Sperm design and variation in the New World blackbirds (Icteridae)

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Abstract

Post-copulatory sexual selection (PCSS) is thought to be one of the evolutionary forces responsible for the rapid and divergent evolution of sperm design. However, whereas in some taxa particular sperm traits are positively associated with PCSS, in other taxa, these relationships are negative, and the causes of these different patterns across taxa are poorly understood. In a comparative study using New World blackbirds (Icteridae), we tested whether sperm design was influenced by the level of PCSS and found significant positive associations with the level of PCSS for all sperm components but head length. Additionally, whereas the absolute length of sperm components increased, their variation declined with the intensity of PCSS, indicating stabilizing selection around an optimal sperm design. Given the diversity of, and strong selection on, sperm design, it seems likely that sperm phenotype may influence sperm velocity within species. However, in contrast to other recent studies of passerine birds, but consistent with several other studies, we found no significant link between sperm design and velocity, using four different species that vary both in sperm design and PCSS. Potential reasons for this discrepancy between studies are discussed.

Introduction

Spermatozoa vary considerably in size and shape between species (Franzén 1970; Cohen 1977; Pitnick et al. 2009), but also, albeit at generally lower levels, within species or between ejaculates of individual males (Ward 1998; Morrow and Gage 2001b; Birkhead et al. 2005; Calhim et al. 2007; Harris et al. 2007). However, while the structure of spermatozoa has been well studied, the causes and consequences of the variation in sperm design and the selective pressures driving its evolutionary divergence are poorly understood.

Sperm design is thought to be shaped by the mode of fertilization and phylogeny (Franzén 1970; Jamieson 1987), but also by post-copulatory sexual selection (PCSS; Sivinski 1980). PCSS consists of sperm competition (Parker 1970) and cryptic female choice (e.g., Thornhill 1983; Eberhard 1991, 1996) and favours sperm characteristics that enhance a male's fertilization success.

Although theoretical models predict that in internal fertilizers sperm size should remain small regardless of the level of sperm competition (Parker 1993; Parker and Begon 1993), empirical studies have often documented considerable variation in sperm size associated with the intensity of PCSS across species. Most of these studies reported positive co-variation between sperm size and PCSS (e.g. Briskie and Montgomerie 1992;

Gage 1994; LaMunyon and Ward 1999; Byrne et al. 2003; but see Gage and Freckleton 2003). In passerine birds, however, this co-variation is positive in some families but negative in others (Immler and Birkhead 2007), and further studies in other taxa are needed for a better understanding of the link between sperm design and PCSS.

In contrast to evolutionary trends across species, we know relatively little about the variation in sperm design within species. Theory predicts that sperm are selected towards specific optima at given levels of sperm competition (e.g. Parker 1993; Parker and Begon 1993). Consequently, the variation of sperm around the optimal design is expected to decline within increasing selection intensity, a phenomenon that has indeed been shown for both intra-specific (Calhim et al. 2007; Kleven et al. 2008) and intra-male variation (Immler et al. 2008; but see Kleven et al. 2008). These three studies on sperm variation were conducted in passerine birds, using species from a large number of different families and with marked variation in sperm competition as reflected by the range of extra-pair paternity levels. One potential reason why Immler et al. (2008) found a negative relationship between intra-male sperm variation and sperm competition but Kleven et al. (2008) did not is that these two studies used different suites of species. Both studies applied rigorous control for phylogenetic effects using the same powerful statistical tools (Freckleton et al. 2002), but most techniques for phylogenetic control currently available rely on the underlying assumption that the traits under examination are exposed to identical modes of evolution across all species in the analysis (Pagel and Harvey 1989; Harvey and Purvis 1991). This assumption may not hold for a broad comparative study on sperm of passerine birds as there is clear evidence that associations between sperm design and sperm competition differ among families (e.g. Immler and Birkhead 2007). Thus, a study within a single family may be less vulnerable to different selection patterns that exist between families.

If the levels of variation in sperm design differ between species in relation to sperm competition, it remains unclear how this affects sperm function. Sperm velocity is an important determinant of male fertilizing success (e.g. Holt et al. 1989; Birkhead et al. 1999; Gage et al. 2004; Malo et al. 2005a), and considering the diversity in sperm size and shape, it seems likely that the design of sperm affects their swimming performance. In fact, theoretical models predict at least three ways through which sperm design may increase sperm velocity: (i) enlarged midpiece size providing elevated energy supply for powering the flagellum (e.g. Cardullo and Baltz 1991; Froman and Feltmann 1998); (ii) a longer flagellum increasing propulsive forces (Katz et al. 1989), which have also been assumed to result in greater velocity (e.g. Gomendio and Roldan 1991; Briskie and Montgomerie 1992; Gage 1994; Byrne et al. 2003); and (iii) longer flagellum length *relative* to head size that can better overcome the drag forces acting on the head (e.g. Higdon 1979; Humphries et al. 2008). A recent study across a

range of passerine species demonstrated that sperm velocity increases with both absolute and relative dimensions of the sperm midpiece and flagellum (Lüpold et al. 2009a). However, while comparative studies are useful for establishing general evolutionary trends, understanding the basis of these trends requires detailed intra-specific analyses (e.g. Garland and Carter 1994; Arnqvist 1997). Consequently, it remains to be established whether the association between sperm design and sperm velocity reported across passerines also exists *within* these species.

