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## Time-resolved Fluorescence and Generalized Polarization: Innovative tools to assess bull sperm membrane dynamics during slow freezing

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35 **Abstract:**

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37 During slow freezing, spermatozoa undergo membrane alterations that compromise their ability  
38 of fertilizing. These alterations are caused either by cold shock or by the use of cryoprotectants  
39 known to be cytotoxic. However, little is known about the membrane changes that occurred  
40 during freezing. Here, we combined Generalized Polarization (GP), **Time-resolved**  
41 **Fluorescence** and laurdan fluorescence properties to investigate the changes in membrane  
42 fluidity and dynamics during slow freezing of bull sperm. We successfully demonstrated that  
43 laurdan may be distributed in three different local environments that correspond to different  
44 membrane lipid composition. These environments won't behave the same way when the cells  
45 will be subjected to either a chemical treatment (adding the cryoprotectants) or a physical  
46 treatment (freezing).

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## 69 Introduction

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71 Artificial insemination is a relevant technique in a dairy and meat cattle breeding  
72 programme. Assisted reproduction and especially artificial insemination has played a central  
73 role in livestock breeding. Hence, the success of artificial insemination requires  
74 cryopreservation of semen for long periods of time.

75 Slow freezing of semen from genetically superior sires allows long storage and  
76 distribution of doses of semen for insemination. Bull sperm cryopreservation techniques have  
77 progressed slowly over the past several decades and the techniques are currently standardized.  
78 The success of cryopreservation is based on adding cryoprotectants (for instance glycerol) to  
79 extenders (diluent which is added to semen to preserve its fertilizing ability) and other  
80 components such as egg yolk, milk, bovine serum albumin, polyvinyl alcohol and liposomes to  
81 minimize the detrimental effects of freezing and thawing [7]. Cryoprotectants protect sperm  
82 from cold shock, osmotic stress, membrane alterations (fluidity and permeability changes) plus  
83 provide energy substrates for spermatozoa survival [7] [19].

84 Spermatozoa are known to be highly sensitive cells. These cells are highly polarized  
85 and show extreme compartmentalization of its plasma membrane. Sperm cells are made of 3  
86 domains : the head, a flagellum and a midpiece. The sperm head can be subdivided in 3 regions :  
87 the acrosome, the equatorial segment and the postacrosome. Since sperm cells are  
88 transcriptionally inactive and cannot synthesize any proteins, they have developed an  
89 alternative strategy of sequestering proteins into different compartments until they are needed.  
90 The process of sequential exposure of sperm proteins must be accurate to ensure successful  
91 fertilization. The acrosome, the equatorial segment, and the postacrosome have distinct  
92 subcellular structures and proteins. A study By Ellis et al. [13] using atomic force microscopy  
93 (AFM) illustrated unique sperm surface topology of each domain that is distinguishable from  
94 that of other domains [13] Maintaining the integrity of these domains after cryopreservation is  
95 crucial for the fertilization process to occur.

96 Therefore, it is of great interest to get into the effect of slow freezing on sperm  
97 functionality. It has been shown that approximately 50% of sperm are immotile when thawed  
98 after cryopreservation and sperm that remain motile have their fertility capacity compromised  
99 due to cryoinjury [34]. Furthermore, while vitrification is commonly used for oocytes

100 cryopreservation, slow freezing is still the only technique to be used in bull breeding program.  
101 Indeed, vitrification requires the use of high concentrations of cryoprotectants and it is known  
102 that various cryoprotectants have a negative impact on spermatozoa [29].

103         The process of freezing and thawing may cause irreversible damages to sperm [15].  
104 Effects of cryopreservation on sperm function and the ability of spermatozoa to fertilize have  
105 been studied extensively in many species, particularly in bull. Cryopreservation alters many  
106 functions such as capacitation (induction of premature capacitation; cryo-capacitation),  
107 acrosomal reaction (premature reaction), mitochondrial function and motility (reduced motility)  
108 were the most significant alterations observed [10]. While most cryoprotectants are known to  
109 show some toxicity to sperm [21], the exact mechanisms by which cryopreservation  
110 (cryoprotectants and cold) affects sperm function are still not well understood.

111 Plasma membrane structure has been shown to have significant impact on spermatozoa  
112 functionality and hence fertility [29]. Indeed, some membrane components such as cholesterol  
113 plays a central role in maintaining the ability of spermatozoon to fertilize the oocyte. During  
114 the cryopreservation process, efflux of cholesterol from sperm plasma membrane along with  
115 influx of  $\text{Na}^+$  and  $\text{Ca}^{2+}$  ions have been described to be major contributors to premature  
116 capacitation (cryocapacitation) [10][11]. In addition, release of reactive oxygen species (ROS)  
117 from dead or moribund spermatozoa present in the frozen semen doses, may alter membranes of  
118 the remaining live sperm subpopulation [18] Therefore, maintaining the integrity of sperm cell  
119 membrane components during the freezing-thawing process is essential to preserve  
120 spermatozoa fertilization potential after thawing.

121 In the present work, we propose to study for the first time the impact of cryopreservation on  
122 bull spermatozoa by advanced biophysical methods (Generalized Polarization and **Time-**  
123 **resolved Fluorescence**) using the fluorophore laurdan. Laurdan is a lipophilic polarity-sensitive  
124 dye that is widely used to study membrane dynamics [5]. Measuring lipid packing of  
125 membranes with GP has been extensively done on cell models [28].

126 Mammalian sperm are strongly laterally organized cells. For example, membrane fluidity and  
127 domain formation in the acrosomal and postacrosomal part of the mammalian sperm cell  
128 membrane are key elements for membrane stabilization before capacitation and creation of  
129 fusion competence after capacitation [30].

130 We believed that laurdan GP and **Time-resolved Fluorescence** could be used as sensitive tools  
131 for evaluation of spermatozoa plasma membrane dynamics after freezing.

132 **Materials and Methods**

133

134 ***Animals, semen collection and semen processing***

135 Semen samples were obtained from Hostein bulls (n=6) who were clinically proven to be free  
136 from any general or genital diseases. The bulls were housed at the Elva Novia agricultural  
137 cooperative (Fontaines, France) and maintained under uniform feeding and housing conditions.  
138 Ejaculates were collected using an artificial vagina. The semen samples were assessed for  
139 volume, sperm concentration and percentage of motile spermatozoa.

