AN ABSTRACT OF THE THESIS OF

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Title: Identification of a Mechanism Underlying Heritable Subfertility in Roosters Homozygous for the Rose Comb Allele.

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David P. Froman

The overall objective of this research was to define the cellular basis underlying heritable subfertility in roosters homozygous for the rose comb allele (*R/R*). Fertilization in the hen is preceded by the ascension of motile sperm through the vagina and sperm sequestration within sperm storage tubules (SST). The objective of the first set of experiments was to determine if reduced sperm sequestration could account for subfertility. Sperm sequestration differed between genotypes following intravaginal insemination ($p < 0.0001$). However, sperm sequestration did not differ between genotypes when sperm were incubated with SST *in vitro* ($p > 0.05$). Therefore, subfertility was attributed to reduced sperm transport within the vagina. To test this hypothesis, an assay was developed to evaluate fowl sperm motility *in vitro*. Based upon this assay, ejaculates from subfertile males contained smaller subpopulations of highly motile sperm than the ejaculates from controls ($p < 0.001$).

The objective of the next set of experiments was to characterize the motility of individual sperm and to identify a mechanism that could account for the genotypic

difference in sperm cell motility. Computer-assisted sperm motion analysis evaluation revealed that ejaculates from controls contained 91% motile sperm whereas ejaculates from subfertile males contained 62% motile sperm ($p < 0.001$). The ATP concentration in sperm from subfertile males was 63% less than that of sperm from controls ($p < 0.001$). A link between sperm ATP concentration and immotility was investigated. First, sperm metabolism was evaluated using motility as an endpoint. The genotypic difference in sperm motility persisted when ATP synthesis was limited to glycolysis ($p < 0.001$). Consequently, mitochondrial respiration could not account for the genotypic difference in sperm motility. In contrast, sperm uptake of [1,2-^{3}H] 2-deoxy-D-glucose did differ between genotypes ($p < 0.001$). The activity of key glycolytic enzymes, creatine kinase, and dynein ATPase did not differ between genotypes ($p > 0.05$). Therefore reduced sperm motility did not appear to be due to ATP synthesis, allocation of high energy phosphate bonds along the axoneme, or ATP consumption ($p > 0.05$). In conclusion, subfertility of roosters homozygous for the rose comb allele was attributed to decreased spermatozoal glucose transport.

Identification of a Mechanism Underlying Heritable Subfertility in Roosters Homozygous for the Rose Comb Allele

by

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CONTRIBUTION OF AUTHORS

Dr. David P. Froman, as major advisor, participated in all phases of the research reported herein. He was active in supervising the design, execution and analysis of each experiment.

Mr. Allen J. Feltmann assisted in semen collection and experiments performed in Chapter 3. He was also instrumental in the fertility trials and motility experiments performed in Appendix B.

Mr. Louis G. Jones, Jr. provided the Hobson SpermTracker™ and supervised the computer assisted sperm motion analysis described in Chapter 4.

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CHAPTER 1

INTRODUCTION

Reproduction in the domestic fowl depends upon events that occur throughout the lifespan of the animal. The gonadal-hypothalamic-pituitary axis becomes functional and the reproductive tracts develop *in ovo*. The male reproductive tract becomes patent within a period of weeks after hatching. As evidenced by gonadal weight, gametogenesis begins at puberty. Likewise, secondary sexual attributes and reproductive behavior are apparent at puberty. Fertilization and the subsequent development of a new organism are dependent on the successful transfer of sperm from the male to the female. Movement of spermatozoa from the vagina to the infundibulum, where fertilization occurs, is dependent upon a tier of prerequisites: (1) passage of sperm through the hen's vagina, (2) sperm sequestration within the sperm storage tubules (SST), (3) passage of sperm from the shell gland to the infundibulum, and (4) gamete recognition.

Fertilization in the hen is complicated by a lack of synchronization of ovulation with copulation. Additionally, a nascent egg often blocks the oviduct following deposition of sperm within the vagina. These apparent blocks to fertilization are overcome by sperm sequestration within SST. The SST are simple, unbranched tubules located in the utero-vaginal junction (Hodges, 1974). The role of SST in fertilization has been recently reviewed by Bakst *et al.* (1994). As stated previously, fertilization in the fowl depends upon a tier of prerequisites. A review of the prerequisites that relate to sperm follows.

While sperm sequestration affords the hen a viable population of sperm within the oviduct, only 1-2% of sperm deposited in the vagina enter the SST (Brillard, 1993). Therefore, several researchers have proposed selection processes in the vagina that allow only competent sperm to reach the SST (Bakst *et al.*, 1994). First, 84-92% of inseminated sperm are lost from the oviduct either by defecation or muscular contractions of the cloaca (Allen and Grigg, 1957; Howarth, 1971). The remnant subpopulation appears to be segregated by two mechanisms. First, a motile subpopulation of sperm are believed to propel themselves to the UVJ by moving over the vaginal surface between mucosal folds (Bakst *et al.*, 1994). This sperm movement is enhanced by the beating action of cilia present on the epithelia cells in the mucosal folds. Second, immotile sperm are believed to be lost from the oviduct when trapped in fluid or debris moving in an abovarian direction. Therefore, factors that affect sperm transport in the vagina are the contractility of the oviduct, the beating of epithelia cilia, and the motility of sperm within the oviduct.

Sperm antigens may also affect sperm transport in the vagina. Steele and Wishart (1992) reported that although semen contains immunoglobulins, sperm are immunoglobulin-free prior to ejaculation. However, immunoglobulins are bound to the surface of many sperm following deposition in the vagina. The majority of sperm characterized by surface bound immunoglobulin were dead, whereas only a small percentage of viable sperm had surface bound immunoglobulin. Steele and Wishart (1992) suggested the binding of immunoglobulin to sperm resulted in sperm death and thus reduced the size of the subpopulation of viable sperm within the oviduct. However, it was

not determined if immunoglobulin binding induced sperm death or if the immunoglobulin binding followed sperm death resulting from some other cause.

In addition to sperm motility and antigens, a third factor affecting vaginal sperm transport appears to be the cell surface. Desialylation of sperm surface glycoproteins or glycolipids decreased fertility following intravaginal insemination (Froman and Thurston, 1984; Lake and Ravie, 1988; Froman and Engel, 1989; Howarth, 1990a; Froman and Thursam, 1994). In contrast, Froman and Engel (1989) reported no deleterious effect on fertility following intramagnal insemination of desialylated sperm. Therefore, Froman and Engel (1989) as well as Froman and Thursam (1994) attributed the effect of desialylation to reduced sperm sequestration rather than aberrant gamete recognition. A second experimental approach that reduces fertility and sperm sequestration is suspension of sperm in a hypertonic medium prior to insemination. Steele and Wishart (1996) proposed that removal of sperm surface proteins impedes sperm passage through the vagina by making treated sperm more susceptible to immunoglobulins present in the vagina. In summary, cell surface properties have a profound affect on sperm passage through the vagina.

Sperm passage through the vagina enables sperm sequestration in the SST. Sperm sequestration entails entry, storage within, and egress from the SST. The movement of sperm into the SST is dependent upon motility. This has been demonstrated *in vitro* by Nash *et al.* (1986), Bakst (1987), and Steele and Wishart (1992). Sperm are maintained within the SST *in vivo* for a period of days to weeks. While storage of sperm within SST is well established, the mechanisms underlying maintenance and egress are unknown (Zavaleta and Ogasawara, 1987; Bakst *et al.*, 1994). Antiperistalsis of the oviduct muscles

is believed to transport sperm from the shell gland to the infundibulum. In this regard, Allen and Grigg (1957) demonstrated that radiolabeled dead sperm moved from the shell gland to the infundibulum within minutes. Likewise, carbon particles and india ink introduced above the vaginal sphincter ascend through the oviduct and reach the infundibulum within minutes (Fujihara *et al.,* 1983; Etches, 1996).

The infundibulum serves as a secondary sperm storage site (Bakst *et al.*, 1994). More importantly, the infundibulum is the site of fertilization in the domestic fowl (Olson and Neher, 1948). Fertilization depends upon sperm binding to the inner perivitelline layer, a subsequent acrosome reaction and sperm penetration of the inner perivitelline layer, and finally fusion of the plasma membranes of the sperm cell and oocyte as reviewed by Howarth (1984). Based upon research performed in the late 1980's and early 1990's, sperm binding of the perivitelline layer and the acrosome reaction appear to be receptor-mediated phenomenon (Koyanagi *et al.*, 1988; Kido and Doi, 1988; Howarth, 1990a; 1990b; 1992; Bramwell and Howarth, 1992; Steele *et al.*, 1994). In review, fertility in the domestic fowl depends upon sperm attributes that must be manifest at specific times within the oviduct. Therefore, failure of sperm to interact with either the oviduct or the oocyte at key steps within the fertilization process results in the production of a nonfertilized egg.

Roosters homozygous for the rose comb allele (*R/R*) are subfertile and therefore less fecund than *r/r* roosters (Crawford and Merritt, 1963). That is, hens inseminated with spermatozoa from *R/R* males, hereafter referred to as subfertile males, lay significantly fewer fertilized eggs than hens inseminated with sperm from fertile males. Subfertility in *R/R* males was first described in the 1950s (Cochez, 1951; Ponsignon, 1951); however, the

cell specific mechanism that results in subfertility has remained undetermined. Many reproductive attributes have been compared between subfertile and fertile males. For example, neither sperm viability nor morphology differed between subfertile and fertile genotypes (Crawford and Smyth, 1964b; Kirby *et al*, 1989). Likewise, neither testicular structure nor semen characteristics differed between genotypes (Buckland and Hawes, 1968).

Investigation of sperm-specific attributes that might account for the subfertility of *R/R* males has yielded contradictory data. Crawford and Smyth (1964a) could not attribute subfertility to poor sperm motility. Likewise, Petitjean and Cochez (1966) reported no difference in the motility of ejaculated sperm between genotypes. However, after *in vitro* sperm storage, the sperm motility of subfertile males was less than that of controls (Petitjean and Cochez, 1966). Studies of sperm metabolism have also generated contradictory information. Crawford and Smyth (1964a) measured sperm metabolism with the methylene blue reduction time assay. These researchers failed to detect a genotypic difference in sperm metabolism. In contrast, Buckland *et al.* (1969) reported sperm from subfertile males had decreased aconitase and fumarase activities. Fumarase activity was significantly correlated with sperm fertilizing ability. However, Petitjean and Servouse (1981) failed to identify any genotypic effect in fumarase activity. Kirby and Froman (1991) and Kirby *et al.* (1994) measured sperm metabolism by formazan production. These researchers concluded sperm from subfertile males had significantly lower metabolic capacity than sperm from fertile controls.

Etches *et al.* (1974) and Kirby *et al.* (1989; 1994) compared sperm movement above and below the vaginal sphincter. The subfertile status of *R/R* males was dependent upon insemination route. The expected incidence of subfertility was only observed following intravaginal insemination. No genotypic difference in fertilizing ability was observed following intramagnal insemination. Likewise, the fertilizing ability of testicular sperm did not differ between genotypes following intramagnal insemination (Kirby *et al.*, 1994). Testicular fowl sperm, unlike mammalian sperm, can fertilize oocytes if deposited in the oviduct above the vaginal sphincter (Howarth, 1983; Kirby *et al.*, 1989). Kirby *et al.* (1994) attributed the subfertile status of *R/R* males to an inability of sperm to pass through the vagina and become sequestered within the SST.

In summary, the prerequisites that enable fowl sperm to fertilize oocytes are known. Of these, three prerequisites are dependent upon spermatozoal properties: (1) passage of sperm through the hen's vagina, (2) sequestration within the SST, and (3) gamete recognition. Therefore, any cellular defect that would result in reproductive failure at one or more of these levels could account for the subfertility associated with *R/R* roosters. Based upon previously published data, either sperm passage through the vagina or sequestration within the SST appeared to be a likely basis for subfertility. The overall objective of this research was to define the cellular basis underlying heritable subfertility in roosters homozygous for the rose comb allele (*R/R*).

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CHAPTER 2

IDENTIFICATION OF A SPERM CELL ATTRIBUTE RESPEONSIBLE FOR SUBFERTILITY OF ROOSTERS HOMOZYGOUS FOR THE ROSE COMB ALLELE[1]

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2.1 ABSTRACT

Fertility, sperm metabolism, sperm filling of oviduct sperm storage tubules (SST), and sperm motility were compared between subfertile roosters homozygous for the rose comb allele (*R/R*) and fertile controls (*r/r* or *R/r* males). As expected, fertility of *R/R* males was less than that of controls ($p < 0.0001$). The metabolic rate of spermatozoa from *R/R* males was also reduced compared with controls ($p < 0.05$). Likewise, filling of SST *in vivo* was lower ($p < 0.0001$) for spermatozoa from *R/R* males when compared with controls. However, *in vitro* filling of SSTs was not different between genotypes ($p > 0.05$). Motility of spermatozoa from *R/R* males was less than that of controls ($p < 0.001$) as determined by an objective spectrophotometric assay. Previous researchers have concluded that subfertility associated with homozygosity for the rose comb allele is attributable to a sperm-specific phenomenon. However, the cellular mechanism(s) responsible for the subfertile status were not defined. In contrast to previous research, we have demonstrated that subfertility of *R/R* males is explicable in terms of reduced sperm transport through the hen's vagina in which reduced sperm motility appears to be the major contributing factor.

2.2 INTRODUCTION

In 1951 Cochez [1] reported that roosters homozygous for the rose comb allele (*R/R*) were subfertile. Subfertility has been reported by several groups and determined to be a sperm-specific phenomenon [2-4]. Thereafter, sperm motility was implicated as a basis for subfertility. Sperm motility is essential for passage through the hen's vagina [5]. Poor sperm motility for *R/R* roosters was reported by Petitjean and Cochez [6]; however, Crawford and Smyth [7], Petitjean [8] as well as Petitjean and Servouse [9], reported normal motility for sperm from homozygous rose comb roosters. These conflicting observations may have been due to subjective estimates of sperm motility.

In contrast to subfertility following intravaginal insemination, intramagnal insemination of sperm from *R/R* males resulted in fertility equivalent to that of fertile males [10,11]; consequently, the authors inferred that subfertility following intravaginal insemination was due to insufficient filling of the sperm storage tubules (SST). Maximal filling of SST occurs during the first 24-48 hours after insemination and is essential for the series of fertilized eggs that typically follow a single insemination [12]. In previous research, neither an objective determination of motility nor an estimation of SST filling was made for sperm from *R/R* males.

Sperm metabolism also has been suspect. Buckland *et al.* [13] associated homozygosity for the rose comb allele with decreased spermatozoal fumarase, aconitase and isocitric dehydrogenase activities. Fumarase activity was significantly correlated with fertility. However, Petitjean and Servouse [9] did not observe a difference in spermatozoal

fumarase activity from subfertile R/R and fertile males. Likewise, estimates of sperm metabolism also have been contradictory. For example, neither Crawford and Smyth [7] nor Petitjean [8] observed a difference in sperm metabolism between subfertile *R/R* and fertile males. However, studies in this laboratory have indicated otherwise [11,14].

The objective of the present work was to provide a definitive explanation for the subfertility associated with roosters homozygous for the rose comb allele. The present research was designed to do the following: (1) compare sperm filling of SST following intravaginal insemination of sperm from *R/R* and fertile males, (2) compare sperm filling of SST following incubation of oviduct explants with sperm from *R/R* and fertile males, and (3) compare spermatozoal motility from *R/R* and fertile males using an objective method. Providing that reduced sperm metabolism would be observed as reported previously [11,14], we believed these objectives would enable us to definitively identify a sperm cell attribute that would account for subfertility in *R/R* males.

2.3 MATERIALS AND METHODS

2.3.1 Evaluation of Fertility

In order to establish homozygosity for the rose comb allele (*R/R*), Silver Laced Wyandotte roosters were bred to Single Comb White Leghorn (SCWL) hens (*r/r*) and the comb phenotype of progeny determined. Roosters were designated homozygous providing they sired only rose comb chicks (n = 25 to 40 chicks per male). New Hampshire roosters

(*r/r*) of equivalent age were used as controls. Groups of 10 roosters per genotype were maintained under identical conditions.

Fertility of individual roosters was determined in order to verify a difference between genotypes. Sperm concentration was determined according to Bilgili and Renden [15] for each male. Semen was diluted to 2 x 10^9 spermatozoa/ml with Beltsville Poultry Semen Extender (BPSE) prepared according to Sexton and Fewlass [16]. Twelve hens per male were inseminated intravaginally with 1 x 10^8 sperm/hen. Eggs were collected throughout a 21-day interval and set for incubation twice weekly. Fertility was determined by breaking eggs open after 4 days of incubation and then examining the contents for embryonic development. Three replicate trials were performed per male. Fertility was analyzed as described by Kirby and Froman [17]. Based upon fertility, representative roosters (n = 3 per genotype) were selected for subsequent evaluation of sperm metabolism, SST filling, and motility.

2.3.2 Evaluation of Sperm Metabolism

Metabolism was determined by a modification of the technique of Chaudhuri and Wishart [18]. Semen was pooled according to genotype. Each semen sample was diluted to 5 x 10^8 spermatozoa/ml in TES buffer containing 111 mM NaCl, 25 mM glucose and 4 mM $CaCl_2$, pH 7.4. A 200-µl volume of sperm suspension was added to 1.0 ml of reaction medium prewarmed to 41°C in a culture tube. The reaction medium contained 200 µM 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl-tetrazolium chloride (INT), 9.2 µM phenazine methosulphate, and 2.4 mM KCN in the buffer described above. Reaction mixtures, in triplicate, were incubated at 41°C for 1,3,5 or 7 minutes. Reduction of INT to formazan

was terminated by the addition of 200 µl of 0.1 M HCl containing 5% (v/v) Triton X-100 (Sigma Chemical Co., St. Louis, MO). Culture tubes were placed in a boiling water bath for 30 seconds and then centrifuged at 700 x g for 10 minutes. Absorbance of the supernatant was measured at 520 nm. Data were evaluated by the method of least squares.

2.3.3 Evaluation of Sperm Storage Tubule Filling *in vivo*

Semen was pooled according to genotype. Semen was diluted with BPSE to 2 x 10^9 spermatozoa per ml. Each hen was inseminated intravaginally with 1 x 10^8 in a volume of 50 µl. Hens were sacrificed 16-18 hours post insemination and oviducts removed. Oviducts were removed from euthanized hens, split longitudinally, and tissue containing the SST was located using the technique of Bakst [19]. Tissue containing SST was excised, and weighed. Tissue explants were mixed with 10 ml homogenization buffer per g of tissue. The buffer contained 0.05% (v/v) Triton X-100 in 0.9% (w/v) NaCl. The tissue was homogenized for 3 minutes in a 25-ml stainless steel microcontainer (Eberbach Corp., Ann Arbor, MI). Formaldehyde, 37% (v/v), was added to bring the final formaldehyde concentration to 0.5% (v/v). This solution was homogenized for 1 additional minute and then stored overnight at 4°C in 10-ml Erlenmeyer flask. Sperm nuclei were counted with a hemacytometer. Six replicate counts were made per sample. The data were analyzed using the Kruskal-Wallis nonparametric test [20].

2.3.4 Evaluation of Sperm Storage Tubule Filling *in vitro*

Tissue explants were procured and weighed as above. Each explant was halved and incubated either with sperm from *R/R* or *r/r* males in a 25-ml Nalgene Erlenmeyer flask. Incubation conditions were based on Nash *et al.* [21], Bakst [22], and Steele and Wishart

[23]. Incubation was performed at 41°C for 2 hours in a humidified chamber filled with air containing 5% CO_2. Additionally, flasks were rotated at 55 rpm. The incubation medium was Eagle's Minimum Essential Medium (MEM; Sigma) prepared as described by Howarth [21]. Pooled semen from each genotype was diluted beforehand with BPSE to 2×10^9 sperm per ml. A volume of sperm suspension was added to the MEM so that the final concentration was 2.5×10^7 sperm per ml. MEM volume was based upon tissue weight: 4 ml MEM per g explant. After incubation, explants were washed in excess isotonic saline for 3 minutes in order to remove sperm associated with the mucosal surface. Washed explants were homogenized, and spermatozoal nuclei counted as described above. Data were analyzed by paired comparison [20].

