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Extender Components and Surfactants Affect Boar Sperm Function and Membrane Behavior During Cryopreservation

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ABSTRACT: To determine how the individual components of extenders affected boar sperm function and membrane structure and to test a new surfactant's cryoprotective ability, boar sperm were cryopreserved in straws in BF5 extender plus or minus egg yolk plus or minus glycerol plus or minus a surfactant (Orvus ES Paste [OEP] or various concentrations of Pluronic F-127). After thawing, sperm function and fluidity of the isolated head plasma membrane (HPM) were determined. Total motility and adenosine triphosphate content (a measure of viability) were superior postthaw in sperm extended in egg yolk plus glycerol ($P < 0.05$); neither surfactant improved function. Egg yolk plus any other ingredients improved normal acrosome morphology, whereas a combined measure of motility and normal acrosome morphology was better in the presence of 0.33% OEP or 0.1% Pluronic F-127 ($P < 0.05$ vs. controls). Head plasma membrane was isolated from freshly collected spermatozoa and spermatozoa cryopreserved in the various extenders. Membrane fluidity was monitored with the probes *cis*-parinaric acid (cPNA), *trans*-parinaric acid (tPNA), and 1,6-diphenyl-1,3,5-hexatriene (DPH). The cPNA and the DPH monitor the fluidity of gel and liquid-crystalline areas of the membrane, whereas the tPNA preferentially monitors the gel-phase domains of the membrane. Additionally, DPH monitors the hydrophobic core of the bilayer. In the HPM from fresh sperm, the fluidity of each domain changed over time in a manner unique to that domain, and the behavior of the DPH domain varied among boars. The fluidity dynamics of each domain responded uniquely to cryopreservation. The cPNA domain was unaffected, the tPNA domain was altered by four of the eight extenders, and all extenders affected the fluidity of the DPH domain. Membrane structure was significantly correlated with cell function for sperm cryopreserved in extenders that preserved viability and motility. Sperm cryopreserved in egg yolk plus glycerol plus either OEP or 0.1% Pluronic F-127 functioned best when the bulk domains were less fluid initially and the gel domain solidified more slowly. Therefore, the behavior of domains in the HPM of boar spermatozoa is affected by cryopreservation and is related to the postthaw function of boar sperm cryopreserved in different extenders.

Key words: Membrane fluidity, ATP, motility, acrosome.
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Artificial insemination programs using cryopreserved bovine, equine, and ovine spermatozoa allow for multiple inseminations from any one ejaculate (Polge, 1980; Foote, 1982; Amann and Pickett, 1987; Watson, 1990; Coulter, 1992; Foote and Parks, 1993), and swine production could benefit from the same efficiency. However, cryopreserved boar spermatozoa have poorer motility, acrosomal morphology, and viability than fresh spermatozoa (Courtens and Paquignon, 1985; Weitze et al, 1986; Clarke and Johnson, 1987; Almlid and Johnson, 1988; Almlid et al, 1989; Hofmo and Almlid, 1991), and the accompanying poor farrowing rates (40–50%) and low-litter size make frozen boar semen impractical for the average swine producer (Johnson, 1985; Almlid et al, 1987; Hofmo and Almlid, 1991; Crabo and Dial, 1992).

Successful fertilization requires that the spermatozoa undergo capacitation and the acrosome reaction in the female tract. During these processes, the nature and amounts of lipids (Nikolopoulou et al, 1986) and proteins, the cholesterol:phospholipid ratio (Langlais et al, 1981; Farooqui, 1983; Langlais and Roberts, 1985), and the products of phospholipase A_2 (Fraser, 1984; Langlais and Roberts, 1985; Nikolopoulou et al, 1986; Fraser and Ahuja, 1988; Dyck and Buhr, 1994) change in the head plasma membrane (HPM) of boar spermatozoa. These normal molecular changes in the HPM are reflected in the fluidity of the membrane (Fraser, 1984; Buhr et al, 1994). The majority of membrane phospholipid is present in either the liquid-crystalline or gel phase. When the phospholipid acyl chains are packed loosely in the liquid-crystalline state, the lateral mobility of lipids and proteins in the bilayer (Pringle and Chapman, 1981; Malhotra, 1983) necessary for normal cellular function (Shinitzky, 1984) is allowed. If packed closely together in the gel phase, lateral mobility is restricted, and fluid lipids and transmembrane proteins are excluded from these gel areas (lateral phase separations; Gadella et al, 1994). Cryopreservation damages the sperm plasma membrane, redistributing HPM proteins (de Leeuw et al, 1990, 1991), disrupting plasma membrane integrity (Almlid and Johnson,

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1988; Almlid et al, 1989), and changing the content and/or distribution of phospholipids and fatty acids in the HPM (Parks and Graham, 1992; Buhr et al, 1994). Head plasma membrane fluidity is modified during cryopreservation (Buhr et al, 1989; Canvin and Buhr, 1989; Buhr and Pettitt, 1996), which may be related to these changes and the reduced postthaw fertility of cryopreserved boar semen.

Successful cryopreservation depends on the extender, the cryoprotectant, and the cooling and warming processes (Fiser, 1991). Extenders for boar spermatozoa commonly contain buffers, egg yolk, sugars, and the surfactant sodium and triethanolamine lauryl sulphate (Orvus ES Paste [OEP]; marketed as Equex STM, Nova Chemical Sales, Inc., Scituate, Massachusetts; Graham and Crabo, 1972; Pursel and Johnson, 1975; Westendorf et al, 1975). The surfactant optimizes postthaw motility, acrosome morphology (Graham and Crabo, 1972; Pursel et al, 1978a), and fertilization rates (Pursel et al, 1978a). The OEP only improves sperm function in the presence of egg yolk (Hofmo and Almlid, 1991), presumably by dispersing egg yolk–lipid conglomerates formed after dilution of the semen (Strzezek et al, 1984) and not through a direct membrane effect (Pursel et al, 1978a; Pontbriand et al, 1989). The current, yet unproven, hypothesis is that the amphipathic properties of OEP allow it to emulsify and disperse egg yolk lipids and make them more available to the sperm plasma membrane.

