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## Is glycerol a good cryoprotectant for sperm cells? New exploration of its toxicity using avian model

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1 **Is glycerol a good cryoprotectant for sperm cells? New exploration of its toxicity using**  
2 **avian model**

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12  
13 **Short title:** glycerol toxic effects on sperm fertilization

14 **Keywords:** cryoprotectant, fertility, glycerol, sperm, sperm storage tubules (SST), toxicity

15 **Word count:** 5000 words, excluding abstract, references and figure legends

16 **In brief:** Glycerol is a commonly used cryoprotectants for sperm cryopreservation in many  
17 species but shows cytotoxicity to decrease fertility. This study demonstrates that glycerol  
18 affects sperm migration and storage in the oviducts as well as negative modifications of  
19 sperm biology.

20

21 **ABSTRACT**

22 Glycerol is a cryoprotectant used worldwide for sperm cryopreservation in animals but  
23 it is associated with decreasing fertility. The mechanism underlying glycerol effects remains  
24 unclear, thus here we aimed to better understand by using the chicken model. First, we  
25 checked the effect of increasing glycerol concentration at insemination on hen fertility,  
26 showing that 2 and 6% glycerol induced partial and total infertility. Subsequently, we  
27 examined sperm storage tubules (SST) colonizing ability during *in vivo* insemination and *in*  
28 *vitro* incubation of Hoechst stained sperm containing 0, 2 or 6% glycerol. Furthermore, we  
29 conducted perivitelline membrane lysis tests and investigated sperm motility, mitochondrial  
30 function, ATP concentration, membrane integrity and apoptosis, after 60 min of incubation  
31 with different glycerol concentrations (0,1, 2, 6 and 11%), at two temperatures to mimic pre-  
32 freezing (4°C) and post-insemination (41°C) conditions. Whereas 2% glycerol significantly  
33 reduced 50% of SST containing sperm, 6% glycerol totally inhibited SST colonization *in vivo*.  
34 On the other hand, *in vitro* incubation of sperm with SST revealed no effect of 2% glycerol  
35 and 6% glycerol showed only a 17% reduction of sperm filled SST. Moreover, glycerol  
36 reduced sperm-egg penetration rates as well as affected sperm motility, bioenergetic  
37 metabolism and cell death at 4°C when its concentration exceeded 6% and caused greater  
38 damages at 41°C, especially decreasing sperm motility. These data altogether reveal  
39 important effects of glycerol on sperm biology, sperm migration, SST colonization, and  
40 oocyte penetration, suggesting at least a part of fertility reduction by glycerol and open the  
41 way for improving sperm cryopreservation.

42

43 Key words: cryoprotectant, fertility, glycerol, sperm, sperm storage tubules (SST), toxicity

44

45 **Introduction**

46 Sperm cryopreservation is a practical strategy to struggle the biodiversity crisis of wild  
47 animals (Bolton *et al.* 2022), to exchange superior genotypes in domestic animals (Bebbere  
48 & Succu 2022), and to preserve fertility in humans (Tao *et al.* 2020). The cryopreservation  
49 involves successive steps: semen collection, cryoprotectant addition, cellular dehydration,  
50 freezing, storage in liquid nitrogen, and thawing prior to sperm use (Gupta *et al.* 2018; Ugur  
51 *et al.* 2019). Cryoprotectants are chemical compounds that protect cells from cryodamages.  
52 Among them, glycerol, an intracellular cryoprotectant, is the most commonly used one for  
53 sperm cells in most species (Villaverde *et al.* 2013; Papa *et al.* 2015; Whaley *et al.* 2021; Ma  
54 *et al.* 2022). However, it is associated with undesirable cytotoxic effects on sperm (Holt  
55 2000; Morrier *et al.* 2002; Abouelezz *et al.* 2017), affecting sperm biological characteristics  
56 and fertility (Fahy 2010; Best 2015) in various species including humans (McLaughlin *et al.*  
57 1992), mice (Katkov *et al.* 1998), donkeys, horses (Demick *et al.* 1976; Vidament *et al.* 2009),  
58 pigs (Wilmut & Polge 1974; Gutiérrez-Pérez *et al.* 2009), sheep (Slavik 1987; Abdelhakeam *et*  
59 *al.* 1991) and marsupials (Taggart *et al.* 1996; Rodger *et al.* 2009).

60 To avoid its cytotoxic effects, different strategies were developed such as mixing  
61 glycerol with other cryoprotectants during cryopreservation process (Yildiz *et al.* 2007; Silva  
62 *et al.* 2012; Blanch *et al.* 2014) or its removal before insemination (Seigneurin & Blesbois  
63 1995; Purdy *et al.* 2009). While these methods can partly alleviate its negative effects, little  
64 is known about the mechanisms involved in glycerol toxicity, directly on sperm biology  
65 and/or through modifications of the female genital tract.

66 In chickens, glycerol present in semen may dramatically induce total infertility with  
67 only half of the concentration used for cryopreservation (Polge 1951; Neville *et al.* 1971).  
68 During the production and use of chicken cryopreserved semen, sperm face different

69 temperatures: 4°C during the glycerol addition, equilibration, decreasing to -196°C during  
70 freezing and storage, 4°C again after thawing, and finally 41°C after insemination into the  
71 female reproductive tract (Lin *et al.* 2022). While this cryopreservation method is quite  
72 efficient, the combination of glycerol presence at 4°C and 41°C may have direct effects on  
73 sperm biology in addition to their journey inside the oviduct, glycerol may impair sperm  
74 migration and storage into the sperm storage tubules (SST), a specific well developed organ  
75 involved sperm selection, survival, and maintenance of fertilizing capacity, similar to the  
76 sperm reservoir of mammalian fallopian tubes (Camara Pirez *et al.* 2020; Mahé *et al.* 2021).  
77 The deficit of sperm storage in SST and its release to reach the oocyte in the oviduct may  
78 impair final fertility (Machado *et al.* 2019; Kölle 2022). Furthermore, glycerol toxic effects  
79 may also reduce sperm ability to penetrate the coverage of the oocyte, known as the inner  
80 perivitelline membrane (IPVM) in birds, analogous to the mammalian zona pellucida (ZP)  
81 (Ichikawa *et al.* 2017), leading to a fertilization failure.

82         Consequently, using the avian model, here we aimed to decipher the effects of glycerol  
83 on sperm fertilizing ability, including direct effects on sperm biology as well as the  
84 interaction of sperm with female genital tract. We firstly determined the critical  
85 concentrations of glycerol in semen associated with chicken fertility reduction by  
86 insemination tests. Subsequently, we explored the sperm capacity to reach and be stored in  
87 the oviduct with both *in vivo* and *in vitro* SST colonization experiments. Then, we  
88 investigated sperm ability to penetrate perivitelline membrane *in vitro*. Finally, we evaluated  
89 the direct effects of glycerol on sperm biology during pre-freezing (4°C) and post-  
90 insemination (41°C) temperature conditions, on sperm motility, mitochondrial activity, ATP  
91 generation, membrane integrity and apoptosis.

92

93

## 94 **Materials and methods**

### 95 **Animal management**

96 All experiments were conducted in accordance with the legislation governing animal  
97 treatment and were approved by the French Ministry of Higher Education, Research and  
98 Innovation, and the Val-de-Loire Animal Ethics Committee (authorization number: N°  
99 APAFIS#4026-2016021015509521 and APAFIS#34415-202112141205965). A total of 20 adult  
100 Sasso T-44 roosters were randomly allocated to 2 groups for fertility tests and sperm biology  
101 parameters study (motility, mitochondrial activity, ATP concentration, viability, sperm-SST  
102 storage and PVM penetration). Two herds of 60 adult Lohmann hens were housed by groups  
103 of 4, one for fertility tests and the other for SST and PVM experiments. All animals were  
104 under a lighting regimen of 14h light:10h dark, controlled temperature at 20°C, feeding with  
105 a standard diet and water *ad libitum* at the INRAE Poultry Experimental unit  
106 (<https://doi.org/10.15454/1.5572326250887292E12>). Animals were between 35 and 85-  
107 week-old during this study.

108

### 109 **Semen collection and processing**

110 Rooster semen was collected in a tube containing 200 µL of Lake PC diluent (Lake &  
111 Ravie 1981) by abdominal massage (Burrows & Quinn 1937). Semen samples were then  
112 pooled and diluted to  $2\ 000 \times 10^6$  cells/mL with Lake PC diluent at room temperature for the  
113 following treatments. The number of all experimental replicates are given in the Figure  
114 legends.

115

### 116 **Fertility tests**

117 After collection, pooling and adjustment of glycerol concentration to 0, 1, 2, 6 and 11%  
118 (v/v), semen was immediately used for intravaginal insemination (4 cm depth), with a dose  
119 of  $100 \times 10^6$  sperm/hen for 2 consecutive days (12 hens/experimental group). Eggs were  
120 collected from day 2-7 after first insemination and stored at 15°C/85% humidity before  
121 incubation at 37.7°C/55% humidity. Fertile and infertile eggs were determined by candling at  
122 the 7<sup>th</sup> day of incubation (Long & Kulkarni 2004). Repeated manipulation with exchanged  
123 group of hens used to confirm the previously observed results (Supplementary Data 1).

