

# Rooster semen cryopreservation: Effect of pedigree line and male age on postthaw sperm function

J. A. Long,<sup>\*1</sup> D. C. Bongalhardo,<sup>\*</sup> J. Pelaéz,<sup>\*</sup> S. Saxena,<sup>†</sup> P. Settar,<sup>†</sup>  
N. P. O'Sullivan,<sup>†</sup> and J. E. Fulton<sup>†</sup>

*\*USDA, Agricultural Research Service, Animal and Natural Resources Institute, Animal Biosciences and Biotechnology Laboratory, Beltsville, MD 20705; and †Hy-Line International, Dallas Center, IA 50063*

**ABSTRACT** The fertility rates of cryopreserved poultry semen are highly variable and not reliable for use in preservation of commercial genetic stocks. Our objective was to evaluate the cryosurvival of semen from 8 pedigreed layer lines at 2 different ages: the onset and end of commercial production. Semen from 160 roosters (20/line) was frozen individually with 11% glycerol at 6 and 12 mo of age. Glycerol was removed from thawed semen by Accudenz gradient centrifugation. The viability of thawed sperm from each male was determined using fluorescent live-dead staining and flow cytometry; sperm velocity parameters were measured using computerized motion analysis. The fertilizing ability of thawed sperm was evaluated *in vitro* by assessing hydrolysis of the inner perivitelline membrane. The postthaw function of sperm from the elite lines varied widely, despite the fact that fresh semen from all of these

lines typically yielded high fertility rates. The percentage of thawed sperm with intact plasma membranes ranged from  $27.8 \pm 2.1$  to  $49.6 \pm 1.9$  and varied among lines and between age groups. Thawed sperm from 2 lines consistently demonstrated the highest and lowest motility parameters, whereas the velocity parameters of the remaining 6 lines varied widely. The mean number of hydrolysis points per square millimeter of inner perivitelline membrane ranged from  $12.5 \pm 4.1$  (line 2) to  $103.3 \pm 30.2$  (line 6). Age effects were observed for 4 out of 8 lines; however, improved postthaw sperm function at 12 mo of age was not consistent for all 3 assays. These results demonstrate variability among pedigreed lines in withstanding glycerol-based semen cryopreservation and provide a model for delineating genotypic and phenotypic factors affecting sperm cryosurvival.

**Key words:** spermatozoa, glycerol, chicken, perivitelline membrane

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## INTRODUCTION

Primary poultry breeders would benefit from the ability to stockpile germplasm from elite lines, especially in the context of disease outbreak; however, fertility rates from frozen-thawed poultry semen are highly variable and unreliable for preservation of genetic stocks. Poultry sperm tolerance to cryopreservation, or freezability, has been shown to vary among genotypic strains of chickens (Bacon et al., 1986; Froman and Bernier, 1987; Tajima et al., 1990; Alexander et al., 1993; Blesbois et al., 2007). In particular, poultry sperm freezability often is poor in lines with low fecundity, requiring more invasive techniques such as intramaginal insemination to produce chicks from frozen-thawed semen (Bacon et al., 1986). It has been suggested that the wide differences

in poultry sperm freezability among lines and strains are linked to heritable physiological or biochemical, or both, attributes (Mitchell et al., 1977).

A series of landmark studies in the 1980s investigated this possibility using a broiler-type line selected for the duration of fertility of frozen-thawed semen with glycerol as the cryoprotectant (Ansah and Buckland, 1983). After 8 generations of selection, both physiological changes and biochemical differences were detected between the selected line and random control lines. The selected line demonstrated a greater uptake of glycerol and carrier-mediated substances than randomly selected control lines (Ansah and Buckland, 1982a). Spermatozoa from the selected line had lower total cholesterol and a lower cholesterol:phospholipid ratio compared with control lines; seminal plasma cholesterol and phospholipid levels also were lower in the selected line (Ansah and Buckland, 1982b). The heritability estimates of these traits ranged from 0 to 0.81. In addition to being more permeable to glycerol, sperm from the selected line also exhibited higher oxygen uptake,

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<sup>1</sup>Corresponding author: Julie.Long@ars.usda.gov

indicating that intracellular organelles were more resistant to freeze-thaw injury (Scott et al., 1980). Selection of males for duration of fertility with glycerol frozen-thawed semen also caused correlative increases in one seminal plasma protein fraction; however, the specific proteins were not identified in this study (Bentley et al., 1984). Most interesting is that 10+ generations of selecting for duration of fertility with frozen-thawed semen ultimately resulted in higher fertility rates of fresh semen from the selected line (Xia et al., 1988; Alexander et al., 1993), as well as higher numbers of sperm trapped between the outer and inner perivitelline membranes (Alexander et al., 1993), than fresh semen from control lines.

Although the broiler line selected for improved fertility of frozen-thawed semen no longer exists, the cumulative data from this decade of research provide strong evidence for the existence of heritable physiological and biochemical factors that influence the freezability of poultry semen. Given the inability of primary breeders to rely on cryopreserved semen for germplasm archiving, our approach was to determine the freezability of semen from multiple elite layer lines by evaluating the function of frozen-thawed spermatozoa from individual males at 2 distinct ages, with the goal of developing a model to further delineate the factors responsible for successful cryopreservation. We present here the initial findings that support the feasibility of this model for understanding the compromised physiology of cryopreserved poultry spermatozoa.

