 100 °C (determined in previous studies) [4]. In the second method (freezing at -
179 80 °C), cryovials were put in a cryo-freeze container (Nalgene, Denmark), which was
180 placed in a -80 °C freezer providing a -1 °C/min cooling rate (manufacturer's
181 specifications). Samples frozen at -80 °C were stored at that temperature.
182 Thawing was carried out after one week. The cryovials were immersed in a 30 °C water
183 bath for 2 min 20 s. After thawing, the spermatophores were washed and dissected as
184 described for experiments 1 and 2. Viability and mitochondrial activity of the sperm
185 mass were assessed immediately. Spermatophores from eight males were used in this
186 experiment. For freezing in LN_2 vapor after 30 min of exposure to cryoprotectant and
187 for freezing at -80 °C, experiment was performed in triplicate.
188
189
190 ***2.4 Evaluation of sperm viability and mitochondrial activity***
191 Sperm viability (plasma membrane integrity) and mitochondrial activity were
192 evaluated using fluorescent probes and flow cytometry. After extracting the sperm mass,
193 it was diluted with 50 µl FSW added on the slide. Twenty-five microliters were added to
194 300 µl of FSW in a polypropylene tube, with 100 nM Mitotracker deep red (MT),
195 100 nM YOPRO1 and 5 µM Hoechst 33342 (H342). The MT and YOPRO1 were
196 purchased from Invitrogen (Barcelona, Spain) and the H342 from Sigma (Madrid,
197 Spain). Our group has successfully tested these stains in fish (unpublished data) and
198 mammalian spermatozoa [28]. Mitotracker deep red (MT) accumulates into
199 mitochondria with high membrane potential, thus discriminating cells with active
200 mitochondria. YOPRO1 can penetrate cells with increased membrane permeability,
201 intercalating into DNA and thus staining the nuclei of these cells. Hoechst 33342
202 (H342) is known to permeate the membranes of mammalian spermatozoa, staining the
203 nuclei of all spermatozoa [30]. However, in the present study we found that this dye

204 could not enter squid spermatozoa with intact membranes (YOPRO1–) and only stained
205 part of the YOPRO1+ subpopulation. Therefore, it seems that H342 stains squid
206 spermatozoa when membranes are damaged. YOPRO1+/H342– spermatozoa were
207 considered membrane-intact, but with increased permeability, whereas YOPRO-
208 1+/H342+ spermatozoa were considered membrane-damaged.
209 After 5 min at ambient temperature, the sample was analyzed by flow cytometry (CyAn
210 ADP, Beckman Coulter. Fullerton, CA, USA), carrying out a multicolor experiment.
211 H342 was excited by a violet laser (405 nm) and the emission collected using a
212 450/50 nm filter; YOPRO1 was excited by a blue laser (488 nm) and the emission
213 collected using a 530/40 nm filter; Mitotracker deep red was excited by a red laser
214 (635 nm) and the emission collected using a 665/20 nm filter. Events were first plotted
215 in a forward scatter vs. sideward scatter plot, and a gate was defined around the cloud of
216 events corresponding to spermatozoa (validated using H342+ events, unequivocally
217 identified with spermatozoa). Only events falling in that gate were considered as
218 spermatozoa for fluorescence analysis. The fluorochrome combination allowed us to
219 distinguish four subpopulations of spermatozoa: H342–/YOPRO1– were considered
220 membrane-intact (viable) spermatozoa; H342–/YOPRO1+ were considered
221 spermatozoa with increased membrane permeability; H342–/YOPRO1–/MT+ were
222 considered viable spermatozoa with intact membranes that also had active
223 mitochondria, and were expressed as the ratio of YOPRO1–/MT+ within YOPRO1–
224 (membrane intact) spermatozoa; H342+ spermatozoa were considered non-viable
225 (damaged membranes). Ten thousand events were read per sample.

226

227 ***2.5 Evaluation of sperm motility***

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

232

233 *2.6 Statistical analysis*

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

241

242 **3. Results**

243 ***3.1 Scanning electron microscopy of Illex spermatozoa***

244 Observations from scanning electronic microscopy showed that *Illex coindetii*
245 spermatozoa have a cylindrical-shaped head and two tails (Figure 2). Thirteen
246 spermatozoa were measured, presenting mean ± SD values. The whole head region
247 (acrosome + nucleus + nuclear appendage or mitochondrial spur) measured 6.83 ±
248 1.39 µm long (n= 13). The acrosome is a tronco-conical structure 0.43 ± 0.17 µm long
249 positioned at the apex of the nucleus. The nucleus is elongated (5.12 ± 1.25 µm long,
250 1.39 ± 0.08 µm wide), being broadest at three-quarters of its length in correspondence
251 with the annular atria (the insertion of the tails into the nucleus-spur border), a funnel-
252 like structure which the long flagella projects. A cone–shaped appendage (1.41 ±
253 0.60 µm long) projects behind the posterior part of the nucleus. The tails were 53.22 ±
254 11.50 µm long and 0.24 ± 0.05 µm wide (n= 22). The structure became more
255 filamentous in the distal part of the tail. The total length of the spermatozoon was
256 58.49±4.40 µm (n=22).

