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Sperm morphology and sperm velocity in passerine birds

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Sperm velocity is one of the main determinants of the outcome of sperm competition. Since sperm vary considerably in their morphology between and within species, it seems likely that sperm morphology is associated with sperm velocity. Theory predicts that sperm velocity may be increased by enlarged midpiece (energetic component) or flagellum length (kinetic component), or by particular ratios between sperm components, such as between flagellum length and head size. However, such associations have rarely been found in empirical studies. In a comparative framework in passerine birds, we tested these theoretical predictions both across a wide range of species and within a single family, the New World blackbirds (Icteridae). In both study groups, sperm velocity was influenced by sperm morphology in the predicted direction. Consistent with theoretical models, these results show that selection on sperm morphology and velocity are likely to be concomitant evolutionary forces.

Keywords: sperm competition; sperm motility; comparative study; New World blackbirds; Icteridae

1. INTRODUCTION

Sperm competition occurs when sperm from different males attempt to fertilize the same set of ova (Parker 1970). A potent evolutionary force, sperm competition, favours traits that enhance male fertilizing ability. Sperm velocity is one such trait and has been shown to determine fertilizing success across a wide range of taxa (e.g. fishes: Gage *et al.* 2004; mammals: Holt *et al.* 1989, Moore & Akhondi 1996, Malo *et al.* 2005; and birds: Birkhead *et al.* 1999, Donoghue *et al.* 1999). Since sperm vary considerably in their size and shape, both between and within taxa (e.g. Cohen 1977; Jamieson 2007; Pitnick *et al.* 2009), it seems likely that sperm velocity may be affected by sperm morphology. Distinct swimming characteristics have been described for different sperm types such as those of non-passerine and passerine birds (Vernon & Woolley 1999), but even within taxa with similar overall sperm structure, the absolute or relative size of different components or overall sperm size can affect sperm velocity, such as in Iberian deer *Cervus elaphus hispanicus* (Malo *et al.* 2006), the zebra finch *Taeniopygia guttata* (Mossman 2008), cichlid fishes (Fitzpatrick *et al.* accepted) and nematodes (LaMunyon & Ward 1998; Zajac 2008). However, the way that diversity in sperm morphology translates into variation in sperm velocity remains unclear.

Theoretical models predict that sperm with increased midpiece size might contain more mitochondria and hence produce more energy for powering the flagellum in the absence of glycolytic support (e.g. Cardullo & Baltz 1991). Energy is provided from the metabolism of cyclic AMP by the mitochondria of the midpiece, and catalysed ATP is shunted along the flagellum to generate motility through the myosin–actin interaction (Bedford & Hoskins 1990). Sperm with a longer midpiece have been reported to produce more ATP in the Atlantic salmon *Salmo salar* (Vladić *et al.* 2002), and in the domestic fowl *Gallus domesticus*, sperm velocity is positively associated with the rate of ATP synthesis (Froman & Feltmann 1998). There is also indirect evidence for a link between energy and sperm velocity from a number of comparative studies that documented positive relationships between absolute midpiece size and sperm competition intensity, suggesting that an enlarged midpiece occurs predominantly in species under intense sperm competition, where sperm velocity may be particularly important (Johnson & Briskie 1999; Anderson & Dixson 2002; Anderson *et al.* 2005; but see Immler & Birkhead 2007). However, there is very little direct empirical evidence for a positive association between midpiece size and sperm velocity, and Malo *et al.* (2006) found a negative correlation between these traits in Iberian deer, contrary to theoretical predictions.

