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Within and between breed differences in freezing tolerance and plasma membrane fatty acid composition of boar sperm

K E Waterhouse[1,2], P O Hofmo[3], A Tverdal[4] and R R Miller Jr[5]

[1]Team Semin, PO Box 8146 Dep., N-0033 Oslo, Norway, [2]The Norwegian School of Veterinary Science, Department of Production Animal Clinical Sciences, PO Box 8146 Dep., N-0033 Oslo, Norway, [3]Norsvin (Norwegian Pig Breeders Association), PO Box 504, N-2304 Hamar, Norway, [4]The Norwegian School of Veterinary Science, Department of Basic Sciences and Aquatic Medicine, PO Box 8146 Dep., N-0033 Oslo, Norway and [5]Hillsdale College, Biology Department, 33 E College, Hillsdale, Michigan 49242, USA

Correspondence should be addressed to K E Waterhouse; Email: karin.waterhouse@veths.no

Abstract

The response of sperm to cryopreservation and the fertility of frozen–thawed semen varies between species. Besides species differences in sperm physiology, structure and biochemistry, factors such as sperm transport and female reproductive tract anatomy will affect fertility of frozen–thawed semen. Therefore, studying differences in sperm cryotolerance between breeds and individuals instead of between species may reveal sources of variability in sperm cryotolerance. In the present study, the effect of cooling, re-warming and freezing and thawing on plasma membrane and acrosome integrity of sperm within and between Norwegian Landrace and Duroc breeds was studied. Furthermore, the relation between post-thaw survival rate and fatty acid composition of the sperm plasma membranes was investigated. Flow cytometry assessments of plasma membrane and acrosome integrity revealed no significant differences between breeds; however there were significant male-to-male variations within breeds in post-thaw percentages of live sperm (plasma membrane intact). The most abundant fatty acids in the plasma membranes from both breeds were palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1, n-9), docosapentaenoic acid (22:5, n-6) and docosahexaenoic acid (22:6, n-3). The ratio of Σ 22:5, n-6 and 22:6, n-3/Σ all other membrane fatty acids was significantly related to survival rate (plasma membrane integrity) of sperm for both Norwegian Landrace (correlation coefficient (r_s) = 0.64, $P < 0.05$) and Duroc (r_s = 0.67, $P < 0.05$) boars. In conclusion, male-to-male differences in sperm survival rate after freezing and thawing may be partly related to the amount of long-chain polyunsaturated fatty acids in the sperm plasma membranes.

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Introduction

Frozen–thawed boar semen is rarely used for routine artificial insemination (AI) in commercial piglet production; however the increasing export of boar semen necessitates improved cryopreservation techniques. When frozen–thawed boar semen is used for AI, the farrowing rates are reduced by 10–25% and the litter sizes are reduced by 1–3 piglets compared with AI with fresh semen (Johnson *et al.* 1981, Almlid & Hofmo 1996). Breed-specific fertility data from field trials with Norwegian boars are limited; however, semen frozen during the 1990s seems to indicate a breed-specific response to cryopreservation. The mean motility of frozen–thawed semen produced in this decade was 36.3% for Norwegian Landrace and 19.6% for Duroc boars (Hofmo & Grevle 1999).

Cryopreservation exposes sperm to physical and chemical stress, and less than 50% of the sperm survive with fertilizing ability maintained. The negative effects of cooling, freezing and thawing are mainly caused by lipid phase transitions, ice crystallization and osmotic-induced water fluxes, and subsequent membrane reorganizations influence membrane integrity, structure and function (for review see Hammerstedt *et al.* 1990). The initial plasma membrane lipid composition may partially affect the cryogenic survival of sperm (De Leeuw *et al.* 1991, Parks & Lynch 1992). Differences in fatty acid composition and sterol levels have been associated with tolerance to cold-shock and cryopreservation, and observed variation among species in survival after freezing and thawing has been connected to such differences (Parks & Lynch 1992, White 1993). Specifically, a higher ratio of unsaturated/saturated membrane fatty acids and lower levels of cholesterol within bull and ram sperm membranes as compared with sperm membranes from humans and dogs have been

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suggested as an explanation for the differences in cold-shock and cryopreservation tolerance observed between these species (White 1993). However, these species-species differences in membrane lipid composition can neither explain the major differences in post-thaw survival and fertility between species such as bull and boar, nor the variation between breeds and the male-to-male variation within breeds (Holt 2000). Furthermore, species differences in the female reproductive tract and differences in the number of sperm needed for successful fertilization also determine fertility of frozen semen. Thus, a comparison of membrane lipid composition at breed and individual level is desirable (Holt 2000).