2.3.5 Evaluation of Sperm Motility

Sperm motility was measured by using a modification of a swim-up technique [22]. Semen was pooled according to genotype. Spermatozoal concentration and viability were determined according to Bilgili and Renden [15]. Semen was diluted to 5×10^8 spermatozoa/ml with 50 mM TES buffer, pH 7.4, containing 111 mM NaCl, 25 mM glucose, 4 mM $CaCl_2$. A 150-µl volume of sperm suspension was layered upon 1.5 ml 6% (w/v) Accudenz® (Accurate Chemical & Scientific Corporation, Westbury, NY) solution prewarmed to 41°C in a disposable cuvette. The cuvette was incubated for 5 minutes in a 41°C water bath. Absorbance was measured at 550 nm one minute after loading the incubated cuvette into a spectrophotometer (DU-640, Beckman Instruments, Fullerton, CA).

Each representative rooster used in the experiments described above was bred to 10 heterozygous rose comb hens (*R/r*). At sexual maturity, male progeny were bred to SCWL hens in order to establish homo- or heterozygosity for the rose comb allele (5 SCWL hens per male). Sperm motility was compared between homozygous (n = 29) and heterozygous (n = 29) rose comb males. Data were analyzed by single classification analysis of variance with the general linear model of SAS [23].

2.4 RESULTS

As shown in Figure 2.1, Silver Laced Wyandotte roosters homozygous for the rose comb allele were subfertile relative to controls. Both initial fertility and duration of fertility were reduced in each replicate trial. These observations were consonant with previous research [4]. Likewise, sperm from the homozygous rose comb roosters were characterized by a metabolic rate that was less than that of the controls ($p < 0.05$). As shown in Figure 2.2, the difference in formazan production observed for subfertile and fertile roosters was comparable to that observed previously [11,14].

At 16-18 hours after intravaginal insemination, SST contained an average 2.9×10^6 sperm in the case of fertile males (Table 2.1). In contrast, SST from hens inseminated with sperm from R/R males contained only 41% ($p < 0.0001$) as many sperm (Table 2.1). However, when oviduct explants were incubated with sperm there was no difference in SST filling between genotypes (Table 2.2).

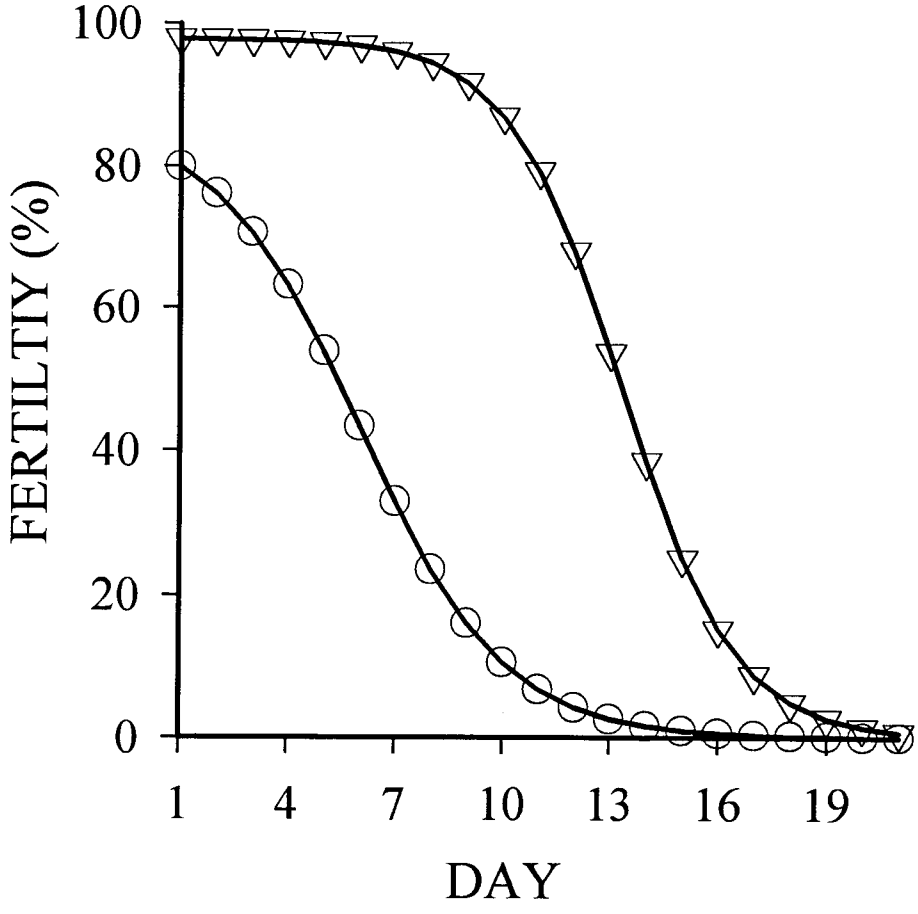

FIGURE 2.1. Duration of fertility after a single insemination of SCWL hens with sperm from a *R/R* male (circles) or *r/r* male (triangles). Solid lines represent the functions

$y(x) = [82.9]/[1 + e^{-.443(7.3-x)}]$, and

$y(x) = [97.8]/[1 + e^{-.664(13.2-x)}]$,

in which 7.3 and 13.2 are estimates of the parameter τ, or time of half-maximal fertility. Each hen (n = 36 per treatment group) was inseminated with 1×10^8 sperm. There was a significant difference ($p < 0.0001$) in τ between R/R and r/r males.

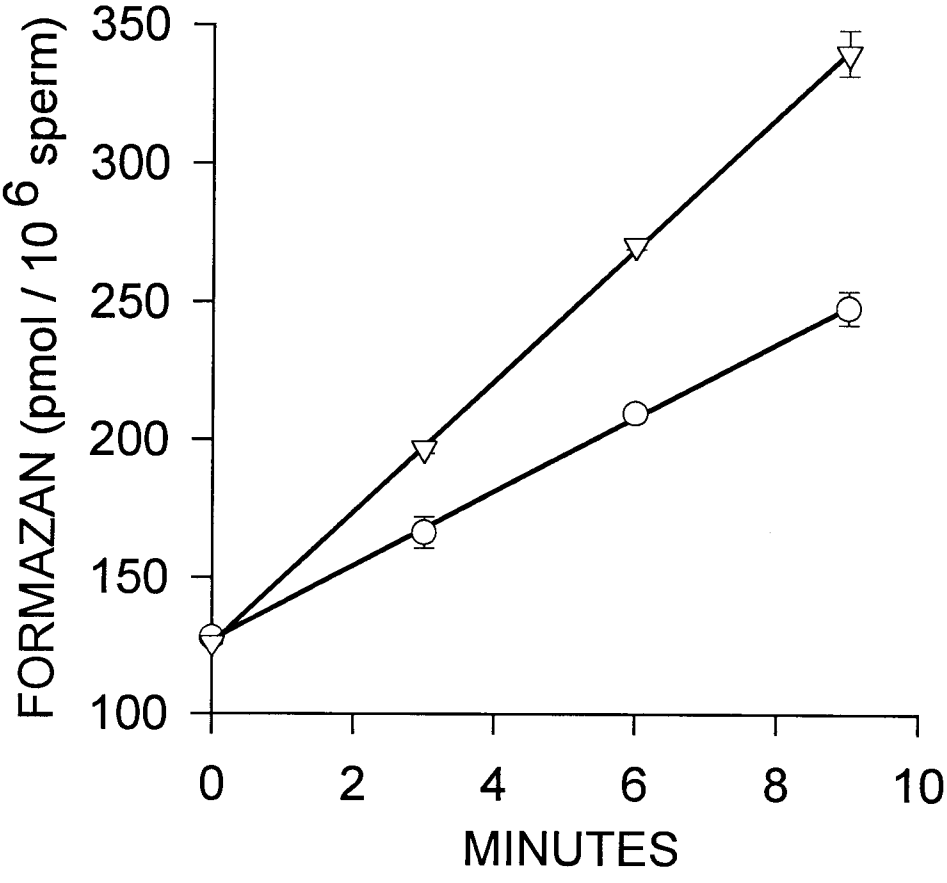

FIGURE 2.2. Formazan production by sperm from *R/R* males (circles) and *r/r* males (triangles) incubated at 41 °C. Solid lines represent the functions: (a) R/R males, y(x) = 127.68 + 13.41(x), $R^2 = 0.99$, and (b) r/r males, y(x) = 126.13 + 23.83(x), $R^2 = 0.99$.

Table 2.1. Spermatozoa from *R/R* and *r/r* roosters present in SST after artificial insemination.

Genotype	Hens (n)	Spermatozoa in SST* (x 10^6)
R/R	17	1.207 ± 0.0539[a]
r/r	17	2.934 ± 0.1811[b]

* Each value is a mean ± SEM.
[a,b] Means differed at $p < 0.0001$.

Table 2.2. Spermatozoa from *R/R* and *r/r* roosters present in SST after incubation *in vitro*.

Genotype	Hens (n)	Spermatozoa in SST* (x 10^6)
R/R	20	2.32 ± 0.305
r/r	20	2.27 ± 0.285

*Each value is a mean ± SEM.

Preliminary research demonstrated that sperm motility was essential for sperm penetration into 6% (w/v) Accudenz®. Sperm immobilized by heat denaturation at 56°C did not penetrate. When a sperm suspension was overlaid on the Accudenz® and the absorbance of the Accudenz® layer was measured as a function of time, data points approximated a logistic function (Figure 2.3). The greatest change in absorbance occurred between 2 and 8 minutes of incubation. Therefore, an incubation interval of 5 minutes was chosen for subsequent motility measurements.

As shown in Table 2.3, the mean absorbance value of samples from *R/R* males was 67% of controls. Thus, the motility of sperm from *R/R* males was less than that of the fertile controls.

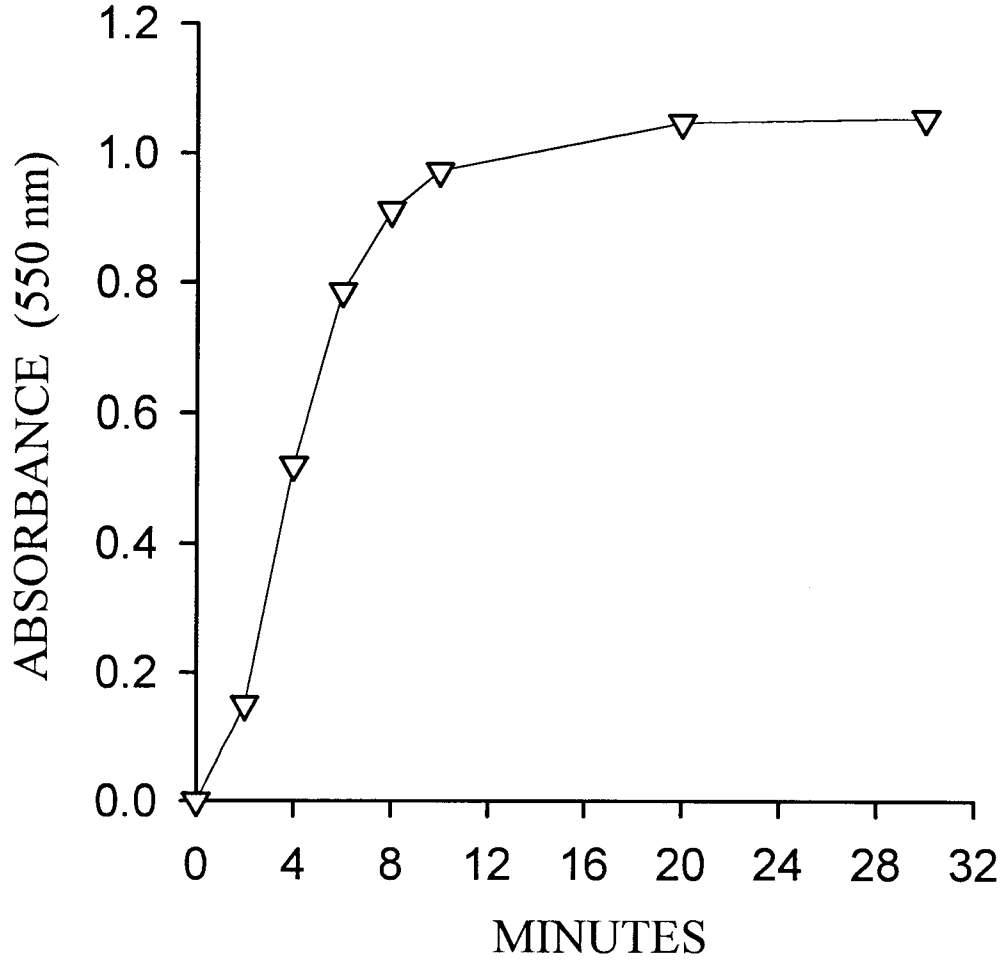

FIGURE 2.3. Absorbance of 6% (w/v) Accudenz® solution through time following an overlay of sperm suspension. The cuvette contained 1.5 ml of Accudnez® pre-warmed to 41°C. After a 0.150 ml volume sperm suspension (5 x 10^8 sperm/ml) was layered upon the Accudenz® and the initial reading taken, the cuvette was maintained at 41°C in a water bath.

Table 2.3. Results of the evaluation of sperm motility for homozygous (*R/R*) and heterozygous (*R/r*) rose comb roosters.

Genotype	Roosters (n)	Absorbance*
R/R	29	0.4962 ± 0.048^{a}
R/r	29	0.7417 ± 0.047^{b}

*Absorbance at 550 nm was directly proportional to the extent to which spermatozoa entered a solution of 6% Accudenz®. A sperm suspension was overlaid upon the Accudenz® within a cuvette. The cuvette was incubated at 41°C for 5 min, then the absorbance of the Accudenz® layer was measured.

[a,b] Means different at $p < 0.001$.

2.5 DISCUSSION

The cellular basis for the subfertility of roosters homozygous for the rose comb allele has perplexed researchers since the 1950s [see 11, for review]. Previous research, when viewed collectively, has generated contradictory results. Nonetheless, sperm motility has been implicated by several groups [6,11] but never objectively measured. Chaudhuri and Wishart [18] correlated sperm metabolic activity with sperm motility in normal roosters. Kirby *et al.* [11] reported that sperm from *R/R* roosters were characterized by reduced metabolic rate and inferred poor sperm motility. Suboptimal filling of sperm storage tubules by sperm from *R/R* males also was inferred from fertility data [11] but, as with sperm motility, objective measurements were not made.

Filling of SST by spermatozoa is paramount for fertility in poultry [12]. However, only 1% of inseminated sperm from fertile roosters reach the SST [27]. In this study, sperm from fertile control roosters were recovered from the SST at a rate of 2.9% after artificial insemination comparable to previously published values [27]. We attribute our slightly higher value to differences among breeds of roosters and hens [28]. In contrast, the recovery rate for sperm from *R/R* males was only 41% ($p < 0.0001$) of that observed for fertile control sperm. We inferred that sperm from *R/R* males had a reduced ability to traverse the vagina to reach the SST. We hypothesized that poor sperm motility accounted for suboptimal SST filling.

An alternative hypothesis was that sperm from *R/R* males traversed the vagina but failed to enter the SST. Consequently, sperm from subfertile and fertile males were

incubated with oviduct explants containing SST. In this case, however, there was no difference in the extent of SST filling between genotypes. Preliminary work (Figure 2.4) using spermatozoa stained with the fluorochrome bis-benzamide [19] and incubated with explants indicated that the majority of spermatozoa associated with the explant following washing, were within the SST. Therefore, we concluded that incubation of sperm with explants followed by explant homogenization and hemacytometer counts of homogenization-resistant sperm nuclei provided an estimate of sperm that had entered the SST. In summary, the alternate hypothesis that sperm from R/R males traversed the vagina but failed to enter the SST was rejected.

Results from the *in vivo* and *in vitro* SST experiments along with the observations of Kirby and Froman [14] as well as Kirby *et al.* [11] with respect to sperm metabolism lead us to believe that decreased sperm motility was the most likely explanation that could account for the subfertility associated with *R/R* roosters. Our initial attempts to test this hypothesis were informative, in that sperm from *R/R* roosters did not penetrate a 6% Accudenz solution to the extent that sperm from fertile controls did. Penetration of the Accudenz layer was dependent on sperm motility (Figure 2.3). We inferred a difference in sperm motility based upon observable differences in sperm cell mobility. However, testing the motility hypothesis required a larger number of roosters. Therefore, each of three homozygous rose comb roosters was bred with approximately 10 heterozygous hens. Male progeny (n=80) were tested for homozygosity for the rose comb allele. Therefore, males heterozygous for the rose comb allele were used as fertile controls in the motility experiment. As shown in Table 2.3, homozygotes were characterized by reduced ($p <$

0.001) motility. While the sperm motility assay did not provide any assessment of variables such as beat frequency or velocity, it did provide an evaluation of the mobility of a sperm cell population. The results from this experiment provided definitive evidence that the motility of sperm from *R/R* males is reduced when compared to fertile controls (Table 2.3). However, the basis for this difference in motility is unknown.

Sperm are highly compartmentalized cells. In the case of rooster sperm under physiological conditions, motility appears to depend upon an influx of extracellular calcium [25,26] in addition to the synthesis of ATP in the midpiece and hydrolysis of ATP by dynein ATPase associated with the axoneme. Therefore, several cellular compartments may warrant investigation in the case of sperm from males homozygous for the rose comb allele. For example, it is not known whether plasma membrane calcium permeability, ATP production, ATP consumption, or axonemal structure differ due to genotype.

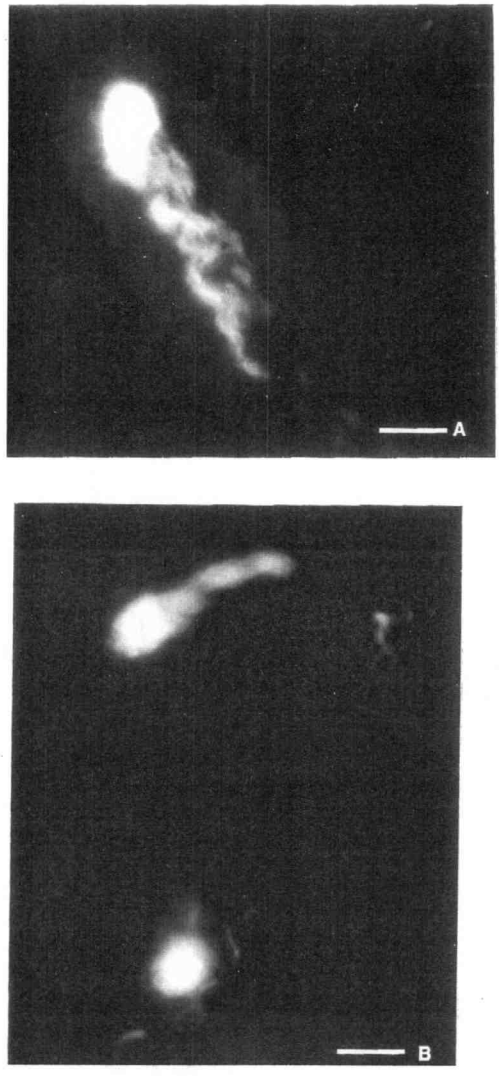

FIGURE 2.4. Fluorescence micrographs of bis-benzamide labeled sperm associated with sperm storage tubules (SST). (A) SST from a hen inseminated intravaginally with 1 X 10^8 sperm and sacrificed 18 hr post artificial insemination. Note fluorescence of sperm nuclei clustered at the distal end and scattered throughout the lumen of the SST. Scale bar = 25 μm. (B) SST from oviduct explant incubated for 2 hr at 41 °C with labeled sperm. Sperm nuclei are located at the distal end of both SST similar to the *in vivo* results in (A). Single sperm can be localized in the lumen of the SST with the addition of white light. Scale bar = 25 μm.

