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PHYSIOLOGY AND ENDOCRINOLOGY SYMPOSIUM: A proteome-based model for sperm mobility phenotype[1,2]

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ABSTRACT: Sperm mobility is defined as sperm movement against resistance at body temperature. Although all mobile sperm are motile, not all motile sperm are mobile. Sperm mobility is a primary determinant of male fertility in the chicken. Previous work explained phenotypic variation at the level of the sperm cell and the mitochondrion. The present work was conducted to determine if phenotypic variation could be explained at the level of the proteome using semen donors from lines of chickens selected for low or high sperm mobility. We began by testing the hypothesis that premature mitochondrial failure, and hence sperm immobility, arose from Ca^{2+} overloading. The hypothesis was rejected because staining with a cell permeant Ca^{2+}-specific dye was not enhanced in the case of low mobility sperm. The likelihood that sperm require little energy before ejaculation and the realization that the mitochondrial permeability transition can be induced by oxidative stress arising from inadequate NADH led to the hypothesis that glycolytic enzymes might differ between lines. This possibility was confirmed by 2-dimensional electrophoresis for aldolase and phosphoglycerate kinase 1. This outcome warranted evaluation of the whole cell proteome by differential detergent fractionation and mass spectrometry. Bioinformatics evaluation of proteins with different expression levels confirmed the likelihood that ATP metabolism and glycolysis differ between lines. This experimental outcome corroborated differences observed between lines in previous work, which include mitochondrial ultrastructure, sperm cell oxygen consumption, and straight line velocity. Although glycolytic proteins were more abundant within highly mobile sperm, quantitative PCR of representative testis RNA, which included mRNA for phosphoglycerate kinase 1, found no difference between lines. In summary, we propose a proteome-based model for sperm mobility phenotype in which a genetic predisposition puts sperm cells at risk of premature mitochondrial failure as they pass through the excurrent ducts of the testis. In other words, we attribute mitochondrial failure to sperm cell and reproductive tract attributes that interact to affect sperm in a stochastic manner before ejaculation. In conclusion, our work provides a starting point for understanding chicken semen quality in terms of gene networks.

Key words: chicken, glycolysis, proteome, sperm, sperm motility

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INTRODUCTION

Hens lay a series of fertilized eggs after a single intravaginal insemination. This ability depends upon sperm ascending the vagina, entering sperm storage tubules (**SST**), residing therein temporarily, and then emerging from the SST. Whereas SST influx occurs within hours after insemination, efflux occurs over an interval of days to several weeks. The most likely mechanism underlying in vivo sperm storage was outlined by Froman (2003). This mechanism was inferred from knowledge of the oviduct of the hen (Bakst et al., 1994) and the in vitro behavior of sperm in experiments prompted by the discovery of sperm mobility, a quantitative trait (Froman and Feltmann, 1998).

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The term "sperm mobility" denotes the net movement of a sperm cell population against resistance at body temperature. This property is measured in vitro by sperm penetration of an Accudenz (Accurate Chemical and Scientific Corp., Westbury, NY) solution (Froman and McLean, 1996). The assay is performed as follows. A cuvette containing 6% (wt/vol) Accudenz is overlaid with a sperm suspension containing a fixed number of sperm. The absorbance of the Accudenz layer is measured with a spectrophotometer after a 5-min incubation at 41°C. Thus, phenotype is expressed in absorbance units. The first link between phenotype and male fecundity was demonstrated by Froman et al. (1997): phenotype was comparable with effective insemination dose. This notion was tested and confirmed by Froman et al. (1999), who demonstrated that male fertility is a function of sperm mobility phenotype. In addition, the effect of phenotype on male fecundity was tested by competitive fertilization (Birkhead et al., 1999). Phenotype had a profound effect upon paternity, and this effect was independent of the dam. The model for in vivo sperm storage proposed by Froman (2003) was tested by Pizzari et al. (2008) by determining paternity through time after a single intravaginal insemination with sperm from each of 2 males. Males within pairs were selected from lines of D. P. Froman with low and high sperm mobility chickens. This experiment demonstrated that the ability of sperm to ascend the vagina, enter the SST, and emerge from the SST is a sire effect.

