REPRODUCTION
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Membrane fluidity and the ability of domestic bird spermatozoa to survive cryopreservation

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Abstract

The ability to survive cryopreservation varies in spermatozoa from different bird species. Among the biological factors potentially responsible for such differences, species variations in membrane fluidity have a role in the restoration of the physiological state after freezing. Membrane fluidity may be assessed by measuring fluorescence polarization anisotropy with a fluorescent dye. Anistropy values are proportional to membrane rigidity and consequently inversely proportional to membrane fluidity. In the present study, polarization anisotropy of spermatozoa originating from species differing in the freezability of their semen (chicken, turkey and guinea fowl) was measured in addition to lipid composition (cholesterol/phospholipid ratio), sperm viability (membrane permeability to eosine) and morphological integrity before and after cryopreservation.
The percentages of viable and normal spermatozoa in fresh sperm were highest in the chicken (87%), lowest in guinea fowl (64%), and intermediate in turkeys (69%). Anisotropy values were highest in guinea fowl (0.205), lowest in chickens (0.155), and intermediate in turkeys (0.180). As a consequence, membrane fluidity was highest in chickens and lowest in guinea fowl. Cryopreservation significantly decreased sperm viability and morphological integrity and increased anisotropy in all species but did not change the inter species hierarchy. Initial cholesterol/phospholipid ratios were lower in chickens than in guinea fowl, and intermediate in turkeys (0.25, 0.26 and 0.29, respectively). Cryopreservation induced a severe decrease in cholesterol/phospholipid ratios in turkeys and guinea fowl.
Sperm membrane fluidity in chickens, turkeys and guinea fowl behaves as an indicator of sperm freezability in these species. Inter species differences for this parameter may be partly explained by differences in initial cholesterol/phospholipids content of spermatozoa. On the other hand, the rigidifying process induced by cryopreservation is not related to lipid damage by the same mechanisms.
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Introduction

Despite its long-proven value for the optimization of male genetic potential, semen preservation in domestic avian species is only rarely used in breeding practice. One of the main reasons is that the success of freezing procedures is highly variable, depending, among other factors, on species and line specificity (reviewed by Hammerstedt 1995, Etches 1996, Massip *et al.* 2004).

However, in the last few years there has been renewed interest in the development of these methods in species where the management of genetic variability has become critical. Indeed, half of the domestic bird species lines are now threatened (Dohner 2001), and massive epidemics of avian influenza in the past few years have emphasized the urgent need for *ex situ* management in domestic bird species. Due to the difficulties of freezing and transferring avian embryos to recipient eggs, alternatives to semen cryopreservation based on long-term storage of embryos are not yet available. Furthermore, the long-term preservation of other reproductive cells (especially primordial germ cells) is being studied in different research laboratories but is not yet considered to be fully appropriate to the *ex situ* management of genetic resources (reviewed by Tajima 2002). Semen cryopreservation is, therefore, the only effective method of *ex situ* management currently available for domestic birds.

One striking observation in domestic birds is that, despite the very similar morphological shapes and ultrastructures of spermatozoa, the freezability of these cells appears to be very different between species, semen freezing being more effective in chickens and ganders than in most other domestic birds (reviewed by Blesbois & Labbé 2003, Massip *et al.* 2004).

Many biological and biophysical factors may affect the ability of sperm cells to prevent the damage caused by freezing–thawing procedures (reviewed by Holt 2000)

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and factors involved in the resistance of the plasma membrane to thermal and osmotic changes appear to be essential. These factors include membrane permeability, lipid composition and fluidity. Inter species variations in spermatozoon membrane permeability have been shown to occur in birds under different osmotic conditions (Blanco *et al.* 2000). Intra-species variations in cholesterol/phospholipids content have also been related to semen freezability in chickens (Ansah & Buckland 1982). However, sperm membrane fluidity that contributes to the restoration of the physiological state of the membrane after freezing has never, to our knowledge, been studied in domestic birds. Membrane fluidity is a submacroscopic approach to the dynamic state of the sperm membrane (Shinitzky & Yuli 1982) that is linked to lipid and protein composition of the membrane. Membrane fluidity may be assessed by measuring the fluorescence polarization anisotropy of sperm membranes with 1,6-diphenyl-1,3,5-hexatriene (DPH), a fluorescent dye inserted in the lipid fraction of plasma membranes, and it has been shown to be a reliable predictor of sperm ability to resist freezing–thawing damage in humans (Giraud *et al.* 2000).