3.2 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).

3.3 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 (Figure 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

285 15% Me2SO (87.4% ± 3.2) in comparison with comparing with the control. Similarly, 286 15% Me2SO was significantly lower than the control and 5% Me2SO (96.1% ± 0.3 287 overall). Nevertheless, we have to consider the higher viability of samples frozen with 288 15% Me2SO, and therefore when considering the absolute proportions of spermatozoa 289 with active mitochondria the differences were not significant (5%: 48.2% ± 3.4; 15%: 290 42.0% ± 2.2; P>0.05). Taking into account the results of Experiment 1, there were few 291 differences between the two concentrations. Therefore we selected the 15% 292 concentration for carrying out Experiment 2. The time-dependent variation of the 293 membrane and mitochondrial status for each cryoprotectant are shown in the Figure 4. 294 There were few differences among cryoprotectants, and membrane status was not 295 significantly affected by exposure time (although there was a trend towards increasing 296 membrane damage with time). However, glycerol yielded a lower proportion of 297 spermatozoa with intact membranes (P<0.05 comparing with Me2SO or ethylene 298 glycol) and a higher proportion of spermatozoa with damaged membranes (P<0.05 299 comparing with ethylene glycol at 5 min; P<0.01 comparing with ethylene glycol and 300 methanol; P<0.001 comparing with ME2SO at 15 min). At 30 min, glycerol yielded the 301 highest proportion of membrane damage (40.5% ± 9.7), although differences were not 302 significant due to an increase in within-replicate variability at that time. Nevertheless, 303 models showed an interaction of cryoprotectant and time for glycerol, indicating a 304 significant decrease in sperm viability with time when using this cryoprotectant. Both 305 membrane permeability and mitochondrial activity were significantly affected by the 306 incubation time, whereas the cryoprotectant type did not show significant effects. Thus, 307 the population of spermatozoa with increased membrane permeability grew from 21.1% 308 ± 1.6 at 5 min to 34.7% ± 2.3 at 30 min (P=0.021). Likewise, almost all spermatozoa 309 with intact membranes showed active mitochondria at 5 min (91.0% ± 1.3), this 310 proportion lowering to 43.5% ± 5.7 at 30 min (P=0.007).

311 ***3.3 Results of the cryopreservation trials***

312 In Experiment 3, we tested the suitability of the cryoprotectants for freezing the
313 spermatophores. Despite the good membrane and mitochondrial status of the
314 spermatozoa in previous experiments, quality decreased considerably after
315 cryopreservation using LN_2 vapours, with membrane integrity and mitochondrial status
316 dropping almost to 0% in most treatments. Only 15% Me2SO, either with 15 or 30 min
317 of incubation before freezing, showed some ability to preserve membrane integrity [5%
318 ± 1.5 vs. 1.5% ± 0.5 (pooled results from the other cryoprotectants); $P<0.001$]. Me2SO
319 also achieved a higher percentage of spermatozoa maintaining active mitochondria
320 (9.4% ± 1.6 vs. 4.4% ± 0.7; $P<0.01$) and a lower percentage of membrane-damaged
321 spermatozoa (85.6% ± 2.8 vs. 94.1% ± 1.1; $P<0.001$). Using a freezer (-80 °C) for
322 freezing the cryovials resulted in less than 1% viable spermatozoa in all treatments.
323 Motility was checked in several samples, and only those frozen with Me2SO and with
324 LN_2 vapours maintained a low proportion of spermatozoa capable to swim after
325 freezing-thawing and activation (5% to 10% of motile spermatozoa).
326 Moreover, freezing/thawing seems to damage the spermatophore. We could
327 observe signs of wear on the spermatophore, and spontaneous spermatophoric reaction
328 when washing the thawed spermatophores. Nevertheless, these observations seemed
329 unrelated to the quality of spermatozoa (data not shown).
330

331 4. Discussion

332 This is the first attempt to explore conditions for spermatophore
333 cryopreservation in Cephalopoda. The interest of this study lies in the fact that some
334 species within this class could be cultured in the near future, and thus the availability of
335 cryopreservation methods for long-term sperm storage would be a valuable tool for
336 farms and to maintain diversity in farmed stocks, or to select and breed desirable traits.
337 Whereas no information is available for cephalopods, there are reports of successful
338 cryopreservation of spermatozoa and spermatophores in other marine invertebrates. As
339 an example, spermatophores and spermatozoa have been cryopreserved in the mud crab