Froman and Feltmann (1998) demonstrated that any given ejaculate contains a mixture of mobile and immobile sperm. However, the size of the mobile subpopulation varied among males within flocks of normal, fertile males. For example, sperm mobility within the randombred flock (n = 271) described by Froman and Feltmann (1998) had a mean and SD of 0.401 and 0.1819 absorbance units. Thus, the CV was 45% within this flock. Subsequent work (Froman and Feltmann, 2000; Froman et al., 2003) used computer-assisted sperm motion analysis to explain phenotype in terms of individual motile sperm cells. Skewed straight line velocity (**VSL**) distributions characterized populations of motile sperm. In this regard, it is noteworthy that distribution shape varied with phenotype. Specifically, sperm mobility phenotype was a linear function (r = 0.997) of the area within the upper tail of a VSL distribution. This area was determined by the number of sperm within an ejaculate with a VSL >30 μm/s. Consequently, immobile sperm could be defined, at least in an operational sense, as those sperm whose VSL was ≤30 μm/s. Thereafter, immobile sperm were proven to contain dysfunctional mitochondria as evidenced by transmission electron microscopy and oxygen consumption (Froman and Kirby, 2005).

Compromised mitochondrial function within immobile sperm was attributed to formation of the mitochondrial permeability transition (**MPT**) before ejaculation (Froman et al., 2006). Calcium overloading is one means by which formation of the MPT is induced (Nicholls and Ferguson, 2002a). This explanation seemed plausible as an underlying mechanism for the following reasons. First, fowl seminal plasma is enriched with glutamate (Freeman, 1985). Second, *N*-methyl-D-aspartic acid (**NMDA**) receptors are ion channels that admit Ca^{2+} in response to glutamate (Purves et al., 2004). Third, NMDA proved to be a fowl sperm motility agonist (Froman, 2003). Fourth, maintenance of fowl sperm motility at body temperature depends upon mitochondrial Ca^{2+} cycling (Froman and Feltmann, 2005). Therefore, Ca^{2+} overloading was deemed to occur via NMDA receptors and sperm mobility to decrease in proportion. We intended to test this hypothesis and, in doing so, describe phenotypic variation at the level of proteins.

MATERIALS AND METHODS

Birds were reared, caged, and used in accordance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 1999) under assurance number 3610 from the Oregon State University IACUC.

Experimental Birds

Semen donors were selected from lines of low- and high-sperm-mobility New Hampshire chickens maintained by the Oregon Agricultural Experiment Station. Sperm concentration and in vitro sperm mobility were measured as outlined by Froman and Feltmann (2010). Cockerels were photostimulated at 20 wk of age, and sperm mobility was measured at 27, 28, and 29 wk of age. Individual sperm mobility phenotype was assigned based on the average of these observations.

Exp. 1

Flow cytometry was performed as described by Froman and Feltmann (2010) using the cell permeant Ca^{2+}-specific dye fluo-3AM (Invitrogen, Carlsbad, CA). Ten replicate males were chosen from the mode of each phenotypic distribution. Data, expressed as number of fluorescent sperm and median fluorescence units, were analyzed by single classification ANOVA (Sokal and Rohlf, 1969a).

Exp. 2

Two-dimensional (**2-D**) electrophoresis was performed with Bio-Rad (Hercules, CA) reagents and instruments, as outlined below. Unless specified, reagents were purchased from Sigma-Aldrich (St. Louis, MO). Semen donors were selected from the mode of each distribution (n = 30 per line) for additional repeated measure analysis (n = 3 assays per male). Sperm mobility was measured on an every-other-day basis. Data were analyzed by nested ANOVA (Sokal and Rohlf, 1969b).

A stock solution of 30% (wt/vol) Accudenz (Accurate Chemical and Scientific Corp.) was prepared with 3 m*M* KCl containing 50 m*M* *N*-tris[hydroxyl-methyl]methyl-2-amino-ethanesulfonic acid (**TES**), pH 7.4, and 12% (wt/vol) Accudenz was prepared with 128 m*M* NaCl containing 50 m*M* TES, pH 7.4 (TES-buffered saline). One protein sample per line was prepared by washing pooled sperm with a modification of the technique described by McLean et al. (1998). A 1-mL volume of 30% (wt/vol) was underlaid beneath a 30-mL volume of 12% (wt/vol) Accudenz in a 50-mL centrifuge tube. Next, a 15-mL sperm suspension (2×10^9 sperm/mL) was overlaid on the 12% (wt/vol) Accudenz. Tubes were centrifuged at 1,200 × *g* for 45 min at 4°C. The infranatant and 2 supernatants were discarded. Washed sperm were suspended in ice-cold Lysis Buffer (Bio-Rad) to a concentration of 0.2 g of sperm/mL. The sperm suspension was placed within a prechilled model 4639 Parr Cell Disruption Bomb (Parr Instrument Co., Moline, IL). The bomb was sealed, charged with N_2 to a pressure of 14.5 kPa, and held on ice for 2 h.

