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Sperm mobility denotes the net movement of a sperm population. Previous work implicated mitochondrial function as a basis underlying phenotypic variation in this quantitative trait. Our objective was to determine if mitochondrial function was indeed critical to expression of phenotype. Phenotype was assigned to roosters within a random bred population (n = 242). A representative subpopulation (n = 40) was used to correlate sperm mobility with oxygen consumption (r = 0.83). In contrast, sperm mobility was independent of mitochondrial helix length in a sample of males (n = 7) representing the range of phenotype observed within the population. Thus, mitochondrial function rather than number appeared to be critical to expression of phenotype. This hypothesis was tested by ultrastructural analysis of sperm midpieces. Males from the lower and upper tails of the distribution were characterized with high and low proportions of sperm containing aberrant mitochondria in 47 and 4% of the cells respectively. When sperm from average males were allowed to segregate into immobile and mobile subpopulations, 40% of immobile sperm contained aberrant mitochondria. In contrast, only 9% of sperm

from the same males contained aberrant mitochondria in non-segregated populations. In conclusion, the mitochondrion is an organelle that may account for phenotypic differences in sperm mobility.

Mitochondrial Function Is a Primary Variable Affecting Sperm Mobility Phenotype in the Domestic Fowl

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Mitochondrial Function Is a Primary Variable Affecting Sperm Mobility in the Domestic Fowl

INTRODUCTION

Various semen evaluation techniques have been employed to evaluate male fertility. Traditional methods still utilized include visual inspection of an ejaculate, sperm concentration, viability, morphology and motility. Unfortunately, some of these measurements are subjective whereas others have never really served as predictors of male fertility. For example, sperm concentration can be readily determined objectively with a hemocytometer, a Coulter counter, or via fluorometric or spectrophotometric measurements. Apart from identifying azoospermic males, these techniques typically cannot discriminate among normal, fertile males. Such limitations prompted the use of sperm function tests in which measurements are based upon the acrosome reaction, sperm binding, or zona penetration. Whereas these tests have been popular with some researchers, they are often quite time consuming and tend to be limited to mammalian sperm. One powerful objective test of sperm quality is computer-assisted sperm motion analysis (CASA), which has been found to be moderately more successful in the identification and selection of sperm donors. This technology has been used to examine either the proportion or number of motile sperm, sperm cell velocity and trajectory. However, this technology is limiting due to the cost of instrumentation.

The assessment of individual sperm can also be performed by microscopy. Light microscopy can be used for gross morphology. Fluorescence microscopy can be used for the counting of live, moribund, and dead sperm. However, only electron microscopy can

be used to either evaluate whole sperm in detail or the organelles that enable sperm to be progressively motile, i.e. mitochondria and the axoneme. Scanning electron microscopy enables the visualization of functional domains on the surface of whole sperm. In contrast, transmission electron microscopy enables one to visualize sperm cell cross-sections, and at high magnification, sections of organelles, structures too small to be resolved by light microscopy.

More recently, molecular methods have been used to diagnose factors that exert a negative influence on male fertility. Continued application of the latest advances in molecular biological technology promises to assist the diagnosis of male infertility and reproductive disease. However, as powerful as these techniques may be, it is doubtful these techniques will differentiate among normal, fertile males. Thus, while profound differences exist among such males, the semen evaluation tests outlined above typically have not been able to consistently identify the most fertile males.

However, a recent test has been developed that, at least in poultry, is predictive of male fertility. The sperm mobility test measures the movement of sperm cell populations by the autosegregation of highly motile sperm from those that are poorly motile or immotile. The sperm mobility test has demonstrated that the size of these subpopulations of motile and immotile sperm shows extreme variation among normal, fertile males. Such categorization is meaningful because sperm mobility is a primary determinant of male fitness. It is presumed that *in vitro* sperm mobility is predictive of *in vivo* sperm mobility because sperm move against resistance in either case.

Previous work has attempted to explain phenotypic differences in sperm mobility. These experiments have found differences in sperm acyl-carnitine content, oxygen

consumption and ATP content between males representing the extremes of a distribution. Sperm cell mitochondria utilize fatty acids as one of their primary energy sources. In order for a fatty acid to enter the mitochondrial matrix, it must first be conjugated to a carnitine molecule. Acyl-carnitine is a term used to describe the activated form of long-chain fatty acids used as endogenous substrates within fowl sperm. Collectively, these data have implicated mitochondrial function as the basis underlying phenotypic variation in sperm mobility. This is because sperm cell ATP is generated by oxidative phosphorylation within the mitochondria, and the energy inherent in ATP is derived from fatty acids.

