Females affect sperm swimming performance: a field experiment with barn swallows *Hirundo rustica*

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Sexual conflict over fertilization may in animals with internal fertilization initiate an evolutionary arms race that causes the female reproductive tract to become hostile to sperm performance if females with more selective reproductive tracts have their eggs fertilized by sperm of superior quality. This hypothesis suggests that sperm should perform better when tested in a physiologically neutral cell culture medium (neutral medium) than in the same "neutral medium" to which fluid derived from the female reproductive tract has been added (female medium). We tested this prediction using in vitro tests on sperm collected from barn swallows *Hirundo rustica* by recording sperm performance on video in neutral medium and "female medium." Sperm performance differed significantly among males but also between neutral and female medium. Sperm performed less well in female medium compared with neutral medium in terms of velocity. A principal component (PC) analysis of the 12 sperm parameters produced 4 PCs that explained 86% of the variance. The difference in the second and the third PC between neutral and female medium, reflecting sperm with a high degree of lateral head displacement and absence of straight and linear movement, many static sperm, and a small fraction of sperm with medium velocity, respectively, was positively related to an indicator of female quality: tail length. The latter result may suggest that high-quality females differentially affected the relative performance of sperm in their reproductive tract, consistent with the theory of sexual conflict. *Key words:* sexual conflict, sexual selection, sperm, swimming performance. *[Behav Ecol]*

Gametes constitute the means by which sexually reproducing organisms transfer their genetic material to the next generation, but the mere fact that sperm are much more abundant than eggs makes the question of which sperm eventually fertilizes an egg crucial. The evolution of anisogamy sets the scene for competition among sperm for fertilization of rare eggs (Queller 1997). This has consequences for optimal timing and allocation of sperm to females, with effects on certainty of paternity (Parker 1984). Therefore, the fitness gains from paternal care are reduced relative to those from mate search (Queller 1997). Particularly, attractive males will enjoy a greater reproductive rate than the average male, making it more beneficial for such males to invest their reproductive effort in mating rather than parental effort (Queller 1997). Simultaneously, females will enjoy a selective advantage from having their eggs fertilized by high-quality sperm, given the relatively small number of eggs and the large number of sperm. Many studies have suggested that females have evolved different kinds of obstacles to fertilization to achieve fertilization by sperm of superior quality (Eberhard 1996). Examples of such potential obstacles include female ejection of ejaculates (Baker and Bellis 1995), elongation of the female tract increasing the distance that sperm have to travel to reach the site of fertilization (Gomendio and Roldan 1993), the chemical environment in which sperm have to swim (e.g., Yanagimachi

1994), and leukocytes actively killing sperm (e.g., Johnson 1973; review in Birkhead, Møller, and Sutherland 1993). However, there are no experimental studies demonstrating that female "obstacles" evolved to provide high-quality males with a fertilization advantage. In sperm competition games, male partners, male nonpartners and females may have different evolutionary interests. Male nonpartners may differ in interests from male partners because male partners prevent fertilization of the eggs of their female by extrapair males. Extrapair males may likewise attempt to fertilize eggs rather than allowing the male partner of a female performing fertilization. Such a conflict may be mediated by females developing immune reactions to the sperm of their partners, but not to sperm of male nonpartners that have not yet deposited sperm in the female reproductive tract. Females and male partners may also differ in their evolutionary interests because females may not necessarily attempt to have their eggs fertilized by their partner, perhaps causing females to produce obstacles to fertilization by their partners. Finally, females and male nonpartners may also be in conflict over fertilization because females may avoid fertilization by unmated males of poor quality while such males may have no other reproductive options than attempting to fertilize the mates of other males. Again, this should result in females producing obstacles to fertilization by poor-quality males and such males attempting to overcome such obstacles. The observations and arguments listed above are evidence of sexual conflict over fertilization (Parker 1979; Rowe and Arnqvist 2005), suggesting that the female reproductive tract and its anatomical, physiological, chemical, and immunological properties may have evolved as means of imposing stringent selection on sperm from one or more males for eventually reaching the site of fertilization.

Numerous studies have indicated that sperm perform differently on different female backgrounds and that the female reproductive tract affects sperm performance (e.g., Eisenbach 2004; Sun et al. 2005; Satake et al. 2006; Suarez and Pacey 2006). However, there are no studies investigating whether females may reduce the performance of sperm and whether such female behavior is related to female fecundity (Birkhead, Møller, and Sutherland 1993; Satake et al. 2006; Birkhead and Brillard 2007). If the environment in which sperm move to achieve fertilization of an egg is hostile to sperm, we can predict that sperm movement will be hampered in an environment characteristic of the female tract, whereas it will be facilitated in a physiologically neutral medium. This simple prediction has to the best of our knowledge never previously been tested experimentally. Furthermore, we can make the prediction that differences in sperm performance between the environment of a female reproductive tract and a neutral environment should depend on male and female characteristics if some females are more fecund than others and if some males have superior abilities to fertilize eggs. Both these latter assumptions are well supported by empirical evidence (differences in fecundity: e.g., Roff 2001; differences in fertilizing ability: Birkhead and Møller 1998). This leads to the third prediction. Performance of sperm should be relatively worse in a female medium from the most fecund females if such females are particularly picky about which sperm fertilizes their eggs and therefore produce particularly strong barriers to sperm. There are no studies that have examined these factors so far, and the mechanisms involved in these processes are still unknown (but for a recent review of female factors affecting fertilization, see Birkhead and Brillard 2007).