140 Computer sperm motility analysis program (CASA, IVOS version, Hamilton thorne  
141 Biosciences, MA, USA) was used to assess sperm motion characteristics. CASA was adjusted  
142 for bovine semen analysis.

143 Ejaculates containing spermatozoa with > 80% forward motility and concentrations greater than  
144  $1.0 \times 10^9$  spermatozoa / ml were used in this study.

145

146 ***Sperm preparation for slow freezing and thawing***

147 After evaluation, semen was diluted with a commercial egg yolk extender (Optidyl® ; Biovet:  
148 BP 62, 32500 Fleurance, France) (composition: Tris diluent, ionised egg yolk, glycerol,  
149 antibiotics) in order to obtain a final concentration of semen of 15 to 20 million /per straw and  
150 settle at RT for 10 minutes. Then, the extended semen was cooled slowly to 4°C over a  
151 minimum of two hours. Subsequently semen was frozen at a programmed rate of -3°C / min  
152 from +4 to -10 °C; -40°C / min from -10 to -100°C; -20°C / min from -100 to -140 °C in a  
153 digital freezing machine (Digitcool, ...). Thereafter, the straws were plunged into liquid  
154 nitrogen. After storage, the straws were thawed in a 37 °C water bath for 30 s immediately  
155 before use. After thawing, spermatozoa motility percentage was assessed using a phase contrast  
156 microscope with a warm slide and the sperm vitality was evaluated using the eosin-nigrosin  
157 stain.

158

159 ***Laurdan Labeling of sperm cells***

160 Laurdan is a polarity probe; upon excitation, its emission undergoes a spectral shift due to the  
161 reorientation of water molecules present in the glycerol backbone region of the membrane. This  
162 shift can be correlated to the lipid phase [24]. In the gel phase, when no water is present, laurdan  
163 emission peaks at 440 nm, whereas in the liquid crystalline phase the spectrum is red-shifted  
164  $\approx 50$  nm. This shift is quantified using the generalized polarization (GP) function. **Laurdan is a**  
165 **fluorescent membrane probe sensitive to changes in the environment polarity (extent of water**

166 penetration into the bilayer surface). As the tighter packing of lipids in ordered compared to  
167 disordered phases leads to less accessibility to water molecules, polarity is also an indicator of  
168 lipid order. Polarity changes are shown by shifts in the Laurdan emission spectrum, which are  
169 quantified by calculating the generalized polarization (GP):  $GP = (I_{440} - I_{490}) / (I_{440} + I_{490})$   
170 where  $I_{440}$  and  $I_{490}$  are the fluorescence emission intensities at 440 and 490 nm respectively.  
171 Higher GP values are attributed to a more ordered lipid environment (sub-band at 440 nm),  
172 lower GP values to a more disordered one (sub-band at 490 nm), with more membrane  
173 hydration.

174 For the labeling experiments, fresh semen was diluted in TRIS buffer solution in order to obtain  
175 the same final concentration than frozen/thawed sperm (10 to 20 times dilution, final  
176 concentration 40-50  $10^6$  spermatozoa / ml). Spermatozoa were labeled with laurdan for 10 min  
177 at 37 °C. Laurdan was dissolved to a concentration of 8mM in dimethylsulfoxide (DMSO) and  
178 stored at - 20°C at the dark. In the laurdan-stained spermatozoa suspension, the amount of  
179 DMSO was 0.02% (v/v) and a final concentration of laurdan of 1.6 $\mu$ M. After staining the  
180 spermatozoa were centrifuged at 200g for 5 min, washed once and a swim-up was performed  
181 as explained below.

182 The same procedure was applied to frozen/thawed spermatozoa. Briefly sperm was diluted in  
183 TRIS buffer solution and labeling was performed as described above.

184

### 185 ***Incubation of fresh semen samples with Optidyl***

186 Experiments were performed in order to evaluate the effect of Optidyl® (Cryoprotectants  
187 mixture) on GP and lifetimes values of fresh spermatozoa. To do so, spermatozoa before  
188 labeling with laurdan were diluted and incubated for 10 min with Optidyl® (dilution factor 15:  
189 same dilution factor than the one used when freezing bull sperm).

190 After incubation, the cells were washed once and resuspended in TRIS buffer and GP and  
191 lifetimes were measured as described below.

192 GP and lifetimes were measured at both 37°C and 23°C. The cell suspensions that were  
193 refrigerated from 37°C to 23°C were warmed from 23°C to 37°C.

194

### 195 ***Swim-up procedure***

196 After centrifugation (washing step), the supernatant was discarded and 4 ml of pre-warmed  
197 TRIS buffer solution were gently layered on the top of the sperm pellet before the sample was  
198 incubated at 37 °C for 30 min in the incubator. After incubation, 3.5 ml of supernatant was

199 aspirated into a 15 ml centrifuge tube to be used for spectroscopy and imaging. This technique  
200 was performed in order to use only motile spermatozoa for the experiments.

201

### 202 ***Generalized Polarization using spectroscopy***

203 Polarity changes are detected by measuring shifts in the laurdan emission spectrum, and the  
204 Generalized Polarization function (GP) was defined as a way of measuring this wavelength  
205 displacement [28]. Laurdan GP is also presented as an accurate and sensitive indicator of lipid  
206 order [2][5]. In the gel phase when no water is present, laurdan emission peaks at 440 nm  
207 whereas in the liquid crystalline phase the spectrum shifts **of about** 50nm.

208 The steady state emission of laurdan was measured by a Fluorolog-3 spectrofluorometer  
209 (HORIBA Jobin Yvon). For the temperature-dependent fluorescence intensity measurements,  
210 the excitation wavelength was 370 nm. Fluorescence emission spectra were recorded between  
211 400 and 600nm. The laurdan GP parameter was calculated using the following equation:

212

$$213 \text{ GP} = (I_{440} - I_{490}) / (I_{440} + I_{490})$$

214

215 where  $I_{440}$  and  $I_{490}$  are the fluorescence emission intensities at 440 and 490 nm respectively.

216 Experiments were conducted both at 37°C and 23°C.

217

### 218 ***Time-Resolved Fluorescence Spectroscopy***

219 The fluorescence lifetime is powerful to probe the molecular environment of a given  
220 fluorophore. Fluorescence lifetime may be understood as the average amount of time that a  
221 fluorophore remains in the excited state following excitation [16][3]. This parameter depends  
222 of the environment and the **structure of the fluorophore**, but is independent of its  
223 concentration. Each individual fluorescent molecule typically exists in a state with its own  
224 characteristic lifetime. Due to the presence of multiple molecules with distinct lifetimes, the  
225 overall fluorescence decay after an excitation pulse is inherently multiexponential. Each  
226 component of the multiexponential decay reflects the fractional contribution of a specific form  
227 of the fluorescent probe.

