

# Sperm Mobility: Phenotype in Roosters (*Gallus domesticus*) Determined by Mitochondrial Function<sup>1</sup>

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## ABSTRACT

Previously, inheritance of sperm mobility entailed a maternal additive genetic effect, and sperm ATP content was correlated ( $r = 0.80$ ) with phenotype. The present study was conducted to determine if mitochondrial function was critical to phenotypic expression. Whereas phenotype was independent of mitochondrial helix length, phenotype was correlated with sperm oxygen consumption ( $r = 0.83$ ) using random-bred roosters. Aberrant mitochondria characterized immobile sperm, as evidenced by transmission-electron microscopy. Such mitochondria were swollen and contained disorganized cristae. Additional experiments were performed with roosters from lines selected for low or high sperm mobility. A threefold difference in sperm oxygen consumption was observed between lines. Single nucleotide polymorphisms were observed in mitochondrial DNA by sequencing replicate mitochondrial genomes from each line. An A-to-G substitution in the gene encoding tRNA<sup>Arg</sup> was inherited consistently, as evidenced by restriction fragment length polymorphism analysis using two male and two female progeny per family group and 14 family groups per line. Motile concentration in semen from low-line males was half that observed in semen from high-line males, as evidenced by computer-assisted sperm motion analysis. Likewise, 47% of sperm from low-line males contained aberrant mitochondria, compared to 4% for high-line males. In summary, sperm mobility phenotype was dependent on mitochondrial function, which in turn was altered by genetic selection. Fowl deferent duct fluid contains a high concentration of glutamate. We propose that variation in sperm mobility phenotype stems from the extent to which glutamate induces excessive mitochondrial Ca<sup>2+</sup> uptake before ejaculation.

gamete biology, glutamate, sperm, sperm maturation, sperm motility and transport

## INTRODUCTION

Sperm mobility denotes the net movement of a sperm cell population against resistance at body temperature. Consequently, this quantitative trait [1] is highly predictive of male fertility in both noncompetitive [2] and competitive [3, 4] contexts. Sperm mobility phenotype is determined by

measuring the absorbance of an Accudenz solution following sperm penetration from an overlaid sperm suspension [1]. Absorbance is proportional to the number of sperm within the sample that have a straight-line velocity (VSL) of greater than 30  $\mu\text{m}/\text{sec}$  [5, 6]. In other words, whereas mobile sperm are necessarily motile, motile sperm are not necessarily mobile.

Sperm mobility phenotype was highly variable among random-bred New Hampshire roosters using media prepared without an exogenous substrate [2]. Likewise, sperm oxygen consumption and acylcarnitine content differed with sperm mobility phenotype. Consequently, mitochondrial function was deemed to be pivotal in the expression of phenotype. Subsequently, this conclusion was substantiated by additional lines of evidence. First, an exclusive maternal additive genetic effect was observed when heritability was estimated [7]. Second, mitochondrial ultrastructure differed between commercial meat-type roosters characterized by either low or high sperm mobility [8]. Specifically, 22% of sperm ejaculated from low-sperm-mobility males contained swollen mitochondria with disorganized cristae. In contrast, only 5% of sperm from high-sperm-mobility males contained aberrant mitochondria. It is noteworthy that fowl sperm motility depends on Ca<sup>2+</sup>-dependent phospholipase A<sub>2</sub> activity, which provides long-chain fatty acids for  $\beta$ -oxidation within the mitochondrial matrix [9].

The present report outlines a series of experiments conducted over the course of several years. Each experiment was designed to address mitochondrial structure or function relative to sperm mobility phenotype. Initial experiments were performed with random-bred New Hampshire roosters. Subsequent experiments used roosters of lines derived from the random-bred population. These lines were produced using a selection scheme in which maternal inheritance played a significant role. Experimental variables included sperm oxygen consumption, mitochondrial helix length, and mitochondrial ultrastructure. The inheritance of a single nucleotide polymorphism (SNP) within the mitochondrial gene encoding tRNA<sup>Arg</sup> also was evaluated. Collectively, our data demonstrate that mitochondrial function is pivotal to expression of sperm mobility phenotype. An unexpected outcome was a new insight pertaining to the composition of fowl deferent duct fluid [10] and the capacity of deferent duct sperm to manifest progressive motility [11].

