

Paternity in mallards: effects of sperm quality and female sperm selection for inbreeding avoidance

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Postcopulatory processes might play an important role in sexual selection. In theory, fertilization success could be controlled by females via selection of particular sperm within their reproductive tract, or it could be determined by sperm competition per se. In practice, these two mechanisms are difficult to disentangle. To assess the relative importance of both mechanisms we used artificial insemination in combination with measurements of sperm quality (swimming speed and motility) in mallards. In this species, females often lack behavioral control over copulations and hence may use postcopulatory mechanisms to optimize their reproductive output. One important factor affecting female fitness may be selection of genetically compatible males. To investigate the influence of sperm quality and parental relatedness on paternity we inseminated 12 groups of related females with a sperm mixture containing equal numbers of sperm from a brother and from an unrelated male. Paternity was independent of the relatedness of the siring male to the female but was significantly affected by long-term sperm swimming speed and motility. No interaction between relatedness and sperm quality on paternity was observed. These results suggest that female mallards are not able to select sperm on a purely genetic basis and emphasize the importance of sperm quality in gaining paternity. *Key words:* *Anas platyrhynchos*, cryptic female choice, mallard, sperm competition, sperm motility, sperm selection, sperm swimming speed. [*Behav Ecol* 16:825–833 (2005)]

Darwin first described sexual selection as an important evolutionary force acting through differential reproductive success of individuals (Darwin, 1871). Traditionally, this process was perceived to be an exclusively precopulatory process (Andersson, 1994). However, as a consequence of female promiscuity—now known to be widespread in animals (Birkhead and Møller, 1998)—sexual selection does not stop at insemination but continues after copulation (Birkhead and Pizzari, 2002). Initially, research focused on male-male competition over fertilization (sperm competition; Parker, 1970). However, in recent years the question of whether and to what extent females are able to bias paternity in favor of a particular male after having copulated with several males (postcopulatory or cryptic female choice; Eberhard, 1996; Pitnick and Brown, 2000; Thornhill, 1983) received growing attention (e.g., Birkhead, 1998; Birkhead and Pizzari, 2002). In particular, the potential ability of females to discriminate and differentially utilize sperm of different males within their reproductive tract, so-called sperm selection or “sperm choice” (Birkhead, 1998), became the focus of an intense scientific debate (Birkhead, 2000; Eberhard, 2000; Kempenaers et al., 2000; Pitnick and Brown, 2000).

One main potential benefit to females of cryptic sperm selection is to increase the genetic quality of their offspring. Females could achieve this by selecting for particular “good genes” or by selecting for a genetically more compatible genome (for reviews see Jennions and Petrie, 2000; Tregenza and Wedell, 2000; Zeh and Zeh, 1996). At the interspecific level numerous studies showed that conspecific sperm have a higher probability of fertilizing an egg than heterospecific

sperm (Dziuk, 1996; Jennions and Petrie, 2000). Similarly, sperm from a male from the same race, population, or strain often have a higher fertilization capacity (Brown and Eady, 2001; Markow, 1997). On the other hand, inbreeding has negative fitness consequences (reviewed in Keller and Waller, 2002), whereas increased individual heterozygosity has positive fitness consequences (e.g., Amos et al., 2001; Foerster et al., 2003). Sperm selection may thus be driven by the costs associated with inbreeding and outbreeding. Selection of sperm based on sperm genotype could be a mechanism to select the genetically most compatible sperm, not only after copulating with two or more conspecific males (e.g., Bretman et al., 2004) but even within a male’s ejaculate (Marshall et al., 2003). In the following paragraphs we will focus on postcopulatory female sperm selection within the female reproductive tract only based on sperm genotype.

Growing support for female sperm choice based on male genotype comes mainly from studies on invertebrates (Bishop, 1996), in particular, insects (Clark et al., 1999; Stockley, 1999; Wilson et al., 1997). However, much of the evidence remains suggestive rather than conclusive because all studies are based on natural matings. In this situation it is not possible to distinguish between differential sperm numbers inseminated, differential sperm uptake during copulation, and sperm selection within the female reproductive tract after copulation (e.g., Bretman et al., 2004; Mack et al., 2002; Nilsson et al., 2003). Recent studies have attempted to control sperm numbers inseminated by kin and nonkin using indirect measures of sperm transfer, such as the duration of spermatophore attachment (Bretman et al., 2004; Jennions et al., 2004; Tregenza and Wedell, 2002), but the evidence for postcopulatory sperm selection against inbreeding is mixed.