We used the New World blackbirds (Icteridae) as a study taxon to test the hypotheses that (i) sperm length and the size of individual sperm components are influenced by the level of sperm competition; (ii) the intra-specific variation in sperm design decreases with increasing sperm competition; and that (iii) sperm design affects sperm velocity within species.

Methods

Sperm collection and analyses

In collaboration with numerous ornithologists, museum collectors, and bird banders in North America and South America, we collected sperm samples from wild populations of 38 different species of Icteridae, using any of four different methods: (i) cloacal massage (e.g. Burrows and Quinn 1937; Samour et al. 1986), (ii) natural ejaculations into the false cloaca of model females (Pellatt and Birkhead 1994), (iii) from faecal samples (Immler and Birkhead 2005), or (iv) by dissection of the distal end of the seminal glomera (i.e. sperm-storage organ at the end of the deferent duct) in species where specimens were collected for other research projects, museums or management programmes. Sperm collected through these different techniques do not differ in their morphological measurements (Immler and Birkhead 2005; Immler et al. 2008). All samples were fixed in 5–10% formalin. A full list of species with their phylogeny and sample sizes for the different analyses is given in the online Supplementary Table S1.

Sperm morphometric analyses

For each sperm sample, we analyzed 5–10 morphologically normal and undamaged sperm, which is a sufficient sample size to capture the within-male variation for comparative studies in passerine birds (Calhim et al. 2007; Immler and Birkhead 2007). We measured samples from digital images taken at magnifications of 250× or 400× (depending upon sperm size). Using computer-assisted image analysis, we measured the following traits of each sperm cell to the nearest 0.1μm: (i) head length, (ii) helical midpiece length, which we used to

calculate straight midpiece length using the formula in Birkhead et al. (2005), (iii) flagellum length, and (iv) total sperm length. We used the within-sample mean of each sperm trait for all further analyses.

Sperm velocity analyses

We measured sperm velocity in sperm samples that we collected using model females, cloacal massage, or immediate dissection of collected birds. After diluting a freshly collected sperm sample in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen Ltd.) to a final concentration of approximately 20×10^6 sperm/ml, we immediately placed 15µl under a phase-contrast microscope at 35±0.1°C and videotaped at a magnification of 200×. We then analyzed all video recordings using computer-aided sperm analysis (Hobson Tracking Systems Ltd, U.K.) and removed non-typical trajectories following the principles of Mossman (2008).

In the intra-specific comparisons of sperm morphology and velocity, we focused on the four species for which we had full datasets for ≥20 males (range 20–120 males; see online Supplementary Table S1): *Agelaius phoeniceus, Xanthocephalus xanthocephalus, Quiscalus quiscula, Molothrus ater*. Within the Icteridae studied so far, these four species span the entire range of sperm lengths (61–145µm) and also most of the range of relative testes size (see Figs. 1 and 2).

To calculate a sperm velocity index, we performed a principal components analysis (PCA) on five motility parameters: (i) curvilinear velocity (total sperm trajectory, VCL), (ii) average-path velocity (smoothed and averaged trajectory, VAP), (iii) straight-line velocity (shortest distance between the start and end point of the trajectory, VSL), (iv) path linearity (departure of actual sperm track from straight line, VSL/VCL), and (v) path straightness (departure of average sperm path from straight line, VSL/VAP). Following a two-factor 'varimax' rotation in the PCA, the speed parameters (i–iii) were maximally loaded on the first principal component (PC1) and the path-shape parameters (iv–v) on the second (PC2; Table 1). We used PC1 for our analyses.

Statistical analyses

We conducted all statistical analyses using the statistical package R v.2.7.1 and normalized all non-normal data distributions by log or square-root transformations to meet the parametric requirements of the statistical models.

To control for phylogenetic effects in all comparative analyses, we constructed a phylogenetic tree (see online Supplementary Table S1), and accounted for statistical non-independence of data points by shared ancestry of species (Felsenstein 1985; Harvey and Pagel 1991), using a generalized least-squares (GLS) approach in a phylogenetic framework (Pagel 1999; Freckleton et al. 2002). This approach allows the estimation

of the phylogenetic scaling parameter λ, with values of λ close to 0 indicating phylogenetic independence, and values of λ close to 1 indicating a complete phylogenetic association of the traits. We used likelihood ratio tests to establish whether the model with the maximum-likelihood value λ differed from models with values of λ=1 or 0, respectively. We indicate the significance levels of these likelihood ratio tests by superscripts following the λ estimates (e.g., $\lambda^{0.1;1.0}$; first superscript: against λ=1; second superscript: against λ=0).

