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PASSIVE SPERM LOSS AND PATTERNS OF SPERM PRECEDENCE IN MUSCOVY DUCKS (*CAIRINA MOSCHATA*)

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ABSTRACT.—We investigated the importance of passive sperm loss in the outcome of sperm competition in captive, wild-type Muscovy Ducks (*Cairina moschata*). In nature, Muscovy Ducks can be expected to experience more intense sperm competition than most other waterfowl because of their non-monogamous mating system. We estimated the instantaneous rate of sperm loss from the reproductive tracts of female Muscovy Ducks as 0.0235 ± 0.0018 h^{-1} [SE], a fairly typical rate in comparison to the few other species of birds in which passive sperm loss has been measured. We also measured sperm precedence in trials in which a captive female was allowed to mate with two males in succession, with either a 24-h or a 72-h lag between matings. Paternity was determined with microsatellite markers. The mean proportion of a female's eggs fertilized by the second male (P_2) was 0.72 ± 0.14 in trials with the 24-h lag and 0.42 ± 0.13 in trials with the 72-h lag. The last-male precedence observed in the 24-h trials can be explained by a quantitative model in which passive sperm loss alone determines average success, but this model is not consistent with the outcome of the 72-h trials. Other factors, including perhaps postcopulatory female choice, must be acting in addition to passive sperm loss in the trials with the longer lag. *Received 18 August 2009, accepted 20 January 2010.*

Key words: *Cairina moschata*, Muscovy Ducks, passive sperm loss, sperm competition, sperm precedence, waterfowl.

Pérdida Pasiva de Esperma y Patrones de Precedencia Espermática en *Cairina moschata*

RESUMEN.—Investigamos la importancia de la pérdida pasiva de esperma en el resultado de la competencia espermática en patos cautivos de tipo silvestre de la especie *Cairina moschata*. En la naturaleza, se espera que los individuos de esta especie experimenten una competencia espermática más intensa que la mayoría de otros anseriformes debido a su sistema de apareamiento no monógamo. Estimamos la tasa instantánea de pérdida de esperma del tracto reproductivo de hembras de esta especie en 0.0235 ± 0.0018 h^{-1} [EE], un valor relativamente típico en comparación con las pocas especies de aves adicionales en las cuales se ha medido la pérdida pasiva de esperma. También medimos la precendecia espermática en ensayos en los que a una hembra cautiva se le permitó aparearse con dos machos consecutivamente, con un intervalo de 24 h o de 72 h entre apareamientos. La paternidad se determinó mediante marcadores microsatélites. La media de la proporción de los huevos de una hembra que fue fertilizada por el segundo macho (P_2) fue 0.72 ± 0.14 en ensayos con intervalo de 24 h y 0.42 ± 0.13 en ensayos con intervalo de 72 h. La precedencia del último macho observada en los ensayos con intervalo de 24 h podría ser explicada por un modelo cuantitativo en el que la pérdida pasiva de esperma por sí sola determina el éxito promedio, pero este modelo no concuerda con el resultado de los ensayos de 72 h. Otros factores aparte de la pérdida pasiva de esperma, incluyendo quizás la selección postcopulatoria por parte de las hembras, deben estar actuando en los ensayos con intervalo mayor.

SPERM COMPETITION CAN occur whenever individual females mate with multiple males in the course of single breeding attempts. Multiple mating by females is common across animals, so it is not surprising that sperm competition acts as an important selective force in a wide range of animal groups (Parker 1970; Ginsberg and Huck 1989; Birkhead and Møller 1992, 1998; Simmons 2001). In birds, multiple mating can be quite common even in socially monogamous species (Griffith et al. 2002, Westneat and Stewart 2003), but other social mating systems, notably promiscuity, are especially conducive to multiple mating and, thus, to sperm competition. In waterfowl (Anatidae), almost all species are socially monogamous (Oring and Sayler 1992), so we can expect sperm competition to be concentrated in the subset of socially monogamous species with a high frequency of extrapair mating (Afton 1985, Sorenson 1994, McKinney and Evarts 1998, Dunn et al. 1999, Cunningham 2003) and in the small minority of species with non-monogamous social systems (Oring and Sayler 1992, Coker et al. 2002). Here, we investigate sperm competition in one of the latter, the Muscovy Duck (*Cairina moschata*), concentrating on the phenomenon of last-male sperm precedence.

