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Sperm mobility: mechanisms of fertilizing efficiency, genetic variation and phenotypic relationship with male status in the domestic fowl, *Gallus gallus domesticus*

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When females are sexually promiscuous, sexual selection continues after insemination through sperm competition and cryptic female choice, and male traits conveying an advantage in competitive fertilization are selected for. Although individual male and ejaculate traits are known to influence paternity in a competitive scenario, multiple mechanisms co-occur and interact to determine paternity. The way in which different traits interact with each other and the mechanisms through which their heritability is maintained despite selection remain unresolved. In the promiscuous fowl, paternity is determined by the number of sperm inseminated into a female, which is mediated by male social dominance, and by the quality of the sperm inseminated, measured as sperm mobility. Here we show that: (i) the number of sperm inseminated determines how many sperm reach the female sperm-storage sites, and that sperm mobility mediates the fertilizing efficiency of inseminated sperm, mainly by determining the rate at which sperm are released from the female storage sites, (ii) like social status, sperm mobility is heritable, and (iii) subdominant males are significantly more likely to have higher sperm mobility than dominant males. This study indicates that although the functions of social status and sperm mobility are highly interdependent, the lack of phenotypic integration of these traits may maintain the variability of male fitness and heritability of fertilizing efficiency.

Keywords: fertilizing efficiency; fowl; maternal inheritance; sperm competition; sperm mobility; social dominance

1. INTRODUCTION

Traits that increase male reproductive success are under strong sexual selection (Darwin 1871; Arnold & Wade 1984), especially in the presence of sperm competition, i.e. when the sperm of different males compete to fertilize the eggs of a female (Parker 1970). However, the way different male traits interact to determine paternity (Pizzari & Birkhead 2001) and the way the heritability of these traits is maintained under directional sexual selection remains unresolved (Pominankowski & Møller 1995; Rowe & Houle 1996). Although theory predicts that genes conferring a male reproductive advantage will increase in frequency and eventually go to fixation, this is not supported by most empirical studies (Houle 1998). The erosion of genetic variation may not occur if positive correlational selection (Lande & Arnold 1983) on male reproductive traits is prevented.

In the domestic fowl, *Gallus gallus domesticus*, a species in which sperm competition is typically intense (e.g. Pizzari & Birkhead 2000; Pizzari 2001), dominant males are likely to inseminate relatively more sperm into individual females: they have more copulation opportunities (Guhl *et al.* 1945; Cheng & Burns 1988), disrupt copulations initiated by their subordinates (Cheng & Burns 1988; Pizzari 2001), and females bias insemination success in their favour both before (Pizzari 2001) and after copulation (Pizzari & Birkhead 2000). Social dominance is heritable in the fowl (Craig *et al.* 1965), as in some other taxa (Dewsbury 1990; Moore 1990), providing scope for sexual selection. In addition to social dominance, the quality of the sperm inseminated also plays a crucial role in sperm competition in this species (Birkhead *et al.* 1999).

We studied domestic fowl to:

(i) examine the mechanism by which high-quality sperm confers a fertilization advantage;
(ii) estimate the heritability of sperm mobility; and
(iii) examine the phenotypic relationship between sperm mobility and social dominance.

2. MATERIAL AND METHODS

(a) *Study population and sperm mobility*

The entire study was carried out on a random-bred population of New Hampshire fowl (base population $n = 242$) housed at Oregon State University (Froman & Feltmann 1998; Froman

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et al. 1999). Sperm quality is measured as 'sperm mobility', an *in vitro* assay which measures the ability of sperm to penetrate a solution of an inert medium (Accudenz: Accurate Chemicals & Scientific Corporation, Westbury, NY, USA), in absorbance units with a spectrophotometer (Froman & Feltmann 1998). Sperm mobility is a normally distributed trait which is stable and significantly repeatable within individual males (Froman & Feltmann 1998).