Such data, however, did not provide direct evidence of the role of mitochondria. Therefore, the objective of this thesis was to find unequivocal evidence that the phenotypic differences in sperm mobility were explicable in terms of mitochondrial function. Oxygen consumption was considered to be the simplest, most telling measurement of the dynamic nature of mitochondrial function. Likewise, transmission electron microscopy was deemed to be a complementary experimental approach for ultrastructural analysis of sperm mitochondria.

LITERATURE REVIEW

Sperm mobility is a primary determinant of male fertility in the fowl and is defined as the net movement of a sperm population. (Froman, 1998). Sperm cell motility is affected by numerous factors, such as chemical and physical environmental factors, structural integrity and metabolic potential (Ishijima et al., 1990). Sperm cell response to environmental conditions is a function of how reproductively successful it will become in the future.

The fowl testes produce 60,000 sperm cells per second. The testes are located internally and are structurally similar to that of the mammalian testes. Spermatogenesis and steroidogenesis in the bird proceed at 41°C. After entry into the spermatogenic cycle, spermatogonia proceed through meiosis to give rise to spermatozoa in 13-15 days. Avian spermatozoa have been found to be capable of fertilization as soon as they emerge from the seminiferous tubules if inseminated surgically above the uterovaginal junction and do not require maturation in the epididymis, as in the mammal. Avian spermatozoa are stored in the deferent duct and do not require capacitation. The ability of sperm to become motile is gained during passage through the deferent duct where the spermatozoa are stored prior to ejaculation.

Sperm motility is vital for a reproductively sound sperm cell. In the 1930's (Hartmann, 1932; Gunn, 1936) it was discovered that mammalian sperm are most active at body temperature but are extremely sensitive to this heat and therefore are not viable for any length of time. The testes and accessory glands in the male fowl however, are located intra-abdominally and their sperm are capable of surviving within the female

reproductive tract for up to 5 weeks post mating (Elford, 1916; Crew, 1926). Munro (1938) hypothesized that fowl sperm are for the most part quiescent in the female reproductive tract and in fact remain immotile in vaginal sperm-storage tubules (SST) for much of their 3-5 week stay (Lake, 1975). The mechanisms in which these cells then exit the SST are unknown. It has been hypothesized that head-to-head agglutination of the sperm within these tubules account for this prolonged storage (Compton & VanKrey, 1981; Froman & Engel, 1989). Furthermore, it has been suggested that the continuous release of these spermatozoa from the SST (Burke & Ogasawara, 1969) during the ovulatory cycle is due to the progressive decrease in the agglutination capacity of the sperm (Froman & Engel, 1989).

Internal fertilization of the domestic hen depends on sperm ascending the tract and penetration of the ovum. This breach into the perivitelline space involves hydrolysis of the proteinaceous layer by sperm acrosomal enzymes (Bakst and Howarth, 1977).

In the 1950's artificial insemination methods were just beginning to surface. Shaffner (1941) was the first to successfully produce fowl chicks from deep-frozen semen. But not until the 1980's was the development of a successful, standardized cryopreservation protocol for semen first introduced into avian production (Lake et al., 1981 and 1986). Since this time, research has been done to study the effects of cryopreservation on cellular integrity and mechanism (Wishart and Palmer, 1985 and 1986; Mazur, 1984). However, very few studies have looked at freezing effects on avian sperm specifically (Ravie and Lake, 1982; and Zavos and Graham, 1983). Although fertilization rates have undoubtedly improved since the 50's, it is unclear if this is attributed to more advanced cryopreservation techniques (Lake and Wishart, 1984;

Chaudhuri and Lake, 1988; Blasbois and Mauger, 1987) or a genetically more superior breed of bird.

There has been a long-term interest in evaluating male fertility. Various semen techniques have been employed over the years to evaluate male fertility. Motility of poultry sperm is evaluated through such methods as spectrophotometry (Wishart, 1984; Ashizawa and Wishart, 1987, 1992; Froman and Thursam, 1994), CASA (Bakst and Cecil, 1992; Froman & Feltmann, 2000), microscopy and a technique using Accudenz to measure sperm penetration and subsequently sperm mobility and fertility (Froman and McLean, 1996). This type of method of sperm evaluation has been used on many different species and was first introduced in the early 90's (Suttiyotin and Thwaites, 1993) however has proved to be extremely successful in mobility and fertility determination in the fowl. It uses the idea that sperm move against resistance in both *in vivo* and *in vitro*. Using a dense suspension of Accudenz overlaid with a semen suspension, motile sperm will enter the solution rapidly, whereas immotile or poorly motile sperm will not. Following sperm migration into the Accudenz, sperm mobility can be quantified using spectrophotometry.