The objectives of the present study were to test 1) whether males are consistent in sperm performance, 2) whether sperm swim less well in female than in neutral medium, and 3) whether female medium from the highest quality females is relatively more effective in hindering sperm performance. Sperm performance may suffer if the female can modulate her reproductive tract or its biochemical and immunological environment to choose sperm of specific males. Therefore, we predict that sperm will perform less well in the most fecund females compared with the least fecund females. To this end, we conducted in vitro tests of sperm performance in the field, using the barn swallow *Hirundo rustica* as a model organism and using tail length of females as a proxy for quality and hence fecundity (see below).

The barn swallow is a approximately 20-g passerine, semicolonial migratory bird with a well-known life history. Sperm competition is common as reflected by frequent extrapair copulations (Møller 1985) and extrapair paternity reaching up to a third in different populations (Møller and Tegelström 1997; Saino et al. 1997; Møller et al. 1998, 2003). Testes size and hence sperm production is large for the body size of the species (Møller 1991; Birkhead, Briskie, and Møller 1993; Møller and Briskie 1995). Males that gain extrapair copulations and fertilizations have long tails (a secondary sexual character; Møller 1988, 1992; Saino et al. 1997). Females with long tails are of superior phenotypic quality in terms of laying date, clutch size, number of clutches, and survival (Møller 1993; Cuervo et al. 2003). Therefore, tail length is an external indicator of phenotypic quality in both sexes (Møller 1994). Because a third of all females produce extrapair offspring and hence engage in extrapair copulations, there is reason to believe that the female tract has been subject to selection for discrimination among sperm but also for sperm from different males to compete by overcoming any such female discrimination for successful fertilization.

MATERIALS AND METHODS

General field procedures

We captured barn swallows in 4 different farms around Chernobyl, Borispil, and Kanev in Ukraine during early June 2006. Using mist nets placed across window and door openings, we captured wild birds at the peak of reproduction just before and during laying for most males. Birds were placed in cloth bags before being measured and weighed. Subsequently, we collected at least 2 sperm samples from all males using a simple massage technique. Sperm were collected in microcapillaries for easy handling. We collected sperm from male barn swallows and recorded their swimming performance in a physiologically neutral cell culture medium (neutral medium) and in medium derived from the reproductive tract of female swallows (see below for details on medium), randomly alternating the order of the 2 tests for each male, while recording sperm performance on video, and using a computer-assisted sperm analysis (CASA) system for performance measurements. G.R. recorded sperm performance blindly with respect to the phenotype of males and females, and G.R. also derived statistics for sperm performance by analysis of video recordings blindly with respect to male and female identities. It is important even to record sperm performance blindly because otherwise bias could arise from when the recording was initiated. We have previously shown that sperm morphology and behavior is related to level of background radiation in Ukraine (Møller et al. 2008). However, this effect was not important for the interpretation of the present study because sperm performance was compared within males.

We recorded 12 standard measurements of external morphological traits with a ruler (length of right and left wing and left and right outermost tail feathers, length of central tail feathers, and wingspan) or a digital caliper (length, height, width of beak, length of tarsus, and length of keel), and we traced the surface of the right stretched wing on a piece of paper. These measurements are accurate and repeatable, as shown by repeat measurements of a number of individuals in the same breeding season (Møller 1994). In the analyses presented here, we only use tail length estimated as the mean of the right and the left outermost tail feather, body mass, and tarsus length. The justification for using these 3 variables was that tail length can be considered a secondary sexual character, body mass an estimate of body condition (especially when entering tarsus length as an additional variable), and tarsus length a measure of structural body size. We checked all individuals for broken or damaged tail feathers by determining whether the tip was rounded, as it is the case for undamaged feathers. Likewise, we checked the base of all tail feathers for any signs of feather sheaths that are present on feathers still growing. A total of 2 individuals with damaged or growing feathers were excluded from the analyses.

We did not assign individuals to nests and hence did not know their exact breeding status, although checks of nests revealed nest contents to mainly be eggs and small nestlings. This time of the breeding cycle is when males copulate with their own females but also with neighboring females (Møller 1985, 1994).