The aim of the present study was to assess sperm membrane fluidity and cholesterol/phospholipid ratios with regard to inter species differences in freezability in domestic birds to provide greater understanding of responses to cryopreservation. Three domestic bird species known to differ widely in the freezability of their semen were chosen: the chicken ('high' freezability), the turkey ('moderate' freezability) and the guinea fowl ('low' freezeability). Membrane fluidity and cholesterol/phospholipid ratios of these three species were measured on fresh and frozen–thawed spermatozoa and compared with viability (membrane permeability by eosine) and morphological integrity. A method of freezing of guinea fowl spermatozoa was also adapted for this purpose.

Materials and Methods

Animals

The chickens used were 48 adult broiler males of the Géline de Touraine breed raised in individual battery cages under a 14L: 10D photoperiod and fed a commercial diet containing 12.4 MJ metabolizable energy/Kg.

The turkeys comprised of 40 turkey males of the BUT Big 6 breed (BUT France, Rennes, France) raised in floor pens under a 14L:10 D photoperiod and fed a commercial diet containing 12.4 MJ metabolizable energy/Kg.

The guinea fowl males used were 48 adult Galor males (Galor, Amboise, France) raised in individual battery cages under a 14L: 10 D photoperiod and fed a commercial diet containing 12.4 MJ metabolizable energy/Kg.

Animal experiments were carried out in accordance with ethical guidelines set out by INRA.

Semen collection and treatment

Semen was collected routinely three times a week by dorso-abdominal massage in each species (Burrows & Quinn 1936). Care was taken to avoid any contamination of semen with cloacal products. Yellow and abnormal semen samples were systematically discarded. For each species, eight to nine pools of semen (each pool containing the semen of three different males) were either diluted (1:2) in a saline-glutamate diluent for further analysis of membrane fluidity, viability and morphological integrity and lipid analysis of fresh semen, or diluted in the freezing diluent for the cryopreservation process (following paragraph). The saline-glutamate diluent was Beltsville Poultry Semen Extender, BPSE (pH adjusted to 7.2; Sexton 1982).

Freezing methods

The method of freezing used was the method giving the highest levels of fertility for each of the species studied in our laboratory conditions. The mean percentages of fertility (% fertile eggs/incubated eggs) obtained with these methods in our laboratory conditions were 80% for chickens (Tselutin *et al.* 1999), 40% for turkeys (Blesbois & Grasseau 2001, Labbé *et al.* 2002) and 20% for guinea fowl (unpublished observations). These results were routinely obtained with intra vaginal insemination of fertile females of the corresponding species (a mean of 4 females per ejaculate), with successive twice a week inseminations and a dose of 6×10^6 spermatozoa/female/insemination. The semen diluents used for each of these methods were glutamate-based diluents containing phosphate buffers and sugars, with neutral ph and initial osmolarity between 350 and 400 mOsm.

Chicken semen

The freezing method employed was the method using the cryoprotectant dimethyl acetamide (DMA) described by Tselutin *et al.* (1999). Briefly, semen samples were diluted 1:2 in freezing diluent and then cooled to 4 °C before the addition of 6% DMA. Pellets of approximately 80 μl of cooled semen were then rapidly plunged in liquid nitrogen for freezing and cryopreservation. For the thawing procedure, semen pellets were quickly thawed on thermoregulated hotplates at 60 °C before use.

Turkey semen

The method used to freeze turkey semen was that described by Blesbois and Grasseau (2001) and Labbe *et al.* (2002) and was an adaptation of the method described for turkeys by Tselutin *et al.* (1995). Briefly, semen samples were diluted 1:4 in the original turkey diluent modified by the addition of NaOH 1 N and TES buffer (N-Tris–(hydroxymethyl)-methyl-2-amino-ethanesulfonic acid) to reach a neutral pH and an osmolarity of

380 mOsm. Samples were than cooled to 4 °C before the addition of 8% DMA. Semen pellets of approximately 80 μl were then frozen rapidly and stored in liquid nitrogen. Frozen pellets were quickly thawed on thermoregulated hotplates at 60 °C before use.