In vertebrates, the evidence for female sperm selection based on the genotype of conspecific sperm is also inconclusive. Two studies in the guppy (*Poecilia reticulata*) showed

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directional postcopulatory sexual selection for more colorful males (Evans et al., 2003; Pilastro et al., 2004). However, superior fertilization success of more colorful males can be the result of such males transferring ejaculates of superior quality (Evans et al., 2003) and/or females accepting more sperm from more colorful males (Pilastro et al., 2004). Whether there is further sperm selection within the female reproductive tract can neither be confirmed nor rejected by these studies. A similar problem applies to studies on sand lizards (*Lacerta agilis*; Olsson et al., 1996, 1997), which show that genetic relatedness explains a significant part of the variation in fertilization success under sperm competition. However, this effect can also be attributed to unrelated males transferring more sperm or females accepting more sperm from unrelated males. An experimental study in the domestic fowl (*Gallus gallus domesticus*) found that paternity success varied across females, which were inseminated with equal numbers of sperm from two males (Birkhead et al., 2004). It remained unclear whether this is the result of cryptic sperm selection or early embryo mortality. Recently Pizzari et al. (2004b) demonstrated that female red jungle fowl (*Gallus gallus*) retained fewer sperm after natural inseminations by brothers despite the fact that in a second experiment male fowl were found to inseminate even more sperm into sisters than into unrelated females. Again it remains unclear whether females differentially ejected sperm or whether sperm selection took place within the female reproductive tract. Support for sperm selection at the level of the egg stems from an in vitro fertilization experiment with mice (Rülicke et al., 1998). This study observed nonrandom fertilization with respect to major histocompatibility complex haplotype. Although there is some further evidence of selective immunological reactions against sperm inside the mammalian reproductive tract (Cohen and Werrett, 1975; Dondero et al., 1978; review in Zeh and Zeh, 1997), several other studies failed to detect any effect of female sperm selection (Cunningham and Cheng, 1999; Stockley, 1997).

The above suggests that the mixed evidence for cryptic sperm selection might partly stem from the difficulties to disentangle female-mediated effects on the outcome of paternity from biases caused by sperm competition (Birkhead, 1998, 2000; Eberhard, 2000; Kempnaers et al., 2000; Pitnick and Brown, 2000). Sperm competition and cryptic female choice are two processes that occur simultaneously. When a female copulates with several males, sperm from these males will compete inside the female's reproductive tract to fertilize her ova (Parker, 1970). Thus, any observed bias in paternity may be purely male mediated due to differences in the amount of transferred sperm (Cook and Wedell, 1996), variation in sperm quality (e.g., motility; Birkhead et al., 1999) or size (Radwan, 1996), or mating order (Birkhead and Parker, 1997). To clearly demonstrate female sperm selection within the female reproductive tract, it is essential to control for these effects. Earlier studies based on natural matings did not control for the numbers of transferred sperm (e.g., Clark et al., 1999; Olsson et al., 1996; Stockley, 1997; Wilson et al., 1997) or did so only in indirect ways via male size and age (Stockley, 1999) or via the presence of a spermatophore (Bretman et al., 2004; Jennions et al., 2004; Tregenza and Wedell, 2002). No information about individual male sperm quality was available in any of these studies.

We studied the relative importance of postcopulatory female sperm selection and sperm competition in mallards (*Anas platyrhynchos* L.). Species like mallards are of particular interest for such studies because females frequently lack behavioral (precopulatory) mechanisms to control the transfer of sperm. Mallards form socially monogamous pairs in autumn, and it is assumed that females base their mate choice

predominantly on indirect (genetic) benefits because drakes do not provide obvious direct benefits such as territories or help with brood care (Cunningham, 1997). Although female mallards show strong preference for their social partner (Bluhm and Gowaty, 2004) and apparently do not incite but strongly resist extrapair copulations (Cunningham, 2003), they commonly suffer from coerced copulations by other males (Cunningham, 1997; Davis, 2002). However, copulations with nonpreferred males lead to significant fitness reduction for female mallards (decreased offspring viability and mother productivity; Bluhm and Gowaty, 2004). Unlike most bird species, drakes possess a penis-like intromittent organ, which allows males to deposit the ejaculate deep inside the female's reproductive tract. This should further reduce female behavioral control because sperm ejection (Pizzari and Birkhead, 2000) might be less likely. Therefore, ducks might have evolved other postcopulatory mechanisms to assure fertilization by the preferred male.

In a previous study of sperm selection in mallards, Cunningham and Cheng (1999) artificially inseminated ducks with a mixture of sperm from males of two different genotypes (white plumage and wild type). This study failed to detect consistent sperm use between inseminations and therefore dismissed cryptic sperm selection by female mallards purely based on genotype. However, in the study of Cunningham and Cheng (1999), sperm of eight different males per genotype was pooled for insemination, and therefore females were inseminated with a mixture of sperm from 16 different males. No information on the representation of each single male in the insemination mixture or about sperm quality was available. Because both sperm density (numbers per unit of ejaculate volume) and quality can vary dramatically between ejaculates in this species (Stunden C, personal communication; this study), variation in composition and quality of the used sperm mixture might have obscured effects of female sperm choice.

The aim of our study was to investigate the relative importance of sperm characteristics and cryptic female sperm selection in determining paternity. Here we present the results of an experiment where female mallards were artificially inseminated with a sperm mixture containing equal sperm numbers from one brother and from one unrelated male. This method allowed us to rule out effects of mating order, to control for the number of transferred sperm, and to measure the quality of the sperm from each male. We used sperm from a brother and an unrelated male because matings between siblings represent an extreme case of inbreeding and reduction in heterozygosity. If mechanisms to avoid inbreeding or to increase offspring heterozygosity have evolved, we expected them to become apparent in such an extreme case.