340 (*Scylla serrata*) [3], in the black tiger shrimp (*Penaeus monodon*) [34,39] and in the
341 giant freshwater prawn (*Macrobrachium rosenbergii*) [1]. For practical reasons, we have
342 only used whole spermatophores or spermatangia, rather than the free sperm mass. The
343 possibility of cryopreserving whole spermatophores offers several advantages, since
344 manipulation is easier and the spermatophores are structures whose function is to
345 protect spermatozoa from stress, and to maintain them in a quiescent state. However,
346 since spermatophores isolate spermatozoa from the environment, they might interfere
347 with the cryopreservation process (delaying slowing cryoprotectant equilibration, for
348 instance). We did not perform a comparison by using free spermatozoa in the three
349 experiments, so this issue remains unsolved for now.

350 This study also presents a working flow cytometry protocol for analyzing squid
351 spermatozoa, which allows the membrane and mitochondrial status of squid
352 spermatozoa to be assessed (adapted from published protocols in other species [30]).
353 Interestingly, Hoechst 33342, which stains the whole sperm population in other species
354 [28], could not permeate the membrane of squid spermatozoa (we cannot exclude the
355 presence of membrane transporters causing Hoechst 33342 excursion), therefore
356 identifying a disrupted-membrane sperm subpopulation.

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

366 One of the most important steps in designing a cryopreservation protocol is the
367 selection of a proper cryoprotectant combination as well as the optimum concentrations

368 and exposure times [11]. Although cryoprotectants prevent cell damage during
369 freezing/thawing, they are usually toxic [36]. In our study we have found that, when
370 applied to spermatophores/a, cryoprotectant exposure exerts few negative effects on
371 *Illex* spermatozoa. We have to take into account that both the addition and removal of
372 permeating cryoprotectants subject the spermatophore and the spermatozoa to fast
373 osmotic shocks [12], which could affect the protective role of the former and the post-
374 thawing viability of the latter.
375 It could be argued that the spermatophores/a may hamper the entrance of
376 cryoprotectants, therefore limiting their toxic effects on spermatozoa. However, in our
377 time exposure experiment we used an equilibration time of up to 30 min, which is fairly
378 long. Indeed, we detected some negative effects on membrane permeability and
379 mitochondria with time, which could possibly be related (since the mitochondrial
380 function would affect membrane permeability [29]). Interestingly, glycerol caused a
381 higher increase in membrane damage, but no differences were observed in
382 mitochondrial status or membrane permeability in comparison with the other
383 cryoprotectants.
384 Our results suggest that *Illex* spermatozoa might be resistant to osmotic insults or to
385 other toxic effects of the cryoprotectants. However, we must be aware of the limitations
386 of our experiments. Moreover, we still have to determine the role of the spermatophore
387 as a barrier for cryoprotectants. Replicating our experiments in released spermatozoa
388 (more complex, though, due to the different procedures for washing free cells) would
389 allow us to estimate the "real" resistance of squid spermatozoa to cryoprotectants.
390 Nevertheless, the results we obtained could be useful for designing future
391 experiments. The toxicity results alone highlight glycerol as the least suitable option.
392 They also indicate that cryoprotectant concentrations around 5% would be less effective
393 (at least when the spermatozoa are kept within the spermatophore) suggesting that a
394 successful protocol should use higher concentrations, while combining this higher

395 concentration with a relatively short exposure time (better results when using 15% and
396 5 min).

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

412 In conclusion, this study provides the first data on the toxic effects of
413 cryoprotectants used in cephalopod spermatozoa, including a very preliminary
414 cryopreservation trial. *Illex* spermatozoa displayed few signs of toxicity when exposed
415 to cryoprotectants, but the attempts at cryopreservation were unsuccessful. The toxicity
416 results showed that glycerol might be inadequate for *Illex* spermatozoa, whereas Me2SO
417 seemed to be the most adequate, especially at 15% and after an exposure treatment of
418 5 min. We have also presented a method to assess membrane and mitochondrial status in
419 cephalopod spermatozoa by using flow cytometry, which was fast and effective.
420 Although this study was limited and our results must be considered with caution, it
421 might help to design future experiments aimed at achieving sperm cryopreservation in
422 Cephalopoda. Freezing free spermatozoa instead of the whole spermatophore should be