## MATERIALS AND METHODS

### Sperm Oxygen Consumption

Experimental animal care was in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching. Roosters were caged (30  $\times$  46  $\times$  63 cm) and maintained on a 14L:

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10D photoperiod. Sperm mobility phenotype was assigned to random-bred New Hampshire roosters ( $n = 242$ ) according to the method outlined by Froman et al. [2]. The Kolmogorov-Smirnov test for goodness of fit [12] was used to determine whether observed frequencies approximated a normal distribution. A representative subpopulation of roosters ( $n = 40$ ) was selected for correlation analysis based on range of scores, group mean, and coefficient of variation. Sperm mobility and sperm oxygen consumption were measured as outlined by Froman et al. [2]. Sperm mobility was correlated with sperm oxygen consumption [13]. Sperm oxygen consumption was measured in a second experiment as outlined above using roosters from lines selected for either low or high sperm mobility ( $n = 28$  per line). In this case, data were analyzed by single-classification ANOVA [14].

### Mitochondrial Helix Length

Seven roosters were selected from a random-bred population ( $n = 242$ ) to represent the range in observed phenotype. Mitochondrial helix length ( $n = 50$  sperm/male) was estimated via immunofluorescence as outlined by Korn et al. [15]. Data were analyzed by single-classification ANOVA [14]. The Student-Newman-Keuls test was used for a posterior comparison among means [16].

### Mitochondrial Ultrastructure

Males characterized by average sperm mobility phenotype ( $n = 10$ ) were selected from a base population ( $n = 242$ ) of random-bred roosters. In each case, a sperm mobility assay was performed [2] before transmission-electron microscopy (TEM). This required preparation of a sperm suspension containing  $5 \times 10^8$  sperm/ml. A subsample was withdrawn to evaluate mitochondrial ultrastructure before separation of mobile from immobile sperm. A second subsample was used to perform the sperm mobility assay, which entailed overlaying the sperm suspension upon prewarmed 6% (w/v) Accudenz (Accurate Chemical and Scientific Corp., Westbury, NY) in a cuvette. After the 5-min incubation at  $41^\circ\text{C}$ , the overlay was recovered with a pipette. This sperm suspension was used to evaluate the mitochondrial ultrastructure of immobile sperm. Each sperm suspension was microcentrifuged for 20 sec at  $15,600 \times g$  to concentrate sperm, and TEM was performed as outlined by Thurston and Hess [17]. Approximately 10 sections, spaced  $125 \mu\text{m}$  apart, were evaluated per male. Midpiece sections ( $n = 500$  per male) were categorized according to mitochondrial ultrastructure. Any midpiece containing mitochondria with well-defined cristae and mitochondria arranged in a compact helix around the axoneme was categorized as normal. Likewise, any midpiece containing swollen mitochondria or mitochondria with disorganized cristae were categorized as aberrant. Proportions of sperm with aberrant mitochondria were converted to logits before statistical analysis, where  $L_i = \ln[(r_i - 0.5)/(n - r_i - 0.5)]$ ,  $r_i$  is the number of aberrant midpieces, and  $n = 500$ . Transformed data were analyzed with the log odds model:

$$p(x) = [1]/[1 + e^{-l(x)}]$$

where  $0 \leq p \leq 1$  and  $l(x) = \mu + \alpha_i + B_j + e_{ij}$  as the model statement, in which  $\alpha$  and  $B$  denote treatment (sperm segregation) and block (male) effects, respectively. Data were analyzed using a randomized complete block design [18].

