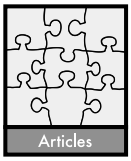

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Between-male variation in sperm size, velocity and longevity in sand martins *Riparia riparia*

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Sperm mobility is known to be an important determinant of a male's sperm competitive ability. Although more debated, sperm length and its relation to sperm swimming ability has also been proposed to determine a male's fertilisation potential. Furthermore, both mobility and length may covary with a male's phenotype, either positively (the phenotype-linked fertility hypothesis) or negatively if, for instance, low-quality males have less access to females but invest more in sperm production. Using dummy females, we collected sperm samples from wild sand martins *Riparia raparia* males. We investigated the relationship between sperm length and sperm swimming speed as measured by sperm straight line velocity (VSL), and determined whether sperm traits are correlated with male body size and condition. We found that total sperm length is repeatable within-ejaculate and shows substantial inter-male variation. Sperm length was associated with sperm velocity: males with short sperm have sperm that swim initially faster but die sooner, whereas males with longer sperm have sperm that swim more slowly but for a longer time. Smaller males produced sperm with higher overall velocity. This correlation between male size and sperm behaviour may reflect alternative fertilisation strategies where small males having less mating opportunities invest more in sperm competitive ability. The existence of such alternative strategies would participate in maintaining variation in sperm length and velocity in this species.

It is now well established that sperm swimming ability influences the outcome of sperm competition. In the sea urchin *Lytechinus variegatus*, males producing faster sperm have higher rates of fertilisation (Levitan 2000), while in boars *Sus scrofa domesticus*, a variety of sperm motility parameters are correlated with male fertility (Holt et al. 1997). In birds, sperm mobility or velocity are primary determinants of male fertility (Froman et al. 1999), and males having highly mobile sperm sire a greater proportion of offspring in competitive fertilisation experiments (Birkhead et al. 1999, Donoghue et al. 1999, Denk et al. 2005). Similar results have been found in fish such as the Atlantic salmon *Salmo salar* (Gage et al. 2004).

Besides sperm swimming ability, sperm length has been hypothesized to also play a role in determining a male's sperm competitive ability. The exact mechanism by which this would operate is unknown but it has been proposed that sperm length is positively related to sperm speed and thus that sperm length should increase with increasing risk of sperm competition (Parker 1998). Comparative studies found some support for this hypothesis in various taxa including insects, frogs, birds and mammals (Gomendio and Roldan 1991, 1993, Briskie and Montgomerie 1992, Gage 1994, Briskie et al. 1997, Byrne et al. 2003; but see Stockley et al. 1997). Within species some empirical data also suggest a positive relationship between sperm size and sperm competitiveness (LaMunyon and Ward 1998, Oppliger et al. 2003). However, the positive relationship between sperm length and sperm swimming speed proposed by theoretical models has seldom been tested (LaMunyon and Ward 1998, Gage et al. 2004, Birkhead et al. 2005). Thus, the degree to which sperm size determines success in sperm competition is currently under debate.

The idea that male phenotype, i.e. social status, body size or the size of sexual ornaments, may reflect sperm quality is also under debate. The phenotype-linked fertility hypothesis proposes that females would benefit from choosing males with elaborated sexual ornaments if they maximize the chances of mating with fertile males (Sheldon 1994). Tests of this hypothesis in birds, fish and mammals have yielded contradictory results with some studies finding a relationship between male phenotype and ejaculate quality (Matthews et al. 1997, Simmons and Kotiaho 2002, Peters et al. 2004, Malo et al. 2005, Schulte-Hostedde and Montgomerie 2006) while others do not (Birkhead and Fletcher 1995, Liljedal et al. 1999, Pilastro and Bisazza 1999). Theoretical developments (Parker 1998) also contradict the phenotype-linked fertility hypothesis in suggesting

that males that have low access to females, e.g. small males, may invest more in sperm expenditure to compensate for a lower number of mating opportunities. Support for this hypothesis comes from the feral fowl *Gallus gallus domesticus* where sub-dominant males, which are less preferred by females and have less access to them, produce more mobile sperm than dominant males (Pizzari et al. 2007).