Compared with somatic cells, sperm plasma membranes have especially high levels of long-chain polyunsaturated fatty acids (PUFAs), in particular docosapentaenoic acid (DPA; 22:5, n-6) and docosahexaenoic acid (DHA; 22:6, n-3). DPA and DHA have been reported to be the predominant fatty acids within the boar sperm plasma membranes (Poulos *et al.* 1973, Parks & Lynch 1992) Recently, variations in PUFAs within sperm plasma membranes, in particular DPA and DHA, have been associated with differences in cryotolerance in sperm isolated from Asian and African elephants (Swain & Miller 2000), common wombats, grey kangaroos and koalas (Miller *et al.* 2004), and blue foxes and silver foxes (Miller *et al.* 2005). Furthermore, Arav *et al.* (2000) found a high PUFAs/saturated fatty acids ratio in cold-resistant bee sperm and lower ratios in cold-sensitive ram and fowl sperm. To our knowledge, no comparable data for fatty acid composition within sperm plasma membranes from different boar breeds exists nor is it known whether there are male-to-male differences. As boar sperm have relatively low levels of sterols in their membranes (Parks & Lynch 1992, Cerolini *et al.* 2001), this study will focus on the fatty acid fraction of the sperm plasma membranes.

The objectives of this study were to investigate plasma membrane fatty acid composition and the effects of cooling, freezing and thawing on plasma membrane and acrosome integrity of sperm from Norwegian Landrace and Duroc boars. The relationship between post-thaw plasma membrane integrity and plasma membrane fatty acid composition was studied. We hypothesized that there are breed and male-to-male differences in plasma membrane and acrosome integrity of sperm after freezing and thawing and, further, that survival rate (plasma membrane integrity) after freezing and thawing was related to the fatty acid composition of the sperm plasma membrane.

Materials and Methods

Animals and semen processing

Sperm-rich fractions were collected once by the gloved hand method from each of 12 mature Norwegian Landrace and 12 mature Duroc boars housed at Norsvin (Hamar, Norway) for routine semen production for AI purposes. Semen was collected at, at least, 5 day intervals from the previous collection. All the boars were fed the same commercial feed diet. During collection the semen was filtered through gauze and only sperm-rich fractions were collected. Within 15 min after collection, semen volume, sperm concentration, morphology and motility were determined.

Determination of sperm concentration was performed in a spectrophotometer (SDM 5, Mini Tub, Tiefenbach, Germany). The semen was diluted in BTS at 32 ± 1 °C to 250 ml and sperm morphology and motility were estimated under a microscope at 200 × magnification. Only ejaculates with ≥80% motile and morphologically normal sperm were included. The diluted semen was then cooled at 15 °C over a 3 h period and centrifuged for 10 min at 800 × ***g*** at 15 °C. Prior to centrifugation, a 5 ml aliquot of the BTS diluted semen was taken for later analyses. After centrifugation, the supernatant was discarded and the pellet was resuspended at 15 °C (1:1) with cooling extender (11% lactose with 20% egg yolk). The sperm concentration was estimated again (hemocytometer) and additional cooling extender was added to adjust the sperm concentration to 1.5×10^9/ml. After cooling to 4 °C over a 2 h period, freezing extender (cooling extender with 12% glycerol and 1.5% Equex-STM (Nova Chemical Sales, Inc, Scituate, MA, USA)) was added amounting to 1/3 of the final volume to give final concentrations of 1×10^9 sperm/ml, 4% glycerol and 0.5% Equex-STM. A 1 ml aliquot of the egg yolk and glycerol diluted (EYG) semen was taken before the semen was frozen in 2.5 ml maxi-straws (Mini Tub, Tiefenbach, Germany) in a controlled rate freezer for 9 min following a programmed freezing protocol. The freezing chamber was pre-cooled to −100 °C. Immediately afterwards the straws were transferred to the chamber which was then warmed at +10 °C/min to −70 °C and held at this temperature for 1 min before lowering the temperature −50 °C/min to −120 °C. The straws were held at −120 °C for 4 min before being transferred to liquid nitrogen. The BTS and EYG semen aliquots were transported to the laboratory in Oslo (2 h transport), at 15 °C and 4 °C respectively, for further analyses.