A second experiment was performed using roosters from lines selected for either low or high sperm mobility ( $n = 3$  roosters/line). Sperm from each rooster were concentrated by microcentrifugation for 20 sec at  $15,600$

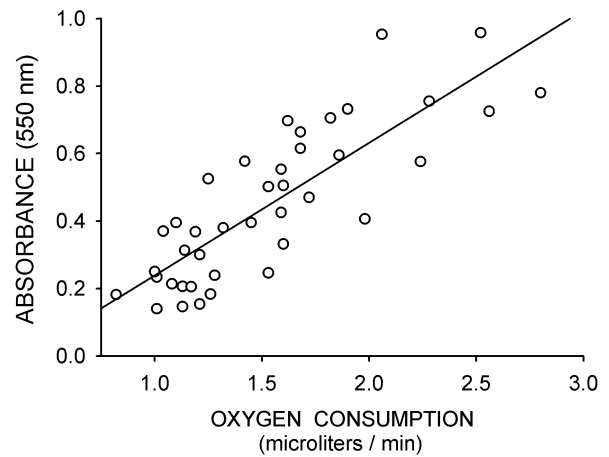


FIG. 1. Correlation of sperm mobility with sperm oxygen consumption. Sperm mobility was quantified as described in *Materials and Methods*. Each datapoint (O) represents a data pair from a single rooster immediately after ejaculation ( $n = 40$  roosters). The solid line denotes the regression equation: absorbance =  $-0.154 + (0.393)(\text{O}_2 \text{ consumption})$ . The product-moment correlation coefficient was 0.83.

$\times g$ , prepared for TEM, and evaluated, and the data were transformed as outlined above. Transformed data were analyzed as described above using the model statement  $l(x) = \mu + \alpha_i + e_p$ , where the parameter  $\alpha$  represents a line effect.

### Mitochondrial Genome Sequencing

Roosters from lines of New Hampshire chickens selected for either low or high sperm mobility were used in this effort. The complete mitochondrial genome from each of six roosters ( $n = 3$  per line) was amplified using polymerase chain reaction (PCR) and sequenced at the University of Arkansas DNA Resource Center on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster, CA) using the primer sets described by Desjardins and Morais [19]. Each genome was sequenced to sixfold coverage, sequencing both strands of three separate clones for each overlapping PCR fragment. Complete consensus mitochondrial genome sequences were deposited in the National Center for Biotechnology Information (NCBI) GenBank database under accession numbers AY235571 (low sperm mobility) and AY235570 (high sperm mobility).

### Inheritance of Mitochondrial SNP

The SNP at nucleotide 11 177 of the mitochondrial genome was identified in the parental generation by comparison of consensus sequence assemblies. This SNP was an A-to-G transition in the mitochondrial Arginine tRNA gene found in the low-sperm-mobility line. This locus was evaluated in two male and two female progeny from each of 14 dams per

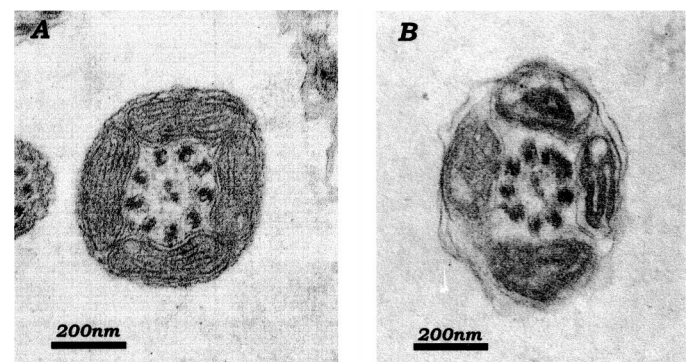


FIG. 2. Transmission-electron micrographs of fowl sperm midpieces. **A**) Cross-section showing mitochondria arranged in a compact helix around the axoneme. Mitochondrial cristae are well-defined. **B**) Cross-section showing mitochondria loosely adherent to the axoneme. Mitochondrial cristae are disorganized. Original magnification  $\times 60,000$ .