We used the intra-specific coefficients of variation (CV), all log-transformed, as indices of intra-specific variation in sperm dimensions or velocity. For intra-specific CV estimates, Calhim et al. (2007) estimated the minimum adequate sample size to be around 10 males. Although we attempted to collect samples from at least 10 males for each species, this was not possible for most species due to limited access to reproductive males or because for some species, many faecal samples contained no sperm. However, Calhim et al.'s (2007) simulations also showed that even 5 males yield intra-specific CV estimates within about 5% of that obtained from 50 males. Including species with ≥6 males increased our sample size from 9 to 15 different species and formed a wider basis for our comparative analyses. We thus performed our analyses of intra-specific variation on all 15 species and confirmed the results using the more conservative, albeit considerably smaller, dataset of 9 species (for species and sample sizes see online Supplementary Table S1). For intra-specific variation in sperm velocity, we had data from 6 species with >5 males (one of these species with <10 males).

To account for the variation in sample sizes across species (6–351 males per species; online Supplementary Table S1), we maximized the efficiency of parameter estimation by weighting our analyses by the corresponding sample sizes (*N*). In brief, similar to the phylogeny matrix (*V*) used in the above GLS approach, we constructed a matrix (*W*) with 1/log(*N)* in the diagonal and 0 in all other positions and combined the two matrices with $Q=V+cW$, where c is a constant (Martins and Hansen 1997; Garamszegi and Møller 2007). By varying c we determined the model with the highest maximum likelihood score. We will report the weighted and unweighted results of our final models.

As an index of sperm competition, we used relative testes size by including both log-transformed combined testes mass (CTM) and body mass as independent variables in our analyses, which is preferable to the use of residuals from a regression between the two variables (e.g. García-Berthou 2001). We obtained testes and body mass data from the published literature, our own measurements, and from museum databases (i.e. Field Museum of Natural History Chicago and Smithsonian National Museum of Natural History), using data only from birds that were likely to be in breeding condition and to have fully developed testes given their geographic location, date of collection and the range of testes sizes in the databases (see Calhim and Birkhead 2007). Although relative testes size as a measure of sperm competition tends to slightly underestimate the sperm

production rate, and thus potentially the level of sperm competition, for species under intense sperm competition (Lüpold et al. 2009b), it was the best index available for the entire collection of species in our study.

In the intra-specific comparisons of sperm morphology and velocity, we used four morphological traits: (i) midpiece, and (ii) flagellum length, (iii) flagellum:head (F:H) ratio, and (iv) midpiece:flagellum (M:F) ratio to account for both absolute and relative sizes of sperm components. For *A. phoeniceus*, we obtained 148 natural ejaculates from 120 different males, using model females. For the 16 males with ≥2 samples, we used intra-male means to avoid pseudoreplication. In the other three species, we obtained a single sample from each male after dissection. The processing time of samples (from collection in the marsh to start of video recording for motility analysis) varied between males due to field conditions, but it had no significant effect on any of our analyses (all $P>0.21$). We thus omitted control for the elapsed time and performed simple regressions in all four species.

Results

We found considerable variation in the size of sperm dimensions across the 38 species of Icteridae, with mean sperm length ranging between 61 and 145µm. In nested ANOVAs, about 98% of the total variation in midpiece, flagellum, or total sperm length was explained by the inter-specific variation, whereas the intra-specific and intra-male variations contributed only around 1.8% and 0.2%, respectively. In contrast, head length varied relatively little among species (range 12.3–15.0µm), such that the inter- and intra-specific variance components shared similar percentages of the total variation (46.0% and 44.2%, respectively), with the remaining 9.8% being explained by the variation within males.

Relationships between sperm traits

Using the means for each of the 38 species, midpiece length was positively correlated with flagellum length ($r=0.97$, $P<0.0001$, $\lambda<0.0001^{<0.001;1.0}$) and with total sperm length ($r=0.96$, $P<0.0001$, $\lambda<0.0001^{<0.001;1.0}$). Head length was also positively related to all other sperm measures (midpiece: $r=0.46$, $P=0.007$, $\lambda<0.0001^{0.15;1.0}$; flagellum: $r=0.50$, $P=0.003$, $\lambda<0.0001^{0.04;1.0}$; total length: $r=0.54$, $P=0.002$, $\lambda<0.0001^{0.03;1.0}$).

The relative increase of the three sperm components (head, midpiece and flagellum length) with total sperm length may indicate metabolic and/or functional regulation of the sperm (e.g., Cardullo and Baltz 1991; Humphries et al. 2008). We thus compared the slopes between the three sperm traits relative to total sperm length in an ANCOVA with trait size as the response variable, and trait and sperm length as the explanatory

variables. All interactions between slopes were statistically significant: flagellum length (slope=1.14) increased significantly more rapidly with total sperm length than did head length (slope=0.15; t=-20.36, P<0.0001), and the slope for midpiece length (slope=1.48) was significantly steeper than that of flagellum length (t=-5.062, P<0.0001; see Fig. 1). Consequently, any elongation of the sperm head would result in a disproportionate increase in flagellum length, and this in turn would entail an even greater increase in midpiece length.