Our experimental procedure allows us to make the following predictions. (1) If fertilization success is solely based on the number of transferred sperm, and neither sperm quality nor cryptic female sperm choice influences the outcome, both competing males should gain equal amounts of paternity. (2) If sperm quality determines fertilization success, the male with the highest sperm quality should gain most paternity, independent of his relatedness to the female. (3) If sperm selection enables female mallards to discriminate against sperm of closely related males in order to avoid negative effects of inbreeding, the unrelated male should gain most paternity.

MATERIALS AND METHODS

Animals

In 2000, we caught nine pairs of wild mallards at Starnberger See (47° 54' N/11° 18' E) and Ammersee (48° 00' N/11° 08' E)

in Southern Germany. Eggs laid by those (presumably) unrelated mallards were artificially incubated, and ducklings were raised in large groups in 2000 and 2001. Parentage of all ducklings was confirmed by microsatellite analysis (see below).

Ducks were kept in two 60-m² outdoor aviaries with a 4-m² concrete pond each and seven outdoor aviaries (54–68 m²) situated at the shore of a small lake with two-third of the area covered by water. Birds were fed commercial duck food (Anseres 3, Kasper Faunafood, Woerden, Netherlands) mixed with wheat, except during the breeding season, when we provided special breeding pellets (Anseres 4). The birds received fresh lettuce at least three times a week.

Experimental design

We used a sperm mixture containing equal numbers of sperm from two unrelated males (sperm donors) to artificially inseminate four sisters of each male, that is, at each insemination eight females were inseminated with the same sperm mixture. We conducted the experiments with 12 groups of four sisters, from a total of nine genetically unrelated families. One male per family was used as the sperm donor. In total we had six different pairs of males whereby three males were used in two different combinations. We performed repeated inseminations using the same individuals (sisters and male pairs) in identical combinations throughout the study.

Between April and June 2002 sperm collection and inseminations took place once a week, and inseminations were conducted when viable sperm of both males could be collected (at least 30% of sperm cells were motile after sperm collection). In total we performed 224 inseminations on the 48 individual females over a period of 8 weeks (4.7 inseminations per female, range: 3–7; SE \pm 0.7). For practical reasons inseminations were conducted for all females on the same dates and could not be matched with individual egg-laying cycles.

Females were housed in groups of four sisters, isolated from males. Groups were kept either separately, or together with another genetically distinct family (not inseminated by the same pair of males), or together with ducks that were not inseminated. Each aviary contained $N = (\text{number of ducks} + 2)$ nesting boxes and nesting material (straw).

Semen collection and artificial insemination

Sperm donors were kept isolated in small aviaries (2.4–6.1 m², including a water tub) together with one female from the end of January 2002 onwards. To prevent copulation shortly before sperm collection, the female was removed the day before each collection and returned afterwards. From the end of April onwards males were kept singly. Food was removed on the evening before sperm collection to minimize fecal contamination of ejaculates and was replaced immediately after collection.

Sperm were obtained according to the massage procedure described in Lake and Stewart (1978), modified by collecting the ejaculate directly in a 1-ml plastic syringe placed at the base of the intromittent organ, where the ejaculate emerges. Sperm samples of the pair of sperm donors were always taken within 30 min and inseminated within 1 h after collection. On average we obtained an ejaculate size of 12.7×10^7 spermatozoa (range: 3.6×10^7 – 47×10^7 ; SE $\pm 1.9 \times 10^7$; ejaculate volume: 50–200 μ l), which is comparable to ejaculate sizes obtained with the same procedure in another mallard study (5.3×10^7 – 10.6×10^7 spermatozoa; Stunden et al., 1998). To prevent sperm dehydration and to facilitate insemination of accurate volumes (and hence sperm numbers) we used Ringer-Lactate (Ringer-Lactate after Hartmann, Z.Nr. 1-19.566, Mayrhofer Pharmazeutika GmbH, Linz, Austria) as sperm extender (Humphrey, 1972; Smyth, 1968). Immediately after collection,

the ejaculate was diluted approximately 1:3 with Ringer-Lactate, and this suspension was used in further procedures.

The concentration of sperm in the suspension was determined in three counts of two dilutions in an improved Neubauer counting chamber (repeatability of counts within ejaculates: $r = .925$, $n_0 = 6$, $p < .001$, $N = 56$ ejaculates; Lessells and Boag, 1987). Immediately prior to insemination we mixed the sperm suspensions so that one insemination volume contained equal numbers of spermatozoa from the two males. Ducks were inseminated with the maximum number of sperm available per male combination; on average 3.18×10^7 sperm (range: 3×10^6 – 1.2×10^8) diluted in 200 μ l Ringer-Lactate. The sperm mixture was put approximately 8–10 cm inside the female reproductive tract using a syringe (Knoll, 1978).