Overall, studies testing the relationship between sperm size and motility and the relationship between male phenotype and sperm quality are scarce. This is undoubtedly because obtaining samples of live sperm from free-ranging animals is difficult. The goal of our study was to investigate how sperm velocity, a measure of sperm swimming ability, sperm length and sperm longevity are related to each other in the sand martin *Riparia riparia*. The sand martin is a colonial, socially monogamous species where extra-pair copulations and fertilizations are common and sperm competition is intense (Nicholls et al. 2001). Sand martin males are known to be prone to copulate with a dummy female, thus allowing the collection of natural ejaculates (Nicholls 2000). To test whether male phenotype may signal sperm characteristics in the sand martin, we examined correlations between both sperm length and velocity and body measures and condition. As intra-ejaculate variation in sperm morphology may be indicative of more or less stable spermatogenesis (Schulte-Hostedde and Montgomerie 2006) we also tested the correlation between intra-ejaculate variance (SD) in sperm length and body measures and condition.

Methods

The study was conducted on a sand martin colony comprising seven sub-colonies situated on the Tisza River, Hungary (48° 11′N, 21° 28′E; Szép 1995) from 27 April to 19 May 2003. Five females found dead at the bottom of the riverbank were stuffed, fitted with a false cloaca and fixed at the end of a fishing rod in copulatory posture to serve as dummies, a method previously used in this species by Nicholls and collaborators (2001). The dummy female was placed in the centre of a 33×25 cm clapnet (Moudrý, Czech Republic) that was triggered from the distance to capture copulating males immediately after they ejaculated.

Dummy females were presented either from 06.15 to 10.50, or from 15.30 to 19.50 (local time), when male sand martins exhibit the highest probability of copulating with a female mount (Nicholls 2000). The five models were used alternatively and presented for 30 min or until a male copulated with it. If no male copulated with the dummy female, we moved to another sub-colony. If a male copulated with the dummy, we collected the ejaculate and immediately recorded sperm motion. The dummy was afterwards presented again in the same colony. The time of copulation and when the sample was videotaped were recorded to the nearest second in order to calculate the time elapsed between ejaculation and sperm analysis.

The model's false cloaca was filled with 5 µl of Dulbecco's Modified Eagle Medium (4500 mg glucose/l, 110 mg sodium pyruvate/l, L-glutamine, Sigma Aldrich) to hydrate the transferred ejaculate and facilitate its collection. The ejaculate collected from the false cloaca was mixed to 995 µl pre-warmed (40°C) Dulbecco's Medium leading to 1ml of sperm/buffer solution. When the ejaculate was deposited outside the false cloaca we used the 5 µl of buffer to hydrate and collect the sample. A 7 µl-sample was then loaded within a prewarmed (40°C) MicroCell (50 µm chamber depth, Conception Technologies, San Diego) and the slide placed under a microscope equipped with a $20 \times$ bright-field objective under a dark-field condition generated with a Ph2 annular phase ring. Sperm motion was recorded for 15 min on a DV cassette using a video camera fitted to the microscope and plugged to a DV handycam used as a recorder. Electricity for the apparatus (microscope, heating stage and controller, video camera) was supplied by a car battery plugged to a 12–230V AC/DC 300W converter. While videotaping sperm motion, we weighed the male (± 0.5 g) and measured its left tarsus ($\pm 1.10^{-2}$ mm), left wing (± 1 mm), left external tail feather (± 1 mm), and sternum ($\pm 1.10^{-2}$ mm). We sometimes failed to take all measures causing sample sizes to vary.