Associations between sperm morphology, variation and sperm competition

Across the 38 species, all morphometric sperm traits except head length increased significantly with relative testes size (Table 2; Fig. 2). The λ values were all very small and not significantly different from 0, indicating that the relationships between these traits are independent of the underlying phylogeny (Freckleton et al. 2002).

The intra-specific CV estimates of sperm components varied 6- to 13-fold across the 15 species (e.g., CV(flagellum)=1.1–6.7%, CV(midpiece)=1.0–13.2%) and were negatively correlated with relative testes size, particularly after weighting for sample sizes within species (Table 3; Fig. 3). Here, the λ value for the variation in head size was much higher than in any of the other analyses, but not significantly different from 0, suggesting again independence of the phylogeny. The same relationships were also significantly negative using only the 9 species with ≥10 males and thus more than the minimal adequate sample sizes within species (all P≤0.04; Fig. 3).

Correlations between sperm morphology and velocity

Within the four species with samples from ≥20 males, sperm velocity (PC1) was not significantly correlated with any of the four morphological sperm measures that are predicted to affect sperm velocity.

In *A. phoeniceus*, the sperm velocity index (PC1) only showed a positive trend with midpiece length (Fig. 4; N=120 males; midpiece: r=0.17, P=0.06; flagellum: r=0.08, P=0.39; F:H ratio: r=-0.01, P=0.96; M:F ratio: r=0.14, P=0.12). Due to the trend with midpiece size, we also tested VAP and VSL separately, which are both frequently used as univariate measures of sperm velocity (e.g., Moore and Akhondi 1996; Burness et al. 2004; Birkhead et al. 2005; Malo et al. 2005a; Cornwallis and Birkhead 2007). VAP increased significantly with midpiece (r=0.18, P=0.05) and flagellum length (r=0.21, P=0.03), but VSL did not (midpiece: r=0.14, P=0.12; flagellum: r=0.11, P=0.72). Both velocity measures were not significantly correlated with the F:H or M:F ratios (all r<0.14, all P>0.11). Overall, we found no consistent pattern in *A. phoeniceus*, but some indication for a

potential link between sperm morphology and velocity, although any effects were at best weak and not significant after accounting for multiple testing.

In *X. xanthocephalus*, we also found no association between the size of sperm components and sperm velocity (*N*=24 males; midpiece: *r*=-0.05, *P*=0.82; flagellum: *r*=-0.09, *P*=0.68; F:H ratio: *r*=0.18, *P*=0.40; M:F ratio: *r*=0.04, *P*=0.84). Likewise, sperm velocity did not co-vary with sperm dimensions in *Q. quiscula* (*N*=20 males; midpiece: *r*=0.05, *P*=0.84; flagellum: *r*=-0.29, *P*=0.23; F:H ratio: *r*=-0.27, *P*=0.26; M:F ratio: *r*=0.24, *P*=0.32) and in *M. ater* (*N*=21 males; midpiece: *r*=0.11, *P*=0.64; flagellum: *r*=0.25, *P*=0.30; F:H ratio: *r*=-0.12, *P*=0.61; M:F ratio: *r*=0.03, *P*=0.91). Using individual parameters of sperm velocity did not change these results qualitatively.

Having found some, but no significant, positive trends, we combined the datasets from all four species and performed an ANCOVA for each sperm trait to establish whether a general pattern existed across species. For each morphometric trait, the interactions between sperm trait and species were not statistically significant (all $P>0.36$) and thus omitted. Furthermore, in the analyses with midpiece and flagellum length, respectively, as explanatory variables, the coefficients did not differ significantly between *A. phoeniceus*, *X. xanthocephalus*, and *Q. quiscula*. Collapsing these three factor levels did not change the explanatory power of the models (ANOVAs between models: $P>0.53$) but lowered Aikake's Information Criterion (AIC) by >3 units, thus indicating a better fit of the simplified models. Using the same criteria, *X. xanthocephalus* and *Q. quiscula* could be combined in the analyses with the ratios between sperm components. All minimal adequate models showed a positive effect of trait size (all partial $r>0.37$, all $P<0.001$) on sperm velocity and significantly different intercepts between species groups (all partial $r>0.17$, all $P<0.03$), but the explanatory power of all models was relatively low (all multiple $r^2=0.19$ to 0.21). In other words, after combining the results among all four species, there is some indication that sperm morphology may influence sperm velocity as predicted by theory.

Finally, we compared the intra-specific variance of sperm velocity with that of sperm morphology and with relative testes size. Across the 6 species with a full dataset for more than 5 males, we combined the log-transformed intra-specific CV's of the 5 motility parameters in a PCA with a 'varimax' rotation and used PC1 as an index of intra-specific variation in sperm velocity. This index was inversely correlated with the variances in sperm components (Fig. 5; midpiece: *r*=-0.93, *P*=0.008; flagellum: *r*=-0.94, *P*=0.009; total sperm length: *r*=-0.96, *P*=0.005; all $\lambda<0.0001^{<0.03;1.0>}$), and it also increased with relative testes size (testes: *r*=0.96, *P*=0.02; body: *r*=-0.89, *P*=0.07; $\lambda<0.0001^{0.03;1.0}$). However, using the intra-specific variation in VSL or VAP (measured as CV's) instead of PC1 (above), the association between the variation in sperm velocity and relative testes size was

no longer significant (all $r<0.75$, all $P>0.11$). In all analyses, the unweighted analyses yielded qualitatively the same results as the unweighted ones.