A.P.M. attempted to collect sperm from all males, and we were successful for 190 of 193 males. When we had collected sperm from a male, we randomly chose a female captured at the same time and site and then collected fluid from her reproductive tract. We used the same massage technique on females as used for collecting sperm from males, with the muscles of the cloaca contracting repetitively before a sample could be collected. We repeatedly attempted to collect fluid from the female tract in the absence of muscle contractions, and we were never successful in more than 20 attempts, thus

excluding the possibility that this fluid was alimentary canal mucus. This procedure produced approximately 1–2 µL of transparent vaginal fluid from the outermost 1 mm of the cloaca; this was subsequently used for the tests. If the fluid was contaminated with feces, which was easily detected from the white and/or brown color of the sample, we discarded the sample and collected a new one. We deliberately placed female fluid in the same video system as described below to search for sperm and fecal matter. There were no sperm present in female fluid in any of the 10 samples that we investigated, thus excluding the possibility that the results were confounded by sperm from more than one male being present in the test tube. Likewise, there was no fecal matter present in any of these 10 samples. Due to the logistic problems of conducting this kind of study under field conditions, the total number of males for which we were able to record videos in the 2 different media was 20, with each male being tested with fluid from a different female because the amount of fluid extracted was insufficient for multiple tests on a single female. We used 166 males to test for repeatability of sperm performance among recordings, and we used 10 males to test for repeatability of sperm performance among ejaculates. Repeatability (R) is a standard estimate of consistency in phenotypic traits among measurements that allows partitioning of the phenotypic variance within and among individuals, ranging from 0 (no consistency) to 1 (all values remain constant) (Falconer and Mackay 1996). Repeatability also has the important property that repeatability sets an upper limit to heritability (Falconer and Mackay 1996). We estimated repeatability and its standard error (SE) using the equations in Becker (1984).

Sperm behavior

We quantified the behavior of sperm by adding sperm either to a neutral medium or to a mixture of fluid from the cloaca of a female and neutral medium, followed by video recording under a microscope. Whereas a third treatment with no neutral medium might have been desirable, this procedure was impossible because of the extremely high concentration of sperm in the ejaculate (we estimated tens of millions of sperm in less than 1 µL of ejaculate), completely hindering free sperm movement and hence recording of sperm behavior. We deliberately avoided initial dilution of all sperm samples because such a general treatment would add another layer of uncontrolled treatment effects to the entire study. The design with 2 treatments that we used ensured that the treatments had the same components with the exception of fluid from the female reproductive tract. Furthermore, the amounts of ejaculate and fluid from the female reproductive tract were so small that both treatments were highly diluted, causing the proportion of neutral medium to exceed 80% in the 2 treatments. This makes it unlikely that factors such as viscosity would differ between treatments.

The sperm sample was diluted in a one-step procedure by placing an aliquot of undiluted sperm on a prewarmed (37 °C) microscope slide with a 80-mm deep chamber (Hamilton Thorne Research, Beverly, MA) and then added either neutral medium (Dulbecco's modified eagle medium [D-MEM]) or female fluid and D-MEM. D-MEM contains 4500 mg/L glucose, 4 mM L-glutamine, and 110 mg/L sodium pyruvate and is manufactured by Gibco. Temperature was maintained constant using a heating aggregate. Immediately after that, we added 9 µL neutral medium, or 1–2 µL female fluid medium and 9 µL neutral medium, and placed the cover glass over the sample. We added 9 µL of neutral medium to both treatments to ensure that the difference in performance was due to the 1–2 µL female fluid rather than any difference in neutral medium. Sperm motility and images were recorded immedi-

ately after activation using a Sony CCD black and white video camera (XC-ST50CE PAL, Sony, Tokyo, Japan) at 50 Hz vertical frequency, mounted on an external negative phase-contrast microscope (Olympus CH30, Olympus, Tokyo, Japan) with a ×10 objective. Video recordings that allowed magnification were stored on mini-DV tapes. The recordings were later analyzed using CASA (HTM-CEROS sperm tracker, CEROS version 12, Hamilton Thorne Research). The image analyzer was set at frame rate 50 Hz, number of frames 25, minimum contrast of 30, and minimum cell size 20 pixels. Cells having a smoothed path velocity (VAP) <10 µm/s and a VSL <5 µm/s were considered to be static. Each motility measurement lasted 0.5 s. Between 7 and 20 replicate measurements for each of 12 different sperm performance, variables were made from each ejaculate (depending on the number of males "queuing" for being tested) (see below for details of the different variables). Variation in number of replicate measurements for each male was due to variation in amount of sperm. For each male, we quantified sperm motility within 2 min after extraction of the sperm, although most were recorded within 1 min, and any effect of storage was tested explicitly using order of treatment as a factor (see below). We kept both males and females until the very moment when female medium had to be used, then extracted the sperm and the fluid, and added this immediately to the test chamber.