As surfactant plays an important role in the cryopreservation of boar spermatozoa, we substituted a surfactant of known chemistry to improve our understanding of the relationship of surfactant structure to spermatozoa function during cryopreservation. Pluronic F-127 (Pluronic; BASF Canada, Inc., Georgetown, Ontario, Canada) is a nonionic, well-characterized surfactant typically used to load fluorescent indicator probes into cells without harming them (Bailey and Buhr, 1993). It consists of a hydrophobic center of propylene oxide plus propylene glycol, which is flanked on either end by ethylene oxide, a hydrophile.

Membranes from fresh boar sperm have at least three identifiable domains whose fluidity is altered by the composition of cryopreservation extender (Buhr and Pettitt, 1996). Domains, which are areas of unique structure within a single membrane, were detected with fluorescence polarization using the probes *cis*-parinaric acid (cPNA), 1,6-diphenyl-1,3,5-hexatriene (DPH), and *trans*-parinaric acid (tPNA). Both cPNA and DPH partition equally into liquid-crystalline and gel-phase domains of the membrane by inserting between the fatty acyl chains of the phospholipids perpendicular to the plane of the membrane (Sklar et al, 1979; Lentz, 1989, 1993). The DPH can also insert into the space between the bilayer leaflets, parallel to the plane of the membrane, whereas the cPNA is restricted to insertion between the acyl chains as its charged end anchors it at the membrane/water interface (Borenstain and Barenholz, 1993; Lentz, 1993). Thus, cPNA and DPH report on two different domains within the bilayer. The tPNA probe preferentially inserts itself into gel-phase areas of the membrane (Sklar et al, 1979) but, like cPNA, is also restricted to locations between the acyl chains (Ben-Yashar and Barenholz, 1991; Borenstain and Barenholz, 1993; Lentz, 1993). Dynamic behavior of these domains in HPMs isolated from boar spermatozoa is known to be exquisitely sensitive to chilling and cryopreservation (Buhr et al, 1989; Canvin and Buhr, 1989). The current study hypothesized that composition of the cryopreservation extender would alter membrane dynamics in one or more domains in a manner related to spermatozoa function, as measured by motility, acrosome morphology, and adenosine triphosphate (ATP) content.

Materials and Methods

Boars and Semen Collection

Five boars, housed and handled according to the regulations of the Canadian Council for Animal Care, were used for all experiments. The spermatozoa-rich fraction from each boar was collected by the gloved-hand method through a layer of gauze into an insulated thermos bottle maintained at 37°C. The concentration of the spermatozoa was estimated using a previously calibrated spectrophotometer. The semen was then filtered through a double layer of Miracloth cloth (Calbiochem, La Jolla, California) at 37°C to remove gel particles.

Fresh Semen

Four ejaculates from each boar were collected, and the HPM fluidity of each fresh ejaculate was analyzed. In addition, membrane fluidity of pooled fresh semen (n = 4) was determined by simultaneously collecting an ejaculate from all five boars, pooling the five ejaculates immediately, and then measuring the HPM fluidity.

Semen Cryopreservation

Four ejaculates from each boar were separately cryopreserved by the method of Pursel and Johnson (1975) as modified by Fiser et al (1993). After adding 0.006 ml of Lincospectin (The Upjohn Company, Orangeville, Ontario, Canada; each milliliter of Lincospectin contains 50 mg of lincomycin hydrochloride USP and 100 mg of spectinomycin sulfate) per milliliter, the semen was returned to the thermos and was cooled to 22°C over 2 hours; conditions were set in pilot experiments to ensure the repeatability of this rate of cooling. Cooled semen was centrifuged at room temperature (800 × g, 10 minutes), and the seminal plasma supernatant was discarded. Each ejaculate was then split into eight portions and received fraction A of one of eight different extenders (n = 20 per extender). Extenders were based on Beltsville BF5 extender (BF5; Pursel and Johnson, 1975; Pursel et al, 1978a), altering the normal BF5 content of egg yolk, glycerol,

and surfactant. The following extenders were used: 1) minimal BF5 extender (mBF5; BF5 extender with no egg yolk, glycerol, or OEP); 2) BF5 extender with 20% (v:v) egg yolk but no glycerol or OEP (EY); 3) BF5 extender with 3% glycerol but no egg yolk or OEP (GLY); 4) BF5 extender with 20% egg yolk and 3% glycerol but no OEP (EYGLY); 5) EYGLY extender with 0.33% (w:v) OEP (EYGLY:OEP); 6) GLY extender plus 0.1% (w/v) Pluronic (GLY:0.1P); 7) EYGLY extender plus 0.1% Pluronic (EYGLY:0.1P); and 8) EYGLY extender plus 0.01% Pluronic (EYGLY:0.01P). The selected concentrations of Pluronic do not damage boar spermatozoa (Buhr and Pettitt, 1996). Each extender was prepared in A and B fractions, with and without glycerol, respectively. For extenders mBF5 and EY, fractions A and B were identical.

Tubes containing semen extended with fraction A were placed in 250-ml beakers filled with 22°C water and were cooled to 5°C over 3 hours by placing in a cold room free of draughts; again, repeatability of cooling rates was predetermined. Extender fraction B was then added in random treatment order, and straws (0.5 ml, catalog no. AA 101, I.M.V. International Corp., Minneapolis, Minnesota) were filled with the extended spermatozoa and were sealed with sealing powder (I.M.V.) or stainless steel sealing balls (Minitube of America, Madison, Wisconsin). The final concentration was 6×10^8 spermatozoa per milliliter of extender. Straws were then frozen in liquid nitrogen vapors at a rate (determined by a thermocouple in a straw) of 30°C/minute from 5°C to a final temperature of −70°C (Fiser et al, 1993). Straws were plunged directly into liquid nitrogen and were stored for at least 48 hours.

