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Article:

Sperm mobility determines the outcome of sperm competition in the domestic fowl

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The aim of this study was to establish whether the mobility of sperm of the domestic fowl, as measured by an *in vitro* assay, predicted the outcome of sperm competition. Thirteen pairs of New Hampshire roosters, comprising one male categorized as having high-mobility sperm and the other as having average-mobility sperm, were used. Each male provided 25×10^6 sperm, which were mixed and artificially inseminated into between four and seven New Hampshire hens, each of which produced 2–11 offspring. The experiment was conducted twice, such that the same pair of males inseminated the same females. Paternity was assigned by using microsatellite markers. There was a clear effect of sperm-mobility phenotype on the outcome of sperm competition: in all 13 pairs the high-mobility male fathered the majority of offspring (73.3% overall; $p < 0.0001$). The proportion of offspring fathered by the high-mobility male within pairs varied significantly between male pairs ($p < 0.0005$). This effect was associated with the difference in sperm-mobility scores between males within pairs: there was a significant positive relationship between the proportion of offspring fathered by the high-mobility male and the ratio of mobility scores between males ($p < 0.05$). In addition, compared with their success predicted from the non-competitive situation, in the competitive situation high-mobility males were disproportionately successful in fertilizing eggs compared with average-mobility males. This may occur because female sperm storage is limited in some way and a greater proportion of high-mobility sperm gain access to the female's sperm storage tubules. There was no evidence that female effects accounted for any of the variation in paternity.

Keywords: sexual selection; reproduction; sperm mobility; sperm competition; domestic fowl

1. INTRODUCTION

Sperm competition, which is defined as the competition between ejaculates of different males for a given set of ova, is widespread throughout the animal kingdom (Parker 1970, 1998; Birkhead & Parker 1997). Among species with internal fertilization, sperm competition occurs when females copulate with and are inseminated by more than one male during a single fertile period. The factors and underlying mechanisms that determine which of several males will fertilize a female's ova are not well known; information that exists for a few insect species shows that mechanisms widely vary (Simmons & Siva-Jothy 1998). In birds, the main factors that determine the outcome of sperm competition appear to be similar in passerines and non-passerines (Birkhead 1998a). When equal numbers of sperm from two male birds are inseminated at different times, the second male usually fertilizes the majority of offspring, and the longer the interval between the two inseminations the greater the last-male sperm-precedence effect (Birkhead *et al.* 1995; Colegrave *et al.* 1995; Birkhead & Biggins 1998). This effect occurs as a consequence of the passive loss of sperm from the

female reproductive tract (Wishart 1987; Lessells & Birkhead 1990): the longer the interval between two otherwise equal inseminations, the greater the proportion of sperm from the first insemination that is lost or used by the time the second insemination takes place (Birkhead 1998b).

Last-male sperm precedence in birds occurs in the laboratory under conditions that are biologically improbable among individuals in the wild. In nature the outcome of sperm competition in birds is likely to be more complex and influenced by a number of factors, including: (i) the number and timing of inseminations; (ii) the timing of inseminations relative to when the female ovulates; (iii) the number of sperm inseminated by each male; and (iv) the fertilizing ability of sperm from each male (Birkhead 1998b).

In the present study we focus on differences between males in the ability of their sperm to fertilize eggs as a factor influencing the outcome of sperm competition. It has been known for a long time by animal breeders that if females are inseminated with a mixture comprising equal numbers of sperm from two males, one male usually fathers more offspring than the other. This effect is referred to as differential fertilizing capacity (DFC) (Lanier *et al.* 1979) and because it is usually consistent across different females, it is assumed to be mediated by differences in the sperm of different males (Dziuk 1996).

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1759

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However, despite several studies linking *in vitro* measures of sperm movement with fertilizing ability (for example, in pigs (Holt *et al.* 1997), rats (Moore & Akhondi 1996) and domestic fowl, *Gallus gallus* (Wishart & Palmer 1986; Chaudhuri & Wishart 1988; Froman & McLean 1996)), these have always been conducted in a non-competitive situation. This is surprising since it is known that 'heterospermic' inseminations, that is, mixtures of sperm, provide the most sensitive way of discriminating between the fertilizing ability of different males (Dziuk 1996). As a consequence, the basis for DFC remains unclear.

