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

Shaliha Bechoua, Pascale Winckler, Audrey Jossier, Caroline Peltier, Frédéric Delize, Noémie Devaux, Jean-Marie Perrier-Cornet, Hélène Simonin

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

Authors : Shaliha Bechoua*[1,2] ; Pascale Winckler*[1,3] ; Audrey Jossier[1] ; Caroline Peltier [1]; Frédéric Delize [4] ; Noémie Devaux [4]; Jean Marie Perrier Cornet [1,3] ; Hélène Simonin[1]

*The authors contributed equally to this work

[1] Univ Bourgogne Franche-Comte, Agrosup Dijon, PAM UMR A02.2012, F21000 Dijon, France
[2] CRB Ferdinand Cabanne, CHU Dijon Bourgogne, Dijon, France
[3] Univ Bourgogne Franche-Comte, Agrosup Dijon, PAM UMR A02.2012, DimaCell Imaging Facility, F21000 Dijon, France
[4] Elva Novia, Fontaines, 71150 France

Corresponding author:
shaliha.bechoua@chu-dijon.fr

Key words: slow freezing, membrane, bull sperm, GP, Time-resolved Fluorescence

Summary statement: GP and Time-resolved Fluorescence can be used as innovative tools to assess membrane fluidity after slow freezing

Abstract:

During slow freezing, spermatozoa undergo membrane alterations that compromise their ability of fertilizing. These alterations are cause either by cold shock or by the use of cryoprotectants known to be cytotoxic. However, little is known about the membrane changes that occurred during freezing. Here, we combined Generalized Polarization (GP), Time-resolved Fluorescence and laurdan fluorescence properties to investigate the changes in membrane fluidity and dynamics during slow freezing of bull sperm. We successfully demonstrated that laurdan may be distributed in three different local environments that correspond to different membrane lipid composition. These environments wont behave the same way when the cells will be subjected to either a chemical treatment (adding the cryoprotectants) or a physical treatment (freezing).

Introduction

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

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

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

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

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

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

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

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

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

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

Materials and Methods

Animals, semen collection and semen processing

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

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

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

Sperm preparation for slow freezing and thawing

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

Laurdan Labeling of sperm cells

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

penetration into the bilayer surface). As the tighter packing of lipids in ordered compared to disordered phases leads to less accessibility to water molecules, polarity is also an indicator of lipid order. Polarity changes are shown by shifts in the Laurdan emission spectrum, which are quantified by calculating the generalized polarization (GP): GP = (I440-I490) / (I440 + I490) where I440 and I490 are the fluorescence emission intensities at 440 and 490 nm respectively. Higher GP values are attributed to a more ordered lipid environment (sub-band at 440 nm), lower GP values to a more disordered one (sub-band at 490 nm), with more membrane hydration.

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

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

Incubation of fresh semen samples with Optidyl

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

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

GP and lifetimes were measured at both 37°C and 23°C. The cell suspensions that were refrigerated from 37^0C to 23^0C were warmed from 23^0C to 37^0C.

Swim-up procedure

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

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

Generalized Polarization using spectroscopy

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

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

$$GP = (I440-I490) / (I440 + I490)$$

where I440 and I490 are the fluorescence emission intensities at 440 and 490 nm respectively. Experiments were conducted both at 37°C and 23°C.

Time-Resolved Fluorescence Spectroscopy

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

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

Laurdan Fluorescence lifetimes were measured using a time-correlated single-photon-counting (TCSPC) system on a FluoroMax-4 spectrofluorometer (HORIBA Jobin Yvon). Fluorescence lifetime decay curves were measured with a pulsed 370-nm diode. Detection was set to 465 nm with a 6 nm bandpass. The lifetime was recorded on a 100 ns scale and measurements were stopped when peak signal reached 10 000 counts. The Instrument Response Function (IRF) was obtained using the diffusion of the excitation light on the sample at 370 nm.

DAS6 v6.1 software was used for TCSPC histogram analysis. As large deviation of chi-squared values from unity (chi-squared > 1.5) occurs when using mono or bi-exponential adjustments, data fitting was established using a three-exponential decay equation. This provides three fluorescent lifetimes with the corresponding fractional contributions.

For this experiment 3ml of a suspension of labeled spermatozoa were used. Experiments were conducted both at 37°C and 23°C.

Statistical analyses

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

The same tests were also computed to detect potential differences between 23^0C and 37^0C.