124

#### 125 **Sperm- SST test**

#### 126 **Sperm staining**

127 Sperm was labeled with the previous methodologies (McDaniel *et al.* 1997; King *et al.*  
128 2002). After collection, pooling and dilution, 40  $\mu$ L of 1 mg Hoechst 33342  $\text{mL}^{-1}$  (Sigma-  
129 Aldrich) was added to 1 mL of diluted semen and gently mixed on an orbital shaker at 4°C for  
130 4 h. The fertilizing ability of stained sperm is given in Supplementary Data 2, showing that  
131 Hoechst labeling did not affect fertility tendency.

#### 132 ***In vivo* insemination**

133 Hoechst stained sperm were divided to 3 groups and added glycerol to a final  
134 concentration of 0 (control), 2 and 6% of glycerol. A total of 3 hens per group were  
135 inseminated twice at 24 h intervals with a dose of  $200 \times 10^6$  sperm/female (King *et al.* 2002)  
136 then slaughtered 24 h after the second insemination and oviducts were isolated. The villi  
137 (n=6 per animal) containing SST were separated randomly from uterovaginal mucosa  
138 (Cordeiro *et al.* 2018). Villi pieces were fixed in 4% paraformaldehyde solution at 37°C for 30  
139 min then mounted in Fluoromount-G™ medium (ThermoFisher) on microscope slides.  
140 Images were acquired using a microscope slide scanner (Axio Scan.Z1, Zeiss) with an

141 objective lens 20X. Fluorescence imaging was performed with X-Cite Illumination System and  
142 emission Band Pass of EM BP 445/50 (DAPI) and EM BP 690/50 (Alexa Fluor 633) used  
143 respectively for Hoechst labeled sperm and SST autofluorescence. The presences of sperm-  
144 filled or sperm-empty SST were identified manually with QuPath image analysis software  
145 and the percentage of SST filled with stained sperm was calculated.

#### 146 ***In vitro* incubation**

147 For this experiment, sperm staining and the uterovaginal villi collection were  
148 performed as previously described. Hoechst labeled sperm were washed in Lake PC diluent  
149 to remove extra staining solution and centrifuged at 600×g for 20 min at 4°C. Sperm pellets  
150 were resuspended and adjusted with Lake PC diluent to  $2\,000 \times 10^6$  cells/mL then mixed  
151 with glycerol to final concentration of 0 (control), 2 and 6% glycerol. For each treatment, 6  
152 pieces of villi were placed individually in 1 mL of Advanced DMEM/F-12 medium  
153 (ThermoFisher, Gibco#12634-010 ) containing 0, 2 and 6% of glycerol in a 4-well culture dish.  
154 Each well was inseminated with  $200 \times 10^6$  sperm and incubated at 39°C for 30 min in 5% CO<sub>2</sub>  
155 and 95% humidity. After incubation, villi pieces were washed in Dulbecco's phosphate-  
156 buffered saline (DPBS) to remove sperm adhering outside the SST, then fixed and analyzed  
157 as previously described.

158

#### 159 **Sperm-PVM perforation test**

##### 160 **Isolation of perivitelline membrane (PVM)**

161 PVM was isolated according to the method described previously (Steele *et al.* 1994;  
162 Bongalhardo *et al.* 2009). Yolk membrane was separated from freshly unfertilized chicken  
163 eggs and washed several times in DPBS to remove adherent yolk. Segments excluding



164 germinal disc area of PVM were cut in the size of 75 mm × 75 mm and placed in Advanced  
165 DMEM/F-12 medium before use.

### 166 **Sperm glycerolized treatment**

167 After collection and pooling, semen was diluted with glycerol-Lake PC diluent to 200 ×  
168 10<sup>6</sup> sperm cells/mL and final concentration of 0 (control), 1, 2, 6 and 11% glycerol then  
169 incubated at 4 or 41°C for 10 min.

### 170 ***In vitro* incubation of PVM and glycerolized sperm**

171 A piece of PVM placed in 1 mL of Advanced DMEM/F-12 medium was incubated with  
172 10 × 10<sup>6</sup> sperm of each condition (0, 1, 2, 6 and 11%) in a 24-well culture dish at 41°C for 20  
173 min. After incubation, PVM segments were washed in DPBS and fixed in 4%  
174 paraformaldehyde solution at 37°C for 1 min (Ichikawa *et al.* 2017). The PVM sections were  
175 then mounted on microscope slides, stained with Schiff's reagent (Sigma-Aldrich) and air-  
176 dried (Akhlaghi *et al.* 2014). The images of holes that formed in the PVM were captured by  
177 the microscope slide scanner with brightfield optics of an objective lens 20X. Three regions  
178 were randomly selected from each PVM and the number of holes was counted manually  
179 with QuPath software to calculate the number of holes/mm<sup>2</sup>.

180

### 181 **Evaluation of sperm quality parameters**

182 After semen collection and dilution, glycerol was added to reach final concentration of 0  
183 (control), 1, 2, 6 and 11% and then incubated at 4 or 41°C for 60 min.

### 184 **Sperm motility**

185 Sperm motility were examined with a computer-assisted sperm analysis system (CASA,  
186 IVOS, IMV Technologies) as previously described (Vitorino Carvalho *et al.* 2021). The  
187 evaluation was performed with a concentration of 30 × 10<sup>6</sup> sperm/mL. Based on several

188 motility parameters, i.e. average path velocity (VAP), straight line velocity (VSL) and  
189 straightness ( $STR = VSL/VAP$ ), motility results were indicated as percentage of motile sperm  
190 and progressive sperm, which were defined as the percentage of sperm showing a VAP > 5  
191  $\mu\text{m}/\text{sec}$  and VAP > 50  $\mu\text{m}/\text{sec}$  with STR > 75%, respectively.

### 192 **Mitochondrial activity**

193 Sperm mitochondrial parameter was evaluated with a fluorescent probe JC-1 (Sigma-  
194 Aldrich, CAS NO.: 3520-43-2), a green-fluorescent monomer at low membrane potential and  
195 forms red-fluorescent aggregates at higher potential (Gliozzi *et al.* 2017). Semen was diluted  
196 to  $1 \times 10^7$  sperm/mL in PBS to final volume of 200  $\mu\text{L}$  and then incubated with 1  $\mu\text{g}/\text{mL}$  of JC-  
197 1 dye at 37°C for 30 min. A total of 5 000 events of each sample were analyzed by Guava®  
198 easyCyte (IMV Technologies). The results were expressed as the percentage of sperm with  
199 mitochondrial membrane depolarization.

### 200 **ATP concentration**

201 A luciferase reaction assay (CellTiter-Glo® Luminescent Assay Kit, Promega#G7570)  
202 was used to measure sperm ATP concentration here (Nguyen *et al.* 2015). After incubation  
203 with glycerol, semen samples were immediately centrifuged at 4°C, 800×g for 10 min,  
204 supernatant was removed and the pellets were frozen at -20°C. Before ATP assay, sperm  
205 pellets were thawed and resuspended in 100  $\mu\text{L}$  PBS in the 96-well white polystyrene  
206 microplate. After 30 min of equilibration at room temperature, 100  $\mu\text{L}$  luciferin/luciferase  
207 reagent was added to each well and plates were placed on an orbital shaker for 2 min to  
208 induce cell lysis then waited for 10 min to stabilize the luminescent signal. The luminescence  
209 was recorded with the luminometer plate reader (CLARIOstar, BMG LABTECH) and  
210 transformed to ATP concentration based on a standard curve.

### 211 **Membrane breakage**

212 This parameter was explored by SYBR 14/Propidium iodide (PI) double fluorescent  
213 staining technique (Molecular Probes, Invitrogen #L7011) combined with a flow cytometer  
214 Guava® easyCyte (Gliozzi *et al.* 2017). PI positive sperm (red fluorescence) were recognized  
215 as membrane damaged sperm and SYBR 14 ones (green fluorescence) were considered as  
216 intact sperm. The results were expressed as percentage of sperm showing membrane  
217 breakage.

## 218 **Apoptosis**

219 Annexin V-binding technique (Hoogendijk *et al.* 2009) was used to determine sperm  
220 apoptotic cells according to the manufacturer's instructions (Novus Biologicals™#NBP2-  
221 29373). Briefly, 1.5 µL semen was washed in 1 mL of PBS twice. Sperm pellets were obtained  
222 by centrifuge at 400×g for 5 min and resuspended in 55 µL of Annexin V-FITC and PI mix  
223 staining buffer. After 20 min of incubation in the dark, 200 µL of assay buffer were added  
224 and samples were analyzed by Guava® easyCyte within 1 h.

225

## 226 **Statistical Analysis**

227 Statistical analyses were performed with GraphPad Prism version 6.07. The impact of  
228 glycerol concentration on fertility was analyzed by Chi-square test and Fisher's exact test.  
229 Percentage of SST filled with sperm were analyzed by Kruskal-Wallis and Dunn's multiple  
230 comparisons tests to evaluate the effect of glycerol concentration. Significant differences  
231 were considered when p-value was under 5%. Sperm-PVM penetration data were analyzed  
232 by two-way ANOVA test to examine the effects of glycerol concentration and exposure  
233 temperature. All sperm quality parameters results were analyzed by two-way ANOVA test to  
234 examine the effects of glycerol concentration and exposure time. Since exposure time had  
235 no effect on all sperm quality parameters at 4°C (Table 1), data from different timepoints

236 were pooled in a mixed model to highlight the effects of glycerol concentration. Differences  
237 in the means were analyzed by Tukey's HSD Post Hoc test.

238

239

## 240 **Results**

### 241 **Impact on animal fertility**

242 Whereas 1% glycerol had no effect on the number of fertilized eggs (Figure 1), the  
243 presence of 2% glycerol significantly reduced the fertility by about 50% while 6% and 11% of  
244 glycerol resulted in total infertility. Same effect was reproduced in the second replicate test  
245 (Supplementary Data 1).