The present study had two objectives: principally, to test the hypothesis that the *in vitro* difference in sperm mobility predicts fertilization success in a competitive situation; and second, to determine whether differential fertilizing success is an entirely male attribute or whether some of the variation in the outcome of sperm competition is attributable to the differential use of sperm by females (Eberhard 1996; Birkhead 1998*c*). In terms of our first objective, because it is well established that high-mobility sperm are more likely to achieve fertilization in a non-competitive situation (Froman & Feltmann 1998; Froman *et al.* 1999), our aim was to distinguish between two possible scenarios: either (i) that in a competitive situation the proportion of offspring fathered by the two males is predictable simply on the basis of their performance in a non-competitive situation, or (ii) that males with sperm of higher mobility were disproportionately more successful in a competitive situation.

2. METHODS

The study was conducted on New Hampshire males and females of the domestic fowl. Sperm competition is likely to be intense in jungle fowl, the ancestor of the domestic fowl, since female domestic fowl routinely copulate with several males (McBride *et al.* 1969; T. Pizzari and T. R. Birkhead, unpublished data; see also Jones & Mench 1991). We used a recently developed *in vitro* assay of sperm performance: sperm mobility, the ability of sperm to penetrate a solution of Accudenz (Accurate Chemicals & Scientific Coporation, Westbury, NY, USA), an inert medium (for details of the assay see Froman & McLean 1996; Froman & Feltmann 1998). Froman & Feltmann (1998) have shown that sperm mobility is a normally distributed trait and is highly stable within males between 30 and 64 weeks of age. They categorized birds as high-mobility phenotypes if their mean Accudenz score was more than 1.5 standard deviations above the population mean, and males whose mean score was between one standard deviation below the mean and the mean were categorized as 'average' sperm-mobility phenotypes.

Sperm mobility is an important determinant of fertility in the domestic fowl in a non-competitive situation: Froman & Feltmann (1998) showed that in a large sample of New Hampshire chickens under standardized conditions males with high-mobility sperm fertilized significantly more eggs than males with low-mobility sperm. Subsequently, using different birds of the same strain Froman *et al.* (1999) showed that the relationship between sperm mobility and the proportion of eggs fertilized is a logistical function, with an asymptote at a mobility score of 0.6 (based on 48 males, each inseminating 10–12 hens, each laying seven eggs; approximately 3700 eggs in total). Because the relationship between mobility and fertility was well established and because we used males from this same population maintained under identical conditions, we assumed that the high- and average-mobility phenotypes used in the present study would show similar differences in fertility.

Sperm mobility was measured by diluting a semen sample to a fixed sperm concentration and then overlaying a 6% solution of Accudenz in a polystyrene cuvette with a fixed volume of sperm suspension. The absorbance of the Accudenz layer was measured after a 5 min incubation at body temperature (41 °C) by means of a Turner model 340 spectrophotometer at 550 nm (Froman & Feltmann 1998). Absorbance is proportional to the size of the sperm population that penetrates the Accudenz layer (Froman *et al.* 1999). Sperm mobility covaries with sperm oxygen consumption, ATP content and several measures of sperm velocity. In addition, mobility is an attribute of sperm and not of seminal fluid: sperm that are washed and resuspended in a tissue medium show no change in mobility (Froman & Feltmann 1998). Overall, these results suggest that the extreme variation in sperm mobility observed between males results from differential rates of mitochondrial ATP synthesis (Froman *et al.* 1999). Sperm mobility predicts fertility in a non-competitive situation more accurately than other measures of sperm performance in the domestic fowl and this appears to be because the assay mimics the situation that sperm experience immediately after insemination: in both situations sperm have to move against some type of resistance (Froman & Feltmann 1998; Froman *et al.* 1999). The positive correlation between mobility and fertilization success appears to arise because a greater proportion of high-mobility sperm enter the sperm storage tubules (Donoghue *et al.* 1998), resulting in both a higher proportion of fertile eggs and a longer period over which fertile eggs are laid (Froman *et al.* 1999).

In the present study we used males selected from a population of 509 individuals whose mean mobility score was 0.476 ± 0.218 s.d. (see Froman *et al.* 1999). Males were categorized as either high- or average-mobility phenotypes, as in Froman's previous studies (above); 15 high- and 15 average-mobility males were used. Mean sperm-mobility scores for each male were derived from between five and seven measurements made between 27 and 61 weeks of age. Repeatability (Lessells & Boag 1987) of mobility scores across all 30 males was significant ($r = 0.64$; $F_{29,175} = 13.0$, $p < 0.0001$). The mean score of the high-mobility males was 0.805 ± 0.077 (range of means 0.716–0.982) and the mean score for average males was 0.334 ± 0.057 (range 0.242–0.455) ($p < 0.0001$). We thus avoided very low-mobility males and did so because these males could have low sperm mobility for a number of reasons. Our analyses are therefore conservative. As in Froman's previous studies, all males used in this study had previously been screened for sperm viability (by the method of Bilgili & Renden 1984) and all showed 100% sperm viability (see Froman & Feltmann 1998).