Percent motile spermatozoa (35 and 22°C only), acrosome morphology (Bailey and Buhr, 1993), and ATP content (a measure of viability; Brooks, 1970; Orlando et al, 1982; Comhaire et al, 1983; Irvine and Aitken, 1985) were measured on 50-µl samples taken at various times: 1) immediately after collection (35°C), 2) immediately prior to adding extender fraction A (22°C), 3) immediately after adding extender fraction A (22°C), 4) immediately prior to adding extender fraction B (5°C), and 5) immediately after adding extender fraction B (5°C). Percent motile spermatozoa for each sample was estimated by adding 2 µl of semen to 2 ml of 0.85% NaCl in a glass watch plate held at 37°C. Fifteen microliters of diluted semen was then added to a warm slide and was covered with a glass coverslip, and at least six fields were observed. Total motility for each sample was estimated in duplicate to the nearest 5%. Acrosome morphology was determined by adding 5 µl of semen to 40 µl of 0.2% glutaraldehyde in phosphate-buffered saline in a glass watch plate held at 37°C. Two 12-µl droplets of diluted semen were added to one warm slide, and each was covered with a glass coverslip. At least 100 spermatozoa were counted per droplet, ensuring that the same area of the slide was not evaluated more than once, and were classified to be either normal or damaged (including ruffled, damaged, or absent). Adenosine triphosphate concentration was determined by a luciferin-luciferase assay (FireZyme Limited, Diagnostic Technologies, Halifax, Nova Scotia, Canada). All 50-µl samples were scored as quickly as possible after removal and were kept at the appropriate temperature until scored.

For postthaw analysis, individual straws were thawed (Fiser et al, 1993) with gentle agitation in a 60°C water bath for 8 seconds, wiped dry, emptied into 3.0 ml of Beltsville Thawing Solution (Pursel and Johnson, 1975) at 33°C, inverted gently, and allowed to equilibrate for 5 minutes. Two 200-µl samples were taken for ATP analysis. An additional 2 ml of Beltsville Thawing Solution (33°C) was then added, and spermatozoa were analyzed for motility and acrosome morphology.

Isolation of HPM

Unless otherwise specified, all procedures, buffers, and equipment were at 22–25°C.

For each extender treatment, 20 straws (6×10^9 spermatozoa in 10 ml total) from each boar were thawed as above and pooled ($n = 4$ per extender). Semen was diluted 1:1 (v:v) with Tris buffer (5 mM Tris-HCl, 0.25 M sucrose, pH 7.4) and centrifuged (2,500 × *g*, 10 minutes), and the pelleted spermatozoa were resuspended to 20 ml in Tris buffer. Fresh semen was diluted with Tris buffer either at 1:1 (v:v) or to a maximum volume of 155 ml (less than 1:1). These ejaculates contained an average of $87.7 \pm 6.3 \times 10^9$ spermatozoa (mean ± SEM). Fresh and frozen-thawed spermatozoa were subsequently subjected to the same procedures. The HPM was then isolated by nitrogen cavitation and differential centrifugation, using the procedure of Gillis et al (1978) as modified by Canvin and Buhr (1989).

Fluidity Analysis

The membrane probes cPNA, tPNA (Molecular Probes, Inc., Eugene, Oregon), and DPH (Sigma Chemical Co., St. Louis, Missouri) were made up as stock solutions (2 mM) in absolute ethyl alcohol every 3 months and were stored under nitrogen in the dark at −20°C. On the day of use, they were diluted to 2 µM in Tris–sodium chloride buffer (10 mM Tris, 0.9% [w:v] NaCl, pH 7.4).

Four quartz cuvettes were prepared, each containing HPM and one of the three fluorescent probes; final concentrations were 1 µM probe and 50 µg of HPM protein per milliliter in a total volume of 3.0 ml of Tris-NaCl buffer. The fourth cuvette contained HPM and Tris-NaCl buffer in place of probe solution and was used to measure background fluorescence. Cuvettes were then incubated for 10 minutes in the dark.

Fluorescence polarization was performed on an Alphascan spectrofluorometer (Photon Technology International, South Brunswick, New Jersey) in a T-format equipped with excitation and dual-emission monochromators and a four-position sample turret with the water jacket set to maintain the cuvette temperature at 25°C. Excitation slit width was 5 nm, and both emission slits were 8 nm. For cPNA and tPNA, excitation and emission wavelengths were 322 and 410 nm, respectively; those of DPH were 358 and 428 nm, respectively. A Teflon-coated magnetic micro stirring bar was placed in each cuvette to mix the contents continuously. For fluidity determination, emission intensity was recorded from each cuvette at 20 records per second for 10 seconds every 2 minutes for 90 minutes.

Statistical Analyses

Microscopic and ATP Data—All microscopic and ATP data were transformed to a standard normal distribution and then were analyzed using analysis of variance within the General Lin-

Table 1. *Adenosine triphosphate (ATP) concentration (μg per 1.2 × 10⁹ spermatozoa) of extended boar spermatozoa prior to cryopreservation and following cryopreservation in 0.5-ml straws**

Extender†	Extender A at 22°‡	Extender a at 5°C‡	Extenders A + B at 5°C‡	Postthaw‡
mBF5	4.73 ± 0.03[b]	2.85 ± 0.06[b]§	2.89 ± 0.07[b]	0.60 ± 0.12[b]‖
EY	4.94 ± 0.03[a]	4.47 ± 0.08[a]§	4.48 ± 0.07[d]	0.87 ± 0.12[b]‖
GLY	4.69 ± 0.04[b]	2.96 ± 0.06[b]§	2.68 ± 0.07[b]	0.76 ± 0.12[b]
EYGLY	4.92 ± 0.03[a]	4.46 ± 0.06[a]§	3.68 ± 0.07[a]¶	1.50 ± 0.12[a]
EYGLY:OEP	4.93 ± 0.03[a]	4.71 ± 0.06[a]	3.78 ± 0.07[a]¶	1.67 ± 0.11[a]‖
GYL:0.1P	4.70 ± 0.03[b]	3.55 ± 0.06[c]§	3.23 ± 0.07[c]	0.84 ± 0.11[b]
EYGLY:0.1P	4.94 ± 0.04[a]	4.50 ± 0.06[a]§	3.60 ± 0.07[a]¶	1.88 ± 0.13[a]‖
EYGLY:0.01P	4.92 ± 0.03[a]	4.51 ± 0.06[a]§	3.63 ± 0.07[a]¶	1.81 ± 0.11[a]