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CHAPTER 3

TRANSFER OF SPERM INTO A CHEMICALLY DEFINED ENVIRONMENT BY CENTRIFUGATION THROUGH 12% (WT/VOL) ACCUDENZ®

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3.1 ABSTRACT

Centrifugation is commonly used to wash sperm. However, most washing techniques do not put sperm in a chemically defined environment. Rather, washing by centrifugation in effect, dilutes seminal plasma components. A 0.5-mL volume of 30% (wt/vol) Accudenz® was layered beneath 5 mL of 12% (wt/vol) Accudenz® in a 15 mL polypropylene centrifuge tube. Diluted semen from individual males (n=10) was overlaid upon the 12% (wt/vol) Accudenz®. After centrifugation at 1250 x *g* at 4 C for 25 min, washed sperm were present at the interface of the Accudenz® layers. Based upon hemacytometer counts, sperm recovery was 83% (CV=12%). Neither sperm viability nor morphology were affected by washing. Efficacy of the washing procedure was evaluated by using extracellular glucose, glutamic acid, Ca^{+2}, and protein as markers. Washing eliminated 99% of the glutamic acid and glucose associated with sperm. Likewise, washing removed 97.5% of the extracellular Ca^{+2} associated with sperm. As evidenced by total protein analysis and SDS-PAGE, washing removed 98% of seminal plasma proteins from sperm. In addition, washing did not affect sperm motility or fertilizing ability. This procedure returns extended sperm to a physiological concentration in a chemically defined environment. By suspending washed sperm in distinct media, we induced differential sperm motility. Therefore, this procedure is suitable for the study of the effect of specific substances upon sperm metabolism and motility.

3.2 INTRODUCTION

Sperm motility is essential for maximal fertility in the fowl (Bakst *et al.*, 1994). Fowl sperm motility is sensitive to factors in seminal plasma and synthetic diluents. For instance, fowl sperm are motile if suspended in a calcium free medium at 30 C. However, if the temperature is increased to 40 C, sperm become immotile (Thomson and Wishart, 1988). Motility is restored at 40 C by 2 mM calcium, 10% seminal plasma, or 10% peritoneal fluid collected at ovulation (Ashizawa *et al.*, 1989). Likewise, sperm respiration is strongly influenced by intracellular calcium concentrations (Ashizawa *et al.*, 1992). Of the substances that reactivate fowl sperm motility, calcium's affect on motility has received the most attention (Thomson and Wishart, 1991; Ashizawa *et al.*, 1994). In fact, calcium was identified as one of two substances in seminal plasma that stimulated motility at 40 C (Ashizawa and Wishart, 1987).

Proteins and serum replacements may also affect sperm activity. Bakst and Cecil (1992a; 1992b) evaluated turkey sperm motility after storage in diluents containing BSA and two commercially available serum replacements using computer-assisted semen analysis. The presence of BSA and serum replacements significantly increased sperm motility (Bakst and Cecil, 1992a; 1992b). In addition, Mohan *et al.* (1995) reported that fowl seminal plasma contains a 75 kDa sperm motility inhibiting factor (SMIF) that inhibits motility. Furthermore, a number of enzymes (Lake, 1984) and a proteinase inhibitor (Lessley and Brown, 1978) have been reported in fowl seminal plasma but the precise biological roles of these proteins are unknown.

Fowl semen contains significant concentrations of proteins, glutamic acid, and inorganic ions (Freeman, 1984). Transparent fluid adds glucose to the seminal plasma (Freeman, 1984). Assessment of sperm function in relation to exogenous nutrients, motility agonists, and antagonists, is necessary for analyzing how sperm react with their environment. Traditionally, centrifugation of sperm has been used as a method for washing sperm free of substances present in seminal plasma. However, this approach merely dilutes the components of the medium in which sperm are suspended. Because of the biochemical complexity of seminal plasma and the potential for interactions between these compounds, it is desirable to study sperm in a chemically defined environment. In previous research (Froman and Thursam, 1994), sperm were washed through 12% (wt/vol) Accudenz® in order to remove an exogenous enzyme. We hypothesized this simple technique could also be used to wash sperm free of seminal plasma components and thereby provide a means to study sperm in a chemically defined environment.

3.3 MATERIALS AND METHODS

3.3.1 Experimental animals

New Hampshire roosters (n = 10) were used as semen donors. All roosters were housed in individual cages, provided feed and water *ad libitum*, and were 26-42 weeks of age when used for the following experiments.

3.3.2 Sperm washing procedure

A 30% (wt/vol) stock solution of Accudenz®[3] was prepared using 3 mM KCl containing 5 mM TES, pH 7.4, as the solvent. A 50 mM TES solution, pH 7.4, containing 130 mM NaCl (hereafter referred to as TES buffer), was prepared. The TES buffer had an osmolality of approximately 315 mmol/kg. A portion of the TES buffer was diluted with deionized water to an osmolality of 275 mmol/kg. The diluted TES buffer was used to prepare 12% (wt/vol) Accudenz® (315 mmol/kg) with the 30% stock solution.

Each experiment was performed with sperm from individual males. Sperm concentration was determined spectrophotometrically. Ejaculates were diluted to 2 x 10^9 sperm/mL with TES buffer. Five mL of the 12% (wt/vol) Accudenz® was placed in a 15-mL polypropylene centrifuge tube. A 0.5-mL volume of 30% (wt/vol) Accudenz® was placed beneath the 12% (wt/vol) Accudenz® with a Pasteur pipet. The sperm suspension was overlaid on the 12% (wt/vol) Accudenz® solution and screw caps secured. Tubes were centrifuged at 1250 x *g* at 4 C for 25 min. After centrifugation, fluid above and below the washed sperm was removed by aspiration.

3.3.3 Evaluation of fertility

Fertilizing ability of sperm from individual roosters (n=10) was determined before and after washing. Sperm concentration in each ejaculate was determined spectrophotometrically. A sample of the ejaculate was diluted to 2 x 10^9 sperm/mL with 50 mM TES, pH 7.4, containing 120 mM NaCl, 10 mM glucose, and 2 mM $CaCl_2$, hereafter referred to as motility buffer. Each sperm suspension was used to inseminate 10

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[3]Accurate Chemical and Scientific Corp., Westbury, NY 11590.

hens with 75 x 10^6 sperm/hen. The remaining neat semen was diluted with TES buffer and washed as described above. Washed sperm were diluted to 2 x 10^9 sperm/mL with motility buffer. Ten hens per male were inseminated as described above. Eggs were collected throughout a 10-day interval and set twice. Fertility was determined by breaking eggs open after 4 days of incubation and then examining the contents for embryonic development. Fertility was analyzed as described by Kirby and Froman (1991).

3.3.4 Evaluation of washing procedure

Sperm viability (n=10 males, 1 ejaculate per male) was determined before and after washing by ethidium bromide exclusion (Bilgili and Renden, 1984). Ejaculates were diluted to 2 x 10^9 sperm/mL with TES buffer and washed as described above. Recovery of washed sperm (n=10 males, 1 ejaculate per male) was determined as follows. Sperm concentration of neat semen was determined using a hemacytometer and each ejaculate was diluted to 2 x 10^9 sperm/mL with TES buffer and washed as described above. The sperm suspension volume was recorded for washed sperm and sperm concentration was determined with a hemacytometer.

Glucose concentration (n=10 males, 1 ejaculate per male) was determined with an assay kit[4]. Neat semen was diluted to 2 x 10^9 sperm/mL with motility buffer. A 0.5-mL sample of the sperm suspension was centrifuged at 12,000 x *g* for 5 minutes and 20 μL of the supernatant assayed for glucose. The residual sperm suspension was washed as described above. Washed sperm were resuspended to 2 x 10^9 sperm/mL with TES buffer and then centrifuged at 12,000 x *g* for 5 minutes. The supernatant was assayed for glucose.

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[4]Sigma Chemical Company, Catalog # 115-A, St. Louis, MO, 63178

Unknown glucose concentrations were determined using a standard curve (r=0.99) with standards ranging from 25 mM to 0.1 mM glucose.

Calcium concentrations (n=10 males, 1 ejaculate per male) were measured spectrophotometrically with the indicator Arsenazo III[5] according to Gratzer and Beaven (1977). A 5 mM Arsenazo III solution was passed 3 times through a column of Dowex 50W and then filtered prior to use. Neat semen was diluted to 2 x 10^9 sperm/mL with motility buffer. A 0.5-mL sample of the sperm suspension was centrifuged at 12,000 x *g* for 5 minutes. A 0.4-mL volume of the supernatant was assayed by adding 0.1 mL of filtered Arsenazo III and then measuring the absorbance at 654 nm. The remaining sperm suspension was washed as described above. Washed sperm were resuspended to 2 x 10^9 sperm/mL with TES buffer and centrifuged at 12,000 x *g* for 5 minutes. The supernatant was assayed for calcium. Unknown calcium concentrations were determined using a standard curve (r=0.99) with standards ranging from 10 mM to 0.1 μM calcium.

Concentrations of glutamic acid (n=5 males, 1 ejaculate per male) were determined by amino acid analysis[6]. Prior to analysis, a 0.5-mL volume of neat semen was centrifuged at 12,000 x *g* for 5 min, and the supernatant was frozen at -20 C. The remaining neat semen was diluted to 2 x 10^9 sperm/mL with TES buffer and washed as described above. The washed sperm were microcentrifuged and the supernatant frozen as above. Protein concentrations (n=10 males, 1 ejaculate per male) were determined using the Bradford

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[5]Sigma Chemical Company, A 9676, St. Louis, MO, 63178

[6]AAA Laboratory, Mercer Island, WA, 98040

protein assay[7]. A 0.25-mL sample of neat semen was centrifuged at 12,000 x *g* for 5 min, and the supernatant was assayed for total protein. The remaining neat semen was diluted to 2 x 10^9 sperm/mL with TES buffer and washed as described above. Washed sperm were microcentrifuged and the supernatant was assayed for total protein as described above. Total protein was also evaluated before and after washing using SDS-PAGE (Laemmli, 1970). Electrophoresis was performed in a vertical slab gel containing 6-20% (wt/vol) acrylamide.

3.3.5 Evaluation of sperm mobility

Sperm mobility was measured using the procedure of Froman and McLean (1996). An ejaculate from each of 10 males was diluted to 5 x 10^8 sperm/mL with motility buffer. A 60-μL volume of sperm suspension was layered upon 0.6 mL of 6% (wt/vol) Accudenz® solution prewarmed to 41 C in a disposable microcuvette. The 6% (wt/vol) Accudenz® solution was prepared by diluting the 30% (wt/vol) Accudenz stock solution with motility buffer diluted to 290 mmol/kg. The cuvette was incubated for 5 min in a 41 C water bath. Absorbance was measured at 550 nm 1 min after the incubated cuvette was loaded into a spectrophotometer. The remaining neat semen was diluted to 2 x 10^9 sperm/mL with TES buffer and washed as described above. The mobility of washed sperm was measured as above, but using each of the following media for sperm resuspension: 1) isotonic buffered saline containing 2 mM Ca^{+2}, 2) isotonic buffered saline containing 2 mM Ca^{+2} and 10 mM glucose, and 3) isotonic buffered saline containing 2 mM Ca^{+2} and 3 mM CN^-. Each medium was also used to prepare 6% (wt/vol) Accudenz®.

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[7]Bio-Rad, 500-0006, Hercules, CA 94547

3.4 RESULTS

Semen diluted to 2 x 10^9 sperm/mL can be layered on top of 12% (wt/vol) Accudenz® without mixing layers. When centrifuged at 1250 x *g*, sperm pass through the 12% (wt/vol) Accudenz® while the solution the sperm were suspended in does not mix with Accudenz® solution. Furthermore, sperm do not penetrate the 30% (wt/vol) Accudenz® solution underlying the 12% (wt/vol) Accudenz®. Therefore, when this procedure is performed as described in the Materials and Methods section, sperm form a band approximately 1 cm deep at the interface of the 12% and 30% (wt/vol) Accudenz solutions. Likewise, a thin white band is present at the interface of the medium in which the sperm were suspended and the 12% (wt/vol) Accudenz® solution. Transmission electron microscopy (TEM) of the material from this band revealed a few intact sperm, cellular debris, and spermiophages (data not shown). Conversely, sperm recovered from the interface of the 12% and 30% (wt/vol) Accudenz® solutions had normal morphology as evidenced by TEM and phase contrast microscopy (data not shown). Likewise, these sperm were viable as evidenced by ethidium bromide exclusion (Table 3.1). As shown in Table 3.1, recovery of washed sperm was 83% (CV=12%). As shown in Table 3.2,

TABLE 3.1. Sperm recovery and sperm viability before and after washing sperm through 12% (wt/vol) Accudenz®

Roosters (n)	Pre-Wash Viability[1] (%)	Post-Wash Viability[1] (%)	Recovery[1] (%)
10	99.75 ± 0.05	99.5 ± 0.05	83.8 ± 4.0

[1]Each value is a mean ± SEM.

TABLE 3.2. Reduction of marker compounds after washing sperm through 12% (wt/vol) Accudenz®

Roosters (n)	Component	Reduction[1] (%)
9	glucose	99.2 ± 0.14
5	glutamic acid	99.8 ± 0.05
10	calcium	97.3 ± 0.13
9	protein	98.3 ± 0.23

[1]Each value is a mean ± SEM.

washing removed 99.8, 99.2 and 97.3% of extracellular glutamic acid, glucose, and Ca^{+2}, respectively. Likewise, washing removed 98.3% of extracellular protein (Table 3.2). The efficacy of the washing procedure was corroborated by SDS-PAGE (Figure 3.1).

The postwash sperm mobility was 97% of prewash mobility when the resuspending medium contained glucose (data not shown). As shown in Table 3.3, the fertilizing ability of washed sperm did not differ from non-washed control sperm ($P \geq 0.05$; Table 3.3). Sperm mobility was 75% of controls when washed sperm were resuspended in glucose free isotonic saline (Figure 3.2). In contrast, when washed sperm were resuspended in a glucose free medium containing 3 mM cyanide, sperm were immotile (Figure 3.2).

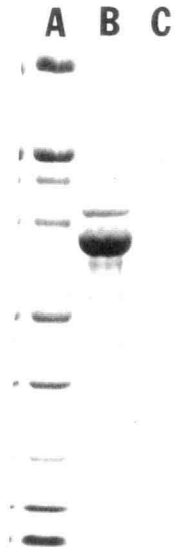

FIGURE 3.1. Polypeptides derived from chicken seminal plasma proteins following SDS-PAGE in a vertical slab gel containing a 6-20% (wt/vol) gradient of acrylamide. Polypeptides were stained with Coomassie blue. Lane A denotes molecular weight markers. Lane B shows proteins associated with nonwashed sperm. After washing sperm through 12% (wt/vol) Accudenz®, sperm were resuspended to a physiological concentration with TES buffer. As demonstrated in lane C, washing removed seminal plasma proteins.

TABLE 3.3. Fertilizing ability of sperm before and after washing through 12% (wt/vol) Accudenz®

Roosters (n)	Treatment	Hens[1] (n)	Eggs[2]	Fertility[3] (n)
8	Nonwashed	70	549	71.9±10.9
8	Washed	61	450	71.1±10.6

[1]Each Single Comb White Leghorn hen was inseminated with a dose of 75 x 10^6 sperm in a volume of 75 μl.

[2]Collected daily and set weekly.

[3]Each value is a mean ± SEM.

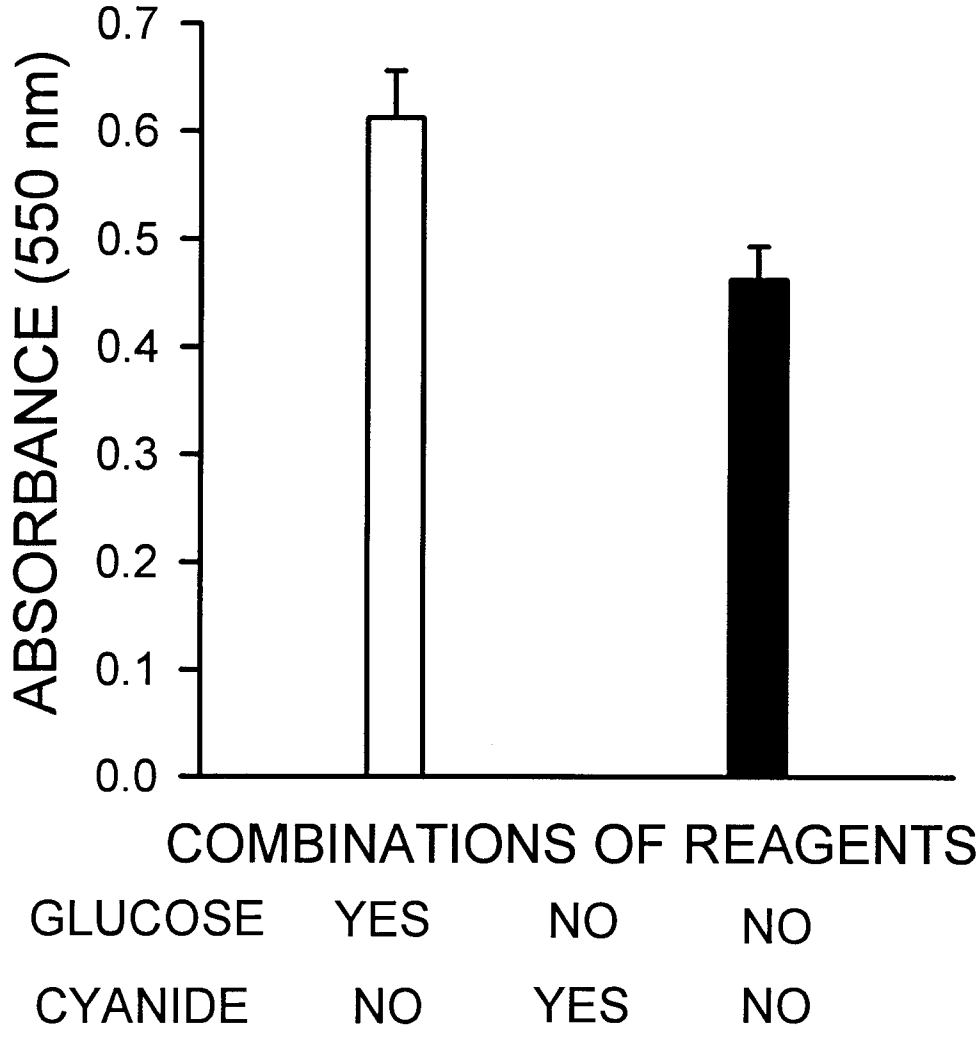

FIGURE 3.2. Sperm mobility as measured by a change in absorbance following an overlay of 6% (wt/vol) Accudenz with sperm suspensions after centrifugation through 12% (wt/vol) Accudenz. Each bar represents the mean and standard error of 10 replicate trials with semen from individual males. In each assay, pooled sperm were washed by centrifugation through 12% (wt/vol) Accudenz prepared with buffered isotonic saline. This procedure washes sperm and returns them to a physiological concentration. Washed sperm were resuspended in each of three media. Each medium contained 2 mM Ca^{+2}. Media differed according to glucose and cyanide content. Combinations of reagents are shown along the X axis. Glucose and cyanide were used at 15 and 3 mM, respectively. Sperm were immotile in the glucose free medium containing cyanide.

3.5 DISCUSSION

Our primary objective was to determine whether we could procure fully functional sperm in a chemically defined environment. In previous research, sperm were centrifuged through an Accudenz® solution in order to wash sperm free of an exogenous enzyme (Froman and Thursam, 1994). We hypothesized this simple technique could also be used to wash sperm free of seminal plasma components so that the effect of specific compounds could be studied on sperm cell function. Attributes of washed sperm were evaluated *in vitro* and *in vivo*. The efficacy of washing was evaluated in terms of the extent sperm could be washed free of small and large molecular weight compounds.

Traditionally, centrifugation has been used as a method to wash sperm. However, this approach merely dilutes the components of the medium in which sperm are suspended. As alluded to above, Froman and Thursam (1994) prepared a sperm suspension containing exogenous neuraminidase, centrifuged the sperm through 12% (wt/vol) Accudenz, and recovered washed sperm at a physiological concentration from the interface between the 12% and an underlying 30% (wt/vol) Accudenz solution. Fertilizing ability was not affected and washed sperm were free of neuraminidase activity. Therefore, the washing procedure effectively removed an exogenous macromolecule from a population of sperm. However, we wanted to establish the efficacy of the procedure for compounds commonly found in extended semen.

The efficacy of washing was tested using the following markers: glutamic acid, glucose, Ca^{+2}, and seminal plasma protein. Glutamic acid is the predominant free amino

acid in fowl seminal plasma (Freeman, 1984). Due to the pK_a of the α-amino group, the α-carboxyl group and the side chain, this molecule is an organic anion at physiological pH. Glucose was used because it is present in semen (Freeman, 1984). Likewise, a hexose is commonly used as a nutrient in extenders. We chose the inorganic cation Ca^{+2} as the third marker because it is found in mM concentrations in seminal plasma and is a motility agonist at body temperature (Ashizawa and Wishart, 1987; Wishart and Ashizawa, 1987). Finally, we used seminal plasma proteins as macromolecular markers.

