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Turkey Sperm Mobility Influences Paternity in the Context of Competitive Fertilization[1]

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ABSTRACT

We have devised a novel means of investigating competitive fertilization in turkeys, using microsatellite genotyping to identify male parentage. Our results demonstrate that sperm mobility is a mechanism responsible in part for paternity efficiency in turkeys. Sperm mobility is composed of several parameters in which sperm motility is a component. Differences between ejaculates in the number of sperm penetrating into a dense, insert, nontoxic solution were measured and used to classify males into high, average, or low sperm mobility phenotypes. Microsatellite genotyping was used to determine parentage of poults after equal numbers of sperm from 10 males (either high or average phenotype, n = 5, mixed with low phenotype, n = 5) were inseminated simultaneously. In a separate study, the numbers of sperm hydrolyzing the perivitelline layer of eggs were compared between hens inseminated with sperm from high-, average-, or low-phenotype males. Overall, heterospermic inseminations resulted in consistently fewer offspring produced by low-mobility phenotype males. This correlated with physiological data in which semen from the low-mobility males had reduced numbers of sperm at the fertilization site as determined by sperm hole counts in the perivitelline layer of eggs. This is the first illustration of a measurable sperm trait predictive of paternity success in a competitive fertilization trial in turkeys, a species that is predominately reproduced by artificial insemination of multiple-sire pools.

INTRODUCTION

Sperm competition is an integral, yet virtually unstudied, physiological event of commercial turkey production. In the United States, commercial production of turkeys relies exclusively on artificial insemination. For a majority of commercial farms, males are managed as flocks, and semen from 10 or more males is pooled for the insemination of multiple hens. Pooling semen is convenient, and it is generally assumed that sperm from each male are equally fecund. While it is known that sperm competition exists, the probability of one male's sperm fertilizing an egg relative to the sperm of another male is ignored. Yet, in a preliminary study, DNA fingerprint analysis of turkey offspring produced from heterospermic inseminations revealed that reproductive fitness was highly skewed among individual males [1]. In one trial, a single male produced 37 of 70 offspring when semen from 10 males was pooled. In an attempt to identify factors that influence the observed reproductive disparity between males, individual ejaculates were evaluated for semen volume, sperm concentration, sperm viability, membrane integrity, and subjective sperm motility. No relationship between these semen parameters and paternity was apparent [1].

Since many semen quality tests for poultry are not predictive of fertility, investigators have begun to focus on identifying physiological sperm characteristics that influence fertility and sperm storage in the female [2–5]. One such assay, the ''sperm mobility test,'' measures the ability of sperm to swim into a dense, inert, nontoxic diluent (Accudenz). Unlike traditional motility assessments in which the percentage of moving sperm is subjectively estimated, this assay is performed at a hen's body temperature (41°C) and objectively measures the proportion of sperm with a powerful and relatively linear forward motion (mobility). Male-to-male variation in sperm mobility phenotype was repeatedly shown to be a normally distributed trait in roosters and toms [3, 6–8]. Sperm mobility is a quantitative trait and is one determinant of fecundity [7]. When males were selected out of a flock on the basis of high and low sperm mobility, and their semen was pooled by sperm mobility phenotype, fertility was higher or lower as predicted [5]. When sperm mobility was measured in roosters over a period of 20 wk [3] and in toms for 22 wk [8], it remained consistent within mobility classification. This is an important finding, since sire selection should take place early in production, and the benefit of improving semen quality could be maintained over the entire breeding period.