Another way sperm morphology might influence sperm velocity is through the flagellum length. Theory predicts that the propulsive forces of a flagellum increase with its length (Katz *et al.* 1989), and it has sometimes been assumed that these propulsive forces are positively related to sperm velocity (e.g. Gomendio & Roldan 1991; Briskie & Montgomerie 1992; Gage 1994; Byrne *et al.* 2003). However, empirical studies are needed to test this assumption and establish how much of the variation in sperm velocity can be attributed to the flagellum length. A sperm moving in a viscous medium is subject to many different forces, including drag that mostly depends on the size of the sperm head and is likely to counteract the thrust of the flagellum (Wu 1977; Higdon 1979; Humphries *et al.* 2008). Moreover, it appears that most of the power needed for swimming is the power required to propel the flagellum itself. This power is proportionate to the flagellum length, as is the total power a flagellum can produce, such that elongation of the flagellum length should be accompanied by an increase in midpiece size

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to compensate for the elevated energy requirements (Cardullo & Baltz 1991). Flagellum length is indeed positively related to the midpiece length among birds and mammals (Cummins & Woodall 1985; Gage 1998; Immler & Birkhead 2007). Recent theory suggests that, given the opposing forces of the drag from the head and the dependence on energy supply from the midpiece, flagellum length may have to be considered relative to other sperm components rather than on its own to test hypotheses of the links between sperm morphology and velocity (Cardullo & Baltz 1991; Humphries *et al.* 2008).

To test whether sperm velocity is determined by sperm morphology, either by the absolute or relative size of sperm components (e.g. midpiece-to-flagellum ratio: Cardullo & Baltz 1991; or head-to-flagellum ratio: Humphries *et al.* 2008), we used two different comparative approaches in passerine birds, using data from (i) a wide range of species and (ii) one particular family, the New World blackbirds (Icteridae). We examined the Icteridae as a separate and additional dataset owing to recent evidence for distinct phylogenetic patterns between passerine families (Immler & Birkhead 2007), such that associations between the closely related species of a family may be less affected by distinct evolutionary trends between taxa and thus are likely to yield clearer results.

2. MATERIAL AND METHODS

(a) *Sample collection*

We collected fresh sperm samples from wild populations of 40 different passerine bird species (1–142 males per species; for species list and phylogeny, see the electronic supplementary material). Although we also had many samples of the bullfinch *Pyrrhula pyrrhula*, we omitted this species owing to its strikingly different sperm morphology from all other passerine species, with a rounded head and a very small and atypical midpiece (Birkhead *et al.* 2006, 2007). We collected all samples using model females (Pellatt & Birkhead 1994), cloacal massage (e.g. Burrows & Quinn 1937; Samour *et al.* 1986), or where birds were collected for other research projects or management programmes, by the dissection of the distal end of the seminal glomera (i.e. sperm-storage organ at the end of the deferent duct). Sperm collected through different techniques do not differ in their morphological measurements (Immler & Birkhead 2005), but within species, sperm from dissected birds tend to be approximately 10 per cent slower than those collected through cloacal massage (S. Lüpold 2006, unpublished data). Thus, we included the sampling method as a factor in all our analyses.

After dilution of the collected semen in Dulbecco's modified eagle medium (Invitrogen Ltd), we immediately placed a small drop (15 µl) under a phase-contrast microscope and videotaped at a magnification of 200× and 35°C. However, for some species that were used as part of concurrent studies, samples were recorded at 37 or 39°C. Across this range, temperature tends to elevate sperm velocity equally for all sperm within a given species, which consequently affects the species mean (Mossman 2008). Thus, we included temperature as a covariate in all analyses except for within the Icteridae, in which all samples were measured at 35°C. Finally, after videotaping, we fixed the remainder of each diluted sperm sample by adding formalin to an end concentration of approximately 5 per cent.

(b) *Sample analyses*

We analysed all video recordings using computer-aided sperm analysis (Hobson Tracking Systems Ltd, UK), filtering out atypical trajectories following the principles of Mossman (2008). We then used the mean of all remaining tracks per sample to calculate mean values for each species.

From all fixed samples, we captured high-resolution digital images at microscope magnifications of 250× or 400×, using a Spot Insight QE camera (Diagnostic Instruments Inc.) mounted onto a Leitz Laborlux S microscope, and analysed 5–10 morphologically normal sperm per sample, which is an adequate sample size for passerine birds owing to their very low variation within ejaculates (e.g. Immler *et al.* 2008). We measured the following four sperm components to the nearest 0.1 µm: the lengths of the (i) head, (ii) straight-line helix of the midpiece (calculated from the midpiece length using the formula in Birkhead *et al.* 2005), (iii) flagellum, and (iv) total sperm length. For each sperm trait, we used the means within individuals to calculate the mean for each species.