High- and average-mobility males were paired at random, other than avoiding pairing individuals known to be first-order relatives. We also avoided assigning males to females known to be first-order relatives and using any females that were first-order relatives. Ejaculates were obtained from each male by means of abdominal massage (Burrows & Quinn 1937) and 25×10^6 sperm from each male of a pair were mixed and inseminated (once) into seven hens (in one case eight). Eggs were collected from the second day after insemination; to maximize the number of offspring we obtained, continued to be collected for 21 days, although most fertile eggs were laid in the first ten days. Eggs were artificially incubated and each female produced a mean of 6.31 ± 2.42 offspring (range 0–11). The experiment

was repeated twice such that the same pairs of males inseminated the same hens. This allowed us to check for an order effect and it also allowed us to establish whether any significant repeatability in relative fertilization success for each male pair existed within females, after controlling for male effects. If this were the case it would provide evidence that females also affected the outcome of sperm competition (see Wilson *et al.* 1997; Birkhead 1998*c*).

Paternity was established as follows. We obtained blood samples from all offspring (*n* = 1204, 669 from experiment 1 and 535 from experiment 2) and from all adult males and females for molecular parentage analysis. We used males and females of the same strain of domestic fowl in conjunction with molecular paternity analysis, rather than using different strains of males and genetic plumage markers (as in most other studies), to avoid the possible confounding effect of strain differences in fertilization success. Blood samples were stored in 100% ethanol until DNA extraction. DNA was obtained by using a standard phenol–chloroform extraction protocol (Bruford *et al.* 1998). Parents were genotyped for a number of tetranucleotide-repeat-containing microsatellite markers until we found at least one marker for which both males and females were different for both alleles at that locus. We then used that marker to genotype all offspring, and to assign each of them to one of the potential sires using the paternal alleles as criteria for parentage assignment. The markers used were LEI0194, LEI0217, LEI0228, LEI0234, LEI0246 and LEI0248 (McConnell *et al.* 1999). Each 10 μl polymerase chain reaction (PCR) contained 25–50 ng DNA, 0.25 units Taq polymerase (Advanced Biotechnologies), 1 mM of each primer, 1.5 mM MgCl$_2$, and 0.2 mM dNTPs in the manufacturer's buffer. PCR amplification was performed in a Hybaid Touchdown thermal cycler with the following reaction profile: 94 °C for 1 min, 50 °C for 30 s and 72 °C for 30 s, for 30 cycles, and a final step of 72 °C for 2 min. We used fluorescent-labelled primers and ran the products in an Applied Biosystems model 377 DNA sequencer. Allele size was determined by using the GeneScan 2.1 (Applied Biosystems) software. In some cases, offspring (*n* = 77; 6.3% of all offspring) could not be assigned to a male because males and females shared alleles; these were excluded from the analyses. We also excluded all families where we did not have at least two offspring from a female in both experiments. The final data set comprised 13 pairs of males, 71 females, and 867 offspring. The number of offspring in a 'brood' fathered by the male with the higher-mobility sperm score was treated as a binomial variable. We used logistical regression (Dobson 1990) to determine the relative importance of male pair, female (after controlling for male pair) and order (i.e. experiment 1 or 2) on the probability that an offspring was fathered by the high-mobility male.

To distinguish between the two scenarios (above), i.e. that in a competitive situation: (i) the proportion of offspring fathered by the two males was predictable solely on the basis of their performance in a non-competitive situation, and (ii) that high-mobility males were disproportionately successful, we compared the predicted proportion of offspring fathered by high-mobility males in a non-competitive situation with the observed proportion in a competitive situation, i.e. in our experiments. If scenario (i) is correct we expected no difference between the predicted and observed values, whereas if scenario (ii) is correct we expect that the high-mobility males would have a significantly greater success in the competitive situation. We estimated the predicted ratio of offspring fathered by the two males in a non-competitive situation by using the mean mobility score for

Table 1. *Results from logistical regression analysis of the importance of different factors in explaining the probability of offspring being fathered by the higher-mobility male in a pair*

factor	Wald statistic[a]	d.f.	*p*
male pair	64.4	12	< 0.0005
female	55.33	58	0.6
order	0.60	1	0.4
male pair × experiment	20.9	12	0.075
overall fit of binomial model	156.8	129	0.05