Discussion

Our main results were that across species, (i) all major sperm components (except head length) increased in size with the level of PCSS, indicating directional selection for longer sperm; (ii) the intra-specific variation in all sperm components decreased with increasing sperm competition, suggesting stabilizing selection around an optimal sperm design within species; (iii) within four species varying in their level of sperm competition, sperm velocity was not significantly correlated with sperm design, but in *A. phoeniceus* we found interesting patterns with midpiece and flagellum length that deserve further investigation; and (iv) the intra-specific variation in sperm velocity was inversely related to the variation in sperm design, while there was no clear pattern for its association with sperm competition.

(i) Sperm design and sperm competition

We found positive relationships between sperm competition and all sperm components (except head size), consistent with previous studies on other taxa (e.g., Gage 1994; Balshine et al. 2001; Anderson and Dixson 2002; Byrne et al. 2003). However, while longer sperm may be advantageous and selected for by PCSS in these taxa, in other taxa shorter sperm appear to be favoured (Immler and Birkhead 2007). The reasons for these inverse evolutionary trends are currently poorly understood, and further insight into the links between sperm design, sperm function, and male and female reproductive organs may help us understand the selective mechanisms and processes.

It also remains to be established why PCSS may favour longer sperm, but at least three potential mechanisms have been proposed: First, sperm size can be selected for by co-evolution with sperm-storage structures within the female reproductive tract, as evinced in various taxa (e.g. Dybas and Dybas 1981; Briskie and Montgomerie 1992; Miller and Pitnick 2002). Second, if a larger midpiece can produce more ATP, longer sperm (with a longer midpiece) may also be more competitive because they are longer lived (Parker 1993, 1998, but see Levitan 2000; Gage et al. 2002; Immler et al. 2007) or swim faster (discussed below, section iii). Finally, larger sperm may also displace smaller rival sperm from the female reproductive tract (LaMunyon and Ward 1998). Which of these mechanisms apply to the Icteridae has not been established, but a combination of different

factors seems likely given the positive association of sperm design with sperm velocity across the Icteridae (Lüpold et al. 2009a) or with the size of female sperm-storage tubules across passerine birds in general (Briskie and Montgomerie 1992; Briskie et al. 1997).

(ii) Variation in sperm design and sperm competition

In addition to selection for longer sperm, PCSS also appears to reduce the intra-specific variation in sperm design in the Icteridae, probably by stabilizing selection around a species-specific optimum (Morrow and Gage 2001b; Calhim et al. 2007). That we found the same pattern across closely related species of a single family as in the sample of species used in Calhim et al.'s (2007) or Kleven et al.'s (2008) studies, where the overall variation in levels of PCSS is much greater, suggests that stabilizing sexual selection may be particularly strong within icterid species. However, the depletion of sperm variation between males in species under intense PCSS raises the question of how much sperm design ultimately contributes to the variation in reproductive success. It would also be interesting to know more about the heritability and selection potential of sperm design in species under intense selection. To date, such information in passerine birds is, to our knowledge, available only for the zebra finch (*Taeniopygia guttata*), a species under low sperm competition, in which sperm design is highly heritable and varies considerably between males (Birkhead et al. 2005).

(iii) Sperm design and velocity

Within each of four icterid species we found no significant association between the size of sperm components and the sperm velocity index, and only very weak positive relationships for individual velocity parameters. We thus found at best little support of theoretical models that have predicted different ways through which sperm design may influence sperm velocity (Higdon 1979; Katz et al. 1989; Cardullo and Baltz 1991; Humphries et al. 2008).

Our results contrast with a recent empirical study across 40 passerine species, including 13 species of Icteridae, where strong positive associations have been observed between sperm velocity and absolute or relative sperm dimensions (Lüpold et al. 2009a), or with Mossman's (2008) study in the zebra finch, where sperm velocity is positively related to and genetically linked with sperm design. Overall, however, the majority of intra-specific studies have found no significant links between sperm design and velocity, in either internal or external fertilizers (e.g. Gage et al. 2002; Burness et al. 2004; Minoretti and Baur 2006; Stoltz and Neff 2006; Fitzpatrick et al. 2007; Locatello et al. 2007; Pitcher et al. 2007), including another passerine species, the red-billed quelea (*Quelea quelea*; S. Calhim and T. R. Birkhead, unpubl. data). Finally, as in the Icteridae, sperm velocity in

cichlid fish is also independent of sperm length in most species, but strongly associated across species (Fitzpatrick et al. 2009).