(c) *Statistical analyses*

We performed all analyses on all 40 species and the 13 icterid species only. After normalizing non-normal data distributions by log- or square-root transformations, we conducted a principal components analysis on five descriptors of sperm motility: (i) curvilinear, (ii) average-path and (iii) straight-line velocities, and (iv) straightness and (v) linearity of sperm trajectories. Using a two-factor 'varimax' rotation, this analysis collated information of all variables into two principal components: the speed parameters (i–iii) in the first and the path-shape parameters (iv–v) in the second principal component (see table S2 in the electronic supplementary material). We used the scores of the first principal component as a measure of sperm velocity (henceforth referred to as 'sperm velocity').

Since we performed all analyses separately, we calculated effect size r (partial correlation coefficient) and 95 per cent non-central confidence intervals from the t-values of our statistical models to establish the strength of the associations (Nakagawa & Cuthill 2007). Confidence intervals excluding zero indicate statistical significance at the α-level of 0.05 (Smithson 2003).

To account for the statistical non-independence of data points by shared ancestry of species (Felsenstein 1985; Harvey & Pagel 1991), we constructed a phylogeny (see the electronic supplementary material), and used a generalized least-squares approach in a phylogenetic framework (Pagel 1999; Freckleton *et al.* 2002). In addition to phylogenetic control, we estimated for each analysis the association of the traits with the phylogeny, expressed by the phylogenetic scaling parameter λ. Values of λ close to 0 indicate phylogenetic independence, and values of λ close to 1 indicate a complete phylogenetic association. We used likelihood-ratio tests to establish whether the model with the maximum-likelihood value of λ differed from the models with values of $\lambda = 1$ or 0, respectively. Superscripts following the λ estimates (e.g. $\lambda^{1.0;0.02}$) denote significance levels of these likelihood-ratio tests (first superscript: $\lambda = 1$; second superscript: $\lambda = 0$).

3. RESULTS

Across all 40 species, sperm length ranged between 46.8 and 287.6 µm, with flagellum length accounting for

Table 1. Interspecific associations (controlled for phylogeny) between sperm velocity and sperm morphology among all 40 species and the 13 icterid species. (The superscripts following λ indicate significance levels of the likelihood-ratio tests of the model against the maximum-likelihood value of $\lambda=1$ (first position) and $\lambda=0$ (second position). The effect size r (partial correlation coefficient) is presented with the non-central 95% confidence intervals (LCL, lower confidence limit; UCL, upper confidence limit). Confidence intervals excluding zero are statistically significant at $p<0.05$. Controlling variables: temperature: all $p<0.001$ for all species, n.a. for Icteridae (see §2). Collecting technique: $p=0.002$–0.09 for all species, all $p>0.2$ for Icteridae.)

					effect size		
traits	slope	t	p-value	λ	r	LCL	UCL
all species (n = *40*)							
midpiece	0.74	5.23	<0.0001	$<0.0001^{<0.001;1.0}$	0.66	0.44	0.79
midpiece[a]	0.75	2.96	0.005	$<0.0001^{<0.001;1.0}$	0.45	0.15	0.65
flagellum	1.13	4.03	0.0003	$<0.0001^{<0.001;1.0}$	0.56	0.30	0.72
total length	1.22	3.95	0.0004	$<0.0001^{<0.001;1.0}$	0.56	0.28	0.72
midpiece : flagellum	1.28	4.83	<0.0001	$<0.0001^{<0.001;1.0}$	0.63	0.40	0.77
midpiece : flagellum[a]	3.39	3.32	0.002	$<0.0001^{<0.001;1.0}$	0.49	0.20	0.68
flagellum : head	1.37	4.00	0.0003	$<0.0001^{<0.001;1.0}$	0.56	0.29	0.72
Icteridae (n = *13*)							
midpiece	1.62	2.79	0.017	$<0.0001^{0.003;1.0}$	0.66	0.14	0.84
flagellum	2.17	2.45	0.032	$<0.0001^{0.008;1.0}$	0.61	0.06	0.83
total length	2.39	2.34	0.040	$<0.0001^{<0.001;1.0}$	0.59	0.03	0.82
midpiece : flagellum	4.84	3.04	0.014	$<0.0001^{<0.001;1.0}$	0.69	0.19	0.86
flagellum : head	3.17	3.37	0.006	$<0.0001^{0.07;1.0}$	0.73	0.27	0.88