Effluent from the cell disruption bomb was collected into a 25-mL Nalgene graduated cylinder on ice. The sperm lysate was subdivided into 2-mL microcentrifuge tubes and centrifuged at 16,000 × *g* for 20 min at 4°C. Supernatants were pooled in a 15-mL centrifuge tube held on ice. Protein concentration was determined with the Bio-Rad DC Protein Assay. The extract was diluted to 1.7 mg of protein/mL with Bio-Rad Rehydration-Sample Buffer. A 0.3-mL volume of diluted extract was placed within each of ten 1.8-mL screw-cap cryotubes and stored in liquid N_2 vapor before isoelectric focusing (**IEF**). Thereafter, a nested experimental design was used with 5 IEF trials per line, 4 immobilized pH gradient (**IPG**) strips per IEF trial, and duplicate polyacrylamide gradient gels per IEF trial per line. First-dimension protein separation was performed with 17-cm nonlinear IPG strips (pH 3 to 10) as follows. A 300-μL volume containing 500 μg of protein was loaded dropwise on the polyacrylamide surface of each IPG strip. Next, 4 strips were covered with mineral oil and incubated at 20°C for 14 h in a Protean IEF Cell (Bio-Rad). Isoelectric focusing was conducted at 20°C with rapid voltage ramping until 64,000 Vh was reached. Focused strips were blotted and stored in screw-cap glass tubes at –20°C before SDS electrophoresis. Second-dimension protein separation was performed by treating 1 focused IPG strip per line with dithiothreitol and then iodoacetamide, each dissolved in a Tris-HCl buffer, pH 8.8, containing 2% SDS and 6 *M* urea. Electrophoresis of low and high line proteins was performed concurrently in 18.3 × 19.3 cm precast gradient gels (8 to 16%) within a Protean II xi cell at 5°C and a constant current of 20 mA/gel. Gels were stained with Bio-Safe Coomassie G-250 (Bio-Rad) for 3 h and then destained in deionized water. Image analysis was performed with a ChemiDoc XRS imager and PDQuest 2-D analysis software (Bio-Rad). Data, expressed as pixel number per spot, were analyzed by nested ANOVA (Sokal and Rohlf, 1969b). Mass spectrometry was performed on excised protein from spots of interest at Applied Biomics (Haywood, CA).

Exp. 3

Differential detergent fractionation and multidimensional protein identification technology were performed, as outlined by Peddinti et al. (2008), using 1.5×10^9 sperm per rooster (n = 3 roosters per line). Mass spectra were evaluated by real, as well as random decoy, database searching (Elias and Gygi, 2007) to enable assignment of *P*-values and false discovery rates <0.05 per identification. Specifically, mass spectra were searched against an in silico trypsin-digested chicken protein database and a reversed version derived by using the reverse database function in Bioworks 3.3.1 (Thermo Fisher Scientific, Waltham, MA). Peptide identifications were discarded if they did not exceed 6 AA, if they were identified with the same scan number in different analyses with a mass to charge ratio within ± 1.5 Thomson units, or had a false discovery rate of <0.01 after Benjamini-Hochberg correction of *P*-values derived from decoy searches from the reversed canine protein database (Benjamini and Hochberg, 1995). Retained peptide identifications unambiguously identified a protein with the same criteria used for a BLAST (Basic Local Alignment Search Tool; National Center for Biotechnology Information) search. Function annotations were assessed with Ingenuity Pathway Analysis software (Redwood City, CA). Gene ontology annotation of the proteome was performed as outlined by McCarthy et al. (2007).