Sperm measurements

Sperm quality was analyzed using a Hobson Sperm Tracker (Hobson Tracking Systems Ltd., Sheffield, U.K.). According to the method of Froman and Feltmann (2000) we used a blood-sperm suspension to keep sperm motile in the sperm swimming chamber. Blood (100 μ l) was obtained from a non-experimental, unrelated female mallard (a different female for each insemination) and diluted in 900 μ l TES-buffered saline (Froman and Feltmann, 2000; TES: Sigma Chemical Co., St. Louis, Missouri, USA). Because blood plasma was sufficient to keep sperm activity constant (Denk and Kempnaers, unpublished data), we used only the clear phase of the blood-TES-buffered saline suspension. The ejaculates in Ringer-Lactate solution (see above) were diluted 10:1 with the blood-TES-buffered saline solution to a final concentration of 3 million cells per milliliter.

We injected the sperm suspension in a preheated (38°C) MicroCell swimming chamber (50 μ m depth; Conception Technologies, San Diego, California, USA). Sperm motility was measured at 38°C, which is slightly below avian body temperature, to reduce the effects of evaporation on sperm behavior. We used a microscope with a 4 \times bright-field objective under pseudo dark-field conditions using a Ph3 annular phase ring and a total magnification of 48. We videotaped sperm for 15 min after injection in the sperm swimming chamber. For each ejaculate three replicates were recorded. We used the Hobson Sperm Tracker to measure straightline velocity (VSL) and the number of tracks (a measure of the percentage of motile sperm), with the “minimum track time” set at 1.2 s for individual sperm (Froman and Feltmann, 2000). Measurements were taken at four time intervals after injection in the swimming chamber: 0–2 min, 4–6 min, 8–10 min, and 12–14 min.

Sperm motility was also estimated visually (an easy and reliable technique commonly used in artificial insemination (AI) for commercial breeding; e.g., Knoll, 1978). At 5 min and 2–2.5 h (identical times for both ejaculates in each pair) after dilution we placed a drop of sperm suspension (diluted as described above) in an improved Neubauer counting chamber at 38°C. We videotaped the sample under 25 times magnification (phase-contrast ring 100) using an Olympus BH-2 microscope under standard bright-field conditions. Per ejaculate we filmed three replicates for 1 min each. Two people independently assessed the percentage of motile sperm (0–100%, in steps of 10%). Values obtained by the two observers were highly repeatable (repeatability $r = .94$, $N = 291$ assessments, $p < .0001$).

To measure sperm length, we stored samples from each ejaculate in formaldehyde (10%; Roti@-Histofix, Carl Roth GmbH, Karlsruhe, Germany). A drop of this solution was placed on a slide and observed under an Olympus BH-2 microscope at 200 \times magnification (phase-contrast ring 40).

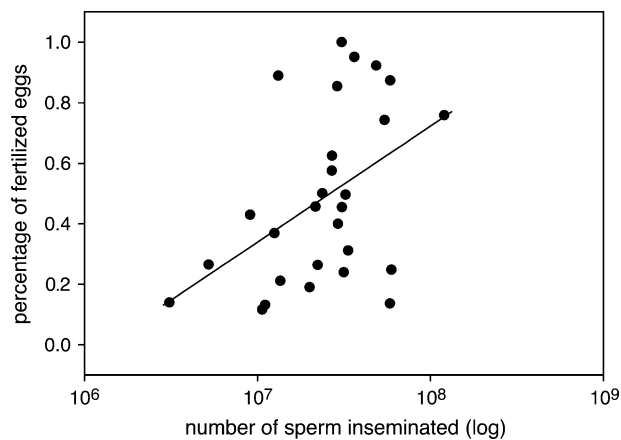


Figure 1

Fertilization success in relation to the number of sperm artificially inseminated (Spearman rank correlation: $r = .42$, $p = .028$; $N = 28$ insemination events of eight females each). Note the logarithmic scale. The solid line represents the regression line. See Materials and Methods for further details.

Three slides were made for each ejaculate, and the total length of 20 sperm per slide was measured using imaging software (Optimas 6.5, Media Cybernetics, Silver Spring, Maryland, USA). Abnormal sperm (e.g., bent heads, heads without tails) were not found.

Fertilization success

Eggs were collected every second day and transferred to an incubator (computer-assisted motor-incubator SV250, J. Hemel Brutgeräte, Verl-Kaunitz, Germany). After 3 days of incubation, embryo development was checked by candling with an ORBAN candling lamp (Tempo Nr. 119). Developing eggs were opened, and the embryo was stored in 70% ethanol. Of 114 developing eggs, three contained visibly dead embryos and two showed disturbed embryonic development (defunct blood vessels). Four of these five eggs were sired by a brother. Undeveloped eggs were left in the incubator for another 3 days before being opened to look for signs of development or early embryonic death (e.g., circular contracted blood vessels; Kosin, 1944, 1945); none of these eggs showed such signs.

Maternity of all fertilized eggs was confirmed by microsatellite analysis (see below) and showed that they belonged to 36 different females, in “clutches” of up to four eggs after an AI event. This clutch size is lower than normal mallard clutch sizes, probably because laying cycles of females were not matched to insemination events (i.e., part of the “clutch” was laid before insemination).