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Last-male sperm precedence is the disproportionate fertilization of eggs by sperm from the last male of a series of two or more to copulate with a female. Last-male precedence is widespread in insects (Ridley 1989) and is usually thought to be the norm in birds as well (Birkhead and Møller 1992, Birkhead 1998). Considerable effort has been put toward finding a mechanism to explain this pattern. In the case of birds, three principal mechanisms have been discussed (Birkhead 1998). "Stratification" proposes that successive ejaculates form layers within the sperm-storage tubules of the female and that the uppermost layer, derived from the last male to copulate, is more available to fertilize eggs than lower layers (Compton et al. 1978, Cheng et al. 1983, McKinney et al. 1984). This mechanism is often described as "last in, first out." A second possibility is "displacement," whereby later ejaculates enter parts of the reproductive tract such as sperm-storage tubules and force out sperm that are already there (Lessells and Birkhead 1990). Third, "passive sperm loss" explains last-male precedence as resulting from the gradual loss of sperm from the female reproductive tract over time; because of such loss, if two ejaculates of equal numbers of sperm are received by a female, fewer sperm of the first will remain to fertilize eggs by the time the second arrives (Lessells and Birkhead 1990). More complicated models can be obtained by combining these mechanisms, for example by pairing stratification with passive sperm loss (Birkhead and Biggins 1998).

Stratification predicts that if a long enough series of eggs is fertilized, the sperm of the last male to copulate will eventually be depleted and the sperm of an earlier male will start to predominate (Lessells and Birkhead 1990). Because little evidence of this pattern has been found in birds, stratification has lost favor as a hypothesis (Birkhead 1998, Birkhead and Biggins 1998). Displacement is a mechanism that has been well demonstrated in insects (Waage 1979, Ono et al. 1989, von Helversen and von Helversen 1991) but for which there is no substantial evidence in birds (Birkhead 1998). In contrast to these first two hypotheses, passive sperm loss has received important support in birds as a general explanation for last-male precedence. The basic assumption that sperm are lost from the female reproductive tract has been verified in several species (Wishart 1988, Birkhead et al. 1993, Cunningham and Cheng 1999, Michl et al. 2002). Moreover, when empirically measured rates of sperm loss have been incorporated into mathematical models, the models have correctly predicted fertilization patterns in certain cases (Birkhead et al. 1995b, Colegrave et al. 1995, Birkhead and Biggins 1998).

The passive-sperm-loss model (Birkhead et al. 1995b) is expressed as $\log_e(P_2/P_1) = d + \mu T - \log_e I$, where P_2 is the proportion of eggs fertilized by the second male, P_1 is the proportion fertilized by the first male, d is the differential fertilizing capacity of the second male in relation to the first, μ is the instantaneous rate of sperm loss from the female reproductive tract, T is the time interval between inseminations, and I is the size of the first insemination in relation to the second. The first term, $\log_e(P_2/P_1)$, measures the success of the second male in relation to the first. This model predicts last-male sperm precedence, as long as the second male does not differ from the first in sperm number or fertilizing capacity per sperm. The model also predicts that the relative success of the second male will increase as the time interval between inseminations increases. This prediction has been supported in several studies (Birkhead and Biggins 1998).

We investigated sperm competition in the Muscovy Duck, which is one of a small minority of waterfowl whose social mating system is considered non-monogamous (Oring and Sayler 1992). The social mating system of Muscovy Ducks has been variously labeled as promiscuous (Delacour 1959, Crawford 1990) or polygamous (Clayton 1984, Todd 1996). An intensive study of free-living, wild-type individuals within their natural range classified the species as promiscuous on the basis of evidence that associations between males and females during the breeding season were generally brief and nonexclusive (Stai 2004). In studying sperm competition in Muscovy Ducks, our first goal was to measure the rate of passive sperm loss, for comparison with other species and as the crucial parameter in the passive-sperm-loss model. Second, we wanted to test for the occurrence of last-male precedence in this species and test the prediction that the magnitude of last-male precedence will increase with the time interval between inseminations. Third, we wanted to determine the quantitative fit between the predictions of the passive-sperm-loss model and the proportion of eggs fertilized by the second male in actual matings. Fourth, we used our genetic data to check whether fertilization success was biased toward males that were less closely related to the female, as has been suggested for other species of birds (Pizzari et al. 2004, Thuman and Griffith 2005).