(b) *Sperm mobility and sperm number*

This part of the study investigated the mechanisms through which sperm mobility conveys a fertilizing advantage to an inseminated ejaculate. The numbers of sperm stored by the female reproductive tract (in the sperm-storage tubules at the uterovaginal junction) were measured by the number of perforations caused by acrosome-reacted sperm on the perivitelline layer (PVL) of eggs (Wishart 1987) laid by 100 random-bred New Hampshire hens. We scored sperm mobility of the random-bred male population ($n = 242$) on four separate occasions between November 1999 and February 2000 and selected 10 individuals with mean (± s.e.) mobility scores ranging between 0.1397 ± 0.037 and 0.7998 ± 0.067, thus avoiding males with very low mobility in order to make our analysis more conservative (see also Birkhead *et al*. (1999)). Semen from the 10 selected males was used to artificially inseminate 100 females (10 females per male) with 100×10^6 sperm and, 15 days later, the same females with 25×10^6 sperm. We collected eggs from the second day after insemination (day 1) and for the next 10 days. For each egg we removed the PVL, examined a 16 mm^2 region centred around the germinal disc–blastodisc using a Leica stage microscope, and recorded the number of perforations in the inner PVL using dark-field optics at magnification ×40 (Wishart 1987). Spermatozoa that reach the ovum but do not fertilize it penetrate the inner PVL (Wishart 1987) and are trapped between the inner and outer PVLs when the latter is deposited around the ovum shortly after fertilization (Bobr *et al*. 1964). Perforations in the inner PVL are caused by acrosome-reacted sperm (Bobr *et al*. 1964) and the number of perforations reliably indicates the number of sperm stored in the female's sperm-storage tubules (Brillard 1993) and the probability that an ovum is fertilized (Wishart 1987; Robertson *et al*. 1998). We therefore used the number of perforations as an index of the number of sperm associated with each ovum at the time of fertilization (Robertson *et al*. (1998); see also Birkhead & Fletcher (1998)).

We investigated the effect of sperm mobility on:

(i) the mean number of sperm reaching the sperm-storage tubules on day 1; and
(ii) the rate of sperm loss from the sperm-storage tubules.

We calculated (i) and (ii) from the regression of ln-transformed (Lessells & Birkhead 1990) mean number of perforations over time:

(i) is the point of intercept of the regression line (intercept, henceforth), and
(ii) is slope of the regression line. The relative importance of sperm mobility and number of sperm inseminated were analysed using ANOVAs with hierarchical sums of squares, where sperm number was a factorial independent variable and sperm mobility the covariate.

(c) *Heritability of sperm mobility*

(i) *Experiment 1*

The aim of this part of the study was to investigate the genetics of sperm mobility and to quantify additive genetic variation in this trait. Seven random-bred males with mean sperm-mobility scores representative of the mobility scores of the whole random-bred population were chosen as semen donors (mean ± s.d. sperm mobility: population = 0.378 ± 0.19, semen donors = 0.440 ± 0.19). Seven groups out of 20 randomly selected, random-bred females were each inseminated every other day for 10 days with the semen of one random-bred male. Eggs were identified by female and stored and incubated together to reduce potential environmental effects. Between 9 and 17 females produced male offspring (from two to five sons per female) in each group (total n of sons = 246). At 18 weeks of age male progeny were photostimulated (14 L : 10 D) and at 25 weeks of age sperm mobility was determined (Froman *et al*. 1999). We used a multiple-trait derivative-free restricted maximum-likelihood (MTDFREMI, Boldman *et al*. (1995)) animal model (a breeding design in which all breeding values for the pedigree are estimated) to estimate the heritability (h^2) of sperm mobility. MTDFREMI is a set of FORTRAN programs designed to estimate variance components using a derivative-free restricted maximum-likelihood algorithm and is particularly useful for our study as it makes use of pedigree data. Use of the animal model also allowed us to incorporate maternal and cytoplasmic effects in addition to the usual additive genetic effects (Lynch & Walsh 1998).

(ii) *Experiment 2*

Four groups of three full-sib sisters were selected on the basis of the sperm mobility of their full-sib brothers. The full-sib brothers of two of the four groups of sisters having significantly higher mean sperm mobility (mean mobility ± s.e.: 'high' group 1 = 0.681 ± 0.097, 'high' group 2 = 0.791 ± 0.043) than the full-sib brothers of the other two groups of sisters ('low' group 1 = 0.196 ± 0.068, 'low' group 2 = 0.238 ± 0.052, $F_{1,30} = 89.03$, $p < 0.0001$). All females were inseminated with the sperm of a single unrelated male of average sperm mobility. Each female was inseminated several times per week over a period of nine weeks. Sperm mobility of the progeny was measured when they were 25–33 weeks old. Data were analysed using a nested ANOVA (Sokal & Rohlf 1969) and *post-hoc* comparisons were performed with the Student–Newman–Keuls test (Sokal & Rohlf 1969).