TABLE 1. Estimates of mitochondrial helix length from roosters representing the range in sperm mobility phenotype within a base population of random bred roosters.

Rooster	Sperm mobility <sup>a</sup> (absorbance units)	Helix length ( $\mu\text{m}$ ) <sup>b</sup>	Coefficient of variation (%)
1	0.130	$3.85 \pm 0.016$	2.9
2	0.208	$3.81 \pm 0.018$	3.2
3	0.415	$3.74 \pm 0.018^c$	3.3
4	0.496	$3.86 \pm 0.017$	3.2
5	0.560	$3.86 \pm 0.014$	2.5
6	0.737	$3.84 \pm 0.014$	2.9
7	0.836	$3.84 \pm 0.014$	3.7

<sup>a</sup> Sperm mobility was quantified as described in *Materials and Methods*.

<sup>b</sup> Each value is a mean  $\pm$  SEM.

<sup>c</sup> Different from all nonsuperscripted means ( $P < 0.05$ ).

TABLE 2. Single nucleotide polymorphisms within mitochondrial DNA from low and high sperm mobility lines of New Hampshire chickens.<sup>a</sup>

Position	Desjardins and Morais [19] <sup>b</sup>	Low line	High line
1292 Phe	T	T	C
2017 12S	G	A	G
8464 COX2	T	C	C
11 177 tRNA <sup>Arg</sup>	A	G	A
13 217 ND5	A	A	G
15 352 Cytb	A	G	A

<sup>a</sup> A sixfold coverage was used to sequence the entire mitochondrial genome from three replicate males per line. Sequences were identical within lines.

<sup>b</sup> Based on pooled DNA from Single Comb White Leghorns.

line. Sperm mobility phenotype was confirmed in male offspring by nested ANOVA [20] using three observations per male. Heparinized blood was provided to the University of Arkansas DNA Resource Center as coded blind samples.

The DNA was extracted with a QIAamp 96 DNA Blood Kit (Qiagen, Valencia, CA). Mitochondrial DNA was amplified by PCR using the following primers (Sigma Genosys, The Woodlands, TX):

Forward: 5'-CAGGGCGGCTAGATGAGCAGA (nucleotides 11 102 to 11 122)

Reverse: 5'-GGGTGGGATTAGGGTTGCTTC (nucleotides -11 873 to -11 895)

Thirty-five cycles of PCR were completed using 30 sec at 94°C for melting, 1 min at 55°C for annealing, and 1 min at 72°C for extension. The first cycle was preceded by 2 min at 94°C, and the final cycle was followed by an additional 7 min at 72°C. Then, samples were held at 8°C. The resulting PCR product was digested with the *Hae*III restriction endonuclease (Promega, Madison, WI) by incubation at 37°C for 1 h. Digested PCR product was electrophoresed through 1.5% (w/v) agarose and stained with ethidium bromide. Phenotype was predicted by banding pattern.

### Properties of Motile Sperm

Computer-assisted sperm motion analysis was performed according to the method described by Froman and Feltmann [5] using roosters from lines selected for either low- or high-sperm-mobility phenotype ( $n = 10$  roosters/line). Ejaculates were pooled by phenotype. The VSL distributions were constructed as outlined by Froman [9]. These distributions, which contained 4290 and 5275 observations for low- and high-sperm-mobility lines, respectively, served as databases for an analysis of the relationship between straightness, which is a measure of sperm cell trajectory [5], and VSL. Datasets approximated logistic functions. Therefore, the parameters of  $y(x) = [\gamma]/[1 + e^{B(\tau-x)}]$  were estimated by iterative least squares [21].