Plasma membrane and acrosome integrity

Liquid semen stored in BTS at 18 °C, in EYG at 5 °C and in EYG re-warmed to 38.5 °C was diluted to approximately 2×10^6 sperm cells/ml in PBS (holding temperature 18 °C, 5 °C and 38.5 °C respectively) containing 2.4 μM propidium iodide (PI; Molecular Probes Europe, Leiden, The Netherlands) and 0.2 μg/ml peanut agglutinin conjugated with phycoerythin, (PNA R-PE; Biømeda, Foster City, CA, USA) to detect plasma membrane and acrosome integrity respectively. Sybr-14 (20 nM) (Molecular Probes Europe) was included in the PBS staining solution to exclude egg-yolk particles and to ensure that only sperm were analyzed for PI and PNE R-PE fluorescence. For frozen semen samples, one straw from each boar was thawed at 50 °C

for 50 s and transferred to a test tube. The semen was diluted as described above in PBS at a holding temperature of 38.5 °C. All the stained samples were incubated in darkness for 10 min at their respective storage temperature (sperm samples in EYG at a holding temperature of 5 °C were incubated at room temperature) before analysis by flow cytometry. Flow cytometric analyses were performed using a Coulter EPICS × L flow cytometer (Beckman Coulter Ltd, Luton, Beds, UK) equipped with a 15 mW argon laser with excitation at 488 nm. Light-scatter data were collected in linear mode, while fluorescence data were collected in logarithmic mode. Side and forward light scatter parameters and Sybr-14 were used to identify sperm events and 10 000 sperm per sample were collected at low sample pressure. Sybr-14 fluorescence was detected using a 505–545 nm BP filter (FL1), PNA R-PE fluorescence was detected using a 560–590 nm BP filter (FL2) and PI fluorescence was detected using a 660–700 nm BP filter (FL4). Unstained samples were used as negative fluorescence controls. Compensation was set each day with unstained sperm and sperm singularly stained with Sybr-14, PNA R-PE and PI. The percentages of live sperm were determined by gating of the negative sperm population (PI negative) in the FL4 histogram after gating the histogram for Sybr-14 positive events (Fig. 1a). A cytogram of PI versus PNA R-PE fluorescence was used to determine the percentages of live acrosome intact (LAI) sperm after gating the cytogram for Sybr-14 positive events (Fig. 1b). Two replicate experiments were performed for each extender and temperature for each semen sample from all boars, and the average values were used in further analyses of the data.

Isolation of sperm plasma membranes

Membrane fatty acid analyses of frozen–thawed sperm were conducted in Hillsdale (MI, USA) where sperm plasma membranes were isolated by a modified version of the technique described by Agrawal *et al.* (1988). One straw (2.5×10^9 sperm) from each boar was thawed at 50 °C for 50 s and washed in 3 ml Tyrode's solution (pH 7.4), and then layered onto 3 ml 10% Percoll in Tyrode's solution and centrifuged at 500 ***g*** for 10 min. The sperm-containing pellets were resuspended in cold 0.1 M phosphate buffer (4 °C, pH 7.2) and the sperm were lysed by N_2 cavitation in a Konte's mini-bomb cell disruption chamber (Kimble-Kontes, Vineyard, NJ, USA) where sperm were equilibrated under N_2 at 500 PSI for 10 min. After equilibration, the sperm suspensions were extruded into cold 0.1 M phosphate buffer (4 °C, pH 6.2). In order to remove cellular debris, extruded suspensions were centrifuged at 6000 ***g*** for 10 min at 4 °C in a Jouan MR 18.12 centrifuge equipped with an Eppendorf rotor (Jouan catalog number 11174622; Westminster, VA, USA). The supernatants, which contained the sperm plasma membranes (Gillis *et al.* 1978, Peterson *et al.* 1980), were layered onto discontinuous sucrose gradients (10%, 20%, 30%, and 40% wt/v sucrose) and centrifuged at 105 000 ***g*** for 2 h at 4 °C in a Beckman Optima TLX centrifuge equipped with a TLA 100.4 rotor (Beckman Instruments, Inc., Palo Alto, CA, USA). The plasma membrane-containing pellets were resuspended in 400 µl 0.1 M phosphate buffer (pH 8.2) and total lipids were extracted from the plasma membranes by using the technique described by Folch *et al.* (1957). All samples were dried under N_2, and stored at −70 °C until further processing.