423 attempted in order to better understand the cryobiology of squid sperm, although the
424 convenience of freezing whole spermatophora remains.
425
426 **5. Acknowledgments** Vanesa Robles and Felipe Martínez-Pastor were supported by the
427 Ramón y Cajal program (RYC-2008-02339 and RYC-2008-02560), and Marta F. Riesco
428 was supported by a Junta de Castilla y León PhD grant (European Social Fund). We
429 thank the Spanish Ministry of Science and Innovation (MICINN) (research projects
430 AGL2009-11546 and AGL2009-06994) and the Fundación Ramón Areces, and Cintia
431 Miranda for her technical support. We gratefully acknowledge Mr. J.M. Fortuño (Institut
432 de Ciències del Mar) for assistance and advice in obtaining SEM images.
433
434 **6. References**
435 1. K. Akarasanon, P. Damrongphol, W. Poolsanguan, Aquaculture Research. 35 (2004)
436 1415-1420.

437 2. C.R. Austin, C. Lutwak-Mann, TProc R Soc Lond B Biol Sci. 161(1964) 143-52.

438 3. S. Bhavanishankar, T. Subramoniam, J Exp Zool. 277(1997) 326–336.

439 4. E. Cabrita , V. Robles , and P. Herráez (Eds) Methods in Reproductive Aquaculture
440 Marine and Freshwater Species, CRC Press, 2008, pp. 505–508

441 5. D. Cuccu, M. Mereu, R. Cannas, M.C. Follesa, A. Cau, P. Jereb, Journal of the
442 Marine Biological Association of the United Kingdom 87(2007) 971-976.

443 6. Development Core Team: A language and environment for statistical computing. R
444 Foundation for statistical Computing, Vienna, Austria. (2010) ISBN 3-900051-07-
445 0, URL http://www.R-project .org/.

446 7. A. Diaspro, F. Beltrame, M. Fato, A. Palmeri, P. Ramoino, Microsc Res Tech.
447 36(1997)159-64.

448 8. P. Domingues, A. Ferreira, L. Marquez, J.P. Andrade, N. Lopez, C. Rosas,
449 Aquaculture International.16 (2008) 215-229.

450 9. A. Dunstan, CJ. Bradshaw, J. Marshall, Plos One. 10 (2011) 6.

451 10. FAO Yearbook 2010. Fishery and Aquaculture Statistics. Capture production. Rome.
452 (2012) 609.

453 11. D.Y. Gao, J. Liu, C. Liu, L.E. McGann, , P.F. Watson, F.W. Kleinhans, P. Mazur,
454 E.S. Critser, J.K. Critser, Hum Reprod. 10 5(1995)1109-22.

455 12. D.Y. Gao, J.K. Critser, ILAR. J 41 4 (2000)187-96.

456 13. P. Gimenez-Bonafé, E. Ribes, P. Sautière, A. Gonzalez, H. Kasinsky, M. Kouach,
457 P.E. Sautière, J. Ausió, M. Chiva, Eur J Cell Biol. 81 (2002) 341-9.

458 14. P. Giménez-Bonafé, F.M. Soler, C. Buesa, P.E Sautière, J. Ausió, M. Kouach, H.E.
459 Kasinsky, M. Chiva, Mol Reprod Dev. 68 (2004) 232-9.

460 15. A.F. González, A.Guerra, Sarsia. 8 (1996)107-118.

461 16. J.M. Healy, Philosophical Transactions of the Royal Society of London Series B-
462 Biological Sciences. 323 (1989) 589-600.

463 17. J.M. Healy, Zoologica Scripta. 19 (1990) 189–193.

464 18. V. Hernández-García, Bulletin of Marine Science. 71 (2002) 347-366.

465 19. H.J.T. Hoving, V. Laptikhovsky, Biological Bulletin. 212 (1995) 177-179.

466 20. H.J.T. Hoving, M.R. Lipinski, J.J. Videler, African Journal of rine Science. 30
467 (2008) 603-612.

468 21. .J.T. Hoving, M.R. Lipinski, J.J. Videler, K.S.R. Bolstad, Marine Biology. 157
469 (2010) 393-400.

470 22. C.L. Huffard, K. Buck, B. Robison. CalCOFI Conference, San Diego, California,
471 November 26-28 Program and Abstracts, Poster-23, (2007) p 65.

472 23. J. Iglesias, F.J. Sanchez, J.G.F. Bersano, J.F. Carrasco, J. Dhont, L. Fuentes, F.
473 Linares, J.L. Munoz, S. Okumura, J. Roo, T. van der Meeren, E.A.G. Vidal, R.
474 Villanueva. Aquaculture 266 (2007) 1-15.

475 24. Y. Ikeda, Y. Sakurai, K. Shimazaki, Invertebrate Reproduction and Development. 23
476 (1993) 39-44.

477 25. V.V. Laptikhovsky, C.M. Nigmatullin , Ruthenica. 6 (1996) 149-159.