228 Laurdan fluorescence lifetime is sensitive to the hydration of the interface region [3] and  
229 displays an **longer fluorescence lifetime** in less hydrated environments [21]. As the tighter  
230 packing of lipids in ordered compared to disordered phases leads to less accessibility to water  
231 molecules, laurdan lifetime is also an indicator of lipid order.

232



233 Laurdan Fluorescence lifetimes were measured using a time-correlated single-photon-counting  
234 (TCSPC) system on a FluoroMax-4 spectrofluorometer (HORIBA Jobin Yvon). Fluorescence  
235 lifetime decay curves were measured with a pulsed 370-nm diode. Detection was set to 465 nm  
236 with a 6 nm bandpass. The lifetime was recorded on a 100 ns scale and measurements were  
237 stopped when peak signal reached 10 000 counts. The Instrument Response Function (IRF) was  
238 obtained using the diffusion of the excitation light on the sample at 370 nm.  
239 DAS6 v6.1 software was used for TCSPC histogram analysis. As large deviation of chi-squared  
240 values from unity ( $\chi^2 > 1.5$ ) occurs when using mono or bi-exponential adjustments,  
241 data fitting was established using a three-exponential decay equation. This provides three  
242 fluorescent lifetimes with the corresponding fractional contributions.  
243 For this experiment 3ml of a suspension of labeled spermatozoa were used. Experiments were  
244 conducted both at 37°C and 23°C.

245

#### 246 *Statistical analyses*

247 A Wilcoxon-Mann-Whitney test was computed with R 3.3.2 to test differences between fresh  
248 and frozen spermatozoa samples on the different variables (GP and lifetimes). This test does  
249 not require the assumption of normal distributions. P-values were calculated for each variable,  
250 indicating significant differences when they are lower than 0.05.

251 The same tests were also computed to detect potential differences between 23<sup>0</sup>C and 37<sup>0</sup>C.

252 Sperm samples of six different bulls were collected. For each bull, one sample of fresh sperm  
253 and one sample of frozen sperm were analyzed.

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267 **Results**

268 ***Steady state spectra and GP of laurdan: fresh versus frozen/thawed bull sperm***

269 The effect of slow freezing and thawing on bull spermatozoa membrane was assessed using  
270 laurdan emission spectral. Figure 1 shows representative laurdan emission spectra measured in  
271 fresh and frozen-thawed samples at two different temperatures, 37°C and 23°C. Spectra from  
272 the frozen-thawed sample show one band with the maximum emission centered at 430 nm with  
273 no shift when temperature decreases from 37°C to 23°C. Spectra from fresh samples were  
274 broader with a shoulder at 490 nm appearing at 37°C. The intensity of the shoulder decreases  
275 when temperature decreases to 23°C, indicating a decreased polarity of the probe  
276 microenvironment. The spectra of fresh samples were thus very different from the frozen-  
277 thawed samples and display a higher intensity of the band centered at 490 nm. Furthermore, the  
278 spectra of fresh samples evolve as the temperature decreases while spectra of frozen thawed  
279 samples were stable with temperature decrease.

280

281 Laurdan GP was then calculated from the emission spectra. Indeed, laurdan GP can be used to  
282 monitor the transition between the two phases gel and liquid-crystalline lipid of the plasma  
283 membrane with good precision.

284 Our results indicate that at 37°C, GP values were significantly increased in frozen / thawed  
285 spermatozoa compared to fresh spermatozoa (0.51 *versus* 0.30,  $p= 0.004$ ). This was also the  
286 case at 23°C (0.53 *versus* 0.4,  $p= 0.044$ ) when comparing frozen / thawed spermatozoa to fresh  
287 spermatozoa (See table 1).

288 When fresh spermatozoa were subjected to temperature variation (from 37°C to 23 °C), GP  
289 value increased significantly (0.30 *versus* 0.40,  $p= 0.04$ ). However, when frozen/thawed  
290 spermatozoa were subjected to the same temperature variation, no significant variation of GP  
291 was observed (0.51 *versus* 0.53,  $p=0.33$ ) (See table 1).

292

293 ***Time-Resolved Fluorescence spectroscopy***

294 The results of the time-resolved fluorescence measurements with laurdan are shown in table 1.  
295 The decay of fluorescence intensity was assumed to be tri-exponential function of 3 lifetimes  
296 (T1, T2, T3). Temperature dependent (for T2) and state dependent (for T3) changes of lifetimes  
297 were observed when comparing fresh *versus* frozen/thawed spermatozoa (Figures 2a and 2b).

298

299 T1 lifetime: no significant difference in terms of temperature and state (fresh versus  
300 frozen/thawed)

301 For fresh and frozen/thawed spermatozoa, no significant variation of T1 lifetimes was observed  
302 when spermatozoa were subjected to a variation of temperature or when fresh spermatozoa  
303 were frozen (Table 1).

304

305 T2 lifetime : temperature dependent correlation of membrane order and dynamics

306 Whatever the state of spermatozoa concerned (fresh and frozen/thawed spermatozoa), a  
307 significant increase of T2 lifetimes when spermatozoa were subjected to a variation of  
308 temperature (from 37°C to 23°C) was observed (fresh spermatozoa:  $3.7 \pm 0.3$  and  $4.07 \pm 0.2$  at  
309 37°C and 23°C respectively;  $p=0.052$  and frozen / thawed:  $3.6 \pm 0.2$  and  $3.9 \pm 0.1$  at 37°C and  
310 23°C respectively;  $p=0.02$ ); see Table 1; Figure 2a

311 No significant differences were observed when comparing fresh versus frozen / thawed at either  
312 37°C ( $p=0.423$ ) and 23°C ( $p=0.229$ ); see Table 1; Figure 2a.

313

314 T3 lifetime : treatment dependent correlation of membrane order and dynamics

315 A treatment dependent change (significant increase) of lifetimes were observed at 37°C and  
316 23°C when comparing fresh versus frozen / thawed spermatozoa at 37 °C ( $9.2 \pm 2.2$  versus  
317  $12.5 \pm 0.4$ ,  $p=0.015$ , % increase of 35.9%) and fresh versus frozen / thawed spermatozoa at 23  
318 °C ( $8.9 \pm 1.4$  versus  $12.8 \pm 0.5$ ,  $p=0.005$ ; % increase of 43.8%). See Table 1, Figure 2b.