* Values are least square means ± SEM of normalized data; $n = 20$ per extender.
† For definitions of extenders, see Materials and Methods.
‡ Within each column, least square means with unlike superscripts are different ($P < 0.05$).
§ Within extender, ATP concentration of sperm in Extender A at 5°C differs from sperm in Extender A at 22°C.
‖ Within extender, ATP concentration of postthaw sperm differs from sperm in Extenders A + B at 5°C.
¶ Within extender, ATP concentration of sperm in Extenders A + B at 5°C differs from sperm in Extender A at 5°C.

ear Models procedure (Statistical Analysis Systems, 1985). An index of spermatozoa function was created by multiplying the mean total motility (MOT) times the normal acrosome morphology (NAR) for each sample (MOT·NAR), thereby combining two types of functional measures into one value that might be more reflective of overall spermatozoa function; this index was analyzed similarly. Statistical models included the following variables: extender, boar, ejaculate, and the step of the cryopreservation process. Differences among treatments were determined by utilizing predicted differences of least squares means and pooled standard errors of the mean for each treatment.

Fluorescent Polarization Data—Prior to statistical analysis, each record was corrected for background fluorescence by subtracting the emissions recorded at the appropriate wavelengths from the blank cuvette. Fluorescent intensity data were transformed into polarization values (PVs) using the Perrin equation (Shinitzky and Barenholz, 1978) as described previously (Canvin and Buhr, 1989). Polarization values are inversely related to fluidity; therefore, the lower the PV, the more fluid the membrane. The PVs were then transformed to a standard normal distribution. The 200 records collected at each 10-second reading were pooled to produce a mean, yielding 45 separate means over the 90 minutes.

To determine the rate of change in fluidity over time, the initial PV for each probe in each replicate was set to zero, and all subsequent PVs were adjusted accordingly (Canvin and Buhr, 1989). Linear regression (Statistical Analysis Systems, 1985) was used to fit a curve to data from each cuvette within each experiment. Residual plots assessed the goodness-of-fit for each curve and determined whether linear, quadratic, or cubic models best described the data. Intercepts were analyzed using analysis of variance within the General Linear Models procedure (Statistical Analysis Systems, 1985). Differences among boars or treatments were determined by utilizing predicted differences using least squares means and pooled standard errors of the mean for each treatment.

To test for differences in the rate of fluidity change among probes, among boars, among extender treatments, or between frozen-thawed and pooled fresh spermatozoa, one selected data set was subtracted from the other data set of interest, and the resulting data set was analyzed by linear regression (Statistical Analysis Systems, 1985). A slope significantly different from zero ($P < 0.05$) indicated that the slopes of the two original data sets were different (Buhr et al, 1993).

Relationships among fluidity and microscopy and ATP data were calculated using linear regression (Statistical Analysis Systems, 1985). Regression coefficients were calculated between the microscopy data and the ATP concentrations (dependent variables) and the fluidity data (independent variables). Regression analyses generating positive or negative slopes significantly different from zero indicated a positive or negative relationship between the dependent and independent variables being tested. Testing the resulting slopes for heterogeneity among treatments identified differences in this relationship among treatments.

Results

Spermatozoal Function

The concentration of ATP in a fresh ejaculate (micrograms per milliliter) at 35 and 22°C differed among boars (data not shown). When this value was converted to correct for cell number (micrograms of ATP per 1.2×10^9 spermatozoa), ATP concentration differed among boars only at 22°C (range: 3.84 to 4.94 ± 0.13; $P < 0.05$). No other functional parameters differed among boars. Mean total motility, ejaculate ATP concentration, and MOT·NAR were significantly less at 22°C than at 35°C (data not shown; $P < 0.05$), although declines in individual boars were seldom significant.

The different extenders were added at 22°C, and sperm were then cooled to 5°C. The concentration of ATP declined from 22 to 5°C in sperm in all extenders, except EYGLY:OEP; the presence of egg yolk lessened this decline (Table 1; $P < 0.05$). There were a few small, but statistically significant, differences in motility and MOT·NAR at 22°C (Table 2). At 5°C, before addition of the glycerol-containing fraction B, sperm in all extenders with egg yolk had similar ATP concentrations (Table 1)

Table 2. *Percentage of spermatozoa with normal acrosomes (NAR), percentage of spermatozoa that were motile (MOT), and the combined functional measure (MOT·NAR) of extended boar spermatozoa prior to cryopreservation and following cryopreservation in 0.5-ml straws**

	NAR†				MOT†‡		MOT·NAR†‡	
Extender§	Extender A at 22°C	Extender A at 5°C	Extenders A + B at 5°C	Postthaw	Extender A at 22°C	Postthaw	Extender A at 22°C	Postthaw
mBFS	96 ± 1^{a}	87 ± 2^{b}‖	89 ± 1^{b}	24 ± 3^{abc}¶	74 ± 1^{abc}	0 ± 1^{b}¶	7076 ± 135^{ab}	0 ± 97^{a}¶
EY	96 ± 1^{a}	93 ± 1^{a}	94 ± 1^{a}	38 ± 4^{ac}¶	69 ± 1^{bcd}	1 ± 1^{b}¶	6600 ± 129^{b}	41 ± 101^{a}¶
GLY	96 ± 1^{a}	86 ± 1^{bc}‖	88 ± 1^{b}	13 ± 4^{c}¶	74 ± 1^{ade}	0 ± 2^{b}¶	7116 ± 135^{ab}	20 ± 105^{a}¶
EYGLY	97 ± 1^{a}	94 ± 1^{a}‖	94 ± 1^{a}	31 ± 3^{abc}¶	73 ± 1^{ce}	8 ± 1^{a}¶	6995 ± 129^{ab}	340 ± 97^{ab}¶
EYGLY:OEP	96 ± 1^{a}	94 ± 1^{a}	95 ± 1^{a}	46 ± 4^{c}¶	75 ± 1^{ae}	13 ± 1^{a}¶	7167 ± 129^{ab}	791 ± 101^{b}¶
GLY:0.1P	96 ± 1^{a}	83 ± 1^{c}‖	86 ± 1^{b}#	16 ± 3^{bd}¶	79 ± 1^{a}	2 ± 1^{b}¶	7516 ± 129^{a}	41 ± 97^{a}¶
EYGLY:0.1P	96 ± 1^{a}	92 ± 1^{a}‖	94 ± 1^{a}	36 ± 4^{ac}¶	75 ± 1^{abc}	12 ± 2^{a}¶	7168 ± 135^{ab}	618 ± 101^{b}¶
EYGLY:0.01P	96 ± 1^{a}	92 ± 1^{a}‖	93 ± 1^{a}	35 ± 3^{ac}¶	77 ± 1^{ae}	8 ± 1^{a}¶	7359 ± 129^{a}	409 ± 97^{ab}¶