246

### 247 **Impact on sperm presence in SST**

248 The concentrations of 0, 2 and 6% glycerol were chosen in SST experiments based on  
249 the fertility results. Indeed, the presence of 2 and 6% glycerol in semen led to a partial and  
250 total loss of fertility, respectively, compared to 0%. We firstly confirmed that this effect was  
251 the same after Hoechst staining protocol (Supplementary Data 2). Subsequently, the  
252 capacity of sperm to reach and be stored in SST was investigated by inseminating Hoechst  
253 stained sperm then SST dissection (Figure 2A). The percentage of sperm filled SST  
254 significantly decreased as the glycerol concentration increased (Figure 2B): 2% and 6%  
255 glycerolized sperm dramatically reduced the percentage of SST containing sperm  
256 respectively by 50% and by nearly 100%.

257 To focus our work on the SST capacity to host sperm (avoiding possible motility effects  
258 preventing sperm to reach SST), same approach was applied with *in vitro* incubation of  
259 uterovaginal villi and glycerolized sperm (Figure 3A). No effect was observed with 2%

260 glycerol (Figure 3B), while 6% glycerol significantly decreased 17% the proportion of SST  
261 colonized by stained sperm.

262

### 263 **Effect on sperm-PVM penetration**

264 In order to evaluate the effect on fertilizing capacity, sperm was incubated 10 min with  
265 different glycerol concentration at 4 and 41 °C and the number of holes generated in PVM by  
266 sperm hydrolysis (Figure 4A) was analyzed. Whereas no interaction between glycerol  
267 concentration and the temperature was detected, each variable independently affected the  
268 number of holes in PVM (Figure 4B), showing less holes with increasing glycerol  
269 concentrations. While a higher reduction of fertilizing ability was observed with the  
270 incubation at 41°C than at 4°C (Supplementary Data 3), no significant difference was found  
271 by pair-wise comparisons.

272

### 273 **Effect on sperm biology with an incubation at 4°C**

274 In order to collect new data relating to sperm biology affected at the specific stages  
275 during the pre-freezing procedure, several aspects of sperm quality parameters were  
276 evaluated including sperm motility, mitochondrial function, ATP concentration, membrane  
277 integrity and apoptosis in presence of various concentrations of glycerol within 1 hour of  
278 incubation at 4°C. At this temperature, no effect of exposure time and its interaction with  
279 glycerol concentration was observed on all evaluated parameters (Table 1). Consequently,  
280 we further combined all timepoints to highlight only the effects of glycerol concentration  
281 (Supplementary Data 4). When compared to 0% glycerol, no effect of 1 and 2% glycerol was  
282 observed on all sperm biological characteristics (Figure 5A-F), except that 2% glycerol  
283 significantly increased ATP concentration (Figure 5D). Furthermore, 6% and 11% glycerol

284 decreased sperm motility and increased the values of all other sperm parameters when  
285 compared to control condition. Moreover, 11% glycerol caused more pronounced negative  
286 influence than 6%, except on mitochondrial function (Figure 5).

287

### 288 **Effect on sperm biology with an incubation at 41°C**

289 We then explored the same sperm biology functions after glycerol exposure at 41°C, to  
290 mimic the impact of temperature after sperm insemination into female reproductive tract,  
291 within 1 hour (the physiological time necessary for sperm to reach the fertilization site in the  
292 oviduct). Based on our preliminary results, sperm motility traits were observed within 30  
293 min of incubation time due to their fast alterations when exposed to glycerol at 41°C. The  
294 tendencies of percentage of motile and progressive sperm were very similar and were both  
295 significantly affected by glycerol concentration, exposure time and their interaction (Table  
296 1). Indeed, whereas no impact of 1, 2 and 6% of glycerol was revealed at 0 min, the addition  
297 of 11% glycerol immediately dropped sperm motility (Figure 6). A similar negative effect was  
298 also induced by 6% glycerol after 10 min of exposure (Figure 6). In addition to the reduction  
299 of sperm motility classically induced by the incubation of chicken sperm at 41°C (decreasing  
300 pattern observed with control condition over time), at 20 and 30 min, all glycerol  
301 concentrations reduced drastically sperm motility in a dose dependent manner, leading to  
302 sperm nearly immobile with 11% of glycerol at 20 min.

303 Significant effects of glycerol concentration, exposure time and their interaction were  
304 observed on mitochondrial function (Table 1). Whereas at 0 min, no difference was observed  
305 in all glycerol concentrations, a significant increase of mitochondria depolarization was  
306 observed at 30 min in absence of glycerol when compared to 0 min. Interestingly,  
307 mitochondria depolarization was higher in 60 min than all other timepoints and it

308 significantly decreased with the increase of glycerol concentration (Figure 7A). Glycerol  
309 concentration, exposure time and their interaction also significantly affected energy  
310 metabolism as revealed by sperm ATP concentration evaluation (Table 1). Indeed, whereas  
311 no significant influence was observed for 1 and 2% glycerol conditions at all timepoints  
312 (Figure 7B), an important increase of ATP concentration was revealed with 11% of glycerol at  
313 30 min as well as 6% of glycerol at 60 min, when compared to control, and to 1 and 2%  
314 glycerol conditions. At 60min, the ATP concentration observed with 11% was higher than the  
315 one obtained with 6% of glycerol.

316 In order to clarify the effect of glycerol on sperm cell survival, two parameters were  
317 evaluated: the presence of sperm membrane breakage and sperm apoptosis. Both  
318 parameters presented very closed patterns with a significant increase of their level due to  
319 glycerol concentration and exposure time, with no significant interaction (Table 1). At 0 min,  
320 no impact was observed on the membrane breakage rate whereas an increase in apoptotic  
321 cell percentage was found immediately after the addition of 11% glycerol. At 30 and 60 min,  
322 the rates of membrane breakage and apoptotic sperm cells significantly increased only for 6  
323 and 11% when compared to the other glycerol concentrations as well as to these  
324 experimental conditions at 0 min (Figure 8).

325

326

## 327 **Discussion**

328 Whereas glycerol is one of the most widely used cryoprotectant to preserve sperm at  
329 very low temperature in animals, its presence is also associated to undesired negative  
330 effects on fertility (Macías García *et al.* 2012). In chickens, this negative influence could lead  
331 to total infertility depending on the glycerol concentration present in inseminated semen

332 (Polge 1951; Seigneurin & Blesbois 1995). However, the cellular and molecular mechanisms  
333 underlying the balance between maintaining sperm integrity and inducing toxic effects when  
334 glycerol acts as a cryoprotectant remain unclear.

335 Here, using insemination program with chicken semen combined with different  
336 glycerol concentrations, we determined that the presence of 2% glycerol harmfully reduced  
337 animal fertility to half (91 to 43%) and that 6% glycerol induced total infertility (Figure 1 and  
338 Supplementary Data 1). These results are consistent with previous studies performed with  
339 different insemination programs and animal genotypes (Polge 1951; Neville *et al.* 1971),  
340 suggesting that glycerol toxic effects appear regardless of animal genetic background, age or  
341 insemination protocols in this species. Whereas 7% glycerol has no effect on pregnancy rates  
342 in cattle (Papa *et al.* 2015), the addition of only 2.2% glycerol in donkey semen induces  
343 infertility (Vidament *et al.* 2009). Consequently, although a cytotoxic glycerol effect could be  
344 presumed for all animal species, some important variability of sensitivity can be observed.  
345 However, very little information is available concerning about the stage and target of this  
346 toxicity, i.e. direct impacts on sperm biology or/and effects on the events occurring inside  
347 the female tract.

348 Once semen is inseminated into hen vagina, a population of sperm first reaches the  
349 SST to be stored before continuing their journey to fertilize the oocyte in the oviduct. Our  
350 data revealed that semen containing 2% glycerol showed a distinguishable decreased ability  
351 to colonize the SST and that 6% glycerol led to sperm absence in SST (Figure 2). This  
352 diminution or absence of sperm in SST, already mentioned previously (Marquez &  
353 Ogasawara 1977), is certainly one of the major causes of fertility reduction induced by the  
354 glycerol presence (Supplementary Data 2). However, this experiment did not discriminate  
355 that sperm absence in SST was due to the failure of sperm migration (from vagina to SST) or



356 of entering and binding into SST. To answer this question, the *in vitro* experiment was  
357 designed to exclude the defect of sperm migration from vagina to UVJ to see if glycerolized  
358 sperm can be found in SST if they are deposited close from SST. Different from *in vivo*  
359 experiment, sperm were present in SST after *in vitro* incubation of sperm with UVJ villi.  
360 Intriguingly found that no impact of 2% glycerol on the rate of sperm filling in SST (Figure 3)  
361 and just a partial reduction of 17% with 6% glycerol, a relatively slight loss when compared  
362 to *in vivo* result (100% reduction), suggesting that small amount of glycerol impairs sperm  
363 travel from vagina to SST *in vivo*, and that higher quantity of glycerol also inhibits sperm  
364 depositing into SST. These negative effects may be due to motility defect and/or sperm  
365 rejected by vagina selection (Brillard 1993; Bakst *et al.* 1994) as well as another barrier at the  
366 SST level acting as a gatekeeper (Brady *et al.* 2022) to exclude glycerolized sperm.