Our hypothesis is that in competitive fertilization, sperm mobility influences sperm sequestration and storage in the hen and subsequent fertility. Since sperm mobility differences are apparent between individual males and are predictive of sperm function in the hen, the semen assessment test was used to classify males into sperm mobility phenotypes. To test our hypothesis, parentage efficiency was determined in offspring produced from heterospermic insemination treatments. Previous evaluations using heterospermic inseminations relied on phenotypic differences between sires to identify offspring (see review [9]). In order to maintain relevance to the turkey industry, we studied a commercial turkey line and determined parentage by microsatellite genotyping. Treatments were designed not only to directly compare sperm competition between phenotypic classes of males but also to investigate competition in the production setting, in which complex heterospermic inseminations (mixing of sperm from more than two males) are an integral part of commercial turkey production. In addition, we evaluated the number of sperm holes in the perivitelline layer of eggs from hens inseminated with sperm from low- and high-mobility phenotype

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TABLE 1. Microsatellite markers used for parentage determination.

Locus	Primer sequences (Forward: top; reverse: bottom)	GenBank access. no.[a]	T[b] (°C)	Alleles detected	Allele size range[c]	Expected heterozygosity[d]	Average exclusion probability[d,e]
TUM20	TCAGTCCTGGCAGTTTAC GTTCCTGTGTAGGACAAT	U79310	58	5	94–154	0.610	0.327
RHT0003	CCGCAGGTTGCTAGAAGC GAAGACATGCCTGGAGAAGC	NA	48	3	199–221	0.542	0.239
RHT0010	TTAACCTATCAGGTCGTTGCG CAGTGCACATGCAGGCAG	NA	48	4	195–223	0.461	0.233
RHT0011	GTGTTTCCTCCTAAGCCATACG GTTCATCTATCTGCTCGCTGC	NA	55	4	148–194	0.593	0.350
RHT0016	ATCACATTCTCTCAAGGCCCC ACTGCTGCAAAGCCTCATATTG	NA	50	11	96–152	0.836	0.668
BTM1	AGGGATGCGTCTCACTGC GCTGCCAGATAAAGTCGAGC	AF021811	58	7	123–145	0.708	0.502
BTM2	CTGTGTTTCCAACTTCTTTGTAGG AGTGTGGCAGTCTGAATGAGG	L47980	58	4	189–197	0.441	0.233
BTM7	TCCGTTCCTTTTAGGTTGTTG AAGATTAGTGCATTCAGTTTCAGC	AF019701	58	5	142–162	0.494	0.276
BTM8	GGTATGATTTCAGTGATGGAGTG TTTCCTGTTTCTCAATTTCCC	AF019705	58	5	202–214	0.707	0.438
Mean totals for 9 loci						0.599	0.987

[a] NA, not applicable.
[b] Annealing temperature used for PCR amplification.
[c] Denoted in base pairs as determined on 5% denaturing polyacrylamide gel from M13 (New England Biolabs, Beverly, MA) sequence size standard.
[d] Data calculated for parents in the study.
[e] Identity of one parent known.

males as an indirect measure of sperm storage in the hen and as a direct measure of sperm numbers at the site of fertilization.

MATERIALS AND METHODS

Animals and Semen Collection

Large White Breeder males and hen poults were purchased from a primary breeder (British United Turkeys of America, Lewisburg, WV) and maintained under standard management conditions during brooding and growing periods. At 28 wk of age, males were photostimulated by increasing light exposure from 12L:12D to 14L:10D to stimulate semen production. Semen was collected using the abdominal massage method [10], beginning at 30 wk of age.

Selection of Semen Donors

Donor selection was initiated at 30 wk and completed by 33 wk of age. Semen donors were selected out of a flock of approximately 100 males in each trial. Males included in the selection process were those evaluated three separate times by the sperm mobility test (n = 79 males). Males were excluded if ejaculates were yellow or contained urates or blood, as recommended by standard guidelines for turkey semen handling [11]. Sperm mobility was measured using the sperm mobility test developed by Froman and McLean [6] and modified for the turkey [12]. Semen from each male was diluted to 1×10^9 sperm/ml with motility buffer, and a 300-μl volume of diluted semen was layered upon 3 ml 2% (w:v) Accudenz (Accurate Chemical and Scientific Corporation, Westbury, NY) solution prewarmed to 41°C in a disposable cuvette. The cuvette was incubated for 5 min in a 41°C water bath, and the percentage transmission was measured 1 min after the cuvette was loaded into a densimeter (Animal Reproduction Systems, Chino, CA). Results were expressed as sperm mobility index (100 − % transmission). Replicate scores were averaged, and males were ranked from highest to lowest mobility by sperm mobility index averages. The sperm mobility test is based on relative rank within a flock and the consistency of sperm mobility index scores after three evaluations. From these rankings, males were designated as high, average, or low mobility (n = 5 males/designation). High- and low-phenotype males were at least one standard deviation away from the mean mobility of the flock.