Exp. 4

Archived testis samples were homogenized in guanidinium isothiocyanate and RNA extracted in acidified phenol (Chomczynski and Sacchi, 1987). Polyadenylated RNA waspurified using the PolyATtract system (Promega Corp., Madison, WI). Purified polyA+ RNA was converted to first-strand cDNA using Superscript II RNaseH- (Invitrogen) using a $CT_{23}V$ primer. First-strand cDNA were subjected to quantitative PCR in a Bio-Rad CFX96 Realtime Thermocycler using SYBRGreen detection (Paton et al., 2000). Primers for genes encoding glucose transporter 3 (**GLUT3**), lactate dehydrogenase subunit B (**LDHB**), and phosphoglucokinase 1 (**PGK1**) were GLUT3 [forward (**F**) 5′-GTTCCTGGTAGAGCGTGCAG; reverse (**R**) 5′-TCTCAAAAAGGGCCACAAAG], LDHB (F 5′-GAACTGGGCCATTGGTCTTA; R 5′-CAGAGGCACTCAGGACACAA), and PGK1 (F 5′-GCTACATGCTGTGCGAAGTGG; R 5′-CAGGAACAGGAACTGGGGTGA). Ribosomal protein S14 (**rpS14**) was used as reference for relative quantification (F 5′-CGTGTGACTGGTGGCATGAAGGTGAAGG; R 5′-CGTCCACGGCGACCACCCTTTCTG). The PCR reactions (20 μL) were run in triplicate and contained

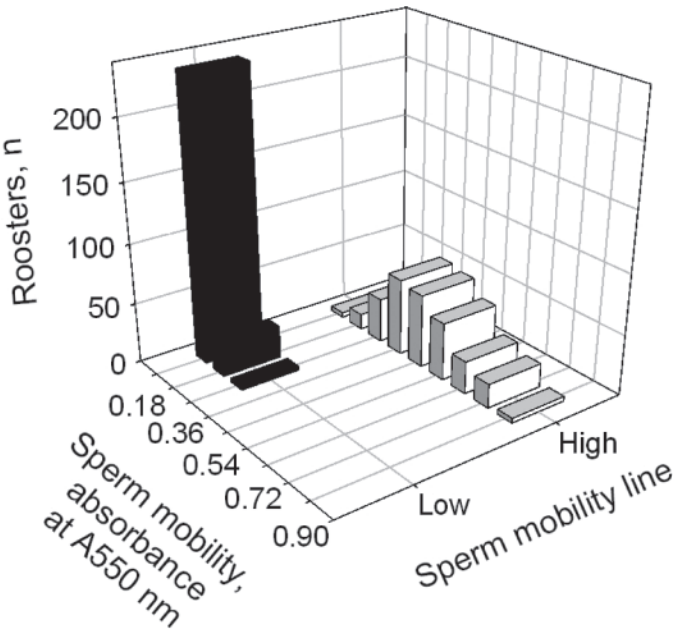

Figure 1. Phenotypic variation within lines of New Hampshire chickens with low (n = 277) and high (n = 293) sperm mobility. Phenotype was determined by photostimulating roosters at 20 wk of age and then performing the sperm mobility assay at 27, 28, and 29 wk of age. The absorbance value assigned per male was the average of these 3 measurements. Males were assigned to categories in increments of 0.090 absorbance units.

50 m*M* TrisCl, pH8.3, 1 m*M* $MgCl_2$, 0.3 mg/mL BSA, 0.2 m*M* dNTP, 1 μ*M* forward and reverse primers, cDNA target (approximately 1 to 3 ng), and 4 U of Taq polymerase. Cycling was 90°C for 30 s, 40 cycles of 90°C for 15 s, 55°C for 15 s, and 72°C for 30 s. After PCR, samples were incubated at 72°C for 180 s and then subjected to melt-curve analysis from 65 to 95°C for evaluation of PCR product homogeneity. Median cycle threshold values for each gene were then normalized relative to the rpS14 median cycle threshold values for that cDNA. Normalized values were analyzed by single classification ANOVA (Sokal and Rohlf, 1969a).

RESULTS AND DISCUSSION

The present work began with the following argument: if sperm cell Ca^{2+} content differs between low- and high-sperm-mobility lines, then mitochondrial Ca^{2+} overloading may help explain phenotypic variation. The putative role of the MPT was based on its induction within motile fowl sperm (Froman et al., 2006). Thus, the mechanism we sought was a variant of neuronal glutamate excitotoxicity (Nicholls and Ferguson, 2002b). In this regard, NMDA receptors were detected by differential detergent fractionation and multidimensional protein identification technology in the present work. Our first experiment used test subjects from the mode of each phenotypic distribution shown in Figure 1. Sperm Ca^{2+} content was estimated with the Ca^{2+}-specific fluorescent dye fluo-3AM and flow cytometry. As shown in Table 1, the percentage of sperm that stained for Ca^{2+} and median fluorescence of stained cells were less in the case of roosters from the low sperm mobility line ($P = 0.018$ and $P = 0.024$, respectively). Therefore, it seemed unlikely that aberrant Ca^{2+} flux induced premature mitochondrial failure. Consequently, an alternative explanation was sought.