## MATERIALS AND METHODS

### *Birds, Semen Collection, and Semen Evaluation*

A total of 160 roosters from 8 pedigree lines ( $n = 20$ /line) were housed individually and maintained under an artificial photoperiod (14L:10D) from 5 to 12 mo of age. Pedigree lines were coded numerically as 1 to 8. Hens from lines 5 and 8 laid brown eggs, whereas the remaining 6 lines laid white eggs. Semen was collected routinely 2 to 3 times each week from each male throughout production (5.5 to 12 mo of age) using the massage method (Burrows and Quinn, 1937). Undiluted, individual semen samples were transported to the laboratory in extruded polystyrene foam coolers to maintain ambient temperature. To ensure the quality of semen before cryopreservation, undiluted semen samples from each male were evaluated for total volume, sperm concentration, and sperm mobility as described previously (Long and Kulkarni, 2004).

### *Semen Cryopreservation and Thawing*

At 6 and 12 mo of age, individual semen samples from each male were cryopreserved using glycerol as

the cryoprotectant. Semen was diluted 1:1 with room temperature Lake's Prefreeze Diluent [magnesium acetate, 3.2 mM; sodium glutamate, 114.0 mM; potassium acetate, 51.0 mM; fructose, 44.0 mM; N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, 4.7 mM; polyvinylpyrrolidone, 0.3%; glutathione, 1.3 mM; pH 7.1; 340 mOsm/kg] and allowed to cool for 10 min in a 4°C walk-in cold room. Cooled semen was further diluted with dropwise addition of 4°C Lake's Diluent containing 33% glycerol to yield a final concentration of 11% glycerol. After 30 min of equilibration at 4°C, semen was loaded into straws. Straws were initially immersed in a prechilled (-20°C) ethanol bath for 30 min then held in liquid nitrogen vapor for 10 min before being plunged into liquid nitrogen. Straws were stored at -196°C for a minimum of 6 mo before thawing. Straws were thawed by immersion in a 3°C ice-water bath for 2 to 3 min.

### *Glycerol Removal*

Glycerol was removed from thawed semen as described by Long and Kulkarni (2004) using a discontinuous Accudenz gradient (Accurate Chemical and Scientific Corporation, Westbury, NY) consisting of a 12% layer (5.0 mL) and a 30% layer (0.5 mL). Semen was gently layered on the top of the gradient and centrifuged ( $1,250 \times g$ ; 4°C) for 25 min with a gradual stop (i.e., without using the brake). After centrifugation, the diluent and glycerol remained above the 12% layer, whereas spermatozoa were present at the interface between the 12 and 30% layers. Recovered spermatozoa were resuspended in Beltsville Poultry Semen Extender (BPSE; Continental Plastic Corporation, Delavan, WI) and assessed for membrane integrity, motility, and hydrolyzing ability.

### *Sperm Viability Assessment*

The membrane integrity of thawed spermatozoa from lines 1 to 8 ( $n = 320$ ; 20 males/line; 2 age groups) was determined using the SYBR-14 propidium iodide (PI) stain combination (Donoghue et al., 1995). In brief, spermatozoa were diluted to  $9 \times 10^6$ /mL in BPSE and incubated for 15 min with 100 nM SYBR-14 and 12 nM PI. A Coulter Epics XL-MCLTM Flow Cytometer (Beckman Coulter, Miami, FL) equipped with a single 488-nm excitation source was used to evaluate 15,000 sperm/sample. Forward and side scatter gating were used to select single sperm from clumps and debris. Fluorescence signals from SYBR-14-stained and PI-stained spermatozoa were collected in FL1 (525 nm bandpass) and FL3 (620 nm bandpass) fluorescence detectors, respectively. Spermatozoa in the 525 nm range excluded PI and were classified as membrane-intact, whereas sperm in the 620 nm range incorporated PI and were classified as membrane-compromised and considered to be nonviable. Sperm exhibiting both fluorescent signals

were classified as partially membrane-compromised and also were considered to be nonviable.

### Sperm Motility Assessment

Computer-aided sperm motility analysis (CASA) was conducted using a Hamilton Thorne IVOS Sperm Analyzer (Hamilton Thorne Biosciences, Beverly, MA). Thawed spermatozoa from lines 1 to 8 ( $n = 320$ ; 20 males/line; 2 age groups) were diluted to  $30 \times 10^6$ /mL in BPSE and warmed to  $41^\circ\text{C}$  before being loaded into a 20- $\mu\text{m}$  Leja 4-chamber slide (Leja Products, Luzernestraat, the Netherlands). A minimum of 800 sperm tracks were evaluated for each sample chamber, averaging  $<10$  s of read time per chamber. The percentage of motile spermatozoa and the following motility parameters were measured: curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL), linearity (LIN), straightness (STR), lateral head displacement amplitude (ALH), and beat-cross frequency (BCF). Computer-aided sperm motility analysis minimum settings were 20.0  $\mu\text{m/s}$  (VAP), 30.0  $\mu\text{m/s}$  (VSL), and an 80% threshold for STR.