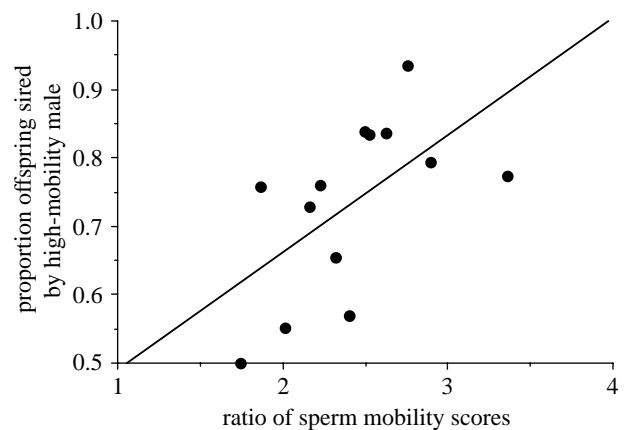[a] χ^2 tables were used to assess significance.

Figure 1. The relationship between the ratio of sperm mobility scores for the two males within a pair and the proportion of offspring fathered by the high-mobility male. The relationship is significant ($y = 0.171x + 0.32$; $r = 0.584$, $n = 13$, $p < 0.05$).

each phenotype (above) and the mean proportion of eggs fertilized by the high-mobility phenotype in a non-competitive situation (derived from the relationship between sperm mobility and fertility presented by Froman *et al.* (1999)), divided by this value plus the mean proportion of eggs fertilized by the average-mobility phenotype. On this basis, high-mobility males (mean score 0.805, see above) were expected to fertilize 98% of eggs under standard conditions, whereas average-mobility males were expected to fertilize 87% of eggs: the predicted fertilization success of high-mobility males was therefore 98/(98+87) = 53% and we compared this with the observed value by using a one-sample *t*-test.

3. RESULTS

There was clear evidence that the probability of an offspring being fathered by the higher-mobility male differed between male pairs ($p < 0.0005$; table 1). There was no evidence for any effect of female after controlling for male pair effects ($p > 0.05$), or order ($p > 0.05$); these results indicate that both experiments gave similar outcomes. Nor was there any significant interaction between male pair and experiment ($p > 0.05$), indicating that the relative success of males was similar in each of the two experiments. The overall fit of the binomial models, allowing for differences between male pairs, is

reasonably satisfactory although there was some evidence for unaccounted variation ($p = 0.05$; table 1).

Within all 13 pairs of males the high-mobility individual fathered a greater proportion of offspring than the average-mobility male, and overall the high-mobility male fathered $73.3\% \pm 12.9$ s.e. of offspring (one-sample t-test of the proportion being 0.5, $t = 6.51$, 12 d.f., $p < 0.0001$). Although it was not one of our original objectives, it is worth noting that the difference between pairs of males (table 1) was associated, in part, with the ratio of their mobility scores: there was a significant positive relationship between the proportion of offspring fathered by the high-mobility male and the ratio of the mobility scores of the males within a pair ($r = 0.584$, $n = 13$, $p < 0.05$; see figure 1).

In addition, the observed proportion of offspring fathered by the high-mobility males in a competitive situation (73.3%; see above) was significantly greater than that predicted by a model that assumes that their fertilization success was entirely predictable from their performance in a non-competitive situation (53%; one-sample t-test, $t = 5.69$, 12 d.f., $p < 0.001$). This confirmed that in a competitive situation the sperm from high-mobility males was disproportionately successful in fertilizing eggs.

4. DISCUSSION

Our main finding was that sperm mobility, as measured by an *in vitro* assay, had a major effect on the proportion of offspring sired in a sperm-competition situation and that sperm from high-mobility males were disproportionately successful. Our results are consistent with, but also provide a clear biological explanation for, the results of previous studies that have used mixtures of sperm from different males to identify high-fertility males and which show that competitive inseminations are over one hundred times more sensitive for this than single, non-competitive inseminations (Dziuk 1996).

Our results raise a number of questions regarding (i) the mechanism(s) that result(s) in the disproportionate fertilization success of high-mobility sperm, (ii) whether similar variation in sperm performance exists in populations of wild animals, and (iii) how variation in sperm performance might be maintained in a population.