* Values are least square means ± SEM of normalized data; n = 20 per extender.
† Within each column, least square means with unlike superscripts are different ($P < 0.05$).
‡ Motility was not determined at 5°C.
§ For definitions of extenders, see Materials and Methods.
‖ Within extender, acrosome morphology of sperm in Extender A at 5°C differs from sperm in Extender A at 22°C.
¶ Within extender and function, postthaw value differs from prefreeze. (Extenders A + B at 5°C for NAR; 22°C for MOT and MOT·NAR).
# Within extender, acrosome morphology of sperm in Extenders A + B at 5°C differs from sperm in Extender A at 5°C.

and higher NAR than those without egg yolk (Table 2; $P < 0.05$). Addition of fraction B immediately reduced sperm ATP concentrations in extenders containing egg yolk and glycerol plus or minus surfactant, as compared to sperm in the egg yolk–only extender (Table 1; $P < 0.05$).

Cryopreservation decreased all functional parameters in all extenders, as compared to prefreeze measures. In cryopreserved sperm, ATP concentration and total motility were higher in extenders containing egg yolk and glycerol ($P < 0.05$), NAR was superior when the extender contained egg yolk, and MOT·NAR was highest in EYGLY:OEP or EYGLY:0.1P extenders (Table 2).

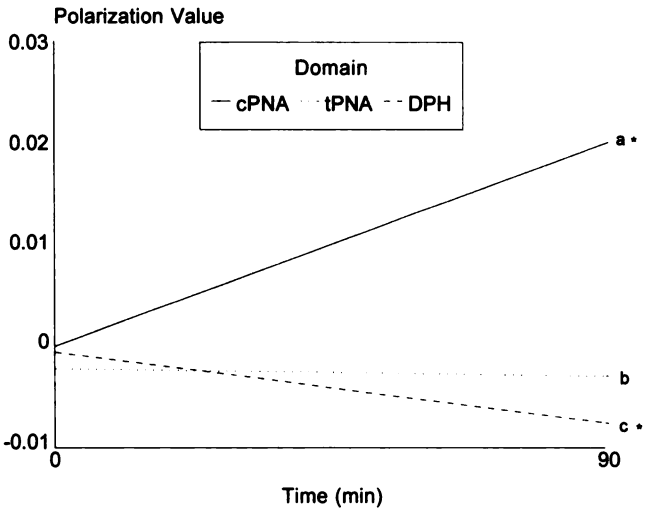

FIG. 1. Change in fluidity of three head plasma membrane domains isolated from pooled fresh boar spermatozoa. Increasing polarization value equals decreasing fluidity. Slopes with different superscripts (a, b, c) differ ($P < 0.05$). Asterisk (*) indicates that the slope differs from zero ($P < 0.05$).

Spermatozoal Membrane Fluidity

Fresh Spermatozoa—The fluidity of the different HPM domains changed over time at differing rates. Rates of change ranked as follows: cPNA > tPNA = DPH ($P < 0.05$) for each individual boar (data not shown) but cPNA > tPNA > DPH ($P < 0.05$) when the HPM was obtained from pooled semen (Fig. 1). The fluidity of the cPNA domain always decreased over time (significant positive slope; $P < 0.05$), the fluidity of the tPNA domain did not change (slope = 0), and the DPH domain fluidity either increased (boars 1, 3, and pooled; $P < 0.05$) or was static. The intercepts from these normalized data, being the mathematical start point of a line, were naturally influenced by subsequent values and, although they often differed from zero, did not differ among boars or domains.

Frozen-Thawed Spermatozoa—Fluidity of the DPH domain in membranes from fresh spermatozoa changed at a different rate than that of any cryopreserved sperm (Figs 2, 3; $P < 0.05$). Fluidity of the tPNA domain of fresh spermatozoa changed at a different rate ($P < 0.05$) from that of sperm cryopreserved in four extenders (mBF5, GLY, EYGLY, and EYGLY:0.01P), whereas cPNA domain fluidity was similar for fresh and cryopreserved sperm.

For the HPM from sperm cryopreserved in any extender, the three domains always changed fluidity at different rates, except for treatments containing OEP or 0.1% Pluronic (Figs. 2, 3). However, extenders did not affect the rate of change for any domain; cPNA fluidity declined and tPNA fluidity was stable for all treatments.