### Sperm Hydrolyzing Assessment

The hydrolyzing ability of thawed spermatozoa from lines 1 to 8 ( $n = 320$ ; 20 males/line; 2 age groups) was determined using the perivitelline assay (Robertson et al., 1997). Briefly, the inner and outer perivitelline layers of fresh eggs were separated by acid hydrolysis. Isolated squares (10  $\text{mm}^2$ ) of the inner perivitelline layer (IPVL) were stored in NaCl-2-[[Tris(hydroxymethyl)methyl]amino]ethanesulfonic acid at  $4^\circ\text{C}$  for 3 to 24 h. Before the assay, IPVL were rinsed with room temperature minimal essential medium (MEM) and transferred to wells containing 600  $\mu\text{L}$  of MEM at  $41^\circ\text{C}$ . Spermatozoa were diluted to  $4 \times 10^8$ /mL with NaCl-2-[[Tris(hydroxymethyl)methyl]amino]ethanesulfonic acid and incubated ( $41^\circ\text{C}$ ) for 30 min. Sperm aliquots (200  $\mu\text{L}$ ) were added to the wells containing MEM-IPVL to yield a final concentration of  $1.25 \times 10^7$ /mL. Sperm dilutions were based on the percentage of membrane-intact spermatozoa, rather than the concentration of sperm, in each thawed sample after glycerol removal. Sperm-IPVL mixtures were incubated ( $41^\circ\text{C}$ ) for 5 min. After incubation, IPVL were removed, washed with 1% NaCl to remove sperm, and carefully spread on slides. Slides were immediately photographed and later evaluated with Picasa software (<http://picasa.google.com>) to determine the number of holes per square millimeter. Thawed sperm from each male were incubated with IPVL isolated from the eggs of 3 different hens with 2 IPVL squares/egg, for a total of 6 IPVL per sperm sample. In addition to calculating the mean number of hydrolysis points, sperm hole data also were grouped into 8 discrete categories: zero holes (0), 1 to 10 holes (1), 11 to 20 holes (2), 21 to 40 holes (3), 41 to 60 holes

(4), 61 to 80 holes (5), 81 to 100 holes (6), and 100+ holes (7) for subsequent data analysis.

### Statistical Analysis

Prefreeze data analyzed were the total semen volume, sperm concentration, and sperm mobility. Post-thaw data analyzed were the percentage of membrane-intact sperm, motility parameters (VSL, VAP, VCL, STR, LIN, BCF, and ALH), the mean number of IPVL hydrolysis points, and hydrolysis point category (0 to 7). Repeated measures ANOVA were used to compare data between 6 and 12 mo of age for all lines combined and for each individual line (1 to 8). When interactions occurred between line and age, lines were compared at 6 and 12 mo separately using the GLM procedure. For hydrolysis categories, logistic regression was used to predict the natural logarithm of the odds for the males inside the lines being in 1 of the 8 categories. Pearson correlation coefficients among all traits were calculated first using the whole set of data and then among lines within each age and between age among each line. All procedures were performed using SAS software (Version 9.2, SAS Institute Inc., Cary, NY).

## RESULTS

### Fresh Semen Parameters

The semen parameters from each pedigreed line at 6 and 12 mo of age are shown in Table 1. Data from all lines were within acceptable quality standards for rooster semen, and each line is known to yield 90% fertility rates after insemination of fresh semen (Settar, unpublished data). Males from line 8 consistently yielded high semen volumes ( $557.9 \pm 33.6 \mu\text{L}$ ), whereas the remaining lines were more uniform in semen volume (range, 201.4 to 382.5  $\mu\text{L}$ ). Sperm concentrations ranged from 5.6 to  $9.5 \times 10^9$  sperm/mL and were similar for most lines between 6 and 12 mo of age ( $P > 0.05$ ); however, males from lines 1 and 4 yielded higher sperm concentrations at 12 mo of age ( $P < 0.05$ ), and males from line 3 produced more sperm at 6 mo of age ( $P < 0.05$ ). Sperm mobility values also varied among lines and between age groups. In particular, sperm mobility was higher for lines 3, 4, 6, 7, and 8 at 12 mo than at 6 mo of age ( $P < 0.05$ ).

### Sperm Viability

The percentage of thawed sperm with intact plasma membranes ranged from  $27.8 \pm 2.1$  to  $49.6 \pm 1.9$  and varied among lines and between age groups (Table 2). Overall, the viability data approximated a normal distribution, with 25% of the lines (2/8) exhibiting the highest number of intact sperm, 62.5% (5/8) demonstrating an average number of intact sperm, and 12.5% (1/8) having the lowest number of intact sperm. The

**Table 1.** Semen quality parameters of fresh rooster spermatozoa from 8 different pedigree lines at the onset and end of commercial production