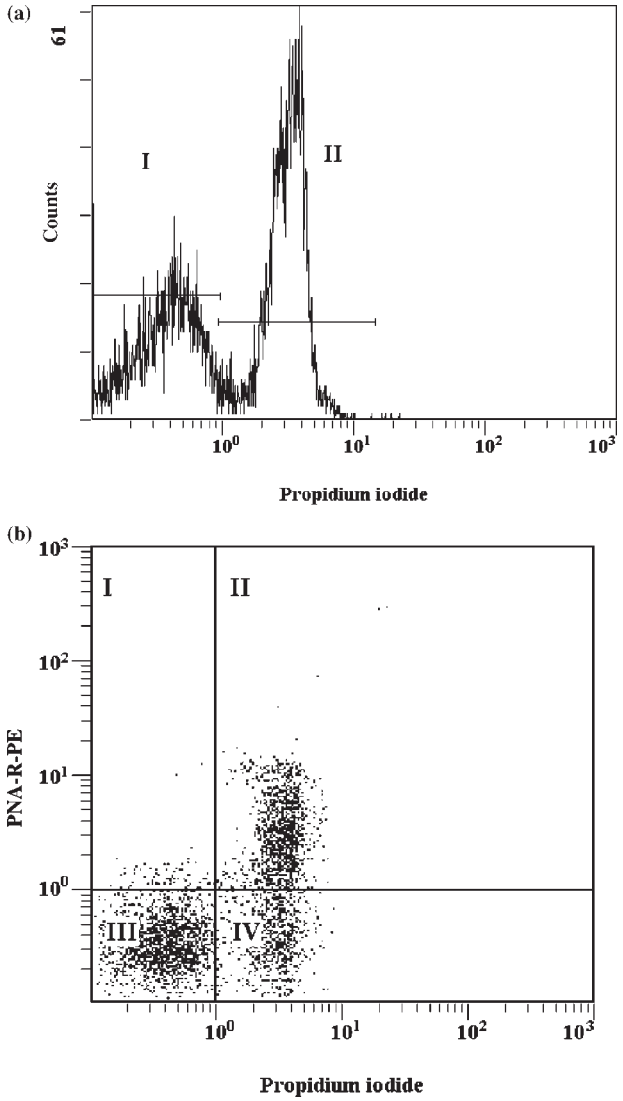

Figure 1 (a) A representative histogram of propidium iodide (PI) fluorescence intensities in frozen–thawed boar sperm showing percentages (of the total sperm population collected) of live and dead sperm. The histogram is gated for Sybr-14 positive events. I, live sperm, negative for PI. II, dead sperm, positive for PI. (b) A representative cytogram of PI versus PNA R-PE fluorescence in frozen–thawed boar sperm. The cytogram is gated for Sybr-14 positive events. I, live acrosome reacted/damaged sperm, negative for PI and positive for PNA R-PE. II, dead acrosome reacted/damaged sperm, positive for PI and PNA R-PE. III, live acrosome intact sperm, negative for PI and PNA R-PE. IV, dead acrosome intact, positive for PI and negative for PNA R-PE.

Membrane fatty acid analyses

Neutral lipids were fractionated from other sperm plasma membrane lipids on silicic acid columns (Dittmer & Wells 1969). Columns were prepared in 6.0 ml fritted SPE (columns) (Restek Inc., Bellefonte, PA, USA) packed with approximately 2 ml per column pre-baked (120 °C) silicic acid (SIL-R, 100–300 mesh; Sigma Chemical Co., St Louis, MO, USA). The columns were washed three times with chloroform. After total lipids were resuspended in 2:1 (v/v) chloroform-methanol and applied to the columns, neutral lipids were eluted with 4 ml chloroform. Membrane lipids, which contained phospholipids, sphingolipids, and some glycolipids, were eluted with 4 ml methanol and dried under N_2.