[a] Since *Garrulus glandarius* was a statistical outlier for midpiece length (figure 1*a*) and the midpiece : flagellum ratio (see §3), with strong leverage on the associations with sperm velocity, we also present the results after excluding this species.

78.6–95.0% of sperm length. The straightened helical midpiece reached a considerable proportion of the flagellum length (mean 86.4%, range 54.8–100%), except for the very short midpiece of the European jay *Garrulus glandarius* (9% of flagellum length). Compared with the sixfold variation in total sperm length, head length varied little between species (10.9–20.6 μm). Straight midpiece length varied 83-fold (range 3.3–272.0 μm) and 10-fold if *G. glandarius* was excluded, while flagellum varied 7.4-fold (36.4–271.5 μm).

(a) *Relationships between spermatozoal components, and with sperm competition*

All sperm components were positively related to one another: head–midpiece ($r=0.63$, $p<0.0001$, $\lambda<0.0001^{<0.001;0.1}$); head–flagellum ($r=0.63$, $p<0.0001$, $\lambda<0.0001^{<0.001;0.1}$); and midpiece–flagellum ($r=0.90$, $p<0.0001$, $\lambda<0.0001^{<0.001;0.1}$). The same was true for the Icteridae alone ($n=13$, all $r>0.75$, all $p<0.005$). To test whether a potential link existed between flagellar activity and energy supply by the midpiece, we calculated the allometric slope between the midpiece and the flagellum length in a reduced major-axis analysis (McArdle 1988). Midpiece length increased disproportionately relative to the flagellum length, with allometric slopes of 1.68 across all species, and 1.44 among the Icteridae (coefficients = 0.78 and 0.90, respectively, both $p<0.0001$). Additionally, flagellum length increased much faster than head size, with allometric slopes of 2.95 for all species and 4.00 for the Icteridae (coefficients = 0.86 and 0.94, respectively, both $p<0.0001$).

Sperm morphology was also positively associated with levels of sperm competition as measured by relative testes size across all species (see table S1 in the electronic supplementary material). The highly significant relationships for the Icteridae are published elsewhere on a much larger dataset than the 13 species used here, which were the only ones for which we had motility data (Lüpold *et al.* submitted).

(b) *Sperm velocity and sperm morphology*

Across all species and among the Icteridae, sperm velocity increased significantly with both absolute sizes of morphological sperm traits and ratios between them (table 1; figure 1). Both the midpiece : flagellum and flagellum : head ratios did not predict sperm velocity better than the midpiece or flagellum length alone, as indicated by the similar effect sizes with wide 95 per cent confidence intervals (table 1). Nonetheless, including flagellum length and head length as two independent variables rather than the ratio between them resulted in a strong negative effect of the head length and positive effect of the flagellum length on sperm velocity in the Icteridae (head: $r=-0.83$ (95% CI: -0.93 to -0.42), $p=0.002$; flagellum: $r=0.91$ (0.65–0.96), $p=0.0002$; $\lambda<0.0001^{1.0;1.0}$; figure S1 in the electronic supplementary material). Across all species, the effect of head size was also negative but not significant (head: $r=-0.10$ (-0.40–0.23), $p=0.57$; flagellum: $r=0.50$ (0.21–0.69), $p=0.002$; $\lambda<0.0001^{<0.001;1.0}$). We did not conduct the same analysis with midpiece and flagellum length owing to the strong intercorrelation between these two variables, as reflected by variance inflation factors (VIF; Marquardt 1970) exceeding 30, far beyond the suggested threshold of 10 (Marquardt 1970; Kleinbaum *et al.* 1998). By contrast, the VIF for the analysis for the head and the flagellum were less than 3.3.