As evidenced by *in vitro* mobility at body temperature and the fertilizing ability of washed sperm *in vivo* (Table 3.2), the washing procedure did not impair sperm function. As shown in Table 3.2, the washing procedure reduced the presence of extracellular markers by an average of 98.7%. As evidenced by a CV of 1.1%, the efficacy of the washing procedure was independent of the chemical nature of the marker. In each case, the postwash concentration of the marker was negligible. Therefore, we concluded the washing procedure did indeed place sperm in a chemically defined environment.

Having shown it was possible to place functional sperm in a chemically defined environment, we conducted an experiment to demonstrate application of the method. We hypothesized that we could control sperm metabolism by controlling the extracellular environment. We chose sperm mobility as an endpoint. Fowl sperm metabolism is affected by glucose (Goldberg and Norman; 1961; Sexton, 1974; Wishart, 1982). Having considered that: (1) we could wash sperm free of glucose, (2) fowl sperm can utilize an endogenous substrate under aerobic conditions (Howarth, 1978; Howarth, 1981), and (3) cyanide inhibits mitochondrial respiration, we formulated the following hypotheses. First,

sperm would be motile in a glucose-free medium. Second, sperm would be immotile in a glucose free medium containing cyanide. As shown in Figure 3.2, these hypothesized outcomes were observed. Therefore, profound differences in sperm function were experimentally induced by controlling the chemical composition of the spermatozoal environment. In conclusion, the procedure described herein represents a simple method to recover large numbers of viable fowl sperm in a chemically defined environment. We propose that it can be applied to the study of such phenomena as sperm metabolism, motility, and the acrosome reaction.

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CHAPTER 4

REDUCED GLUCOSE TRANSPORT IN SPERM FROM ROOSTERS (*Gallus domesticus*) WITH HERITABLE SUBFERTILITY

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4.1 ABSTRACT

Roosters homozygous for the rose comb allele (*R/R*) are subfertile. In previous research, these subfertile roosters were characterized with limited sperm mobility as measured with an *in vitro* sperm penetration assay. The objective of the present study was to further characterize sperm motility and account for an underlying mechanism. Reduced sperm mobility was explicable in terms of reduced sperm motility ($p < 0.001$) as measured with a Hobson SpermTracker. The concentration of intracellular ATP in sperm from subfertile roosters was less than that from fertile controls ($p < 0.001$). The genotypic difference in sperm mobility was maintained when ATP production was dependent on anaerobic glycolysis ($p < 0.001$). In this case, sperm were incubated with exogenous glucose and cyanide. Consequently, we could not attribute the genotypic difference in sperm mobility to mitochondrial respiration. In contrast, glucose transport, as measured by the uptake of [1,2-^{3}H] 2-deoxy-D-glucose, was reduced in sperm from subfertile roosters ($p < 0.001$). Neither hexokinase nor glyceraldehyde-3-phosphate dehydrogenase activity differed between genotypes ($p > 0.05$). Likewise, lactate dehydrogenase activity did not differ between genotypes ($p > 0.05$). As evidenced by creatine kinase activity and dynein ATPase activity, neither the potential for energy transfer nor utilization within the axoneme differed between genotypes ($p > 0.05$). Therefore, we attribute the subfertility of roosters homozygous for the rose comb allele to decreased spermatozoal glucose transport.

4.2 INTRODUCTION

The production of a series of fertilized eggs by a hen after a single insemination is dependent on several events occurring in the hen's reproductive tract. First, sperm traverse the vagina and enter the sperm storage tubules (SST), which are located near the vaginal sphincter [1]. Sperm must be motile to reach and enter the SST [1,2]. Thereafter, sperm are released from the SST and apparently transported by antiperistalsis to the infundibulum where fertilization occurs. Sperm motility does not appear to be necessary for sperm transport above the vaginal sphincter or for fertilization [2-4].

Heritable male subfertility in the domestic fowl has been reported in two models [5,6]. Single comb (*r/r*) roosters carrying the dominant sperm degeneration allele (*Sd*) are subfertile due to a defect acquired by sperm during their passage through the excurrent ducts of the testis resulting in premature sperm degeneration [7]. Roosters homozygous for the rose comb allele (*R/R*) are also subfertile [5,8]. Froman *et al.* [9] demonstrated that these two traits have a cumulative effect. Unlike sperm from *Sd* roosters, sperm from *R/R* roosters are viable and have normal morphology [10]. Behavior of sperm from *R/R* males within the hen's oviduct has been well characterized [4,8,11]. While intravaginal insemination of ejaculated sperm from *R/R* males resulted in subfertility [4,8], no difference in fertility was observed after intramagnal insemination of ejaculated sperm [4,11]. Likewise, no difference in fertility was observed following intramagnal insemination of testicular sperm from *R/R* males [4]. McLean and Froman [8] reported that sperm ejaculated by *R/R* males do not enter the SST after artificial insemination (AI) to the same

extent as sperm from fertile controls. However, there is no genotypic difference in sperm penetration of SST when sperm are incubated with oviduct explants [8]. Viewed collectively, these observations implicate poor sperm motility as a possible factor responsible for subfertility of *R/R* males, as was suspected by Kirby *et al.* [4]. This hypothesis was not rejected by McLean and Froman [8], who demonstrated sperm from *R/R* males had reduced mobility as evidenced by *in vitro* sperm penetration of Accudenz.

Poor sperm motility can be associated with several factors. Among these are abnormal axonemal structure due to mutations, defects in sperm biochemistry, and immobilizing antisperm antibodies [12]. Metabolism of sperm from *R/R* males has been suggested as having a role in subfertility of these roosters [4,13]. Most notably, fumarase activity was reported to be less in sperm from *R/R* males than controls and the activity of this enzyme was correlated to fertility [13]. However, this finding was later contradicted [14]. Kirby *et al.* [4] and McLean and Froman [8] used a test of overall metabolic activity to show metabolism of sperm from *R/R* males was less than controls. This test did not, however, identify the specific metabolic pathway or pathways responsible for the decreased activity observed.

The objective of the current research was to further characterize sperm motility and key biochemical attributes underlying motility. The present research was designed to do the following: 1) confirm decreased mobility of sperm populations, 2) evaluate motile properties of individual sperm by computer assisted sperm motion analysis, 3) identify a mechanism that might account for compromised sperm motility.

4.3 MATERIALS AND METHODS

4.3.1 Experimental Animals

All comparisons were made between sperm from subfertile *R/R* and fertile *r/r* roosters. All roosters were F2 having been derived from an orginal cross of a Silver Laced Wyandotte (*R/R*) male and 10 Single Comb White Leghorn hens (*r/r*). Males were individually caged, received feed and water *ad libitum* and were used as semen donors when they were 26-42 weeks of age.

4.3.2 Evaluation of sperm mobility

Sperm mobility was measured with an objective spectrophotometric method that quantitates the number of sperm that enter a 6% (v/v) Accudenz [15]. Semen was obtained from individual males (n = 40/genotype) and sperm concentration determined. Semen was diluted to 5 x 10^8 sperm/ml with 50 mM TES buffer, pH 7.4, containing 120 mM NaCl, 10 mM glucose, and 2 mM $CaCl_2$. This buffer will be referred to as motility buffer. A 60 µl volume of sperm suspension was layered upon 0.6 ml 6% Accudenz (Accurate Chemical & Scientific Corporation, Westbury, NY) solution prewarmed to 41°C in a disposable microcuvette. The cuvette was incubated for 5 min in a 41°C water bath. Absorbance was measured at 550 nm 1 min after the incubated cuvette was loaded into a spectrophotometer (DU-640, Beckman Instruments, Fullerton, CA).

4.3.3 Evaluation of sperm motility

Motility variables of individual sperm were determined using computer assisted sperm motion analysis. Motility of sperm from individual *R/R* males and fertile controls

was evaluated with a Hobson SpermTracker (Biogenics, Napa, CA). Semen was obtained from individual males and sperm concentration determined. Semen was diluted to 17 x 10^8 sperm/ml with motility buffer. Approximately 3 µl of sperm suspension was injected into a 20 micron MicroCell slide (Conception Technologies, San Diego, CA). In each case, sperm motility variables were estimated using a 40X objective, negative phase contrast microscopy. Three fields of approximately 50 sperm per field were evaluated for a total of 150 sperm tracks per sample at 25°C.

4.3.4 Evaluation of Sperm Biochemical Properties

ATP concentrations in freshly ejaculated sperm (n = 9 per genotype) were determined using the FireZyme Ltd. (Halifax, Nova Scotia) luciferase method. Semen was diluted to 5 x 10^8 sperm/ml with a proprietary isotonic diluent. A 50 μl volume of the sperm suspension was diluted further to lyse cells and stabilize ATP. This solution, in turn, was mixed with a proprietary solution containing luciferin and luciferase. Light output was measured immediately with a luminometer (FireZyme Ltd). ATP concentrations were determined from a standard curve.

Sperm mobility based upon ATP derived from anaerobic glycolysis was measured as follows. Semen was pooled according to genotype (n = 10 males per genotype) and five replicate experiments performed. In each case sperm concentration was determined spectrophotometrically. Semen was diluted to 2 x 10^9 sperm/ml with 50 mM TES, pH 7.4, containing 130 mM NaCl. Five ml of 12% (w/v) Accudenz was placed in a 15 ml polypropylene centrifuge tube. A 0.5 ml volume of 30% (w/v) Accudenz was placed beneath the 12% Accudenz with a Pasteur pipet. The sperm suspension was overlayed on

the 12% Accudenz solution. Tubes were centrifuged at 1250 x *g* for 25 min at 4°C. After centrifugation, fluid above and below the pelleted sperm was removed by aspiration. A stock solution of isotonic buffered saline containing 2 mM $CaCl_2$ was used to prepare each of the following media: 1) 15 mM glucose, 2) 15 mM glucose and 3 mM cyanide, and 3) 3 mM cyanide. Each medium was used to prepare a 6% (w/v) Accudenz solution. Mobility of sperm from each genotype was measured using the mobility assay as described above.

Lactate dehydrogenase (LDH), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and hexokinase (HK) activities were determined from a crude sperm extract. Semen was pooled according to genotype (n = 10 males per genotype) and 12 replicate experiments performed. Pooled semen was centrifuged through 12% (w/v) Accudenz as described above. Washed sperm were diluted to 1 x 10^9 sperm/ml with PBS containing 0.1% (v/v) Triton X-100 (Sigma). The sperm suspension was transferred to a 15-ml dounce homogenizer and disrupted by homogenization. Sperm were lysed with the loose and tight clearance pestle (10 strokes each) and lysates were centrifuged at 12,000 x *g* for 5 min. Supernatants constituted crude enzyme extracts. GAPDH and HK activities were determined according to the procedures of Krebs [16] and Schulze *et al.* [17], respectively. LDH activity was determined according to the procedure described by Gutmann and Wahlfeld [18].

Dynein ATPase activity was determined with the procedure of McConnell *et al.* [19]. Semen was pooled according to genotype and diluted 1:10 with 50 mM TES buffer, pH 7.4 containing 130 mM NaCl. This sperm suspension was centrifuged at 700 x *g* for

10 min at 4°C. The sperm pellet was resuspended in demembranation buffer containing 20 mM Tris, pH 7.9, 0.2 M sucrose, 1 mM $MgSO_4$, 1 mM dithiothreitol (DTT), 0.5 mM PMSF, and 0.1% (v/v) Triton X-100. After centrifugation, demembranated sperm were washed once with detergent-free demembranation buffer and recentrifuged. Dynein ATPase was solubilized by resuspending the demembranated sperm in a 20 mM Tris buffer, pH 7.9, containing 0.6 M NaCl, 1 mM DTT, 4 mM $MgCl_2$, 0.5 mM EDTA, and 0.5 mM PMSF for 10 min at 4°C. This suspension was centrifuged at 100,000 x *g* for 30 min at 4°C. The supernatant was used as the crude dynein extract.

Dynein ATPase activity was assayed by the hydrolysis of ATP. ATPase activity was determined by incubating 0.25 ml of crude dynein extract with 1 ml of 20 mM Tris, 4 mM $MgSO_4$, 0.1 mM ATP, pH 7.9, for 30 min at 41°C. The mixture was boiled to stop the reaction. ATP concentration was determined using bioluminescence as described above.

Creatine kinase activity was determined from a crude sperm extract. Semen was pooled according to genotype (n = 10 males per genotype) and 12 replicate experiments performed. Pooled semen was centrifuged through 12% (w/v) Accudenz as described above. Washed sperm were diluted to 2 x 10^9 sperm/ml with 50 mM TES buffer, pH 7.4, containing 130 mM NaCl. A 0.01 ml sample of the sperm suspension was diluted into 0.39 ml of 0.15 M Tris, pH 8.0, containing 0.15 M HEPES, 5 mM $MgCl_2$, 150 mM KCl, 0.5

mM EDTA, 1 mM DTT, and 0.05% (v/v) Triton X-100. A 0.01 ml sample of this disrupted sperm suspension was diluted with 0.09 ml of detergent free Tris buffer. Creatine kinase activity in the extract was determined with a creatine phosphokinase kit (Sigma).

Hexose uptake was determined with the procedure of Hiipakka and Hammerstedt [20]. Sperm from individual ejaculates (n = 15 per genotype) were diluted to 1 x 10^9 sperm/ml with 50 mM TES, pH 7.4, containing 130 mM NaCl and centrifuged at 700 x *g* for 20 min at 4°C. Washed sperm were diluted to 1.5 x 10^9 sperm/ml with 50 mM TES, pH 7.4, containing 120 mM NaCl, 1 mM 2-deoxy-D-glucose, and 2 mM $CaCl_2$. A 0.1 ml volume of sperm suspensions was mixed with an equal volume of buffer containing [1,2-^{3}H] 2-deoxy-D-glucose (1 μCi/μl). The resultant sperm suspensions were incubated at 41°C for 16 min. Uptake of 2-deoxy-D-glucose was stopped by the addition of 0.5 ml of a 0.1 M glucose solution. Samples were centrifuged at 12,000 x *g* for 5 min, supernatants discarded, and pellets resuspended in 0.5 ml of 50 mM TES, pH 7.4, containing 120 mM NaCl and 0.1% (v/v) Triton X-100. Each sperm extract was incubated 56°C for 10 min and then transferred to scintillation vials containing 5 ml of CytoScint ES scintillation fluid (ICN, Costa Mesa, CA). Radioactivity was determined in a Beckman LS 6000SE scintillation counter (Beckman Instruments, Fullerton, CA). Cytochalasin B (Sigma) was used to inhibit glucose uptake and thus provide an estimate of radioactivity in the extracellular space within the sperm pellet.

4.3.5 Statistical Analysis

Single classification ANOVA was used to analyze the following data sets: sperm mobility as measured by penetration of Accudenz, sperm motility variables estimated by computer assisted sperm motion analysis, ATP concentrations, and 2-deoxy-D-glucose uptake. Enzyme activities were analyzed by t-test.

4.4 RESULTS

As shown in Table 4.1, sperm mobility differed between genotypes ($p < 0.0001$). As shown in Table 4.2, the percentage of motile sperm may account for the reduced sperm mobility of *R/R* roosters. In contrast, variables derived from motile sperm, e.g. straight line velocity, did not differ between genotypes ($p > 0.05$). An additional genotypic difference was sperm ATP concentration (Table 4.3). Sperm from subfertile *R/R* roosters had an ATP concentration that was 37% of that from fertile controls ($p < 0.0001$).

Preliminary research with sperm from fertile roosters demonstrated that washing sperm through 12% (v/v) Accudenz followed by resuspension in 15 mM glucose had no effect on sperm mobility. In other words, the postwash mobility was equivalent to the prewash mobility. However, as shown in Table 4.4, the postwash mobility from fertile roosters differed when washed sperm were resuspended in different media. When washed sperm were resuspended in a glucose free medium containing 3 mM KCN, sperm did not penetrate the Accudenz solution from the sperm suspension overlay. Evaluation of such sperm suspensions by phase contrast microscopy confirmed that these sperm were

TABLE 4.1. Sperm mobility measured by sperm penetration of 6% (w/v) Accudenz®.

Genotype	Roosters (n)	Absorbance*
R/R	40	0.101 ± 0.09[a]
r/r	40	0.500 ± 0.19[b]

*Absorbance at 550 nm is proportional to the extent to which sperm penetrate the Accudenz layer from a sperm suspension overlay. Each value is a mean ± SEM.

[a,b] Means different at $p < 0.0001$.

TABLE 4.2. Variables[†] estimated with computer assisted sperm motion analysis.

Genotype*	Straight line Velocity (μm)	Average path Velocity (μm)	Beat cross frequency (hz)	ALH[§] (μm)	Motile Sperm (%)
R/R	18 ± 5.7	31 ± 7.1	10.7 ± 3.1	7.2 ± 1.1	62 ± 20[a]
r/r	20 ± 6.3	34 ± 5.6	12.1 ± 2.3	6.3 ± 1.3	91 ±6.3[b]

[†]Each value is a mean ± SEM

*n = 12 males per genotype

[§]Amplitude of lateral head displacement

[a,b]Means different at $p < 0.001$.

TABLE 4.3. Concentrations of ATP in sperm from males homozygous for the rose comb allele (*R/R*) and controls (*r/r*).

Genotype	Roosters (n)	ATP* (nmole/10^9 sperm)
R/R	9	31.84 ± 8.2^a
r/r	9	85.41 ± 10.6^b

*Each value is a mean ± SEM.

[a,b]Means different at $p < 0.001$.

TABLE 4.4. Genotypic difference in sperm mobility when ATP production was limited to anaerobic glycolysis.

Combinations of Reagents in TES buffer*		Absorbance by Genotype†	
Glucose	Cyanide	R/R	r/r
+	-	0.145 ± 0.040[a]	0.705 ± 0.043[b]
-	+	0.005 ± 0.001	0.007 ± 0.006
+	+	0.125 ± 0.029[a]	0.675 ± 0.066[b]

*TES buffer consisted of: 50 mM TES, pH 7.4, 120 mM NaCl, 2 mM $CaCl_2$, and when present, 15 mM glucose, and 3 mM KCN.

†Absorbance at 550 nm is proportional to the extent to which sperm penetrate the Accudenz® layer from a sperm suspension overlay. Each value is a mean ± SEM.

[a,b] Means within rows differed at $p < 0.0001$.

immotile. In contrast, when washed sperm from fertile roosters were resuspended in medium containing 15 mM glucose and 3 mM KCN, sperm mobility was 83% (Table 4.4) of that KCN free medium containing glucose. Therefore, sperm from fertile roosters used anaerobic glycolysis to generate ATP required for progressive motility.

As also shown in Table 4.4, the genotypic difference was evident when washed sperm were resuspended in medium containing either 15 mM glucose or 15 mM glucose with 3 mM KCN. However, the genotypic difference was lost in the medium containing just 3 mM KCN. Therefore, the genotypic difference persisted when anaerobic glycolysis was the only means of ATP production.

As shown in Figue 4.1, uptake of [1,2-^{3}H] 2-deoxy-D-glucose differed between genotypes ($p < 0.001$). Uptake of 2-deoxy-D-glucose (dGlc) by sperm from controls was 84.4 pmole 2dGlc/10^{8} sperm. In contrast, uptake of dGlc by sperm from *R/R* males was only 52.25 pmole dGlc/10^{8} sperm, or 62% of the control value. In addition, hexokinase, glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase, creatine kinase, and dynein ATPase activities did not differ between genotypes ($p > 0.05$; Table 4.5).