Guinea fowl semen

Fresh semen samples were diluted in IGGK diluent (Suraï & Wishart 1996) to reach the final concentration of 3×10^9 spermatozoa/ml. Because the use of DMA did not provide fertile samples after freezing–thawing following either the chicken or the turkey procedures used previously, or various adaptations of these methods (unpublished observations), samples of semen were then cooled to 4 °C before the addition of 6% dimethyl formamide (DMF). Semen freezing was not more successful with pellets than with straws in this species (unpublished observations). Semen was therefore packaged in 0.5 ml straws and frozen at 15 °C/min down to −145 °C in a programmable freezer (IMV, L'Aigle, France). Semen was then plunged in liquid nitrogen. Samples were plunged in a water bath at 50 °C for 5 sec before use as frozen–thawed semen.

Viability and morphological integrity of spermatozoa

Morphological integrity and viability of the spermatozoa from the three species were assessed on the same slides with eosine-nigrosin smears (Blom 1950). Briefly, 60 million previously diluted spermatozoa (see semen collection and treatment and freezing methods sections) were mixed with a dye composed of 1.6% eosine and 6% nigrosin diluted in BPSE (20 μl diluted semen in 2 ml dye solution) and incubated for 2 min before spreading on a slide, drying and observation under the microscope. Spermatozoa were considered to be viable and normal when they were not stained and when morphology was normal from head to tail. All other shapes were considered to be abnormal. Eosin-stained spermatozoa were considered to be dead. The proportions of dead, viable-abnormal cells and viable-normal spermatozoa were then evaluated under light microscopy (Zeiss Axioplan 2, Zeiss Gruppe, Jena, Germany) at × 600. Two slides were observed per sample for both techniques and 300 spermatozoa counted per slide. All evaluations were made by the same person.

Membrane fluidity

Membrane fluidity was assessed by measuring the fluorescence anisotropy with the fluorescent dye DPH inserted in the lipid fraction of the plasma membranes according to an adaptation of the method described by Giraud *et al.* (2000).

To avoid any diluent–seminal plasma interaction, fresh and frozen–thawed semen samples were centrifuged for 15 min at 700 *g* at room temperature. The supernatant was replaced by the same volume of BPSE diluent. After homogenisation, spermatozoa concentrations were estimated using a photometer (Jenway, Donmow, England) at a wavelength of 600 nm. The washed spermatozoa were then suspended at a concentration of 1×10^6 spermatozoa/ml in 3 ml PBS with DPH (10^{-6} mol/l, prepared from a DPH stock solution of 2×10^{-3} mmol/l in tetrahydrofurane). The suspension was incubated for 20 min at room temperature and then 10 min at the temperature chosen for the evaluation of anisotropy (40 °C for standardized evaluation because 40 °C is the usual body temperature in birds). The length of contact between the dye and the spermatozoa was chosen to be long enough to ensure stable insertion of the dye in the lipid bilayer of the plasma membrane of spermatozoa, and short enough to avoid any risk of 'internalisation' of the dye in the cytoplasm or mitochondrial fraction of the cells. The DPH/phospholipid molar ratio was lower than 1/2000 in order to minimize probe to probe interactions and probe-induced disturbance of the lipid bilayer. Sperm suspensions containing no DPH were similarly assessed to check light scattering. Analyses were performed with a spectrofluorimeter Quanta Master (PTI, Monmouth, Junction, New Jersey, USA) equipped with two motorised polarizers that allow instantaneous measurement of vertical and horizontal fluorescence. The samples were excited with vertically polarized light (365 nm). Emission (430 nm) was measured through the polarizer both parallel and perpendicular to the excitation polarizer. The parallel (Ipa) and perpendicular (Ipe) fluorescence intensities were recorded and permitted calculation of anisotropy (rf): $rf = (Ipa - Ipe)/(Ipa + 2Ipe)$. Anisotropy is directly proportional to membrane rigidity and consequently inversely proportional to fluidity, the lower the rf, the more fluid the membrane.