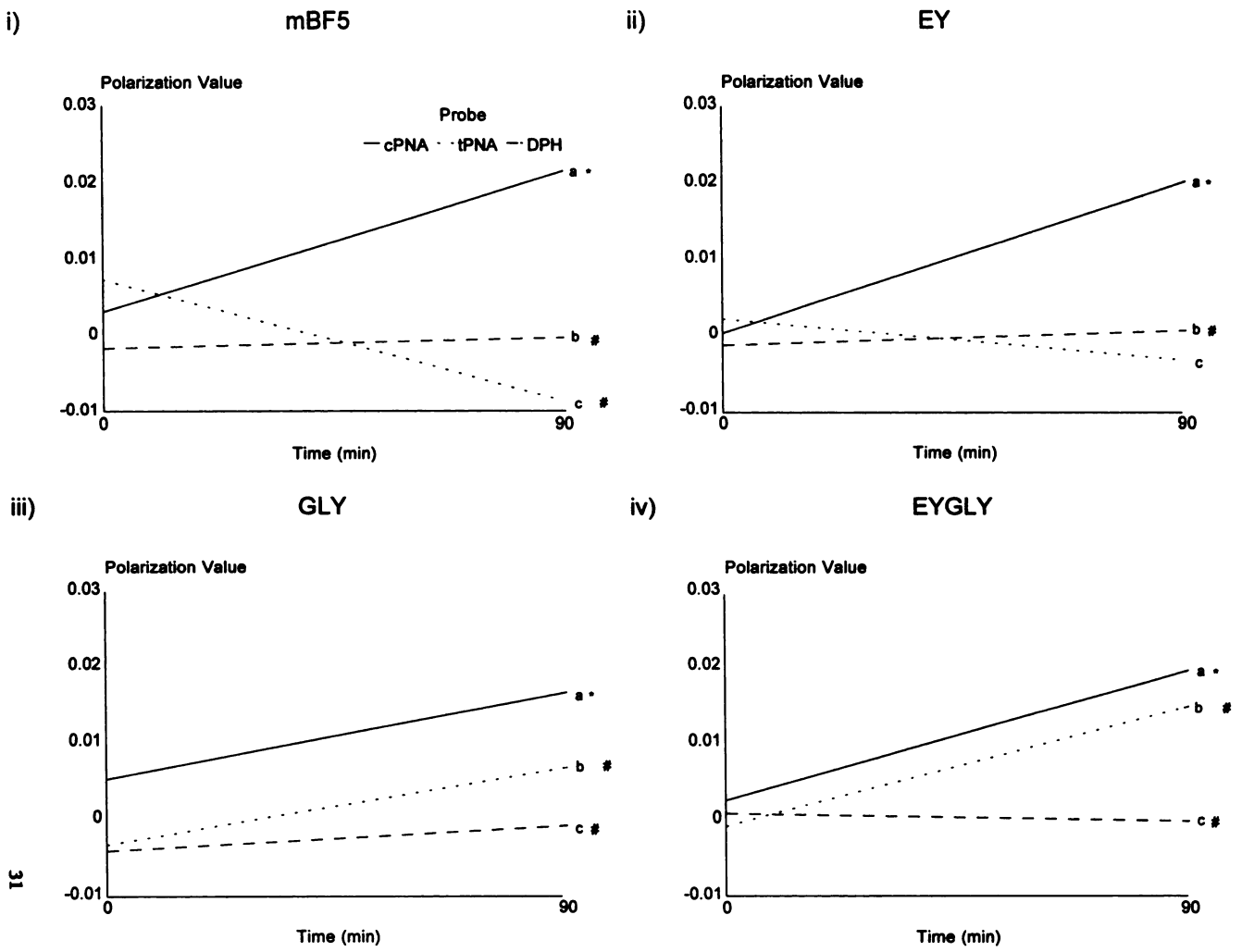

FIG. 2. Change in fluidity of three head plasma membrane domains isolated from boar spermatozoa cryopreserved in straws in four different extenders (n = 4 per extender). Minimal BF5 (mBF5) is BF5 extender with no egg yolk, glycerol, or surfactant; EY is BF5 extender with 20% egg yolk but no glycerol or surfactant; GLY is BF5 extender with 3% glycerol but no egg yolk or surfactant; EYGLY is BF5 extender with egg yolk and glycerol but no surfactant. Increasing polarization value equals decreasing fluidity. Slopes with different superscripts (a, b, c) differ ($P < 0.05$). Asterisk (*) indicates that the slope differs from zero ($P < 0.05$). Number sign (#) indicates that the slope differs from the slope of the domain in fresh sperm ($P < 0.05$; see Fig. 1).

Although DPH fluidity increased significantly in EYGLY:0.1P and was otherwise stable, overall, the rates of change in the DPH domain did not differ among treatment extenders. As expected, the intercepts from these normalized data did not differ among domains or treatments.

Relationship of Membrane Structure to Sperm Function

The postthaw function of sperm cryopreserved in extenders containing egg yolk plus glycerol plus surfactant was significantly correlated with membrane fluidity parameters (Table 3). The initial fluidity (intercept) of the cPNA domain of sperm cryopreserved in either EYGLY:OEP or EYGLY:0.1P was positively related to ATP, motility, and MOT·NAR, meaning that improved function was related to lower initial fluidity (higher PV) of the bulk lipid domain monitored by cPNA. Improved MOT·NAR of sperm in these same two extenders was similarly correlated to lower initial fluidity of DPH, which also monitors bulk-phase membrane lipids. In contrast, function of sperm cryopreserved in EYGLY:0.01P was better when the cPNA domain was more fluid initially. The rate of change of the gel-phase (tPNA) domain was negatively related to function, indicating that the more rapid the rate of solidification of the gel domain, the poorer was the function.

Discussion

Extender composition clearly affected sperm function during and after the cryopreservation process. Furthermore, the fluidities of HPM domains in frozen-thawed sperm differed from that of fresh sperm and were unique-