367       After 30 min *in vitro* incubation of sperm with UVJ villi, we found that 6% glycerol did  
368 not impair sperm motility (Supplementary Data 5), showing a completely different result  
369 from another sperm motility observation. In that experiment, sperm were incubated with  
370 with 6% glycerol without UVJ villi, leading sperm to lose almost their entire motility within  
371 30 min (Figure 6). This finding may suggest that UVJ tissues provide a positive effect on  
372 motility maintenance, which has been proposed in a previous study (Spren *et al.* 1990).

373       In addition to affect sperm transport and storage, glycerol may also directly influence  
374 sperm fertilizing ability including its potentiality to release proteolytic enzymes to hydrolyze  
375 the perivitelline membrane (PVM), a crucial step to penetrate and fertilized the oocyte  
376 (Lemoine *et al.* 2011; Priyadarshana *et al.* 2020). The higher the glycerol concentration was,  
377 the lesser the sperm hydrolysis activity was (Figure 4), proving that glycerol impairs chicken  
378 sperm fertilizing capacity and, this effect was more pronounced at 41°C than at 4°C  
379 (Supplementary Data 3), suggesting a synergic effect between temperature and glycerol

380 concentration. To our best knowledge, these data are the first revealing glycerol effects  
381 directly on sperm-egg penetration in birds. However, a different observation was reported in  
382 rams, indicating that glycerol accelerates egg penetration and polyspermy (Slavik 1987),  
383 suggesting a species specific effect remaining to be confirmed.

384         The sperm processing for freezing, thawing and insemination involves mainly two  
385 temperatures: low temperature (4°C) used during sperm preparation and physiological body  
386 temperature (41°C) after insemination into the female tract. These temperatures were  
387 considered here to decipher the direct impacts of glycerol on sperm biology to better  
388 understand which stages of the process the sperm are affected. At 4°C, despite the presence  
389 of less than 2% of glycerol had no effect on sperm biology within 60 min, more than 6% of  
390 glycerol significant decreased sperm motility and viability, and increased mitochondrial  
391 activity and ATP concentration (Figure 5), revealing that the modification for current freezing  
392 protocol might be useful to reduce undesired glycerol effects since the most used glycerol  
393 concentration for chicken sperm cryopreservation is more than 8% (Mocé *et al.* 2010; Zong  
394 *et al.* 2022). Decreasing the amount of glycerol to less than 2% may be a possible solution by  
395 replacing its requirement with other cryoprotectants. However, cytotoxic effects of other  
396 cryoprotectants, such as DMA and DMSO, cannot be ignored (Best 2015; Mosca *et al.* 2019).  
397 An option would be to mix several cryoprotectants to have a sufficient cryoprotection while  
398 maintaining each individual under its own toxicity threshold. Designing a satisfactory and  
399 harmless sperm cryopreserved method remains an endeavor and a challenge even after 70  
400 years of development. Another finding in our study was the absence of effect of exposure  
401 time on sperm biology at 4°C within 1 h (Table 1 and Figure 5). This information could be  
402 very useful to add more flexibility to insemination programs, by keeping semen at 4°C

403 maximum 1 hour in farm environment. However, *in vivo* data on animal fertility are still  
404 needed to verify this potential procedure modifications.

405 At 41°C (physiological body temperature), the situation is dramatically different.  
406 Indeed, sperm motility was rapidly decreased by glycerol in a concentration dependent way,  
407 leading to a strong motility reduction more than 90% within 20 min in the presence of 11%  
408 glycerol (Figure 6). Consequently, this severe disorder of sperm motility could disturb normal  
409 sperm progression to reach SST or the fertilizing site in the oviduct since this whole journey  
410 takes only 1 hour *in vivo* (Bakst *et al.* 1994). Short-term exposure of glycerol at room  
411 temperature in 20 min also led to a significant reduction of sperm motility in humans  
412 (McLaughlin *et al.* 1992) and mice (Katkov *et al.* 1998), suggesting that it's not a chicken  
413 specific problem. At this temperature, higher concentration of glycerol (6 and 11%) also  
414 increased sperm membrane breakage, a typical feature of necrosis, and apoptosis as  
415 evidenced by Annexin V test (Figure 8). Whereas similar results were observed for  
416 membrane breakage of stallion sperm under 5% glycerol condition, no effect was observed  
417 on apoptotic rate (Macías García *et al.* 2012), suggesting a different sensitivity of glycerol  
418 effects on sperm cell death patterns depending on the species. Alternatively, modifying a  
419 glycerol freezing diluent for chicken sperm by adding apoptotic regulators such as  
420 agonist/antagonist of death receptor signaling or caspase inhibitors (Fischer & Schulze-  
421 Osthoff 2005) might be a possible way to limit glycerol cell death induction. Furthermore,  
422 this increase of sperm death may also be one of the causes of the reduction of sperm  
423 presence in SST. Since SST are known to select and maintain higher quality sperm, sperm  
424 presenting death signals may be rejected (Brillard 1993; Blesbois & Brillard 2007).

425 Lastly, glycerol changes chicken sperm energy metabolism with a dramatic increase of  
426 ATP concentration and a lower mitochondrial depolarization (Figure 7), corresponding to

427 previous studies in stallions (Macías García *et al.* 2012) and boars (Malcervelli *et al.* 2020).  
428 During ATP synthesis via mitochondrial respiration, the displacement of protons generates a  
429 voltage gradient to polarize the membrane with positive charge outside, defined as  
430 mitochondrial membrane potential (Wang *et al.* 2022). Thus mitochondria depolarization,  
431 refers to sperm with low mitochondrial membrane potential, generally associates with ATP  
432 deficiency and lower sperm motility (Alamo *et al.* 2020). Here sperm incubated at 41°C for  
433 30 min showed no difference of mitochondria depolarization in each glycerol condition  
434 (Figure 7A), theoretically generating a comparable ATP content in each of them (Pandey *et*  
435 *al.* 2021). However, glycerol concentrations of 6 and 11% led to a 25 and 60-fold higher ATP  
436 concentrations than the other conditions (Figure 7B), raising the hypothesis that excessive  
437 ATP is synthesized via glycolysis or pentose phosphate pathways (Setiawan *et al.* 2020).  
438 However, further studies will be needed to demonstrate direct evidence of the activity of  
439 these pathways.

440 Dynein, a specific ATPase uses energy from ATP hydrolysis and distributes it along the  
441 axonemal microtubules, the central cytoskeletal structures of sperm flagellum, to generate  
442 sperm movement (Sengupta *et al.* 2020). Theoretically, sperm with higher ATP contents  
443 show higher motility (Alamo *et al.* 2020). However, here we observed that at 30 min ATP  
444 accumulation in sperm under 6 and 11% glycerol conditions (Figure 7B) contrastingly  
445 decreased their motility performance (Figure 6). This finding raises another hypothesis that  
446 glycerol may disrupt dynein enzymatic activity or flagellar axonemal structure, leading to the  
447 accumulation of unused ATP.

448 To conclude, we used the chicken model to demonstrate that glycerol affects severely  
449 sperm capacity to interact with female reproductive tract, disturbing sperm migration and  
450 storage (Figure 9) and reducing sperm ability to penetrate the ovum and achieve fertilization

451 in the oviduct. We also proved that these effects occur mainly at the physiological body  
452 temperature, suggesting a good sperm capacities preservation by the glycerol during  
453 cryopreservation/thawing process. Consequently, although understanding the entire  
454 mechanisms of glycerol toxicity remains a challenge, it is clear that removing glycerol prior to  
455 insemination is a valuable solution to avoid fertility reduction in chickens and should be also  
456 considered in mammalian species.

457

#### 458 **Declaration of interest**

459 The authors declare that there are no conflicts of interest.

460

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464

#### 465 **Author contribution statement**

466 PM, EB and AVC conceived the original idea. H-LHL and IG performed the experiments.  
467 H-LHL and AVC analyzed the data. J-PB, NG, KR and L-RC aided in interpreting the results. H-  
468 LHL, PM, and AVC wrote the manuscript. All authors discussed the results, commented on  
469 the manuscript and approved the submitted version.

470

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475

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688  
689

690 **TABLE**

691 **Table 1. Effect of glycerol concentration (GLY) and exposure time (T) on *in vitro* sperm**  
 692 **quality parameters.**

parameter	two-way ANOVA test (p-value)					
	4°C			41°C		
	GLY	T	GLY × T	GLY	T	GLY × T
motile sperm	< <b>0.0001</b>	0.9103	0.8219	< <b>0.0001</b>	< <b>0.0001</b>	< <b>0.0001</b>
progressive sperm	< <b>0.0001</b>	0.9633	0.9839	< <b>0.0001</b>	< <b>0.0001</b>	< <b>0.0001</b>
mitochondria activity	<b>0.0192</b>	0.0676	0.9894	< <b>0.0001</b>	< <b>0.0001</b>	<b>0.0004</b>
ATP concentration	< <b>0.0001</b>	0.9520	0.9767	< <b>0.0001</b>	<b>0.0049</b>	< <b>0.0001</b>
membrane breakage	<b>0.0041</b>	0.4032	0.9609	< <b>0.0001</b>	< <b>0.0001</b>	0.3196
apoptosis	< <b>0.0001</b>	0.0851	0.9361	< <b>0.0001</b>	< <b>0.0001</b>	0.5577