Maintenance of fowl sperm mobility is highly contingent upon successful mitochondrial function. The midpiece of fowl sperm is a helical array of 25-30 densely packed mitochondria that surround the axoneme and is responsible for energy production (Fawcett, 1975). During spermiogenesis, mitochondria aggregate about the midpiece following annulus migration and formation of the flagellar structural elements. During mammalian spermiogenesis, germ cell mitochondria undergo various protein composition and shape modifications (Hecht and Bradley, 1981; Demartino et al., 1979; Clermont et

al., 1990). Additional changes occur during sperm maturation in the epididymis. First, the outer membranes become crosslinked with disulfide bond linkages (Calvin and Bedford, 1971); second, mitochondrial capsule proteins (MCP) are synthesized and incorporated in the membrane during spermiogenesis (Calvin et al., 1987; Kleene et al., 1990). These MCPs along with the disulfide bonds together help stabilize the outer mitochondrial membranes (Pallini et al., 1979; Calvin et al., 1981). The relationship of these changes to mitochondrial function in the mature gamete is unclear.

Spermatazoal ultrastructure of the domestic chicken, guinea fowl, and turkey have been well characterized (Nagano, 1960; Bakst et al., 1975; Lake et al, 1968; Marquiz et al., 1975 and Tingari, 1973). The ability to analyze sperm on the surface as well as internally via microscopic means was first developed in the early 1970's. Scanning and transmission electron microscopy have been utilized for studying many features of spermatozoa, as well as the extent of morphological abnormalities within a semen sample.

Mitochondria are comprised of two compartments, an outer or intermembrane compartment and an inner or matrix compartment. The outer compartment is responsible for the exclusion of protein molecules and the free passage of metabolite molecules. Within the mitochondria there are large enzyme complexes, cytochrome c and ubiquinone molecules, all which comprise the respiratory chain system. Complex V or ATP synthase complex is responsible for the synthesis of ATP from ADP and inorganic phosphate. Fertilization in the hen depends upon motile sperm and motility depends upon energy supply. This energy is provided in the form of ATP, which is generated via oxidative phosphorylation. Mitochondria in the sperm cell lie adjacent to dynein ATPase

in the axoneme which ATP energy is delivered and is ultimately responsible for flagellar motility (Cardullo, 1991). In humans, evidence suggests that the presence of a shorter midpiece and therefore fewer mitochondria lead to male infertility (Mundy et al., 1995).

Sperm cell mitochondria use long chain fatty acids as their energy to generate ATP. Long-chain fatty acid oxidation is dependent on the activity of a carnitine carrier in the inner membrane, which transports activated fatty acids from their site of formation at the outer membrane to their site of oxidation in the matrix.

Disruption of mitochondrial enzymatic activities is usually associated with mitochondrial dysfunction and may provide biochemical correlation between mitochondrial dysfunction and male infertility. Disorders of mitochondrial metabolism are mainly due to mutation, age and oxidative stress which all can affect mitochondrial functions in numerous ways. Defects can occur during protein synthesis and in the enzymes and or receptors functional during post-translational processing of the proteins or mitochondrial functions can be impaired by inadequate protein transport through the membranes. Some mitochondrial disorders are caused by deletions in the mitochondrial genome. A recent report done by Ruiz-Pesini et al (1998) show that sperm motility depends upon mitochondrial energy production in humans. In addition to these findings, PCR amplification of mtDNA show a significantly higher rate of mtDNA deletions in asthenozoospermic patients compared to normal fertile patients (Kao et al., 1995).

Mitochondria are the centers for oxygen reduction and can be susceptible to toxic oxygen radicals formed during metabolism. Examples include superoxide and hydroxyl radicals. Oxygen radicals cause significant cell damage in various different ways.

Structurally and metabolically healthy mitochondria have their own defense mechanisms against these oxygen radicals.

In the fowl, data have been collected that provide evidence that sperm mobility may also be related to mitochondrial function. These experiments have found differences in sperm acyl-carnitine content (Froman et al., 1999); oxygen consumption and ATP content (Froman and Feltmann, 1998) between males of high and low fertility. These data however have yet to show direct evidence that mitochondrial dysfunction is correlated with male infertility. Therefore, the overall objective of this thesis is to evaluate phenotypic differences in sperm mobility and to find direct evidence relating mitochondrial function and fertility.