478 26. R.L. Levin, J Biomech Eng. 104 (1982) 81-6.

479 27. Y.Z. Li, H. Pan, J. Xu, X.W. Fan, X.C. Song, Q. Zhang, J. Xu, Y. Liu, J Hazard
480 Mater.179 (2010) 266-75.

481 28. F. Martínez-Pastor, E. Aisen, M. Fernández-Santos, M. Esteso , A. Maroto-Morales,
482 O. García-Alvarez, J. Garde, Reproduction Cambridge England. 137 (2009) 225-
483 235.

484 29. F. Martínez-Pastor, M.R. Fernández-Santos, E. del Olmo, A.E. Dominguez-
485 Rebolledo, M.C. Esteso, V. Montoro, J. Garde, Reprod Fertil Dev. 20 5 (2008) 547–
486 556.

487 30. F. Martínez-Pastor, M. Mata-Campuzano, M. Álvarez-Rodríguez, M. Álvarez, L
488 .Anel, P. de Paz, Reprod Domest Anim 45 Suppl. 2 (2010) 67-78.

489 31. M.J. Naud, J.N. Havenhand, Marine Biology. 148 (2006) 559-566.

490 32. K.N. Nesis, Ruthenica 6 (1995) 23-64.

491 33. C. Nigmatullin, R.M. Sabirov, V.P. Zalygalin, Berliner Palaeobiologische
492 Abhandlungen. 3 (2003) 225-240.

493 34. S. Nimrat, T. Sangnawakij, V. Vuthiphandchai , J. World Aquac. Soc 36 (2005) 76–
494 86.

495 35. G.J. Pierce, A.L. Allcock, I. Bruno, P. Bustamante, A.F. González, A. Guerra, P.
496 Jereb, E. Lefkaditou, S.K. Malham, A. Moreno, J. Pereira, U. Piatkowski, M.
497 Rasero, P. Sánchez, M.B. Santos, M. Santurtun, S. Seixas, I. Sobrino, R. Villanueva
498 Cephalopod biology and fisheries in Europe. In: Anderson, E.D. (Ed.), ICES
499 Cooperative Research Report. International Council for the Exploration of the Sea
500 (2010) Copenhagen pp. 175.

501 36. V. Robles, E. Cabrita, M.P. Herráez, Zebrafish. 6 (2009) 281-93.

502 37. C. Rosas, J. Tut, J. Baeza, A. Sanchez, V. Sosa, C. Pascual, L. Arena, P.,Domingues,
503 G. Cuzon. Aquaculture 275 (2008) 291-297.

504 38. Y. Sakurai, R.E. Young, J. Hirota, K. Mangold, Veliger 38(1995) 185–191.

505 39. M. Salazar, M. Lezcano, C. Granja Protocol for cryopreservation of *Penaeus*
506 *vannamei* sperm cells in E. Cabrita , V. Robles , and P. Herráez (Eds) Methods in
507 Reproductive Aquaculture Marine and Freshwater Species, CRC Press, 2008, pp.
508 505–50.

509 40. R. Villanueva, D. J. Staafb, J. Argüellesc, A. Bozzanoa, S. Camarillo-Coopd, et al,
510 Aquaculture. 342–343 (2012) 125–133.

511 41. K. Watanabe, Y. Sakurai, S. Segawa, T. Okutani, American Malacological Bulletin. 13
512 (1996) 73–88.

513

514

515

516

517 **7. Figure captions**

518

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

522

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

526

527 Figure 3. Spermatozoa plasma membrane status (intact, increased permeability and
528 damaged) and mitochondrial status after subjecting the *Illex coindetii* spermatophores to
529 5% or 15% of different permeating cryoprotectants (ETG: ethylene glycol, GLY:
530 glycerol, MET: methanol, PPD: 1,2-propanediol) for 5 min, or to medium without
531 cryoprotectant (Control). Medians in each treatment are indicated by vertical lines.
532 When only significant main effects were detected, they are indicated in the inset text. If
533 interactions between cryoprotectant type and concentration were significant, we carried
534 out a pairwise comparison among treatments. In this case, lines join significantly
535 different treatments (P value showed). Active mitochondria ratio is the proportion of
536 spermatozoa with active mitochondria within the intact membrane population.