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

Results

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

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

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

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

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

Time-Resolved Fluorescence spectroscopy

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

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

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

T2 lifetime : temperature dependent correlation of membrane order and dynamics

Whatever the state of spermatozoa concerned (fresh and frozen/thawed spermatozoa), a significant increase of T2 lifetimes when spermatozoa were subjected to a variation of temperature (from 37^{O}C to 23^{O}C) was observed (fresh spermatozoa: 3.7 ± 0.3 and 4.07 ± 0.2 at 37^{O}C and 23^{O}C respectively; p=0.052 and frozen / thawed: 3.6 ± 0.2 and 3.9± 0.1 at 37^{O}C and 23^{O}C respectively; p=0.02); see Table 1; Figure 2a

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

T3 lifetime : treatment dependent correlation of membrane order and dynamics

A treatment dependent change (significant increase) of lifetimes were observed at 37^{O}C and 23^{O}C when comparing fresh versus frozen / thawed spermatozoa at 37 ^{O}C (9.2 ± 2.2 *versus* 12.5 ± 0.4, p= 0.015, % increase of 35.9%) and fresh versus frozen / thawed spermatozoa at 23 ^{O}C (8.9 ± 1.4 *versus* 12.8 ± 0.5, p=0.005; % increase of 43.8%). See Table 1, Figure 2b.

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

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

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

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

Effect of Optidyl on fresh spermatozoa GP and lifetimes

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

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

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

Regarding T2, the behavior was the same for the three types of spermatozoa, fresh, fresh incubated with Optidyl and frozen-thawed. As already observed for the fresh and frozen-thawed spermatozoa a significant increase of T2 was observed with temperature decrease to 23°C for spermatozoa incubated with Optidyl (fresh without optidyl 37 °C: 3.52, *versus* Fresh without optidyl 23 °C: 3.98) (fresh with optidyl at 37°C: 3.53 *versus* with optidyl at 23°C: 3.74). Furthermore, the T2 variation was reversible with warming again to 37°C; see Figure 4a.
The measurement of T3 gave interesting results as the T3 value increases for the fresh (9.83 at 37°C), incubated with Optidyl (12.14 at 37°C) and frozen-thawed spermatozoa (12.82 at 37°C) respectively. T3 was independent of temperature variation for fresh spermatozoa and the ones incubated with Optidyl; see Figure 4b.

Discussion

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

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

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

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

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

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

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

Interestingly, the cryoprotectant mixture Optidyl does not increase the GP values however it prevents the increase of GP observed during a temperature change.
The extender used for semen dilution (Optidyl) maintains the biological environment necessary for survival of spermatozoa and prevents membrane damages during cryopreservation. Egg yolk (rich in phosphatidylcholine) in the semen extender is thought to regulate the efflux of integral protein, phospholipids and cholesterol, and thus protects the plasma membrane against temperature-related injury [14].
Studies conducted over the past two decades by Bergeron et al. [8] have revealed that a family of lipid- binding proteins (BSP proteins) present in bull seminal plasma is detrimental to sperm preservation as they induce cholesterol and phospholipid depletion from the plasma membrane. Interestingly, low- density lipoproteins (LDL) present in egg yolk interact with the BSP proteins, minimizing lipid removal from the sperm membrane. This phenomenom will influence sperm storage in liquid or frozen states.

In the present work, we found that lifetime decay correlates well with a three exponential model, meaning that laurdan molecules may be distributed in three different local environments. We can argue that laurdan could be distributed in the three environments anterior acrosomal region, posterior acrosomal region and equatorial segment, and that each environment corresponds to a specific lifetime value. This difference in lifetimes values (T1: 0.7; T2: 3.7; T3: 9.2) is presumably a reflection of the different lipid composition of the membranes in spermatozoa.
In particular, cholesterol in spermatozoa plays a crucial role in the biological processes that occur during the fertilization process. Hence, the variation in cholesterol/phospholipids ratio has been linked to the ability to survive cryopreservation [12].
It has been shown by freeze fracture [31] that cholesterol is densely packed in the anterior acrosomal region as well as in the postacrosomal region while the equatorial region contains very low level of cholesterol.