## RESULTS

Sperm mobility was correlated with oxygen consumption ( $r = 0.83$ ) when roosters represented a random-bred population and media were prepared without an exogenous substrate (Fig. 1). In contrast to oxygen consumption, mitochondrial helix length was discounted as a variable af-

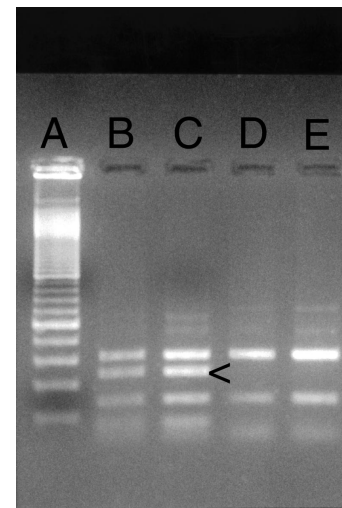


FIG. 3. Results of RFLP analysis for an SNP within the mitochondrial gene for tRNA<sup>Arg</sup>. The DNA was amplified as described in *Materials and Methods*. Males from a line of chickens characterized by low sperm mobility were identified with a 245-bp band (arrow). Lane A: 100-bp DNA ladder; lanes B and C: DNA fragments from low-line males; lanes D and E: DNA fragments from high-line males.

fecting phenotypic expression (Table 1). As evidenced by coefficients of variation, mitochondrial helix length was uniform within males and averaged 3.8  $\mu$ m. When mitochondrial ultrastructure was evaluated before the separation of mobile from immobile sperm, the majority of sperm contained mitochondria comparable to the one shown in Figure 2A. Such mitochondria appeared to be compact, with well-defined cristae. However, a minority of sperm contained aberrant mitochondria comparable to those shown in Figure 2B. The subpopulation of sperm containing aberrant mitochondria increased from  $9\% \pm 2.2\%$  to  $40\% \pm 6.7\%$  (mean  $\pm$  SEM  $P < 0.001$ ) once mobile sperm were separated from immobile sperm. This response was uniform among males; no block effect was observed ( $P > 0.05$ ).

As reported in the NCBI GenBank database [22, 23], the mitochondrial genome from the low- and high-sperm-mobility lines of New Hampshire chickens contained 16 782 and 16 784 base pairs (bp), respectively. As shown in Table 2, multiple SNPs were found within the coding region of mitochondrial DNA. The SNP at position 11 177 within the Arginine TRNA gene was selected for study within the filial generation.

Phenotype of male progeny was evaluated by nested ANOVA (Table 3). As expected, the effect of line was highly significant ( $P < 0.0001$ ). When sperm mobility estimates were based on the average of triplicate observations per male, means and SDs were  $0.124 \pm 0.0580$  and  $0.587 \pm 0.0885$  absorbance units for low- and high-sperm-mobility lines, respectively. Thus, test subjects represented distinct

TABLE 3. Summary of nested ANOVA accompanying RFLP analysis of DNA from chickens within lines selected for either low or high sperm mobility.<sup>a</sup>

Source of variation	Degrees of freedom	Sum of squares	Mean square	F	Variance component (%)
Line	1	8.711893	8.711893	325.98 <sup>b</sup>	89.1
Dam	26	0.694850	0.026725	2.57 <sup>c</sup>	2.3
Male progeny	28	0.290613	0.010379	1.07	0.2
Error	112	1.089128	0.009724	—	8.4

<sup>a</sup> Sperm mobility was measured in triplicate for each of two male progeny from each of 14 dams per line.

<sup>b</sup>  $P < 0.0001$ .

<sup>c</sup>  $P < 0.05$ .



TABLE 4. Comparison of sperm oxygen consumption from roosters with in lines selected for either low or high sperm mobility.

Line	Roosters (n)	Sperm oxygen consumption per $10^9$ sperm ( $\mu\text{L}/\text{min}$ ) <sup>a</sup>
Low sperm mobility	28	$0.8 \pm 0.04^b$
High sperm mobility	28	$2.5 \pm 0.15^c$

<sup>a</sup> Each value is a mean  $\pm$  SEM.