Sperm velocity was not directly associated with relative testes size (i.e. log(combined testes mass) with log (body mass) as a covariate), both across all species (testes: $r=-0.14$, $p=0.39$; body: $r=-0.23$, $p=0.17$; $\lambda=0.27^{<0.001;0.26}$) and among the Icteridae (testes: $r=0.14$, $p=0.68$; body: $r=-0.63$, $p=0.03$; $\lambda=0.49^{0.002;0.26}$).

Figure 1. Associations of sperm velocity with (*a*) midpiece, (*b*) flagellum length and (*c*) the flagellum : head ratio. Each data point represents a species (filled circles, Icteridae; open circles, all other species). After controlling for phylogeny, all associations were significant among the Icteridae ($p=0.017$, 0.032 and 0.006, respectively) and across all species ($p=0.005$, 0.0003 and 0.0003, respectively). Further details are given in table 1.

4. DISCUSSION

We show that sperm velocity is positively associated with absolute and relative midpiece and flagellum length across passerine birds, both in a wide range of avian taxa and among closely related species of a single family. Although flagellum length corrected for head size did not predict sperm velocity better than flagellum length alone (see Humphries *et al.* 2008), the negative effect of head size on sperm velocity indicates that accounting for head size is important.

Since sperm velocity is an important predictor of the outcome of sperm competition (e.g. Holt *et al.* 1989; Birkhead *et al.* 1999; Gage *et al.* 2004), and sperm competition is one of the forces driving the rapid and divergent evolution of sperm morphology (reviewed in Pitnick *et al.* 2009), a link between sperm morphology and velocity has long been assumed but rarely found in empirical studies. In simple terms, the most frequently used theoretical predictions for such a link are that increased midpiece or flagellum length results in greater sperm velocity owing to more energy available or greater propulsive forces (Katz *et al.* 1989; Cardullo & Baltz 1991). Our results support both these predictions, showing positive associations between sperm velocity and the lengths of the midpiece and flagellum, but also total sperm length, across passerine birds. Similar results have previously been obtained in mammals (Gomendio & Roldan 1991; reanalysed with phylogenetic control by Gomendio & Roldan 2008).

However, sperm velocity is effectively the result of thrust and drag, and thus depends on the balance between flagellum length and head size (Humphries *et al.* 2008). In our study, the ratio between the flagellum and the head length yielded largely the same results as the absolute flagellum length (table 1), probably owing to relatively small head size combined with almost fourfold variation in flagellum length compared with that in head size. Nevertheless, the negative effect of head size on sperm velocity became particularly apparent in the Icteridae, when the head and the flagellum length were included as independent variables. This finding suggests that even if longer flagella can generate greater thrust than shorter ones (Katz *et al.* 1989), this advantage may be outweighed by the size of the head and the resulting drag forces (Wu 1977; Humphries *et al.* 2008), such that flagellum length has to respond by elongation at some point. This idea is also supported by the disproportionate increase in flagellum length compared with head size. Flagellum length may thus not simply be under selection for increased thrust but also to overcome the drag of the head, which emphasizes the consideration of the sperm head in studies of sperm morphology and velocity (Humphries *et al.* 2008).