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

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

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

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

-anterior acrosomal region (apical ridge)

-equatorial segment

-posterior acrosomal region

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

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

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

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

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

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

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

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

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

Acknowledgments

We would like to thank the cooperative Elva Novia (Fontaines, FRANCE) for proving us in bull sperm and also the Regional Council of Bourgogne – Franche Comté and the "Fonds Européen de DEveloppement Régional (FEDER) for funding this project.

Competiting interests

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

Funding

This work was supported by the Regional Council of Bourgogne – Franche Comté, the "Fonds Européen de DEveloppement Régional (FEDER)"

References

[1] V. Ahnonkitpanit et al., « Role of egg sulfolipidimmobilizing protein 1 on mouse sperm-egg plasma membrane binding », Biol. Reprod., 61 (1999) 749-756

[2] M. Amaro, R. Šachl, P. Jurkiewicz, A. Coutinho, M. Prieto, et M. Hof, « Time-Resolved Fluorescence in Lipid Bilayers: Selected Applications and Advantages over Steady State », *Biophys. J.*, 107 (2014) 2751‑2760

[3] M. Amaro, F. Reina, M. Hof, C. Eggeling, et E. Sezgin, « Laurdan and Di-4-ANEPPDHQ probe different properties of the membrane », *J. Phys. Appl. Phys.*, 50 (2017), 134004

[4] A. Ambrosini, G. Zolese, G. Balercia, E. Bertoli, G. Arnaldi, et F. Mantero, « Laurdan fluorescence: a simple method to evaluate sperm plasma membrane alterations », *Fertil. Steril.*, 76 (2001) 501‑505

[5] L. A. Bagatolli, T. Parasassi, G. D. Fidelio, et E. Gratton, « A model for the interaction of 6-lauroyl-2-(N,N-dimethylamino)naphthalene with lipid environments: implications for spectral properties », *Photochem. Photobiol.*, 70 (1999) 557‑564

[6] L. A. Bagatolli, « Monitoring Membrane Hydration with 2-(Dimethylamino)-6-Acylnaphtalenes Fluorescent Probes », *Subcell. Biochem.*, 71 (2015), 105‑125

[7] F. Batellier *et al.*, « Advances in cooled semen technology », *Anim. Reprod. Sci.*, 68 (2001) 181‑190

[8] A. Bergeron et P. Manjunath, « New insights towards understanding the mechanisms of sperm protection by egg yolk and milk », *Mol. Reprod. Dev.*, 73 (2006). 1338‑1344

[9] S. Büyükleblebici *et al.*, « Cryopreservation of bull sperm: Effects of extender supplemented with different cryoprotectants and antioxidants on sperm motility, antioxidant capacity and fertility results », *Anim. Reprod. Sci.*, 150 (2014) 77‑83

[10] N. Cormier, M. A. Sirard, et J. L. Bailey, « Premature capacitation of bovine spermatozoa is initiated by cryopreservation », *J. Androl.*, 18 (1997) 461‑468

[11] N. Cormier et J. L. Bailey, « A differential mechanism is involved during heparin- and cryopreservation-induced capacitation of bovine spermatozoa », *Biol. Reprod.*, 69 (2003) 177‑185

[12] A. Darin-Bennett et I. G. White, « Influence of the cholesterol content of mammalian spermatozoa on susceptibility to cold-shock », *Cryobiology*, vol. 14 (1977) 466‑470,

[13] D. J. Ellis et al., « Post-testicular development of a novel membrane substructure within the equatorial segment of ram, bull, boar, and goat spermatozoa as viewed by atomic force microscopy », Journal of Structural Biology, 138 (2002) 187-198

[14] M. Forouzanfar *et al.*, « In vitro comparison of egg yolk-based and soybean lecithin-based

extenders for cryopreservation of ram semen », *Theriogenology*, 73 (2010), 480‑487

[15] H. M. Harshan, L. P. Singh, A. Arangasamy, M. R. Ansari, et S. Kumar, « Effect of buffalo seminal plasma heparin binding protein (HBP) on freezability and in vitro fertility of buffalo cauda spermatozoa », *Anim. Reprod. Sci.*, 93 (2006) 124‑133

[16] H. C. Ishikawa-Ankerhold, R. Ankerhold, et G. P. C. Drummen, « Advanced fluorescence microscopy techniques--FRAP, FLIP, FLAP, FRET and FLIM », *Mol. Basel Switz.*, 17 (2012) 4047-4132

[17] M. B. Khalil *et al.*, « Sperm capacitation induces an increase in lipid rafts having zona pellucida binding ability and containing sulfogalactosylglycerolipid », *Developmental Biology*, vol. 290, n° 1, p. 220-235, févr. 2006.