Anisotropy was also measured between 0 and 40 °C in order to observe the potential presence of a lipid transition phase during cooling before freezing. For this purpose, decreasing temperatures were obtained with a cooling water bath and analyses were performed with the same samples for all temperatures tested.

Relationship between the proportion of dead spermatozoa and membrane fluidity

Because of the possibility of a relationship between the proportion of dead spermatozoa and membrane rigidity (and consequently membrane fluidity), we investigated whether there was a direct relationship between these two parameters. Samples with 100% dead spermatozoa were prepared by rapid and successive freezing–thawing procedures without addition of any diluent or cryoprotectant. Known fractions of these 100% dead spermatozoan samples were added to normal samples of semen to obtain samples with percentages of dead spermatozoa between 4 and 100%. The results did not show any relationship between the proportion of dead

spermatozoa and anisotropy values (non-significant coefficient of correlation $r = -0,2$) and are not reported in the results section.

Lipid analysis

Aliquots of 350 µl of diluted semen were centrifuged (600 *g*, 15 min, 4 °C) to separate spermatozoa and seminal plasma. The pellets contained the spermatozoa. Lipids from this spermatozoon suspension were extracted with chloroform/methanol (v/v: 1/2) according to Bligh and Dyer (1959) and lipid classes were further analysed as described in Douard *et al.* (2000). Briefly, lipid classes were separated by thin-layer chromatography on silica rods. The migration conditions comprised 20 min in a solvent composed of hexane/ethyl-ether/acetic acid (v/v/v, 87/13/0.75). Detection and quantification were performed on an Iatroscan (Iatron, Tokyo, Japan).

Statistical analysis

Statistical analyses were performed with the Statview software (Abacus Concepts Inc. Berkeley, CA, USA). Changes in anisotropy, lipid components, viability and morphological integrity of spermatozoa were evaluated by one- to three-way analysis of variance according to the number of factors involved in each experiment, and then followed by the Fisher Protected Least Significant Difference Test.

The evolution of anisotropy with temperature and species was evaluated by analysis of covariance using the temperature effect. Temperature-dependent variations were parallel between species in the range 10–40 °C. The effect of species was then analysed as main effect.

Results

Effects of species and freezing on sperm viability, morphological integrity and membrane fluidity

Viability and morphological integrity of spermatozoa (Fig. 1a and b)

The percentages of dead and morphologically abnormal spermatozoa of fresh semen were lower in chickens than in turkeys and guinea fowl. Consequently, the percentages of viable and morphologically normal spermatozoa were significantly higher in chickens than in turkeys and guinea fowl (87 ± 2.1, 69 ± 5.0 and 64 ± 8.9% respectively).

Cryopreservation induced a highly significant decrease in viability and morphological integrity of spermatozoa in all species, with a particularly dramatic increase in the proportion of dead spermatozoa (multiplied by three in chicken, five in guinea fowl and seven in turkeys). However, the differences between species were increased by semen freezing. The significantly highest percentage of viable and morphologically normal spermatozoa in cryopreserved semen was observed in the chicken (32 ± 7.0%) and the lowest in the guinea fowl (13 ± 2.5%). Turkey semen gave intermediate results (25 ± 1.0%), which were significantly different from those observed in the chicken and guinea fowl. The percentage of dead spermatozoa was also higher in frozen–thawed spermatozoa of guinea fowl.

Membrane fluidity

Anisotropy values measured on fresh semen (Fig. 2) were significantly lower in the chicken (0.155 ± 0.016) than in the other two species ($P < 0.05$). Guinea fowl gave the

