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

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### ► To cite this version:

Hsiu-Lien Herbie Lin, Pascal Mermillod, Isabelle Grasseau, Jean-Pierre Brillard, Nadine Gérard, et al.. Is glycerol a good cryoprotectant for sperm cells? New exploration of its toxicity using avian model. *Animal Reproduction Science*, 2023, 258, pp.107330. 10.1016/j.anireprosci.2023.107330 . hal-04230575v1

**HAL Id: hal-04230575**

**<https://hal.inrae.fr/hal-04230575v1>**

Submitted on 6 Oct 2023 (v1), last revised 10 Oct 2023 (v2)

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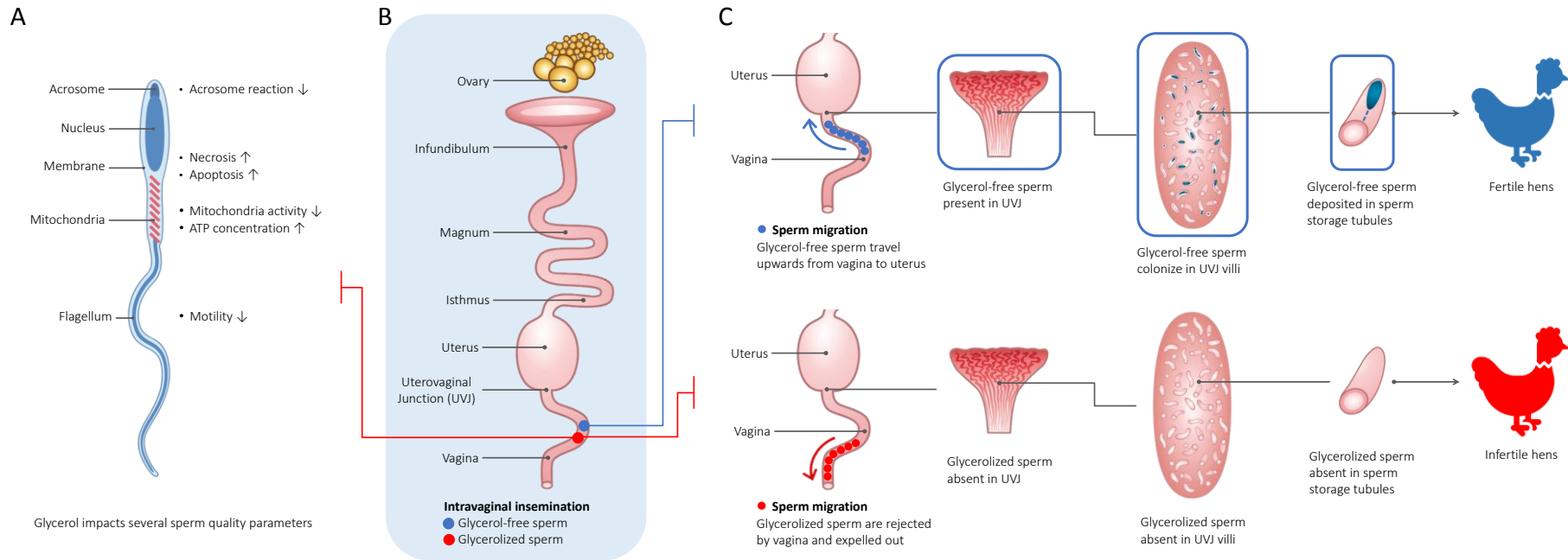


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## Highlights

1. Glycerol provides excellent cryoprotection and shows cytotoxicity to sperm cells.
2. In hens, 2% or 6% glycerol added into semen induce partial or total infertility.
3. Glycerol affects sperm migration and storage in the oviduct.
4. Glycerol causes more negative modifications of sperm biology at 41°C than at 4°C.
5. Removing glycerol prior to insemination is the most valuable solution so far to avoid fertility reduction.

**Graphical Abstract**



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

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

2 **avian model**

3

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12

13 **ABSTRACT**

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

34

35 Key words: Cryoprotectant; Fertility; Glycerol; Sperm storage tubules (SST); Toxicity

36

37 **1. Introduction**

38 Sperm cryopreservation is a practical strategy to struggle the biodiversity crisis of wild  
39 animals (Bolton et al., 2022), to exchange superior genotypes in domestic animals (Bebbere  
40 and Succu, 2022), and to preserve fertility in humans (Tao et al., 2020). The cryopreservation  
41 involves successive steps: semen collection, cryoprotectant addition, cellular dehydration,  
42 freezing, storage in liquid nitrogen, and thawing prior to sperm use (Ugur et al., 2019).  
43 Cryoprotectants are chemical compounds that protect cells from cryodamages. Among  
44 them, glycerol, an intracellular cryoprotectant, is the most commonly used one for sperm  
45 cells in most species (Villaverde et al., 2013; Papa et al., 2015). However, it is associated with  
46 undesirable cytotoxic effects on sperm (Holt, 2000; Morrier et al., 2002), affecting sperm  
47 biological characteristics and fertility (Fahy, 2010; Best, 2015) in various species including  
48 humans (McLaughlin et al., 1992), mice (Katkov et al., 1998), donkeys, horses (Vidament et  
49 al., 2009), pigs (Gutiérrez-Pérez et al., 2009), sheep (Abdelhakeam et al., 1991) and  
50 marsupials (Taggart et al., 1996).

51 To avoid its cytotoxic effects, many studies have proposed glycerol alternatives during  
52 cryopreservation process (Yildiz et al., 2007; Silva et al., 2012), but still lacking encouraging  
53 fertilization success (Abouelezz et al., 2015, 2017; Gloria et al., 2019) or fail to fit safety  
54 requirements and clear identification for cryobanking (Tselutin et al., 1999). Another  
55 strategy is related to glycerol removal before insemination (Seigneurin and Blesbois, 1995;  
56 Purdy et al., 2009). While these methods can partly alleviate its negative effects, little is  
57 known about the mechanisms involved in glycerol toxicity, directly on sperm biology and/or  
58 through modifications of the female genital tract.

59 In chickens, glycerol present in semen may dramatically induce total infertility with  
60 only half of the concentration used for cryopreservation (Polge, 1951; Neville et al., 1971).

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

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

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## 86 **2. Materials and methods**

### 87 **2.1. Animal management**

88 All experiments were conducted in accordance with the legislation governing animal  
89 treatment and were approved by the French Ministry of Higher Education, Research and  
90 Innovation, and the Val-de-Loire Animal Ethics Committee (authorization number: N°  
91 APAFIS#4026-2016021015509521 and APAFIS#34415-202112141205965). A total of 20 adult  
92 T44 roosters (*Gallus gallus domesticus*, SASSO, France) were randomly allocated to 2 groups  
93 for fertility tests and sperm biology parameters study (motility, mitochondrial activity, ATP  
94 concentration, viability, sperm-SST storage and PVM penetration). Two herds of 60 adult  
95 Lohmann hens were housed by groups of 4, one for fertility tests and the other for SST and  
96 PVM experiments. All animals were under a lighting regimen of 14h light:10h dark,  
97 controlled temperature at 20°C, feeding with a standard diet and water *ad libitum* at the  
98 INRAE Poultry Experimental unit (<https://doi.org/10.15454/1.5572326250887292E12>).  
99 Animals were between 35 and 85-week-old during this study.