(iii) *Experiment 3*

Two groups of full-sib sisters whose full-sib brothers had either significantly higher (mean ± s.e.: 0.54 ± 0.01) or lower (0.16 ± 0.02; $F_{17,148} = 19.42$, $p < 0.0001$) sperm mobility were inseminated with the sperm of nine cockerels (two groups of sisters per cockerel). To minimize variation in sperm mobility due to fathers we chose males of similar sperm mobility (hence, we did not expect fathers to have a significant effect on sons' sperm mobility). Sperm mobility of the male progeny was then measured and its variation analysed as in experiment 2 (§ 2c(ii)).

(d) *Sperm mobility and social status*

We created 33 pairs of males comprising a high and a low sperm-mobility individual (mean ± s.e. of high and low mobility, respectively = 0.553 ± 0.013, 0.167 ± 0.012; paired-$t_{32} = 21.30$, $p < 0.0001$). Males were scored high or low based on three successive sperm-mobility trials (Froman *et al*. 1999). To avoid any

potentially confounding effect of body size, the difference between pair members was minimized (mean ± s.e. body mass (kg) of 'high' versus 'low' males: 3.062 ± 0.060 versus 3.052 ± 0.039, paired-$t_{32} = 0.17$, $p = 0.863$). Males were released with four Single Comb White Leghorn females in 3 × 4 m pens with pine bedding, *ad libitum* food and water and a 14 L : 10 D regime. Each male pair was observed in 30 min observation periods randomly distributed among pairs every day between 16.00 and 19.00 (the period when male fowl are sexually most active (Upp 1928; Craig & Bhagwat 1974; Cheng & Burns 1988; Pizzari & Birkhead 2001) for 2 days, starting from the day after the birds were released in the pen. Social dominance between male-pair members was assessed on the basis of the number of times one male avoided the other. The male that was significantly more likely to be avoided was regarded as dominant (Guhl *et al.* 1945). The dominance relationship in 30 of the 31 pairs (two pairs, in which a male was slightly injured during handling potentially impairing his competitive ability, were excluded from the experiment) was unequivocal (least-significant male pair, $\chi^2_1 = 6.0$, $p = 0.014$). We also recorded the frequency of three other behaviours known to correlate with social dominance in male fowl: frequency of (i) crowing (Leonard & Horn 1995), (ii) wing flapping (Leonard & Zanette 1998), and (iii) vigilance (Sullivan 1991). Dominant males performed these behaviours significantly more than subordinates (mean ± s.e. behaviour frequency per trial, dominant versus subordinate, crowing: 6.16 ± 1.23 versus 0.12 ± 0.12 (paired *t*-tests), $t_{29} = 5.05$, $p < 0.0001$; vigilance: 1183.21 ± 41.99 versus 625.70 ± 75.41s, $t_{29} = 6.97$, $p < 0.0001$; wing flapping: 3.88 ± 0.48 versus 1.14 ± 0.26, $t_{29} = 5.47$, $p < 0.0001$). In the remaining pair one male accounted for 65% (11 out of 17) of the observed avoidance cases ($\chi^2_1 = 1.47$, $p = 0.22$), but performed behaviours (i)–(iii) significantly less often than the other male (crowing: $t_6 = 3.56$, $p = 0.012$, vigilance: $t_6 = 3.79$, $p < 0.01$, wing flapping: $t_6 = 2.26$, $p = 0.06$, $n_{\text{trials}} = 7$) and was thus regarded as the subdominant one of the pair.

3. RESULTS

(a) *Sperm mobility and sperm number*

As expected, the number of sperm inseminated affected the number of sperm stored by females (see Brillard 1993) and the mobility of sperm influenced the rate at which sperm were lost from the female oviduct. More mobile ejaculates retained their ability to fertilize eggs for longer because they were lost at a significantly slower rate. Significantly more sperm reached the storage sites following inseminations of 100×10^6 sperm ($F_{1,17} = 6.72$, $n_{\text{males}} = 10$, $n_{\text{females}} = 100$, $p = 0.019$; figure 1). Sperm mobility did not influence the number stored ($F_{1,17} = 1.77$, $p = 0.200$), but high-mobility sperm were lost at a significantly slower rate ($F_{1,17} = 4.51$, $p = 0.049$) and hence retained their fertilizing capacity for longer. The number of sperm inseminated also had a weak effect on the rate of sperm loss ($F_{1,17} = 4.12$, $p = 0.058$). When 25×10^6 sperm were inseminated, sperm mobility had a significant, positive effect on the number of sperm stored ($R^2 = 0.33$, $p = 0.048$).