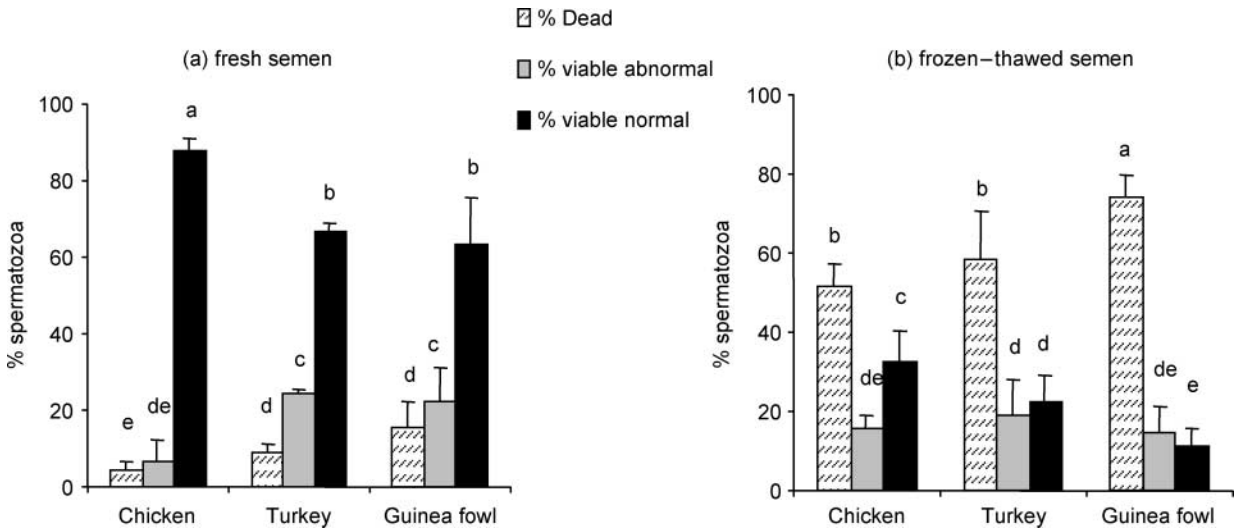

Figure 1 Effects of species and freezing on viability and morphologic integrity of chicken, turkey and guinea fowl spermatozoa. (a) dead, viable abnormal and viable morphologically normal fresh spermatozoa. (b) dead, viable abnormal and viable morphologically normal frozen–thawed spermatozoa. Results are expressed as means±S.E. $n = 9$; ^{a,b,c,d,e} indicate significant differences ($P < 0.05$).

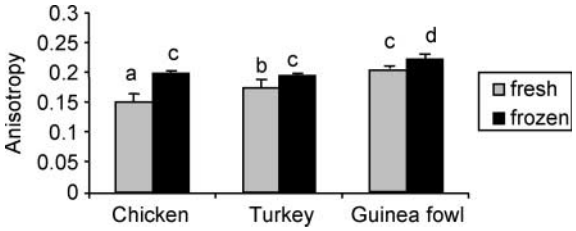

Figure 2 Effects of bird species and freezing on membrane fluidity of chicken, turkey and guinea fowl spermatozoa measured at 41 °C. Results are expressed as means±S.E. $n = 9$; ^{a,b,c,d} indicate significant differences ($P < 0.05$).

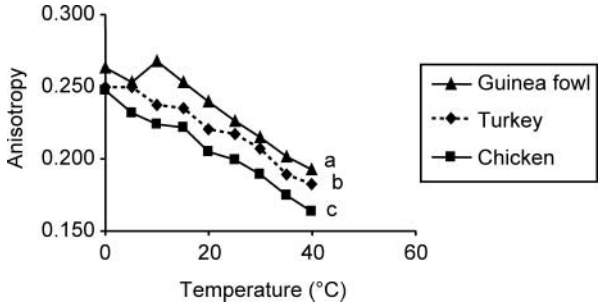

Figure 3 Effects of temperature on the evolution of membrane anisotropy of chicken, turkey and guinea fowl spermatozoa. $n = 6$; S.E. were between 0.007 and 0.016 anisotropy values; ^{a,b,c} indicate significant differences ($P < 0.05$).

highest values (0.205 ± 0.009) and turkeys showed intermediate results (0.180 ± 0.017), and these were significantly different from those obtained in the other two species ($P < 0.05$).

Consequently, the highest fluidity (the lowest anisotropy value or lowest membrane rigidity) was observed with chicken spermatozoa while the lowest fluidity (or highest rigidity) was observed with guinea fowl spermatozoa. Turkey spermatozoa showed intermediate membrane fluidity.