Video recordings of sperm motion were later analysed using the Hobson Sperm Tracker System at the Max Planck Institute for Ornithology, Seewiesen, Germany. For each sample, we took five measures of straight line velocity (VSL) using a 60 s sampling interval and a 60 s pause between intervals. This allowed us to monitor the dynamics of sperm velocity over a period of several minutes after ejaculation and thus to derive sperm longevity from statistical models as the rate at which sperm velocity declines with time (Froman and Feltmann 2000). The minimum track time was 1.2 s, which minimized the risk that the tracking system would count a moving particle as a motile sperm (Froman and Feltmann 2000). Among the various sperm motility parameters computed by the Hobson Sperm Tracker, we chose to use the straight line velocity (VSL) because it has been previously shown to be a good predictor of male fertility in several taxa (reviewed in Pizzari et al. 2004) including birds (Denk et al. 2005), and it provides a measure of sperm motility that can readily be used to test the relationship between sperm speed and sperm length. Ten to 28 (mean $\pm$ SE: 19 ± 2) sperm were measured for their total length from steady video pictures.

We captured 60 males that copulated with dummy females. Some males did not ejaculate during the copulation ($n = 22$, 36.6%). In eight cases (13.3%) the dummy attracted several males that successively copulated with it, resulting in a mixture of ejaculates that could not be used. Eventually, we obtained measurements of sperm length for 28 males and, due to technical problems, video recordings of motile sperm from 14 males.

The males that were prone to copulate with our dummies could form a biased subset of the male population. If they were, those males should differ in their mean morphology and should exhibit smaller variance in those traits compared to the whole male population. We thus compared mean values and variances of all traits between our sample (measures taken by ZN and FH) and a larger sample of males (ranging from 202 to 580 individuals, measures taken by TS) captured during the intensive ringing campaign later in the season.

VSL was square-root transformed prior to analyses to match modelling assumptions. Analyses involving repeated measures within the same male were conducted with

Generalized Linear Mixed Models using the Restricted Maximum-Likelihood estimation method (REML-GLMM, Littell et al. 2006) and assuming normal distribution of the error. The male's identity was declared as the random subject using an unstructured R matrix and intercepts and slopes were allowed to vary among males. Degrees of freedom for fixed effects were estimated with the Satterthwaite approximation, which may yield non-integer numbers. Modelling assumptions were validated by plotting model residuals against predicted values and by testing the residuals' normality. Analyses were conducted using the SAS, version 9.1. All tests are two-tailed with significance level set at $\alpha = 0.05$.

Results

Males who copulated with dummy females did not statistically differ in size from males captured during the intensive ringing campaign later in the season (all $t < 1.85$, $P > 0.065$). However, they differed in their body mass ($t = 5.50$, $P < 0.001$, $df = 611$). Yet, this difference is likely to be mostly due to a seasonal change in body mass. Our sample also showed similar variances in all morphological traits measured (all $F < 2.09$, $P > 0.15$). The males who copulated with our dummies can thus be considered as a representative sample of the whole population.

Sperm length showed variation within and among males (Table 1, Fig. 1). The average time elapsed between male ejaculation and the start of the 15 min video recording was 9 min 35 s (range: 5 min 39 s to 11 min 17 s). A first analysis modelling VSL as a function of time elapsed since ejaculation and using male identity as a random factor (REML-GLMM) revealed that: 1) VSL significantly declined with time ($b \pm SE = -0.018 \pm 0.007$, $F_{1,13} = 5.70$, $P = 0.03$), and that 2) males differed in their initial sperm velocity (different intercepts) and in the rate at which their sperm velocity declined (different slopes). Entering the males' mean sperm length in this model resulted in VSL to be significantly explained by an interaction between sperm length and time elapsed since ejaculation ($F_{1,11.6} = 4.81$, $P < 0.05$). In other words, sperm size was correlated with sperm longevity, with males having the shortest sperm exhibiting: 1) the highest initial sperm velocity, and 2) the steepest decline in velocity, whereas males having longer sperm showed stable intermediate velocity (Fig. 2).