(b) *Heritability of sperm mobility*

(i) *Experiment 1*

Both mothers and fathers had an important genetic influence on the sperm mobility of their sons. First, we found significant additive variation (direct (animal) additive genetic effect) in the sperm mobility of the male progeny produced by 140 hens inseminated by 7 cockerels (20 hens per cockerel; animal model, $h^2 = 0.30$, animal model versus model with no animal effect: likelihood-ratio test = 28.29, d.f. = 4, $p < 0.001$; figure 2), and a significant exclusively maternal genetic contribution (maternal additive genetic effect, $h^2 = 0.15$, model with fixed animal and environmental effects versus model with no fixed parameters: likelihood-ratio test = 5.62, d.f. = 1, $p = 0.018$). The observed maternal genetic effect had a relatively low (0.08) genetic correlation with the animal additive effect, indicating that a maternally transmitted genetic element, independent from autosomal genes, is involved in determining sperm mobility.

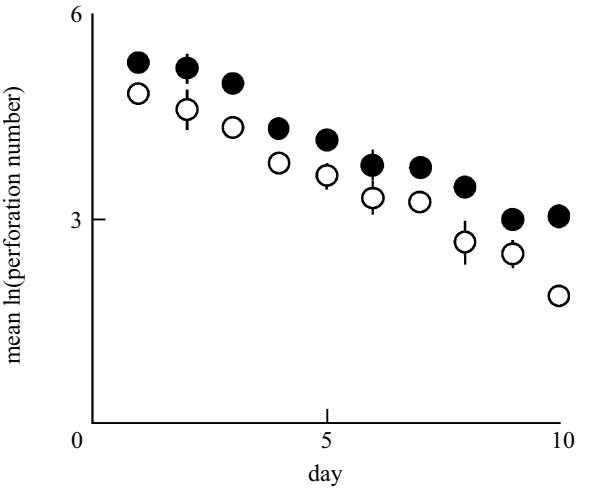

Figure 1. Sperm mobility and fertility. Decline over time of mean number of sperm stored by females inseminated with 25×10^6 (open circles) and 100×10^6 (filled circles). Vertical bars represent s.e. values.

(ii) *Experiment 2*

Second, consistent with the previous result, we found that the sperm mobility of sons produced by mothers that had either high or low sperm-mobility brothers and were inseminated with sperm from a single male with average sperm mobility differed significantly in a way predicted by the sperm mobility of their maternal uncles (mean ± s.e. of two high sperm-mobility female groups versus two low female groups = 0.416 ± 0.035, 0.471 ± 0.023 versus 0.268 ± 0.024, 0.233 ± 0.028, $F_{3,528} = 13.92$, $p < 0.01$, Student–Newman–Keuls test, $p < 0.05$), confirming an additive genetic component of sperm mobility.

(iii) *Experiment 3*

Last, we investigated the variance in sperm mobility of the male progeny produced by full-sib sisters whose full-sib brothers had either high or low sperm mobility and that were artificially inseminated with the semen of nine average sperm-mobility males. We again found that the mean sperm mobility of maternal uncles significantly explained variation in the sperm mobility of the progeny (maternal uncles' effect: $F_{1,108} = 22.81$, $p < 0.001$, paternal effect: $F_{8,108} = 1.53$, $p = 0.156$; $n_{\text{sires}} = 9$, $n_{\text{females}} = 109$).