Parentage Analysis

Methods used to obtain microsatellite markers from turkey genomic libraries are described elsewhere (TUM [13] and RHT series [14, 15]; Table 1). Primer pairs for the BTM series of markers were designed from unique turkey microsatellite sequences in GenBank [16] that contained (GT) > 11. Polymerase chain reaction conditions, allele sizes, heterozygosity, and exclusionary power for each marker were determined with parental DNA (Table 1). Genomic DNA was extracted from whole blood of potential sires, inseminated hens, and offspring, using a modified phenol lysis method [17, 18]. Genomic DNA (50 ng) from each animal was aliquoted into 96-well v-bottom microtiter plates (USA Scientific, Ocala, FL). Microsatellite loci were amplified according to a slightly modified protocol developed for bovine microsatellites [19]. Thermocycling was performed on an MJ Research PTC 200 Thermocycler (MJ Research, Watertown, MA), so cycle step times were reduced from 1 min to 15 sec. Radiolabeled products were analyzed on the Storm 850 PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Parental and progeny genotypes were independently scored for each marker genotype, and paternal determination was performed with CERVUS 1.0 (http://helios.bto.ed.ac.uk/evolgen [20]). For inseminations of pooled ejaculates from 10 toms, a range of 4–9 marker genotypes was generated for each offspring to reach a 99% confidence level for sire candidate identity.

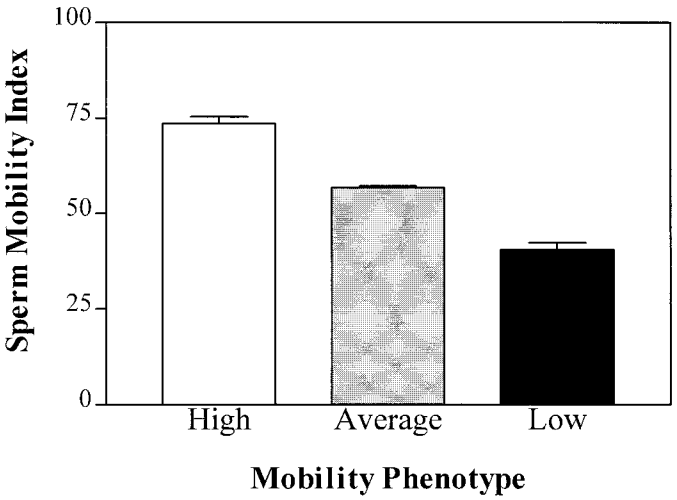

FIG. 1. Classification of high-, average-, and low-mobility phenotype males used in the competitive fertilization trials. Males were selected out of 79 individuals and ranked in triplicate for sperm mobility.

Competitive Fertilization Trials between Two Males with High or Low Sperm Mobility Phenotypes

Semen was collected from high- and low-mobility males, and sperm concentration was determined and adjusted to 5 $\times$ 10^9 sperm/ml in Beltsville Poultry Semen Extender (BPSE; Continental Plastics Corp., Delavan, WI) for each ejaculate. Ejaculates from males were paired (n = 6 pairs) according to sperm mobility phenotype (high or low) and marker-genotyped, such that male parentage could be determined with a single marker genotype from each offspring. Equal volumes (25 μl) of semen from each male were placed in an artificial insemination straw and used to inseminate hens. As a control, ejaculates from high-mobility males were paired and hens were inseminated as described above. Turkey hens were photostimulated at 28 wk of age by increasing the duration of light exposure daily from 6L:18D to 14L:10D to stimulate ovarian development and egg production. Hens (n = 5 per male-male pair) were inseminated at 42 wk of age. Eggs were collected for the first week after insemination and incubated, and poult blood was collected from the hatchlings.