We recorded 12 different measures of sperm performance with CASA. Because there is only very limited information about the sperm performance factors determining fertilization in birds, even in chickens, with sperm motility and swimming speed being the only exceptions (Birkhead et al. 1999; Froman et al. 1999), and none of these having been based on extensive video recordings, we decided to be prudent and obtain data on each of 12 different variables. We recorded mean values of VAP (smoothed path velocity [microns/second]), VSL (straight-line velocity [microns/second]), VCL (track velocity [microns/second]), ALH (amplitude of lateral head displacement [microns]), BCF (beat cross frequency [Hertz]), LIN (linearity [VSL/VCL]), and STR (straightness [VSL/VAP]) for each sample. The actual numbers of cells are counted in all field of analysis. As the velocity measurements are calculated for each cell, we were able to estimate percentage of static sperm cells (fraction of all cells that are not moving at all), sperm with slow velocity (fraction of all cells moving with VAP <10 µm/s or VSL <5 µm/s), with medium velocity (fraction of all cells moving with VAP between 10 and 50 µm/s), sperm with rapid velocity (fraction of all cells moving with VAP >50 µm/s), and the percentage of progressive sperm (fraction of all sperm that moved with STR >80 and VAP >50 µm/s) (see Technical Guide, Hamilton Thorne Biosciences, Beverly, MA, for more information on variables and their definitions).

Statistical analyses

The proportion of progressive, static, rapid, medium, and slow sperm was square root arcsine transformed before analyses. We analyzed sperm performance in neutral medium from different frames of video recordings using analyses of variance (ANOVAs), with each frame containing different sperm. Male identity was entered as a fixed factor in this analysis. For the 20 males analyzed in 2 kinds of medium, we tested for order of treatment effects (neutral medium first, followed by neutral medium or the reverse) on sperm performance in 1-way ANOVAs, with order as a factor, and none of these tests for order effects reached statistical significance. Hence, this factor was subsequently eliminated. Finally, in analyses of sperm behavior in neutral medium, we included date to control for differences among days and time of day recorded to the nearest

Table 1

PC analysis of sperm traits, with the 5 first components accounting for 85.8% of the variance

Variable	PC1	PC2	PC3	PC4
Eigenvalue	5.51	2.27	1.50	1.01
Percentage of variance	45.95	18.89	12.53	8.46
VAP	**0.39**	0.17	0.14	−0.04
VSL	**0.41**	0.03	0.10	−0.04
VCL	0.22	**0.50**	0.24	0.02
ALH	0.13	**0.50**	0.02	−0.19
BCF	−0.08	0.02	0.12	**0.95**
STR	0.29	**−0.43**	−0.10	−0.02
LIN	**0.32**	**−0.41**	−0.07	−0.07
Progressive	**0.38**	0.07	−0.26	0.11
Rapid	**0.37**	0.22	−0.25	0.09
Medium	**−0.32**	0.04	**−0.41**	−0.12
Slow	−0.18	−0.08	−0.20	0.11
Static	−0.08	−0.21	**0.73**	−0.12

Loadings exceeding 0.30 were considered as being important and they are shown in bold.

Figure 1
Mean performance measured as the first PC (PC1) for sperm of 20 male barn swallows in neutral and female fluid medium. The line $Y = X$ indicating that the sperm perform equally well in the 2 media is shown.

minute to account for diurnal variation in copulation activity and hence age of sperm. The latter is known to peak in the early morning with a much smaller peak in the evening (Møller 1985). None of these variables entered as significant predictors.

Because the number of sperm performance parameters was large, we reduced these to a small number of independent measures in a principal component (PC) analysis. This analysis was based on the transformed original variables (as described above), using the correlation matrix and orthogonal rotation (Table 1). Four PCs with eigenvalues exceeding 1.00 were subsequently identified and used for further analyses. We included all 4 PCs because they accounted for considerable amounts of variation (PC4 accounted for more than 8% of the variance and could thus potentially be important). These PCs could readily be interpreted based on the factor loadings of the original variables (Table 1), assuming that loadings exceeding 0.30 are significant (Tabachnick and Fidell 1996). More specifically, PC1 had positive loadings exceeding 0.30 for VAP, VSL, LIN, and the proportion of progressive and rapid sperm and a negative loading on sperm with medium velocity. This PC is thus an indicator of high straight-line velocity with progressive movement. PC2 had strong positive loadings for VCL and ALH and strong negative loadings for STR and LIN. Therefore, this PC reflected sperm with a high degree of lateral head displacement and absence of straight and linear movement. PC3 had a strong positive loading for the proportion of static sperm and a negative loading for the proportion of sperm with medium velocity. Thus, this PC reflected sperm with slow or static movement. Finally, PC4 had a strong positive loading on BCF, implying sperm with high beat cross frequency.

We rescaled estimates of the PCs relative to medium by rotating the coordinate system 45° to the right (see Figure 1), so the Y axis coincided with the line where performance in the 2 media was similar (i.e., $Y = X$). We subsequently calculated orthogonal deviations of the 4 PCs from this new Y axis as estimates of relative performance in the 2 media. The null assumption is that mean deviation should be zero for all 4 PCs and that deviations for the 4 PCs should constitute a homogeneous sample. Thus, we tested if means differed from zero in 1-sample t-tests, and we tested whether deviations differed among the 4 PCs in a 1-way ANOVA with post hoc comparisons of means.