Undeveloped eggs could not be unambiguously attributed to individual females, and molecular assignment of maternity of undeveloped eggs on the basis of genetic markers is difficult (e.g., Arnold et al., 2003). Therefore, we estimated fertilization success, taking into account the fact that ducks require AI every 4–5 days to keep producing fertile eggs (Lake and Stewart, 1978; Smyth, 1968). The total number of eggs that could potentially have been fertilized ($N = 294$) was then determined as the total number of eggs laid in the aviary during the first 4 days after an insemination event, multiplied by the proportion of ducks inseminated in an aviary. Fertilization success after AI was on average 48% (range: 13–100%, Figure 1). This is probably an underestimate because some inseminations took place after the period when the next day's egg could have been fertilized.

Fertilization success of our AI was low compared to fertilization success in clutches of free-living mallards (96%; Denk AG

and Kempnaers B, unpublished data) and somewhat lower than reported in other AI studies (11.4% complete failure, Cunningham and Cheng, 1999; 66%, Knoll, 1978; 70%, Stunden et al., 1998). However, these studies used 2–12 times as much sperm as we did. In our experiment, females were inseminated once a week with 25% of a male's ejaculate, whereas free-living female mallards copulate on average twice a day (Cunningham, 1997). Thus, our reduced fertilization success might have been caused by sperm depletion. Indeed, we found a positive relationship between the number of inseminated sperm and the percentage of fertilized eggs (Figure 1; general linear mixed models [GLMMs]—response variate: number of fertilized eggs 4 days postinsemination; binomial denominator: total number of eggs; explanatory variate: number of sperm inseminated; random factor: experimental group; Wald $F = 3.21$, $df = 1$, $p = .073$). To verify whether undeveloped eggs resulted from sperm depletion, rather than early embryo mortality, we examined the perivitelline membranes in a random sample of 20 undeveloped eggs from clutches which contained at least one fertilized egg, according to the method described by Birkhead et al. (1994). Sperm were stained with the fluorescent Hoechst dye 33258. Observations on naturally fertilized eggs suggested that sperm are evenly distributed over the entire perivitelline membrane (Wishart and Staines, 1999). Our own examination of 12 naturally fertilized mallard eggs (from four broods) indicated a higher sperm density on areas other than the blastodisc region (mean number of sperm/mm² \pm SE: blastodisc: 19 ± 4.8 ; other region: 32 ± 7.7 ; restricted maximum likelihood [REML] analysis: response variate: number of sperm per square millimeter; fixed factor: membrane area (blastodisc or other region), Wald $F = 6.69$, $df = 1$, $p = .01$; random factors: duck identity, $p = .041$; egg identity nested in duck identity $p = .23$). Therefore we searched for trapped sperm at three randomly chosen regions of the perivitelline membrane (at 200 \times magnification), checking 5–10% of the total membrane area. No sperm cells were detected on any of the 20 eggs. Because the probability of fertilization is correlated with the number of sperm found on the membrane (Wishart and Staines, 1999), our findings suggest that the eggs failed to develop because of a lack of sperm.

Parentage analysis

To extract DNA from embryos we used DNeasy® Tissue Kit (Qiagen, Hilden, Germany). Parentage was assigned by using seven polymorphic microsatellite markers (*APL 2*, *APL 11*, *APL 12*, *APL 14*, *APL 23*, *APL 26*, *APL 36*; Denk et al., 2004). Amplified fragments were resolved on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Weiterstadt, Germany). In the nine unrelated males used in this study, we found 6–11 alleles per locus, leading to a combined exclusion probability of more than 99.98% (Jamieson and Taylor, 1997). All offspring alleles were unequivocally assigned to one of the females and to one of the two males used in an insemination.

Statistical analyses

We calculated the repeatability of measurements of sperm characteristics both within an ejaculate (measurement repeatability) and between ejaculates (seasonal repeatability, Table 1). Measurement repeatability was high ($r = .59$ – $.85$, all $p < .001$), suggesting that our methods were reliable and measurement error was low. Sperm size was not significantly correlated with swimming performance (VSL, motility; all $p > .07$), so they were considered independent variables in all models.

Because we performed repeated inseminations of related females our data were structured, with repetition at three

Table 1
Repeatability of sperm measurements within males across ejaculates (seasonal repeatability)

Sperm characteristics	Seasonal repeatability			
	Statistic	<i>r</i>	<i>n</i> ₀	<i>p</i>
Size	$F_{8,47} = 20.45$.76	6.17	<.001
Swimming				
VSL (0–2 min)	$F_{8,48} = 2.34$.18	6.26	.033
VSL (4–6 min)	$F_{8,47} = 2.21$.17	6.16	.044
VSL (8–10 min)	$F_{8,42} = 2.48$.21	5.70	.027
VSL (12–14 min)	$F_{8,40} = 2.06$.16	5.39	.064
Track (0–2 min)	$F_{8,48} = 1.22$.05	6.26	.27
Track (4–6 min)	$F_{8,47} = 0.84$	-.03	6.16	.57
Track (8–10 min)	$F_{8,42} = 1.21$.04	5.70	.32
Track (12–14 min)	$F_{8,40} = 1.42$.07	5.39	.22
Overall motility (5 min)	$F_{8,20} = 2.10$.26	3.12	.085
Overall motility (2 h)	$F_{8,44} = 2.01$.15	5.98	.067

*n*₀, average number of repeated measures; VSL, straightline velocity; track: number of tracks recorded in 2 min; motility: percentage motile cells as scored by eye (see Materials and Methods for details). Significant results are indicated in bold.

levels: (1) the female (repeated insemination of each female), (2) the family (group of related females), and (3) pairs of males used for repeated insemination. Such structured data are best analyzed using mixed models, where the random term specifically models the pseudoreplication associated with the structure (Grafen and Hails, 2002: Chapter 12). To analyze changes in sperm swimming traits over time, we used REML mixed models with male identity as a random factor (to control for multiple ejaculates per male). We compared the number of observed tracks and sperm swimming speed (VSL) at each of two consecutive time intervals (see Figure 2).