Line	Volume ( $\mu\text{L}$ )		Sperm concentration ( $\times 10^9/\text{mL}$ )		Sperm mobility (optical density)	
	6 mo of age	12 mo of age	6 mo of age	12 mo of age	6 mo of age	12 mo of age
1	298.8 $\pm$ 13.7 <sup>ab,A</sup>	381.3 $\pm$ 18.3 <sup>b,B</sup>	7.4 $\pm$ 0.39 <sup>b,A</sup>	8.3 $\pm$ 0.36 <sup>bc,B</sup>	0.311 $\pm$ 0.05 <sup>b</sup>	0.277 $\pm$ 0.03 <sup>a</sup>
2	334.5 $\pm$ 22.4 <sup>b</sup>	382.5 $\pm$ 21.4 <sup>b</sup>	6.2 $\pm$ 0.24 <sup>ab</sup>	6.2 $\pm$ 0.37 <sup>a</sup>	0.377 $\pm$ 0.04 <sup>b</sup>	0.498 $\pm$ 0.07 <sup>c</sup>
3	260.5 $\pm$ 22.1 <sup>a</sup>	223.9 $\pm$ 22.3 <sup>a</sup>	9.5 $\pm$ 0.31 <sup>d,A</sup>	7.4 $\pm$ 0.63 <sup>b,B</sup>	0.330 $\pm$ 0.04 <sup>b,A</sup>	0.584 $\pm$ 0.05 <sup>cd,B</sup>
4	265.8 $\pm$ 20.3 <sup>a</sup>	201.4 $\pm$ 20.1 <sup>a</sup>	7.8 $\pm$ 0.34 <sup>bc,A</sup>	8.7 $\pm$ 0.36 <sup>c,B</sup>	0.201 $\pm$ 0.03 <sup>a,A</sup>	0.402 $\pm$ 0.06 <sup>b,B</sup>
5	301.3 $\pm$ 22.5 <sup>ab</sup>	340.3 $\pm$ 22.3 <sup>b</sup>	5.6 $\pm$ 0.30 <sup>a</sup>	6.0 $\pm$ 0.33 <sup>a</sup>	0.350 $\pm$ 0.05 <sup>b</sup>	0.273 $\pm$ 0.05 <sup>a</sup>
6	317.2 $\pm$ 33.5 <sup>ab</sup>	331.9 $\pm$ 30.7 <sup>b</sup>	8.3 $\pm$ 0.24 <sup>c</sup>	8.1 $\pm$ 0.36 <sup>bc</sup>	0.221 $\pm$ 0.03 <sup>a,A</sup>	0.613 $\pm$ 0.05 <sup>cd,B</sup>
7	333.8 $\pm$ 24.4 <sup>b,A</sup>	256.8 $\pm$ 20.8 <sup>a,B</sup>	7.6 $\pm$ 0.23 <sup>bc</sup>	7.7 $\pm$ 0.40 <sup>b</sup>	0.312 $\pm$ 0.04 <sup>b,A</sup>	0.513 $\pm$ 0.04 <sup>cd,B</sup>
8	618.8 $\pm$ 27.6 <sup>c,A</sup>	497.1 $\pm$ 39.5 <sup>c,B</sup>	5.9 $\pm$ 0.17 <sup>a</sup>	6.2 $\pm$ 0.22 <sup>a</sup>	0.264 $\pm$ 0.04 <sup>ab,A</sup>	0.495 $\pm$ 0.07 <sup>bc,B</sup>

<sup>a-d</sup>Within columns, different superscripts denote significantly different ( $P < 0.05$ ) means.

<sup>A,B</sup>Within line pairs (6 and 12 mo of age), different superscripts denote significantly different ( $P < 0.05$ ) means.

distribution of 2 of the lines within this normal population, however, was affected by age. For lines 3, 7, and 8, the percentage of viable sperm was higher for semen frozen at 12 mo than that at 6 mo of age ( $P < 0.05$ ). Line 7 shifted from the average group at 6 mo of age ( $37.3 \pm 1.3\%$ ) to the high group at 12 mo of age ( $47.6 \pm 1.5\%$ ); likewise, line 8 shifted from the lowest group at 6 mo ( $27.8 \pm 2.1\%$ ) to the average group at 12 mo of age ( $40.4 \pm 2.2\%$ ). Line 3 was in the highest group at both 6 ( $42.0 \pm 2.1\%$ ) and 12 ( $49.6 \pm 1.9\%$ ) mo of age, despite the improved sperm viability observed at 12 mo.

### Sperm Motility

Mean CASA velocity parameters of thawed spermatozoa are shown in Table 3. There were no differences among lines or between age groups for BCF ( $P > 0.05$ ). For lines 1, 2, 3, 4, 5, and 8, no differences ( $P > 0.05$ ) occurred between the 6- and 12-mo samples for any velocity or linearity parameters; therefore, these data were combined to evaluate line differences. For line 6, both VCL and ALH were higher ( $P < 0.05$ ) for 6-mo ( $105.2 \pm 3.4 \mu\text{m/s}$ ;  $6.3 \pm 0.6 \mu\text{m}$ ) than for 12-mo ( $89.0$

$\pm 5.0 \mu\text{m/s}$ ;  $3.6 \pm 0.5 \mu\text{m}$ ) samples. For line 7, ALH also was higher ( $P < 0.05$ ) at 6 mo ( $4.2 \pm 0.7 \mu\text{m}$ ) than 12 mo ( $1.9 \pm 0.5 \mu\text{m}$ ) of age. Line 6 consistently demonstrated the highest motility parameters and line 8 consistently exhibited the poorest motility parameters, whereas the velocity and linearity parameters of the remaining lines varied widely (Table 3). For example, spermatozoa from line 5 exhibited high ALH but low VAP and VCL values. The percentage of motile sperm from 3 lines was correlated with the percentage of membrane-intact sperm at both 6 and 12 mo of age (line 1,  $r = 0.55$ ,  $P = 0.001$ ; line 5,  $r = 0.62$ ,  $P = 0.002$ ; line 8,  $r = 0.39$ ,  $P = 0.019$ ).