319

320 **Weights of T1, T2 and T3: B1, B2 and B3**

321 The weights of T1, T2 and T3 were defined as B1, B2 and B3.

322

323 When comparing the weights of B1, B2 and B3 for fresh spermatozoa subjected to a variation  
324 of temperature (37°C to 23°C), a significant difference was observed only for B3 (21.3% versus  
325 31.6%;  $p=0.04$ ), Table 1 and Figure 2c.

326 However, no significant variation was observed for frozen thawed spermatozoa subjected to a  
327 variation of temperature (37°C to 23°C) whatever the proportion considered, Table 1 and  
328 Figure 2c.

329

330 **Effect of Optidyl on fresh spermatozoa GP and lifetimes**

331 The effect of the cryoprotectant mixture Optidyl was measured on membrane dynamics so as  
332 to differentiate the membrane modifications due to the cryoprotectants from the ones due to

333 freezing-thawing. For this purpose, spermatozoa were incubated with Optidyl and washed  
334 before laurdan staining.

335

336 While incubation of spermatozoa with Optidyl had no effect on GP value measured at  
337 37°C, it clearly impacted GP evolution during a temperature variation from 37 to 23°C and  
338 subsequent warming to 37°C (37°C bis). Indeed, in fresh spermatozoa GP increases from 0.32  
339 to 0.41 with decreasing temperature to 23 °C and decreases again to 0.30 when temperature get  
340 back to 37°C. In fresh spermatozoa incubated with Optidyl, GP value remains stable at around  
341 0.32 during the temperature variation. As already observed in [Table 1](#), frozen-thawed  
342 spermatozoa had a significant higher GP value at 37°C (0.58) than fresh spermatozoa (0.32)  
343 and fresh spermatozoa incubated with Optidyl (0.32); [Figure 3](#). Furthermore, their GP value  
344 was stable (around 0.56) during the temperature variation as observed for fresh spermatozoa  
345 incubated with Optidyl, see [Figure 3](#).

346

347 Regarding T2, the behavior was the same for the three types of spermatozoa, fresh, fresh  
348 incubated with Optidyl and frozen-thawed. As already observed for the fresh and frozen-thawed  
349 spermatozoa a significant increase of T2 was observed with temperature decrease to 23°C for  
350 spermatozoa incubated with Optidyl (fresh without optidyl 37 °C: 3.52, *versus* Fresh without  
351 optidyl 23 °C: 3.98) (fresh with optidyl at 37°C: 3.53 *versus* with optidyl at 23°C: 3.74).  
352 Furthermore, the T2 variation was reversible with warming again to 37°C; see [Figure 4a](#).

353 The measurement of T3 gave interesting results as the T3 value increases for the fresh (9.83 at  
354 37°C), incubated with Optidyl (12.14 at 37°C) and frozen-thawed spermatozoa (12.82 at 37°C)  
355 respectively. T3 was independent of temperature variation for fresh spermatozoa and the ones  
356 incubated with Optidyl; see [Figure 4b](#).

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367 **Discussion**

368 Spermatozoa are very specific cells that are made of 5 regions known as the acrosome,  
369 equatorial segment, postacrosome, midpiece and principal piece. They are polarized and  
370 polarity is established during spermiogenesis in the testis. Spermatozoa plasma membrane  
371 contains protein and lipids (free cholesterol and phospholipids represent 80% of sperm lipids).  
372 Among the phospholipids, a high proportion of polyunsaturated fatty acids are susceptible to  
373 oxidative stress (ROS: Reactive Oxygen Species).

374 Membrane lipids are correlated to specific functions, because they promote the creation of  
375 microdomains with different fluidity and permeability characteristics required for the  
376 fertilization process [33][30]. In addition, lipids are major determinant of motility, viability and  
377 cell membrane integrity [27]. It has been shown in cat sperm, that cryopreservation significantly  
378 impaired the plasma membrane integrity including the acrosome [32] that is why developing  
379 techniques to study membrane integrity are of importance.

380 In the present work, we measured the fluorescence properties of laurdan in spermatozoa  
381 membranes. Laurdan, a membrane dye [6], presents a large excited state dipole moment, which  
382 results in its ability to report the extent of water penetration into the bilayer surface due to the  
383 dipolar relaxation effect. Because water penetration has been correlated with lipid packing and  
384 membrane fluidity [25], laurdan has been extensively used in membrane studies.

385 In our study, spermatozoa have undergone different treatments but are all motile because  
386 selected by a swim-up procedure after staining. We thus assumed that the variations of  
387 fluorescence properties of laurdan reflect modifications in the membrane lipids organization  
388 and/or dynamics. Hence, spermatozoa, in which membrane traffic is virtually absent, offer a  
389 good mammalian model in which laurdan properties can be exploited to study surface lipid  
390 dynamics, since the absence of membrane traffic in sperm precludes label internalization [20].

391 Previous works demonstrated that a higher intensity of the band centered at 490 nm  
392 indicates a higher polarity and a more fluid lipid phase [23]. Membranes of fresh sperm cells  
393 respond to cooling to 23°C by a decrease of the emission spectra of laurdan at 490 nm resulting  
394 in a higher GP value at 23°C than at 37°C. Thus, fresh sperm cells have functional membranes  
395 able to respond to modifications of their environment by a decrease of fluidity.

396 Our results indicate that frozen-thawed spermatozoa display a decreased membrane  
397 fluidity (higher GP value) in comparison to fresh spermatozoa at 37°C. Furthermore, the  
398 emission spectrum of laurdan is not modified by a temperature decrease to 23°C. These results  
399 indicate that membrane is altered by the freezing-thawing process.

400 Membrane fluidity is an important feature of spermatozoa fertility. An increased fluidity  
401 of the sperm membrane has been shown to be associated with the acquisition of its fertilizing  
402 ability [14]. Compositional and physicochemical alterations of bilayer features can greatly  
403 affect the fertilizing ability of spermatozoa, for instance, modifying structural and functional  
404 properties of proteins embedded in the bilayer [4]. Thus, modification of membrane fluidity  
405 and behavior after freeze-thawing may lead to fertilization ability decrease of spermatozoa.