MATERIALS AND METHODS

Correlation Analysis

Sperm mobility of random bred New Hampshire roosters (n = 242) was determined according to Froman et al. (1999). Based upon a single mobility score, each 27-wk-old male was assigned to one of 11 frequencies, and the Kolmogorov-Smirnov test for goodness of fit (Sokal and Rohlf, 1969a) was used to determine whether observed frequencies approximated a normal distribution. A representative subpopulation (n = 40 roosters), as evidenced by range of scores and coefficient of variation, was selected from the base population. A semen sample from each rooster was diluted so that a 2-ml suspension was procured containing 2.5×10^8 sperm/ml. Semen was diluted with 50 mM *N*-Tris-[hydroxy-methyl]methyl-2-amino-ethanesulfonic acid (TES; Sigma Chemical Co., St. Louis, MO), pH 7.4, containing 128 mM NaCl and 2 mM $CaCl_2$ (TES-buffered saline). Oxygen consumption of diluted sperm was measured over a 3-min interval at 41°C with a Model 5300 YSI Biological Oxygen Monitor (Yellow Springs Instruments, Yellow Springs, OH). A second sample from each ejaculate was used to measure sperm mobility (Froman et al. 1999). Sperm mobility was correlated with oxygen consumption (Sokal and Rohlf, 1969b).

Mitochondrial Helix Length

Seven roosters were selected from the base population based upon ranked mobility scores. Collectively, phenotypes represented the population range. A semen sample from each male was diluted 1:40 in neutral buffered formalin. Fixed sperm were washed by diluting a 200-µl volume of sperm suspension to 1.5 ml with PBS followed by

centrifugation at 10,000 rpm for 1 min. Supernatants were discarded. Sperm were resuspended in 1.5 ml PBS and centrifuged again. A 10-µl volume of washed sperm was placed on a slide, smeared, and air-dried. A 1-ml volume of supernatant from a hybridoma cell line containing a monoclonal antibody was used to cover the slide. This antibody previously was shown to bind an epitope on the mitochondria of galliform birds (Korn et al. 2000). Each slide was incubated with primary antibody for 1 h at room temperature. Thereafter, the antibody solution was discarded, the slide washed twice with PBS, and then covered with a 1:50 dilution of a goat anti-mouse IgG conjugated to fluorecein isothiocyanate (FITC; Sigma Chemical Co., St. Louis, MO) in PBS containing 1% (w/v) BSA for 10 to 30 minutes. The slide was washed twice with PBS containing 1% (w/v) BSA and washed once with PBS. Sperm were counterstained with ethidium bromide (12 µg / ml PBS) by covering the slide with stain for 1 min and washed with PBS. Sperm were examined at 100x under oil immersion with a Nikon Optiphot 2 microscope equipped with an epifluorescent illuminator. Excitation wavelength was 450 to 490 nm. Measurements of helix length were made on the scale of centimeters from color slides projected onto a flat surface. Observed values were converted to micrometers. Fifty observations were made per male. Data were analyzed by single classification ANOVA (Sokal and Rohlf, 1696c).

Mitochondrial Ultrastructure

Roosters from the tails of the population distribution, i.e. low and high sperm mobility phenotypes, were re-evaluated by the sperm mobility assay. Three males were selected from each phenotype. Males were selected based upon consistency of their sperm mobility scores. The phenotypic difference was confirmed by ejaculating males

on an every-other-day basis (n = three ejaculates per male) and measuring sperm mobility in triplicate per ejaculate. Data were evaluated by nested ANOVA (Sokal and Rohlf, 1969d).

Samples were prepared for transmission electron microscopy (TEM) as follows. Each ejaculate was microcentrifuged for 20 seconds in order to concentrate spermatozoa. A column of pelleted spermatozoa was extruded from a Pasteur pipet into 3 ml of 2% (v/v) glutaraldehyde in Millonig's phosphate buffer, pH 7.35. Sperm were fixed for 3 hours at room temperature, rinsed with fresh Millonig's buffer, and then post-fixed in 2% (w/v) osmium tetroxide for 3 hours. Each sample was dehydrated by passage through a graded series of acetone solutions. Dehydrated samples were placed in a 1:1 mixture of acetone and Spurrs epoxy plastic for 3 hours. Thereafter, a second volume of epoxy plastic was added with mixing and specimens left to sit overnight. Subsamples of the cylinder of compacted cells were transferred to fresh epoxy plastic in Beam capsules, and the plastic polymerized by heating at 64°C for 1 hour. Once trimmed, blocks were ultrathin sectioned using a diamond knife. A series of 7 to 10 serial sections per male were retained and placed on a 200 mesh copper grid. Each set of serial sections was placed on a grid. Sets of serial sections were approximately 150 μm apart to ensure each set contained different sperm cells. Each grid was then stained with uranyl acetate and lead citrate and a single section from each set was chosen, and TEM analysis performed with a CM12 Phillips transmission electron microscope. Midpiece sections (n = 500 per male) were categorized according to mitochondrial ultrastructure, i.e. normal or abnormal. Proportions of sperm with abnormal mitochondria were converted to logits