537

538 Figure 4. Time dynamics of membrane and mitochondrial status of *Illex coindetii*
539 spermatozoa (mean±SEM at 5, 15 and 30 min), after submitting the spermatophores to
540 15% of the cryoprotectants Me2SO, ethylene glycol (ETG), glycerol (GLY), methanol

541 (MET) or 1,2-propanediol (PPD). When only significant main effects were detected,
542 they are indicated in the inset text. If interactions between cryoprotectant type and
543 incubation time were significant, we carried out a pairwise comparison among
544 treatments within each time. In this case, different letters indicate incubation times when
545 treatments were significantly different (differences detailed in the inset text). Active
546 mitochondria ratio is the proportion of spermatozoa with active mitochondria within the
547 intact membrane population.
548

549

Figure(s)

Figure 1

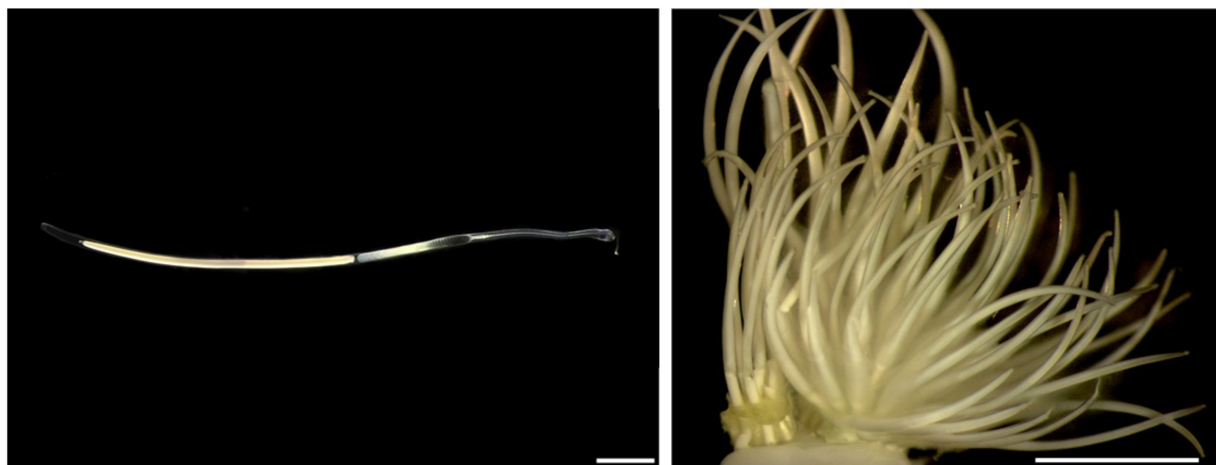
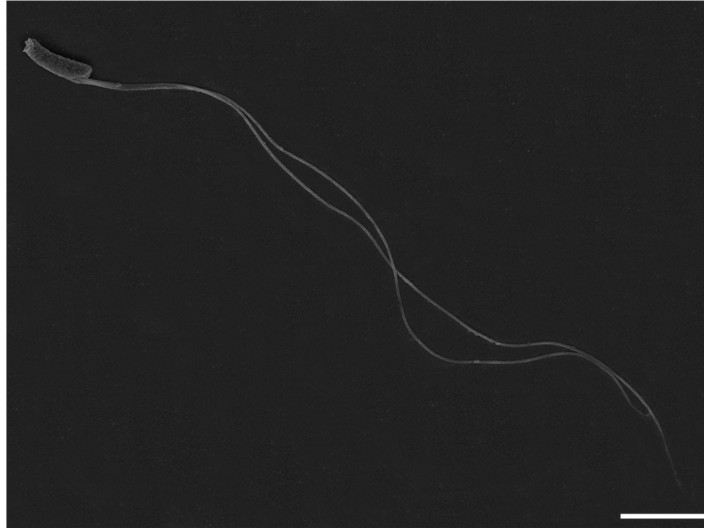
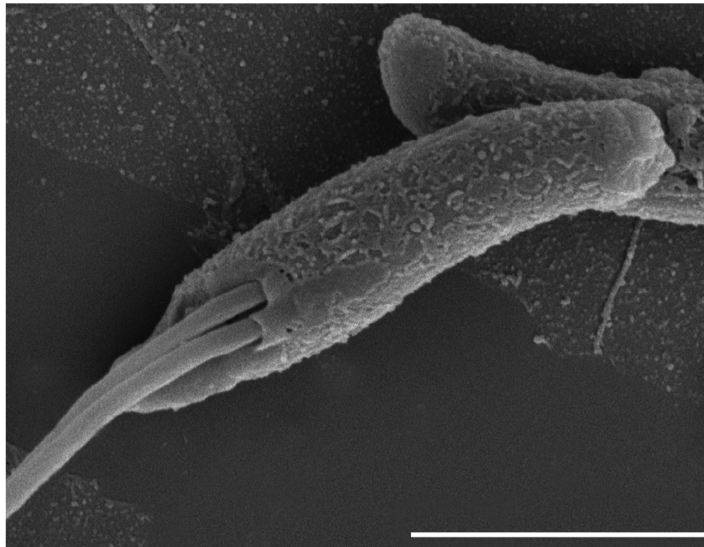