Fatty acid methyl esters (FAMEs) were prepared as described by Metcalfe *et al.* (1966). FAMEs were separated on an Omega-wax capillary column (30 m × 0.53 mm × 0.5 μm film thickness; Supelco Incorporation, Bellefonte, PA, USA) in a Trace GC (Thermo-Electron Corp., Austin, TX, USA). The injector temperature was 225 °C and the flame ionization detector was 250 °C with a He carrier gas rate of 20–25 ml/min. The initial column temperature was 185 °C. After a 5 min delay, the column temperature was increased to 205 °C at a rate of 2 °C/min. A split ratio of 3:1 was used for all analyses. Individual FAMEs within each boar sample were identified by retention times as compared with the retention times of known fatty acid methyl ester standards.

To check if possible individual differences in fatty acid composition were due to different amounts of live sperm and dead sperm after freezing and thawing, live and dead sperm were separated by layering frozen–thawed samples on a gradient of 45% Percoll over 90% Percoll followed by centrifugation at 700 ***g*** for 30 min. Live sperm were found in the pellets whereas dead sperm were found at the interphase of the 45% and 90% Percoll layers (Swain & Miller 2000). Live and dead sperm from 2 Landrace and 2 Duroc boars were isolated and subjected to plasma membrane isolation and membrane fatty acid analyses as previously described.

Statistical analyses

Sperm data are presented as mean values with standard deviations (S.D.) for both breeds ($n = 12$ for both breeds). ANOVA was used to compare membrane integrity variables of sperm at different storage temperatures and the relative amounts of each fatty acid and the ratios of Σ unsaturated/Σ saturated, Σ 22:5, n-6 and 22:6, n-3/Σ all other membrane fatty acids, and 22:6, n-3/22:5, n-6 between the two breeds. The fatty acid data (% g) was arcsine transformed when testing equality in ANOVA. However, untransformed means are presented in Table 2. The F-test was used to compare the between-boar variation within breed at 18 °C with between-boar variation after cooling, re-warming and after freezing and thawing for sperm membrane integrity variables. A paired *t*-test was used to make paired comparisons in membrane integrity variables of sperm for different storage temperatures within breed. Spearman's rank correlation was used to assess the relationship between the percentage of live sperm after freezing and thawing and the ratios of Σ unsaturated/Σ saturated, Σ 22:5, n-6 and 22:6, n-3/Σ all other membrane fatty acids, and 22:6, n-3/22:5, n-6 within breed. A paired *t*-test was used to make paired comparisons of ratios; Σ unsaturated/Σ saturated, Σ 22:5, n-6 and 22:6, n-3/Σ all other membrane fatty acids, and 22:6, n-3/22:5, n-6 between post-thaw Percoll-separated live and dead sperm. Statistical analyses were conducted using JMP version 5.0.1 for Windows (SAS Institute Inc, Cary, NC, USA). *P* values less than 0.05 were considered statistically significant.

Results

Sperm membrane integrity

Neither percentages of live (PI-negative) nor LAI (PI and PNA R-PE negative) sperm were significantly different between the breeds for sperm stored at 18 °C (BTS), at 5 °C (EYG), re-warmed from 5 °C to 38.5 °C (EYG) or for frozen–thawed sperm (Table 1). Cooling of sperm to 5 °C induced a small, yet significant, reduction in the percentage of live sperm compared with 18 °C for both breeds. For Landrace the reduction was 3.2% ($P < 0.0001$) and for Duroc the reduction was 1.6% ($P < 0.0001$). After cooling to 5 °C, the percentage of LAI sperm was reduced by 3.5% for Landrace ($P < 0.0002$) and by 0.6% for Duroc breeds ($P < 0.57$) compared with 18 °C. At 5 °C, percentages of live and LAI sperm were still above 90% for both breeds (Table 1).

Re-warming of sperm to 38.5 °C after storage at 5 °C significantly decreased the percentages of live and LAI sperm for both Landrace and Duroc breeds. For Landrace, the percentages of live and LAI sperm decreased by 5.5% ($P < 0.0001$) and 32.7% ($P < 0.0001$) respectively compared with sperm held at 18 °C. For Duroc, the percentage of live sperm decreased by 7.1% ($P < 0.0001$) and the percentage of LAI sperm decreased by 22.5% ($P < 0.0001$) compared with sperm held at 18 °C (Table 1).

Table 1 Percentages of live and live acrosome intact (LAI) sperm at different storage temperatures for Norwegian Landrace ($n = 12$) and Duroc ($n = 12$) boars. Values are presented as means (S.D.).