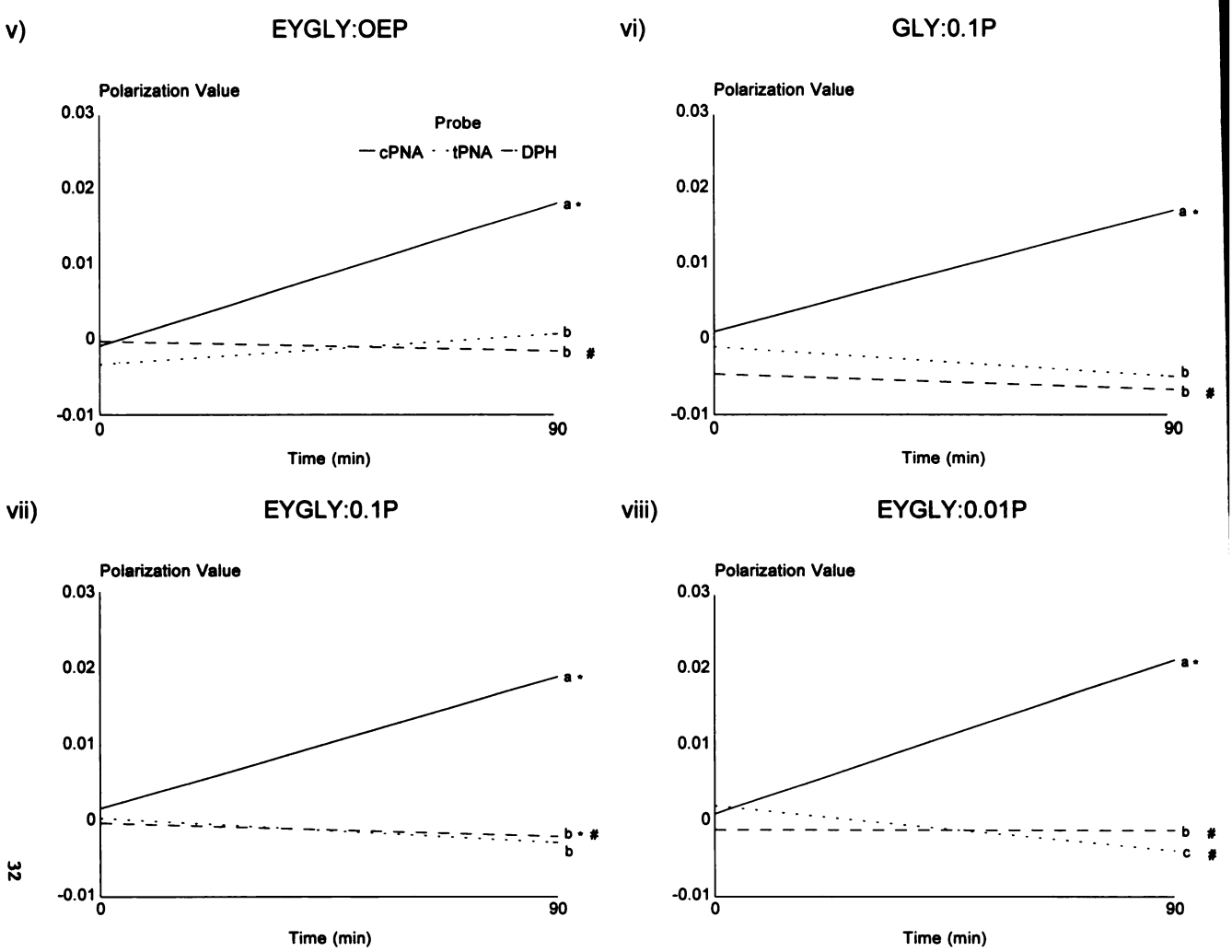

FIG. 3. Change in fluidity of three head plasma membrane domains isolated from boar spermatozoa cryopreserved in straws in four different extenders (n = 4 per extender). EYGLY:OEP is BF5 extender with 20% egg yolk, 3% glycerol, and 0.33% Orvus ES Paste; GLY:0.1P is BF5 extender with no egg yolk, 3% glycerol, and 0.1% Pluronic; EYGLY:0.1P or EYGLY:0.01P is BF5 extender with 20% egg yolk, 3% glycerol, and 0.1% Pluronic or 0.01% Pluronic, respectively. Increasing polarization value equals decreasing fluidity. Slopes with different superscripts (a, b, c) differ ($P < 0.05$). Asterisk (*) indicates that the slope differs from zero ($P < 0.05$). Number sign (#) indicates that the slope differs from the slope of the domain in fresh sperm ($P < 0.05$; see Fig. 1).

ly related to both the treatments given the whole sperm and the postthaw sperm function. Differences in fluidity characteristics of boar sperm membranes have been shown before (Buhr et al, 1989; Buhr and Pettitt, 1996), but the current linkage of the behavior of three domains with the function of intact sperm provides valuable insights, both into how membrane structure and cell function are related and into the specific effects of the various extender components.

Adenosine triphosphate concentration appeared to be a sensitive functional parameter as it alone detected male-to-male differences in fresh, unextended semen and permitted detection of many extender-related effects in cooled and cryopreserved sperm. The immediate decline in cellular ATP seen on addition of glycerolated extender was expected (Pursel et al, 1978b) and apparently occurred only in extenders with egg yolk plus surfactant, because cellular ATP in the absence of egg yolk was already so low that glycerol had no further impact. The osmotic tolerance limits for boar spermatozoa during glycerol addition have recently been established (Gilmore et al, 1998), and it would be interesting to determine the relationship of spermatozoal ATP and membrane fluidity to differing osmotic stress during cryopreservation.

Pluronic with or without egg yolk improved or maintained sperm function during and after cryopreservation, whereas OEP improved postthaw sperm function only in the presence of egg yolk (Pursel et al, 1978a; Hofmo and Almlid, 1991). Although not directly investigated in this study, the different effects between surfactants may be due to their different chemistries. Low-affinity, nonionic surfactants, like Pluronic, insert into a membrane and

Table 3. *Relationships among postthaw sperm function and head plasma membrane domain fluidity**

Extender†	Spermatozoa function	Fluidity parameter	Relationship (sign and *P* value)	R^2
EYGLY:OEP	% Motile	cPNA intercept	+0.0001	0.96
		tPNA slope	−0.0028	0.88
	ATP	cPNA intercept	+0.0144	0.92
	MOT·NAR	cPNA intercept	+0.0001	0.96
		DPH intercept	+0.0004	0.87
		tPNA slope	−0.0008	0.84
EYGLY:0.01P	% Motile	cPNA intercept	−0.0054	0.96
	ATP	cPNA intercept	−0.0209	0.92
	MOT·NAR	cPNA intercept	−0.0027	0.96
EYGLY:0.1P	% Motile	cPNA intercept	+0.0001	0.96
		tPNA slope	−0.0208	0.88
	ATP	cPNA intercept	+0.0002	0.92
	MOT·NAR	cPNA intercept	+0.0001	0.96
		DPH intercept	+0.0277	0.87

ATP, adenosine triphosphate; MOT·NAR, combined functional measure of motility (MOT) and normal acrosomes (NAR); cPNA, *cis*-parinaric acid; tPNA, *trans*-parinaric acid; DPH, 1,6-diphenyl-1,3,5-hexatriene.

* Regression coefficients were calculated between the functional and fluidity parameters, and the resulting slopes were tested for positive or negative relationships and heterogeneity among treatments.