[18] E. de Lamirande, C. Tsai, A. Harakat, et C. Gagnon, « Involvement of reactive oxygen species in human sperm arcosome reaction induced by A23187, lysophosphatidylcholine, and biological fluid ultrafiltrates », *J. Androl.*, 19 (1998) 585‑594

[19] C. M. O. Medeiros, F. Forell, A. T. D. Oliveira, et J. L. Rodrigues, « Current status of sperm cryopreservation: why isn't it better? », *Theriogenology*, 57 (2002) 327‑344,.

[20] G. J. Morris, K. Faszer, J. E. Green, D. Draper, B. W. W. Grout, et F. Fonseca, « Rapidly cooled horse spermatozoa: loss of viability is due to osmotic imbalance during thawing, not intracellular ice formation », *Theriogenology*, 68 (2007) 804‑812

[21] L. Olexikova, M. Miranda, B. Kulikova, A. Baláži, et P. Chrenek, « Cryodamage of plasma membrane and acrosome region in chicken sperm », *Anat. Histol. Embryol.*, vol. 48 (2019) 33‑39

[22] D. M. Owen, A. Magenau, D. Williamson, et K. Gaus, « The lipid raft hypothesis revisited – New insights on raft composition and function from super-resolution fluorescence microscopy », *BioEssays*, 34 (2012) 739‑747

[23] S. Palleschi et L. Silvestroni, « Laurdan fluorescence spectroscopy reveals a single liquid-crystalline lipid phase and lack of thermotropic phase transitions in the plasma membrane of living human sperm », *Biochim. Biophys. Acta BBA - Biomembr.*, vol. 1279 (996) 197‑202

[24] T. Parasassi, E. K. Krasnowska, L. Bagatolli, et E. Gratton, « Laurdan and Prodan as Polarity-Sensitive Fluorescent Membrane Probes », *J. Fluoresc.*, 8 (1998) 365‑373

[25] T. Parasassi et E. Gratton, « Membrane lipid domains and dynamics as detected by Laurdan fluorescence », *J. Fluoresc.*, 5 (1995) 59‑69

[26] D. E. Pegg, « Principles of cryopreservation », *Methods Mol. Biol. Clifton NJ*, vol. 1257 (2015) 3‑19

[27] J. A. Rooke, C. C. Shao, et B. K. Speake, « Effects of feeding tuna oil on the lipid composition of pig spermatozoa and in vitro characteristics of semen », *Reprod. Camb.*

Engl., 12 (2001) 315‑322

[28] S. A. Sanchez, M. A. Tricerri, et E. Gratton, « Laurdan generalized polarization fluctuations measures membrane packing micro-heterogeneity in vivo », *Proc. Natl. Acad. Sci. U. S. A.*, 109 (2012) 7314‑7319

[29] H. Sieme, H. Oldenhof, et W. Wolkers, « Sperm Membrane Behaviour during Cooling and Cryopreservation », *Reprod. Domest. Anim.*, 50 (2015) 20‑26

[30] C. D. Stubbs et A. D. Smith, « The modification of mammalian membrane polyunsaturated fatty acid composition in relation to membrane fluidity and function », *Biochim. Biophys. Acta*, 779 (1984) 89‑137