METHODS

Acquisition and maintenance of birds.—We acquired 12 male and 10 female Muscovy Ducks, all young of the year, from Northwest Wildfowl (Everett, Washington) in November 1999. They were transferred to the Smithsonian Institution's Conservation and Research Center (CRC) in Front Royal, Virginia, in May 2000. Two ducklings, one male and one female, hatched in September 2000 and were raised to adulthood, and two more yearling females were acquired from Northwest in March 2001. Thus, the total captive population size was 26 birds at its maximum. All subjects were wild-type birds, descended from a stock that was originally acquired from Paraguay in the early 1970s (Stai and Hughes 2003).

Ducks were housed year-round in a set of covered outdoor pens on a south-facing slope at CRC. Individual pens were 12.2 × 18.3 m enclosures with 4.6-m^2 ponds in the center. Abundant natural vegetation grew in the pens, providing cover and ground-nesting sites. Wooden nest boxes were added to individual nesting pens during the 2000 season. Ducks had free access to running water and commercial duck feed and were given occasional supplements of mealworms. Females were supplemented with oyster shells during egg laying. Shelters with straw bedding and heat lamps were provided during the winter months.

During the nonbreeding season, all birds were held in a single flock that occupied multiple adjoining pens. From the onset of egg laying in February or March until August or September, males and females were separated into same-sex flocks. The flock of males occupied multiple adjoining pens and had visual and auditory access to females through the wire fencing. Females were held in a flock adjacent to the males until late spring, when they were distributed into individual pens in preparation for trials and nesting.

Experiment 1: Measurement of passive sperm loss rate.—Ten pairs consisting of one male and one female Muscovy Duck were placed each in a separate nesting pen on 27 June 2000. Pairs were

allowed to copulate ad libitum until the first egg was laid, following Cunningham and Cheng (1999). The male was then removed to the holding pen. Each egg was collected on the day it was laid and replaced with a dummy egg until the female stopped laying. The last clutch was completed on 27 July. Eggs were stored at 4°C until dissection.

The number of sperm trapped in the egg's perivitelline layer declines with successive eggs in a clutch, reflecting the decline in sperm numbers in the sperm-storage tubules (Wishart 1987, Brillard and Antoine 1990, Brillard and Bakst 1990). Thus, the $\log_e$ of sperm number found in the perivitelline layer, regressed against time, produces a slope equal to the rate of passive sperm loss (μ). We prepared the perivitelline membrane of each egg for sperm counting according to Wishart (1987). The fluorescent DNA probe (4,6-diamidino-2-phenylindole [DAPI]) and the rinse solution ($Ca^{2+}Mg^{2+}$-free Dulbecco's phosphate-buffered saline [PBS]) were obtained from Sigma-Aldrich (St. Louis, Missouri). We used a 1% solution of DAPI in PBS to stain the membranes and Krystalon to seal the mount. Prepared slides were stored in darkness at 4°C.

We followed Wishart's (1987) counting technique, using fluorescence microscopy and a ×20 objective, to make six scans per slide. Each scan was 10 mm long and 0.940 mm wide (i.e., the diameter of the field of view); thus, all sperm in a 56.4-mm^2 area were counted for each egg. We performed a simple linear regression of $\log_e$ of sperm numbers against time to estimate the rate of sperm loss. Because complete uptake of sperm by the storage tubules did not always occur immediately after the male was removed (as reflected by a lag in the peak number of trapped sperm), the first data point in each regression was the egg with the maximum number of trapped sperm within its own clutch (Birkhead and Petrie 1995).

Experiment 2: Sperm competition trials.—We ran two sets of sperm competition trials, one set with a 24-h lag between matings and the other with a 72-h lag. Females were allowed no physical contact with any male for at least 29 days before participation in a trial so that they would have no or virtually no viable sperm remaining from any earlier insemination. Female Muscovy Ducks will lay eggs without fertilization, as is true of many species of birds (Romanoff and Romanoff 1949). We conducted trials by introducing a male into the female's nesting pen between 1 and 7 h after egg laying, thus avoiding a period of low fertility between −4 to +1 h surrounding egg laying and putting all trials within the +1 to +7 h period of high fertility shown by Raud and Faure (1990) in domestic female Muscovy Ducks. If no egg was laid on a trial day, either the female was palpated before male introduction to rule out the presence of an egg in the oviduct (which would block sperm uptake from a new insemination), or the male was introduced after 1500 hours (i.e., at least 2 h beyond the latest time of day that females were observed laying eggs). The mating of male 1 of a trial took place on the day the female laid the second (infertile) egg of a new clutch, and the mating of male 2 occurred 24 h or 72 h afterward (while maintaining a minimum of 1 h after egg laying before male introduction).