Since a heritability of $h^2 = 0.30$ for sperm mobility was observed (Froman et al., 2002), the discovery of underlying genes, in particular those that compromise sperm mobility, has been an experimental goal (D. D. Rhoads and D. P. Froman, unpublished data). According to Ashizawa and Sano (1990), rooster sperm are immotile before ejaculation even though they possess the potential for progressive motility. Consequently, ADP generation within the axoneme is most likely negligible before ejaculation. Thereafter, regeneration of ATP from ADP within the axoneme depends upon the phosphocreatine shuttle (Wallimann et al., 1986, 1992), which is driven by mitochondrial oxidative phosphorylation. This process is fueled in motile sperm by long-chain fatty acids released by phospholipase A_2, which is activated by mitochondrial Ca^{2+} cycling (Froman, 2003; Froman and Feltmann, 2005). Thus, motile fowl sperm do not require exogenous energy, at least for an interval of minutes to hours after ejaculation. Collectively, these observations implied that ATP is regenerated within fowl sperm by distinct pathways before and after ejaculation. This realization was intriguing because glycolysis yields NADH in addition to ATP, and NADH depletion is one form of oxidative stress that can induce the MPT (Nicholls and Ferguson, 2002b).

Table 1. Summary of flow cytometry experiment[1] performed with sperm[2] stained with the calcium-specific dye fluo-3AM

	Sperm mobility phenotype	
Variable	Low	High
Semen donors, n	10	10
Stained cells,[3] %	85 ± 7.8	91 ± 4.3
Median fluorescence, [4] arbitrary units	542 ± 74.8	626 ± 74.8

[1]Each value is a mean ± SD.
[2]A total of 10,000 sperm were counted per semen donor.
[3]Means differed at $P = 0.0177$.
[4]Means differed at $P = 0.0244$.

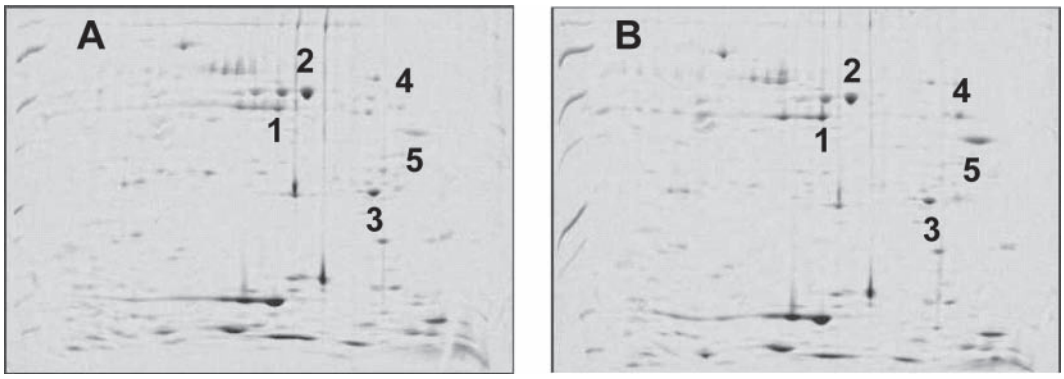

Figure 2. Proteins resolved by 2-dimensional electrophoresis from an extract of sperm prepared with a Parr Cell Disruption Bomb (Parr Instrument Co., Moline, IL) and a proprietary lysis buffer for procuring soluble proteins. Each image represents a precast 18.3 × 19.3 gradient gel stained with Bio-Safe Coomassie G-250 (Bio-Rad, Hercules, CA). Gels A and B contained proteins from sperm ejaculated by low and high sperm mobility males, respectively. The numbers 1, 4, and 5 denote brain-type creatine kinase, aldolase, and phosphoglycerate kinase 1, each of which differed between phenotypes. The numbers 2 and 3 denote enolase and triose phosphate isomerase, which did not differ between phenotypes.

These insights yielded the following possibility: if glycolytic enzyme content is deficient within sperm from low line roosters, then such sperm might be at risk during passage through the excurrent ducts of the testis.