and transformed date evaluated by single classification ANOVA (Sokal and Rohlf, 1969c).

A second experiment was performed with ejaculates from males categorized by average sperm mobility. In this case, the proportion of sperm with abnormal mitochondria was determined for extended semen overlaid on Accudenz before and after incubation at 41°C. Samples were prepared and data transformed as outlined above. Transformed data were analyzed with a paired comparison (Sokal and Rohlf, 1969e).

RESULTS

Frequencies of sperm mobility phenotypes are shown in Figure 1. The hypothesis that observed frequencies approximated a normal distribution was not rejected ($P > 0.05$). As shown in Figure 2, sperm mobility was correlated ($r = 0.83$) with oxygen consumption using a subpopulation of roosters representative of the base population. A photomicrograph of a rooster sperm cell labeled with mouse monoclonal anti-mitochondrial antibody is shown in Figure 3. Immunofluorescence was used to estimate spermatozoal mitochondrial helix length. Mean helix length for roosters representing the range of sperm mobility phenotypes observed in the base population are shown in Table 1. As evidenced by coefficients of variation, little variation in helix length was observed within males. Whereas ANOVA detected a significant variation in helix length among males ($P < 0.01$), this difference was limited to a single average male. Thus, the difference could not account for the variation in sperm mobility phenotype observed among males within a population.

In contrast, the evaluation of mitochondrial ultrastructure revealed a structural defect that provided an insight into the variation in sperm mobility observed among males within a population (Fig. 1) as well as variation in sperm oxygen consumption observed among males (Fig. 2). Figures 4, 5 and 6 show representative sections of spermatozoal midpieces from males characterized by low sperm mobility. Whereas the majority of sperm from low sperm mobility males were characterized by midpieces containing mitochondria with well-organized cristae and a closely adherent plasma membrane (Fig. 4), 47% of the sperm from such males had midpieces that contained

aberrant mitochondria (Fig. 4 and 5). In contrast, only 4% of sperm from high sperm mobility males contained midpieces with aberrant mitochondria Table 2). Regardless of sperm mobility phenotype, the anomaly appeared to be indicative of a degenerative process in that aberrant mitochondria ranged from compact organelles with disorganized cristae to grossly swollen organelles lacking discernible cristae (see Fig. 5 versus 6). The phenotypic difference in mitochondrial ultrastructure ($P < 0.05$) appeared to be independent of sperm viability. Sperm viability was estimated by ethidium bromide exclusion at 95 ± 4.5 and $99 \pm 0.3\%$ for low and high sperm mobility males, respectively. Due to the small sample size used in the initial TEM experiment, sperm mobility phenotype was confirmed by repeated measure analysis. As shown by the outcome of the ANOVA in Table 3, experimental males did indeed represent extremes in sperm mobility phenotype. The outcome of the second TEM experiment is shown in Table 4. In this case, the autosegregation of mobile sperm enhanced the proportion of sperm with aberrant mitochondria over four-fold ($P < 0.001$) in the subpopulation of immobile sperm.