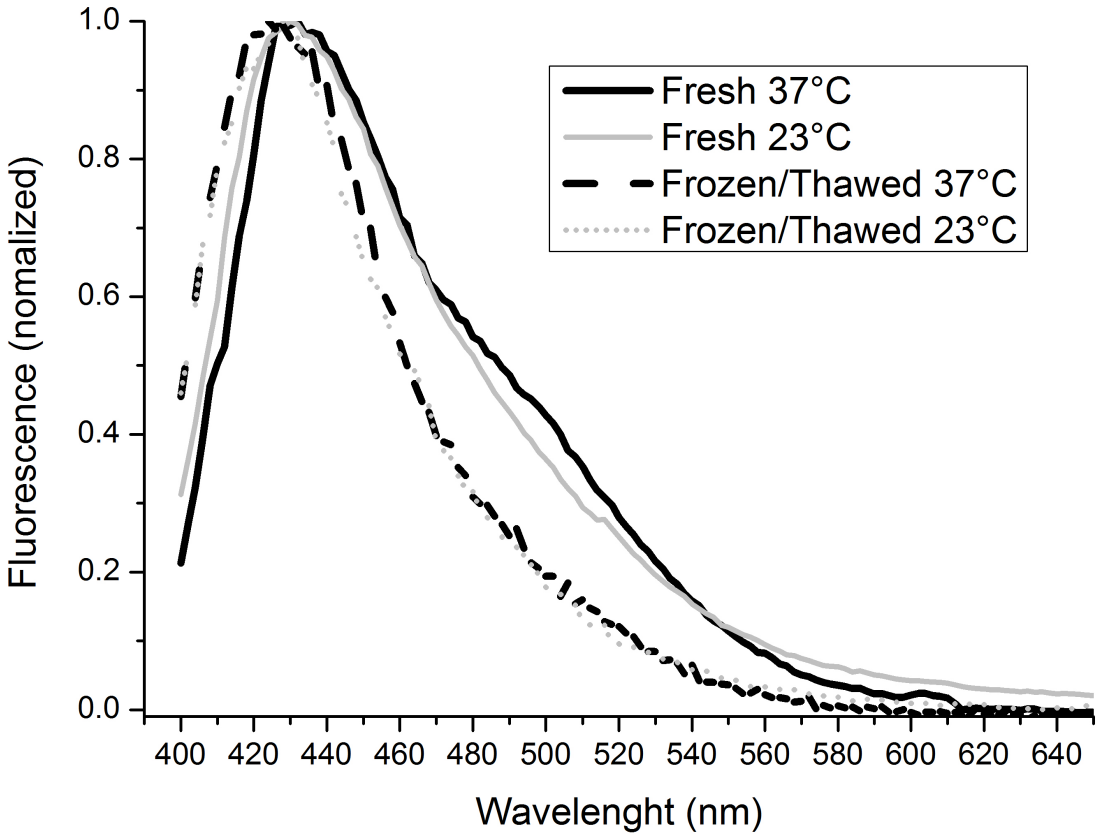
[31] J. Tesařík et J.-E. Fléchon, « Distribution of sterols and anionic lipids in human sperm plasma membrane: Effects of in vitro capacitation », *J. Ultrastruct. Mol. Struct. Res.*, 97 (1986) 227‑237

[32] A. I. S. B. Villaverde *et al.*, « Cryoprotective effect of different glycerol concentrations on domestic cat spermatozoa », *Theriogenology*, 80(2013) 730‑737

[33] S. R. Wassall et W. Stillwell, « Polyunsaturated fatty acid-cholesterol interactions: domain formation in membranes », *Biochim. Biophys. Acta*, 1788 (2009) 24‑32

[34] P. F. Watson, « The causes of reduced fertility with cryopreserved semen », *Anim. Reprod. Sci.*, vol. 60‑61, n° Supplement C, (2000) 481‑492

Figure 1 : Representative normalized steady-state emission spectra of Laurdan labelled fresh sperm cells and frozen/thawed sperm cells. GP measurements were conducted both at 37^{O}C and 23 ^{O}C



GP images of spermatozoa labelled with Laurdan using a false color scale

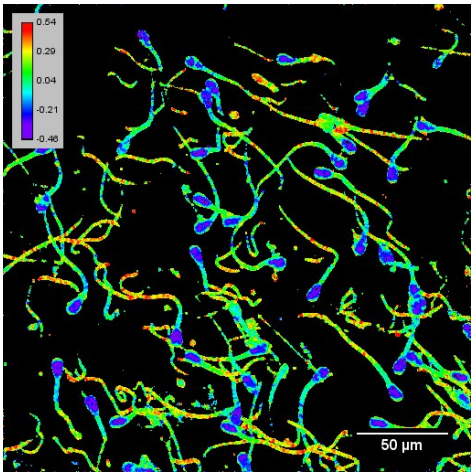
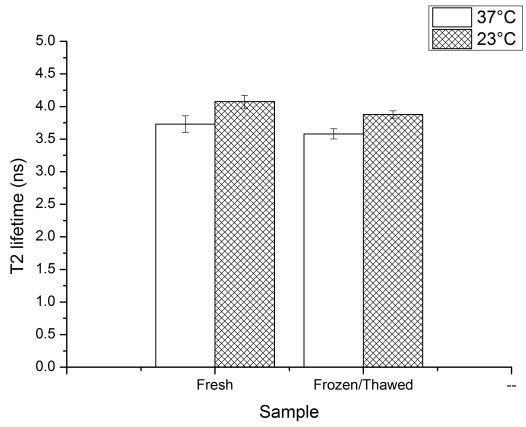