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### 101 **2.2. Semen collection and processing**

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

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### 108 **2.3. Fertility tests**



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

## 117 **2.4. Sperm- SST test**

### 118 **2.4.1. Sperm staining**

119 Sperm was labeled with the previous methodologies (McDaniel et al., 1997; King et al.,  
120 2002). After collection, pooling and dilution, 40 µg of Hoechst-33342 (bisbenzimidazole, Sigma-  
121 Aldrich) was added to 1 mL of diluted semen and gently mixed on an orbital shaker at 4°C for  
122 4 h. The fertilizing ability of stained sperm is given in Supplementary Data 2, showing that  
123 Hoechst labeling did not affect fertility tendency.

### 124 **2.4.2. *In vivo* insemination**

125 Hoechst stained sperm were divided to 3 groups and added glycerol to a final  
126 concentration of 0 (control), 2 and 6% of glycerol. A total of 3 hens per group were  
127 inseminated twice at 24 h intervals with a dose of  $200 \times 10^6$  sperm/female (King et al., 2002)  
128 then slaughtered 24 h after the second insemination and oviducts were isolated. The villi ( $n$   
129 = 6 per animal) containing SST were separated randomly from uterovaginal mucosa  
130 (Cordeiro et al., 2021). Villi pieces were fixed in 4% paraformaldehyde solution at 37°C for 30  
131 min then mounted in Fluoromount-G™ medium (ThermoFisher) on microscope slides.  
132 Images were acquired using a microscope slide scanner (Axio Scan.Z1, Zeiss) with an

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

### 138 **2.4.3. *In vitro* incubation**

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

150

## 151 **2.5. Sperm-PVM perforation test**

### 152 **2.5.1. Isolation of perivitelline membrane (PVM)**

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

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

### 158 **2.5.2. Sperm glycerolized treatment**

159 After collection and pooling, semen was diluted with glycerol-Lake PC diluent to 200 ×  
160 10<sup>6</sup> sperm cells/mL and final concentration of 0 (control), 1, 2, 6 and 11% glycerol then  
161 incubated at 4 or 41°C for 10 min.

### 162 **2.5.3. *In vitro* incubation of PVM and glycerolized sperm**

163 A piece of PVM placed in 1 mL of Advanced DMEM/F-12 medium was incubated with  
164 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  
165 min. After incubation, PVM segments were washed in DPBS and fixed in 4%  
166 paraformaldehyde solution at 37°C for 1 min (Ichikawa et al., 2017). The PVM sections were  
167 then mounted on microscope slides, stained with Schiff's reagent (Sigma-Aldrich) and air-  
168 dried (Akhlaghi et al., 2014). The images of holes that formed in the PVM were captured by  
169 the microscope slide scanner with brightfield optics of an objective lens 20X. Three regions  
170 were randomly selected from each PVM and the number of holes was counted manually  
171 with QuPath software to calculate the number of holes/mm<sup>2</sup>.

## 173 **2.6. Evaluation of sperm quality parameters**

174 After semen collection and dilution, glycerol was added to reach final concentration of  
175 0 (control), 1, 2, 6 and 11% and then incubated at 4 or 41°C for 60 min.

### 176 **2.6.1. Sperm motility**

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

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

### 184 **2.6.2. Mitochondrial activity**

185 Sperm mitochondrial parameter was evaluated with a fluorescent probe JC-1 (Sigma-  
186 Aldrich, CAS NO.: 3520-43-2), a green-fluorescent monomer at low membrane potential and  
187 forms red-fluorescent aggregates at higher potential (Gliozzi et al., 2017). Semen was diluted  
188 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-  
189 1 dye at 37°C for 30 min. A total of 5 000 events of each sample were analyzed by Guava®  
190 easyCyte (IMV Technologies). The results were expressed as the percentage of sperm with  
191 mitochondrial membrane depolarization.

### 192 **2.6.3. ATP concentration**

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

### 203 **2.6.4. Membrane breakage**

204 This parameter was explored by SYBR 14/Propidium iodide (PI) double fluorescent  
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3 205 staining technique (Molecular Probes, Invitrogen #L7011) combined with a flow cytometer  
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5 206 Guava® easyCyte (Gliozzi et al., 2017). PI positive sperm (red fluorescence) were recognized  
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8 207 as membrane damaged sperm and SYBR 14 ones (green fluorescence) were considered as  
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10 208 intact sperm. The results were expressed as percentage of sperm showing membrane  
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13 209 breakage.

### 15 210 **2.6.5. Apoptosis**

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18 211 Annexin V-binding technique (Hoogendijk et al., 2009) was used to determine sperm  
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20 212 apoptotic cells according to the manufacturer's instructions (Novus Biologicals™#NBP2-  
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23 213 29373). Briefly, 1.5 µL semen was washed in 1 mL of PBS twice. Sperm pellets were obtained  
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26 214 by centrifuge at 400×g for 5 min and resuspended in 55 µL of Annexin V-FITC and PI mix  
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28 215 staining buffer. After 20 min of incubation in the dark, 200 µL of assay buffer were added  
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31 216 and samples were analyzed by Guava® easyCyte within 1 h.

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### 36 218 **2.7. Statistical Analysis**

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38 219 Statistical analyses were performed with GraphPad Prism version 6.07. The impact of  
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41 220 glycerol concentration on fertility was analyzed by Chi-square test and Fisher's exact test.  
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44 221 Percentage of SST filled with sperm were analyzed by Kruskal-Wallis and Dunn's multiple  
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46 222 comparisons tests to evaluate the effect of glycerol concentration. Significant differences  
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49 223 were considered when p-value was under 5%. Sperm-PVM penetration data were normally  
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52 224 distributed and analyzed by two-way ANOVA test to examine the effects of glycerol  
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54 225 concentration and exposure temperature. All sperm quality parameters results were  
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57 226 analyzed by two-way ANOVA test to examine the effects of glycerol concentration and  
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59 227 exposure time. Since exposure time had no effect on all sperm quality parameters at 4°C  
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228 (Table 1), data from different timepoints were pooled in a mixed model to highlight the  
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3 229 effects of glycerol concentration. Differences in the means were analyzed by Tukey's HSD  
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5 230 Post Hoc test.  
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### 10 232 **3. Results**

#### 13 233 **3.1. Impact on animal fertility**

15 234 Whereas 1% glycerol had no effect on the number of fertilized eggs (Figure 1), the  
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18 235 presence of 2% glycerol significantly reduced the fertility by about 50% while 6% and 11% of  
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21 236 glycerol resulted in total infertility. Same effect was reproduced in the second replicate test  
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23 237 (Supplementary Data 1).  
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#### 28 239 **3.2. Impact on sperm presence in SST**

31 240 The concentrations of 0, 2 and 6% glycerol were chosen in SST experiments based on  
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33 241 the fertility results. Indeed, the presence of 2 and 6% glycerol in semen led to a partial and  
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36 242 total loss of fertility, respectively, compared to 0%. We firstly confirmed that this effect was  
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39 243 the same after Hoechst staining protocol (Supplementary Data 2). Subsequently, the  
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41 244 capacity of sperm to reach and be stored in SST was investigated by inseminating Hoechst  
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44 245 stained sperm then SST dissection (Figure 2A). The percentage of sperm filled SST  
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46 246 significantly decreased as the glycerol concentration increased (Figure 2B): 2% and 6%  
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49 247 glycerolized sperm dramatically reduced the percentage of SST containing sperm  
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51 248 respectively by 50% and by nearly 100%.