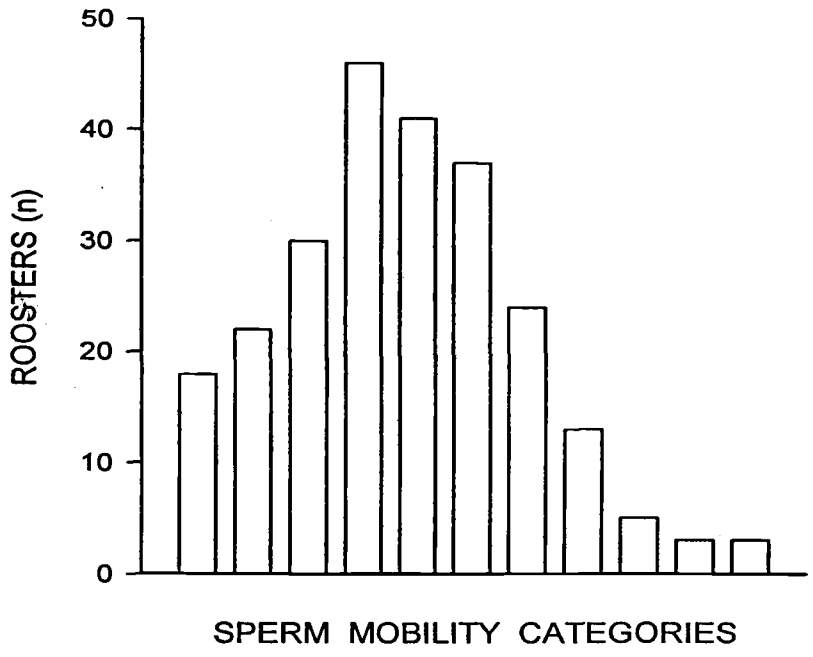

FIGURE 1. Frequency distribution following categorization of New Hampshire roosters (n = 242) according to sperm mobility scores. A single observation was made per rooster. Each category denotes an increment of 0.090 absorbance units from a baseline of zero. Thus, the category with the maximal number of roosters denotes a range of 0.27 to 0.36 absorbance units. Scores ranged from 0.008 to 0.990 absorbance units. The hypothesis that observed frequencies approximated a normal distribution was not rejected ($P > 0.05$). Estimates of the population mean and standard deviation were 0.38 and 0.19 absorbance units, respectively.

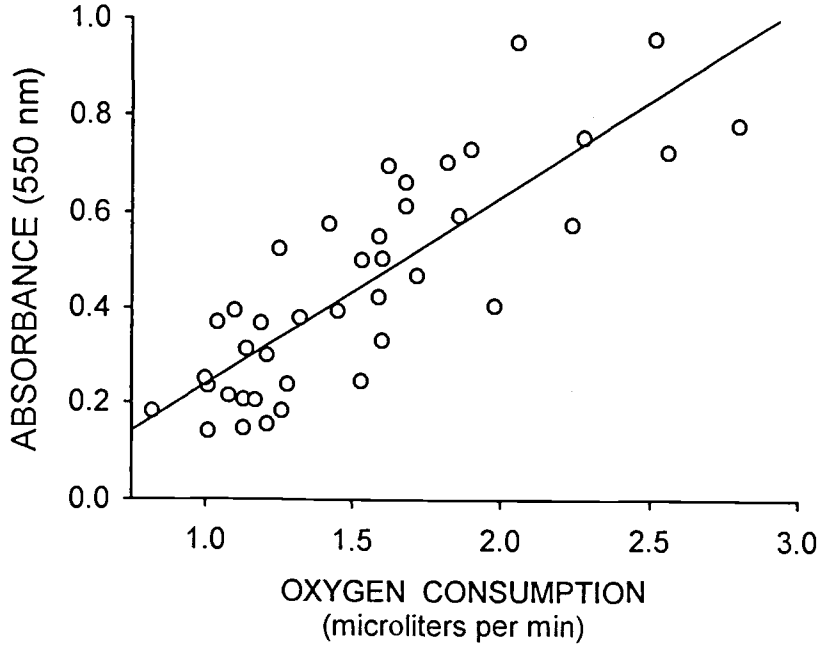

FIGURE 2. Correlation of sperm mobility and oxygen consumption. Each data point (o) represents data collected from a single rooster immediately after ejaculation (n = 40 roosters). The solid line denotes the regression equation: absorbance = -0.154 + (0.393)(O_2 consumption). The product-moment correlation coefficient was 0.83.

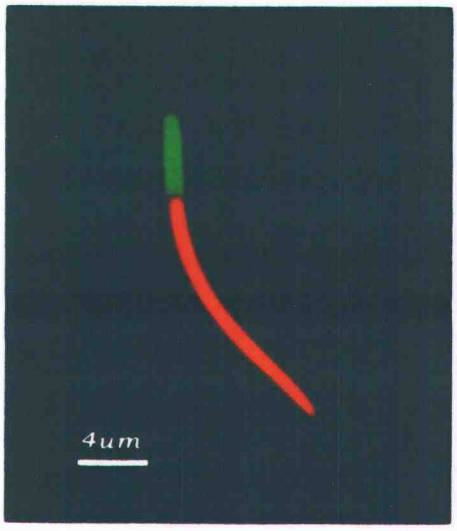

FIGURE 3. Epifluorescence micrograph of a rooster sperm labeled with a mouse monoclonal antibody specific for an epitope on the outer mitochondrial membrane. The mitochondrial helix, 3.9 μm in length, appears green due to FITC labeling of the secondary antibody. The nucleus was counterstained red with ethidium bromide.