Males were assigned to trials according to the following criteria: (1) each individual was both first male and second male at least once in a set of trials; (2) at least 2 days had passed since the male's previous copulation; (3) a dyad of males was not used together in the same trial more than once in a set of trials; and (4) males were paired such that paternity of offspring would be distinguishable with the available microsatellite genotypes (see below). On occasions when the preassigned male showed no intent to initiate a copulation attempt (i.e., by approaching female, pecking at dorsal feathers) within 30 min of introduction, it was removed from the female's pen and replaced with another male (always maintaining criteria 2 and 4, above, and observing 1 and 3 to the extent possible). In 36 of the 42 trials (86%), a copulation was initiated within 3 min of male introduction to the pen; in the remaining 6 trials, the lag to copulation was 4–49 min. We videotaped all trials and reviewed the videotapes if direct observation was ambiguous to ensure that copulations were behaviorally complete. A copulation was labeled complete when the sequence of copulatory events (mount, grasp, tread, tail bend) ended with a single ejaculatory thrust by the male (McKinney et al. 1983, Sorenson 1994). Only one copulation was allowed per trial; any male that initiated a second attempt was removed immediately. If no second copulation was attempted, the male was left in the female's pen for up to 11 min (mean = 4.5 min) before removal.

Females proceeded to lay eggs until the clutch was complete. We collected eggs on the day they were laid and replaced each with a dummy egg. Eggs were incubated in a Petersime incubator at 37.5°C and 86% relative humidity until ~7 days of embryonic development. We briefly froze and then dissected eggs to collect tissue for genetic analysis (see below) or to confirm lack of development or embryonic death. Tissue samples were preserved in 75 mM NaCl/25mM EDTA/1% SDS.

We report here on 21 successful trials, 8 of which were 24-h trials and 13 of which were 72-h. Trials were conducted during July–August 2000 ($n = 1$), June–August 2001 ($n = 13$), and May 2002 ($n = 7$). Trials involved 10 females (1–3 trials each; mean = 2.1) and 13 males (1–5 trials each; mean = 3.2).

Genetic analysis and paternity assignment.—All adults were genotyped at four microsatellite loci (Stai and Hughes 2003) on an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, California). The mean number of alleles (n_a) and mean heterozygosity (H) for these four loci were lower for individuals from the captive, wild-type stock used in the present study ($n_a = 3.8$, $H = 0.47$) than for a free-living population in Brazil ($n_a = 13.0$, $H = 0.88$; Stai and Hughes 2003). The level of genetic variation found in our captive stock resembled closely the level found in domestic Muscovy Ducks in Brazil (Stai and Hughes 2003). To provide additional genetic information when needed, some adults were genotyped at an additional two loci (APH16 and APH17; Maak et al. 2003) using acrylamide gels. Alleles resolved on the ABI were sized using GENOTYPER, version 2.1, and alleles visualized on acrylamide gels were scored by three independent observers. Offspring were similarly genotyped at the one or more loci for which the possible combinations of parental genotypes allowed paternity assignment (but not at the loci that could not be informative given the genotypes of the possible parents). Because adult genotypes had not been determined when the earliest mating trials occurred, there were seven clutches for which criterion 4 (see above) could not be fully observed. Paternity in those clutches was indistinguishable for 1–4 eggs, whereas 55–78% of the offspring were assignable. The remaining 14 clutches were 100% assignable.

Following Thuman and Griffith (2005), we calculated the genetic similarity between females and their mating partners using

the *R* (total relatedness) parameter from the program Mer (Wang 2002). *R* was estimated using the four microsatellite loci at which all adults were genotyped. We calculated difference in relatedness as the *R* value for the female and male 2 in a given trial minus the *R* for the female and male 1.