54 249 To focus our work on the SST capacity to host sperm (avoiding possible motility effects  
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57 250 preventing sperm to reach SST), same approach was applied with *in vitro* incubation of  
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59 251 uterovaginal villi and glycerolized sperm (Figure 3A). No effect was observed with 2%

252 glycerol (Figure 3B), while 6% glycerol significantly decreased 17% the proportion of SST  
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3 253 colonized by stained sperm.  
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### 7 255 **3.3. Effect on sperm-PVM penetration**

10 256 In order to evaluate the effect on fertilizing capacity, sperm was incubated 10 min with  
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13 257 different glycerol concentration at 4 and 41 °C and the number of holes generated in PVM by  
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15 258 sperm hydrolysis (Figure 4A) was analyzed. Whereas no interaction between glycerol  
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18 259 concentration and the temperature was detected, each variable independently affected the  
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21 260 number of holes in PVM (Figure 4B), showing less holes with increasing glycerol  
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23 261 concentrations. While a higher reduction of fertilizing ability was observed with the  
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26 262 incubation at 41°C than at 4°C (Supplementary Data 3), no significant difference was found  
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28 263 by pair-wise comparisons.  
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### 33 265 **3.4. Effect on sperm biology with an incubation at 4°C**

36 266 In order to collect new data relating to sperm biology affected at the specific stages  
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39 267 during the pre-freezing procedure, several aspects of sperm quality parameters were  
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41 268 evaluated including sperm motility, mitochondrial function, ATP concentration, membrane  
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44 269 integrity and apoptosis in presence of various concentrations of glycerol within 1 hour of  
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47 270 incubation at 4°C. At this temperature, no effect of exposure time and its interaction with  
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49 271 glycerol concentration was observed on all evaluated parameters (Table 1). Consequently,  
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52 272 we further combined all timepoints to highlight only the effects of glycerol concentration  
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54 273 (Supplementary Data 4). When compared to 0% glycerol, no effect of 1 and 2% glycerol was  
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57 274 observed on all sperm biological characteristics (Figure 5A-F), except that 2% glycerol  
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59 275 significantly increased ATP concentration (Figure 5D). Furthermore, 6% and 11% glycerol  
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276 decreased sperm motility and increased the values of all other sperm parameters when  
277 compared to control condition. Moreover, 11% glycerol caused more pronounced negative  
278 influence than 6%, except on mitochondrial function (Figure 5).

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### 3.5. Effect on sperm biology with an incubation at 41°C

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

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



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

9  
10 In order to clarify the effect of glycerol on sperm cell survival, two parameters were  
11 evaluated: the presence of sperm membrane breakage and sperm apoptosis. Both  
12 parameters presented very closed patterns with a significant increase of their level due to  
13 glycerol concentration and exposure time, with no significant interaction (Table 1). At 0 min,  
14 no impact was observed on the membrane breakage rate whereas an increase in apoptotic  
15 cell percentage was found immediately after the addition of 11% glycerol. At 30 and 60 min,  
16 the rates of membrane breakage and apoptotic sperm cells significantly increased only for 6  
17 and 11% when compared to the other glycerol concentrations as well as to these  
18 experimental conditions at 0 min (Figure 8).

#### 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 **4. Discussion**

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49 Whereas glycerol is one of the most widely used cryoprotectant to preserve sperm at  
50 very low temperature in animals, its presence is also associated to undesired negative  
51 effects on fertility (Macías García et al., 2012). In chickens, this negative influence could lead  
52 to total infertility depending on the glycerol concentration present in inseminated semen  
53 (Polge, 1951; Seigneurin and Blesbois, 1995). However, the cellular and molecular

324 mechanisms underlying the balance between maintaining sperm integrity and inducing toxic  
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3 325 effects when glycerol acts as a cryoprotectant remain unclear.

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5 326 Here, using insemination program with chicken semen combined with different  
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7 327 glycerol concentrations, we demonstrated that the presence of 2% glycerol harmfully  
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10 328 reduced animal fertility to half (91 to 43%) and that 6% glycerol induced total infertility  
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13 329 (Figure 1 and Supplementary Data 1). These results are consistent with previous studies  
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15 330 performed with different insemination programs and animal genotypes (Polge, 1951; Neville  
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18 331 et al., 1971), suggesting that glycerol toxic effects appear regardless of animal genetic  
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20 332 background, age or insemination protocols in this species. Whereas 7% glycerol has no effect  
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23 333 on pregnancy rates in cattle (Papa et al., 2015), the addition of only 2.2% glycerol in donkey  
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26 334 semen induces infertility (Vidament et al., 2009). Consequently, although a cytotoxic glycerol  
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28 335 effect could be presumed for all animal species, some important variability of sensitivity can  
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31 336 be observed. However, very little information is available concerning about the stage and  
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34 337 target of this toxicity, i.e. direct impacts on sperm biology or/and effects on the events  
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36 338 occurring inside the female tract.