	18 °C[a]	5 °C[b]	38.5 °C[b]	Post-thaw[b]
Live sperm (%)				
Landrace	95.5 (1.5)	92.4 (1.1)	87.3 (2.9)	48.8 (10.1)
Duroc	94.3 (2.6)	92.8 (2.0)	87.6 (3.6)	51.3 (11.0)
LAI sperm (%)				
Landrace	94.4 (1.7)	91.1 (1.5)	63.5 (18.9)	44.7 (10.5)
Duroc	92.5 (4.4)	91.9 (2.2)	71.7 (12.8)	45.0 (8.8)

[a]Sperm diluted and stored in BTS; [b]sperm diluted and stored in freezing extender containing 20% egg yolk and 4% glycerol.

After freezing and thawing, the percentages of live and LAI sperm were significantly reduced by 48.9% ($P < 0.0001$) and 52.6% ($P < 0.0001$) respectively compared with fresh sperm stored at 18 °C for the Landrace breed. For frozen-thawed Duroc sperm the percentages of live and LAI sperm were significantly reduced by 45.6% ($P < 0.0001$) and 54.4% ($P < 0.0001$) respectively compared with fresh sperm stored at 18 °C ($P < 0.0001$).

A significant increase in male-to-male variation was observed for percentages of live ($P < 0.0001$) and LAI ($P < 0.0001$) sperm after freezing and thawing compared with fresh sperm stored at 18 °C for both Landrace and Duroc breeds. A significant male-to-male variation for both breeds was also observed for the percentage of LAI sperm after re-warming from 5 °C to 38.5 °C compared with fresh sperm stored at 18 °C ($P < 0.0001$).

Plasma membrane fatty acid composition of frozen–thawed sperm

Except for palmitoleic acid (16:1) levels, there were no significant differences between the two breeds in the relative levels of individual fatty acids (Table 2). Low levels of palmitoleic acid (16:1) were detected in Duroc sperm plasma membranes, while palmitoleic acid was not detected in Landrace sperm plasma membranes. In both breeds, the most abundant saturated fatty acids were palmitic acid (16:0) and stearic acid (18:0) and the most abundant unsaturated fatty acids in both breeds were oleic acid (18:1, n-9), docosapentaenoic acid (DPA; 22:5, n-6) and docosahexaenoic acid (DHA; 22:6, n-3). For some specific fatty acids, namely DPA, DHA and 20:4, n-3, relatively high standard deviations were observed for both breeds.

Analyses of frozen–thawed sperm indicated no significant differences when the ratios of Σ unsaturated/Σ saturated membrane fatty acids ($P > 0.89$), Σ 22:5, n-6 and 22:6, n-3/Σ all other membrane fatty acids ($P > 0.15$), and 22:6, n-3/22:5, n-6 ($P > 0.24$) were compared between the two breeds. Furthermore, analyses of frozen–thawed sperm demonstrated no significant differences in sperm plasma membrane fatty acid ratios when comparing Percoll-separated live and dead sperm: Σ unsaturated/Σ saturated membrane fatty acids ($P > 0.22$), Σ 22:5, n-6 and 22:6, n-3/Σ all other membrane fatty acids ($P > 0.11$), and 22:6, n-3/22:5, n-6 ($P > 0.82$).

Significant correlation coefficients were observed when the ratios of Σ 22:5, n-6 and 22:6, n-3/Σ all other membrane fatty acids were correlated with the percentage of live sperm after freezing and thawing, a correlation coefficient (r_s) of 0.64 was observed in the Landrace breed ($P = 0.024$) and a correlation coefficient (r_s) of 0.67 was observed in the Duroc breed ($P = 0.017$) (Table 3).

Table 2 Percentage gram fatty acids found in phospholipids of sperm plasma membrane from Norwegian Landrace ($n = 12$) and Duroc ($n = 12$) boars. Values are presented as means (S.D.).