We used the difference in sperm parameters between the neutral medium and the female medium in tests designed
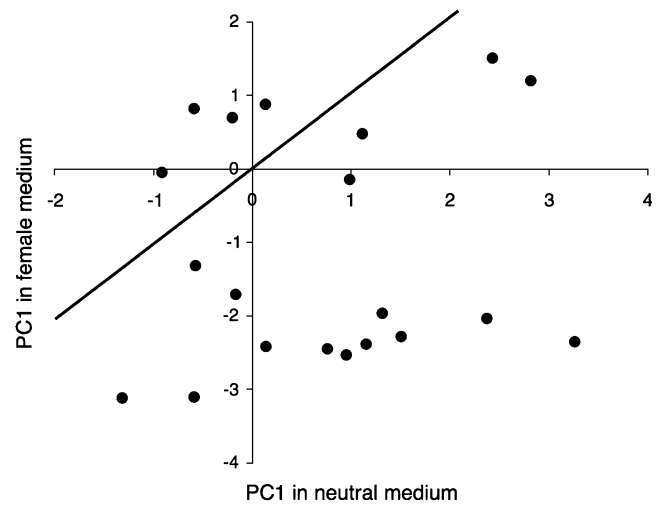
to investigate the relationships between difference in sperm performance and male and female external morphology. In these analyses, we included male and female tail length, body mass, and tarsus length (a measure of body size) as predictor variables. We used the Akaike information criterion (AIC) and change in AIC to identify the best-fit models (for procedures, see Burnham and Anderson 2002), assuming that a difference between models of AIC >2.00 is biologically significant.

All values reported are means (SE), and we used a significance level of 5%.

RESULTS

Sources of variance of sperm performance

We were able to obtain sperm from 190 of 193 male barn swallows, or 98.4%. Repeatability of sperm parameters within and among samples was moderate with only one value not being statistically significant (Table 2). Whereas all within-sample repeatabilities ranged from 0.07 to 0.48, they were much smaller at 0.01 to 0.33 for repeatability among different samples taken from the same male (paired t-test, $t = 6.25$, degrees of freedom [df] = 15, $P < 0.0001$). These results imply that sperm parameters were repeatable based on multiple estimates of the same sample but also that individual males were repeatable in their sperm behavior among ejaculates.

Sperm performance in different media

Sperm performed less well in female than in neutral medium with respect to velocity. Rescaled PC1 differed significantly from rescaled PC2, PC3, and PC4 (Tukey post hoc tests, $P < 0.0001$), whereas all other differences were not significant (Tukey post hoc tests, $P > 0.10$). The rescaled estimate of PC1 relative to medium was −1.36 (SE = 0.33), differing significantly from zero (1-sample t-test: $t = -4.16$, df = 19, $P = 0.0005$), whereas the means for PC2, PC3, and PC4 did not differ significantly from zero. Sperm from 16 of 20 males performed better in neutral medium (Figure 1). For comparison, similar data for PC2 demonstrate no clear difference in sperm performance between neutral and female medium for this component of sperm behavior (Figure 2). According to

Table 2

Mean (mean of means estimated for each sequence of video analyzed and SE) and within- and among-sample repeatability of sperm parameters from barn swallows from Ukraine

Variable	Mean (SE)	Within-sample F	Within-sample R (SE)	Among-sample F	Among-sample R (SE)
Pct. static	36.25 (1.67)	10.21***	0.48 (0.04)	9.81***	0.33 (0.02)
Pct. slow	5.10 (0.29)	1.73***	0.07 (0.02)	1.25	0.01 (0.01)
Pct. medium	24.29 (0.81)	4.74***	0.27 (0.04)	5.95***	0.22 (0.02)
Pct. rapid	34.36 (1.36)	9.89***	0.47 (0.04)	8.34***	0.29 (0.02)
Pct. progressive	29.84 (1.23)	8.98***	0.44 (0.04)	7.38***	0.27 (0.02)
LIN	64.75 (1.16)	7.82***	0.41 (0.04)	2.01*	0.05 (0.01)
STR	83.33 (1.02)	5.36***	0.30 (0.04)	3.69**	0.15 (0.02)
BCF (Hz)	21.48 (0.35)	3.50***	0.20 (0.03)	3.26**	0.14 (0.02)
ALH (µm)	3.85 (0.11)	5.30***	0.30 (0.04)	9.03***	0.31 (0.02)
VSL (µm/s)	53.81 (0.88)	6.08***	0.25 (0.03)	3.66**	0.15 (0.02)
VCL (µm/s)	78.85 (1.51)	7.92***	0.41 (0.04)	5.61***	0.21 (0.02)
VAP (µm/s)	57.15 (1.17)	8.25***	0.42 (0.04)	5.50***	0.20 (0.02)
PC1	−0.00 (0.07)	13.09***	0.52 (0.04)	8.23***	0.28 (0.02)
PC2	0.00 (0.05)	5.36***	0.28 (0.04)	3.65**	0.15 (0.02)
PC3	−0.00 (0.04)	9.06***	0.42 (0.04)	8.23***	0.28 (0.02)
PC4	0.00 (0.03)	2.63***	0.13 (0.03)	2.02*	0.07 (0.01)

Sample size is 166 males with repeat recordings for a single ejaculate, whereas 10 males were analyzed for 2 different ejaculates. Pct. = %.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.0001$.

the loading of sperm parameters on PC1 (Table 1), these results for PC1 imply that smoothed path velocity, straight-line velocity with rapid and progressive movement, and linearity of movement were greater in neutral than in female medium.