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39 339 Once semen is inseminated into hen vagina, a population of sperm first reaches the  
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41 340 SST to be stored before continuing their journey to fertilize the oocyte in the oviduct. Our  
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44 341 data revealed that semen containing 2% glycerol showed a distinguishable decreased ability  
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46 342 to colonize the SST and that 6% glycerol led to sperm absence in SST (Figure 2). This  
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49 343 diminution or absence of sperm in SST, already mentioned previously (Marquez and  
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51 344 Ogasawara, 1977), is certainly one of the major causes of fertility reduction induced by the  
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54 345 glycerol presence (Supplementary Data 2). However, this experiment did not discriminate  
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57 346 that sperm absence in SST was due to the failure of sperm migration (from vagina to SST) or  
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59 347 of entering and binding into SST. To answer this question, the *in vitro* experiment was  
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3 348 designed to exclude the defect of sperm migration from vagina to UVJ to see if glycerolized  
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5 349 sperm can be found in SST if they are deposited close from SST. Different from *in vivo*  
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7 350 experiment, sperm were present in SST after *in vitro* incubation of sperm with UVJ villi.  
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9 351 Intriguingly found that no impact of 2% glycerol on the rate of sperm filling in SST (Figure 3)  
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11 352 and just a partial reduction of 17% with 6% glycerol, a relatively slight loss when compared  
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13 353 to *in vivo* result (100% reduction), suggesting that small amount of glycerol impairs sperm  
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15 354 travel from vagina to SST *in vivo*, and that higher quantity of glycerol also inhibits sperm  
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17 355 depositing into SST. These negative effects may be due to motility defect and/or sperm  
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19 356 rejected by vagina selection (Brillard, 1993; Bakst et al., 1994) as well as another barrier at  
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21 357 the SST level acting as a gatekeeper (Brady et al., 2022) to exclude glycerolized sperm.  
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25 358         After 30 min *in vitro* incubation of sperm with UVJ villi, we found that 6% glycerol did  
26  
27 359 not impair sperm motility (Supplementary Data 5), showing a completely different result  
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29 360 from another sperm motility observation. In that experiment, sperm were incubated with  
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31 361 6% glycerol without UVJ villi, leading sperm to lose almost their entire motility within 30 min  
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33 362 (Figure 6). This finding may suggest that UVJ tissues provide a positive effect on motility  
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35 363 maintenance, which has been proposed in a previous study (Spren et al., 1990).  
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41 364         In addition to affect sperm transport and storage, glycerol may also directly influence  
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43 365 sperm fertilizing ability including its potentiality to release proteolytic enzymes to hydrolyze  
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45 366 the perivitelline membrane (PVM), a crucial step to penetrate and fertilized the oocyte  
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47 367 (Lemoine et al., 2011; Priyadarshana et al., 2020). The higher the glycerol concentration was,  
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49 368 the lesser the sperm hydrolysis activity was (Figure 4), proving that glycerol impairs chicken  
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51 369 sperm fertilizing capacity and, this effect was more pronounced at 41°C than at 4°C  
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53 370 (Supplementary Data 3), suggesting a synergic effect between temperature and glycerol  
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55 371 concentration. To our best knowledge, these data are the first revealing glycerol effects  
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372 directly on sperm-egg penetration in birds. However, a different observation was reported in  
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3 373 rams, indicating that glycerol accelerates egg penetration and polyspermy (Slavik, 1987),  
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5 374 suggesting a species specific effect remaining to be confirmed.  
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8 375 The sperm processing for freezing, thawing and insemination involves mainly two  
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10 376 temperatures: low temperature (4°C) used during sperm preparation and physiological body  
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12 377 temperature (41°C) after insemination into the female tract. These temperatures were  
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14 378 considered here to decipher the direct impacts of glycerol on sperm biology to better  
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16 379 understand which stages of the process the sperm are affected. At 4°C, despite the presence  
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18 380 of less than 2% of glycerol had no effect on sperm biology within 60 min, more than 6% of  
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20 381 glycerol significant decreased sperm motility and viability, and increased mitochondrial  
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22 382 activity and ATP concentration (Figure 5), revealing that the modification for current freezing  
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24 383 protocol might be useful to reduce undesired glycerol effects since the most used glycerol  
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26 384 concentration for chicken sperm cryopreservation is more than 8% (Mocé et al., 2010; Zong  
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28 385 et al., 2022). Decreasing the amount of glycerol to less than 2% may be a possible solution by  
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30 386 replacing its requirement with other cryoprotectants. However, cytotoxic effects of other  
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32 387 cryoprotectants, such as DMA and DMSO, cannot be ignored (Best, 2015; Mosca et al.,  
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34 388 2019). An option would be to mix several cryoprotectants to have a sufficient cryoprotection  
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36 389 while maintaining each individual under its own toxicity threshold. Designing a satisfactory  
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38 390 and harmless sperm cryopreserved method remains an endeavor and a challenge even after  
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40 391 70 years of development. Another finding in our study was the absence of effect of exposure  
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42 392 time on sperm biology at 4°C within 1 h (Table 1 and Figure 5). This information could be  
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44 393 very useful to add more flexibility to insemination programs, by keeping semen at 4°C  
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46 394 maximum 1 hour in farm environment. However, *in vivo* data on animal fertility are still  
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48 395 needed to verify this potential procedure modifications.  
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396 At 41°C (physiological body temperature), the situation is dramatically different.  
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3 397 Indeed, sperm motility was rapidly decreased by glycerol in a concentration dependent way,  
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5 398 leading to a strong motility reduction more than 90% within 20 min in the presence of 11%  
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7 399 glycerol (Figure 6). Consequently, this severe impairment of sperm motility could disturb  
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10 400 normal sperm progression to reach SST or the fertilizing site in the oviduct since this whole  
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13 401 journey takes only 1 hour *in vivo* (Bakst et al., 1994). Short-term exposure of glycerol at  
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15 402 room temperature in 20 min also led to a significant reduction of sperm motility in humans  
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18 403 (McLaughlin et al., 1992) and mice (Katkov et al., 1998), suggesting that it's not a chicken  
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21 404 specific problem. At this temperature, higher concentration of glycerol (6 and 11%) also  
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23 405 increased sperm membrane breakage, a typical feature of necrosis, and apoptosis as  
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26 406 evidenced by Annexin V test (Figure 8). Whereas similar results were observed for  
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28 407 membrane breakage of stallion sperm under 5% glycerol condition, no effect was observed  
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31 408 on apoptotic rate (Macías García et al., 2012), suggesting a different sensitivity of glycerol  
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33 409 effects on sperm cell death patterns depending on the species. Alternatively, modifying a  
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36 410 glycerol freezing diluent for chicken sperm by adding apoptotic regulators such as  
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39 411 agonist/antagonist of death receptor signaling or caspase inhibitors (Fischer and Schulze-  
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41 412 Osthoff, 2005) might be a possible way to limit glycerol cell death induction. Furthermore,  
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44 413 this increase of sperm death may also be one of the causes of the reduction of sperm  
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46 414 presence in SST. Since SST are known to select and maintain higher quality sperm, sperm  
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49 415 presenting death signals may be rejected (Brillard, 1993; Blesbois and Brillard, 2007).

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51 416 Lastly, glycerol changes chicken sperm energy metabolism with a dramatic increase of  
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54 417 ATP concentration and a lower mitochondrial depolarization (Figure 7), corresponding to  
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57 418 previous studies in stallions (Macías García et al., 2012) and boars (Malcervelli et al., 2020).

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59 419 During ATP synthesis via mitochondrial respiration, the displacement of protons generates a  
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420 voltage gradient to polarize the membrane with positive charge outside, defined as  
421 mitochondrial membrane potential (Wang et al., 2022). Thus mitochondria depolarization,  
422 refers to sperm with low mitochondrial membrane potential, generally associates with ATP  
423 deficiency and lower sperm motility (Alamo et al., 2020). Here sperm incubated at 41°C for  
424 30 min showed no difference of mitochondria depolarization in each glycerol condition  
425 (Figure 7A), theoretically generating a comparable ATP content in each of them (Pandey et  
426 al., 2021). However, glycerol concentrations of 6 and 11% led to a 25 and 60-fold higher ATP  
427 concentrations than the other conditions (Figure 7B), raising the hypothesis that excessive  
428 ATP is synthesized via glycolysis or pentose phosphate pathways (Setiawan et al., 2020).  
429 However, further studies will be needed to determine direct evidence of the activity of these  
430 pathways.