Figure 2





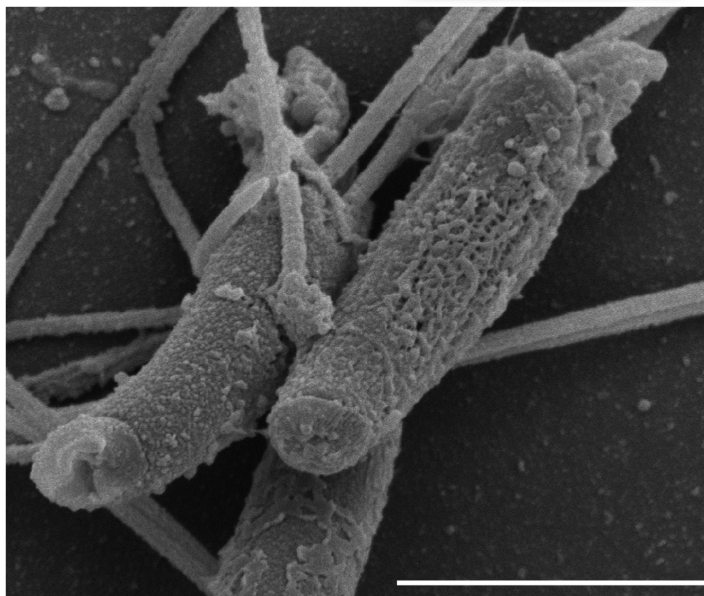
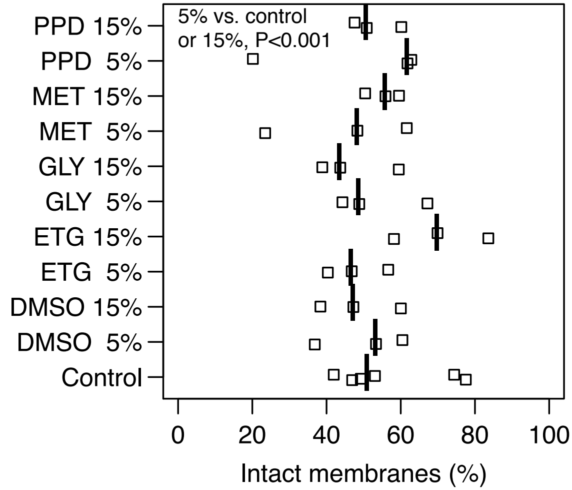
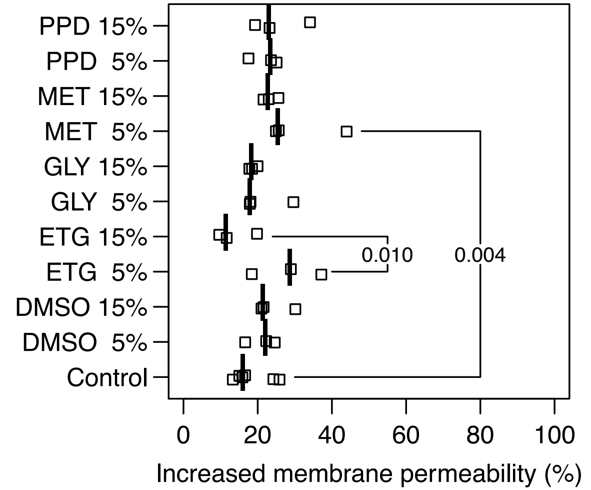