Sperm performance and adult phenotype

We tested whether male and female phenotypic characters could predict relative sperm performance in female medium compared with neutral medium. Relying on 4 PCs, there was no significant model retained for difference in PC1 between neutral and female medium. Difference in PC2 was significantly positively related to female tail length (Figure 3), implying that sperm had higher amplitude of lateral head displacement, higher track velocity, and nonstraight and nonlinear movement in female medium compared with neutral medium when the female had a long tail (Table 3). In other words, sperm moved in straighter lines in neutral medium,

especially when females had long tails. This result also suggests that the lack of difference in PC2 (and PC3) between the 2 media was because of interactions with male and female phenotypes obscuring any main effects. For the difference in PC3 between neutral and female medium, female tail length (Figure 4), female tarsus length, and male tarsus length entered as predictors in a model that accounted for 56% of the variance (Table 3). The difference in PC3 increased with female tail length and tarsus length and decreased with male tarsus length (Table 3). This implies that in female medium, fewer sperm had medium velocity and more were static (not moving) than in neutral medium when the female medium is derived from a long-tailed female, even after controlling for female and male tarsus length (which indicates body size) (Table 3). Finally, the difference in PC4 increased with male body mass (Table 3), implying that beat cross frequency was greater in female than in neutral medium when the male that produced the sperm was heavy.
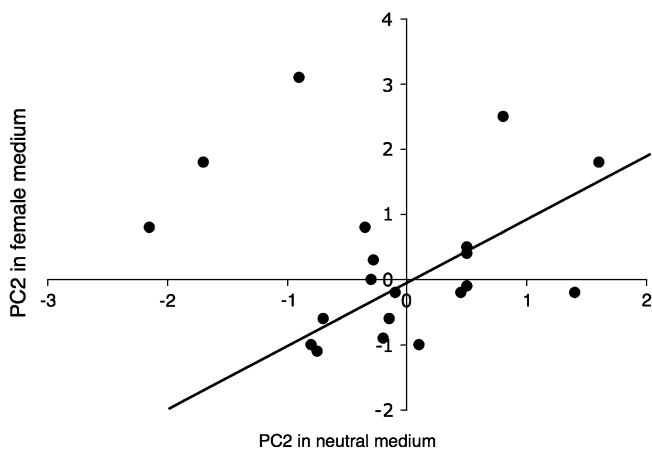
Figure 2
Mean performance measured as the second PC (PC2) for sperm of 20 male barn swallows in neutral and female fluid medium. The line $Y = X$ indicating that the sperm perform equally well in the 2 media is shown.
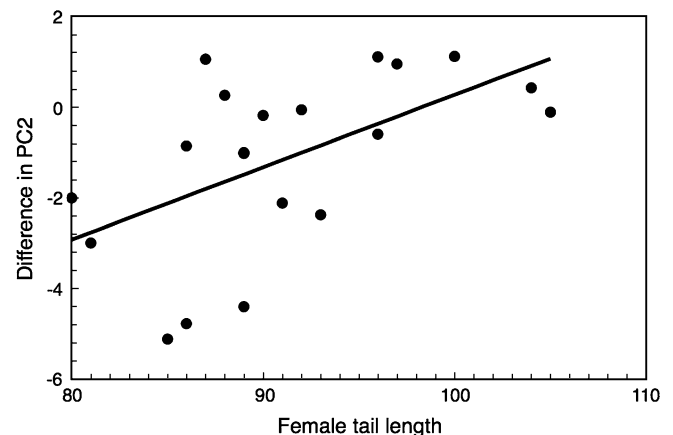
Figure 3
Difference in performance measured as the second PC (PC2) for sperm of 20 male barn swallows in neutral and female fluid medium in relation to female tail length.