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431 Dynein, a specific ATPase uses energy from ATP hydrolysis and distributes it along the  
432 axonemal microtubules, the central cytoskeletal structures of sperm flagellum, to generate  
433 sperm movement (Alamo et al., 2020). Theoretically, sperm with higher ATP contents show  
434 higher motility. However, here we observed that at 30 min ATP accumulation in sperm under  
435 6 and 11% glycerol conditions (Figure 7B) contrastingly decreased their motility performance  
436 (Figure 6). This finding raises another hypothesis that glycerol may disrupt dynein enzymatic  
437 activity or flagellar axonemal structure, leading to the accumulation of unused ATP.

## 5. Conclusion

440 To conclude, we used the chicken model to demonstrate that glycerol affects severely  
441 sperm capacity to interact with female reproductive tract, disturbing sperm migration and  
442 storage and reducing sperm ability to penetrate the ovum and achieve fertilization in the  
443 oviduct. We also proved that these effects occur mainly at the physiological body

444 temperature, suggesting a good sperm capacities preservation by the glycerol during  
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3 445 cryopreservation/thawing process. Consequently, although understanding the entire  
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5 446 mechanisms of glycerol toxicity remains a challenge, it is clear that removing glycerol prior to  
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7 447 insemination is a valuable solution to avoid fertility reduction in chickens and should be also  
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10 448 considered in mammalian species.

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#### 14 15 450 **CRedit authorship contribution statement**

16  
17  
18 451 **Hsiu-Lien Herbie Lin:** Conceptualization, Methodology, Formal analysis, Investigation,  
19  
20 452 Data curation, Writing - original draft. **Pascal Mermillod:** Conceptualization, Writing -  
21  
22 original draft, Visualization, Supervision. **Isabelle Grasseau:** Methodology, Investigation,  
23 453 Data curation. **Jean-Pierre Brillard:** Methodology, Writing - review & editing. **Nadine Gérard:**  
24  
25 454 Methodology, Writing - review & editing. **Karine Reynaud:** Methodology, Writing - review &  
26  
27 editing. **Lih-Ren Chen:** Writing - review & editing. **Elisabeth Blesbois:** Conceptualization,  
28 455 Supervision. **Anaïs Vitorino Carvalho:** Conceptualization, Formal analysis, Writing - original  
29  
30 draft, Visualization, Supervision.  
31 456  
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#### 41 460 **Declaration of interest**

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44 461 The authors declare that there are no conflicts of interest.  
45  
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#### 49 463 **Funding**

50  
51 464 This study was performed with the financial support of CRB-Anim [ANR-11-INBS-0003].  
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#### 57 466 **Acknowledgments**

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467 We thank Maryse Meurisse and Marie-Claire Blache for their help with image analysis.

468 We thank the staffs of the UE-PEAT, INRAE, Nouzilly, France for all assistance with animal  
469 care and experiments.

470

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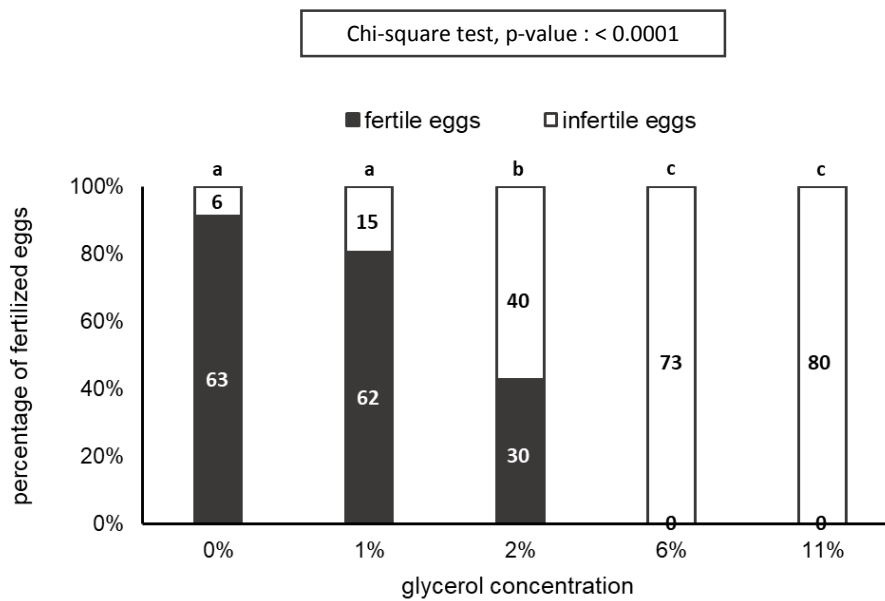
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1 **Figures**

2

3

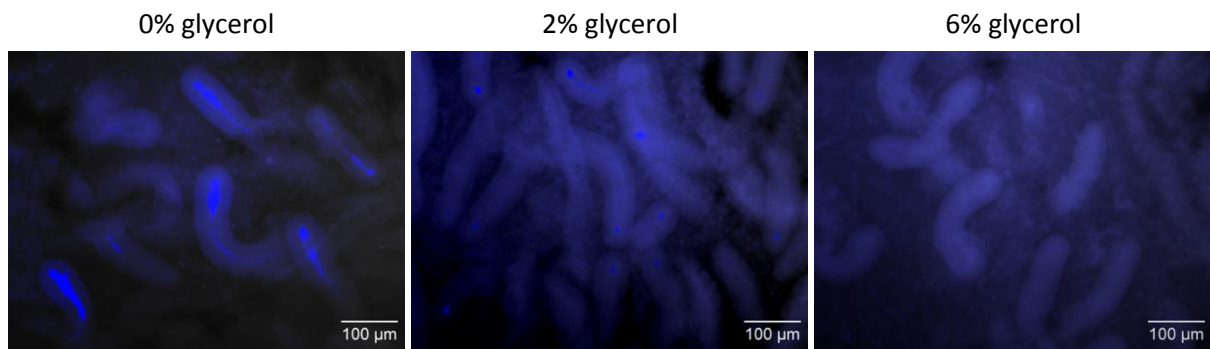


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5 **Figure 1. Effect of glycerol presence in semen samples on sperm fertility capacity.** Semen  
6 was collected and pooled from 10 roosters. Number of hens=12 for each experimental  
7 condition. Eggs were collected between 2<sup>nd</sup> and 7<sup>th</sup> day after first insemination in a dose of  
8  $100 \times 10^6$  sperm per hen for 2 consecutive days. Black and white bars represent the  
9 percentage of fertile and infertile eggs. The numbers of considered eggs are indicated on the  
10 bars. Different letters indicate significant differences (p-value < 0.05).