406  
407 Interestingly, the cryoprotectant mixture Optidyl does not increase the GP values  
408 however it prevents the increase of GP observed during a temperature change.  
409 The extender used for semen dilution (Optidyl) maintains the biological environment necessary  
410 for survival of spermatozoa and prevents membrane damages during cryopreservation. Egg  
411 yolk (rich in phosphatidylcholine) in the semen extender is thought to regulate the efflux of  
412 integral protein, phospholipids and cholesterol, and thus protects the plasma membrane against  
413 temperature-related injury [14].

414 Studies conducted over the past two decades by Bergeron et al. [8] have revealed that a family  
415 of lipid- binding proteins (BSP proteins) present in bull seminal plasma is detrimental to sperm  
416 preservation as they induce cholesterol and phospholipid depletion from the plasma membrane.  
417 Interestingly, low- density lipoproteins (LDL) present in egg yolk interact with the BSP  
418 proteins, minimizing lipid removal from the sperm membrane. This phenomenon will  
419 influence sperm storage in liquid or frozen states.

420  
421 In the present work, we found that lifetime decay correlates well with a three exponential model,  
422 meaning that laurdan molecules may be distributed in three different local environments. We  
423 can argue that laurdan could be distributed in the three environments anterior acrosomal region,  
424 posterior acrosomal region and equatorial segment, and that each environment corresponds to  
425 a specific lifetime value. This difference in lifetimes values ( $T_1: 0.7$ ;  $T_2: 3.7$ ;  $T_3: 9.2$ ) is  
426 presumably a reflection of the different lipid composition of the membranes in spermatozoa.

427 In particular, cholesterol in spermatozoa plays a crucial role in the biological processes that  
428 occur during the fertilization process. Hence, the variation in cholesterol/phospholipids ratio  
429 has been linked to the ability to survive cryopreservation [12].

430 It has been shown by freeze fracture [31] that cholesterol is densely packed in the anterior  
431 acrosomal region as well as in the postacrosomal region while the equatorial region contains  
432 very low level of cholesterol.

433

434 In addition, besides cholesterol, seminolipid is also of high importance in spermatozoa. Indeed,  
435 seminolipid or SGG (SulfoGalactosylGlycerolipid) is present selectively and substantially in  
436 mammalian germ cells. SGG consists of glycerol with a sn-1 alkyl and sn-2 acyl chain as the  
437 lipid backbone, and a (3'sulfo)-galactopyranose  $\beta$ -linked to the sn-3 position. Throughout  
438 mammalian species, C16:0/C16:0 (palmitoyl) SGG is the main molecular species.

439 In spermatozoa, it is an integral component of lipid rafts and it represents 10mol% of total  
440 lipids. Since SGG is only present in sperm head, the % in this area accounts for more than  
441 10mol%. SGG has been described to play a crucial role in mammalian male reproduction and  
442 to be involved in sperm-zonal pellucida binding and sperm-egg plasma membrane binding [1].  
443 The presence of SGG (which is known to be an ordered lipid) in sperm DRMs (detergent  
444 resistant membranes) is due to its propensity to interact hydrophobically with cholesterol, a  
445 pertinent component of DRMs [17]. Overall SGG would contribute to the biophysical  
446 properties of the sperm head plasma membrane.

447 On none-reacted and none-capacitated sperm cells, SGG is present primarily at the apical ridge  
448 subdomain of the plasma membrane of the spread head.

449 Hence, in terms of lipid composition, if we take not only into account cholesterol but also SGG,  
450 the sperm head presents 3 lipid subdomains :

451 -anterior acrosomal region (apical ridge)

452 -equatorial segment

453 -posterior acrosomal region

454 The different lifetimes measured could thus be associated with the 3 head-regions.

455 T1, the shorter fluorescence lifetime, could be related to the presence of tightly packed lipid  
456 domains, with less water accessibility. Due to their more dehydrated composition, the domains  
457 associated to T1 are most probably less affected by the addition of cryoprotectants and freezing.

458

459 In our experiments, we observed that T3 does not vary with a variation of temperature. while  
460 the freezing process affects T3 (increase of T3). The explanation to that, would be that in fresh  
461 spermatozoa, cholesterol will stabilize the plasma membrane preventing any lifetime changes  
462 during temperature changes and that the region that contains higher amount of cholesterol  
463 would be associated to T3.

464 In addition, we observed that T3 irreversibly increased in frozen/thawed spermatozoa compared  
465 to fresh spermatozoa. This could be explained by irreversible modification of lipid distribution  
466 in membrane that took place during cryopreservation. This could be caused by the severe  
467 osmotic stresses, sperm cells encounter during cryopreservation and thawing. This occurs with

468 addition and removal of cryoprotective agents, as well as during freezing and thawing [26].  
469 Sperm are exposed to hypertonic conditions upon extracellular ice formation, because the solute  
470 concentration in the extracellular unfrozen fraction increases. These results in cellular  
471 dehydration due to water transport out of the cell to retain equilibrium between the intra- and  
472 extracellular solute concentrations. Dehydration especially occurs when low cooling rates are  
473 used for freezing. During thawing, the reverse process takes place, and sperm are exposed to  
474 hypotonic conditions resulting in water uptake and swelling.

475 Such osmotic stress has been shown to be the predominant cause of cell damage [20].  
476 Furthermore, T3 also increased with the Optidyl contact to spermatozoa. It can be hypothesized  
477 that some components of Optidyl diffused into the plasma membrane. This would partly explain  
478 the T3 increase.

479

480 Morris et al. (2007)[20] used cryo-scanning electron microscopy and freeze substitution to  
481 show that no intracellular ice is formed in sperm. They claim that cell damage predominantly  
482 results from an osmotic imbalance encountered during thawing. Hence, conducting to membrane  
483 deshydration after freezing.

484

485 We have shown that laurdan fluorescence lifetime can be either treatment dependant (T3) or  
486 temperature dependant (T2) and that optidyl along with the freezing process are going to  
487 prevent the plasma membrane dynamics during a change of temperature (no variation of the  
488 proportion of lifetimes). Our results indicate that plasma membrane environments associated to  
489 each fluorescence lifetime value wont behave the same way when the cells will be subjected to  
490 either a chemical treatment (adding the cryoprotectants) or a physical treatment (freezing). As  
491 for T1

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493

494 The clear-cut relationships between laurdan spectral properties and membrane structure  
495 and dynamics makes laurdan spectroscopy a useful tool in investigating the correlations  
496 between the physical properties of the membrane environment and sperm functions. Our work  
497 demonstrated for the first time that Fluorescence Life Time Spectroscopy and Generalized  
498 Polarization could be used as innovative tools to evaluate bull's plasma membrane dynamics  
499 during the freezing process.