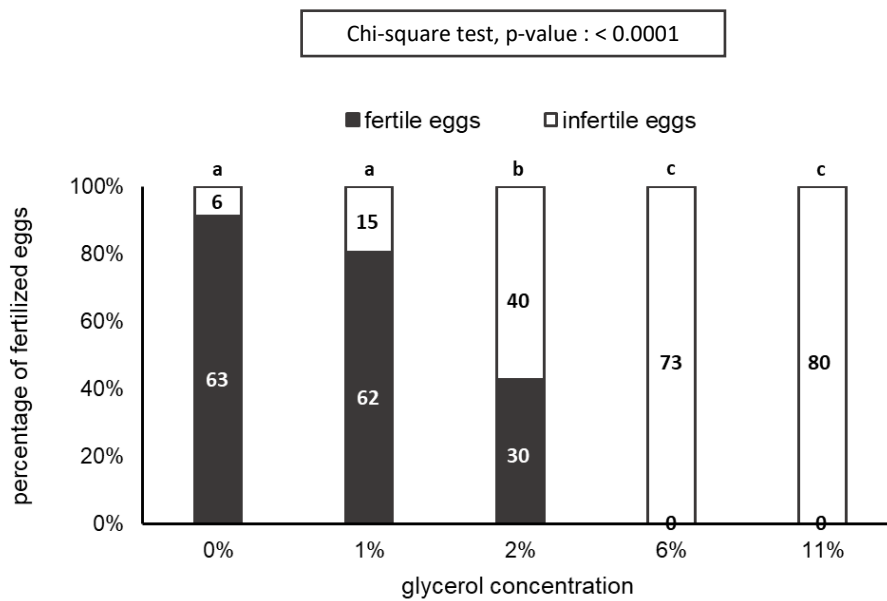
693 P-values lesser than 0.05 were considered as significant (in bold). Data of motile and progressive sperm  
 694 were observed at 0, 10, 20 and 30 min and the other parameters were observed at 0, 30 and 60 min.  
 695



696 **Figures**

697

698

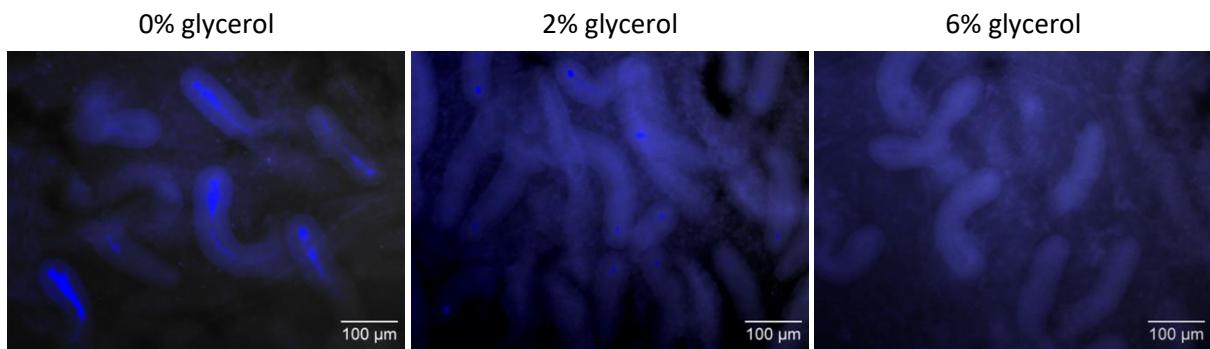


699

700 **Figure 1. Effect of glycerol presence in semen samples on sperm fertility capacity.** Semen  
701 was collected and pooled from 10 roosters. Number of hens=12 for each experimental  
702 condition. Eggs were collected between 2<sup>nd</sup> and 7<sup>th</sup> day after first insemination in a dose of  
703  $100 \times 10^6$  sperm per hen for 2 consecutive days. Black and white bars represent the  
704 percentage of fertile and infertile eggs. The numbers of considered eggs are indicated on the  
705 bars. Different letters indicate significant differences (p-value < 0.05).

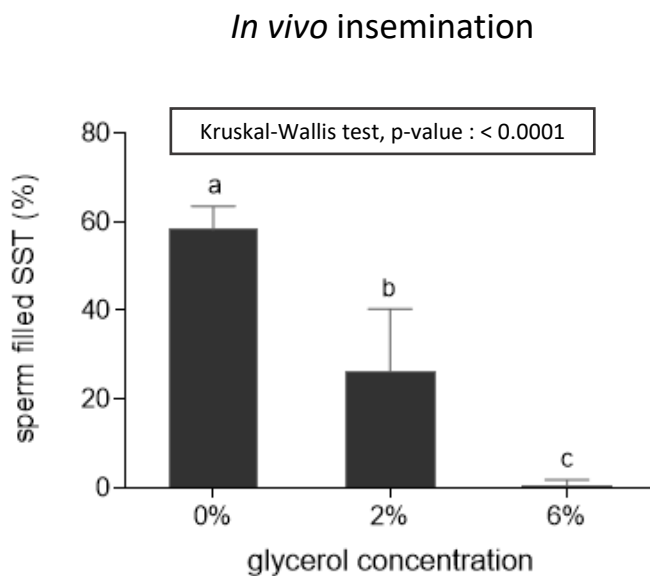
706

707 A



708

709 B

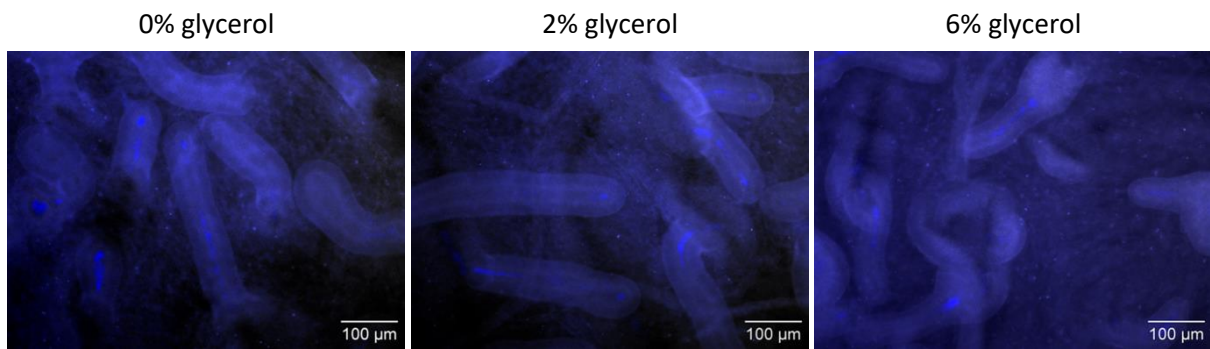


710

711 **Figure 2. The presence of sperm in sperm storage tubules (SST) after *in vivo* insemination.** A  
712 and B represent sperm identification (A) and the percentage of SST containing sperm (B) after  
713 *in vivo* insemination with 0, 2 and 6% glycerolized semen. Sperm stained with Hoechst 33342  
714 at 4°C for 4 h then mixed with glycerol just before insemination. SST were detected by  
715 autofluorescence. Data collected from 6 villi of each treatment. The bars and lines correspond  
716 to the mean (3 experimental replicates) and the standard deviation of the mean. Different  
717 letters indicate significant differences (p-value < 0.05).

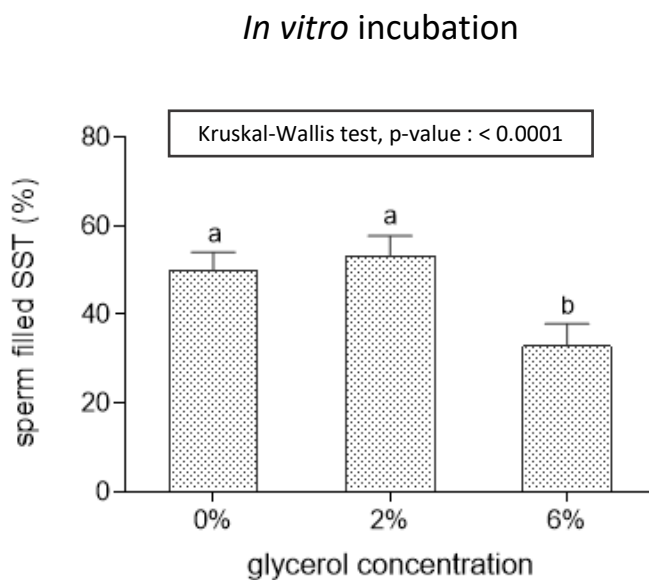
718

719 A



720

721 B

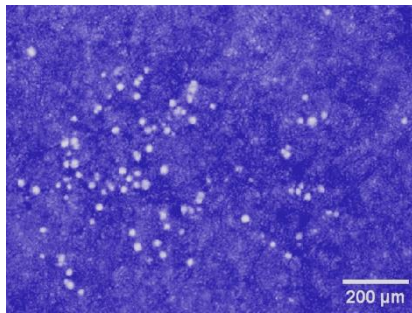


722

723 **Figure 3. The presence of sperm in sperm storage tubules (SST) after *in vitro* incubation.** A  
724 and B represent sperm identification (A) and the percentage of SST containing sperm (B) after  
725 *in vitro* incubation of sperm and uterovaginal villi in 0, 2 and 6% glycerol medium. Sperm  
726 stained with Hoechst 33342 at 4°C for 4 h and washed in Lake PC diluent then mixed with  
727 glycerol just before incubation. SST were detected by autofluorescence. Data collected from  
728 3 villi of each treatment. The bars and lines correspond to the mean (5 experimental  
729 replicates) and the standard deviation of the mean. Different letters indicate significant  
730 differences (p-value < 0.05).