Cryopreservation of the gametes significantly increased anisotropy values (from 0.02 to 0.05, $P < 0.05$) and hence membrane rigidity in each species studied. Cryopreservation therefore lowered the membrane fluidity of spermatozoa in the three species studied.

Effects of temperature variations (from 0 to 40°C) on the evolution of membrane fluidity

Anisotropy increased progressively when the temperature decreased from 40 °C to 0 °C (Fig. 3). The increase in anisotropy was linear between 40 °C and 10 °C and parallel between species ($r = 0.96$). However, the anisotropy = f (temperature) curve showed significantly higher mean values for the guinea fowl than for the chicken, the turkey curve being intermediate and significantly different from the other two curves ($P < 0.01$). There was a slowdown in the increase in anisotropy/rigidity in the guinea fowl curve between 5 and 0 °C.

Effects of species and freezing on the lipid classes of chicken, turkey and guinea fowl spermatozoa

The lipid profiles of the spermatozoa of the three species studied were similar to each other (Fig. 4). Phospholipids were the major lipids (73–75% of total lipids), followed by cholesterol (20–25% of the total lipids), while cholesterol esters and triglycerides were very minor classes. However, there were differences between the three species in the proportions of cholesterol. As a consequence, the mean proportions of cholesterol/phospholipids were significantly higher in guinea fowl than in chickens (0.33 ± 0.03 and 0.26 ± 0.02, respectively; $P < 0.05$, Table 1) while values in turkeys were intermediate (0.29 ± 0.03).

Cryopreservation had a minor effect on the proportions of each lipid class in chicken spermatozoa, but dramatically changed proportions in turkey and guinea fowl spermatozoa (Fig. 5). The proportion of cholesterol was dramatically decreased in turkey and guinea fowl spermatozoa and the proportion of phospholipids

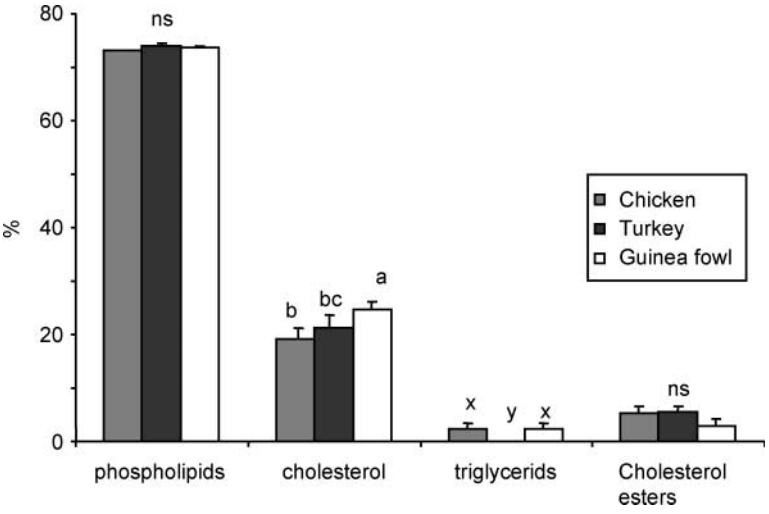

Figure 4 Lipid classes of fresh spermatozoa from chickens, turkeys and guinea fowl. $n = 9$; ^{ns} indicates no significant difference between species within each lipid category; $^{a,b,c,\cdots}$ indicate significant differences within each category of lipid ($P < 0.05$).

Table 1 Effects of species and cryopreservation on the cholesterol/ Phospholipid ratios (Chol/Pl, %) of chicken, turkey and guinea fowl spermatozoa. Results are expressed as means ± S.E.M. $n = 9$.

	Chol/Pl, %	
Species	Fresh spermatozoa	Frozen spermatozoa
Chicken	0.26 ± 0.02^{b}	0.25 ± 0.02^{b}
Turkey	0.29 ± 0.01^{ab}	0.08 ± 0.01^{d}
Guinea fowl	0.33 ± 0.03^{a}	0.14 ± 0.04^{c}

^{a,b,c,d} Indicate significant differences ($P < 0.05$).

correspondingly increased. As a consequence, the cholesterol/ phospholipid ratio was decreased by approximately 65% in turkeys and 50% in guinea fowl ($P < 0.05$, Table 1).