### Sperm Hydrolyzing Ability

Frozen-thawed spermatozoa from lines 1–8 varied widely with respect to hydrolyzing ability (Table 4). The hydrolyzing ability of spermatozoa from lines 1, 2, 4, 5, and 8 was not affected ( $P > 0.05$ ) by the age of the male; however, the mean number of hydrolysis points per square millimeter of inner perivitelline membrane ranged widely from  $12.5 \pm 4.1$  (line 2) to  $96.5 \pm 25.0$  (line 5), clearly indicating potential differences in the fertilizing ability of frozen-thawed sperm among these 5 lines. Grouping the hydrolysis data from these lines into the 8 sperm hole categories further illustrates these differences (Figure 1). The odds of thawed sperm creating 40+ hydrolysis points per square millimeter of inner perivitelline membrane increase ( $P < 0.05$ ) if the roosters are from lines 4 and 5. Conversely, roosters from line 2 have a significant chance ( $P = 0.0009$ ) of creating fewer than 20 hydrolysis points per square millimeter of inner perivitelline membrane.

The hydrolyzing ability of frozen/thawed sperm from Lines 3, 6 and 7 was affected by the age of the male (Table 4), with sperm from 6-mo-old males hydrolyzing fewer holes per  $\text{mm}^2$  of inner perivitelline membrane than sperm from 12-mo-old males ( $P < 0.05$ ). For these 3 lines, sperm from 12-mo-old roosters created 3 times as many holes through inner perivitelline membrane sections than sperm from the same males at 6 mo of age. Figure 2 illustrates the age-specific shift

**Table 2.** Membrane integrity of frozen-thawed rooster spermatozoa from 8 different pedigree lines at the onset and end of commercial production

Line	% membrane-intact sperm (mean $\pm$ SEM)	
	6 mo of age	12 mo of age
1	36.3 $\pm$ 2.3 <sup>ab,A</sup>	41.1 $\pm$ 1.6 <sup>a,A</sup>
2	33.1 $\pm$ 1.3 <sup>ac,A</sup>	32.4 $\pm$ 1.0 <sup>b,A</sup>
3	42.0 $\pm$ 2.1 <sup>b,A</sup>	49.6 $\pm$ 1.9 <sup>c,B</sup>
4	38.3 $\pm$ 2.4 <sup>ab,A</sup>	41.4 $\pm$ 1.6 <sup>a,A</sup>
5	37.3 $\pm$ 2.4 <sup>ab,A</sup>	40.5 $\pm$ 1.4 <sup>a,A</sup>
6	40.1 $\pm$ 1.9 <sup>b,A</sup>	40.8 $\pm$ 1.8 <sup>a,A</sup>
7	37.1 $\pm$ 1.3 <sup>ab,A</sup>	47.6 $\pm$ 1.5 <sup>c,B</sup>
8	27.8 $\pm$ 2.1 <sup>c,A</sup>	40.4 $\pm$ 2.2 <sup>a,B</sup>

<sup>a-c</sup>Within columns, different superscripts denote significantly different ( $P < 0.05$ ) means.

<sup>A,B</sup>Within rows, different superscripts denote significantly different ( $P < 0.05$ ) means.

**Table 3.** Mean computer-aided sperm motility analysis velocity and linearity parameters ( $\pm$ SEM) of thawed rooster sperm from 6- and 12-mo age groups<sup>1</sup>