731

732 A



733

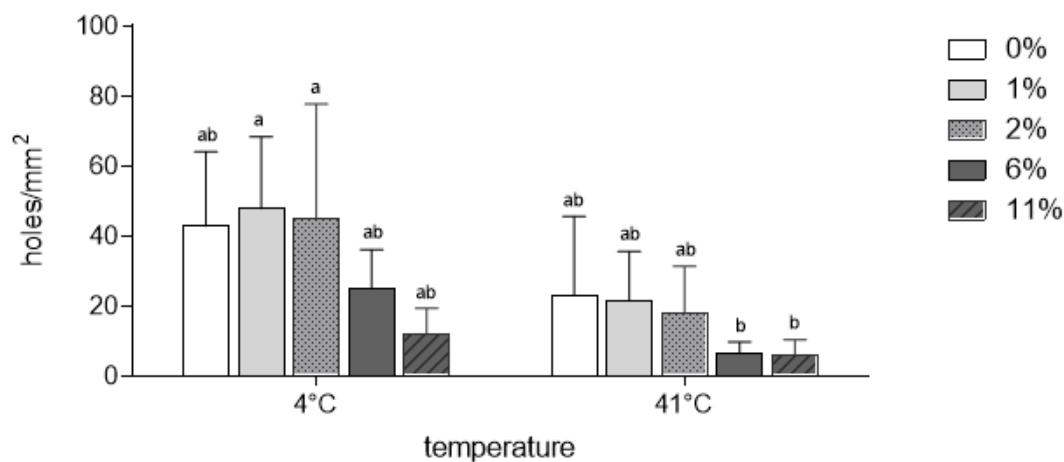
734

735 B

two-way ANOVA test (p-value)			
	GLY	temperature	GLY × temperature
number of holes in PVM	<b>0.0044</b>	<b>0.0003</b>	0.6720

736

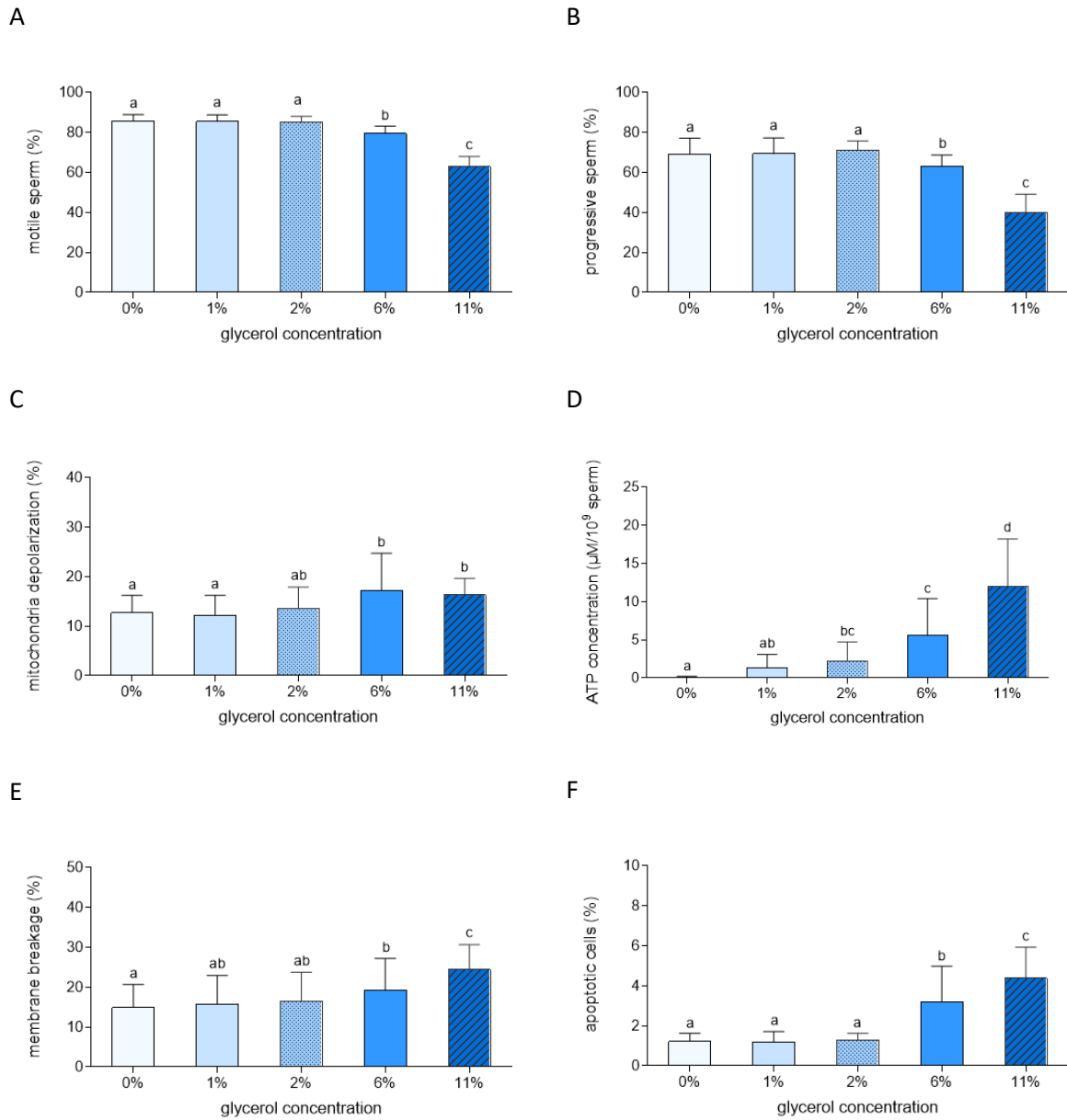
Abbreviation: GLY=glycerol concentration.



737

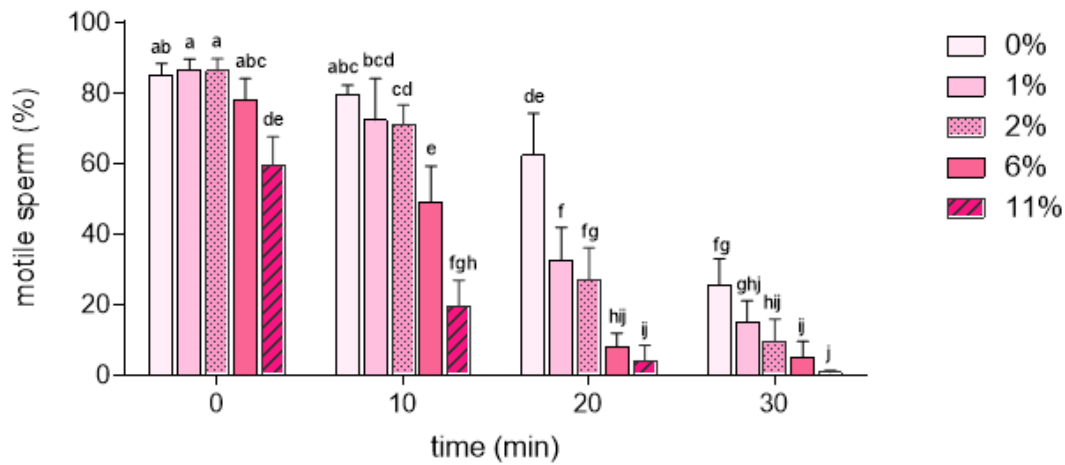
738 **Figure 4. Sperm penetration in perivitelline membrane (PVM) after pre-incubation with**  
739 **different glycerol concentrations.** A and B represent the image (A) and the number of holes  
740 (B) after PVM incubated with glycerolized sperm. Semen samples were collected and pooled  
741 from 10 roosters and subjected to 0, 1, 2, 6 and 11% glycerol at 4 or 41°C for 10 min before  
742 incubation with PVM (41°C for 20 min). The bars and lines correspond to the mean (5  
743 experimental replicates) and the standard deviation of the mean. Different letters indicate  
744 significant differences (p-value < 0.05).

745



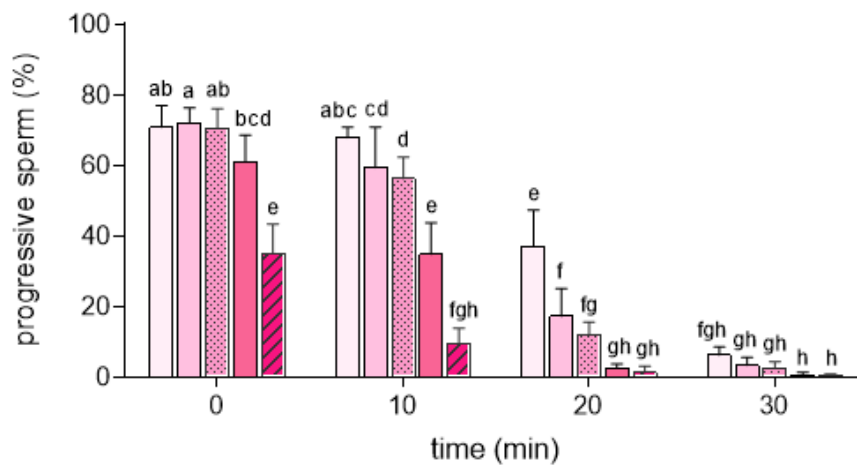
746 **Figure 5. Sperm quality parameters of glycerolized sperm at 4°C.** A-F represent evaluations  
 747 of different sperm quality parameters. Semen samples were collected and pooled from 10  
 748 roosters. The bars and lines correspond to the mean (5 experimental replicates) and the  
 749 standard deviation of the mean. Different letters indicate significant differences (p-value <  
 750 0.05).  
 751