Table 3

Models of the relationship between difference in PC scores for sperm performance between neutral and female fluid medium and female and male external morphology

Variable	df	Sum of squares	F	P	Slope (SE)	Effect size	Delta AIC
PC2	1,18	15.48	7.59	0.013			
Female tail length	1	15.48	7.59	0.013	0.133 (0.048)	0.54	6.48
Error	18	36.73					
PC3	3,16	24.44	6.66	0.004			
Male tarsus length	1	7.50	6.13	0.025	−0.013 (0.005)	0.53	4.48
Female tail length	1	15.03	12.29	0.0029	0.133 (0.038)	0.66	9.39
Female tarsus length	1	6.27	5.12	0.038	0.011 (0.005)	0.49	3.55
Error	16	19.57					
PC4	2,17	3.09	4.20	0.033			
Male body mass	1	2.89	7.85	0.012	0.014 (0.005)	0.56	5.59
Female tarsus length	1	1.27	3.47	0.080	0.006 (0.003)	0.41	1.71
Error	17	6.25					

AICs for the full models were 17.59, 7.57, and −17.25. Effect size was Pearson's product–moment correlation coefficient. Delta AIC was the difference between the full model and the model excluding a given variable from the model.

DISCUSSION

An important finding of this study was that sperm behavior differed between neutral and female medium but also that sperm performance differed significantly among males. Second, not all sperm performance variables showed effects of medium, implying that this effect was not ubiquitous among sperm performance traits. Finally, the difference in sperm performance between neutral and female medium was significantly related to tail length of females, an indicator of female quality and other female traits, but less so to the size of male traits.

Analyses of performance of sperm collected from wild barn swallows showed significant male and treatment effects. Repeatabilities were generally intermediate to small, with among-ejaculate repeatabilities being significantly smaller than within-ejaculate repeatabilities. The higher within-ejaculate repeatabilities imply that we are able to estimate sperm behavior relatively accurately, whereas the small among-ejaculate estimates of repeatability imply that there is considerable variation among ejaculates, although there is also evidence of significant consistency among males. The significant male effects imply that males are consistent in most measures of performance of their sperm that we recorded both when multiple estimates were obtained for the same ejaculate but also for different ejaculates and that males differed significantly in terms of performance of their sperm. Such consistent differences are necessary assumptions for evolution of ejaculate features, but they are also requirements for predictions of fertilization ability. Recent studies suggest that size, motility, and swimming speed of sperm may predict fertilization probability to some extent in both external and internal fertilizers (e.g., Lahnsteiner et al. 1998; Birkhead et al. 1999; Froman et al. 1999; Levitan 2000; Kupriyanova and Havenhand 2002; Gage et al. 2004; Garcia Gonzales and Simmons 2005; Rudolfsen et al. 2008).

The main objective of this study was to test for differences in sperm performance in neutral medium and in medium with fluid derived from the female reproductive tract. One of the 4 components of sperm performance behaved differently among treatments. This shows that effects are not ubiquitous for all performance traits because only one of 4 PCs showed a significant treatment effect. Closer inspection of Figure 1 revealed that the position of the line $Y = X$ deviated from a random distribution by 1.4 standard deviations (1 standard deviation = 1.47 and displacement of the line $Y = X$ to $Y = -2 + X$ results in half of the 20 observations being below and half above the latter line). This must by all means be considered

a large effect (sensu Cohen 1988). The surprising feature of this result is that sperm performed "less well" in their "natural environment" (fluid from the female reproductive tract) than in artificial neutral medium, begging the question why that should be the case. The actual components of the female fluid that affected sperm performance remain to be determined. A simple explanation is that fluid taken directly from females is more viscous than the cell culture medium. We consider this possibility to be unlikely given the high degree of dilution that we used in the experiments (see Materials and Methods). However, as the female reproductive tract in humans is able to mount effective immune responses against infectious agents and, occasionally, sperm cells (Naz and Menge 1994), we speculate that immunoglobulins can bind to sperm in birds and hence directly influence swimming behavior of sperm (for a review of the effect of antisperm antibodies on mammalian fertility, see Chiu and Chamley 2004). A previous study of barn swallows has shown an elevated level of circulating immunoglobulins around the timing of egg laying in adult female barn swallows, but not in males (Saino et al. 2001). The magnitude of this peak in immunoglobulins is positively related to the tail length of the male partner, but not the tail length of
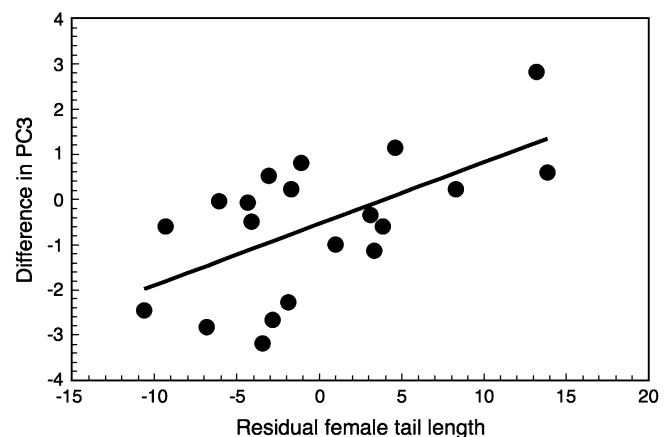

Figure 4
Difference in performance measured as the third PC (PC3) for sperm of 20 male barn swallows in neutral and female fluid medium in relation to residual female tail length (after controlling statistically for male and female tarsus length). The line is the linear regression line.

the female, and female barn swallows deposit immunoglobulins into their eggs (Saino et al. 2001).