Line	VAP ( $\mu$ m/s)	VSL ( $\mu$ m/s)	VCL ( $\mu$ m/s)	STR (%)	LIN (%)	ALH ( $\mu$ m)	BCF (Hz)
1	41.9 $\pm$ 2.8 <sup>bc</sup>	28.3 $\pm$ 2.5 <sup>bc</sup>	86.3 $\pm$ 4.1 <sup>b</sup>	62.5 $\pm$ 2.5 <sup>b</sup>	35.5 $\pm$ 2.2 <sup>b</sup>	4.0 $\pm$ 0.8 <sup>a</sup>	34.3 $\pm$ 1.7 <sup>a</sup>
2	42.5 $\pm$ 3.6 <sup>bc</sup>	30.1 $\pm$ 3.8 <sup>bc</sup>	81.7 $\pm$ 4.2 <sup>b</sup>	62.9 $\pm$ 4.4 <sup>ab</sup>	37.2 $\pm$ 3.4 <sup>a</sup>	2.6 $\pm$ 0.5 <sup>bc</sup>	33.7 $\pm$ 2.5 <sup>a</sup>
3	47.7 $\pm$ 3.1 <sup>ab</sup>	31.0 $\pm$ 2.6 <sup>bc</sup>	99.2 $\pm$ 3.8 <sup>a</sup>	60.5 $\pm$ 3.3 <sup>b</sup>	34.2 $\pm$ 2.4 <sup>b</sup>	4.8 $\pm$ 0.5 <sup>a</sup>	33.5 $\pm$ 1.7 <sup>a</sup>
4	51.7 $\pm$ 4.3 <sup>a</sup>	37.3 $\pm$ 4.0 <sup>ab</sup>	93.5 $\pm$ 5.0 <sup>a</sup>	63.6 $\pm$ 3.5 <sup>ab</sup>	38.8 $\pm$ 2.8 <sup>a</sup>	3.9 $\pm$ 0.6 <sup>a</sup>	33.9 $\pm$ 2.3 <sup>a</sup>
5	47.0 $\pm$ 3.1 <sup>ab</sup>	34.7 $\pm$ 3.5 <sup>b</sup>	87.2 $\pm$ 3.6 <sup>b</sup>	68.1 $\pm$ 3.1 <sup>a</sup>	40.5 $\pm$ 2.9 <sup>a</sup>	2.3 $\pm$ 0.4 <sup>c</sup>	28.5 $\pm$ 2.2 <sup>a</sup>
6	55.7 $\pm$ 2.6 <sup>a</sup>	41.2 $\pm$ 2.5 <sup>a</sup>	— <sup>2</sup>	68.7 $\pm$ 2.2 <sup>a</sup>	42.0 $\pm$ 1.9 <sup>a</sup>	—	31.1 $\pm$ 1.1 <sup>a</sup>
7	45.0 $\pm$ 3.4 <sup>bc</sup>	30.8 $\pm$ 3.4 <sup>bc</sup>	90.8 $\pm$ 3.2 <sup>ab</sup>	62.9 $\pm$ 1.6 <sup>b</sup>	34.1 $\pm$ 1.6 <sup>b</sup>	—	34.1 $\pm$ 1.4 <sup>a</sup>
8	44.3 $\pm$ 2.9 <sup>bc</sup>	29.9 $\pm$ 3.1 <sup>bc</sup>	84.4 $\pm$ 3.7 <sup>b</sup>	58.8 $\pm$ 3.6 <sup>b</sup>	34.8 $\pm$ 2.6 <sup>b</sup>	2.6 $\pm$ 0.5 <sup>bc</sup>	35.3 $\pm$ 1.9 <sup>a</sup>

<sup>a-c</sup>Within columns, different superscripts indicate significant differences ( $P < 0.05$ ).

<sup>1</sup>VAP = average path velocity; VSL = straight-line velocity; VCL = curvilinear velocity; STR = straightness; LIN = linearity; ALH = lateral head displacement amplitude; BCF = beat-cross frequency.

<sup>2</sup>Data were not combined for analysis because the values for these lines between 6 and 12 mo were different ( $P < 0.05$ ).

in sperm hole category for line 3. The odds of obtaining fewer than 20 sperm holes per square millimeter of inner perivitelline membrane increase ( $P < 0.05$ ) when the roosters are 6 mo of age and decrease ( $P < 0.05$ ) when the roosters are 12 mo of age. For line 3, positive correlations were found between the percentage of membrane-intact sperm and the number of hydrolysis points per square millimeter of inner perivitelline membrane at both 6 ( $r = 0.81$ ,  $P = 0.008$ ) and 12 ( $r = 0.71$ ,  $P = 0.02$ ) mo of age. For line 7, a negative correlation was found between the percentage of motile sperm and the number of hydrolysis points per square millimeter of inner perivitelline membrane at 12 mo of age ( $r = -0.48$ ,  $P = 0.04$ ).

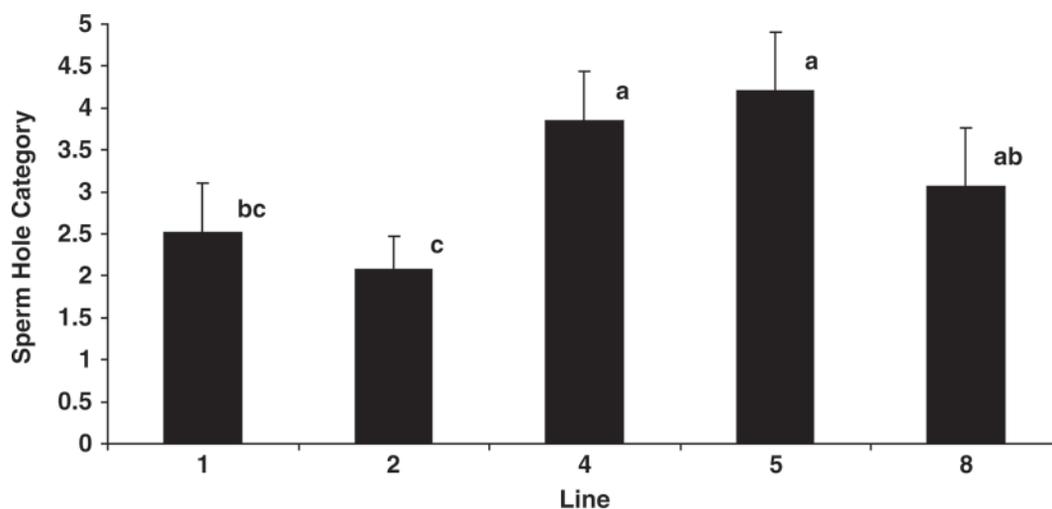
## DISCUSSION

The existence of quantitative differences among poultry lines or strains, or both, is an important determinant of the fertility of cryopreserved semen. We report the first investigation of postthaw sperm function of elite layer lines at the primary breeder level, with the

ultimate goal of identifying lines at both ends of the freezability spectrum to generate a model for determining the effect of genetics on sperm cryosurvival.