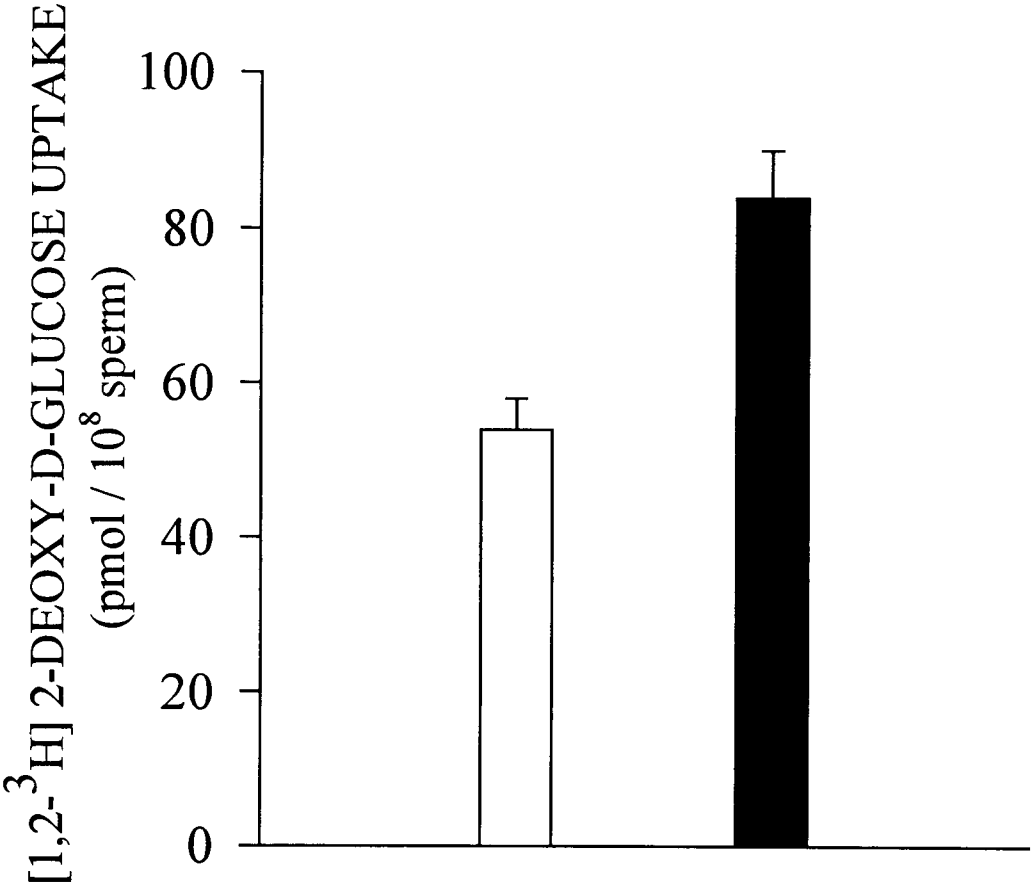

FIGURE 4.1. Uptake of [1,2-^{3}H] 2-Deoxy-D-glucose by sperm from *R/R* (open) and *r/r* (filled) males. Each bar represents the mean and standard error of 14 replicate observations from individual males. Means were significantly different ($p < 0.001$).

Table 4.5. Sperm enzyme activities* (Units/mg protein) by semen donor genotype.

Genotype	Hexokinase	GAPDH[a]	Lactate Dehydrogenase	Creatine Kinase	Dynein ATPase
R/R	0.62 ± 0.1	0.20 ± 0.01	7.9 ± 0.27	9.2 ± 1.1	2.03 ± 0.7
r/r	0.66 ± 0.1	0.20 ± 0.01	7.4 ± 0.21	9.0 ± 1.3	1.94 ± 0.7

* n = 12 replicates using pooled semen from 10 males per genotype. Each value is a mean ± SEM.

[a]Glyceraldehyde-3-phosphate dehydrogenase

4.4 DISCUSSION

Roosters homozygous for the rose comb allele (*R/R*) are subfertile and ejaculate sperm that are characterized by poor mobility [8]. Mobility was measured with an objective spectrophotometric method that quantitates the number of sperm that enter a 6% (v/v) Accudenz solution [15] from a sperm suspension overlay. Therefore, this method is descriptive of sperm cell populations but does not describe the attributes of individual sperm within these populations. We confirmed our initial observations on sperm mobility [8] using two populations of roosters (Table 4.1). Attributes of individual sperm can be determined using computer assisted sperm motion analysis [21-23]. Evaluation of individual ejaculates with such a system indicated the percentage of motile sperm was less in ejaculates from *R/R* roosters than those of fertile controls (Table 4.2). However, variables based upon motile cells, such as straight line velocity, average path velocity, amplitude, and beat cross frequency, did not differ between genotypes. We attributed the decreased sperm mobility of *R/R* males to a lower percentage of motile sperm.

Sperm mobility data sets were transformed into frequency distributions (unpublished data). As previously reported [24], frequencies from fertile roosters approximated a normal distribution. However, the distribution for the subfertile *R/R* roosters was skewed. Subsequent experiments were based on males selected from the portion of each distribution that was most representative of the genotype.

Ford and Rees [25] state that 40-60% of the ATP generated in mammalian sperm cells is used for the purpose of cell motility. Therefore, ATP synthesis is critical to motility.

As shown in Table 4.2, sperm from *R/R* males had lower ATP concentrations than sperm from fertile controls. The measurement of intracellular ATP concentration, as described in the materials and methods, provides an averaged value for all cells within the sample. Based upon the Hobson SpermTracker data (Table 4.2), subfertile and fertile males have 38 and 9% immotile cells, respectively. Therefore, the subfertile males constitute an extreme example of heterogeneity within a sperm cell population from a given male. However, because of the size of the subpopulation of immotile sperm in ejaculates from subfertile males, we hypothesized that we could detect a difference based upon averaged values of biochemical variables. Due to the complex nature of the regulation of ATP synthesis in sperm [25], the basis for the observed differences in ATP concentration were not obvious. Thus, we took advantage of the highly compartmentalized nature of sperm and inferred that mitochondrial respiration did not account for the genotypic difference in sperm motility (Table 4.4). Based upon the results shown in Table 4.4, sperm motility in the presence of cyanide was dependent on exogenous glucose. Cyanide blocks mitochondrial respiration by inhibiting cytochrome oxidase. Sperm were immotile in the presence of cyanide when glucose was absent. However, when sperm were suspended in a medium containing cyanide and glucose, the genotypic difference was apparent. Consequently, mitochondrial function cannot account for the genotypic difference in motility.

The experimental results shown in Table 4.4 provided us with four avenues of investigation: (1) glucose uptake, (2) ATP synthesis by glycolysis, (3) allocation of energy along the axoneme via the phosphorylcreatine shuttle [26], (4) utilization of energy by

dynein ATPase. Glucose uptake is dependent on glucose transporters in the plasma membrane [27]. Glucose uptake was measured using 2-deoxy-D-glucose which enters cells, is phosphorylated but is not metabolized [20]. Uptake of 2-deoxy-D-glucose by sperm from *R/R* males was only 62% of controls (Figure 4.1). In contrast, the production, allocation and utilization of energy within sperm cells did not differ between genotypes (Table 4.5).

Fowl sperm can produce ATP under anaerobic conditions to maintain motility and fertilizing ability [28,29]. Even though there is not a sperm specific lactate dehydrogenase (LDH) in fowl sperm [30-32], LDH is critical for ATP production by anaerobic glycolysis [25]. However, LDH activity did not differ between genotypes. Two additional enzyme activities related to glycolysis were measured: hexokinase and glyceraldehyde-3 phosphate dehydrogenase. Hexokinase is the initial enzyme of glycolysis that phosphorylates glucose within the cytoplasm, and glyceraldehyde-3-phosphate dehydrogenase is the rate limiting glycolytic enzyme of fowl sperm [33]. However, in either case, no difference was observed between genotypes (Table 4.5). Consequently, we did not attribute the genotypic difference in sperm motility to glycolytic ATP synthesis.

Based upon the presence of creatine kinase along the length of fowl sperm axoneme [26] high energy phosphate bonds may be allocated along the axoneme with the phosphorylcreatine shuttle [34,35]. Once the high energy phosphate bonds have been transfered to ADP, the ATP generated is hydrolyzed by dynein ATPase [36]. However, there was neither a difference in creatine kinase activity nor dynein ATPase activity between genotypes (Table 4.5). Specific inhibitors were used in each case to validate

enzyme data. In summary, we rejected the hypothesis that energy allocation or utilization was responsible for the genotypic difference in motility.

Reduced glucose uptake, in part, appears to be responsible for reduced metabolism and poor motility of sperm from *R/R* males. Glucose transport in sperm has been investigated in bull, ram, rat, and human sperm [20,37,38]. To date, six glucose transporters have been identified [27]. Two glucose transporters, Glut1 and Glut3, have been associated with rat and human testis and Glut3 has been associated with both rat and human sperm [39,40]. Glut5 has also been associated with human sperm, however, it is considered a fructose, rather than a glucose, transporter [40]. Chicken glucose transporters have been studied by several groups [41,42]. We used a rabbit anti-Glut3 antibody (a gift from Dr. Martyn White, East Carolina Medical School, Greenville, NC), and have demonstrated the presence of Glut3 in fowl sperm by western blotting. Analysis of the structure and function of Glut3 from sperm of *R/R* roosters may provide a molecular basis for the subfertility associated with this genotype.

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CHAPTER 5

CONCLUSIONS

This research demonstrated that heritable subfertility associated with homozygosity for the rose comb (*R/R*) allele is due to reduced sperm glucose transport. Reduced glucose transport would be expected to limit sperm metabolic rate. Consequently, this could account for the reduced ATP concentrations observed within sperm from subfertile males. Additionally, reduced glucose uptake could account for reduced sperm motility. The percentage of immotile sperm in ejaculates from subfertile males was 38% compared to 9% for fertile males, as evaluated using computer assisted sperm motion analysis. Conversely, the properties of motile sperm do not differ between genotypes. This may explain why *R/R* males are subfertile rather than infertile.

This thesis validated the application of a sperm penetration assay as well as computer assisted sperm motion analysis for the evaluation of fowl sperm motility. Whereas computer assisted sperm motion analysis provides information based upon individual sperm within a population, the sperm penetration assay estimates the size of a highly mobile subpopulation. The sperm penetration assay is based upon the ability of motile sperm to penetrate an Accudenz® solution. Sperm penetration is measured spectrophotometrically. Absorbance at 550 nm is directly proportional to the number of sperm that enter the Accudenz® solution from an overlay of extended semen. When applied to sperm from *R/R* males, the assay lead to the discovery that ejaculates from these males contained a significantly smaller subpopulation of highly mobile sperm than do

ejaculates from fertile controls. In addition to using Accudenz® to measure sperm mobility, this reagent was found useful for placing sperm in a chemically defined medium. This experimental capability afforded the demonstration that the genotypic difference in sperm mobility was present when ATP synthesis was limited to anaerobic glycolysis.

Viewed collectively, these data demonstrate how an aberrant sperm cellular mechanism can account for the subfertility associated with roosters homozygous for the rose comb allele. As stated earlier, reduced glucose transport would be expected to limit ATP synthesis. In a cell characterized by high energetic demands, such a condition would be expected to compromise the cell's function. Thus, it is more apparent why sperm from subfertile males do not ascend the vagina and enter SST to the extent that sperm from fertile males do. It is noteworthy that the subfertility of *R/R* males is a function of insemination route. As explained previously, the genotypic difference in fertility is essentially eliminated by use of intramagnal insemination (Etches *et al.*, 1974; Kirby *et al.*, 1994). Sperm placed in this region of the oviduct do not have to traverse the vagina and enter SST. Therefore, two critical prerequisites for fertilization in the fowl are circumvented by intramagnal insemination. Nonetheless, sperm from subfertile males appear to bind perivitelline layer proteins, undergo an acrosomal reaction, and the fuse with the oocyte plasma membrane as do sperm from fertile controls.

This research demonstrated the importance of sperm motility to fertility in the domestic fowl using a genetic model. In contrast to research such as Wishart and Palmer (1986) who reported a correlation between sperm motility and fertility, the present research demonstrated a cause and effect relationship between the sperm motility and fertility.

Homozygosity for the rose comb allele provides avenues for additional research. It should be noted that the objective of this research was to find a sperm specific attribute that could account for subfertility. Demonstration of reduced glucose transport in sperm from *R/R* males may be sperm specific. To date, 7 members of the glucose transporter gene family have been identified (Mueckler, 1994). Of these, 4 are believed to transport glucose through the plasma membrane. The expression of glucose transporter isoforms is often tissue specific and hormonally regulated. It has been shown that Glut3 is the only glucose transporter present in rat sperm (Burant and Davidson, 1994). Preliminary research in this study demonstrated that Glut3 is present in fowl sperm.

The expression of Glut3 during spermatogenesis may explain why sperm from *R/R* males have reduced glucose transport. Molecular probes exist that could be used to determine mRNA levels of Glut3 in the testes. Decreased Glut3 mRNA levels in testes from *R/R* males may indicate that Glut3 expression is affected by this genotype. Several steps of gene regulation could be affected. These include the initiation of transcription or translation, post-translational modifications to the protein, or mRNA storage in the cytoplasm. During spermatogenesis, translation of mRNA from several sperm-specific proteins occurs up to 8 days after all transcription stops in elongating spermatids (Kleene *et al.*, 1984). This is possible due to the storage of mRNA in the cytoplasm in translationally inert ribonucleoprotein particles. There are other examples of various mechanisms of gene regulation utilized during spermatogenesis such as alternative splicing of mRNA. The complex regulation of gene expression for sperm-specific proteins may provide an explanation of how sperm from *R/R* males have altered Glut3 expression.

Other mechanisms involved in gene expression or protein dispersion during spermatogenesis could be studied with the rose comb model. For example, Braun *et al.* (1989) demonstrated, through the use of transgenic mice, that haploid spermatids exchange mRNA and proteins through intracellular bridges connecting developing spermatids in a cohort. Thus, while a sperm cell may not contain a copy of an allele, it can contain the gene product. These findings relate to a major assumption in early research with *R/R* males. Crawford and Smyth (1964a) demonstrated that sperm carrying the *R* allele derived from heterozygote males are fully functional. However, based on the results of Braun *et al.* (1989), sperm carrying either the *R* allele or a linked allele that may be responsible for subfertility, may contain a fully functional gene product. This could explain why sperm carrying the *R* allele from heterozygotes fertilize oocytes as well as sperm carrying the *r* allele. Thus, roosters heterozygous for the rose comb allele may represent a model that could be used to test the working hypothesis of Braun *et al.* (1989). According to this hypothesis, sperm from heterozygotes would contain functional and dysfunctional gene products.

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APPENDICES

Poultry Science 75, 776-784 (1996)

APPENDIX A

Objective Measurement of Sperm Motility Based upon Sperm Penetration of Accudenz®

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A.1 ABSTRACT

When a suspension of rooster sperm was overlaid upon 6% (wt/vol) Accudenz®, immotile sperm did not enter but motile sperm entered rapidly. The absorbance of the Accudenz® layer increased as a result. These phenomena were used to measure sperm motility objectively at body temperature. The intra-assay coefficient of variation (CV) was 2.6% (n=3). When roosters (n=36) were ejaculated repeatedly and sperm motility data analyzed by two-way ANOVA, a male effect was observed ($P \leq .001$). When roosters were ranked by mean motility scores (n=3 evaluations per male) and representative males selected as semen donors, a difference in fertility ($P \leq .001$) was observed between males characterized by minimal and maximal sperm motility. Frequency analysis with data from a second flock of roosters (n=100) revealed a normal distribution. Roosters categorized by average sperm motility (n=18) or sperm motility greater than a standard deviation above the mean (n=17) were selected for further analysis by repeated measurements. A split plot ANOVA revealed a difference between categories ($P \leq .0001$) and variation among males within a category ($P \leq .0001$). In contrast, sperm motility was independent of time and there was no interaction between category and time ($P \geq .05$). Thereafter, 5 roosters from each group were ejaculated weekly and inter-assay CVs estimated with semen pooled by category (n=3 observations per category). During this interval, sperm motility of average roosters was 55 ± 5.9% of that of roosters within the high motility category. Inter-assay CVs were 18.1 and 9.2% for roosters originally categorized by average and high sperm

motility, respectively. The assay described has potential for: (1) selecting males based on sperm motility, and (2) standardizing the measurement of poultry sperm motility.

A.2 INTRODUCTION

The motility of poultry sperm has been measured objectively by four principal means: spectrophotometry, videomicroscopy, digital image analysis, and sperm movement from one medium into another. Wall and Boone (1973), Atherton *et al.* (1980), as well as Wishart and Ross (1985) described modifications of a spectrophotometric technique originally used by Timourian and Watchmaker (1970) for measuring the motility of sea urchin sperm. Spectrophotometry has been used predominantly to evaluate sperm motility in pooled semen samples (Wishart, 1984a,b; Ashizawa and Wishart, 1987; Wishart and Ashizawa, 1987; Thomson and Wishart, 1988; Ashizawa *et al.*, 1989a,b; Ashizawa *et al.*, 1990; Ashizawa and Wishart, 1992; Thomson and Wishart, 1991; Froman and Thursam, 1994). In contrast, videomicroscopy has been applied principally towards the study of axonemal function (Ashizawa *et al.*, 1989c; Ashizawa and Hori, 1990; Ashizawa and Sano, 1990; Ashizawa *et al.*, 1992a,b; Ashizawa *et al.*, 1993; Ashizawa *et al.*, 1994a,b). The limited use of digital image analysis (Bakst and Cecil, 1992a,b) is most likely attributable to the cost of instrumentation.

As reviewed recently by Suttiyotin and Thwaites (1993), sperm migration from one medium into another has been used as a measure of sperm motility for numerous mammalian species. In contrast, this phenomenon has received little attention as a means

of measuring the motility of poultry sperm (Birrenkott *et al.*,1977; McLean and Froman, 1995). The latter researchers sought a sperm attribute that would account for the subfertility of roosters homozygous for the rose comb allele. In comparison to fertile heterozygotes, subfertile homozygotes were characterized by decreased sperm motility when sperm suspensions from individual roosters were overlaid upon 6% (wt/vol) Accudenz®[3]. Furthermore, sperm motility was measured at body temperature utilizing standard laboratory equipment, the assay was rapid, and the results were easily interpreted and analyzed. Therefore, the objectives of the present research were to: (1) determine intra-and inter-assay coefficients of variation for the sperm penetration test, (2) test for differences in sperm motility among normal, fertile males, and (3) to determine whether a cause and effect relationship could be demonstrated between *in vitro* sperm motility and fertility.

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[3]Accurate Chemical & Scientific Corporation, Westbury, NY 11590.

A.3 MATERIALS AND METHODS

A.3.1 Intra-assay Coefficient of Variation

Four solutions were required for the sperm penetration assay. First, a 30% (wt/vol) stock solution of Accudenz® was prepared with 3 mM KCl containing 5 mM N-tris-[hydroxymethyl]methyl-2-amino-ethanesulfonic acid (TES), pH 7.4, as the solvent. Second, another TES-based buffer, henceforth designated as *motility buffer*, contained 111 mM NaCl, 25 mM glucose, and 4 mM $CaCl_2$ in 50 mM TES, pH 7.4. The osmolality of the motility buffer was 320 mmol/kg. Third, a portion of the motility buffer was diluted to 290 mmol/kg with deionized water. Fourth, a 6% (wt/vol) Accudenz® solution was prepared by diluting the stock solution with diluted motility extender. The pH and osmolality of the 6% (wt/vol) Accudenz® solution were 7.35 pH units and 323 mmol/kg, respectively.

A 1.5-mL volume of the 6% (wt/vol) Accudenz® solution was pipetted into each of three polystyrene cuvets held within a 41 C water bath. After the Accudenz® solution had reached thermal equilibrium, semen was procured from each of 10 New Hampshire roosters. Ejaculates were pooled and the semen thoroughly mixed. Sperm concentration was determined fluorometrically according to Bilgili and Renden (1984). Semen was diluted with motility buffer to a concentration of 5×10^8 sperm per mL. At 3-min intervals, 150-μL volumes of sperm suspension were overlaid upon 1.5-mL volumes of Accudenz® layers. Each cuvet was removed from the water bath after a 5-min incubation and then placed within a spectrophotometer. Absorbance at 550 nm was recorded after a 1-min

interval. This process was repeated for sperm that had been immobilized by heating at 56 C for 10 min. The intra-assay coefficient of variation (CV) was calculated for motile sperm by dividing the observed standard deviation by mean absorbance and then multiplying the proportion by 100.