Sperm from a minority of males performed better in female medium than in the neutral control fluid, whereas sperm from most males performed worse in female medium (Figure 1). In contrast, there was no such consistent difference for PC2–PC4, suggesting that an experimental artifact did not cause the results for PC1. Given that sperm performance was consistent among males for multiple ejaculates, this suggests that sperm from different males varied in performance between the 2 kinds of medium. Interpretations of this result include coincidental significant relationships, interaction effects between males and females, or superior performance of certain males in multiple media. We can exclude the first explanation because we only performed a very restricted number of statistical tests to avoid problems of significance arising by chance. Obviously, it would have been more appropriate to assess sperm performance in medium derived from several females by using an experimental design that crossed all males with all females. We were unable to perform such an experiment for logistical reasons because each male and each female at most produced a couple of microliters of liquid. As an ethical note, we would also like to emphasize that all birds were left unharmed, allowing them to be released alive. Thereby, we will be able to continue our studies on surviving individuals in the years to come.

We predicted that sexual conflict over fertilization should affect relative performance of sperm in neutral and female medium and that indicators of male and female phenotypic quality might predict part of this variation (see Table 2 for evidence of differences in sperm performance among males). If the external phenotype of males reflected differences in performance of their sperm and differences in performance among media, we predicted that males with large secondary sexual characters with a condition-dependent expression should have sperm with superior performance in female medium compared with neutral medium. Such a difference in performance could be due to sperm produced by males with large secondary sexual characters having superior overall performance. Alternatively, sperm of such males may be better able to cope with the biochemically hostile cloacal environment of females because past selection has produced adaptations in sperm to cope with the environment of the reproductive tract of females (for a similar argument, see Birkhead, Møller, and Sutherland 1993). This might especially apply to sperm from males that are likely to be found in sperm competition situations more frequently than expected by chance, as is the case in the barn swallow (Saino et al. 1997). We found no evidence consistent with such effects of male phenotype because male tail length did not enter as a significant predictor in any of the 4 tests. However, there was a weak positive relationship between male tarsus length and PC3 of relative sperm performance and a weak positive relationship between male body mass and PC4 (Table 3).

If fecund females were particularly choosy in terms of fertilization of their eggs, we should expect relative sperm performance in female medium to decrease with indicators of female quality. Tail length of female barn swallows is such a quality indicator, reliably reflecting earliness of breeding, clutch size, annual fecundity, and viability (Møller 1993; Cuervo et al. 2003). Indeed, the difference in PC2 and PC3 between neutral and female medium was partly explained by variation in female phenotype. Female tail length accounted for variation in PC2, with sperm having higher amplitude of lateral head displacement, higher track velocity, and nonstraight and nonlinear movement in female medium compared with neutral medium when the female had a long tail (Figure 3). Furthermore, female tail length accounted for

variation in PC3, with fewer sperm with medium velocity and more static sperm in female medium compared with neutral medium when the female medium derived from a long-tailed female, suggesting that sperm performed less well in medium derived from long-tailed females (Figure 4). This reduction in relative performance of sperm in female medium when the female is of superior quality is as expected if fecund females produce greater obstacles to fertilization of their eggs. However, the mechanisms underlying such a potentially reduced sperm performance remain unknown (Eady 1994; Bernasconi et al. 2002). An explanation for the results in that long-tailed females may have produced larger volumes of fluid, making a larger difference between neutral and female fluid (the larger volume might actually be the way that high-quality females are more effective in hindering the movement of sperm). There were no obvious consistent differences among females in terms of volume of fluid, although quantification of the volume of fluid must await future studies. Here we have shown experimental evidence for individual variation in the extent to which sperm performance is reduced when sperm swim in female medium rather than neutral medium.

These findings are consistent with published information on extrapair paternity in barn swallows. Long-tailed males lose little paternity in their own nests, but gain paternity in neighboring nests, as shown by tail manipulation experiments (Saino et al. 1997). In fact, long-tailed high-quality females are generally mated to long-tailed males (Møller 1993), and such females produce no or few extrapair offspring (Møller and Tegelström 1997; Saino et al. 1997). Therefore, the findings reported here may provide a new hypothesis for how intraspecific differences in extrapair paternity may come about: high-quality females mated to the most attractive males are the least likely to produce extrapair offspring, even if engaging in extrapair copulations, because such females have the strongest defenses against fertilization of their eggs by males of poor quality.

In conclusion, we have shown that fluid from the female reproductive tract affects sperm performance under standardized in vitro conditions, with variation among sperm from different males. The reduced sperm performance due to female fluid was significantly related to an indicator of female quality, suggesting that the most fecund females may produce the largest obstacles to have their eggs fertilized. This provides evidence consistent with the theory of sexual conflict.

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