Therefore, the second experiment was performed to determine if glycolytic enzymes might differ between lines. Although 2-D electrophoresis can afford a means of discovery, one must address several critical variables beforehand. These include the magnitude of the pH gradient in the first dimension, the molecular weight range in the second dimension, the means by which proteins are extracted, the relative amounts of proteins within this extract, the means by which proteins will be stained, and repeatability (Westermeier and Naven, 2002). Consequently, a single pH 3 to 10 gradient was used for IEF rather than a series of overlapping gradients, a large gradient gel format was used for SDS electrophoresis to obtain maximal resolution of focused proteins, a mass spectrometry-compatible dye was chosen with a sensitivity of 10 ng per spot, and it was hoped that sampling from a protein pool would enhance repeatability. Nested ANOVA was performed with sperm mobility data from potential semen donors to confirm that phenotype differed between lines but not among males within a pool. As shown in Table 2, each constraint was realized.

Proteins resolved by 2-D electrophoresis are shown in Figure 2. Three spots differed between lines based upon nested ANOVA. These spots were excised and proteins therein identified by mass spectrometry. These included brain-type creatine kinase ($P = 0.0025$), aldolase ($P = 0.0013$), and phosphoglycerate kinase 1 ($P = 0.0004$). Each of these proteins affects sperm cell energy metabolism. For example, brain-type creatine kinase is the isozyme found in association with fowl sperm axoneme (Wallimann et al., 1986). As such, it enables regeneration of ATP within the axoneme using phosphocreatine generated within the midpiece as a substrate. In contrast, aldolase and phosphoglycerate kinase 1 are glycolytic enzymes. We found the difference in sperm content of phosphoglycerate kinase 1 of particular interest because it is the first enzyme in the glycolytic pathway that generates ATP. This experimental outcome served as the basis for the third experiment in which differential detergent fractionation was performed using sperm from individual roosters to evaluate the sperm cell proteome.

Not only did mass spectrometry confirm the between-line differences observed with 2-D electrophoresis, bioinformatics analysis identified key pathways in which these proteins function. Our experiment was a first attempt to characterize and compare proteins within the fowl sperm proteome. The utility of the method outlined by Peddinti et al. (2008) was confirmed, with 7,139 proteins identified, of which the expression of 894 differed between lines. However, our work differed from Peddinti et al. (2008) in that semen donors had been selected for a specific trait known to determine male fertility as opposed to using semen donors known to vary with respect to fertility. In addition to differences

Table 2. Summary of nested ANOVA used to confirm distinct phenotypes between lines and equivalent phenotype among males within lines[1]

Source of variation	df	Sum of squares	Mean square	*F*-value	Total variance, %
Line	1	9.9776	9.9776	3,582.14****	98
Males within line	58	0.1615	0.0028	1.34	0.2
Observations	120	0.2498	0.0021	—	1.8

[1]Replicate observations (n = 3) were made for each of 30 roosters per line.
****$P < 0.0001$.

Table 3. Glycolytic enzymes and related proteins identified by mass spectrometry after differential detergent fractionation of sperm from roosters selected for low and high sperm mobility

GenInfo identifier[1]	Protein (*Gallus gallus*)	*P*-value[2]	Expression[3]
46048651	Facilitated glucose transporter member 3	<0.0001	Up
45383904	Hexokinase 1	—	—
45383696	Hexokinase 2	—	—
57524920	Phosphoglucose isomerase	<0.0001	Up
71895485	Phosphofructokinase	<0.0001	Up
157951672	Aldolase	<0.0001	Up
45382061	Triose phosphate isomerase	<0.0001	Up
46048961	Glyceraldehyde-3-phosphate dehydrogenase	<0.0001	Up
45384486	Phosphoglycerate kinase 1	<0.0001	Up
71895985	Phosphoglycerate mutase	<0.0001	Up
46048768	α-Enolase	<0.0001	Up
46048765	β-Enolase	<0.0001	Up
45382393	γ-Enolase	0.0263	Up
45382651	Pyruvate kinase, muscle	<0.0001	Up
45384208	Lactate dehydrogenase, A chain	<0.0001	Up
4538766	Lactate dehydrogenase, B chain	<0.0001	Up

[1]Values listed are assigned by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

[2]Probability that protein expression differed between lines. In the case of missing values, the protein in question was detected but the amount was inadequate to ensure a valid test between lines.

[3]Denotes high line relative to low line. In the case of missing values, the protein in question was detected but the amount was inadequate to confirm a difference in expression between lines.

reported herein (Table 3; Figure 3), 596 proteins were more abundant in high-line sperm, whereas 298 proteins were more abundant in low-line sperm. Differentially expressed proteins included membrane as well as soluble proteins. Table 3 outlines differentially detected sperm cell proteins critical to glucose uptake, phosphorylation of glucose, conversion of glucose 6-phosphate into pyruvate, and conversion of pyruvate into lactate. The list includes proteins that differed between lines following by 2-D electrophoresis (e.g., aldolase and phosphoglycerate kinase 1), as well as those that did not (e.g., triose phosphate isomerase and enolase). This inconsistency was attributed to different efficiencies in protein extraction and recovery, factors known to limit proteome analysis by 2-D electrophoresis.