To test the effects of relatedness and sperm quality on fertilization success, we constructed GLMMs with a binomial error, using “number of eggs sired by male A” as response variable and “clutch size” (all fertilized eggs laid after a single insemination) as the binomial denominator. One of the males represented in each sperm mixture was randomly (male with the lower leg band number) assigned “male A,” and his gain of paternity (proportion of eggs sired) was analyzed (as in Evans et al., 2003). In all analyses, the difference in sperm quality between males A and B was used as the explanatory variable. Relatedness, sperm size, and one measure of sperm quality (either number of tracks, VSL, or percentage motile cells) were fitted as fixed effects. Sperm quality measurements made at different time intervals (see above) were analyzed in separate models. We included “female id,” “family,” and “male pair” as random effects to account for repeated inseminations of groups of related females with sperm from the same pair of males. Female id and family were never significant (all *p* > .70), but the effect of male pair sometimes was (.3 > *p* > .02). Inclusion or exclusion of these factors did not change any of the conclusions, nor did the inclusion of the random factor “insemination event” (to control for differences among insemination events).

We initially constructed full models containing all explanatory variables. Nonsignificant terms were dropped from the model until the final model only contained variables with *p* < .10. All eliminated terms were then readmitted to the final model to confirm their lack of significance, and these *p* values are reported here. We included the interaction term relatedness × sperm quality measure in the model to test the hypothesis that sperm have to be of higher quality (swim faster) to be successful if the male is closely related to the female. Other interac-

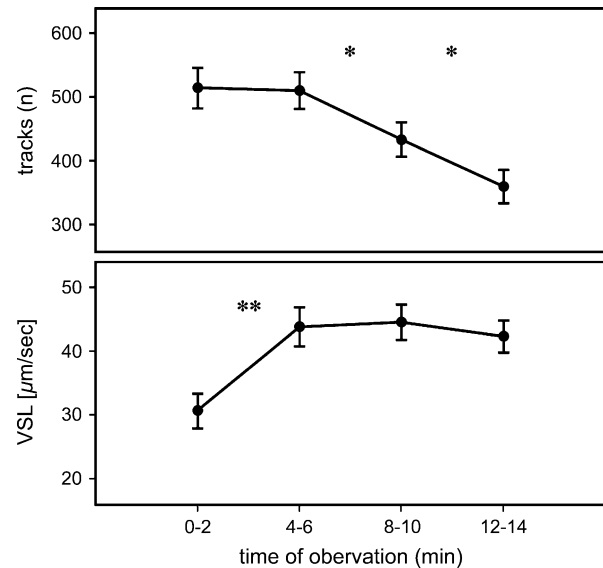


Figure 2
 Change of sperm swimming behavior (number of tracks and straightline velocity [VSL]) over time (*N* = 56 ejaculates). Changes are significant at *p* < .05 (*) and *p* < .001 (**) based on REML analyses controlling for male effects (*N* = 9 males; see text for details). Error bars indicate SE values.

tion terms could not be tested due to low sample size. We used Genstat 6.1.0.200 for all statistical analyses.

RESULTS

Sperm characteristics

Taking each ejaculate as a data point (*N* = 56), mallard sperm showed a mean VSL (\pm SE) of $41.77 \pm 1.56 \mu\text{m}/\text{s}$ (range 4.83–88.20) and an overall percentage of motile sperm (\pm SE) of $48.1 \pm 3.3\%$ (range 5–88.67%). The VSL and number of recorded tracks varied significantly over the observation period (REML; VSL: Wald $\chi^2 = 8.05$, *df* = 1, *p* < .001; number of tracks: Wald $\chi^2 = 6.60$, *df* = 1, *p* < .001). VSL increased between the first two observation periods and remained at a constant level thereafter (see Figure 2). The increase in VSL was not simply caused by a reduction in the number of recorded tracks because the number of tracks decreased only after 6 min (see Figure 2). The average percentage of motile sperm did not change significantly between 5 min and 2–205 h after dilution.

During the course of the study (8 weeks) sperm size was highly repeatable among males, but the seasonal repeatability of measurements of sperm quality was much lower, and not always significant (see Table 1).

Paternity

A male's fertilization success was related to long-term sperm performance. Both the number of tracks and VSL after 8 min or more significantly predicted paternity (Table 2; Figure 3A). In general, sperm motility was the best predictor of paternity (Table 3; Figure 3B), while sperm size did not affect fertilization success (Tables 2 and 3). Given the absence of significant random effects and the large individual variation in sperm quality, we also present simple correlations between sperm quality measurements and fertilization success: VSL 8–10 min, *r* = .261, *p* = .028, *N* = 73; VSL 12–14 min, *r* = .297, *p* = .012, *N* = 70; motility 2 h, *r* = .373, *p* = .002, *N* = 69.