Experiment 1 was replicated as follows. The bottom of each of 3 polystyrene cuvets was perforated with a red-hot stainless steel probe. A 7-mm length of polyethylene capillary tubing (1.9 mm outer diameter) was attached to the cuvet with Silastic® Medical Adhesive[4] so that the upper end of the tubing protruded 1 mm above the plane of the bottom of the cuvet. The adhesive was allowed to cure overnight. Prior to loading each cuvet with 6% (wt/vol) Accudenz® as above, the lower end of each capillary tube was sealed with a stainless steel sealing plug. Thereafter, cuvets were placed in a 41 C waterbath. Semen was collected and manipulated as above. At 3-min intervals, 100-μL volumes of sperm suspension were overlaid upon the Accudenz® layers. After incubating for 10 min at 41 C, each cuvet was removed from the water bath, the stainless steel plug removed, and the Accudenz® layer collected into a 1.5-mL microcentrifuge tube. Accudenz® layers containing sperm were centrifuged at 15,600 x g for 1 min. Each supernatant was removed with a Pasteur pipet, a 40-μL volume of motility buffer was added to the microcentrifuge tube, the pelleted cells were resuspended, and the final volume of the sperm suspension recorded. Likewise, residual sperm suspensions were recovered from cuvets and volumes recorded. Sperm concentrations in top and bottom layers, i.e. extended semen overlay and 6% (wt/vol) Accudenz®, were determined as above. Sperm

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[4]Dow Corning Corp., Medical Products Division, Midland, MI 48640

recovered from the Accudenz® layer were expressed as a percentage of the total sperm recovered from each cuvet. A mean percentage was calculated and the CV calculated as above.

A.3.2 Difference in Sperm Motility Among Males

Repeated measurements were made on individually caged males as follows. Manual ejaculation of 48-wk-old New Hampshire roosters (n=36) was initiated on an every-other-day basis. Roosters were ejaculated randomly on each of 3 consecutive semen collection days. The following steps were performed sequentially for each rooster. Immediately after ejaculation, sperm concentration was determined as above, the ejaculate diluted to 5 x 10^8 sperm per mL with pre-warmed motility buffer, a 300-μL volume of the sperm suspension overlaid upon 3 mL of pre-warmed 6% (wt/vol) Accudenz® held in a polystyrene cuvet, the cuvet incubated for 5 min at 41 C, the cuvet placed within a photometer[5], and a reading made after a 1-min interval. Photometric data were analyzed by two-way ANOVA (Sokal and Rohlf, 1969a).

A.3.3 In Vitro Sperm Motility and Fertility

Roosters were ranked according to their mean motility scores. A fertility trial was performed in which Single Comb White Leghorn hens (n=45 per treatment group) were inseminated with sperm obtained from roosters categorized as having minimal, average, or maximal sperm motility. Roosters within a category (n=3) were manually ejaculated, their semen pooled, sperm concentration measured as above, and pooled semen extended to 5 x 10^8 sperm per mL with motility buffer. Each hen was inseminated intravaginally with 5

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[5]Model 534A Densimeter, Animal Reproduction Systems, Chino, CA 91710

x 10^7 sperm. Egg collection, incubation, and data analysis were performed according to Kirby and Froman (1990).

This experiment was replicated as follows. The true difference in fertility between roosters categorized as having minimal or maximal sperm motility was assumed to be 5 percentage units based upon the results of the first fertility trial. The number of eggs per treatment group needed to detect this difference with 90% certainty at a significance level of α=.05 was calculated according to Sokal and Rohlf (1969b). Thus, only roosters categorized by minimal or maximal sperm motility were used as semen donors in the second fertility trial. Males within one category (n=3) were manually ejaculated and their semen processed as above. Prior to insemination, sperm motility was measured as outlined in the first experiment. Then, each of approximately 130 Leghorn hens was inseminated intravaginally with 5 x 10^7 sperm. Thereafter, this process was repeated for males within the second category and the hens constituting their corresponding treatment group. Egg collection, incubation, and data analysis were performed as above.

A.3.4 Analysis of Males Categorized by Sperm Motility

Repeated measurements were made on males categorized by sperm motility as follows. Males were selected from a second flock of New Hampshire roosters based upon a single measurement of sperm motility and frequency analysis. Individually caged 25-wk-old roosters (n=100) were assigned randomly to be ejaculated on one of three consecutive days. Sperm motility was measured as described above. Data were analyzed by single classification ANOVA (Sokal and Rohlf, 1969c) in order to determine whether observations were independent of a time effect. The Kolmogorov-Smirnov test for

goodness of fit was used to determine whether observed frequencies of pooled data approximated a normal distribution (Sokal and Rohlf, 1969d).

Males were ranked by their sperm motility scores. Males with scores near average (n=18) were categorized as average. Males with scores greater than one standard deviation above the mean (n=17) were categorized as high sperm motility males. Manual ejaculation of categorized roosters was initiated on an every-other-day basis. Roosters were randomized by cage number and sperm motility measured by photometric analysis on each of 3 days. Photometric data were analyzed by split plot design ANOVA (Sokal and Rohlf, 1969e).

A.3.5 Inter-assay Coefficient of Variation

Five representative roosters were selected per sperm motility category. Each rooster was ejaculated on a weekly basis. Semen was pooled by category as roosters were ejaculated, and duplicate measurements of sperm motility were made by spectrophotometric analysis per pool per week. A different batch of reagents was used each week. Inter-assay CVs were estimated from the sample means of each category.

A.4 RESULTS

A.4.1 Intra-assay Coefficient of Variation

Sperm rendered immotile by heating to 56 C did not penetrate the Accudenz® layer. In contrast, motile sperm entered the Accudenz® layer rapidly and, as a consequence, absorbance increased as a function of time. A representative plot of absorbance versus time is shown in Figure A.1. In preliminary experiments, we found the rate of sperm penetration to be most rapid during the initial 5 min of incubation. Likewise, once cuvettes were placed within the spectrophotometer, results were most consistent when measurements were made after a slight delay. We attributed this effect to physical stabilization of the Accudenz® layer. Therefore, we adopted a 5-min incubation interval and a 1-min delay between cuvette transfer and making a measurement as standard operating procedures. When these conditions were used with a sperm suspension derived from pooled semen containing 5 x 10^8 sperm per mL, the mean absorbance, standard deviation, and intra-assay CV (n=3) were 0.9385, 0.0244, and 2.6%, respectively. When the repeatability of the assay was estimated in terms of the percentage of sperm recovered from the Accudenz® layer after 10 min of incubation at 41 C, the mean and intra-assay CV were 82% and 6.2%, respectively.

A.4.2 Difference in Sperm Motility Among Males

When sperm penetration into Accudenz® was measured with a photometer (Figure A.2), a pattern comparable to that obtained with a spectrophotometer (Figure A.1) was observed. When sperm motility was tested repeatedly for each of 36 New Hampshire roosters, the effect of time was nonsignificant. However, a difference ($P \leq 0.001$) in sperm

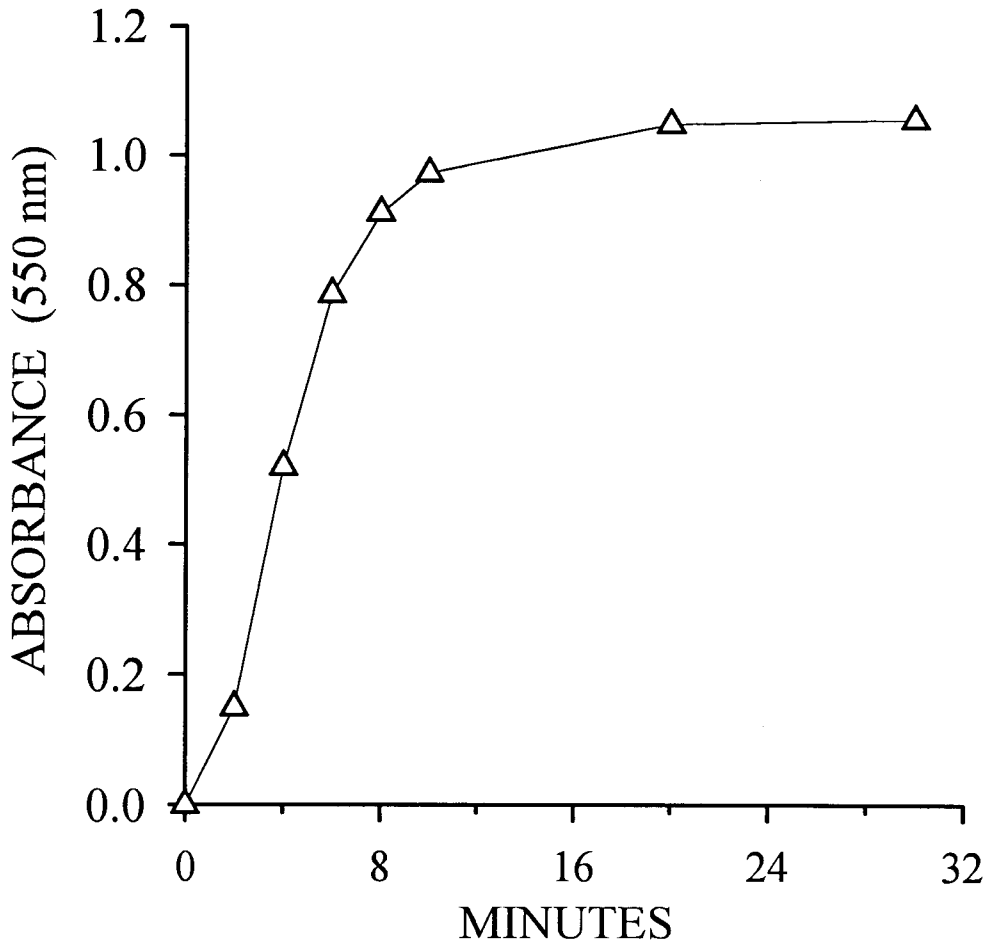

FIGURE A.1. Absorbance of 6% (wt/vol) Accudenz® (△) after overlay with a sperm suspension containing motile sperm. Time zero denotes the time at which a 150-μL volume of sperm suspension, containing 5 x 10^8 rooster sperm per mL, was overlaid upon a 1.5-mL volume of Accudenz® pre-warmed to 41 C. After the initial reading was made, the cuvet was incubated in a 41 C water bath. The cuvet was removed from the water bath and absorbance measured at 550 nm after 2, 4, 6, 8, 10, 20, and 30 min of incubation. When this procedure was repeated with sperm immobilized by pre-heating to 56 C, the absorbance remained at zero over the time course shown.

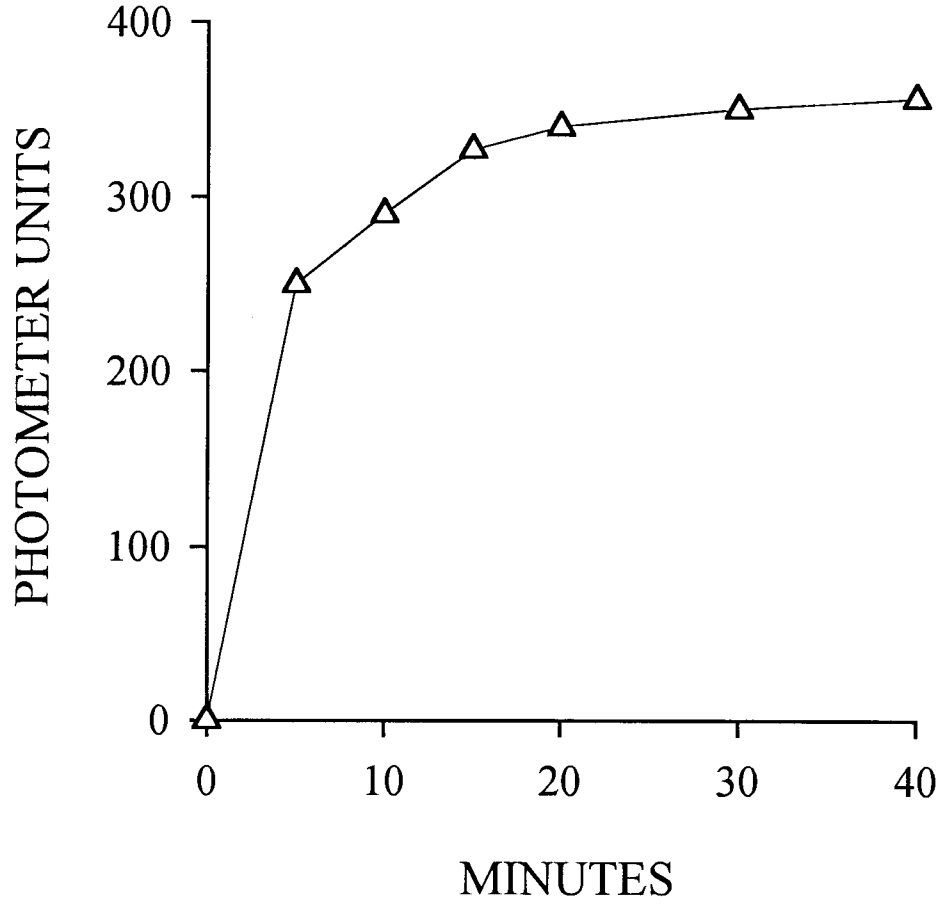

FIGURE A.2. Increase in photometer units (△) due to sperm penetration of 6% (wt/vol) Accudenz®. Time zero denotes the time at which a 300-μL volume of a sperm suspension, containing 5 x 10^8 rooster sperm per mL, was overlaid upon a 3-mL volume of Accudenz® pre-warmed to 41 C. After the initial reading was made, the cuvette was incubated in a 41 C water bath. The cuvette was removed from the water bath and measurements made after 5, 10, 20, 30 and 40 min incubation at 41 C.

motility was found among roosters. The ANOVA is summarized in Table A.1. When males were ranked by mean scores, the maximal sperm motility score was 5 times greater than the minimal score.

A.4.3 In Vitro Sperm Motility and Fertility

Data from the initial fertility trial are summarized in Table A.2. No difference in fertility was observed among treatment groups when hens were inseminated with sperm from males categorized *a priori* by minimal, average, or maximal sperm motility. Fertility averaged 54, 53, and 49% for these treatment groups, respectively. In contrast, a difference in fertility ($P \geq 0.001$) was observed between hens inseminated with sperm from males categorized by minimal or maximal sperm motility in the second fertility trial (Table A.3). Graphical analysis of this data set (Figure A.3) revealed that the difference was attributable to lower initial fertility in the case of hens inseminated with sperm characterized by minimal motility. In the second fertility trial, differential sperm motility was confirmed using extended semen just minutes before insemination. After a 5-min incubation interval at 41 C, the absorbance of the 6% (wt/vol) Accudenz® was 0.4423 and 0.8470 for males categorized *a priori* as having minimal and maximal sperm motility.

A.4.4 Analysis of Males Categorized by Sperm Motility

Measurements of sperm motility from individual roosters made over a 3-d interval were independent of time. Therefore, data were pooled and a frequency analysis performed (Figure A.4). The hypothesis that observed frequencies approximated a normal distribution was not rejected. The solid line in Figure A.4 represents the shape of the predicted

TABLE A.1. Summary of two-way ANOVA following repeated measurements of rooster sperm motility[1]

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	*F*-value
Day	2	8,002	4,001	1.0959
Rooster	35	507,096	14,488	3.9682***

[1]Each of 36 New Hampshire roosters was ejaculated on an every-other-day basis. Three consecutive measurements were made per rooster.

***$P \leq 0.001$

TABLE A.2. Summary of first fertility trial.

Roosters (n)	Sperm Motility[1]	Hens[2] (n)	Eggs[3] (n)	Fertility[4] (%)
3	Maximal	43	697	54 ± 1.6
3	Average	43	686	53 ± 2.0
3	Minimal	44	741	49 ± 2.6

[1]*A priori* categorization based upon sperm penetration of 6% (wt/vol) Accudenz®. Roosters (n=36) were ranked by motility scores, and 3 representative roosters were chosen for each category.

[2]Each hen was inseminated intravaginally with a single dose of 50×10^6 sperm.

[3]Collected over a 21-d interval.

[4]Each value is a mean ± SEM

TABLE A.3. Summary of second fertility trial

Roosters (n)	Sperm Motility[1]	Hens[2] (n)	Eggs[3] (n)	Fertility[4] (%)
3	Maximal	135	2,590	52 ± 1.0^{A}
3	Minimal	129	2,485	44 ± 1.4^{B}

[1]*A priori* categorization based upon sperm penetration of 6% (wt/vol) Accudenz®. Roosters (n=36) were ranked by motility scores, and 3 representative roosters were chosen for each category. Differential sperm motility was confirmed prior to insemination. Sperm from "minimal" roosters penetrated Accudenz® to only 51% of the extent to which sperm from "maximal" roosters did.

[2]Each hen was inseminated intravaginally with a single dose of 50×10^6 sperm.

[3]Collected over a 21-d interval.

[4]Each value is a mean ± SEM.

[A,B]Different at $P \leq .0001$.

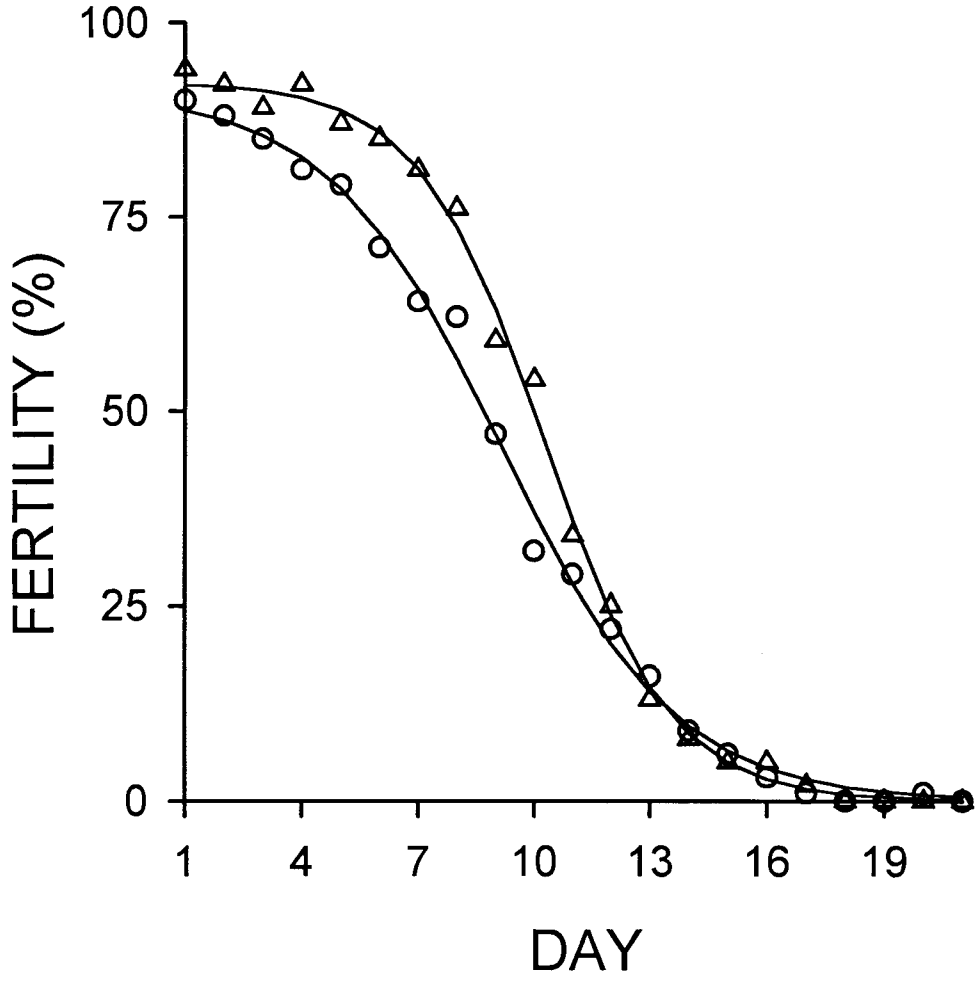

FIGURE A.3. Duration of fertility after a single insemination of Single Comb White Leghorn hens with sperm from New Hampshire roosters categorized as having maximal (△) or minimal (○) sperm motility. Designations were based upon the ranked scores of 36 roosters after sperm penetration into 6% (wt/vol) Accudenz® from an overlaid sperm suspension. Each hen was inseminated intravaginally with 5×10^7 sperm. Solid lines represent the functions

$$y(x) = [98]/[1 + e^{-.5866(11.6-x)}], \text{ and}$$
$$y(x) = [92]/[1 + e^{-.4279(10.4-x)}],$$

in which 98 and 92 are estimates of the parameter γ, the initial percentage of fertilized eggs.