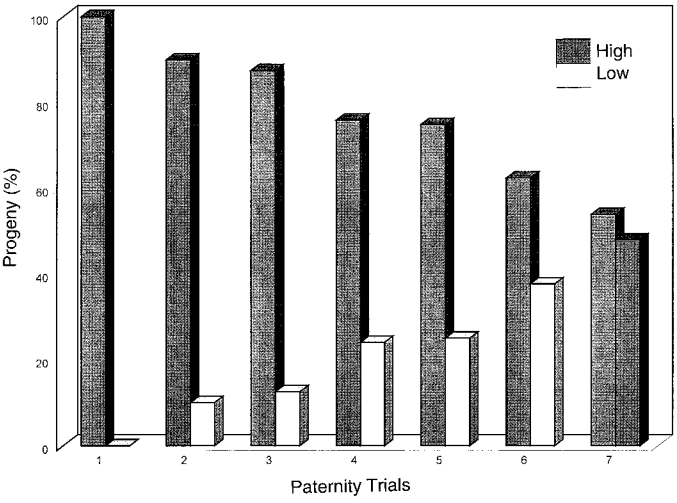

FIG. 2. Results of paired competitive fertilization trials between high- and low-mobility phenotype males. Trials 1–7 represent a total of 5, 41, 16, 51, 16, 32, and 13 poults, respectively. Semen from two high-mobility phenotype males were mixed in trial 7.

Competitive Fertilization Trials between Multiple Pooled Ejaculates from Potential Sires with High, Average, or Low Sperm Mobility Phenotypes

Treatments were 1) high+average mobility, 2) high+low mobility, and 3) average+low mobility phenotypes. Semen was collected from high-, average-, and low-mobility males, and sperm concentration was determined and adjusted to 5 $\times$ 10^9 sperm/ml in BPSE for each ejaculate. A total of 400 μl of diluted semen from each ejaculate was pooled and distributed into a 10-ml Erlenmeyer flask depending on the treatment (total of 10 ejaculates/treatment, 5 from each mobility phenotype) and mixed at 150 rpm for 15 min at room temperature before insemination. Turkey hens were photostimulated as described above. Hens (n = 12/treatment) were inseminated on 14, 17, and 21 days after initiation of photostimulation with 150 $\times$ 10^6 sperm. Eggs were collected for the first week after insemination and incubated, and blood was collected from offspring. Blood samples were also collected from potential candidate sires and inseminated hens and frozen until DNA extraction.

Determination of Sperm Holes in Freshly Laid Eggs after Insemination with Semen from Males with High, Average, or Low Sperm Mobility Phenotype

Semen was collected from males (n = 5 males/phenotype) and pooled by phenotype and diluted as described above. Hens were inseminated 14 and 20 days after the initiation of photostimulation with 50 μl each of pooled semen from either high-, average-, or low-mobility males (n = 8 hens/treatment). All eggs were collected for 1 wk beginning 3 days after the last insemination. The perivitelline layer over the vicinity of the germinal disc was removed and stained using a modified procedure [21] of Bramwell and coworkers [22]. Using a $\times$10 objective on a brightfield microscope, the germinal disc was centered in the field of view, and all holes in the field (circular unstained areas in the perivitelline layer) were counted.

Statistical Analysis

Differences in progeny production by sires were evaluated by chi-square analysis. ANOVA was performed on the sperm hole data with the MIXED procedure [23]. In order to satisfy the normality assumption, data were transformed [ln(y+1)] before ANOVA. Correlations among the residuals (repeated measures) was modeled by running various covariance structures and using Akaike's information criteria [24] to ascertain which of the covariance structures fit best.