None of the morphological traits we measured correlated with total sperm length or intra-ejaculate variation in sperm length (within ejaculate/male standard deviation) (all $|r| < 0.32$, $P > 0.10$, $n = 22$–28). Body condition (body mass accounted for tarsus length used as an explanatory variable in the model) did not covary with sperm length (mass: $F_{1,18} = 0.06$, $P = 0.80$; tarsus: $F_{1,18} = 0.02$, $P = 0.89$), or with intra-ejaculate variation (SD) in sperm length (mass: $F_{1,18} = 0.01$, $P = 0.92$; tarsus: $F_{1,18} = 0.00$, $P = 0.98$).

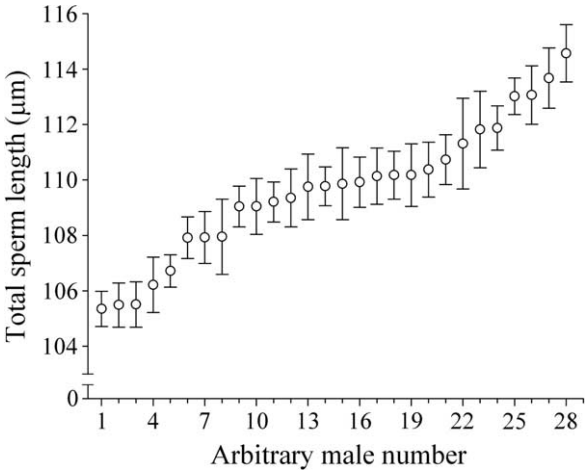

Figure 1. Within- and among-male variation in total sperm length. Circles represent individual means (±SE) in total sperm length based on 10 to 28 spermatozoa per male. Males are numbered and ranked from small to large average sperm length.

Morphological traits did also not covary with sperm velocity (REML-GLMM: time since ejaculation: all $F > 5.90$, $P < 0.032$; morphological traits: all $F < 3.49$, $P > 0.09$), except for tarsus length which was negatively correlated with sperm velocity (REML-GLMM: time since ejaculation: $F_{1,11.7} = 5.62$, $P = 0.036$; tarsus length: $F_{1,11.2} = 5.31$, $P = 0.04$). Males having small tarsi had sperm with higher overall velocity (Fig. 3).

Our modest sample may prevent us from detecting existing relationships between sperm length, variation in sperm length or sperm velocity and male morphology. This is reflected in the low power of all analyses (ranging from 0.05 to 0.48), with the exception of the analysis testing for a correlation between body mass and sperm velocity (0.79). Thus, we may have been unable to detect existing relationships that would be revealed with a larger sample.

Discussion

Theoretical developments investigating the circumstances under which sperm competition would select for increased sperm length have relied on the intuitive assumption that sperm length is positively associated with sperm swimming speed (Gomendio and Roldan 1991, Parker 1998), an assumption that has seldom been tested. We did not find a direct correlation between sperm length and sperm velocity or that sperm longevity negatively covaried with sperm length. We found that short sperm have a higher initial velocity but a shorter lifespan whereas long sperm have a lower initial velocity but a higher longevity. This contradicts the general assumption that sperm size is traded against sperm longevity (Cardullo and Baltz 1991, Immler and

Table 1. Variation in total sperm length (μm) among samples of 28 male sand martins.

Mean	SE	Min.	Max.	CV	Within-male repeatability[a]	N_0	P
109.6	0.5	105.4	114.2	2.2%	0.20	20.7	<0.001

[a]Sensu Lessells and Boag (1987).