Table 2
Results from GLMM analyses of factors determining fertilization success and correcting for repeated measures

Independent variants	0–2 min		4–6 min		8–10 min		12–14 min	
	Wald <i>F</i>	<i>p</i>	Wald <i>F</i>	<i>p</i>	Wald <i>F</i>	<i>p</i>	Wald <i>F</i>	<i>p</i>
Relatedness	0.06	.80	0.10	.76	0.21	.65	0.07	.79
Sperm size	0.64	.42	0.75	.39	1.57	.21	1.68	.19
Number of tracks	2.25	.13	3.46	.063	6.39	.011	5.87	.015
Relatedness	0.05	.82	0.13	.72	0.02	.89	0.03	.87
Sperm Size	0.52	.47	0.56	.45	0.60	.44	1.19	.28
VSL	2.48	.12	1.91	.17	4.55	.033	6.18	.013

Four separate models were constructed for each measurement time (time after injection in the sperm swimming chamber). These models were built separately for number of tracks and straightline velocity (VSL; see Materials and Methods for details). The Wald statistic is shown, which follows a chi-square distribution with $df = 1$. Significant results are indicated in bold.

Whether or not a male was related to the female did not have any effect on fertilization success (see Tables 2 and 3; Figure 3C). This remained true if only insemination events were analyzed in which the ejaculates from both males showed similar VSL or percentage of motile cells (maximum difference: ± 4 SE, $N = 26$ clutches, details not shown). Ejaculates from three males were used in two experiments (different male pairs). Reducing the data set so that each male appeared in only one comparison did not change the significance levels despite smaller sample size ($N = 49$ clutches, details not shown).

We did not find evidence that fertilization success depended on an interaction between sperm quality and relatedness (in all cases, $p > .05$).

DISCUSSION

Our experiment shows a clear effect of variation in sperm quality (swimming speed and sperm motility) on fertilization success in mallards. The degree of genetic similarity between parents did not influence paternity when ducks were artificially inseminated with a sperm mixture of a first-degree relative and an unrelated male. This may suggest that female mallards were not able to select sperm purely based on the sperm's genotype. Furthermore, unlike a study on sedge warblers (*Acrocephalus schoenobaenus*; Marshall et al., 2003), the allele frequency of the successful sperm did not deviate from a distribution expected by chance ($p = .29$, details not shown).

The results of the present study emphasize the importance of sperm quality for fertilization success (Snook, 2005). In our study, long-term sperm behavior was the important factor predicting paternity. The effect sizes of number of active sperm, sperm swimming speed, and overall motility increased over time and only became significant after eight or more minutes. Birkhead et al. (1999) also found that sperm quality determined paternity under sperm competition. They artificially inseminated domestic fowl, *G. gallus*, with a sperm mixture containing ejaculates from one male with low- and one with high-mobility sperm. Males with high-mobility sperm fathered the majority of offspring. Birkhead et al. (1999) found no female effect on variation in paternity, highlighting the importance of sperm competition for fertilization success.

Although studies on insects and lizards suggest that females might be capable of sperm selection based on compatible sperm genotypes (Clark et al., 1999; Stockley, 1999; Wilson et al., 1997), our findings corroborate the conclusions from previous studies on birds and mammals (mallards: Cunningham and Cheng, 1999; common shrew, *Sorex araneus*: Stockley, 1997). Even though a detailed assessment of sperm quality

was missing in the latter two studies, no effect of sperm selection was detected, and fertilization success could be explained in terms of sperm competition.

Even in the absence of female sperm selection, sperm competition per se might be a mechanism by which females select the "best" male. If ejaculate quality reflects male quality, females might have the majority of their offspring sired by the high-quality males. However, there is little evidence that genetic quality translates into sperm quality. In a mammal (lion, *Panthera leo*) genetic quality (individual genetic diversity) influenced sperm quality (e.g., incidence of abnormal sperm; Wildt et al., 1987). In mallards, sperm swimming speed (VSL at 8 min or more) correlated with a measure of the carotenoid-based beak color (Peters et al., 2004), a sexually selected trait in this species (Omeland, 1996a,b). However, other studies failed to find a correlation between preferred male phenotypic traits and sperm quality in birds (Birkhead and Fletcher, 1995; Birkhead and Petrie, 1995; Birkhead et al., 1997).

By ensuring fertilization by the faster and more motile sperm, females might also increase the chances that their sons will produce competitive sperm, if sperm quality is a heritable trait passed on by the father (sexually selected sperm hypothesis; Keller and Reeve, 1995). An experimental study on fowl, *G. g. domesticus*, showed that sperm mobility was highly heritable (Froman et al., 2002). However, the study also suggested that sperm mobility may be largely under the control of an independent maternally inherited element (Froman et al., 2002). In that case, fertilization by the faster sperm would not influence the competitive ability of sons. To our knowledge nothing is known about the heritability of sperm quality in mallards.