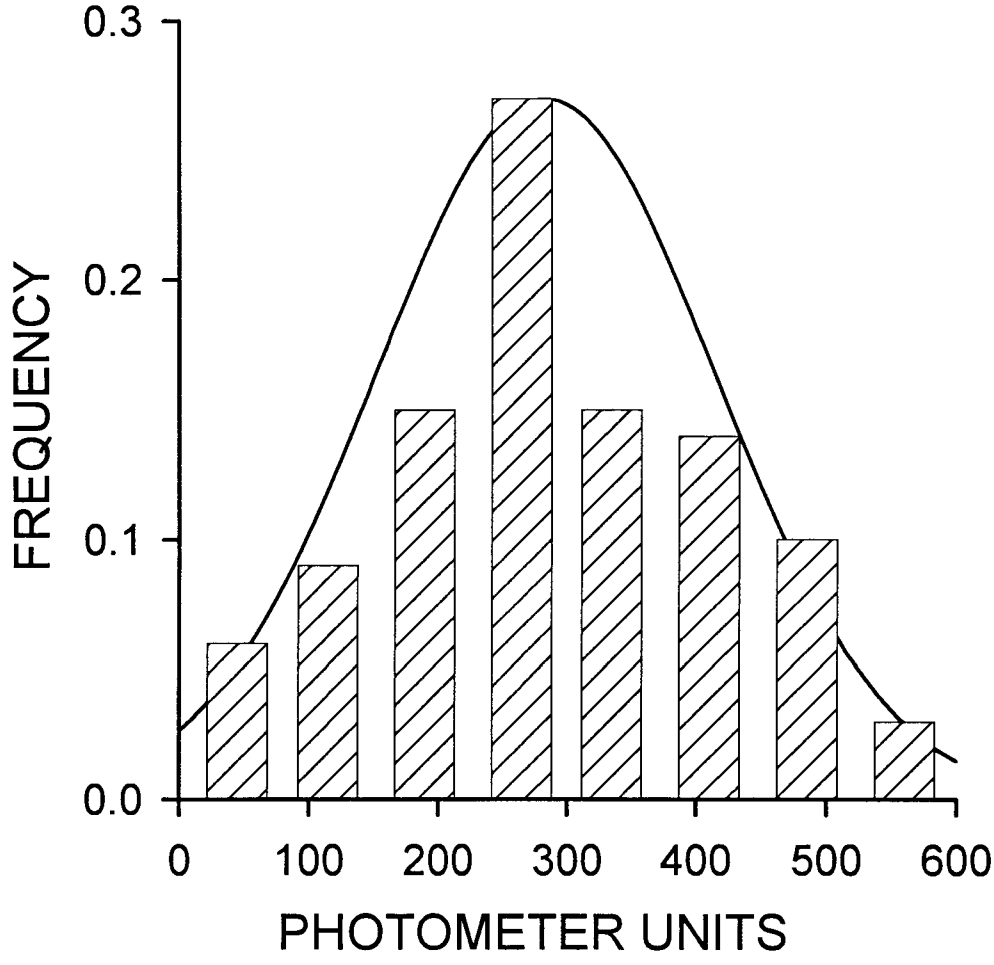

FIGURE A.4. Frequency analysis of sperm motility from individual New Hampshire roosters (n=100). Bars denote categories based upon the extent to which sperm penetrated 6% (wt/vol) Accudenz® as measured by a photometer. Each category represents an increment of 75 photometer units. Bars are centered upon interval midpoints. Thus, the first bar, which is centered on 37.5 photometer units, represents the frequency of observations that were greater than $0 \leq 75$ photometer units. The hypothesis that observed frequencies approximated a normal distribution was not rejected ($P \geq .05$). The solid line represents the normal distribution as determined by the normal probability density function and estimates of 283 and 131.4 for μ and σ, respectively.

distribution using 283 and 131.4 as estimates of μ and σ, respectively. When males were ranked by motility scores, the maximum was 30.5 fold greater than the minimum. Analysis of repeated measurements from males categorized by average or high sperm motility demonstrated highly significant differences ($P \leq 0.0001$) between categories and among males within categories (Table A.4). In contrast, neither time nor a category by time interaction exerted an effect on sperm motility.

A.4.5 Interassay Coefficient of Variation

Mean absorbance, standard deviation, and inter-assay CV (n=3) for roosters categorized by photometric analysis as having average sperm motility (see Figure A.4) were 0.5614, 0.10133, and 18.0% when the sperm motility of pooled semen was evaluated by spectrophotometric analysis. Likewise, these statistics were 1.0082, 0.09256, and 9.2% for roosters categorized by high sperm motility. Sperm from average roosters penetrated the Accudenz® layer to only 55 ± 5.9% of the extent to which sperm from high sperm motility roosters did.

TABLE A.4. Summary of split-plot ANOVA following repeatedmeasurements of sperm motility from roosters categorized *a priori* by average or high sperm motility[1]

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-value
Category	1	813,875	813,874	70.54****
Male within category	33	1,465,375	44,405	3.85****
Time	2	12,033	6,016	0.52
Category by time	2	28,010	4,005	1.21

[1]Each of 35 New Hampshire roosters was ejaculated on an every-other-day basis. Roosters had been characterized beforehand as having average (n=18) or high sperm motility (n=17). Three consecutive measurements of sperm motility were made per rooster.

****$P \leq 0.0001$

A.5 DISCUSSION

The objective analysis of poultry sperm motility has been hampered by a number of factors. These include the very nature of poultry semen, lack of proximity between semen donors and analytical equipment, and the cost of computer-assisted analysis. As inferred from a recent review of sperm interaction with the oviduct (Bakst *et al.*, 1994), the motility of sperm populations within the oviduct, the vagina in particular, should be viewed in terms of millions of sperm dispersed over a ciliated epithelial surface in oviducal fluid at body temperature. Based upon alternative techniques reviewed for this report, the spectrophotometric method originally adapted by Wall and Boone (1973) for poultry sperm has been used most frequently. In this assay, sperm motility is determined in a volume of synthetic diluent containing 5 to 20 million sperm per mL. Furthermore, in 50% of the reports cited, sperm motility was measured at ≤ 30 C. Meaningful information may be derived from the measurement of sperm motility under non-physiological conditions. However, it is known with certainty that the motility of chicken sperm under physiological conditions depends upon an interaction between temperature and extracellular Ca^{+2} (Ashizawa and Wishart, 1987; Wishart and Ashizawa, 1987; Thomson and Wishart, 1988; Ashizawa *et al.*, 1989a; Ashizawa and Sano, 1990; Thomson and Wishart, 1991; Ashizawa *et al.*, 1992; Ashizawa and Wishart, 1992; Ashizawa *et al.*, 1994). Consequently, routine estimation of sperm motility under conditions that approximated body temperature and extracellular fluid electrolyte composition seemed warranted.

The spectrophotometric assay cited most frequently (Wishart and Ross, 1985) can be performed at 40 C using a range of Ca^{+2} concentrations that stimulate sperm motility at body temperature (Wishart and Ashizawa, 1987). This assay utilizes three basic principles. First, absorbance is proportional to the concentration of sperm within a sperm suspension. Second, if a suspension of *motile* sperm is passed through a flow cell and the flow is stopped abruptly, then absorbance decays exponentially as a function of time (see Wishart and Ross, 1985 for further details). Third, the extent to which absorbance decreases is proportional to the percentage of motile sperm in the suspension. Therefore, change in absorbance is the key variable to be estimated. This value can be expressed as an index (Wall and Boone, 1973; Atherton *et al.*, 1980) or a parametric estimate (Wishart and Ross, 1985; Froman and Thursam, 1994).

In a prior study (McLean and Froman, 1995), differential sperm penetration of an Accudenz® solution was used to distinguish roosters with heritable subfertility from fertile males. This method was developed after the method of Wishart and Ross (1985) failed to provide consistent results. Accudenz® is a non-ionic, biologically inert cell separation medium, and the absorbance of Accudenz® at 550 nm increases when motile sperm enter the medium from an overlaid sperm suspension. Unlike previously described spectrophotometric methods for measuring the motility of poultry sperm, the technique did not require a flow cell. We hypothesized that sperm penetration of Accudenz® could be used to detect differences in sperm motility among normal, fertile males.

Our first objective was to determine the intra-assay coefficient of variation. This was determined by overlaying a suspension of rooster sperm upon 6% (wt/vol) Accudenz® in a disposable cuvet, incubating the cuvet at 41 C for a pre-determined interval, and then measuring the absorbance of the Accudenz® layer. This method yielded an intra-assay coefficient of 2.6% (n=3). In a subsequent experiment, the coefficient of variation was based upon sperm recovered *from* the Accudenz® layer. In this case, the coefficient of variation was 6.2% (n=3). We attributed the greater variability to experimental error associated with sperm recovery prior to determination of sperm concentration. In either case, sperm penetration of Accudenz® was found to be a repeatable phenomenon.

Our next objective was to test for differences in sperm motility among males. This was accomplished with two different flocks of New Hampshire roosters. As evidenced by ANOVA (Tables A.1 and A.4) and ranked motility scores, appreciable differences in sperm motility were observed among males. These experiments were performed with a portable photometer rather than a spectrophotometer. Therefore, the photometer afforded an assessment of sperm motility immediately after ejaculation. Patterns of sperm penetration were comparable between instruments (Figures A.1 and A.2). We concluded that differences among males were not attributable to differential *loss* of motility due to inconsistent conditions prior to measurement.

Another objective was to determine whether a cause and effect relationship could be demonstrated between *in vitro* sperm motility and fertility. In the first fertility trial, males characterized by maximal, average, or minimal sperm motility were selected as semen donors (n=3 males per category). As shown in Table A.3, no difference was observed

among groups of hens. In view of the work of Wishart and Palmer (1986), who reported a correlation coefficient of .82 between fertility and the sperm motility of individual males, we attributed the trial's outcome to inadequate sample size. In the first trial, fertility for the maximal and minimal sperm motility groups differed by only 5 percentage points (Table A.3). This difference was viewed as an estimate of the true difference between these two categories of males. We determined that a minimum of 2100 eggs would be needed per group of hens to detect this difference with a 90% certainty at a significance level of α=0.05 (Sokal and Rohlf, 1969b).

In contrast to the first fertility trial, a difference in fertility was detected between maximal and minimal sperm motility males in the second trial (Table A.4). When these data sets were analyzed graphically (Figure A.3), the initial level of fertility, as estimated by the parameter γ , differed by 6 percentage units, and this disparity increased over the course of a week. In fact, fertility during this interval was 84% (n=842 eggs) and 96% (n=868 eggs) for males categorized by minimal and maximal sperm motility, respectively. Thus, while insemination doses were equivalent, the effective insemination doses were not. It is noteworthy that the designations *minimal* and *maximal* must be viewed in context because they only refer to the ranked motility scores of the 36 roosters used in the initial test of variability among males. Thus, the roosters categorized by minimal and maximal sperm motility in the second fertility trial may have been comparable to the roosters used to determine inter-assay CVs, which were categorized by average and high sperm motility, respectively. Based upon spectrophotometric analysis, the absorbance value associated with the "maximal" roosters was 84% of the mean observed for the high sperm motility

roosters. Sperm from "minimal" roosters penetrated Accudenz® to only 51% of the extent to which sperm from "maximal" roosters did. In contrast, sperm from average roosters penetrated Accudenz® to only 55 ± 5.9% of the extent to which sperm from high sperm motility roosters did. Therefore, it is likely that the difference in fertility shown in Table A.3 reflects a comparison of average versus above average sperm motility rather than genuine extremes as implied by the terms *minimal* and *maximal.*

In summary, our overall experimental goal was to determine whether we could develop an objective sperm motility assay that would: (1) approximate physiological conditions, (2) require simple, portable equipment, (3) be applicable to individual males, and (4) yield repeatable, biologically significant results. Each of these stipulations were met. Furthermore, we found that the assay, with minor modifications, could be applied to turkey sperm (Figure A.5). Even so, measurement of poultry sperm motility by sperm penetration of Accudenz® does not afford information about individual sperm. Indeed, it is an assessment of the *mobility* of a sperm population. Nonetheless, it is sperm mobility rather than sperm motility per se that enables sperm sequestration within the hen's sperm storage tubules. In conclusion, our experiments, in conjunction with the single step technique by which sperm can be washed by centrifugation through Accudenz® (Froman and Thursam, 1994), have demonstrated the potential for: (1) studying the effects of chemically defined environments on sperm motility, (2) studying attributes of highly motile or largely immotile subpopulations of sperm, (3) selecting semen donors based upon sperm motility, and (4) the establishment of simple, objective criteria for assessing the quality of semen sold as a commodity.

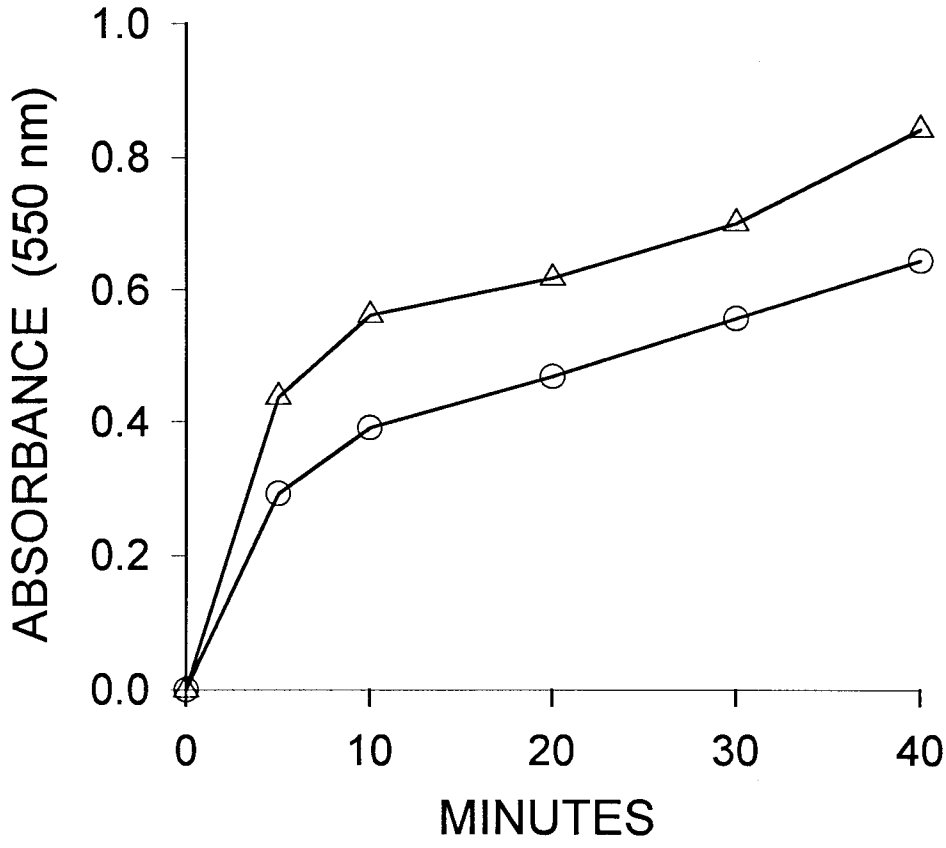

FIGURE A.5. Absorbance of 4% (wt/vol) Accudenz® after overlay with pooled turkey semen (n=10 Beltsville Medium White toms) diluted with 3 mM caffeine in TES-buffered isotonic saline, pH 7.4, containing glucose and Ca^{+2} (△) or the buffer alone (○). In each case, semen was diluted to 1 x 10^9 sperm per mL. Three 150-μL volumes of each sperm suspension were overlaid upon three 1.5-mL volumes of Accudenz®. In each case, the Accudenz® solution had been pre-warmed to 41 C within a polystyrene cuvet. Overlays were staggered by several minutes. After an initial reading was made, each cuvet was incubated in a 41 C water bath. Thereafter, each cuvet was removed from the water bath and absorbance at 550 nm measured after 5, 10, 20, 30, and 40 min of incubation. With or without a motility agonist, turkey sperm penetrated the Accudenz® layer much more slowly than did rooster sperm as evidenced by the magnitude and dispersion of absorbance values over time relative to the number of overlaid sperm. Each symbol represents a mean (n=3). Error bars denote standard deviations. The motility of turkey sperm *in vitro* was enhanced by caffeine.

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APPENDIX B

INCREASED FECUNDITY RESULTING FROM SEMEN DONOR SELECTION BASED UPON *IN VITRO* SPERM MOTILITY

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[1]Oregon State University Technical Paper Number 11,001.

B.1 ABSTRACT

Semen donors were selected from a population of 100 roosters based upon the extent to which sperm penetrated 6% (wt/vol) Accudenz® from an overlay of extended semen. Semen donors categorized by average or high sperm motility (n=5 per phenotype) were ejaculated weekly, their ejaculates pooled by phenotype, and pooled semen extended. A subsample of each sperm suspension was overlaid on 6% (wt/vol) Accudenz® in a cuvette, the cuvette was placed in a 41 C water bath, and the absorbance of the Accudenz® layer was measured after a 5-min incubation. The remainder of the sperm suspension was inseminated (n=55 hens per phenotype). Each hen was inseminated weekly with 50 x 10^6 sperm for 14 wk. The hatchability of eggs laid by hens inseminated with sperm from the high motility phenotype was 10% greater ($P \leq 0.001$) than that of hens inseminated with sperm from the average phenotype. The difference in fecundity was explicable in terms of fertility ($P \leq 0.001$). A replicate experiment tested the effect of sperm motility as well as insemination dose on fertility. Roosters were treated as above, and hens (n=41 to 45 per phenotype) were inseminated weekly with 25, 50, or 100 x 10^6 sperm per hen for 3 wk. Two-way ANOVA detected a sperm motility effect ($P \leq 0.0001$) but did not detect a dose effect ($P \geq 0.05$) or a motility by dose interaction ($P \geq 0.05$). *A posteriori* comparison among means revealed that the maximal fertility obtained with sperm from average roosters was 9% less ($P \leq 0.05$) than that obtained with only 25% as many sperm from the high motility

phenotype. These experiments demonstrated that the fecundity of artificially inseminated hens can be increased when sperm penetration of Accudenz® is used as a selection criterion for semen donors.

B.2 INTRODUCTION

Sperm cells, in essence, are self-propelled DNA-delivery vehicles. Motility is critical for sperm ascension through the hen's vagina to the region where the oviduct's sperm storage tubules are located (Bakst *et al.*, 1994). Sperm sequestration within these tubules affords a reservoir of viable sperm that, upon their release, may passively ascend the oviduct to the site of fertilization over the course of days. Consequently, the extent to which sperm are motile within the hen's vagina appears to be a primary determinant of fecundity. Nonetheless, when compared to other variables that affect poultry reproduction, sperm motility has not been studied extensively.

Froman and McLean (1996) demonstrated that sperm motility is a normally distributed variable when the trait is measured by sperm penetration of Accudenz®[3]. We hypothesized that this technique could be used to select semen donors. Therefore, the objective of the present research was to evaluate such potential.

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[3]Accurate Chemical and Scientific Corp., Westbury, NY 11590.

B.3 MATERIALS AND METHODS

B.3.1 Selection of Semen Donors

Semen donors (n=5 per phenotype) were selected from two groups of individually caged 30-wk-old New Hampshire roosters categorized as having average (n=18) or highly motile sperm (n=17) by repeated measure analysis (Froman and McLean, 1996). Selection criteria were as follows: consistent motility scores as determined by coefficient of variation and a mean motility score (n=3 observations per male) that fell within the ranges shown in Figure B.1. During the week in which roosters were selected, ejaculates were pooled by phenotype and differential sperm motility confirmed. Thereafter, the motility of sperm in pooled, extended semen was measured on a weekly basis prior to insemination.