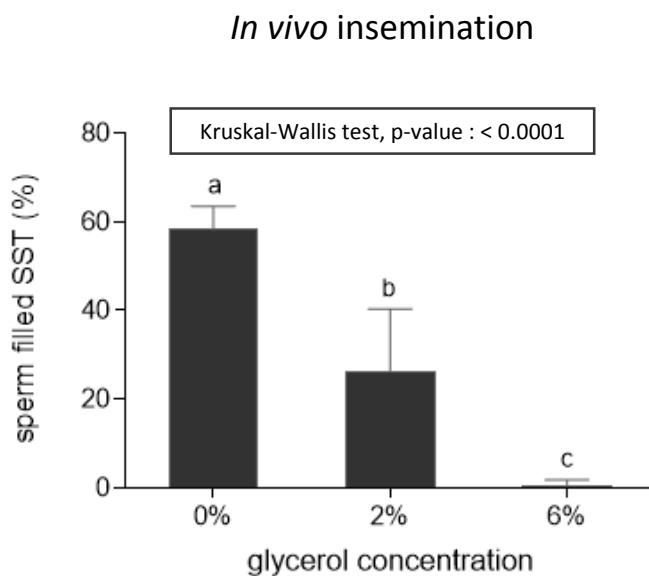
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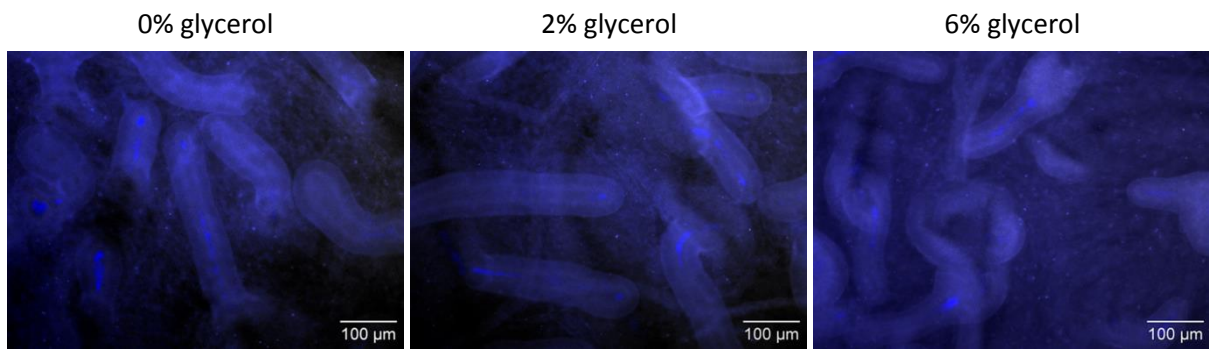


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16 **Figure 2. The presence of sperm in sperm storage tubules (SST) after *in vivo* insemination.** A  
17 and B represent sperm identification (A) and the percentage of SST containing sperm (B) after  
18 *in vivo* insemination with 0, 2 and 6% glycerolized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

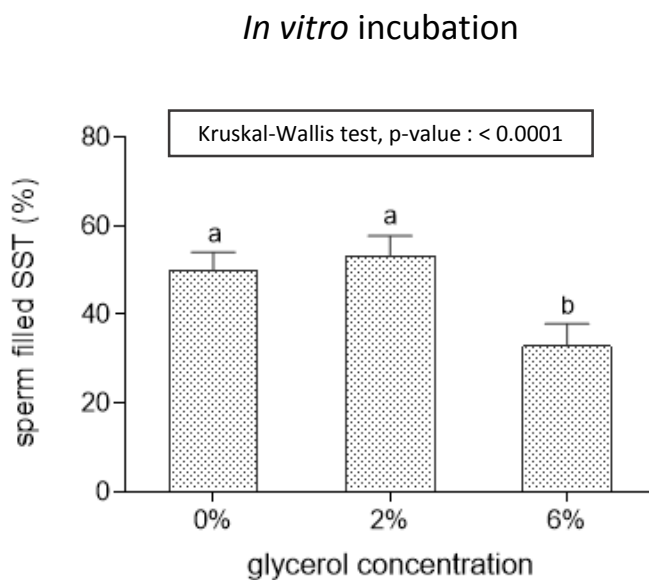
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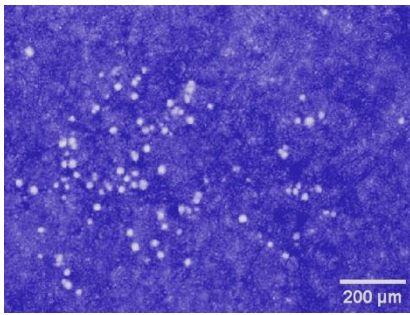


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28 **Figure 3. The presence of sperm in sperm storage tubules (SST) after *in vitro* incubation.** A  
29 and B represent sperm identification (A) and the percentage of SST containing sperm (B) after  
30 *in vitro* incubation of sperm and uterovaginal villi in 0, 2 and 6% glycerol medium. Sperm  
31 stained with Hoechst 33342 at 4°C for 4 h and washed in Lake PC diluent then mixed with  
32 glycerol just before incubation. SST were detected by autofluorescence. Data collected from  
33 3 villi of each treatment. The bars and lines correspond to the mean (5 experimental  
34 replicates) and the standard deviation of the mean. Different letters indicate significant  
35 differences (p-value < 0.05).

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37 A



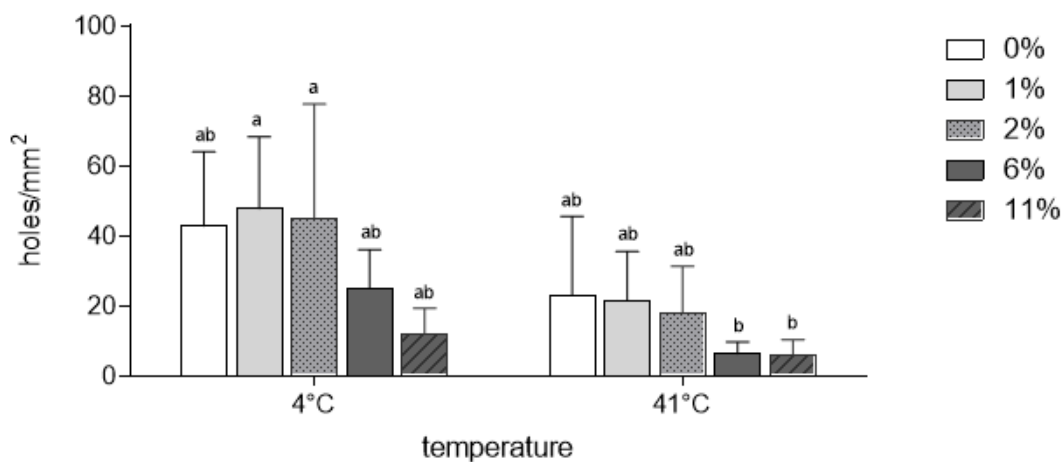
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40 B