One important distinction relative to this approach was the assessment of individual, rather than pooled, semen samples. Semen cryopreservation and postthaw function assessments were conducted with the same individual males at both 6 and 12 mo of age. Given the potential male-to-male variation and within-male variation that is often associated with pooled semen, examining individual males not only minimized experimental variation but also increased the power of the assessment. We chose assays known to accurately assess viability (Donoghue et al., 1995), motility (King et al., 2000; Blesbois et al., 2008), and hydrolyzing ability (Wishart, 1985; Robertson et al., 1997) to evaluate the postthaw function of poultry sperm in the absence of fertility trials because it was not possible in this study to assess the actual fertility of frozen-thawed semen from these pedigreed lines.

As would be expected from elite layer lines averaging 90% fertility (P. Settar, unpublished data), semen



**Figure 1.** Sperm hole category ( $\pm$ SEM) for pedigree lines showing similar ( $P > 0.05$ ) hydrolyzing ability between age groups (6 and 12 mo of age). The 8 discrete categories were zero holes (0), 1 to 10 holes (1), 11 to 20 holes (2), 21 to 40 holes (3), 41 to 60 holes (4), 61 to 80 holes (5), 81 to 100 holes (6), and 100+ holes (7). Different letters indicate differences ( $P < 0.05$ ) among lines.

**Table 4.** Average number of hydrolysis points per square millimeter of inner perivitelline membrane incubated with frozen-thawed rooster sperm (mean ± SEM)

Line	6 mo of age	12 mo of age
1	16.7 ± 5.6 <sup>bc,A</sup>	30.4 ± 8.4 <sup>c,A</sup>
2	14.3 ± 5.1 <sup>bc,A</sup>	12.5 ± 4.1 <sup>d,A</sup>
3	31.4 ± 15.2 <sup>ab,A</sup>	96.6 ± 26.1 <sup>ab,B</sup>
4	49.6 ± 15.1 <sup>a,A</sup>	54.5 ± 10.6 <sup>b,A</sup>
5	49.6 ± 18.7 <sup>a,A</sup>	96.5 ± 25.0 <sup>ab,A</sup>
6	43.6 ± 9.4 <sup>a,A</sup>	131.8 ± 30.2 <sup>a,B</sup>
7	26.7 ± 6.8 <sup>ab,A</sup>	105.1 ± 41.2 <sup>a,B</sup>
8	37.0 ± 17.0 <sup>ab,A</sup>	60.8 ± 15.3 <sup>b,A</sup>

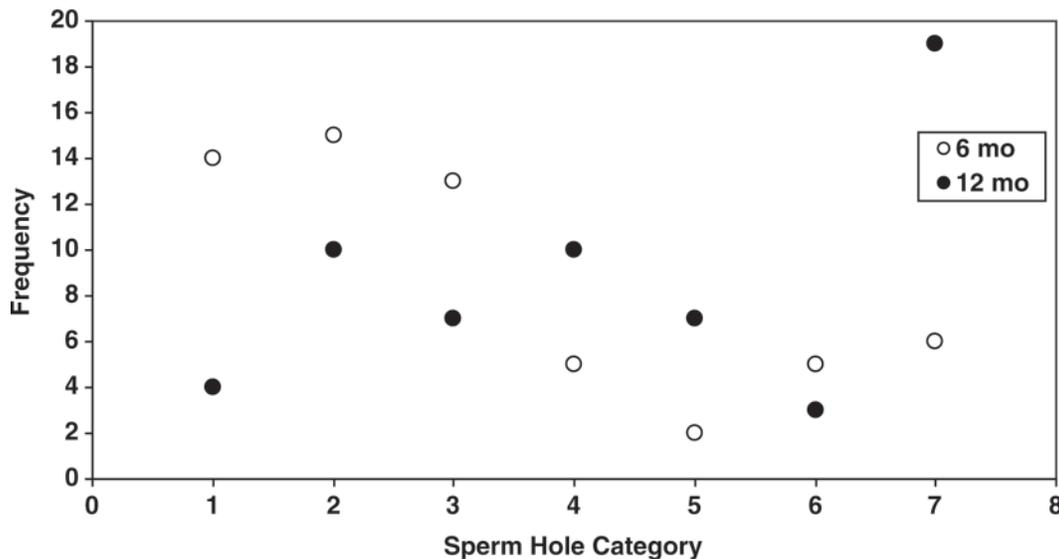
<sup>a-d</sup>Within columns, different superscripts indicate significant differences ( $P < 0.05$ ).