The reasons for the difference between the intra- and inter-specific studies in passerine birds remain unclear, but the discrepancy may at least in part be attributed to the variation in both sperm design and velocity. For example, both *A. phoeniceus* and *Q. quelea* are under intense sperm competition and thus show very low variation in sperm design, whereas zebra finches exhibit much greater variation (Birkhead et al. 2005; Calhim et al. 2007). Therefore, the low variation of the first two species may compromise the detectability of a potential relationship between sperm design and velocity despite large sample sizes (120 and 113 males, respectively), compared to the zebra finch or the inter-specific comparison. Due to the inverse relationship between the intra-specific variances in sperm design and velocity, the other species of this study may in turn have too little variation in sperm velocity for enough statistical power, particularly given their relatively low sample sizes (N=20–24). In fact, these three analyses exhibited low statistical power, and indeed the majority of intra-specific studies that found no link between sperm phenotype and swimming performance are based on fewer than 30 males (e.g., Burness et al. 2004; Minoretti and Baur 2006; Stoltz and Neff 2006; Locatello et al. 2007). The only study that found significant effects with such a small sample size is Malo et al. (2006) with 36 males, but their association may be driven mainly by three extreme values. Overall, it appears that even if sperm design affects sperm velocity, such a pattern may be detected only through a large sample size; when sample sizes are small, any pattern may be obscured too much by confounding factors.

Particularly for internal fertilizers, the environment in which sperm operate is difficult to simulate. Sperm performance is typically measured under standardized conditions (e.g., temperature and medium, such as DMEM), but within the female reproductive tract, sperm locomotion may be affected by wall effects and vaginal fluid, or by components of the seminal fluid. Standardizing the conditions for sperm analysis can control for some confounding effects, particularly for the examination of biomechanical links between form and function, but we do not know whether ejaculates from different male birds perform equally well in artificial media compared to their own seminal fluid. To test this possibility, we would need to separate seminal fluid from sperm and use this to re-suspend a subpopulation of the sperm contained in the same ejaculate. In passerine birds, this approach would be technically difficult because ejaculates are highly viscous and contain very little fluid, and they have to be greatly diluted to obtain a measurable concentration (pers. obs.).

Recent studies in the fowl (*Gallus gallus*) also show that sperm velocity can decrease over successive copulations (Cornwallis and Birkhead 2007), and that sperm performance may depend on social status (Pizzari et al. 2007). Sperm velocity has also been reported to vary with male body condition or quality (Malo et al. 2005b;

Pitcher et al. 2007; Urbach et al. 2007), female attractiveness (Cornwallis and Birkhead 2007), or male mating tactic (Fitzpatrick et al. 2007; Locatello et al. 2007). In contrast, sperm design is highly heritable (Beatty 1970, 1972; Morrow and Gage 2001a; Birkhead et al. 2005) and may thus be limited in intra-male variation compared to sperm velocity. Although a genetic link between sperm design and motility exists in the zebra finch (Mossman 2008), sperm velocity is likely the result of a combination of factors that can depend on, or interact with, other ejaculate parameters (e.g., see Snook 2005 and references therein). Therefore, future research should be directed at identifying these factors, which would allow us to better control for confounding effects and establish whether sperm design translates into sperm velocity as predicted by theoretical models.

Conclusions

Our data suggest that post-copulatory sexual selection favours longer sperm components and reduces the intra-specific variation in sperm design among the Icteridae. Although theoretical models and a comparative study across the Icteridae indicate that selection on sperm design might be associated with selection for faster sperm, sperm design does not appear to dictate swimming performance within these species, or presently unknown factors may confound such a pattern in these and previously studied species. It is crucial that future research resolves the significance of sperm design for sperm function by understanding how both these sperm characteristics are influenced by environmental or conditional effects and controlling for them.

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References

Anderson MJ, Dixson AF (2002) Motility and the midpiece in primates. Nature 416:496

Arnqvist G (1997) The evolution of animal genitalia: distinguishing between hypotheses by single species studies. Biol J Linn Soc 60:365-379

Balshine S, Leach BJ, Neat F, Werner NY, Montgomerie R (2001) Sperm size of African cichlids in relation to sperm competition. Behav Ecol 12:726-731

Beatty RA (1970) The genetics of the mammalian gamete. Biol Rev 45:73-119

Beatty RA (1972) The genetics of size and shape of spermatozoan organelles. In: Glueckson-Waelsch S (ed) The Genetics of the Spermatozoa. Edinburgh University Press, Edinburgh, pp 97-115

Birkhead TR, Martínez JG, Burke T, Froman DP (1999) Sperm mobility determines the outcome of sperm competition in the domestic fowl. Proc R Soc Lond B 266:1759-1764

Birkhead TR, Pellatt EJ, Brekke P, Yeates R, Castillo-Juarez H (2005) Genetic effects on sperm design in the zebra finch. Nature 434:383-387

Briskie JV, Montgomerie R (1992) Sperm size and sperm competition in birds. Proc R Soc Lond B 247:89-95

Briskie JV, Montgomerie R, Birkhead TR (1997) The evolution of sperm size in birds. Evolution 51:937-945