Data analysis.—The proportion of eggs fertilized by the first (P_1) and second (P_2) males was calculated only for eggs for which paternity was assignable and that were potentially fertilizable by either male (i.e., for those eggs laid on or after the day following mating by the second male). Mean P_2 was calculated separately for the set of trials with a 24-h lag between matings and for the set with a 72-h lag. Although no two trials within a set used the same combination of individuals, some individuals were used more than once in the same position (e.g., twice as the second male with a different female and first male). To limit pseudoreplication, we recalculated mean P_2 by grouping trials within a set by the identity of the second male, calculating the mean per male, and averaging those means. We calculated 95% confidence intervals (CI) for observed P_2 and compared them with the P_2 expected according to the PSL model based on sperm-loss data from experiment 1. The other variables in the PSL equation were controlled in a statistical sense: because the same males served in both the first and second roles within sets of trials and because individual males had no way of determining whether they were the first or second male within a trial, both fertilizing capacity and size of the insemination should have been, on average, the same for first and second males for both time lags. Accordingly, we used a simplified version of the PSL model in which both d (the difference in fertilizing ability) and $\log_e I$ (the log of the ratio of insemination sizes) equal zero, causing those terms to drop out of the equation for second-male advantage. The terms d and $\log_e I$ should approximate zero only over sets of trials, on average; for any single trial, nonzero values are possible, contributing to error in predicting second-male advantage based on the rate of sperm loss alone.

To test the prediction that the male less closely related to the female should dominate paternity, we calculated Spearman correlations (r_s) between P_2 and the difference in relatedness of the female to male 2 and male 1.

Ethical considerations.—These experiments were conducted with the approval of the Institutional Animal Care and Use Committees of the University of Miami and the Smithsonian Institution Conservation and Research Center.

RESULTS

Experiment 1: Measurement of the rate of passive sperm loss.—Seven of 10 females laid fertile clutches with a mean clutch size of 11.9 eggs (range: 9–19 eggs). Sperm uptake was assumed to be complete for a given female on the day that the maximum number of sperm was found on the perivitelline membrane for that female. Complete sperm uptake took from 1 to 4 days (mean = 2 days) after the day of male removal. After sperm uptake was complete, all 7 females showed a log-linear decline of sperm numbers with time (Fig. 1). The regression of $\log_e$ mean sperm numbers against number of days since complete uptake was highly significant for each female (mean $r^2 = 0.925 \pm 0.022$; results are presented as means ± SE), and the overall mean instantaneous rate of sperm loss was $0.0235 \pm 0.0018\ h^{-1}$ ($n = 7$ females). From a mean of 507 ± 223 on the day of complete uptake, mean sperm numbers in the sampled area of membrane decreased to 4.8 ± 2.1 by the 10th day after male removal ($n = 7$ females), and to 0.3 ± 0.1 for the last egg of clutches with >10 eggs ($n = 3$). The last fertilized egg of the three largest clutches was laid an average of 17 days after male removal (range: 13–23 days), which indicates that few sperm remain viable in the reproductive tract of female Muscovy Ducks for more than ~3 weeks.

Experiment 2: Sperm competition trials.—Average clutch size ($n = 21$) was 12.6 eggs (range: 7–15 eggs). An average of 64%