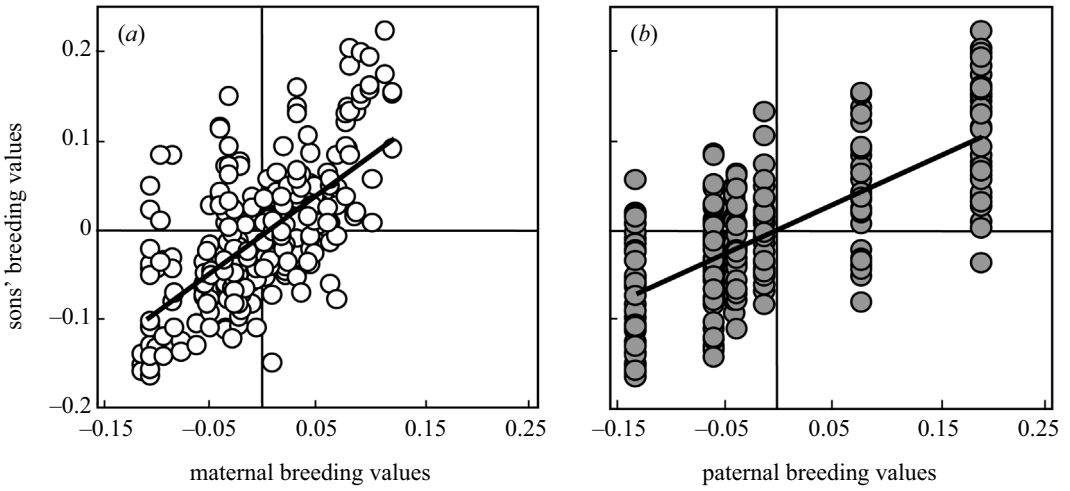

Figure 2. Paternal and maternal influence on sperm mobility. The additive maternal genetic effect on sons' sperm mobility was stronger than the animal additive (autosomal) effect. The regression slope of the maternal predicted additive genetic values (breeding values) on the breeding values of the progeny ((*a*) sons = 0.89 ± 0.07 (maternal effect) $- 0.002 \pm 0.004$, $R^2 = 0.40$, $n_{\text{mothers}} = 140$, $n_{\text{sons}} = 246$, $p < 0.0001$) was significantly steeper than the regression of the paternal breeding values on sons' breeding values ((*b*) sons = 0.56 ± 0.04 (paternal effect) $- 0.0001 \pm 0.004$, $R^2 = 0.48$, $n_{\text{fathers}} = 7$, $n_{\text{sons}} = 246$, $p < 0.0001$, mothers per sons regression slope versus fathers per sons regression slope: $t_{244} = 4.09$, $p < 0.001$, breeding values derived from model considering both the additive genetic effect and the maternal genetic effects).

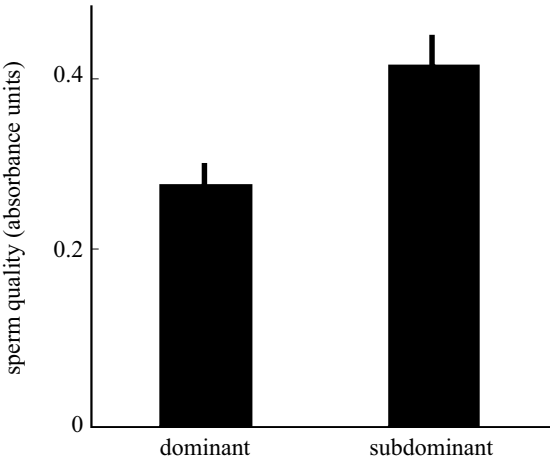

Figure 3. Mean sperm mobility of dominant and subdominant members of experimental male pairs. Vertical bars represent s.e. values.

(c) *Sperm mobility and social dominance*

We did not find a positive phenotypic correlation between social dominance and sperm mobility, as one would expect if there were positive correlational selection (Lande & Arnold 1983) on these traits. In fact, dominant males had significantly lower sperm mobility than their subordinates (proportion of dominants with low sperm mobility = 68% (21 out of 31), $\chi^2_1 = 6.45$, $p = 0.04$; dominants versus subordinates: $t_{30} = 2.09$, $p = 0.045$; figure 3).

4. DISCUSSION

In the present study we have:

(i) identified the potential mechanisms through which both the number and the quality of sperm inseminated differentially affect fertilizing efficiency;
(ii) demonstrated that sperm mobility can be heritable; and
(iii) found a negative phenotypic correlation between sperm mobility and social dominance, the trait which influences the number of sperm that a male inseminates into a female.