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

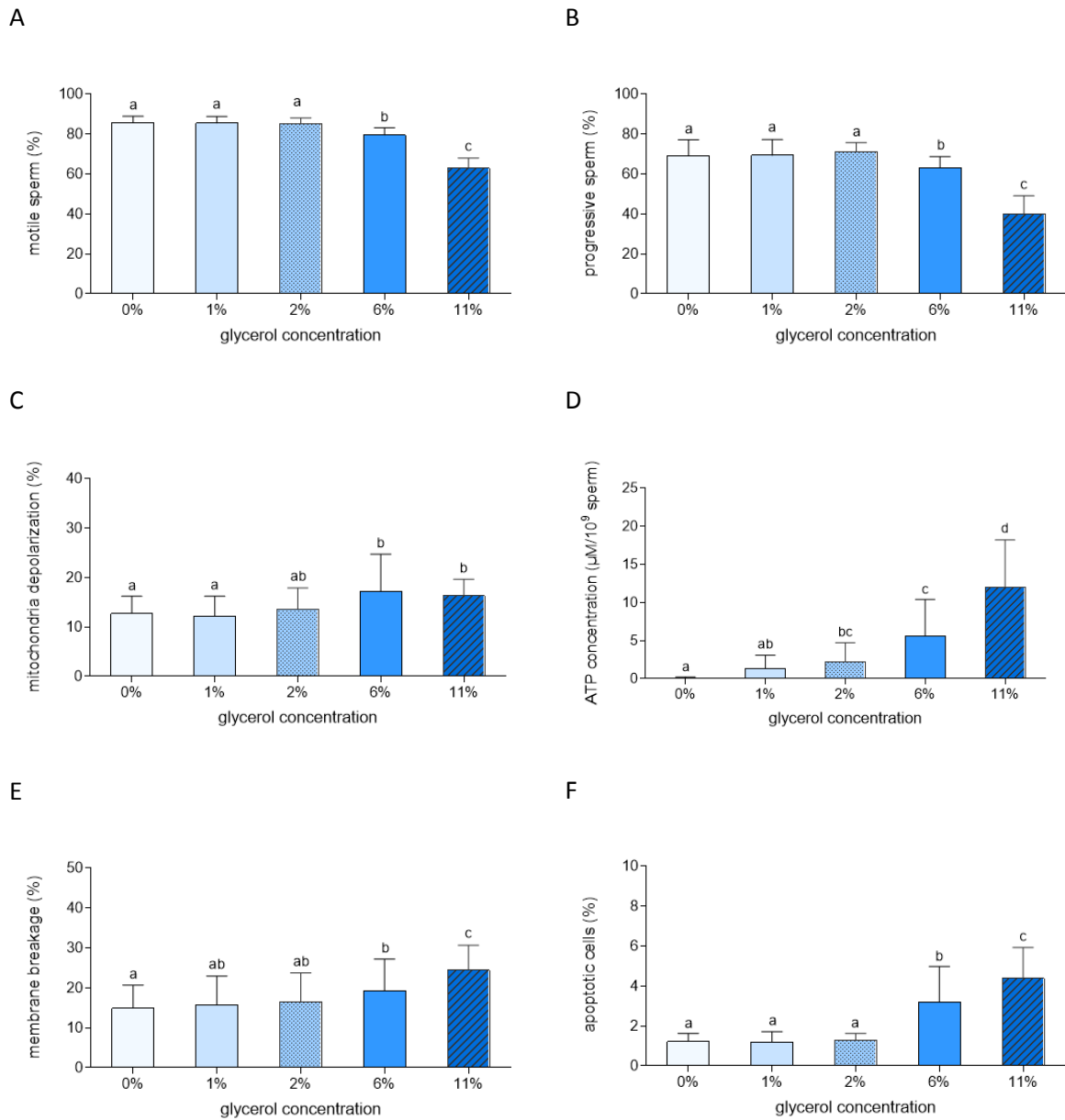
41 Abbreviation: GLY=glycerol concentration.



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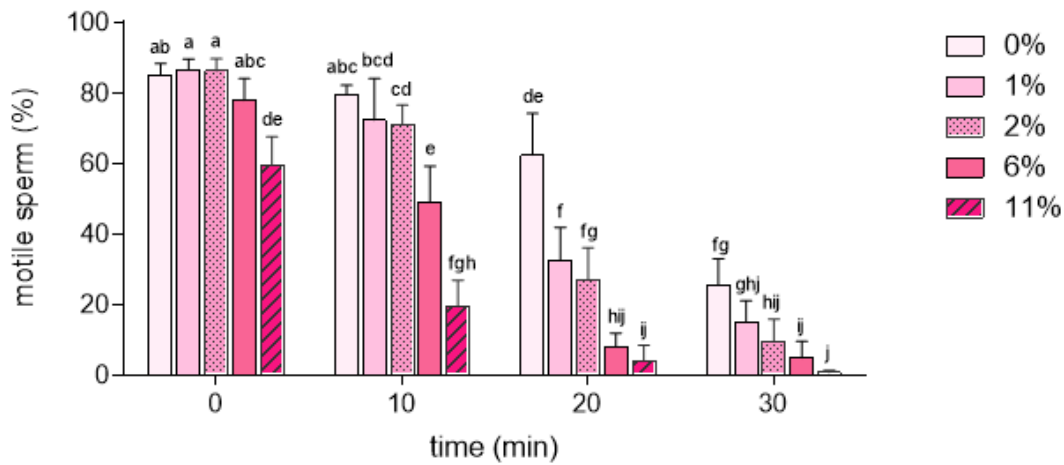
43 **Figure 4. Sperm penetration in perivitelline membrane (PVM) after pre-incubation with**  
44 **different glycerol concentrations.** A and B represent the image (A) and the number of holes  
45 (B) after PVM incubated with glycerolized sperm. Semen samples were collected and pooled  
46 from 10 roosters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

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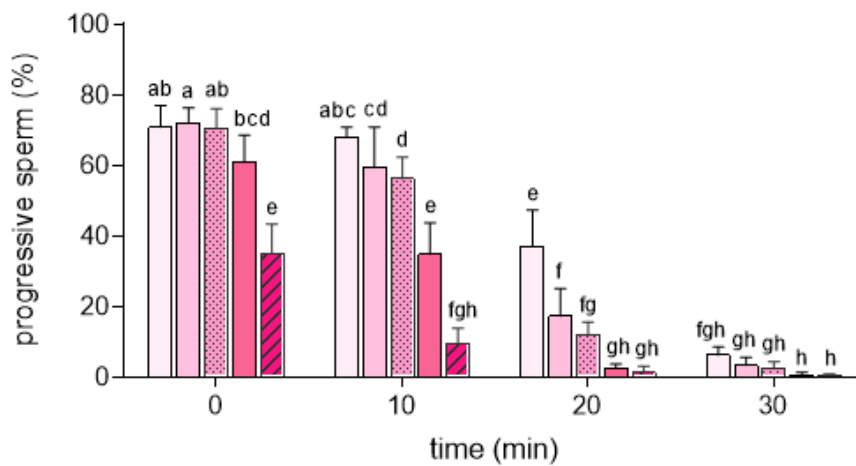
51 **Figure 5. Sperm quality parameters of glycerolized sperm at 4°C.** A-F represent evaluations  
 52 of different sperm quality parameters. Semen samples were collected and pooled from 10  
 53 roosters. The bars and lines correspond to the mean (5 experimental replicates) and the  
 54 standard deviation of the mean. Different letters indicate significant differences (p-value <  
 55 0.05).  
 56