RESULTS

Ranking of Males by Sperm Mobility Phenotype

Males in the flock evaluated in triplicate for sperm mobility index were ranked. Five males that consistently produced ejaculates with the highest sperm mobility index at least one standard deviation above the flock mean were classified as high-mobility phenotype males (Fig. 1). Five males consistently producing ejaculates with low mobility indexes at least one standard deviation below the flock mean were classified as low-mobility phenotype males (Fig.

TABLE 2. Number of offspring sired by males with high, average or low sperm mobility phenotype after heterospermic inseminations of pooled ejaculates.[a]

Sire phenotype	Insemination treatments[b]		
	high+average	high+low	average+low
High	34[c] (51%)	45[c] (67%)	—
Average	33[c] (49%)	—	50[c] (67%)
Low	—	22[d] (33%)	25[d] (33%)

[a] A total of 200 offspring met the 99% criterion for parentage determination; for 9 offspring, parentage probability was > 95%.
[b] Each insemination contained equal numbers of sperm from 10 toms (5 toms/phenotype).
[c,d] Means within columns with no common superscripts differ significantly ($p < 0.05$).

1). Males of average mobility phenotype were those that consistently ranked in the middle of the flock distribution for sperm mobility (Fig. 1).

Competitive Fertilization between Two Potential Sires with High or Low Mobility Phenotypes

Males identified as high-mobility phenotypes produced more offspring in competitive fertilization trials with low-phenotype males (Fig. 2, trials 1–6 with a range of 62.5–100% of the offspring). Competitive fertilization trials between males of the same phenotype classification resulted in an offspring ratio of 1:1 (Fig. 2, trial 7).

Competitive Fertilization between Multiple Pooled Ejaculates from Potential Sires with High or Low Mobility Phenotypes

Heterospermic inseminations of multiple ejaculates from high+average mobility males resulted in no difference between phenotypes in the proportion of offspring produced (Table 2). When the heterospermic inseminations were comprised of high+low or average+low mobility males, a majority of the offspring were sired by high or average sire candidates (Table 2). One low-mobility male produced the majority of offspring for this phenotype, accounting for 11 of 19 (58%) of the poults produced by the low-phenotype candidates in the high+low trial and 14 of 21 (67%) of the poults in the average+low trial resulting from the low-phenotype classification.

Determination of Sperm Holes in Freshly Laid Eggs after Insemination with Semen from High-, Average-, or Low-Mobility Phenotype Males

For the week following insemination, more sperm holes were observed in the perivitelline layer of eggs collected from hens inseminated with high- and average-mobility males than from those inseminated with semen from low-mobility males (Table 3). Compared to the low-mobility group, the mean numbers of sperm holes for the high- and average-mobility treatments at 2 and 3 days postinsemination were 7- and 5-fold, respectively (Table 3). Within the high- and average-mobility groups, the mean number of sperm holes declined from Days 2/3 to Days 8/9, which is consistent with previous reports [4].

DISCUSSION

Elegant studies on sperm competition in both avian and mammalian species have repeatedly demonstrated the paternity dominance of one sire over another [25–30]. This phenomenon is also true in turkeys, in which the majority of offspring are produced by a few males after insemination of pooled semen from seven to ten males [1]. Heterospermic insemination experiments in cattle have correlated semen quality with sire potential [29]. However, in turkeys, none of the many semen parameters investigated were predictive of paternity [1]. In heterospermic trials of roosters, Martin and coworkers [27] observed that the proportion of offspring sired was not influenced by the total number of sperm inseminated, season, breed of hen, or interval from insemination to fertilization. These investigators stated that the ratio of offspring produced was dependent on the ratio of competing sperm. However, identifying which sperm are competitive is difficult in any species [31].