TABLE 1. Estimates of mitochondrial helix length from roosters representing the range of sperm mobility phenotypes observed in a base population.

Rooster	Sperm mobility* (absorbance units)	Helix length (μm)†	Coefficient of Variation (%)
1	0.130	3.85 ± 0.02	2.9
2	0.208	3.81 ± 0.02	3.2
3	0.415	3.74 ± 0.02‡	3.3
4	0.496	3.86 ± 0.02	3.2
5	0.560	3.86 ± 0.01	2.5
6	0.737	3.84 ± 0.01	2.9
7	0.836	3.84 ± 0.01	3.7

*Sperm mobility was quantified as described in *Materials and Methods*.
† Each value is a mean ± SEM.
‡ Different from non-superscripted means ($P < 0.01$).

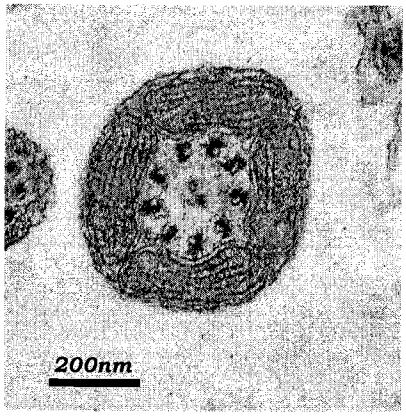

FIGURE.4 Cross-section of a normal midpiece from a rooster categorized as a low mobility phenotype .

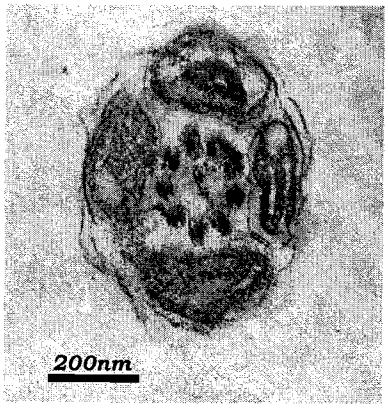

FIGURE 5. Cross-section of a midpiece with moderate abnormalities from a rooster categorized as a low mobility phenotype.

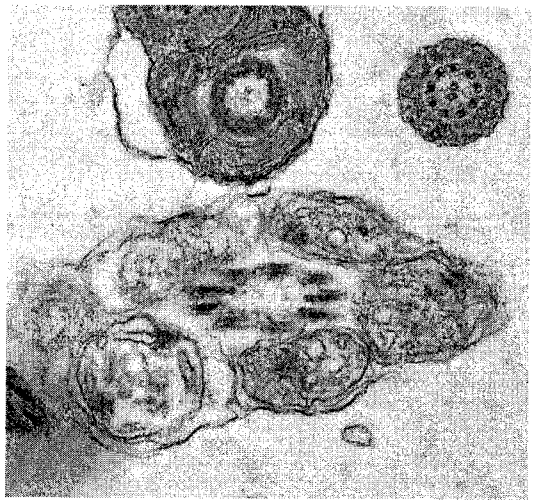

FIGURE 6. Cross-section of a highly abnormal midpiece from a rooster categorized as a low mobility phenotype.

TABLE 2. Percentages of sperm with aberrant mitochondria from roosters categorized by sperm mobility phenotype.

Phenotype	Males (n)	Sperm with Aberrant mitochondria (%)[†]
Low	3	47 ± 8.4^{a}
High	3	4 ± 2.8^{b}

[†] Each value is a mean ± SEM.
[a,b] Means within a column differ ($P < 0.05$).

TABLE 3. Summary of nested ANOVA used to confirm phenotypic difference between males (n = 3 per phenotype) used for ultrastructural analysis of sperm mitochondria.

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-value	Variance component (%)
Phenotype	1	3.4222	3.4222	832.54****	95.9
Male	4	0.0164	0.0041	0.53	0.2
Ejaculate	12	0.0934	0.0079	1.83	0.9
Replicate	36	0.1528	0.0042	--	3.0

**** ($P < 0.0001$).

TABLE 4. Incidence of sperm with aberrant mitochondrial morphology following autosegregation.