57 A



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59 B

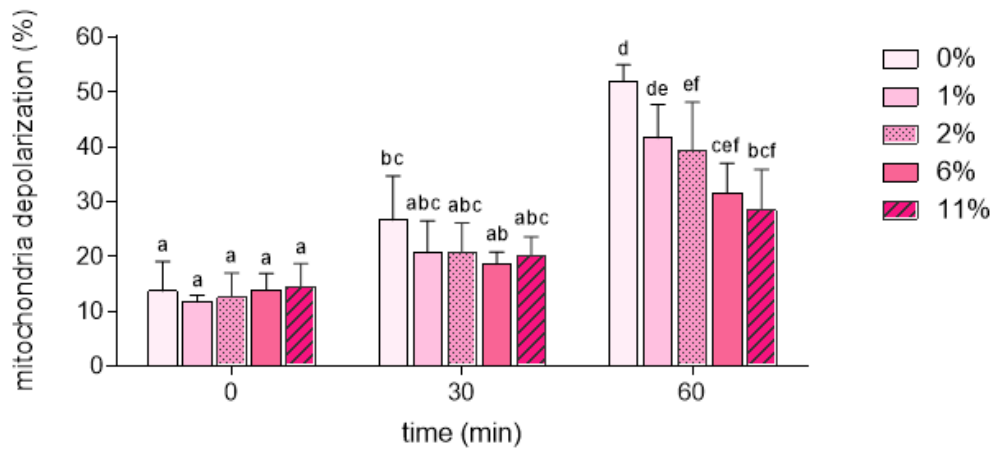


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61 **Figure 6. Sperm motility of glycerolized sperm at 41°C.** A and B represent the percentages of  
62 motile (A) and progressive sperm (B). Semen samples were collected and pooled from 10  
63 roosters. The bars and lines correspond to the mean (5 experimental replicates) and the  
64 standard deviation of the mean. Different letters indicate significant differences (p-value <  
65 0.05).

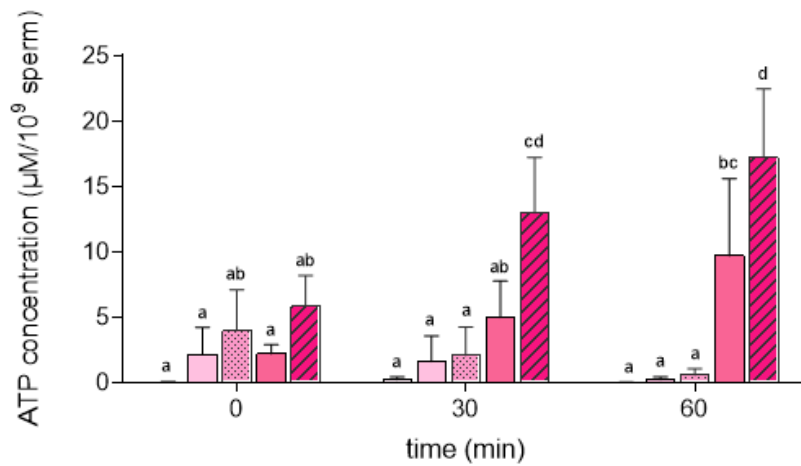
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67 A



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69 B



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

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

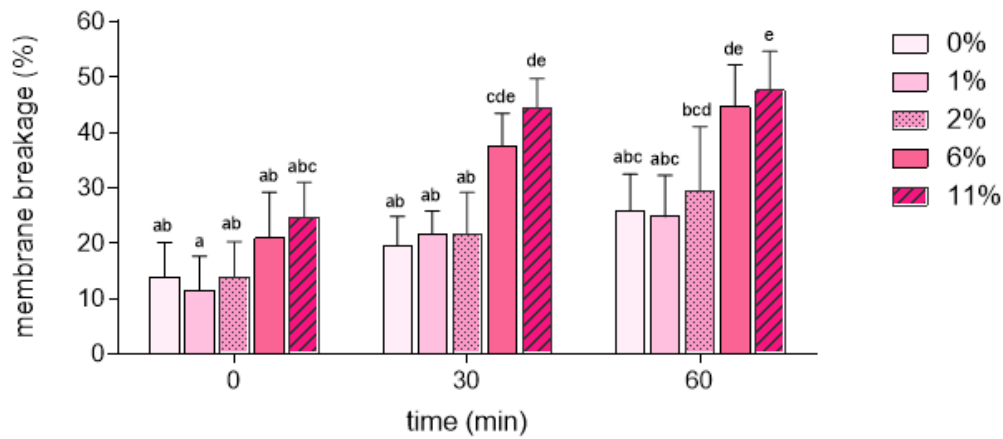
73 (B). Semen samples were collected and pooled from 10 roosters. The bars and lines

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

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

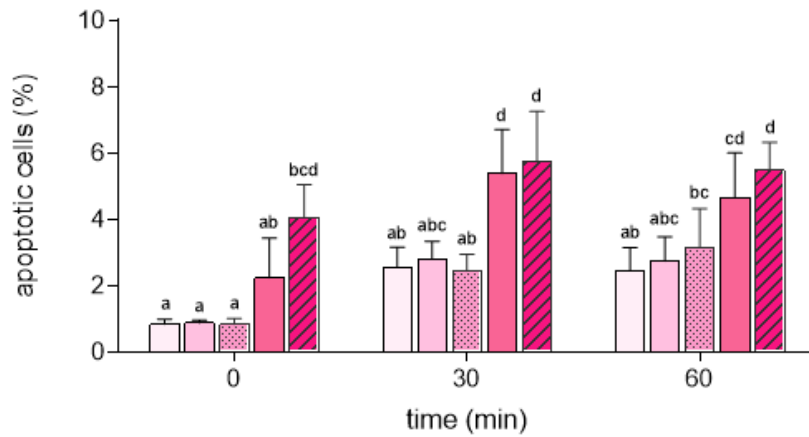
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77 A



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80

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



1 **TABLE**

2 **Table 1. Effect of glycerol concentration (GLY) and exposure time (T) on *in vitro* sperm**  
 3 **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

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



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## **Author declaration**

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

We further confirm that any aspect of the work covered in this manuscript that has involved experimental animals has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

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