In turkeys, it is important to understand what factors influence paternity and to eliminate those males that contribute little or nothing to the gene pool. Here we demonstrate that sperm mobility is a trait that differs between individual males and influences paternity. Sperm mobility is a critical component of successful fertilization. Sperm must be able to traverse the vagina to enter the sperm storage tubules (SST) in the hen and ultimately reach the site of fertilization [32]. Possibly mimicking the physiological conditions of the hen's reproductive tract, the sperm mobility test identifies differences in populations of sperm from individual males. Highly mobile populations of sperm are more likely to reach and occupy storage sites when ejaculates are pooled with semen with lower mobility. The mechanics of poultry sperm motility has been studied extensively using demembranated sperm [33–36]. However, the use of a functional assay, the sperm mobility test, may improve our understanding of what makes certain sperm more competitive in vivo than others. Repeatedly, sperm from those males with low mobility phenotype were compromised in their ability to reach the site of fertilization (as determined by fewer sperm hydrolysing the perivitelline layer) and produce offspring. When sire candidates were preselected with the sperm mobility test, high- and average-mobility males produced, as predicted, the majority of the offspring compared with low-mobility males.

Interestingly, within each phenotype, one or two males produced more offspring than the other candidate sires. For the high and average phenotype toms, mobility may account for this reproductive fitness; however, other fac-

TABLE 3. Sperm holes observed in eggs from hens inseminated with sperm from high-, average-, and low-mobility phenotype males.

Phenotype	Days after artificial insemination			
	Days 2, 3	Days 4, 5	Days 6, 7	Days 8, 9
High	102.9 ± 28.2[a]	47.0 ± 13.7[a]	33.5 ± 8.5[a]	29.8 ± 12.0[a,b]
Average	75.7 ± 21.2[a]	45.5 ± 10.3[a]	58.6 ± 17.3[a]	35.2 ± 13.5[a]
Low	15.5 ± 6.0[b]	8.7 ± 2.4[b]	15.3 ± 4.0[b]	12.7 ± 3.3[a,b]

[a,b] Means within columns with no common superscripts differ significantly ($p < 0.05$); statistical differences based on log transformation of data.

tors may also contribute to fertilization success. For example, in our multiple-insemination trials, one tom identified as a low-mobility candidate produced 50% and 56% of the low-mobility offspring in the high+low and average+low trials, respectively. Although this male's sperm mobility was reduced compared to that of a majority of the flock, sperm traits such as improved sperm storage or survival in the reproductive tract or enhanced sperm-egg binding may have improved his reproductive fitness. Differences in sperm binding in an in vitro assay correlates with fertility in chickens and turkeys [2]. Determining the importance of these factors in competitive fertilization trials may identify other predictable attributes of fertilization success.

Critical to fecundity in turkeys is sperm sequestration within the oviductal SST. These specialized structures are located at the juncture of the vagina and uterus. The number of holes hydrolysed through the perivitelline layer of the egg is informative because it is highly correlated with the number of sperm stored in the SST, allowing an indirect estimate of SST capacity without killing the hen [37]. Even without the competition of sperm from high-mobility males, sperm from the low-mobility males seem to be compromised in their ability to reach the SST and subsequently the site of fertilization. Since sperm storage in the hen insures prolonged intervals of fertile eggs, reduced numbers of sperm in the SST is most likely responsible for lower fertility, an observation made on long-term fertility trials with low-mobility phenotype sires [12].

Because of their reduced potential fertility, males with low sperm mobility could be eliminated to improve the reproductive fitness of a flock. Although fertilization is a complex process, sperm mobility is a measurable trait that predicts paternity after competitive fertilization and could be used to increase reproductive efficiency in commercial flocks. By combining sperm mobility phenotype as a predictor of sire potential with genomic microsatellite markers to identify paternity, we have devised a novel means of investigating aspects of competitive fertilization in turkeys. Since heterospermic insemination is inherent to reproduction in turkeys, this strategy will permit improved understanding of sire potential in the competitive fertilization process.

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