Burness G, Casselman SJ, Schulte-Hostedde AI, Moyes CD, Montgomerie R (2004) Sperm swimming speed and energetics vary with sperm competition risk in bluegill (*Lepomis macrochirus*). Behav Ecol Sociobiol 56:65-70

Burrows WH, Quinn JP (1937) The collection of spermatozoa from domestic fowl and turkey. Poult Sci 16:19-24

Byrne PG, Simmons LW, Roberts JD (2003) Sperm competition and the evolution of gamete morphology in frogs. Proc R Soc Lond B 270:2079-2086

Calhim S, Birkhead TR (2007) Testes size in birds: quality versus quantity - assumptions, errors, and estimates. Behav Ecol 18:271-275

Calhim S, Immler S, Birkhead TR (2007) Postcopulatory sexual selection is associated with reduced variation in sperm morphology. PLoS ONE 2:e413. doi: 10.1371/journal.pone.0000413

Cardullo RA, Baltz JM (1991) Metabolic regulation in mammalian sperm: mitochondrial volume determines sperm length and flagellar beat frequency. Cell Motil Cytoskelet 19:180-188

Cohen J (1977) Reproduction. Butterworths, London, UK

Cornwallis CK, Birkhead TR (2007) Changes in sperm quality and numbers in response to experimental manipulation of male social status and female attractiveness. Am Nat 170:758-770

Dybas LK, Dybas HS (1981) Coadaptation and taxonomic differentiation of sperm and spermathecae in featherwing beetles. Evolution 35:168-174

Eberhard WG (1991) Copulatory courtship and cryptic female choice in insects. Biol Rev 66:1-31

Eberhard WG (1996) Female Control: Sexual Selection by Cryptic Female Choice. Princeton University Press, Princeton, New Jersey

Felsenstein J (1985) Phylogenies and the comparative method. Am Nat 125:1-15

Fitzpatrick JL, Desjardins JK, Milligan N, Montgomerie R, Balshine S (2007) Reproductive-tactic-specific variation in sperm swimming speeds in a shell-brooding cichlid. Biol Reprod 77:280-824

Fitzpatrick JL, Montgomerie R, Desjardins JK, Stiver KA, Kolm N, Balshine S (2009) Female promiscuity promotes the evolution of faster sperm in cichlid fishes. Proc Natl Acad Sci USA 106:1128-1132

Franzén Å (1970) Phylogenetic aspects of the morphology of spermatozoa and spermiogenesis. In: Baccetti B (ed) Comparative Spermatology. Academic Press, New York, pp 29-45

Freckleton RP, Harvey PH, Pagel M (2002) Phylogenetic analysis and comparative data: a test and review of evidence. Am Nat 160:712-726

Froman DP, Feltmann AJ (1998) Sperm mobility: a quantitative trait of the domestic fowl (*Gallus domesticus*). Biol Reprod 58:379-384

Gage MJG (1994) Associations between body size, mating pattern, testis size and sperm lengths across butterflies. Proc R Soc Lond B 258:247-254

Gage MJG, Freckleton RP (2003) Relative testis size and sperm morphometry across mammals: no evidence for an association between sperm competition and sperm length. Proc R Soc Lond B 270:625-632

Gage MJG, Macfarlane C, Yeates S, Shackleton R, Parker GA (2002) Relationships between sperm morphometry and sperm motility in the Atlantic salmon. J Fish Biol 61:1528-1539

Gage MJG, Macfarlane CP, Yeates S, Ward RG, Searle JB, Parker GA (2004) Spermatozoal traits and sperm competition in Atlantic salmon: relative sperm velocity is the primary determinant of fertilization success. Curr Biol 14:44-47

Garamszegi LZ, Møller AP (2007) Prevalence of avian influenza and host ecology. Proc R Soc Lond B 274:2003-2012

García-Berthou E (2001) On the misuse of residuals in ecology: testing regression residuals vs. the analysis of covariance. J Anim Ecol 70:708-711

Garland T, Carter PA (1994) Evolutionary physiology. Annu Rev Physiol 56:579-621

Gomendio M, Roldan ERS (1991) Sperm competition influences sperm size in mammals. Proc R Soc Lond B 243:181-185

Harris WE, Moore AJ, Moore PJ (2007) Variation in sperm size within and between ejaculates in a cockroach. Funct Ecol 21:598-602

Harvey PH, Pagel MD (1991) The comparative method in evolutionary biology. Oxford University Press, Oxford, UK

Harvey PH, Purvis A (1991) Comparative methods for explaining adaptations. Nature 351:619-624

Higdon JJL (1979) A hydrodynamic analysis of flagellar propulsion. J Fluid Mech 90:685-711

Holt WV, Shenfield F, Leonard T, Hartmann TD, North RD, Moore HDM (1989) The value of sperm swimming speed measurements in assessing the fertility of human frozen semen. Hum Reprod 4:292-297

Humphries S, Evans JP, Simmons LW (2008) Sperm competition: linking form to function. BMC Evol Biol 8:319 doi:10.1186/1471-2148-8-319