B.3.2 Effect of Male Phenotype on Fecundity

Individually caged 30-wk-old New Hampshire hens (n=120) were assigned randomly to be inseminated with pooled, extended semen from either the average or high sperm motility phenotype. Hens were inseminated weekly for 14 consecutive weeks. On each occasion, ejaculates were pooled by phenotype in a graduated 15-ml glass centrifuge tube. Semen was transported to the laboratory at a temperature of 20 to 25 C. Upon arrival in the laboratory, sperm concentration was determined fluorometrically (Bilgili and Renden,1984), and neat semen was diluted to 0.5 x 10^9 sperm per mL with 111 mM NaCl buffered with 50 mM N-tris-[hydroxymethyl]methyl-2-amino-ethanesulfonic acid (TES),

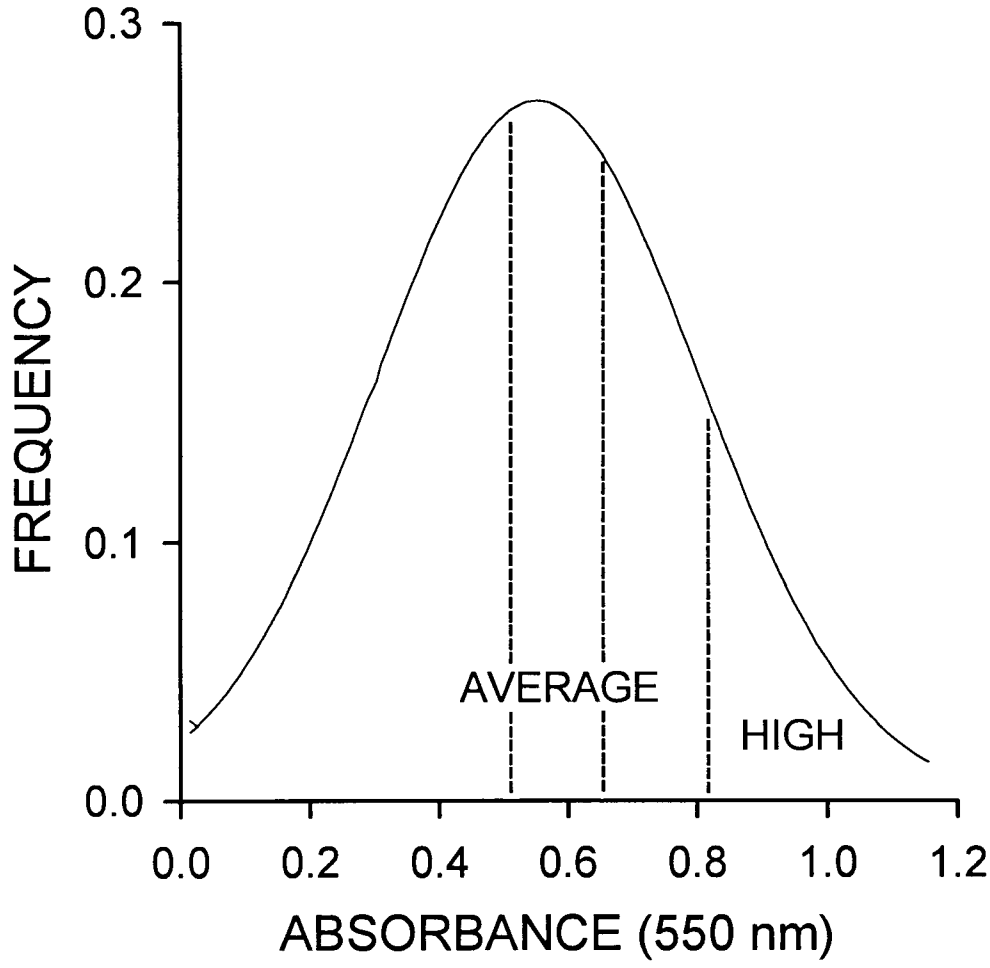

FIGURE B.1. Frequency distribution based on the analysis of sperm motility from 100 New Hampshire roosters. Sperm motility was based upon sperm penetration of 6% (wt/vol) Accudenz® at 41 C from an overlay of extended semen. Absorbance was proportional to the extent to which motile sperm entered the Accudenz® solution. Areas under the curve denoted by dashed lines represent subpopulations of roosters that were categorized as average (n = 18) or high (n = 17) sperm motility phenotypes. Adapted from Froman and McLean (1996). Reproduced with permission of the Poultry Science Association, Savoy, Illinois.

pH 7.4, containing 25 mM glucose and 4 mM $CaCl_2$ (Froman and McLean, 1996). The TES-buffered saline was at room temperature when it was mixed with semen.

Prior to semen collection, 1.5-mL volumes of 6% (wt/vol) Accudenz® were pipetted into each of 2 standard polystyrene cuvettes, each cuvette covered with a 1.5 cm^2 piece of Parafilm®[4], and each cuvette placed in a 41 C water bath. After semen was extended, sperm motility measurements were made in duplicate according to Froman and McLean (1996). Each cuvette was removed from the water bath and then tapped on the counter top to remove any adherent air bubbles that had formed on the interface between the Accudenz® solution and the inner wall of the cuvette during pre-incubation. Then, the cuvette was blanked at 550 nm. Thereafter, a 150-μL volume of sperm suspension was overlaid on the Accudenz® solution, and the cuvette was returned to the 41 C water bath. After a 5-min interval, the cuvette was transferred to the spectrophotometer and absorbance at 550 nm recorded after a 1-min interval.

Each sperm suspension was transported to a caged layer facility at 20 to 25 C in a graduated 15-mL glass centrifuge tube. On the average, approximately 30 min elapsed between the start of semen collection and the arrival of extended semen at the caged layer facility. Each hen within a treatment group was inseminated with 50 x 10^6 sperm in a volume of 100 μL. All hens within a treatment group were inseminated within 20 min, and the centrifuge tube containing the sperm suspension was hand-held during this interval. Following the insemination of hens in one treatment group, semen from the other phenotype was processed and corresponding hens inseminated as above.

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[4]VWR Scientific, Seattle, WA 98124

Eggs were collected daily, set on a weekly basis, and incubated for 22 d. Hatchability, hatch of fertilized eggs, and fertility were determined at the end of the experiment for each hen that remained in lay over the course of the 14-wk egg collection interval. Each proportion was transformed to a modified logit, weighting variables calculated, and each transformed data set analyzed with a log odds model according to Kirby and Froman (1991).

B.3.3 Effect of Male Phenotype and Insemination Dose on Fertility

Individually caged 47-wk-old Single Comb White Leghorn hens (n=260) were assigned randomly to be inseminated with 25, 50, or 100 x 10^6 sperm from either the average or high motility phenotype. Hens were inseminated weekly for 3 consecutive weeks. On each ocassion, semen was processed and sperm motility measured as above with the following exceptions. Once sperm concentration was determined, a 50-μL sample of neat semen was removed with an M-250 Microman positive displacement pipet[5]. This semen was extended to 0.5 x 10^9 sperm per mL in a borosilicate culture tube with TES-buffered saline containing 25 mM glucose and 4 mM $CaCl_2$. This sperm suspension was used to conduct the motility assay.

The remaining neat semen was extended to 1.0 x 10^9 sperm per mL in a graduated 15-mL glass centrifuge tube, and this sperm suspension was used for insemination. Each hen was inseminated with a volume of 25, 50, or 100 μL. Egg collection, incubation, and data analysis were performed as above with one exception: fertility was determined by examining the contents of eggs for embryonic development

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[5]Rainin Intrument Co., Inc., Woburn, MA 01888

after 4 days of incubation. Transformed data were analyzed with a two-way ANOVA. *A posteriori* comparisons among means were made with the Student-Newman-Keuls test (Sokal and Rohlf, 1969).

B.3.4 Analysis of Sperm Motility Data

Mean sperm motility was plotted as a function of time for each phenotype. Because plots appeared linear, the slope and Y-intercept were estimated by the method of least squares for each data set using: $Y_i = \alpha + \beta x_i + \varepsilon$, as the model statement and the General Linear Models procedure (SAS Institute, 1987). An extra sums of squares F test was made to test whether estimates of the Y-intercepts were equivalent.

B.4 RESULTS

B.4.1 Effect of Male Phenotype on Fecundity

As shown in Table B.1, the high sperm motility phenotype was more fecund ($P \leq 0.001$) than the average sperm motility phenotype. Hatchability was 10% greater over the course of a 14-wk egg collection interval. This effect was attributed to differential fertility ($P \leq 0.001$). As shown in Figure B.2, the phenotypic difference in sperm motility observed at the onset of the experiment persisted over the duration of the experiment.

B.4.2 Effect of Male Phenotype and Insemination Dose on Fertility

As shown in Table B.2, the phenotypic difference in fertility observed in the initial experiment was also observed ($P \leq 0.0001$) in a replicate experiment in which the effect of insemination dose was evaluated as well. However, neither an insemination dose effect nor an interaction between sperm motility and insemination dose was detected ($P \geq 0.05$). Based upon *a posteriori* comparisons means, the maximal insemination dose, i.e., 100×10^6 sperm per hen, increased fertility ($P \leq 0.05$) by 7% beyond that observed with the minimal dose of 25×10^6 sperm per hen in the case of the average sperm motility phenotype (Table B.3). Nonetheless, the fertility obtained with the maximal insemination dose from the average phenotype was 9% less than ($P \leq 0.05$) that obtained with the minimal insemination from the high sperm motility phenotype.

Table B.1. Differential hatchability and fertility achieved byselecting semen donors based upon *in vitro* sperm motility[1]

Semen Donor Phenotype	Hens[2] (n)	Eggs[3] (n)	Hatchability[4] (%)	Hatch of Fertilized Eggs[5] (%)	Fertility[6] (%)
Average Sperm Motility	55	3818	77 ± 1.9^{A}	90 ± 1.2	85 ± 1.6^{A}
High Sperm Motility	55	4122	87 ± 1.2^{B}	92 ± 1.0	95 ± 0.7^{B}

[A,B]Means within a column lacking a common superscript differ significantly ($P \leq 0.001$).

[1]Measured by sperm penetration of 6% (wt/vol) Accudenz® at 41 C from an overlay of extended semen.

[2]Each New Hampshire hen was inseminated weekly for 14 conse-cutive weeks with a dose of 50×10^6 sperm in a volume of 100 μL.

[3]Collected daily and set weekly.

[4, 5, 6]Each value is a mean ± S. E. M.

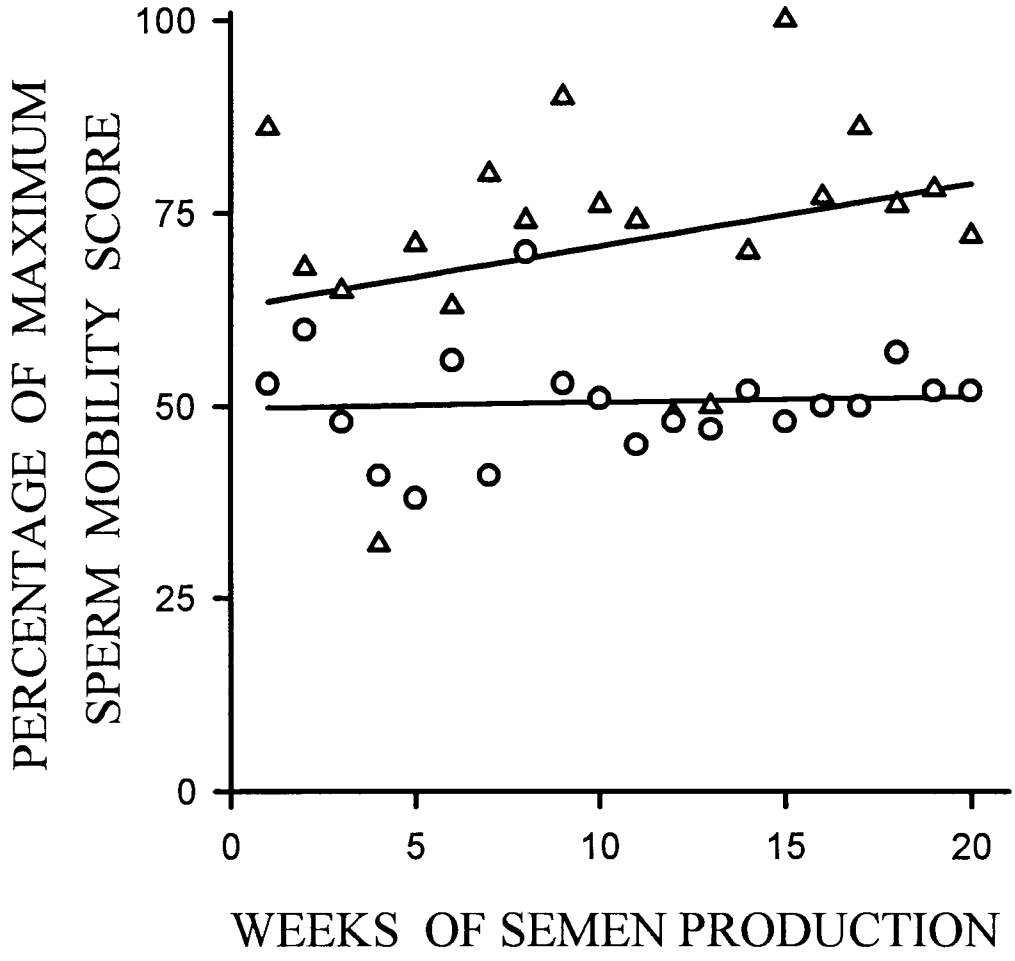

FIGURE B.2. Normalized data from average (o) and high sperm motility (△) phenotypes plotted as a function of time. Roosters (n=5 per phenotype) were ejaculated weekly, and ejaculates were pooled by phenotype. Sperm motility was measured by diluting neat semen with a buffer, overlaying the sperm suspension upon pre-warmed 6% (wt/vol) Accudenz® in a polystyrene cuvette, incubating the cuvette at 41 C for 5 min, and then measuring the absorbance of the Accudenz® at 550 nm. Each data point denotes a mean of duplicate samples normalized against the maximal absorbance value observed over the course of the evaluation period (1.273). Solid lines represent the functions:

$$y(x) = 63 + 0.7925(x)$$

and

$$y(x) = 50 + 0.0737(x).$$

Neither slope differed significantly from zero ($P \geq 0.05$).

TABLE B.2. Summary of two-way ANOVA testing the effects of sperm motility and insemination dose on fertility[1]

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-value
Sperm Motility	1	66.256	66.256	114.42****
Insemination Dose	2	3.102	1.551	2.68
Motility by Dose	2	0.462	0.231	0.40

[1]Each of 260 Single Comb White Leghorn hens was inseminated with 25, 50, or 100 x 10^6 sperm from one of two sperm suspensions on a weekly basis for 3 consecutive weeks. Sperm suspensions were prepared by extending pooled semen from roosters categorized as having average or highly motile sperm (n=5 roosters per phenotype).

****$P \leq 0.0001$.

TABLE B.3. Comparative fertility of rooster phenotypes according to insemination dose

Semen Donor Phenotype[1]	Insemination Dose (x 10^{-6})	Hens[2] (n)	Eggs[3] (n)	Fertility[4] (%)
Average Sperm Motility	25	42	827	79 ± 1.9^{c}
	50	41	811	81 ± 2.2^{c}
	100	42	828	86 ± 1.8^{b}
High Sperm Motility	25	45	898	95 ± 0.9^{a}
	50	45	887	95 ± 0.8^{a}
	100	45	902	96 ± 0.8^{a}

[a,b,c]Means within a column lacking a common superscript differ significantly ($P \leq 0.05$) based upon an *a posteriori* comparison among means.

[1]Based upon sperm penetration of 6% (wt/vol) Accudenz® at 41 C from an overlay of extended semen.

[2]Each Single Comb White Leghorn hen was inseminated weekly for 3 consecutive weeks with 25, 50, or 100 μL of a sperm suspension con-taining of 1×10^{9} sperm per mL.

[3]Collected daily and set weekly over a 3-wk interval.

[4]Each value is a mean ± S. E. M.

B.4.3 Analysis of Sperm Motility Data

Average and high sperm motility phenotypes were characterized by correlation coefficients of 0.9601 and 0.9816, respectively. Thus, each plot approximated a linear relationship. Data points and predicted lines are shown in Figure B.2. Although neither slope was different from zero ($P \geq 0.05$), Y-intercepts differed from one another ($P \leq 0.001$). Therefore, each phenotypic distinction was independent of time over the course of the entire experimental interval.

B.5 DISCUSSION

Previous experimentation (McLean and Froman, 1996; Froman and McLean, 1996) established the validity and suitability of measuring sperm motility based upon *in vitro* sperm penetration of Accudenz®. In brief, the assay was repeatable, was performed at physiological temperature, and was applicable to individual males. In the latter regard, the variable of sperm motility was distributed normally. Furthermore, when a repeated measure analysis was performed with males initially categorized with average or high sperm motility by a single evaluation per male, the categories were indeed distinct. These two phenotypes are shown in Figure B.1 relative to the frequency distribution. We hypothesized that fecundity could be increased by inseminating hens with pooled semen obtained from roosters charac-terized by high sperm motility.

Therefore, in the present work, representative males were selected from the average and high sperm motility phenotypes identified by Froman and McLean (1996). After confirming differential sperm motility when ejaculates were pooled by phenotype in preliminary work, a long-term fertility trial was initiated in which the primary end-point was hatchability. As shown in Table B.1, a difference in hatchability was observed between phenotypes, and this effect was attributable to fertility. The effect of sperm motility on fertility was evaluated in a replicate experiment in which the effect of insemination dose was evaluated as well. Our data (Tables B.2 and B.3) corroborate those of Allen and Champion (1955), Cooper and Rowell (1958), Kamar (1960), McDaniel and Craig (1962), and Wishart and Palmer (1986) in that each of these data sets illustrate the principle that fecundity is more dependent upon sperm *quality* than sperm *quantity*. With this in mind, it is noteworthy that phenotypic distinctions were independent of time (Figure B.2).

In view of the sperm cell's function, the relationship between sperm motility and fertility is self-evident. Thus, reports of a correlation coefficient ≥ 0.70 between sperm motility and fertility are not surprising (Allen and Champion, 1955; Cooper and Rowell, 1958; Kamar, 1960; McDaniel and Craig, 1962; Wishart and Palmer, 1986). Likewise, sperm motility may account for the results of two distinct types of experiments. First, many competitive fertilization experiments, in particular those using sperm from roosters homozygous for the rose comb allele (Allen and Champion, 1955; Etches *et al.*, 1974), may be explained in terms of sperm motility. For example, sperm from subfertile *R/R* males have been characterized by poor sperm motility and a limited ability to enter

sperm storage tubules following intravaginal insemination (McLean and Froman, 1996). Second, testicular sperm are essentially immotile at body temperature (Ashizawa and Sano, 1990). And while sperm need not be motile to ascend the oviduct above the vaginal sphincter, they must be motile to ascend the hen's vagina and reach the sperm storage tubules (Bakst *et al.*, 1994). Consequently, insemination of testicular sperm above the vaginal sphincter yields fertilized eggs whereas intravaginal insemination does not (Howarth, 1983; Kirby *et al.*, 1990). In summary, sperm motility within the hen's vagina appears to be a primary determinant of fecundity.

And yet, even though sperm motility is essential to the production of poultry, the molecular mechanisms that impart motility are only beginning to be understood (Wishart and Ashizawa, 1987; Ashizawa and Sano, 1990; Ashizawa *et al.*, 1992; Ashizawa *et al.*, 1994; Ashizawa *et al.*, 1995). Furthermore, it is not known what accounts for the variable size of the highly motile subpopulations of sperm observed in ejaculates of different males (Figures B.1 and B.2). The sperm penetration assay actually measures the size of motile subpopulations of sperm; for the overlay contains a fixed number of sperm, the incubation time is a constant, and the absorbance of the Accudenz® layer is directly proportional to the number of sperm that enter from the overlay. Even so, it is unknown whether such differences are attributable to spermatogenesis, extra-gonadal sperm maturation, or an interaction between inherent and acquired cellular properties. In any case, variability among males is under-utilized in reproductive management (see Bakst and Wishart, 1995).

Amann and Hammerstedt (1993) categorized *in vitro* methods of semen evaluation by the following goals. First, when evaluating males whose reproductive potential is unknown and by using only a few semen samples per male, identify those *males* that would be subfertile if they were used as semen donors. Second, when evaluating any given male whose reproductive potential has been established, identify those *semen samples* characterized by superior fertilizing potential. Third, when evaluating males whose reproductive potential is unknown and by using only a few semen samples per male, identify those *males that have the potential to be fecund.* These three goals were deemed to be already possible with existing methods, within reach due to the advance of technology, and highly unlikely, respectively. However, the present research has demonstrated that the third goal may be possible with roosters when sperm motility, as measured by sperm penetration of 6% (wt/vol) Accudenz®, is the selection criterion.

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