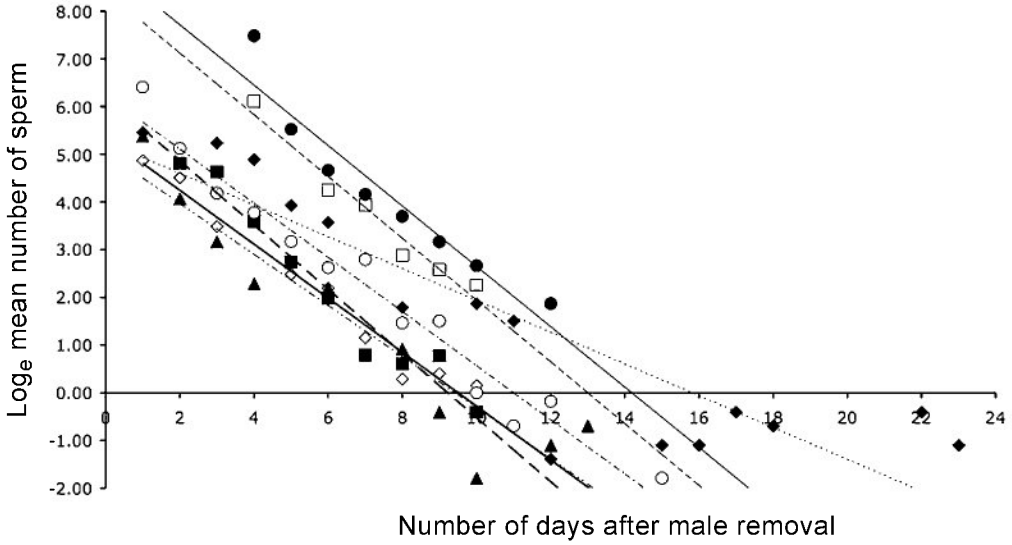

FIG. 1. Linear regressions of sperm numbers found on the sampled area of an egg's perivitelline membrane against number of days after copulation for 7 Muscovy ducks. Each symbol represents a separate female.

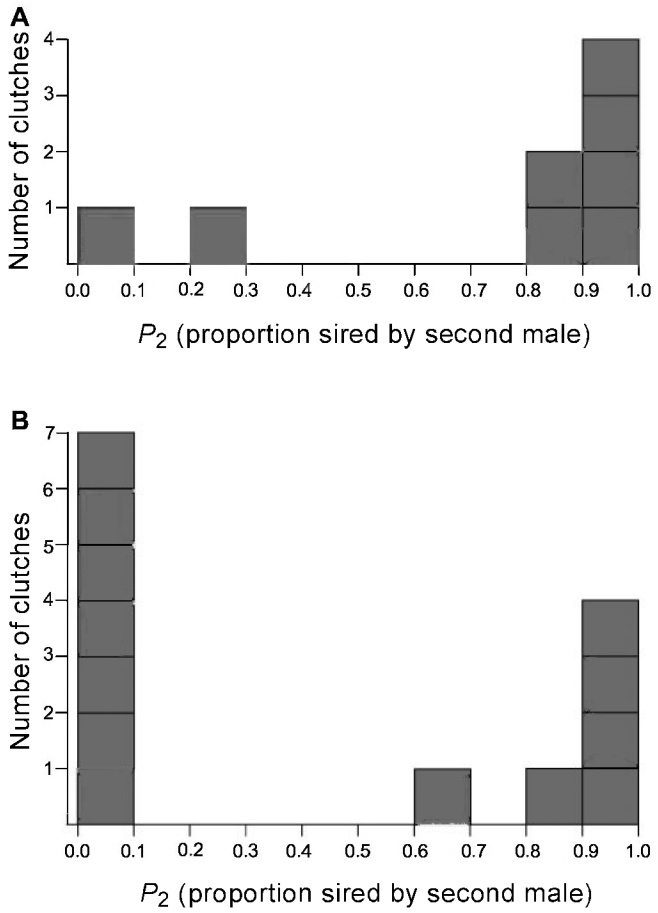

FIG. 2. Frequency distribution of the proportion of potentially fertilizable eggs sired by the second-male Muscovy Duck in sperm competition trials with (A) a 24-h lag between the two matings and (B) a 72-h lag between the two matings.

(range: 33–86%) of the total number of eggs was potentially fertilizable by either male.

In the eight trials with a 24-h lag between matings, mean P_2 was 0.72 ± 0.14. Each of the eight trials used a different male as the second male, so we did not need to recalculate mean P_2 to control for second-male identity. In six of the eight 24-h trials, P_2 was ≥0.8; in the other two, P_2 was 0.22 and 0 (Fig. 2A). In the 13 trials with a 72-h lag between matings, mean P_2 was 0.42 ± 0.13. Two males were used twice as the second male in this set; when mean P_2 was recalculated after first averaging the trials for these two males, the overall mean P_2 was 0.38 ± 0.14. P_2 was 0.0 in seven of the 13 trials and 1.0 in four, with only two intermediate values (Fig. 2B).

By incorporating the mean rate of sperm loss (0.0235 h^{-1}) estimated in experiment 1, the model in which passive sperm loss alone determines fertilization success predicts a P_2 value of 0.64 for 24-h trials and a P_2 value of 0.84 for 72-h trials. The value of P_2 predicted for the 24-h trials (0.64) was close to the observed mean value (0.72) and was well within the 95% CI around that mean (0.45–0.99). By contrast, the value of P_2 predicted for the 72-h trials (0.84) was much higher than the observed mean (0.42) and was outside the 95% CI (0.16–0.68). The predicted mean was also outside the 95% CI around the observed mean recalculated to control for second-male identity.