Discussion

Species-specific differences in the ability of spermatozoa to resist the various stresses caused by cryopreservation have long been a major limitation to the extension of animal genetic resources to preserve biodiversity. Despite close resemblances in the spermatozoon morphology of corresponding species of domestic birds, there is strong evidence that the direct application of semen freezing procedures which are successful in a model species (here the chicken) to other species is not feasible (Blanco *et al.* 2000, Blesbois & Labbé 2003, Saint Jalme *et al.* 2003). In order to improve the cryopreservation of spermatozoa in species with low semen freezability it is important to understand the biological bases that may explain such species differences. This study performed with semen from three avian species (chicken, turkey and guinea fowl) differing in their degree of semen cryopreservation ability showed that the general pattern of sperm membrane fluidity is similar to that observed for sperm freezability in corresponding species. This may partly be explained by differences in initial cholesterol/phospholipid ratios in the spermatozoa of different species.

This study also showed that the freezing–thawing procedure increased spermatozoon membrane rigidity in the three species studied and in many cases induced a decrease in cholesterol/phospholipid ratio.

Membrane fluidity has previously been measured in the spermatozoa of many mammalian species (Hinkovska-Galcheva & Srivastava 1993, Ladha 1998, Giraud *et al.* 2000). It has been suggested that intra species differences in membrane fluidity are linked to lipid composition, and membrane fluidity has been shown in humans to be related to the restoration of membrane viability and motility after freezing. In the current study, species-specific variations in membrane fluidity in birds were compared in fresh and frozen–thawed spermatozoa. Initial membrane fluidity, sperm viability and morphological integrity of spermatozoa before and after freezing were highest in chicken and lowest in guinea fowl spermatozoa. These results support the hypothesis that differences in semen freezability between bird species may at least partly originate from a difference in the initial fluidity of spermatozoon membranes and are in agreement with the general concept that cold tolerance is associated with membrane fluidity.

Membrane fluidity is known to be dependent on the cholesterol content and the degree of saturation of membrane phospholipid fatty acids (Ladha 1998) because cholesterol and saturated fatty acids are rigidifying components of the membranes. In the present study, the proportion of cholesterol found in the lipids of spermatozoa was lower in chicken than in guinea fowl spermatozoa while turkey spermatozoa were intermediate. The same order was found for membrane fluidity. As previous findings have indicated no major differences in the degree of saturation of fatty acids in sperm membranes from guinea fowl, turkeys and chickens (Suraï *et al.* 1998, Blesbois & Hermier 2003), it can be postulated that differences between species in initial spermatozoon membrane fluidity probably at least partly originate from differences in the cholesterol content of the membranes.

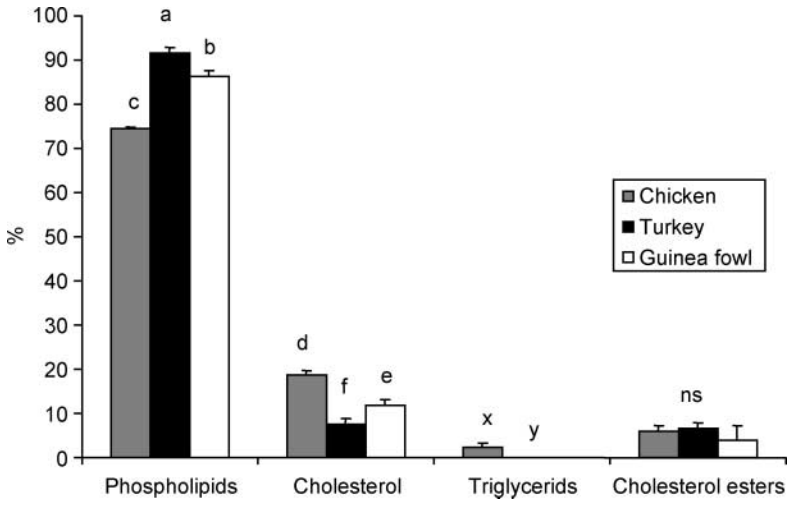

Figure 5 Lipid classes of frozen–thawed spermatozoa from chickens, turkeys and guinea fowl. $n = 9$; ^{ns} indicates no significant difference between species within each lipid category. ^{a,b,c,d,e,f,x,y} indicate significant differences within each lipid category ($P < 0.05$).