752 A



753

754 B

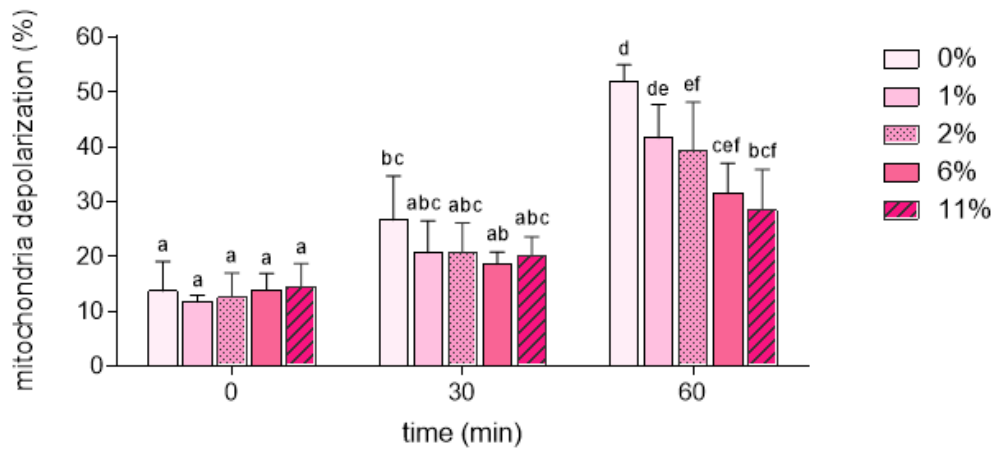


755

756 **Figure 6. Sperm motility of glycerolized sperm at 41°C.** A and B represent the percentages of  
757 motile (A) and progressive sperm (B). Semen samples were collected and pooled from 10  
758 roosters. The bars and lines correspond to the mean (5 experimental replicates) and the  
759 standard deviation of the mean. Different letters indicate significant differences (p-value <  
760 0.05).

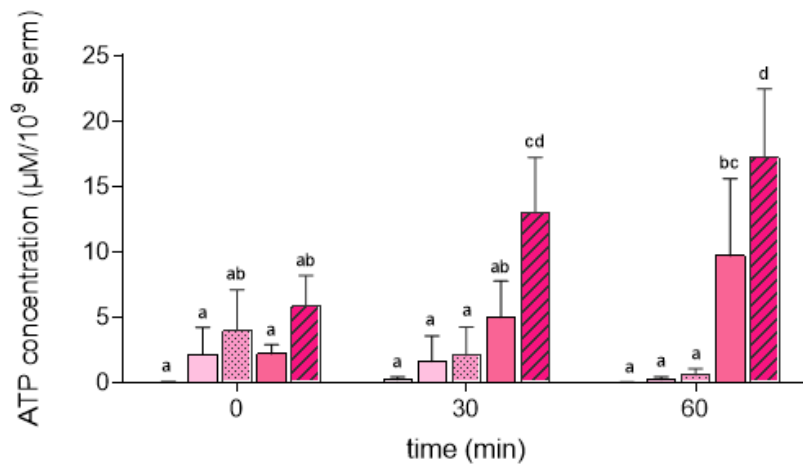
761

762 A



763

764 B



765

766 **Figure 7. Sperm mitochondria function and ATP concentration of glycerolized sperm at 41°C.**

767 A and B represent the percentages of mitochondria depolarization (A) and ATP concentration

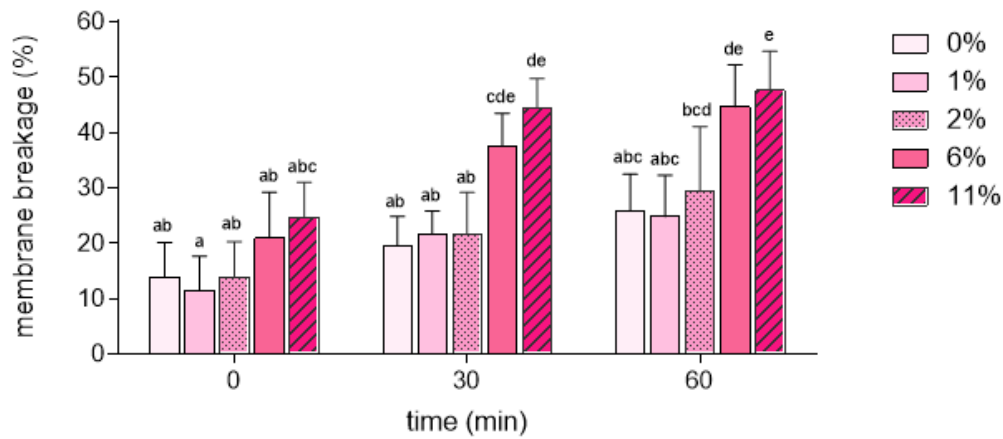
768 (B). Semen samples were collected and pooled from 10 roosters. The bars and lines

769 correspond to the mean (5 experimental replicates) and the standard deviation of the mean.

770 Different letters indicate significant differences (p-value < 0.05).

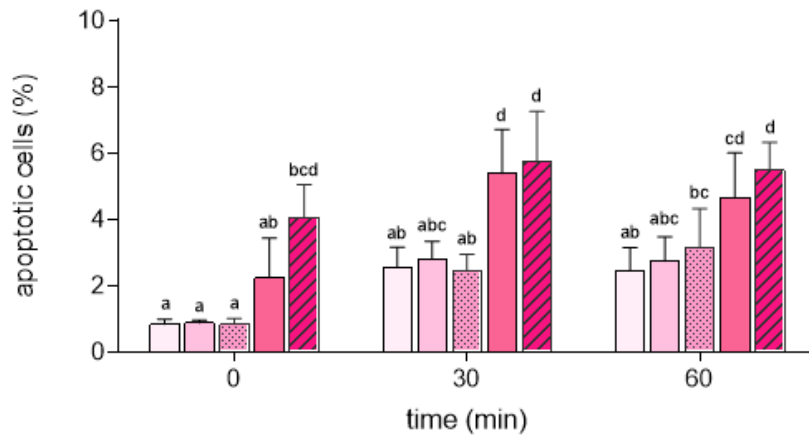
771

772 A



773

774 B

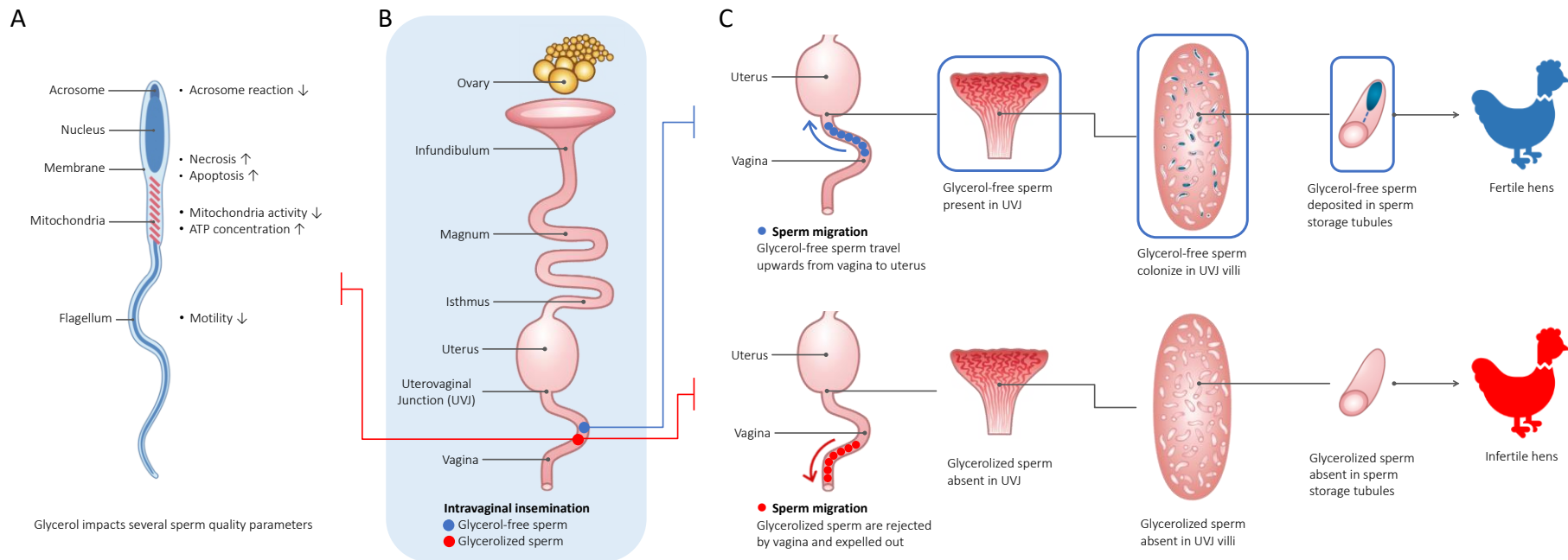


775

776 **Figure 8. Sperm membrane breakage and apoptosis of glycerolized sperm at 41°C.** A and B  
777 represent the percentages of membrane breakage (A) and apoptosis (B). Semen samples were  
778 collected and pooled from 10 roosters. The bars and lines correspond to the mean (5  
779 experimental replicates) and the standard deviation of the mean. Different letters indicate  
780 significant differences (p-value < 0.05).