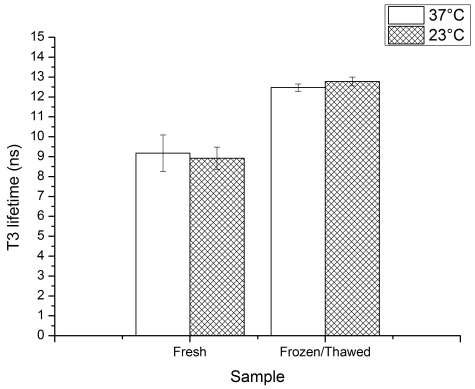
Figure 2 : Time-Resolved Fluorescence Spectroscopy (T2 and T3) in fresh and frozen:thawed spermatozoa at 23^0C and 37^0C (2a and 2b)

Weights B1, B2 and B3 of T1, T2 and T3 respectively in fresh and frozen/thawed spermatozoa at 37^0C and 23^0C, 2c

a)



b)



c)

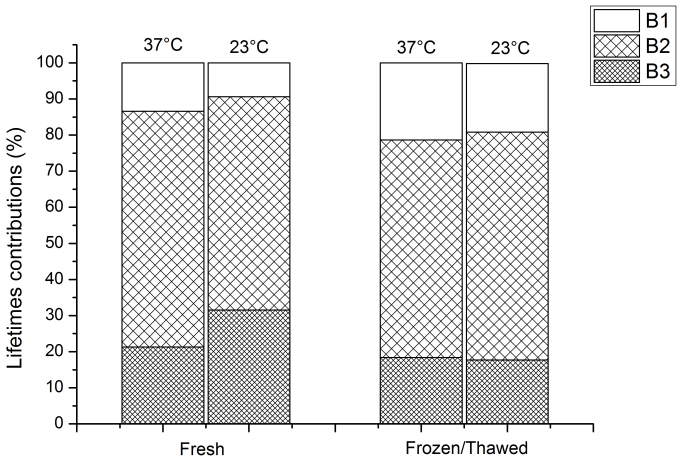


Figure 3 : Effect of Optidyl on GP. GP was measured on spermatozoa incubated first at 37^{O}C, then at 23^{O}C and again at 37^{O}C. (37^{O}C bis). For comparison, results obtained on frozen/thawed spermatozoa were presented.