The present study is also the first to describe the lipid classes of guinea fowl spermatozoa and to compare the three species in the same experimental conditions. The lipid profile of guinea fowl spermatozoa is similar to the profiles found in chickens and turkeys, while chicken and turkey profiles confirmed previous results (Blesbois *et al.* 1997, Kelso *et al.* 1997, Douard *et al.* 2000). All these profiles are consistent with the membrane origin of the lipids of these cells that contain virtually no intra-cytoplasmic lipid structure.

Membrane fluidity is also known to decrease with decreasing temperature. This decrease is assumed to be linear during cooling before the appearance of a marked phase transition in the membranes. One hypothesis to explain the low freezability of guinea fowl spermatozoa might also be that a transition phase exists during cooling before freezing, which would require special attention to cooling rates during this phase. As the membrane rigidifying process in the current study followed a quasi-linear pattern between 40 °C and 0 °C, it can be concluded that a transition phase is not likely to occur at this stage. The fall in the slope of guinea fowl fluidity between 10 °C and 0 °C was not relevant and could not indicate a lipid transition phase that should, in contrast, have been expressed by a sudden increase in the slope. Moreover, the fact that the rigidifying process in sperm membranes during cooling in the three species studied here followed parallel but non-superposed patterns confirms the existence of species-specific differences in sperm membrane fluidity.

In agreement with previous observations made in humans (Giraud *et al.* 2000), the current study indicates that the freezing–thawing procedure induces a membrane rigidifying process in birds, whatever the species. Moreover, this rigidifying process was accompanied by a dramatic decrease in the cholesterol/phospholipid ratio in turkey and guinea fowl spermatozoa. This decrease was not present in chicken spermatozoa in the present study, although a previous study on a different line of chickens showed such a decrease (Blesbois *et al.* 1997). In addition, preliminary studies in turkeys indicated no significant change in the total phospholipid content of fresh or frozen–thawed spermatozoa (a mean of 550 μg/10^9 spermatozoa, measured by phosphorus content, unpublished observations).

This could suggest a genetically determined sensitivity of sperm membranes to freezing–thawing procedures which might be revealed by a greater or lesser tendency to release cholesterol during freezing–thawing. Moreover, if freezing induces an overall tendency for concomitant evolution of membrane fluidity and cholesterol/phospholipid ratio, a proportional direct evolution was not found. This is because membrane cholesterol and phospholipid have an important place in defining membrane fluidity but are not the only factors. The nature and the level of insertion of the proteins in the membrane lipid bilayer also have an effect on membrane fluidity (Shinitzky & Yuli 1982). Furthermore, spermatozoon freezing does not correspond to homogenous changes in membrane composition and various responses to freezing may occur.

Whatever the cause, the decrease in the cholesterol/-phospholipid ratio that occurs with freezing in turkey and guinea fowl spermatozoa, and in some cases in chicken spermatozoa, has also been described in mammalian species (reviewed by Travis & Kopf 2002). The decrease is thus suspected of being linked to cholesterol loss and could represent abnormal activation of capacitation induced by the freezing–thawing procedure for mammalian spermatozoa. However, cholesterol is not equally distributed throughout the plasma membrane of spermatozoa (reviewed by Cross 1998). Moreover, among the processes that may play a role in capacitation and fertilization and that may be altered by freezing, changes in sterol-rich regionalized structures of the plasma membranes called 'rafts' (Simons & Ikonen 1997, Cross 2003, 2004) are strongly suspected. The existence of capacitation is in itself still debatable in bird spermatozoa and future studies are needed in this area to improve understanding of the fertilisation process in birds and the tolerance of bird spermatozoa to freezing–thawing processes.

In conclusion, the present study clearly indicates that spermatozoa membrane fluidity differs between species of domestic birds. These differences partly reflect differences in cholesterol content and may be related to species differences in semen freezability. Further studies are needed in birds to explain how the rigidifying process represented by the freezing–thawing procedure is related to cholesterol loss and how it would be possible to limit such changes to increase the efficacy of semen cryopreservation.

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