Male fertilizing efficiency is likely to result from the effect of multiple male traits (Pizzari & Birkhead 2001). Our study supports this view and indicates that both sperm number and sperm mobility are likely to play an important role in fertilization in the fowl. The influence of sperm mobility and sperm number on paternity is particularly strong when sperm competition occurs and amplifies variance in male reproductive success (Dziuk 1996; Birkhead *et al.* 1999). To the extent to which social dominance mediates the number of sperm inseminated, social dominance and sperm mobility have highly interdependent functions. Therefore, intuitively one would expect male fertilizing efficiency to result from the integration of social dominance and sperm mobility, and these two male traits to be simultaneously favoured by correlational sexual selection (Lande & Arnold 1983). Results consistent with this scenario have been found in a study of domestic mice where dominant males produce relatively competitive ejaculates (Koyama & Kamimura 2000). The results of our study, on the other hand, are in striking contrast with the idea that sperm mobility and social dominance work in unison. First, not only we did not find a positive phenotypic relationship between social dominance and sperm mobility, but we also showed that subdominant males tended to produce more competitive ejaculates. Consistent with the idea that directional sexual selection may not elicit a strong evolutionary response in both sperm mobility and social dominance, we found that sperm mobility, like social dominance (Craig *et al.* 1965) is heritable. A similar situation occurs in the cockroach *Nauphoeta cinerea*, where both male–male competition and female choice are mediated by a male sex pheromone consisting of three different compounds (Moore 1997). Two of these compounds, which together signal male competitive

ability and are therefore functionally interdependent, show high genetic and phenotypic integration (Moore 1997). However, the third compound, which mediates male attractiveness to females, is independent from the other two components of the pheromone (Moore 1997). This prevents intra- and inter-sexual pressures from acting in unison on the pheromone, translating into an overall balancing of sexual selection and the maintenance of genetic variation in this trait (Moore & Moore 1999).

The results of heritability experiment 1 indicate the possibility that sperm mobility may be to a large extent under the control of an independent, maternally inherited element. Experiments 2 and 3 produced results which are consistent with this hypothesis. However, the unbalanced design of these experiments does not allow us to test the extent to which maternally transmitted genes may control sperm mobility. Nevertheless, our study suggests the counterintuitive possibility that mothers may have an important genetic influence over the sperm mobility of their sons. One mechanism by which mothers may influence sperm mobility is through mitochondrial genes (Kao *et al*. 1998; Ruiz-Pesini *et al*. 2000; Pizzari & Birkhead 2001). In the fowl, sperm mobility is positively correlated with sperm ATP content and with sperm oxygen consumption (Froman & Feltmann 1998), suggesting that sperm mobility may be determined by the ATP-synthetic ability of sperm mitochondria, which is partly controlled by mtDNA (Cummins 1998). A maternal influence on sperm traits has been previously suggested to act either through the X chromosome (Ward 2000; Morrow & Gage 2001*a*; Wang *et al*. 2001) or through mitochondrial DNA (Kao *et al*. 1998; Ruiz-Pesini *et al*. 2000), but these studies have not identified an explicit causal relationship between these sperm traits and fertilization efficiency (e.g. Morrow & Gage 2001*b*). In contrast, our study indicates a potential maternal influence on a sperm trait which determines fertilization success, particularly in sperm competition (Birkhead *et al*. 1999), and is thus of obvious biological importance. Studies have identified maternal genetic effects controlling the expression of male reproductive traits, such as sperm morphology and development, due to the X chromosome (Ward 2000; Morrow & Gage 2001*a*; Wang *et al*. 2001) in taxa where males are the heterogametic sex. Due to the fact that, in birds, males are homogametic, our results cannot be explained by a similar mechanism. The evolutionary implications of X-linked control of male traits are fundamentally different from those involving mitochondrial control. Males inherit the X chromosome from mothers, and can transmit their X chromosome to their daughters, but they are typically passive carriers of mitochondrial DNA. Therefore, while the X chromosome is an ideal reservoir for genes controlling male sexual traits (Reinhold 1998) and especially for male-beneficial–female-detrimental genes (Rice 1984), the exclusively maternally transmitted mitochondrial genome (Frank & Hurst 1996; Pominankowski 1999) prevents selection against male-detrimental genes. Mitochrondrial genes controlling male traits may thus result in the absence of selection for these traits or, paradoxically, in the selection for their expression in females. Therefore, to the extent to which they control sperm mobility, males are effectively at an 'evolutionary dead end' and selection through the paternal line is prevented from reducing the variance in male traits.

Despite much interest (Rowe & Houle 1996), the reason why male fitness remains variable under consistent directional selection is unresolved. Our study suggests that the lack of phenotypic integration of functionally interdependent sexually selected traits, possibly mediated by maternally transmitted genes, may help explain this paradox.

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