Fatty acid	Landrace	Duroc
Saturates, total	37.3 (7.15)	37.4 (6.0)
12:0	0.35 (0.52)	0.05 (0.17)
14:0	2.15 (3.30)	1.11 (0.51)
16:0	18.35 (4.03)	18.93 (3.10)
18:0	16.44 (4.12)	17.31 (3.46)
Monounsaturates, total	12.4 (3.7)	13.4 (3.0)
14:1	0.67 (1.18)	0.21 (0.59)
16:1	nd	0.48 (0.84)
18:1, n-9	11.75 (3.67)	12.71 (3.48)
Polyunsaturates, total	50.3 (7.1)	49.1 (5.9)
18:2, n-6	6.32 (2.16)	6.83 (2.43)
18:3, n-3	1.59 (4.55)	0.30 (0.89)
20:4, n-3	6.21 (4.53)	8.76 (10.36)
22:3, n-6	1.55 (2.17)	0.30 (0.70)
22:4, n-6	1.87 (2.63)	0.75 (1.16)
22:5, n-6	15.40 (6.01)	13.92 (5.41)
22:5, n-3	0.45 (0.86)	0.26 (0.43)
22:6, n-3	16.91 (6.25)	18.03 (5.95)
Fatty acid ratios		
Unsaturates/saturates	1.76 (0.46)	1.73 (0.38)
22/rest of fatty acid	0.60 (0.23)	0.52 (0.18)
22:6, n-3/22:5, n-6	1.20 (0.43)	1.39 (0.61)

nd, not detected.

Discussion

Male-to-male differences in freezing and thawing tolerance of sperm have been reported for several species (Larsson & Einarsson 1976, Parkinson & Whitfield 1987, Pickett & Amann 1993, Thurston *et al.* 2001). Individual cryotolerance of sperm amongst boars within the Norwegian Landrace and Duroc breeds was also observed in this study. Differences in percentages of live sperm before freezing were not significant between males within breed; however there was a significant male-to-male variation in percentages of live sperm after freezing and thawing. Our hypothesis that there was a relationship between sperm survival rate (plasma membrane integrity) after freezing and thawing and plasma membrane fatty acid composition was confirmed. We have demonstrated a significant relationship between post-thaw amounts of DPA and DHA in the sperm plasma membranes and survival rate as measured by plasma membrane integrity. Specifically, as post-thaw sperm membrane DHA and DPA levels increase, greater numbers of boar sperm seem to survive the freezing and thawing process. In this study, we were not able to assess initial (raw semen) fatty acid composition of the sperm membranes due to the great

Table 3 Correlation coefficients between percentages of live sperm after freezing and thawing and fatty acid ratios of frozen-thawed sperm from Norwegian Landrace ($n = 12$) and Duroc ($n = 12$) boars.

Ratio	**Live sperm** (%), **Landrace**	**Live sperm** (%), **Duroc**
Unsaturates/saturates	0.42	−0.04
22/rest of fatty acid	0.64*	0.67*
22:6, n-3/22:5, n-6	−0.52	−0.24

*$P < 0.05$.

geographical distance between the boars and the laboratory where the quantification of fatty acids was performed. As sperm plasma membranes lose lipids during the cryopreservation process (White 1993, Cerolini *et al.* 2001), the fatty acid composition should ideally be studied prior to semen processing, i.e. in raw semen. However, the ratios of Σ unsaturated/Σ saturated fatty acids, Σ 22:5, n-6 and 22:6, n-3/Σ all other membrane fatty acids and 22:6, n-3/22:5, n-6 fatty acids for Percoll-separated live sperm compared with dead sperm were not significantly different. Hence, the significant relationship between percentages of live sperm and long chain PUFAs does not seem to be a consequence of individual differences in post-thaw percentages of live and dead sperm, but seems to be related to the individual boar. Differences in cryotolerance have previously been related to a high cholesterol/phospholipid ratio and low unsaturated/saturated fatty acids ratio in sperm membranes; however this relationship cannot explain the observed differences in cryotolerance between species such as bull and boar, or between individuals. In this study, the ratio of Σ unsaturated/Σ saturated fatty acids of sperm plasma membranes, for both Landrace and Duroc boars, was actually comparable to the ratios found in 'good freezers' such as human and rabbit (White 1993). Recent studies have associated cryogenic success and failure of sperm collected from different species with variations in levels of the long-chain PUFAs, DPA and DHA in their sperm plasma membranes (Swain & Miller 2000, Miller *et al.* 2004, 2005). While there were no significant differences in fatty acid composition of sperm plasma membranes from the Landrace and Duroc breeds in the present study, increased standard deviations of the most abundant long-chain PUFAs, DPA and DHA indicated male-to-male variations for both breeds.