<sup>A,B</sup>Within rows, different superscripts indicate significant differences ( $P < 0.05$ ).

parameters measured before cryopreservation demonstrated that each line produced good quality semen. Subsequent work found a high percentage of membrane-intact sperm (91.4 to 98.6%) in fresh semen from all lines (D. C. Bongalhardo and J. A. Long, unpublished data). In the current study, the postthaw function of sperm from the 8 elite lines varied widely, despite the fact that none of the lines produced semen of inferior quality before cryopreservation. The membrane integrity of thawed sperm, which ranged from  $27.8 \pm 2.1$  to  $49.6 \pm 1.9\%$  viable sperm, is similar to published data for roosters (Sexton, 1973; Chalah et al., 1999; Blesbois et al., 2005). The low variation within each line at both age groups suggests that the propensity for sperm membrane stability after a freeze-thaw cycle may be a characteristic trait. Although the percentage of viable thawed sperm was a characteristic unaffected by age for most of the lines, the age of the male did influence membrane integrity for 3 of the 8 lines. Overall cryosurvival rates of chicken, turkey, and guinea fowl sperm

have been correlated with sperm membrane integrity of thawed sperm in combination with the percentage of morphologically normal sperm postthaw (Blesbois et al., 2005). In our study, the relationship between sperm viability and sperm freezability was apparent for only 1 of the 8 lines. A closer scrutiny of sperm viability that accounted for both membrane integrity and normal morphology may have yielded more associations between line and sperm freezability.

The greatest variation among lines occurred with sperm velocity and linearity parameters, with only BCF being similar among lines. Most lines retained similar sperm velocity parameters, however, irrespective of the age of the male. Computer-aided sperm motility analysis parameters have been proposed as predictors of sperm cryosurvival in chickens (Blesbois et al., 2008), and in turkeys, the CASA parameters VSL, LIN, and BCF were positively correlated with sperm mobility in freshly collected semen (King et al., 2000). Unexpectedly, the BCF data for thawed sperm from all of the elite layer lines in our study (range,  $28.5 \pm 2.2$  to  $35.3 \pm 1.9$  Hz) were substantially higher than the data reported for freshly collected turkey sperm from high ( $11.4 \pm 0.9$  Hz) or low ( $8.2 \pm 0.8$  Hz) mobility toms (King et al., 2000). Likewise, the LIN parameters were higher for elite layer lines (range,  $34.1 \pm 1.6$  to  $42.0 \pm 1.9\%$ ) than for high-mobility toms ( $29.3 \pm 2.1\%$ ); VSL parameters appeared to be similar for frozen-thawed chicken sperm (range,  $28.3 \pm 2.5$  to  $41.2 \pm 2.5$   $\mu\text{m/s}$ ) compared with freshly collected turkey sperm (range,  $22.0 \pm 3.0$  to  $32.6 \pm 3.2$   $\mu\text{m/s}$ ). Sperm mobility phenotype has been positively correlated with fertility in both chickens (Birkhead et al., 1999; Froman et al., 1999) and turkeys (Donoghue et al., 1998, 1999) when freshly collected semen was used for insemination. Further, it has been shown that VSL must be  $>30$   $\mu\text{m/s}$  for



**Figure 2.** Combined frequency of sperm hole category from the 3 pedigree lines showing significant ( $P < 0.05$ ) differences at 6 and 12 mo of age. Open circles represent 6-mo and closed circles represent 12-mo data.

sperm to penetrate an Accudenz solution (Froman and Feltmann, 2000). Thawed semen samples from 4 of the 8 elite lines in our study met this criterion; however, due to limited sample volumes, we were unable to directly measure sperm mobility in concert with the viability and hydrolyzing ability assays.

The ability of thawed sperm from each line to hydrolyze the inner perivitelline membrane is perhaps the most significant assessment of fertilizing ability. Adjusting the sperm dose to reflect the number of membrane-intact sperm further improved the power of this assay by ensuring a uniform number of viable sperm for each male evaluated. As with the other sperm function assays, there was significant line-to-line variation and an age effect for 3 of the 8 lines; however, individual male differences were more evident with this assay. The degree of male-to-male variation within line was reflected in the high SE associated with most of the lines. Two of the lines exhibited relatively low male-to-male variation, and the overall hydrolyzing ability of sperm from these 2 lines was poor compared with the other 6 lines. Although a negative correlation was found for 1 line between the percentage of motile sperm and the number of hydrolysis points, it should be kept in mind that the perivitelline assay does not require highly motile sperm because the small volume of media results in a close association between the isolated IPVL and sperm. The more interesting observation here is that although sperm motility was adversely affected by cryopreservation, membrane integrity was not compromised, (e.g., acrosin content was preserved during the freeze-thaw process). In a fertility trial, however, the low sperm motility would prevent the membrane-intact sperm from reaching the ovum. These data give us insight as to how and why fertility results from frozen-thawed sperm would be compromised for this particular line.

Four lines showed improvement for 1 or 2 of the sperm function assays at 12 mo of age. It was anticipated that older males near the end of production might exhibit a decrease in sperm viability, motility, and hydrolyzing ability. Although the primary breeder routinely trains roosters for semen collection before 6 mo of age, it is possible that males from these 4 lines were not in prime semen production when samples were collected for the study. Nonetheless, the data clearly indicate line variations in sperm freezability irrespective of whether the age effect was an artifact of male condition.

In summary, our initial investigation provides conclusive evidence that sperm from elite layer lines differ in the ability to withstand cryopreservation and retain sperm function. These findings support the idea that a model can be developed from existing pedigree lines to delineate genotypic and phenotypic factors affecting sperm cryosurvival. The assays used in this study evaluated specific sperm functions that are integral parts of the fertilization process (e.g., membrane integrity, sperm motility, and hydrolysis activity) and provide a foundation necessary to further explore the ultimate measurement of sperm cryosurvival, fertility.

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