There was no obvious within-male consistency in fertilization success rate. Nine of 12 males that copulated in more than one trial had mixed success, dominating fertilization in at least one trial but losing the majority of fertilizations in at least one other trial. Only one male was consistently successful (in four trials), whereas two males were consistently unsuccessful (in three trials each). If each male in a pair had a 50% chance of dominating, a run of four successes would occur one 16th of the time, so one such run among 12 males is not unexpected. Similarly, the probability of three failures in a row is 1 in 8, not much different from the observed 2 in 12. Although sample size was small, there was also little indication of consistency within females who mated with the same male in different trials ($n = 5$). In three cases, the male dominated paternity in one trial but not the other (twice as male 2 and once as male 1). In the fourth case, the male dominated both times (once in each position), and in the fifth case, the male dominated in neither case (male 1 in both). Again, these observed patterns are similar to ones that would be expected by chance.

Latency to copulation (time between male release and copulatory mount) averaged 3.1 min (range: 0.1–49.1 min), and the mean duration of copulation (time between mount and thrust) was 2.5 min (range: 0.7–4.6 min). We compared the within-trial, between-male differences in latency and duration to the difference in proportion of paternity (arcsine transformed), but there was no significant relationship between either variable and fertilization success.

Combining the 24-h and 72-h trials, fertilization was dominated by the male that was less closely related to the female in 10 cases and by the male that was more closely related to the female in 9. In two cases, there was no difference in relatedness between the female and the two males in a trial. Differences in the relatedness of the female to the second male and the first were not correlated with P_2 for the 24-h trials ($r_s = -0.12$, $P = 0.75$), the 72-h trials ($r_s = -0.30$, $P = 0.30$), or both sets of trials combined ($r_s = -0.24$, $P = 0.28$).

DISCUSSION

Passive sperm loss is inevitable, because the alternative would require immortal sperm; therefore, the real questions in investigating passive sperm loss are the rate at which loss occurs and the importance of this rate in relation to other processes. For Muscovy Ducks, we estimate that sperm are lost from the female reproductive tract at a rate of ~0.024 h^{-1}. This rate falls in the middle of the range estimated in other species of birds: considerably below the rate (0.053 h^{-1}) in Bearded Tits (*Panurus biarmicus*; Sax et al. 1998), considerably above the rate (0.003 h^{-1}) in Wild Turkeys (*Meleagris gallopavo*; Wishart 1988), and quite similar to the rate (0.026 h^{-1}) in Zebra Finches (*Taeniopygia guttata*; Birkhead et al. 1993) and the rate (0.019 h^{-1}) in Collared Flycatchers (*Ficedula albicollis*; Michl et al. 2002).

Complete sperm uptake took up to 4 days in our female Muscovy Ducks. Delayed uptake might lead to mixing of sperm from successive inseminations before they reach the sperm-storage tubules, thus diminishing the likelihood of stratification

and increasing the degree of shared paternity within clutches. We had few cases in which paternity was shared by the two males in a sperm competition trial, however. Thus, delayed uptake does not seem to have played a large role in determining fertilization patterns in our study.

Despite the fact that sperm loss definitely occurs in Muscovy Ducks, the pared-down version of the passive-sperm-loss model was only partially successful in explaining patterns of male precedence in fertilization. The mean fertilization success of second males after a 24-h lag was quite close to that predicted by the model, but the mean success after a 72-h lag was much different from the observed and was outside the 95% CI. In addition, passive sperm loss predicts that the degree of second-male advantage should be higher after a 72-h lag than after a 24-h lag, whereas we observed the opposite. Finally, the assumptions of the passive-sperm-loss model—that sperm from different inseminations mix within the female and are available in proportion to the numbers that remain alive at the time that an egg is fertilized—would lead one to expect more intermediate values of P_2, clustered between 0.5 and 1.0, rather than the large number of the extreme values of 0 and 1 that we observed (Fig. 2). A bimodal pattern of fertilization success such as we observed has also been reported in other studies of sperm precedence in birds, both with natural mating (Warren and Kilpatrick 1929) and artificial insemination (Cunningham and Cheng 1999).