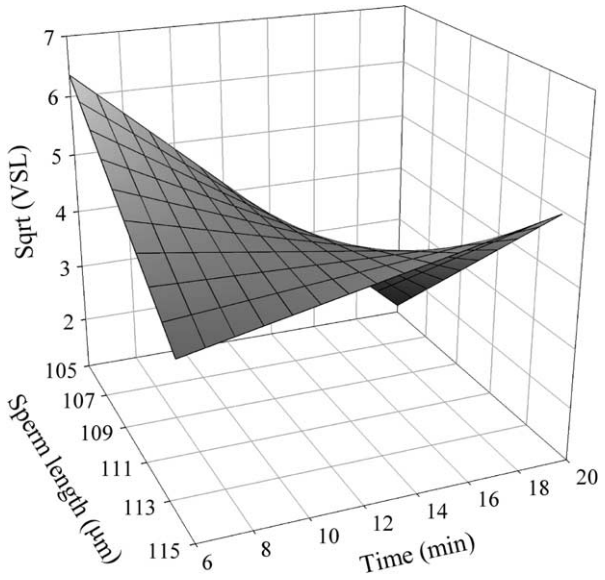

Figure 2. Sperm straight line velocity (VSL, square-root transformed) as a function of the time since ejaculation and the male mean total sperm length. Data are predicted values from a REML-GLMM including the male's identity as a random factor, allowing intercepts and slopes to vary among males.

Birkhead 2007, Immler et al. 2007). It also contrasts with previous studies within (LaMunyon and Ward 1998), and across species (Gomendio and Roldan 1991, Stockley et al. 1997) that found long sperm to have higher maximum velocity and/or reduced longevity. It is however consistent with results found in the sea urchin where sperm velocity and longevity trade off against each other (Levitan 2000). One explanation why long sperm swim more slowly but live longer may be that long sperm are slowed down by higher friction forces in the media due to their larger surface. Yet,

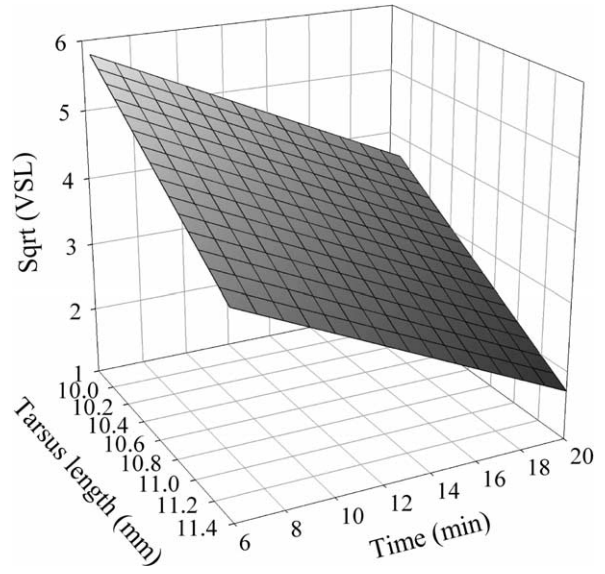

Figure 3. Sperm straight line velocity (VSL, square-root transformed) as a function of the time since ejaculation and the male tarsus length. Data are predicted values from a REML-GLMM including the male's identity as a random factor, allowing intercepts and slopes to vary among males.

their bigger total length may be correlated with a longer midpiece. In our study, we were not able to measure midpiece length. Across bird species, a positive allometry between midpiece length and flagellum length seems to be the rule: midpiece length increases more per unit of flagellum length and is positively correlated with total sperm length (Immler and Birkhead 2007, Immler et al. 2007). However, within species, midpiece size generally shows no association with other components of sperm length (Morrow and Gage 2001a, Birkhead et al. 2005, Malo et al. 2006). Nonetheless, if longer sperm have a higher mitochondrial load per unit of size, this may translate into higher ATP content allowing greater longevity (Froman and Kirby 2005).