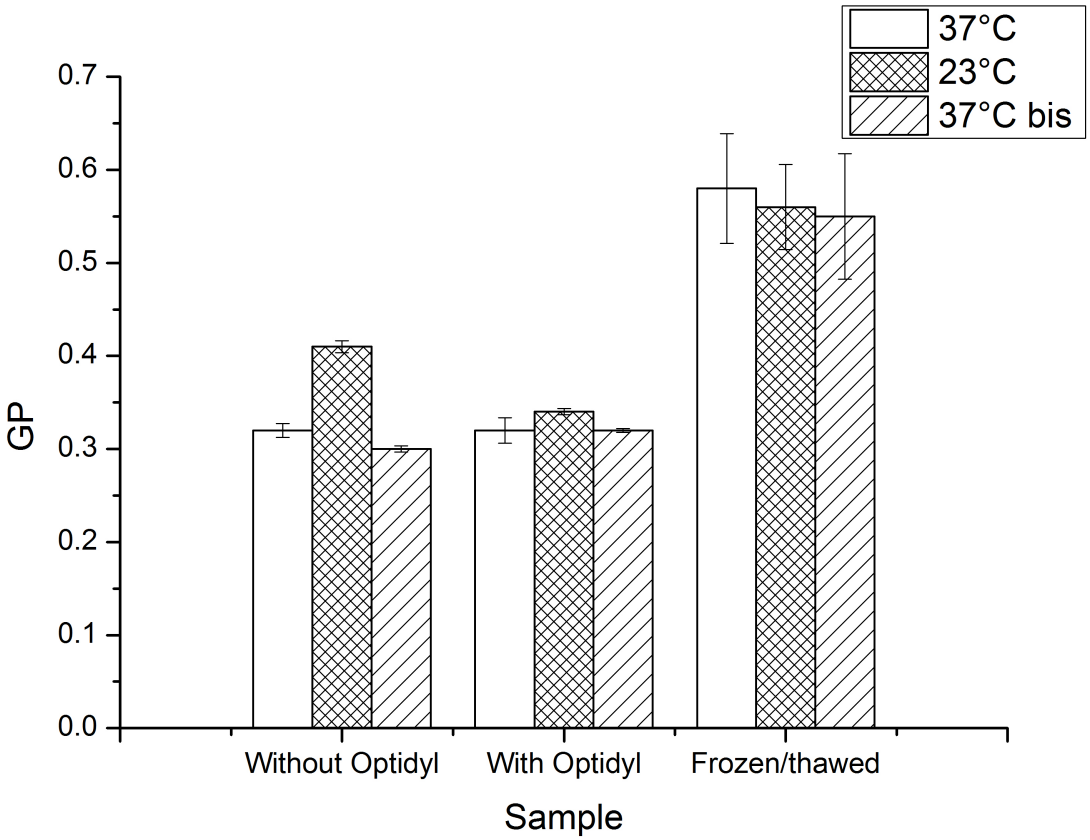
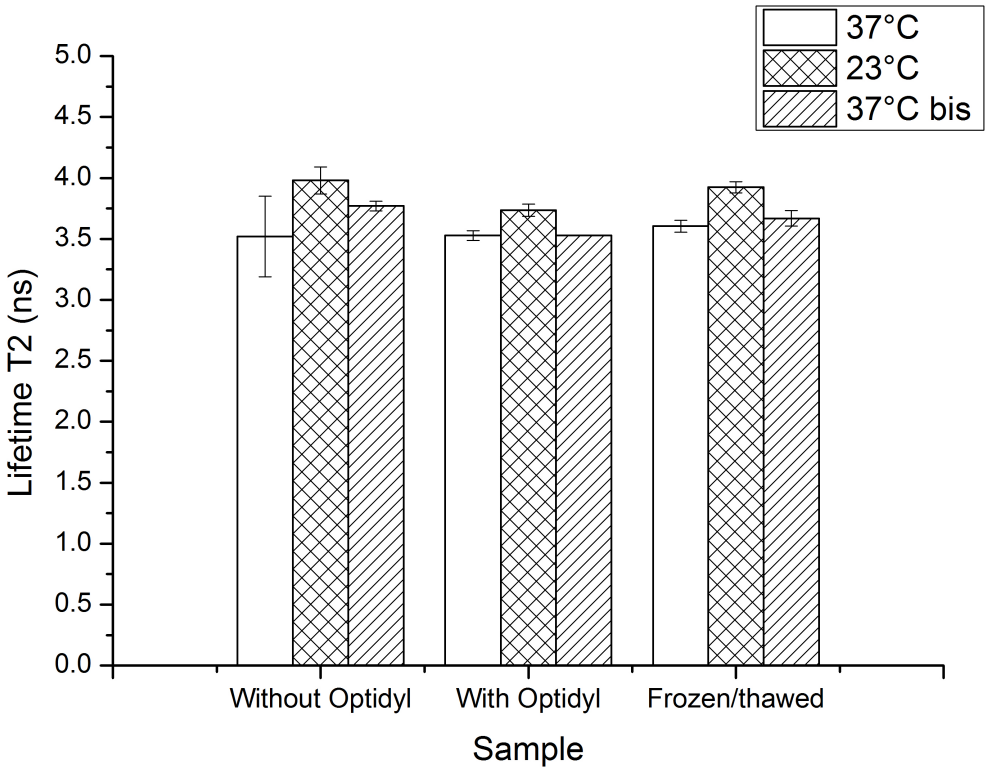
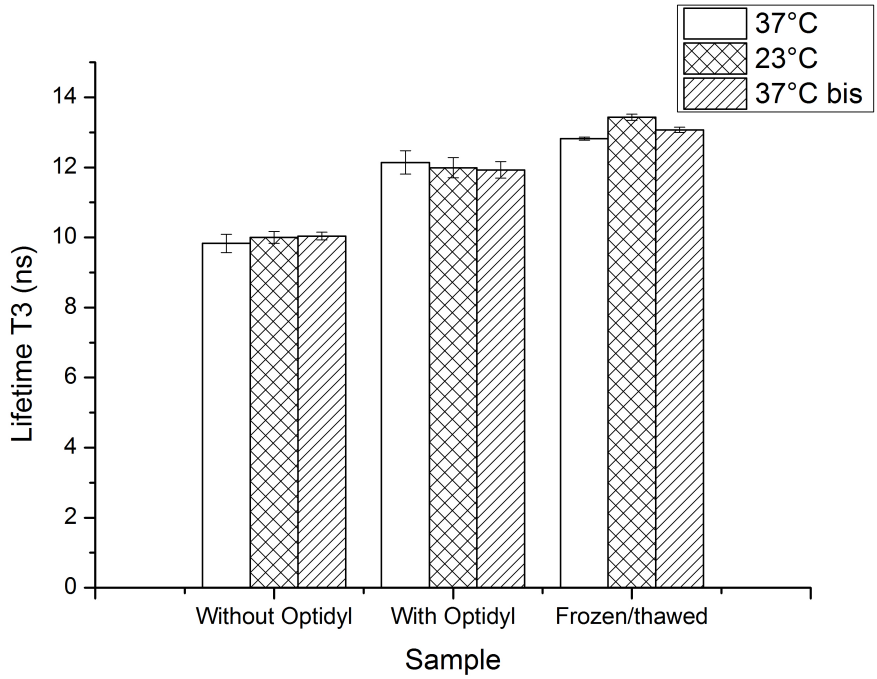