781





782

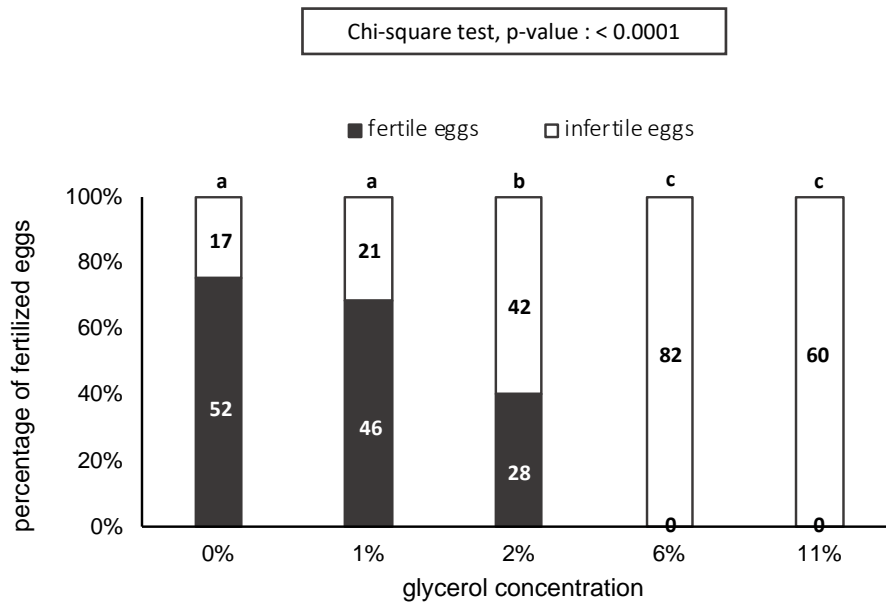
783 **Figure 9. Proposed model of glycerol impacts on sperm fertilizing capacity in chickens.** (A) indicates glycerol impacts on sperm biology:  
 784 increasing cell deaths and ATP contents, and decreasing acrosomal reaction, motility and mitochondria activity. (B) exhibits the intravaginal  
 785 insemination of glycerol-free or glycerolized sperm in hen's oviduct. (C) illustrates glycerol-free sperm deposited in sperm storage tubules of  
 786 uterovaginal junction, leading hens to produce fertile eggs. While glycerolized sperm are rejected and expelled by vagina, leading sperm absence  
 787 in sperm storage tubules and infertile hens.

788

789 **Supplementary Data**

790

791

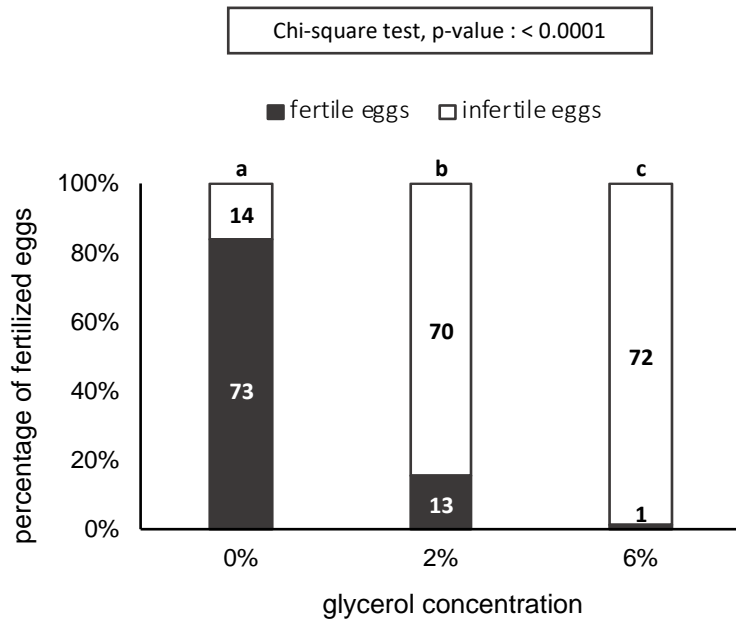


792

793 **Supplementary Data 1. Effect of glycerol presence in semen samples on sperm fertility**  
794 **capacity.** Repeated experiment of Figure 1. Semen was collected and pooled from same  
795 roosters but hens for each treatment were exchanged. Eggs were collected between 2<sup>nd</sup> and  
796 7<sup>th</sup> day after first insemination in a dose of  $100 \times 10^6$  sperm per hen for 2 consecutive days.  
797 Black and white bars represent the percentage of fertile and infertile eggs. The numbers of  
798 considered eggs are indicated on the bars. Different letters indicate significant differences (p-  
799 value < 0.05).

800

801

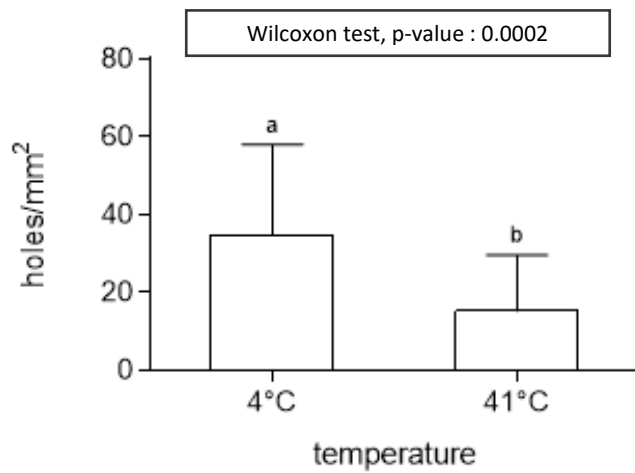


802

803 **Supplementary Data 2. Fertility of Hoechst 33342-stained glycerolized chicken sperm.**

804 Semen collected and pooled from 10 roosters. Number of hens=8 for each treatment (2  
805 experimental replicates). Eggs were collected between 2<sup>nd</sup> and 7<sup>th</sup> day after insemination in a  
806 dose of  $200 \times 10^6$  sperm per hen for 2 consecutive days. Black and white bars represent the  
807 percentage of fertile and infertile eggs. The numbers of considered eggs are indicated on the  
808 bars. Different letters indicate significant differences (p-value < 0.05).

809



810

811 **Supplementary Data 3. The number of holes after PVM incubated with glycerolized sperm.**

812 Semen samples were collected and pooled from 10 roosters and subjected to 0, 1, 2, 6 and  
813 11% glycerol at 4 or 41°C for 10 min before incubation with PVM. Pieces of PVM were collected  
814 individually from one egg for each experiment and then incubated with glycerolized sperm at  
815 41°C for 20 min. The bars and lines correspond to the mean (5 experimental replicates) and  
816 the standard deviation of the mean. Different letters indicate significant differences (p-value  
817 < 0.05).

818

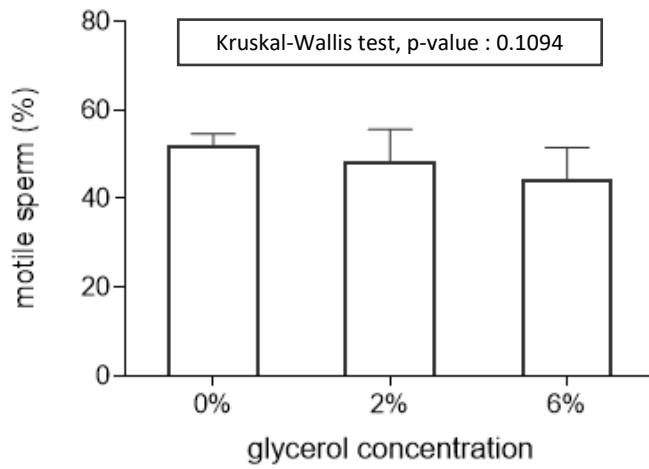
819 **Supplementary Data 4. Effect of glycerol concentration on *in vitro* sperm quality**  
820 **parameters at 4°C**

	one-way ANOVA test (p-value)
motile sperm	<b>&lt; 0.0001</b>
progressive sperm	<b>&lt; 0.0001</b>
mitochondria activity	<b>0.0017</b>
ATP	<b>&lt; 0.0001</b>
membrane breakage	<b>&lt; 0.0001</b>
apoptosis	<b>&lt; 0.0001</b>

821

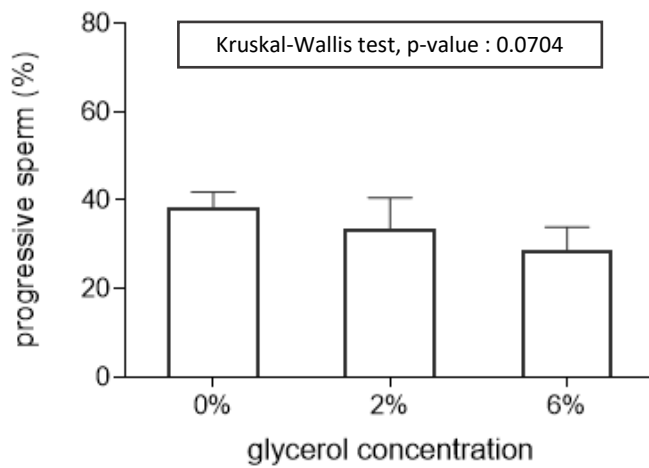
822

823 A



824

825 B



826

827 **Supplementary Data 5. Glycerolized sperm motility after *in vitro* incubation with sperm**  
828 **storage tubules (SST) for 30 min.** A and B represent the percentages of motile (A) and  
829 progressive sperm (B). The bars and lines correspond to the mean (5 experimental  
830 replicates) and the standard deviation of the mean.