500

501



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506

507 **Competiting interests**

508 The author(s) declare(s) that there is no conflict of interest regarding the publication of this  
509 article.

510

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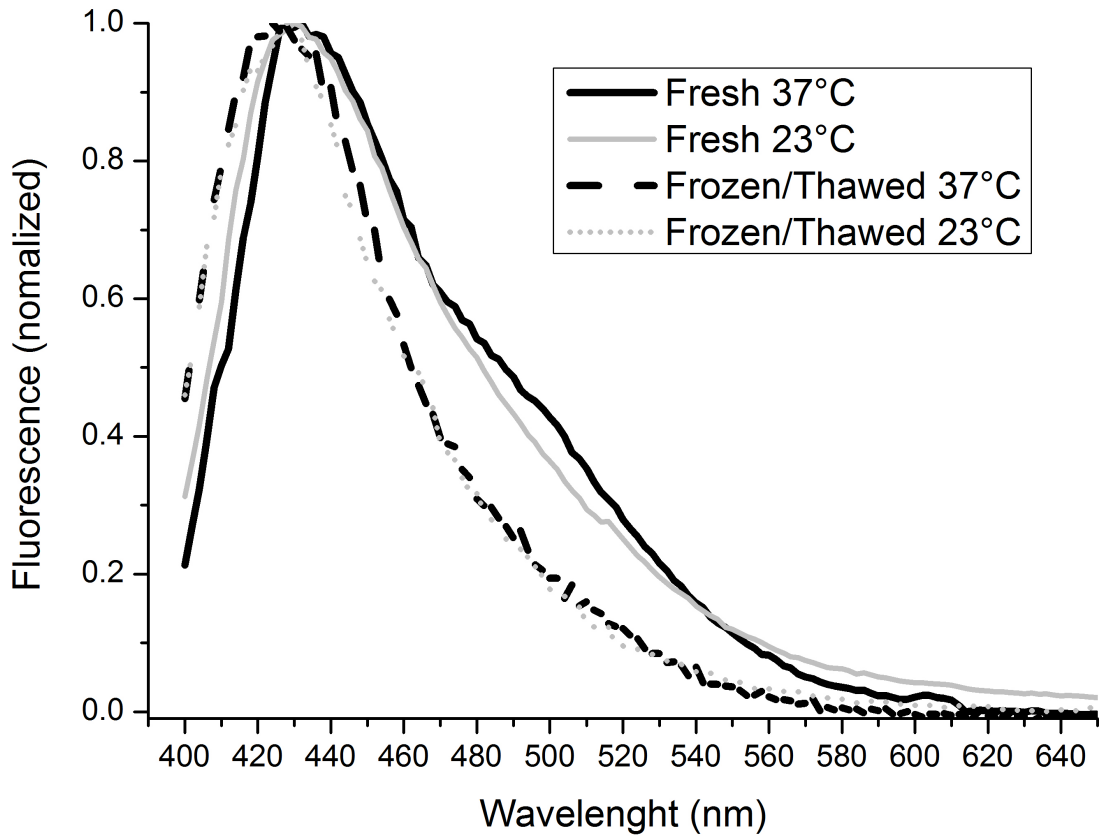
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674 Figure 1 : Representative normalized steady-state emission spectra of Laurdan labelled fresh  
675 sperm cells and frozen/thawed sperm cells. GP measurements were conducted both at 37°C  
676 and 23 °C  
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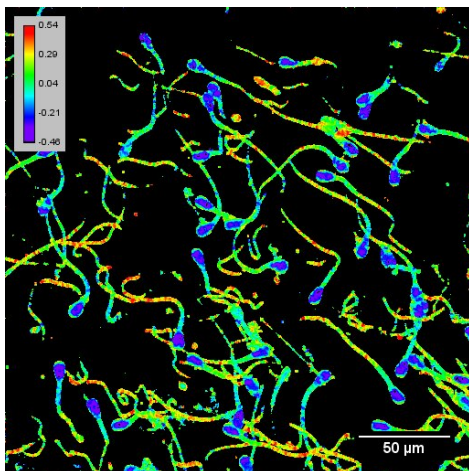
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679 GP images of spermatozoa labelled with Laurdan using a false color scale

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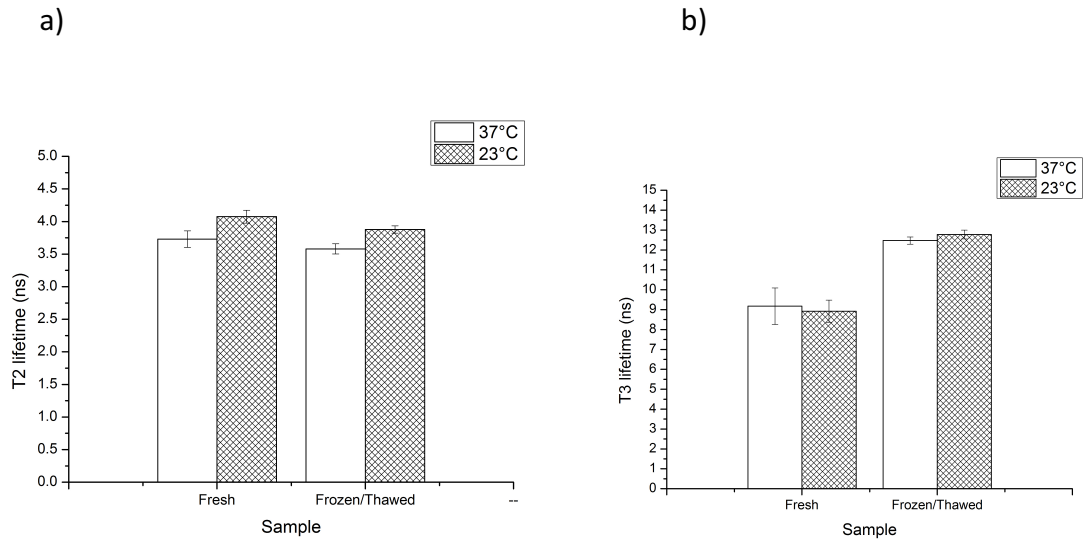
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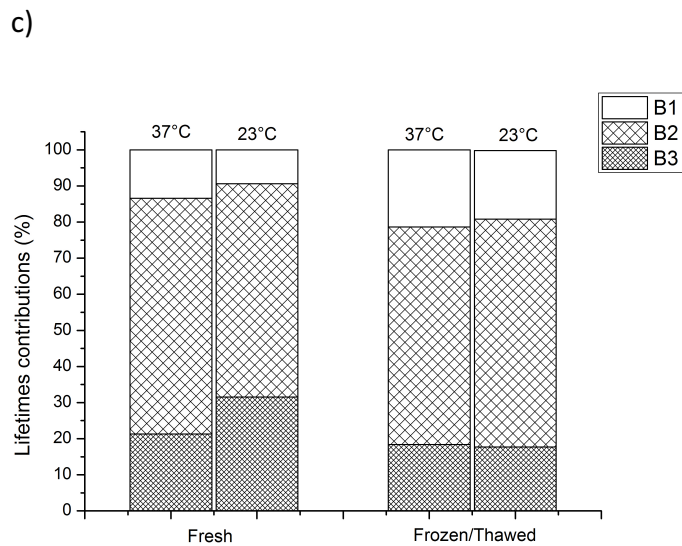
686 **Figure 2 : Time-Resolved Fluorescence Spectroscopy (T2 and T3) in fresh and frozen:thawed**  
687 **spermatozoa at 23<sup>0</sup>C and 37<sup>0</sup>C (2a and 2b)**