During our study, sperm quality (sperm swimming speed and motility) showed high variability within individual drakes (see Table 1), in agreement with observations in passerine species (Birkhead and Fletcher, 1995) but in contrast to domestic fowl (Froman and Feltmann, 2000; Froman et al., 1999). Variation in sperm quality between ejaculates might stem from difficulties to collect sperm samples from drakes, related to their penis-like intromittent organ. Fecal contamination (although not visible in the samples used in our experiments) or excess lymph fluid might affect sperm behavior (Denk AG and Holzmann A, personal observations). An alternative explanation for variability in sperm quality is that it reflects natural fluctuations. Regardless, the artificial or natural fluctuation of sperm quality is not expected to affect the results of our study because each insemination event was analyzed separately. However, it emphasizes the importance of careful measurement of sperm quality in experiments on cryptic female sperm choice.



Figure 3
Mean percentage of eggs sired after a single insemination by a male, when sperm from this male showed (A) a lower or higher straight-line velocity (VSL at 12–14 min) ($N = 70$ clutches) or (B) a higher or lower overall motility (percentage motile cells at approximately 2–2.5 h after dilution) than his competitor ($N = 69$ clutches) or (C) when the male was a brother of the female or unrelated ($N = 73$ clutches). Error bars indicate SE values. p Values are based on GLMMs (see text for details).

It is still possible that cryptic female sperm selection occurs in mallards, but we were unable to demonstrate it, for the following reasons. A general problem with experiments such as ours arises if a bias in paternity due to early embryo mortality is erroneously interpreted as cryptic female choice.

Table 3

Results from GLMM analyses of the effects of motility (the percentage motile cells, 5 min and 2–2.5 h after dilution with sperm extender) on fertilization success, correcting for repeated measures (see Materials and Methods for details)

Independent variants	5 min		2 h	
	Wald F	p	Wald F	p
Relatedness	0.06	.81	0.00	.98
Sperm size	0.04	.85	0.13	.72
Motility	0.30	.58	8.23	.004

The Wald statistic is shown, which follows a chi-square distribution with $df = 1$. Significant results are indicated in bold.

However, the results of our study are unlikely to be an effect of cryptic female choice favoring the unrelated male, unless early embryo mortality was more frequent when the unrelated male fertilized the egg and remained undetected. Only this unlikely scenario would result in apparent equal paternity of the related and unrelated male.

Second, one could argue that our experiment lacked the power to detect sperm selection because of the low sample size. This might be the case, but (1) the data do not show the slightest tendency for sperm selection based on relatedness (see Figure 3C) and (2) despite the limited sample size, significant effects of sperm quality were found (see Figure 3A,B). Thus, our results at least indicate that within the female reproductive tract, sperm competition plays a much more important role in determining fertilization success than postcopulatory female sperm selection based on sperm genotype.

Third, as a concession to the experimental setup, females were inseminated with only a fourth of a natural ejaculate once a week, and females may thus have been sperm limited (see Materials and Methods). This would be a problem if sperm selection mechanisms only work when sufficient numbers of sperm are present.

Finally, it is possible that female sperm selection occurs after natural copulations, but not after AI. Maybe one or more aspects intrinsic to the AI technique (contamination of the sample, buffer properties, storage) compromise the sperm in such a way that cryptic female choice can no longer operate at the level of the sperm. Furthermore, during natural copulations females might obtain additional information about their partners, which might affect the success of different ejaculates. For example, Pizzari and Birkhead (2000) showed that female feral fowl can control paternity by selectively ejecting sperm from nonpreferred males, which is another postcopulatory female mechanism to ultimately bias male fertilization success. Because we used a sperm mixture in our experiment, female mallards lacked the possibility to selectively eject sperm. Whether sperm ejection is possible in species such as mallards, where males have an intromittent organ and deposit the ejaculate further into the female reproductive tract, is unknown. If sperm ejection occurs after forced (artificial) insemination, it could account for the low fertilization success in this study. In chicken and turkey, females eject 80–90% of (artificially) inseminated sperm (Birkhead et al., 1993, and citations therein). Moreover, natural copulations may result in the sequential filling of sperm-storage tubules (Briskie, 1996; King et al., 2002). Segregation of ejaculates from different males within the female reproductive tract might be an important prerequisite to selectively use sperm. Inseminations with mixed ejaculates would prevent the operation of such a mechanism. Nonetheless, despite these problems, AI is the only way to experimentally control for male adjustment of

ejaculate size, for example, in response to the risk of sperm competition (Cook and Wedell, 1996) or in response to female traits like age, body size, or reproductive investment (e.g., Cook and Gage, 1995; Pizzari et al., 2003; Shapiro et al., 1994; Wedell, 1992).

In conclusion, we found that long-term sperm performance is an important determinant of fertilization success under direct sperm competition in this wild bird species. Although it is likely that offspring sired by a close relative suffer a higher risk of embryonic death, we did not observe evidence that sperm selection purely based on sperm genotype occurs within the female reproductive tract. Whether the success of faster and more motile sperm is a mechanism by which females ensure fertilization by the best male (female sperm selection favoring males producing faster sperm), or whether males are ahead in the intersexual conflict over fertilization, and why female mallards have not yet developed effective mechanisms to resist forced copulations needs further investigation.

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