† For definitions of extenders, see Materials and Methods.

bind to the hydrophobic segments of the glycoproteins with little change to the characteristics of the membrane or function of the glycoprotein (Helenius and Simons, 1975). Even at very high concentrations, nonionic detergents release peripheral proteins but will not dissociate the lipid bilayer (Helenius and Simons, 1975; de La Maza et al, 1992; Inoue et al, 1992; Jones, 1992). Alkyl ionic detergents, like OEP (Helenius et al, 1979), on the other hand, bind to membranes with high affinity, expose hydrophobic portions of membrane proteins, and disperse the lipid bilayer into mixed micelles (Helenius and Simons, 1975; de La Maza et al, 1992; Inoue et al, 1992; Jones, 1992). Presumably, these different associations between surfactant, egg yolk, and membrane constituents affect how the membrane is affected (or not) by the cryopreservation process.

As expected, glycerol and egg yolk provided optimal postthaw survival. All functional measures (ATP, NAR, MOT, and MOT·NAR) found egg yolk–glycerol, with or without surfactant, to give equal, maximal protection. The surfactant OEP is known to improve postthaw survival of boar semen frozen in pellets (Pursel et al, 1978a), but it is possible that the reduced functional damage typical of straw freezing (Adler et al, 1968; Pickett and Burndtson, 1974) has made this effect less evident.

The initial, unadjusted fluidity (PV) of the HPM domains was much more sensitive to extender component and boar (Buhr and Pettitt, 1996) than was the rate of change within that domain. Similarly, the fluidity intercepts were related to spermatozoal function (Table 3) much more than was the rate of change within that domain. The initial fluidity may reflect the physiological status of the membrane. Capacitation and the acrosome reaction alter membrane composition (Langlais and Roberts, 1985; Nikolopoulou et al, 1986), and a recent report suggests that cryopreservation induces premature capacitation of bovine sperm, which is evident immediately on thawing (Cormier et al, 1998). Therefore, initial fluidity of these domains might appear to be a useful diagnostic tool of HPM status.

Despite these relationships of initial absolute fluidity with function, we have elected to report solely the correlations found with the intercepts and slopes, i.e., the dynamic behavior of the various domains, for several reasons. Firstly, we have shown in other species that the rate of change of membrane-based activities, and not the absolute starting point, is related to *in vivo* fertility (Bailey et al, 1994). Secondly, absolute PVs provide a limited snapshot-in-time of the membrane's fluidity at that particular instant and, statistically speaking, are more subject to random variation. The intercepts reflect initial starting values as influenced by the subsequent changes in fluidity over time (slopes) and, thus, are influenced by the membrane's dynamic behavior. Slopes have the statistical advantage of incorporating the entire measurement period and are more reliable and less subject to random variation due to the large data set. Most importantly, slopes and intercepts have the biological advantage of representing the unique kinetic behavior of each domain, which would seem to be more important to the processes of membrane change involved in capacitation and the acrosome reaction. In the HPM from fresh sperm, the two bulk lipid domains changed fluidity over time, with the cPNA domain becoming less fluid and the DPH domain becoming more fluid. Presumably, the space between the bilayer is fluidizing since both probes are supposed to partition

equally into gel and fluid domains (Sklar et al, 1979; Lentz, 1989, 1993), but only DPH partitions near this site (Borenstain and Barenholz, 1993; Lentz, 1993).

After cryopreservation, the DPH domain could no longer fluidize, regardless of treatment, but freezing did not affect the dynamic behavior of the gel domain and the other bulk domain. Therefore, the inner membrane core appears to be very sensitive to the detrimental effects of cryopreservation of the intact sperm, perhaps because the core is less readily accessed by the protective components of the egg yolk/surfactant. The different extenders did not affect the dynamics of the DPH domain, even though egg yolk and OEP interacted to affect the initial, unadjusted fluidity (Buhr and Pettitt, 1996). Therefore, extender components affect the absolute fluidity of the DPH domain, whereas freezing has an overwhelming effect on this domain's dynamics.

Even though the core of the membrane appears most drastically affected by the cryopreservation process, it was the bulk lipids of the bilayer leaflet, monitored by cPNA, that appeared most linked to function of the whole sperm. Interestingly, all correlations of membrane structure with cell function were in sperm cryopreserved in extenders that preserved viability and motility, and, in these sperm, the cPNA bulk lipid domain was correlated with more aspects of sperm function than the DPH domain. Basically, sperm cryopreserved in egg yolk–glycerol plus OEP or 0.1% Pluronic functioned best when the bulk domains were less fluid initially and the gel domain solidified more slowly. In contrast, no fluidity characteristic of fresh sperm was correlated with any functional measure. This may indicate that the optimal domain fluidity in cryopreserved sperm differs from the optimal fluidity of HPM domains in fresh spermatozoa. Alternatively, the lack of correlations in fresh sperm may result from the large percentage of robust sperm overwhelming the contributions due to less healthy cells. The greater range of quality in frozen-thawed sperm allows a statistically significant correlation to be detected. The only correlations were with sperm cryopreserved in treatments that maintained some viability and motility, confirming that a wide range of function provides the greatest power to detect statistical relationships; neither the all-healthy fresh sperm nor the all-dead frozen sperm had their structure and function related.

In conclusion, the behavior of domains in the HPM of boar spermatozoa is complicated and affected by cryopreservation processes and extender composition. Cryopreservation of intact sperm had an overwhelming effect on the postthaw function and the HPM fluidity, whereas the presence or absence of egg yolk, glycerol, and/or surfactant in cryopreservation extenders had varying effects on different structural and functional parameters. No extender maintained membrane structure in a state identical to that of fresh sperm, and, as some extenders resulted in apparently normal spermatozoa following freezing and thawing, such maintenance might not be desirable. The initial fluidity of a domain after thawing is more sensitive to the extender components than are changes in fluidity of that domain. Initial fluidity of the bulk lipids and changes in fluidity of the gel-phase lipids in surfactant-containing extenders are related to spermatozoa function, indicating that HPM fluidity and its behavior over time are important components in proper spermatozoa function postthaw. Further work is required to completely understand the relationships between extender composition and membrane status/behavior following cryopreservation, but, based on this work, 0.1% Pluronic appears to be an acceptable substitute for 0.33% OEP in the BF5 extender.

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