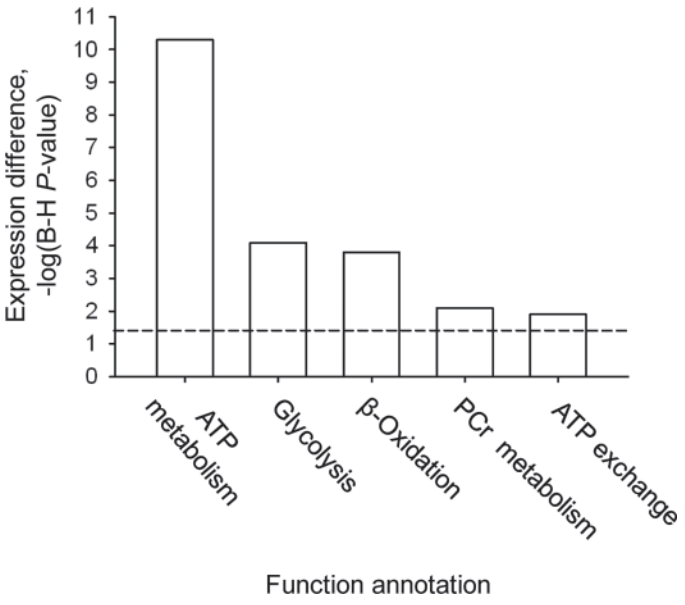

Figure 3. Differentially expressed proteins between high and low sperm mobility lines. The y-axis shows the negative log of the *P*-value from the Benjamini-Hochberg (B-H) correction, an algorithm used to assess differences in protein expression between proteomes. The horizontal dashed line denotes the threshold value of 1.3, which is equivalent to $P = 0.05$. Function annotations are plotted on the x-axis. Vertical bar height denotes high line relative to low line. PCr = phosphocreatine, a substance that shuttles energy from the mitochondrial helix of motile sperm to the axoneme where energy is consumed.

Experiment 4 was performed to ascertain whether genes implicated by difference in protein expression might be implicated by quantitative PCR as well. In view of Table 3, we chose genes encoding proteins that enable 1) glucose uptake (GLUT3), 2) ATP production within the glycolytic pathway (PGK1), and 3) glycolysis when oxygen is limiting (LDHB). Testicular mRNA was purified from roosters used as semen donors for the 2-D electrophoresis experiment, converted to cDNA, and subjected to quantitative PCR (data not shown). No differences were observed between lines ($P = 0.7306$, $P = 0.8820$, and $P = 0.6238$ for GLUT3, PGK1, and LDHB, respectively). In other words, the total testis transcriptome did not predict the sperm cell proteome in this experiment. We attribute this outcome to the possibility that 1) the sperm cell transcriptome is a minor component of the total testis transcriptome, 2) microRNA mediate pathway-specific downregulation of translation in the low sperm mobility line, or 3) post-translational protein degradation is enhanced in the low sperm mobility line.

Data analysis after mass spectrometry enabled us to link mitochondrial failure with specific sperm cell attributes. For example, the predominant difference between lines involved ATP metabolism (Figure 3). This experimental outcome was consistent with 1) the correlation of r = 0.80 observed between sperm [ATP] and sperm