A larger flagellum requires more energy, which is provided by the mitochondrial sheath of the midpiece (Bedford & Hoskins 1990), and elongation of the flagellum should thus encompass an associated increase in the midpiece length (Cardullo & Baltz 1991). We found a disproportionate increase in the midpiece length relative to the flagellum length. This positively allometric slope contrasts with that in mammals, in which Gage (1998) found negative allometry between the midpiece and the flagellum length, while the midpiece volume was independent of the flagellum length. However, compared with mammals, most passerine sperm have an enormously elongated helical midpiece coiled around the flagellum (Jamieson 2007), which also explains their 'twist-drill' motility (Vernon & Woolley 1999). The positive allometry, combined with the positive relationship between sperm velocity and the midpiece : flagellum ratio, underlines the importance of the midpiece length for sperm motility in passerines, but it is unclear whether energy demand is the sole reason for the unusual midpiece size or whether the midpiece may also have a stabilizing function for the mode of locomotion in this taxon. Either way, our results highlight that proportions between sperm components are important and that selection may not act on sperm components independently to maximize sperm performance.

Given the fertilization advantage of fast sperm in situations of sperm competition (e.g. Birkhead *et al.* 1999), we might expect a direct relationship between sperm competition and sperm velocity. In our dataset, we did not find such a direct link, but the strong associations of sperm morphology with both sperm velocity and sperm competition are indicative of an indirect link between sperm velocity and sperm competition in passerine birds. Avian sperm are subject to various selection pressures related to the competition between rival ejaculates and female sperm storage, such that the lack of a direct

relationship between sperm competition and sperm velocity may not be surprising. However, our results show that post-copulatory sexual selection shapes the morphology of sperm in a way that enhances sperm velocity as one important determinant of the outcome of sperm competition, but the direct link may be confounded by other effects such as sperm longevity or female reproductive environment (see below).

If there is associated evolution between sperm morphology and velocity, we would also expect to find evidence for it within species where any variation in sperm morphology affecting sperm velocity could directly lead to differential fertilizing success among competing males. However, little consistency exists across intraspecific studies. For example, in the zebra finch, Mossman (2008) found strong positive relationships between various sperm components and sperm velocity, and even a genetic link between these traits, whereas Lüpold *et al.* (submitted) found no evidence for an association between sperm morphology and sperm swimming speed within each of four different species of Icteridae. Likewise, in cichlid fishes, Fitzpatrick *et al.* (accepted) found little evidence for a link between sperm length and velocity within species, but a strong positive association between species. Many other intraspecific studies in various taxa also found no relationship between sperm morphology and velocity in both internal and external fertilizers (e.g. Gage *et al.* 2002; Burness *et al.* 2004; Minoretti & Baur 2006; Pitcher *et al.* 2007). However, Malo *et al.* (2006) reported a *negative* association between sperm velocity and midpiece length, i.e. contrary to the predicted positive relationship, but also demonstrated positive relationships between sperm velocity and head shape or relative flagellum components. The reasons for the discrepancy between studies remain unclear but it seems likely that sperm morphology is only one of a combination of factors that influence sperm velocity, such as seminal, vaginal or oviducal fluid, presence or absence of female sperm storage, or sperm metabolism. The importance of these different factors may vary between species, and controlling for them may reveal a link between sperm morphology and velocity in one or the other species in which previous studies have found none.

Another possible explanation is that, owing to the techniques currently available, sperm morphology and velocity are measured for different sperm populations within samples. Whereas normal sperm are typically selected for morphological measurements, abnormal sperm that show a relatively normal locomotion trajectory cannot be excluded from analyses of sperm velocity because their fast movements prevent detailed examination of sperm morphology. Consequently, if sperm vary within males, measurements of different subsets of samples could mask relationships between sperm morphology and velocity, particularly if sample sizes are small, as is the case for most intraspecific studies so far.

Finally, in at least some of the intraspecific studies mentioned above, a further explanation for the lack of a significant effect of sperm morphology on velocity could be that sperm traits were examined independently. Using relative instead of absolute measures of sperm components might reveal a significant relationship in some of these studies.

In conclusion, we show that sperm morphology affects sperm velocity in passerine birds, consistent with theoretical predictions. We also demonstrate, at least in the Icteridae, that the sperm head has a negative effect on sperm velocity, which corroborates recent theoretical models (e.g. Humphries *et al.* 2008), predicting that selection acts primarily on proportions between sperm components and suggesting that head size should be taken into account for studies of the links between sperm morphology and velocity.

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