Time*	Sperm with aberrant mitochondria (%)†
Prior to autosegregation of sub-Populations of mobile and immobile sperm	9 ± 2.2^{A}
After autosegregation of sub-Populations of mobile and Immobile sperm	40 ± 6.7^{B}

*See *Materials and Methods* for details.
† Based on 500 sperm from each of 10 roosters categorized beforehand with average sperm mobility.
^{A,B}Means ± SEM within a column differ ($P<0.001$).

DISCUSSION

The objective of the present research was to confirm a pivotal role for mitochondria in the expression of sperm mobility, a new quantitative trait (Froman and Feltmann, 1998). Previous work demonstrated that sperm mobility phenotype was fully expressed when washed sperm were resuspended in a nutrient-free medium (Froman and Feltmann, 1998). This observation unequivocally demonstrated that fowl sperm use an endogenous substrate to generate ATP by mitochondrial respiration. The substrate was subsequently shown to be *n* – octadecanoic acid (Froman et al., 1999). Having correlated sperm ATP content with sperm mobility ($r = 0.80$) in previous work, we expected to observe a similar relationship between sperm mobility and sperm oxygen consumption. As shown in Figure 2, this was indeed the case ($r = 0.83$). Therefore, we concluded that phenotypic differences in sperm mobility were associated with mitochondrial function.

However, the correlation of either sperm ATP or oxygen consumption with sperm mobility presented an interpretive limitation in that measurements of either ATP or oxygen consumption presented *averaged* values for populations of sampled sperm. This limitation was significant because computer-assisted sperm motion analysis (Froman and Feltmann, 2000) had revealed that motile concentration and straight-line velocity were the two most important variables that accounted for phenotypic differences in sperm mobility. Furthermore, distributions of straight line velocity ranged from 2 to 140 μm/s but were skewed towards a central tendency of 21 to 30 μm/s (unpublished data). Thus by inference, the rate of ATP synthesis within sperm cells necessarily differed within and among males within a population. This realization was a refinement of the relationship

between mitochondrial ATP synthesis and ATP consumption at the level of the axoneme (Halangk et al., 1985); for mitochondrial ATP synthesis appeared to show extreme variation among sperm within populations of viable cells.

Research with mammalian sperm has addressed the relationship between mitochondrial volume and sperm motility (Cardullo and Baltz, 1991). Therefore, we tested the possibility that phenotypic differences in sperm mobility might be due to differences in mitochondrial helix length using a monoclonal antibody specific for mitochondria (Fig. 3). Whereas variation in helix length was observed (Table 1), such variation did not account for the range in sperm mobility phenotype. We therefore suspected that mitochondrial function was essential to phenotypic expression.

While the measurement of mitochondrial enzyme complex activities is a routine procedure and a deletion in the human mitochondrial genome has been shown to affect sperm motility (Kao et al., 1995), we deemed the ultrastructural analysis of the sperm midpiece to be a more conservative experimental approach. We argued that if TEM could be used to demonstrate differences in mitochondrial ultrastructure between sperm mobility phenotypes, then mitochondrial function would be a contributing variable relevant to expression of phenotype. As shown in Table 2 and Figure 4, differences in mitochondrial structure were found that afforded an insight into relationships observed between either sperm ATP content (Froman and Feltmann, 1998) or sperm oxygen consumption (Fig. 2) and sperm mobility. Because ultrastructural analysis necessarily requires the use of small sample sizes, precaution was taken to insure that semen donors did indeed represent distinct phenotypes (Table 3). In view of the initial TEM experiment and sperm mobility being a quantitative trait, we hypothesized that the

autosegregation of mobile from immobile sperm during the sperm mobility assay would afford an enrichment of sperm with aberrant mitochondria in the immobile subpopulation. The hypothesis was tested using males characterized by average sperm mobility in a paired comparison. As shown in Table 4, sperm with aberrant mitochondria were associated with the immobile subpopulation. In summary, the mitochondrion is an organelle that clearly affects expression of sperm mobility phenotype in the domestic fowl.

However, this discovery prompts three key questions. First, what mechanisms account for the heterogeneity of spermatozoal mitochondrial ultrastructure observed among and within males? Based upon estimates of 4, 9, and 47% for sperm with aberrant mitochondria from high, average, and low sperm mobility males, respectively, heterogeneity among males may include differences in kind in addition to degree. Second, when does this defect first appear? Is it evident during spermiogenesis, or does it develop as sperm traverse the excurrent ducts of the testis, or does it accompany the onset of sperm motility at ejaculation? Finally, is this phenomenon heritable?

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