Figure 4 : Effect of Optidyl on lifetimes (T2 and T3) and B weights on fresh spermatozoa. T and B were measured on spermatozoa incubated first at 37°C, then at 23°C and again at 37°C. (37°C bis). For comparison, results obtained on frozen/thawed spermatozoa were presented.

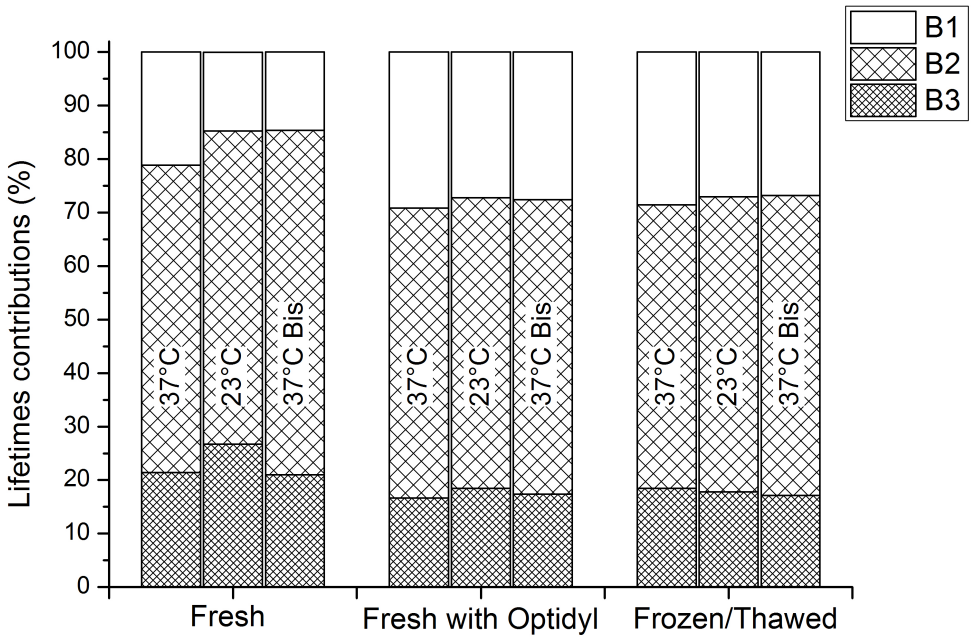
a)



b)



c)


B1
B2
B3
Lifetimes contributions (%)
100
90
80
70
60
50
40
30
20
10
0
37°C
23°C
37°C Bis
37°C
23°C
37°C Bis
37°C
23°C
37°C Bis
Fresh
Fresh with Optidyl
Frozen/Thawed

772 Table 1: table presenting T values ± SD in fresh versus frozen/thawed spermatozoa at 37^0C and 23^0C

773

774

	Fresh				Frozen/Thawed				P-values fresh vs Frozen/Thawed		P-values 37°C vs 23°C	
	37°C		23°C		37°C		23°C					
	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation	37°C	23°C	Fresh	Frozen/Thawed
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

776