688 **Weights B1, B2 and B3 of T1, T2 and T3 respectively in fresh and frozen/thawed spermatozoa**  
689 **at 37<sup>0</sup>C and 23<sup>0</sup>C, 2c**

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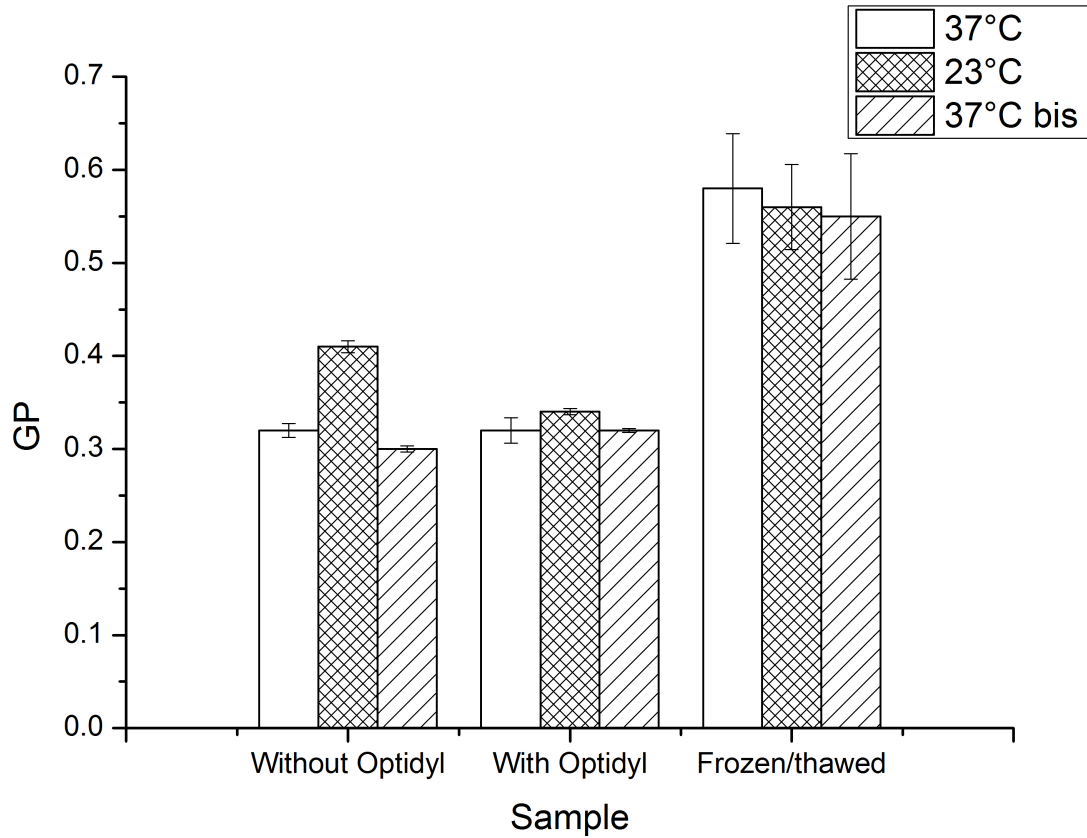