(a) *Mechanisms*

The basic mechanism that results in the disproportionate fertilization success of high-mobility sperm appears to be that a greater proportion of high-mobility sperm enter the sperm-storage tubules. Donoghue *et al.* (1998) provide support for this by showing in turkeys, *Meleagris gallopavo*, that as well as high-mobility sperm fertilizing a greater proportion of eggs in a non-competitive situation, females inseminated with high-mobility sperm had a significantly greater number of sperm associated with the perivitelline layers of their eggs than females inseminated with the same number of lower-mobility sperm. Other studies of turkeys (and domestic fowl) have shown that the numbers of sperm on the perivitelline layers of laid eggs reflect the numbers of sperm originally stored in the sperm-storage tubules (Brillard & Antoine 1990; Brillard & Bakst 1990). Because only a few per cent of the inseminated sperm are retained and stored by the

female (the rest are ejected), and because space in the sperm-storage tubules is rarely limiting (see Bakst *et al.* 1994), one plausible explanation for a higher proportion of high-mobility sperm entering the storage tubules is that the time available for sperm to enter the tubules is limited. The avian vagina is an extremely hostile environment to sperm (Wishart & Steele 1992; Bakst *et al.* 1994), and sperm appear to be able to survive there for only a limited time. A greater proportion of high-mobility sperm may therefore be able to 'escape' the vagina and enter the storage tubules before damage ensues or ejection occurs.

High-mobility sperm also appear to have at least one other attribute that may contribute to their greater competitiveness. Donoghue et al. (1998) found that a greater proportion of high-mobility sperm penetrated the inner perivitelline layer of eggs and hence were more likely to locate and fuse with the female pronucleus than low-mobility sperm. High-mobility sperm may also be more competitive in another way, if, for example they can travel more rapidly between the sperm-storage tubules and the infundibulum (the site of fertilization) than low-mobility sperm. It is generally assumed that when sperm leave the storage tubules they are carried passively by cilia along the oviduct to the infundibulum (see Bakst *et al.* 1994), but it is possible (and remains to be established) that high- and low-mobility sperm differ in their speed of travel; again, any time constraint, such as that between the oviposition of one egg and the fertilization of the next (Bakst *et al.* 1994), may give faster sperm a competitive advantage.

(b) *Similar variation in sperm performance in other taxa?*

As far as we are aware no other study has reported differences in sperm mobility or velocity affecting fertilization success in a sperm-competition situation. However, in the bulb mite *Rhizoglyphus robini* and the nematode *Caenorhabditis elegans*, both of which have amoeboid sperm, larger sperm outcompete smaller sperm in a competitive situation (Radwan 1996; LaMunyon & Ward 1998).

(c) *Maintenance of variability in sperm mobility*

In species in which sperm competition occurs it seems likely that if sperm mobility is heritable it would be subject to strong directional selection and go to fixation. There are several explanations for how variation in a trait such as sperm mobility, which is (potentially at least) closely correlated with fitness, might be maintained within a population (Clark & Begun 1998). Here we briefly consider two explanations.

(i) *Antagonistic pleiotropy*

This is the idea that the competitive advantage of high sperm mobility might be offset by some other trait, such as reduced sperm number via differential fitness in different female genotypes. We have not yet explored this possibility, but other studies suggest that the conditions under which antagonistic pleiotropy operates are rather restrictive (Clark & Begun 1998).

(ii) *Differential fitness in specific female genotypes*

This hypothesis is similar to the idea of cryptic female choice: that females differentially use the sperm of

different males (Eberhard 1996). The only studies to have demonstrated differential sperm use all suggest that the effect is mediated by genetic compatibility or incompatibility between sperm and the female reproductive tract (Markow 1997; Birkhead 1998c; Clark & Begun 1998; see also Zeh & Zeh 1996, 1997). However, the results of the present study provide no evidence for a female effect of any sort on the outcome of sperm competition. The fact that the vaginas of domestic fowl are 'hostile' to sperm, and the idea that there may be a time-limit for sperm movement out of the vagina into the sperm-storage tubules, implies that differences in the degree of 'hostility' between females could determine the proportion of sperm entering the storage tubules and hence constitute a mechanism of cryptic female choice. The fact that we detected no consistency within females in differential fertilization success of the two males suggests that females did not differ in this respect. On the other hand, the relatively low number of offspring per female, and the fact that we were able to repeat the experiment only twice, make our experiment only a relatively weak test of female effect. Other studies that have failed to detect a female effect (see, for example, Stockley 1997; Cunningham & Cheng 1999) may also suffer from a lack of statistical power. However, studies of *Drosophila* have shown that male and female traits associated with sperm competition and sperm choice, respectively, coevolve and that female effects may be extremely subtle, requiring very sensitive experimental methods to detect them (Rice 1996).

(d) *Conclusions*

In conclusion, sperm mobility has a marked effect on the outcome of sperm competition under our controlled experimental conditions. Further studies are required to establish how sperm mobility interacts with other traits, such as sperm numbers also known to be important in sperm competition, and we also need to establish whether sperm mobility is heritable and how it is maintained.

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