Assuming that sperm size increases sperm survival, theory also predicts that, if the timing between insemination and fertilisation increases, long sperm will be favoured (Parker 1998). Conversely, if the risk of sperm competition decreases, i.e. if the time between insemination and fertilisation decreases, short sperm should be selected (Parker 1998). Thus males producing small, fast and short-lived versus long, slow and long-lived sperm may have different selective advantages depending on the timing of insemination relative to fertilisation. When fertilisation occurs shortly after insemination, fast swimming sperm may be able to outcompete rivals' sperm in reaching the storage organs and the ovum (Birkhead et al. 1999). When fertilisation occurs long after insemination, slow swimming, long-lived sperm may have the selective advantage of being passively lost from the female's sperm storage glands at a lower rate and therefore be more numerous by the time of fertilisation (Froman et al. 2002).

Sperm length and sperm velocity showed substantial variation among males, as found in other animals (Morrow and Gage 2001a, Gage et al. 2002, Oppliger et al. 2003, Birkhead et al. 2005, Malo et al. 2006, Schulte-Hostedde and Montgomerie 2006). However, sperm velocity and viability and/or longevity have been found to be important determinants of a male's sperm competition ability and are hypothesised to be under directional selection (Holt et al. 1997, Birkhead et al. 1999, Donoghue et al. 1999, Hunter and Birkhead 2002, García-Gonzáles and Simmons 2005). Additionally, there is good evidence that sperm length has co-evolved with female reproductive anatomy (Briskie et al. 1997, Miller and Pitnick 2002) and it is hypothesised to be under stabilizing selection (Calhim et al. 2007). Thus, what maintains variation in both sperm velocity and size is currently unclear. A first hypothesis is that sperm length and sperm velocity are partially maternally inherited (Pizzari and Birkhead 2002). This hypothesis has recently been substantiated by studies showing that variations in sperm design or motility have a partially sex-biased inheritance (Ward 2000, Morrow and Gage 2001b, Froman et al. 2002, Birkhead et al. 2005, Dowling et al. 2007). However, although maternal genetic effects contribute to variations in sperm traits, their heritability also shows a direct paternal genetic component (Simmons and Kotiaho 2002, Birkhead et al. 2005, Simmons and Roberts 2005, Dowling et al. 2007).

A second hypothesis is that sperm characteristics are condition-dependent sexually selected traits (Rowe and Houle 1996). Condition-dependence has indeed been found in sperm swimming speed and sperm length

(Simmons and Kotiaho 2002, Malo et al. 2005, Schulte-Hostedde and Montgomerie 2006, García-Gonzáles and Simmons 2007). However, we found no significant correlations between body condition and sperm characteristics, and even found that males with shorter tarsi produced sperm with higher overall velocity (Fig. 3).

This latter result is in agreement with another hypothesis that trade-offs exist between sperm traits that determine a male's sperm competitive ability and some other fitness related traits, which may promote variation in sperm traits. Theory (Parker 1998) predicts that males differing in their access to fertile females should invest differently in their ejaculate expenditure with disfavoured males investing more. Parker (1998) reviewed some empirical examples supporting this hypothesis. A more recent example is provided by the feral fowl where dominant males monopolise fertile females but produce less mobile sperm compared to subdominant males (Froman et al. 2002, Pizzari et al. 2007). Our results suggest that such a trade-off might exist in sand martins. Small males may have less mating opportunities and thus invest more in sperm competitive ability by producing faster swimming sperm. Such a trade-off would participate in maintaining variation in sperm velocity and length. The lesser access of small males to females however remains to be investigated.

In summary, we found that total length of sand martins' sperm is repeatable within a male's ejaculate and shows substantial variation between males. Sperm length was associated with sperm velocity: males with shorter sperm have sperm that swim initially faster but die sooner. This result contradicts the intuitive idea that long sperm swim faster. Such a trade-off between maximum velocity and longevity among sperm of different sizes may promote different but equally successful insemination strategies. Additionally, small males produced sperm with higher average velocity. If smaller males have a lower access to females this may be a strategy to compensate for their lower mating opportunities. Our sample is modest and further studies are welcome to confirm our results. Studies on a variety of species where male phenotype preconditions access to fertile females are also needed to test whether males develop alternative fertilisation strategies by producing sperm of different length, velocity and/or longevity.

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