<sup>b</sup> Denote samples with different variances ( $P < 0.01$ ).

populations, even though variation among dams within lines was a significant effect ( $P < 0.05$ ). As evidenced by PCR restriction fragment length polymorphism (RFLP) (Fig. 3), each male and female within the low-sperm-mobility line tested positive for a 245-bp fragment arising from the presence of two *Hae*III sites within the low-line PCR product because of the A-to-G transition at position 11 177.

As shown in Table 4, a threefold difference in sperm oxygen consumption was observed between low- and high-sperm-mobility lines. This difference denoted distinct populations of roosters, because variances differed ( $P < 0.01$ ). Transmission-electron microscopy was performed with a subsample of three nonrelated, representative males per line. Percentages of sperm with aberrant mitochondria were  $47\% \pm 8.4\%$  and  $4\% \pm 2.8\%$  for low- and high-sperm-mobility lines, respectively. As expected, a significant line effect was observed ( $P < 0.05$ ). The VSL distributions are shown in Figure 4. Computer-assisted sperm motion analysis was performed with sperm suspensions containing  $1.2 \times 10^6$  sperm/ml. Motile concentration was estimated to be  $0.51 \times 10^6$  and  $1.01 \times 10^6$  sperm/ml for low- and high-sperm-mobility lines, respectively. Thus, the percentage of motile sperm differed by half between lines (43% vs. 84%), but a threefold difference was found in the number of sperm with a VSL of greater than  $30 \mu\text{m}/\text{sec}$ . Nonetheless, the trajectory of motile sperm did not differ between lines (Fig. 5).

## DISCUSSION

Our work represents a long-term effort directed at identifying a basis for variation in sperm mobility phenotype at the subcellular level. Mitochondria appeared to play a critical role based on biochemical data in initial experiments [1, 2]. A subsequent heritability study added weight to this argument [7]. A central role for mitochondria was plausible in light of the relationship between mitochondrial DNA and disease [24], which includes compromised sperm motility in humans [25–27]. Therefore, our objective was to provide a coherent explanation for phenotypic variation in sperm mobility that took into account mitochondrial DNA [7] and

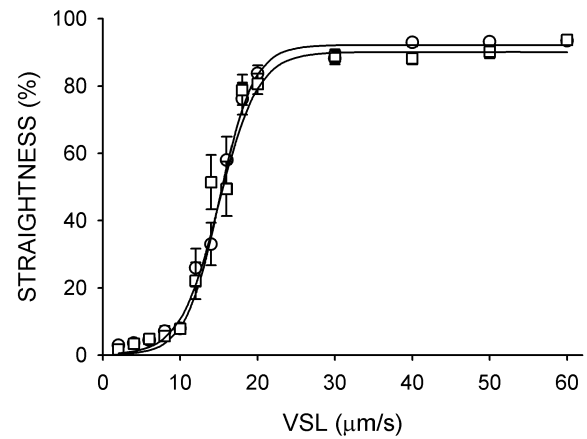


FIG. 5. Effect of VSL on sperm cell trajectory. Straightness denotes the quotient between the straight-line distance between the first and last point of a sperm cell's smoothed path and the length of the smoothed path. Each dataset approximated a logistic function. Each symbol denotes a mean  $\pm$  SEM. Open squares represent data from a sperm cell population characterized by low sperm mobility ( $n = 20$  observations/mean). Open circles represent data from a sperm cell population characterized by high sperm mobility ( $n = 30$  observations/mean).

the role of the mitochondrial  $\text{Ca}^{2+}$  cycle in fowl sperm oxidative phosphorylation [9, 28].