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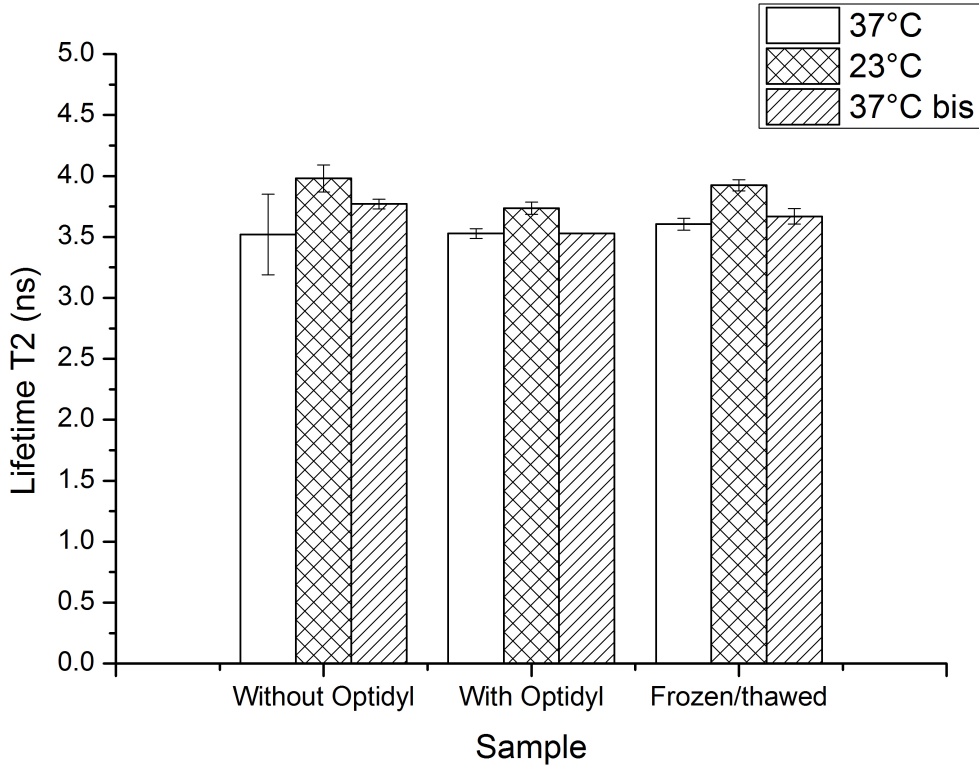
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711 **Figure 3** : Effect of Optidyl on GP. GP was measured on spermatozoa incubated first at 37°C,  
712 then at 23°C and again at 37°C. (37°C bis). For comparison, results obtained on frozen/thawed  
713 spermatozoa were presented.  
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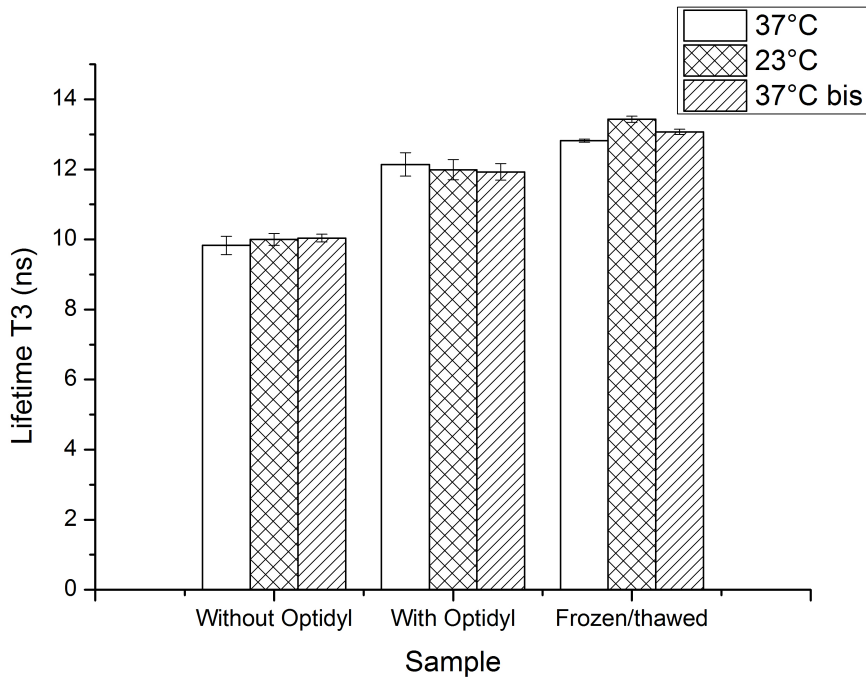


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737 **Figure 4** : Effect of Optidyl on lifetimes (T2 and T3) and B weights on fresh spermatozoa. T and  
 738 B were measured on spermatozoa incubated first at 37°C, then at 23°C and again at 37°C.  
 739 (37°C bis). For comparison, results obtained on frozen/thawed spermatozoa were presented.  
 740  
 741 a)



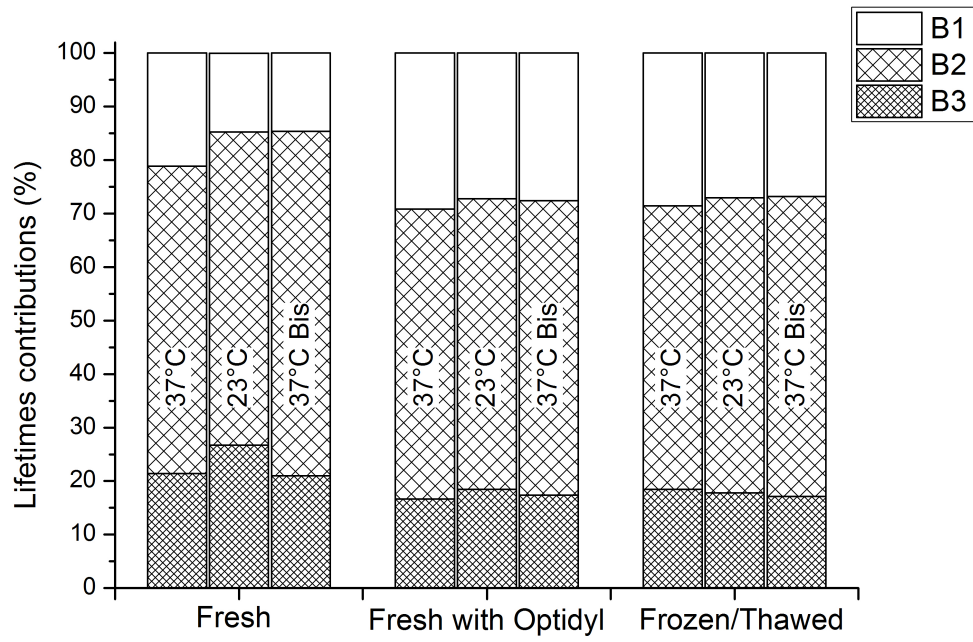
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772 Table 1: table presenting T values  $\pm$  SD in fresh versus frozen/thawed spermatozoa at 37<sup>0</sup>C and 23<sup>0</sup>C

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	Fresh				Frozen/Thawed				P-values fresh vs Frozen/Thawed		P-values 37°C vs 23°C	
	37°C		23°C		37°C		23°C		37°C	23°C	Fresh	Frozen/Thawed
	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation				
T1 (ns)	0,7	0,3	0,8	0,3	0,6	0,2	0,7	0,2	0,748	0,470	0,589	0,377
T2 (ns)	3.7*	0,3	4.07*	0,2	3.6**	0,2	3.9**	0,1	0,423	0,229	0,052	0,020
T3 (ns)	9,2	2,2	8,9	1,4	12,5	0,4	12,8	0,5	0,015	0,005	0,937	0,470
B1 (%)	13,4	8,4	9,4	4,5	21,3	6,1	19,0	6,8	0,093	0,030	0,485	0,470
B2 (%)	65,3	8,7	59,0	7,3	60,3	7,4	63,3	7,8	0,310	0,485	0,240	0,485
B3 (%)	21.3*	7,7	31.6*	9,8	18,4	2,3	17,7	2,3	0,173	0,002	0,041	0,688
GP	0.3*	0,02	0.4*	0,03	0.51*	0,09	0,53	0,08	0,005	0,045	0,005	0,334

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