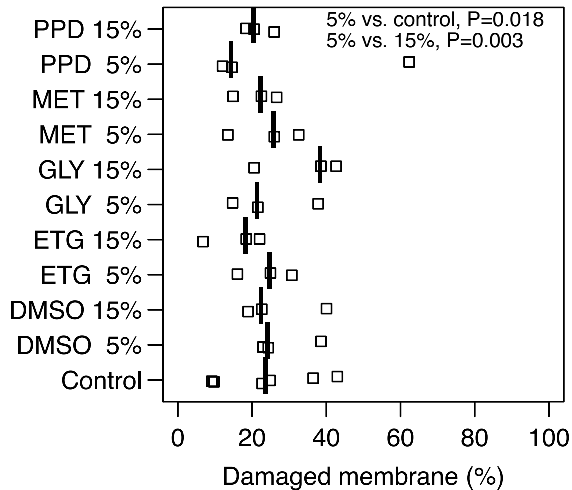
Figure 3



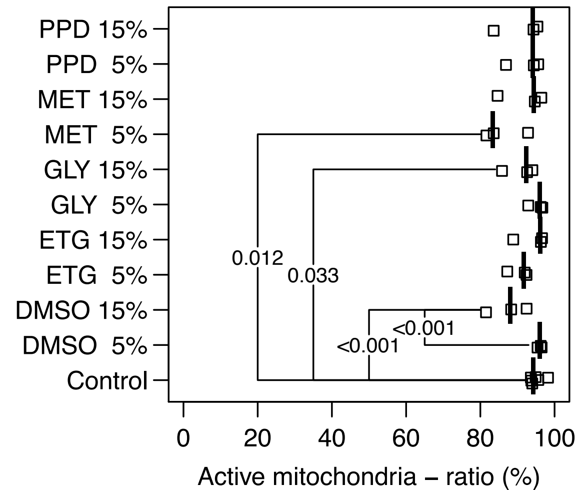
(a) Viability (YO-PRO-1–/H342–)



(b) Membrane permeability (YO-PRO-1+/H342–)

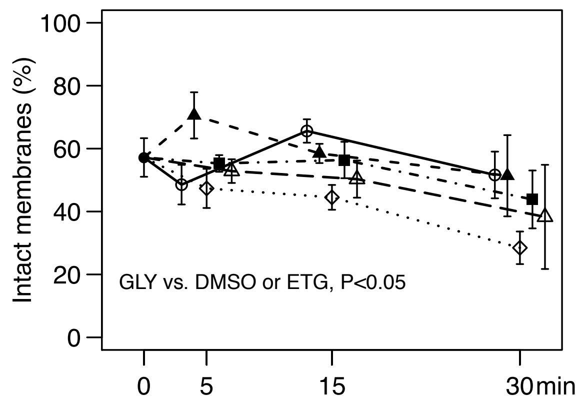


(c) Damaged membrane (YO-PRO-1+/H342+)

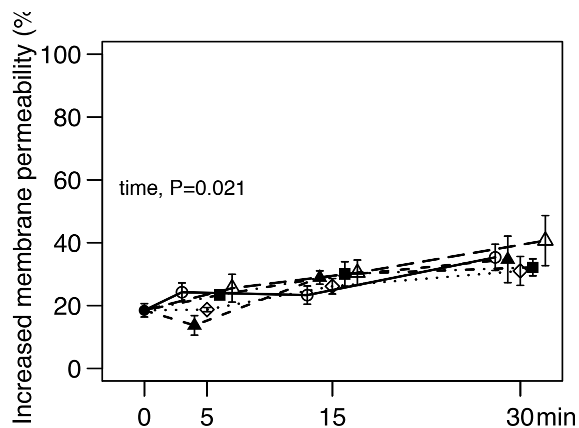


(d) Active mitochondria (Mitotracker+)

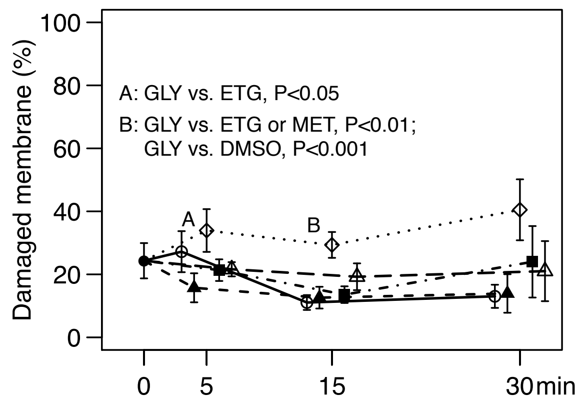
Figure 4



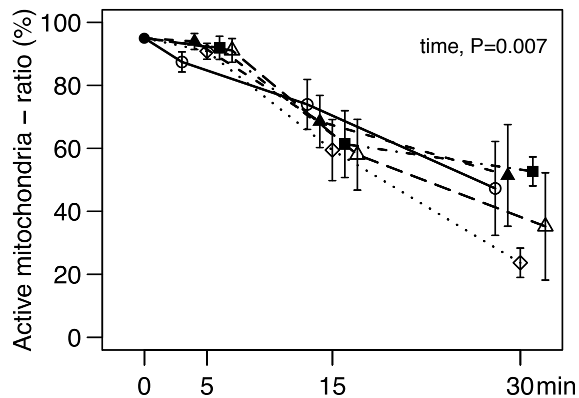
(a) Viability (YO-PRO-1–/H342–)



(b) Membrane permeability (YO-PRO-1+/H342–)



(c) Damaged membrane (YO-PRO-1+/H342+)



(d) Active mitochondria (Mitotracker+)

CTL DMSO ETG GLY MET PPD

**Statement of Funding*

This work has been funded by the Spanish Ministry of Science and Innovation (MICINN) research projects AGL2009-11546 and AGL2009-06994 and Fundación Ramón Areces.