Mitochondrial function can be assessed by variables such as the P:O ratio, proton leakage, ion fluxes, volume, and formation of reactive oxygen species [29]. However, oxygen consumption was deemed to be best for two reasons. First, oxidative phosphorylation is the means whereby fowl sperm convert a portion of the energy inherent to fatty acids into the energy of ATP [9, 28, 30, 31]. Second, selection for low- and high-sperm-mobility lines was performed with exogenous substrate-free media. The demonstration that sperm mobility was correlated with sperm oxygen consumption ( $r = 0.83$ ) (Fig. 1) in a random-bred population corroborated a correlation between sperm mobility and sperm ATP content ( $r = 0.80$ ) using an ancestral random-bred population [1]. More importantly, the range in sperm oxygen consumption (Fig. 1) afforded a means for assessing the efficacy of selection at the level of an organelle. The importance of mitochondrial function in phenotypic expression was advanced by two additional experiments. First, mitochondrial helix length was rejected as a variable affecting phenotypic expression (Table 1). Second, the percentage of sperm with aberrant mitochondria (Fig. 2) increased 4.4-fold ( $P < 0.001$ ) because of the separation of mobile from immobile sperm. We propose that immobile sperm with dysfunctional mitochondria are apoptotic [32].

The mitochondrial genome was sequenced for three rea-

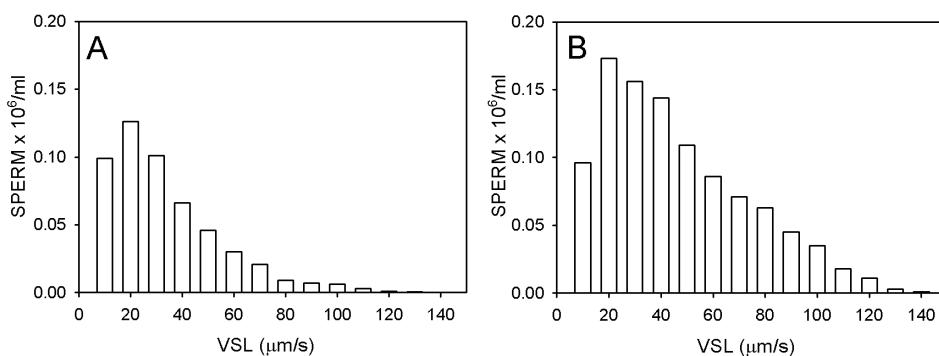


FIG. 4. The VSL distributions from lines of chickens characterized by low (A) or high (B) sperm mobility. Frequencies were constructed from the observed VSL of individual sperm cells within suspensions diluted to  $1.2 \times 10^6$  sperm per ml. In the case of the low-sperm-mobility line, the number of sperm with a VSL of greater than  $30 \mu\text{m}/\text{sec}$  was 32% of that observed for the high-sperm-mobility line.

sons. First, a significant maternal additive genetic effect was observed when heritability was estimated, and this effect had a low genetic correlation with the animal additive effect [7]. Thus, the mitochondrial genome not only may affect male fitness but also sexual selection [33]. Second, selection for low- and high-sperm-mobility lines used a scheme in which dams played a critical role. Specifically, female siblings of roosters characterized by either low or high sperm mobility were bred to nonrelated males with a phenotype comparable to that of their brothers. Third, heritable mitochondrial disease may stem from either deletions or point mutations within the mitochondrial genome [34]. Thus, a conservative approach, in which mitochondrial DNA was sequenced from multiple individuals of known phenotype per line, was deemed to be best.

The SNPs detected within the coding region of mitochondrial DNA recovered from blood cells are outlined in Table 2. Of these, the SNP at position 11 177 was selected for further study. This SNP was uniformly inherited, as evidenced by RFLP analysis (Fig. 3). The 795-bp PCR product contained an *Hae*III site (GGCC) that yielded a 300-bp fragment in the case of the high-sperm-mobility line. In contrast, the A-to-G transition at position 11 177 resulted in a second *Hae*III site within the PCR product in the case of the low-sperm-mobility line. Consequently, a 245-bp fragment was derived from the 300-bp fragment. This 245-bp fragment uniformly identified male and female progeny from the low-sperm-mobility line. Thus, in this experimental case, RFLP analysis served the same purpose as the sperm mobility assay for identifying males by line (Table 3).