mobility phenotype (Froman and Feltmann, 1998); 2) the correlation of r = 0.83 observed between sperm O_2 consumption and sperm mobility phenotype (Froman and Kirby, 2005); and 3) mitochondrial ultrastructure within immobile sperm (Froman and Kirby, 2005). A difference between lines was also observed with respect to mitochondrial β-oxidation of long-chain fatty acids. It is likely that this difference accounts for the premature sperm efflux from the SST observed when low sperm mobility males were used as semen donors (Froman et al., 2006; Pizzari et al., 2008). To date, the most parsimonious explanation of in vivo sperm storage (Froman, 2003) is as follows: 1) sperm ascension of the vagina and penetration of SST is powered by oxidation of endogenous long-chain fatty acids, presumably from the outer mitochondrial membrane; 2) sperm residence within the SST, in which sperm maintain position in a fluid stream, is powered by oxidation of exogenous fatty acids released from SST epithelial cells (Bakst et al., 1994); and 3) sperm efflux arises from decreased sperm cell velocity as sperm mitochondria become senescent. Ejaculates from low-sperm-mobility males do indeed contain mobile sperm. These mobile subpopulations, however, are substantially smaller than those within ejaculates from high-sperm-mobility males. Thus, mobile sperm from low-sperm-mobility males can ascend the vagina and reside within the SST, but larger numbers emerge from the SST sooner than do resident sperm from high-sperm-mobility males (Froman et al., 2006). Consequently, the fecundity of the low-sperm-mobility phenotype is compromised in 2 ways. First, the effective insemination dose is reduced, because the preponderance of ejaculated sperm are immobile and, therefore, cannot ascend the vagina. Second, those sperm that do ascend the vagina have mitochondria that fail or become senescent prematurely, which results in premature efflux from the SST.

The observed range of sperm mobility phenotype (see Figure 1) denotes highly variable proportions of mobile and immobile sperm within ejaculates among males. The basis for such variation was explained in terms of VSL distributions (Froman et al., 2003; Froman and Kirby, 2005). Nonetheless, Froman and Kirby (2005) tested whether sperm cell trajectory might be a contributing factor in addition to VSL. An a posteriori analysis was performed based upon 4,290 and 5,275 independent computer-assisted sperm motion analysis observations for low- and high-mobility sperm, respectively. Sperm cell trajectory, as measured by straightness, was plotted as a function of VSL. Coincident logistic functions were observed in which an asymptotic value of 90% was approached at high VSL. Consequently, Froman and Kirby (2005) concluded that the quality of sperm motion is comparable between lines for those sperm that are moving. We contend that protein expression levels for 2 pathways critical to sperm motion, metabolism of phosphocreatine and exchange of ATP (Figure 3), are consistent with this conclusion. Therefore, the principal unresolved issue was the quantity of immobile sperm within any given ejaculate. In this regard, a key observation was made by Bowling et al. (2003). These authors reported a 4-fold difference (16 vs. 4%) in the percentage of aberrant mitochondria within sperm sampled from the deferent ducts of broiler breeders with low and high sperm mobility.

The model for phenotypic variation proposed by Froman et al. (2006) linked formation of the MPT with Ca^{2+} overloading before ejaculation. In other words, deferent duct fluid glutamate was thought to act upon sperm NMDA receptors to promote mitochondrial Ca^{2+} uptake. Parenthetically, it has yet to be determined whether this is the means by which fowl sperm acquire the potential for progressive motility. Nonetheless, this possibility and the relationship between Ca^{2+} overloading and MPT formation (Nicholls and Ferguson, 2002a) provided a simple explanation for mitochondrial failure before ejaculation. However, our first experiment (Table 1) made this hypothesis untenable. A more nuanced explanation took into account the following: 1) the likelihood that immotile sperm within the deferent duct have a minimal energy demand; 2) the fact that glycolysis yields NADH as well as ATP; and 3) the relationship between NADH depletion and formation of the MPT. In view of these guiding principles and the differential expression of glycolytic enzymes observed between low- and high-sperm-mobility lines (Table 3, Figures 2 and 3), we propose that phenotypic variation is due to a genetic predisposition that puts sperm cells at risk of premature mitochondrial failure as they pass through the excurrent ducts of the testis; that is, mitochondrial failure stems from a set of conditions that interact to affect sperm in a stochastic manner. In other words, premature failure of sperm mitochondria occurs within the excurrent ducts of roosters at large. However, the extent to which this happens varies among roosters and has a genetic basis.

SUMMARY AND CONCLUSIONS

The experiments presented herein afford an explanation of phenotypic variation in sperm mobility at the molecular level. Our work complements previous work in which phenotypic variation was explained at the level of the sperm cell (Froman and Feltmann, 2000) and the mitochondrion (Froman and Kirby, 2005). The model we propose is consistent with bioenergetic theory in general and factors that affect mitochondrial integrity in particular. Moreover, our model is testable. For example, if phenotype is inversely proportional to deferent duct transit time, then a reproductive tract feature will be identified that puts sperm cells at risk before ejaculation. Likewise, if primordial germ cell transfer from low-line donors to high-line recipients yields male progeny with poor sperm mobility, then genes affecting sperm cell features rather than reproductive tract attributes may be pivotal to phenotypic variation. In any event, our work sets the stage for a gene-based definition of semen quality for the domestic fowl.

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