The terms 'good freezer' and 'bad freezer' have existed for a long time, and it seems to be a trait of the individual boar rather than a characteristic of individual ejaculates. In fact, newly published results from Thurston *et al.* (2002) indicate that there is a genetic basis for the variation in cryopreservation-induced injuries, which included disrupted plasma and acrosomal membranes and poor motility, between individuals categorized as 'good freezers' and those categorized as 'bad freezers'. While the underlying mechanism(s) for the genetic differences related to cryopreservation-induced injuries is unknown, it has been suggested that these male-to-male differences may represent differences in sperm lipid and protein composition (Holt *et al.* 2005). Since the boars in the present study were all on the same diet, the observed male-to-male differences in long-chain PUFAs in the sperm membranes may be related to differences in desaturation and elongation rates of dietary linoleic acid (18:2, n-6) and linolenic acid (18:3, n-3). Sæther *et al.* (2003) have shown that it is the Sertoli cells that are most active in converting the dietary essential fatty acids to long-chain PUFAs in the rat testis, which supports their hypothesis of a lipid transport system between Sertoli cells and developing germ-line cells. Male-to-male differences in the rates of lipid transport and male-to-male differences in the elongation and desaturation rates of dietary linoleic acid (18:2, n-6) to DPA (22:5, n-6) and of linolenic acid (18:3, n-3) to DHA (22:6, n-3) may have a genetic basis and affect the fatty acid composition of sperm plasma membranes. Other sources of variation in freezability between individual boars have been reported; the proportion of distinct morphologically different subpopulations of sperm in fresh ejaculates varies between boars and is correlated with sperm quality after freezing and thawing (Thurston *et al.* 2001). In humans, significant relationships between oxidative defence enzymes and antioxidants and motility after freezing and thawing have been demonstrated (Meseguer *et al.* 2004).

Higher levels of long-chain PUFAs, such as DPA and DHA, have been associated with increased levels of membrane fluidity (Quinn 1985, Salem *et al.* 1986). For humans, sperm with a high degree of initial membrane fluidity demonstrated superior post-thaw motility compared with sperm with a lower degree of initial membrane fluidity (Giraud *et al.* 2000) and Aboagla & Terada (2003) found that trehalose-induced increase in plasma membrane fluidity was related to improved motility and acrosome integrity after freezing and thawing. Even though the fatty acid composition in the membrane is changed as a result of the cryopreservation process (White 1993), we speculate that boars with higher levels of DPA and DHA in their sperm membranes after freezing and thawing were more cryotolerant due to initially higher membrane fluidity. Breed-specific differences in survival rate (plasma membrane integrity) after freezing and thawing were not observed in this study. However, previous observations may suggest a significant relationship between post-thaw motility and breed (Hofmo & Grevle 1999). Subjective motility assessments of frozen–thawed sperm in the present study corresponded to previously reported results, with significant differences between the breeds (results not shown). We speculate that mechanisms or structures involved in regulation and/or generation of motility may have been affected differently in the two breeds during the cryopreservation process.

Cooling sperm to 5 °C caused only minor reductions in the percentage of live and LAI sperm compared with 18 °C. This was somewhat surprising, since the literature describes boar sperm as being sensitive to cold shock (Johnson *et al.* 2000). Our results indicate that Landrace and Duroc sperm resist cold shock-induced alterations and maintain intact plasma and acrosome membranes after cooling to 5 °C. One could argue that the dyes (PI, Sybr-14 and PNA R-PE) have slower incorporation at this low temperature; however PI incorporation into sperm DNA has been observed during cryomicroscopy at −20 °C (Medrano *et al.* 2002). The re-warming of cooled sperm (5 °C) to 38.5 °C further induced significant disruption of the plasma membrane and especially of the acrosome as compared with 5 °C. The freezing and thawing further induced marked reductions in both live and LAI

sperm for both breeds, and these reductions correspond to those of previously published results (Maxwell & Johnson 1997, Cerolini *et al.* 2001).

In conclusion, the results of our study indicate that it is the individual male and not the breed that is decisive for the survival rate, measured as plasma membrane integrity, after freezing and thawing of boar sperm. Furthermore, the male-to-male differences in sperm survival after freezing and thawing seem to be partially related to the amounts of long-chain PUFAs in the plasma membrane after freezing and thawing. Future work will compare initial and frozen–thawed fatty acid composition of the sperm plasma membranes and will study the relationship with survival rates after cryopreservation.

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