The difference in sperm mobility between lines (Table 3) was explicable in terms of three interrelated variables: sperm oxygen consumption, mitochondrial ultrastructure, and motile concentration. In the case of oxygen consumption, mean values (Table 4) represented extremes of the range observed with random-bred roosters (Fig. 1). As evidenced by means and variances (Table 4), genetic selection for low and high sperm mobility resulted in extremes in mitochondrial function and ultrastructure (Fig. 2). Whereas only 4% of sperm from high-line males contained aberrant mitochondria, 47% of sperm from low-line males contained mitochondria that were swollen and contained disorganized cristae ( $P < 0.05$ ). Thus, the extent of mitochondrial dysfunction appeared to be the variable that accounted for the difference in motile concentration observed between low and high lines ( $0.51$  vs.  $1.01 \times 10^6$  sperm/ml). It is noteworthy that these values represent extremes in the range observed in an ancestral random-bred population [5]. However, the nature of motile sperm did not differ between lines, as evidenced by the relationship between VSL and straightness (Fig. 5). Rather, it was the number of highly motile sperm that differed (Fig. 4). This conclusion is consistent with the observation that sperm mobility of commercial meat-type chickens was a function of the number of sperm with a VSL of greater than  $30 \mu\text{m/s}$  when test males represented the phenotypic range [6].

Our research has three implications. First, male fitness in galliform birds can be altered by genetic selection when sperm mobility is the selection criterion. Second, the effect of mitochondrial DNA may warrant consideration in such an endeavor. The SNP that we investigated may not contribute to phenotypic expression, but our interest in mitochondrial DNA stemmed from strong empirical evidence before selection. In addition to heritability data, this evidence included producing replicate low- and high-sperm-

mobility family groups by crossing a single sire characterized by average sperm mobility with full-sib dams whose brothers expressed a low- or high-sperm-mobility phenotype [7]. In other words, the phenotype of male progeny was predicted by that of maternal uncles rather than by that of the sire. If this SNP contributes to phenotypic expression by altering the structure of mitochondrial tRNA<sup>Arg</sup>, then this mutation may not be universally applicable for phenotypic prediction based on RFLP analysis. Nonetheless, our experiments demonstrate the potential for using RFLP analysis to identify males at hatch with distinct reproductive potentials. Likewise, females with the potential to produce subfertile male offspring also might be identified. Whereas the association of mitochondrial DNA with male subfertility is not novel [25–27], our research illustrates a novel application of DNA-based technology in the context of animal breeding.

The third implication of our research pertains to sperm maturation, which is understood poorly in the fowl. According to Lake [10], fowl deferent duct fluid contains less than  $1 \text{ mM Ca}^{2+}$ ,  $30\text{--}40 \text{ mM K}^{+}$ , and approximately  $80 \text{ mM}$  glutamate. In contrast, blood plasma contains  $5 \text{ mM Ca}^{2+}$ ,  $5 \text{ mM K}^{+}$ , and only  $0.2\text{--}0.3 \text{ mM}$  glutamate [35]. Fowl sperm express glutamate channels, as evidenced by the effect of *N*-methyl-D-aspartic acid (NMDA) on fowl sperm VSL [9]. The NMDA receptor is permeable to  $\text{Ca}^{2+}$  and  $\text{K}^{+}$  in addition to  $\text{Na}^{+}$  [36]. We propose that mitochondrial uptake of  $\text{Ca}^{2+}$  and loss of  $\text{K}^{+}$  are phenomena related to sperm maturation and that glutamate channels mediate the flux of these ions at the plasma membrane level. Furthermore, we propose that variation in sperm mobility phenotype depends on the extent to which glutamate contributes to excessive mitochondrial uptake of  $\text{Ca}^{2+}$  among sperm within a population moving through the excurrent ducts of the testis. This proposal is consistent with the role of extracellular  $\text{Ca}^{2+}$  in the demise of mitochondria arising from glutamate-mediated toxicity [37, 38], and it explains why any given ejaculate contains a mixture of immobile and mobile sperm cells.

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