Clearly, then, fertilization success in our study was strongly affected by one or more processes other than passive sperm loss. Some of the possible processes are male-driven. Both ejaculate size (the number of sperm per insemination) and ejaculate quality (usually assessed by sperm motility) vary within and between male birds (Wishart and Palmer 1986, Birkhead et al. 1995a, Froman et al. 2002, Cornwallis and Birkhead 2007) and can have a large effect on fertilization success (Birkhead et al. 1999, Denk et al. 2005). Some of the within-male variation is attributable to a decline in ejaculate size and quality with repeated inseminations over short time intervals (Birkhead et al. 1995a); such short-term declines should not have been a factor in our experiments because we were careful to rest males for at least 2 days between matings. Nevertheless, ejaculate size and quality undoubtedly varied between matings and could have played a considerable role in creating variation in fertilization success. These male-driven processes, however, are unlikely to explain one of our more salient results: the lower success of second males with a 72-h lag than of those with a 24-h lag. Between-male differences in ejaculate size and quality cannot explain this trend because males alternated between the first-male and second-male role for both mating intervals. Within-male variation is also unlikely to explain this trend, because males had no obvious means of assessing the length of the lag between matings and so should not have been able to adjust their ejaculate to the length of the lag.

Females, by contrast, had every opportunity to assess the length of the lag between matings. Thus, female-driven processes may have caused the trend toward lower success after a longer lag, though at present we cannot explain why female preferences would vary as a function of mating interval. Female-driven processes may also have caused some or all of the wide variation in second-male success seen within trials with the same lag. One level at which females might influence success is through their behavior during copulation. In our study, a male released into a female's pen often approached the latter so rapidly that it was difficult to discern during the event whether the female had moved into the receptive prone posture before being reached by the male. We were able to confirm female receptivity from video replay, however, and in all trials the copulation appeared to be behaviorally complete. Nevertheless, it is possible that females were able to affect insemination through changes in their behavior too subtle for us to discern. Another possibility is that females exercise some form of postcopulatory choice. Much attention has been given to the possibility of postcopulatory choice in a variety of animal groups (Ward 2000, Bussière et al. 2006, Rosengrave et al. 2008), including birds (Cunningham and Cheng 1999, Pizzari and Birkhead 2000, Denk et al. 2005, Birkhead and Brillard 2007). Postcopulatory choice has been controversial, in part because it is often difficult to see a mechanism by which postcopulatory choice could be exerted. In birds, one simple mechanism has been demonstrated: female domestic chickens are able to accomplish selective ejection of sperm from particular males after copulation (Pizzari and Birkhead 2000). Male waterfowl possess an intromittent organ (Coker et al. 2002), which may make sperm ejection more difficult for females (Denk et al. 2005); however, anatomical specializations of the vagina found in some waterfowl species (Brennan et al. 2007) may prevent males from penetrating deeply into the female reproductive tract and thus maintain sperm ejection as an option. Another possible mechanism for postcopulatory choice is through storage of the sperm of successive male partners in different regions of the female's reproductive tract. Harvey and Parker (2000) showed through computer simulation that intraspecific variation in sperm precedence could arise through a lack of mixing of sperm during storage, and King et al. (2002) used labeled sperm to show that sperm from different inseminations were stored in different storage tubules in domestic chickens and turkeys. Segregation of ejaculates during storage might result in bimodal patterns of sperm precedence, in which either the first or the second male to mate dominates fertilization, even if females are unable to choose which male dominates.

Relatedness might provide a criterion for postcopulatory choice, with females biasing fertilization toward the sperm of less closely related males (Griffith and Immler 2009). We found no evidence of such a bias in our results, but given that our measure of relatedness was based on a relatively small number of loci, we believe that the possibility of such a bias deserves further investigation.

In conclusion, we have demonstrated that even though passive sperm loss occurs in Muscovy Ducks at a rate typical for birds, last-male precedence is not always observed, which suggests that processes other than passive loss are also important. Male-driven variation in ejaculate size and quality undoubtedly has some importance in determining which male dominates fertilization, but the evidence also suggests a role for female-driven processes. Female-driven processes that might be at work in Muscovy Ducks include variation in female behavior during copulation, selective ejection of male sperm, and segregation of ejaculates in different storage areas in the female reproductive tract. Given the promiscuous mating system of this species, these sperm competition processes may be especially important in this species in determining patterns of mating success and, thus, the outcome of sexual selection.

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