Immler S, Birkhead TR (2005) A non-invasive method for obtaining spermatozoa from birds. Ibis 147:827-830

Immler S, Birkhead TR (2007) Sperm competition and sperm midpiece size: no consistent pattern in passerine birds. Proc R Soc Lond B 274:561-568

Immler S, Calhim S, Birkhead TR (2008) Increased postcopulatory sexual selection reduces the intramale variation in sperm design. Evolution 62:1538-1543

Immler S, Saint-Jalme M, Lesobre L, Sorci G, Roman Y, Birkhead TR (2007) The evolution of sperm morphometry in pheasants. J Evol Biol 20:1008-1014

Jamieson BGM (1987) The Ultrastructure and Phylogeny of Insect Spermatozoa. Cambridge University Press, Cambridge

Katz DF, Drobnis EZ, Overstreet JW (1989) Factors regulating mammalian sperm migration through the female reproductive tract and oocyte vestments. Gamete Res 22:443-469

Kleven O, Laskemoen T, Fossøy F, Robertson RJ, Lifjeld JT (2008) Intraspecific variation in sperm length is negatively related to sperm competition in passerine birds. Evolution 62:494-499

LaMunyon CW, Ward S (1998) Larger sperm outcompete smaller sperm in the nematode *Caenorhabditis elegans*. Proc R Soc Lond B 265:1997-2000

LaMunyon CW, Ward S (1999) Evolution of sperm size in nematodes: sperm competition favours larger sperm. Proc R Soc Lond B 266:263-267

Levitan DR (2000) Sperm velocity and longevity trade off each other and influence fertilization in the sea urchin *Lytechinus variegatus*. Proc R Soc Lond B 267:531-534

Locatello L, Pilastro A, Deana R, Zarpellon A, Rasatto MB (2007) Variation pattern of sperm quality traits in two gobies with alternative mating tactics. Funct Ecol 21:975-981

Lüpold S, Calhim S, Immler S, Birkhead TR (2009a) Sperm morphology and sperm velocity in passerine birds. Proc R Soc Lond B:doi:10.1098/rspb.2008.1645

Lüpold S, Linz GM, Rivers JW, Westneat DF, Birkhead TR (2009b) Sperm competition selects beyond relative testes size in birds. Evolution:doi:10.1111/j.1558-5646.2008.00571.x

Malo AF, Garde JJ, Soler AJ, Garcia AJ, Gomendio M, Roldan ERS (2005a) Male fertility in natural populations of red deer is determined by sperm velocity and the proportion of normal spermatozoa. Biol Reprod 72:822-829

Malo AF, Gomendio M, Garde J, Lang-Lenton B, Soler AJ, Roldan ERS (2006) Sperm design and sperm function. Biol Lett 2:246-249

Malo AF, Roldan ERS, Garde J, Soler AJ, Gomendio M (2005b) Antlers honestly advertise sperm production and quality. Proc R Soc Lond B 272:149-157

Martins EP, Hansen TF (1997) Phylogenies and the comparative method: a general approach to incorporating phylogenetic information into the analysis of interspecific data. Am Nat 149:646-667

Miller GT, Pitnick S (2002) Sperm-female coevolution in *Drosophila*. Science 298:1230-1233

Minoretti N, Baur B (2006) Among- and within-population variation in sperm quality in the simultaneously hermaphroditic land snail *Arianta arbustorum*. Behav Ecol Sociobiol 60:270-280

Moore HDM, Akhondi MA (1996) Fertilizing capacity of rat spermatozoa is correlated with decline in strait-line velocity measured by continuous computer-aided sperm analysis: epididymal rat spermatozoa from the proximal cauda have a greater fertilizing capacity *in vitro* than those from the distal cauda or vas deferens. J Androl 17:50-60

Morrow EH, Gage MJG (2001a) Artificial selection and heritability of sperm length in *Gryllus bimaculatus*. Heredity 87:356-362

Morrow EH, Gage MJG (2001b) Consistent significant variation between individual males in spermatozoal morphometry. J Zool 254:147-153

Mossman J (2008) The Role of Mitochondrial Genetic Variation on Sperm Function: Empirical Tests of the Frank and Hurst Hypothesis. PhD thesis. University of Sheffield, Sheffield

Pagel M (1999) Inferring the historical patterns of biological evolution. Nature 401:877-884

Pagel MD, Harvey PH (1989) Comparative methods for examining adaptation depend on evolutionary models. Folia Primatol 53:203-220

Parker GA (1970) Sperm competition and its evolutionary consequences in the insects. Biol Rev 45:526-567

Parker GA (1993) Sperm competition games: sperm size and sperm number under adult control. Proc R Soc Lond B 253:245-254

Parker GA (1998) Sperm competition and the evolution of ejaculates: towards a theory base. In: Birkhead TR, Møller AP (eds) Sperm Competition and Sexual Selection. Academic Press, London, pp 3-54

Parker GA, Begon ME (1993) Sperm competition games: sperm size and sperm number under gametic control. Proc R Soc Lond B 253:255-262

Pellatt EJ, Birkhead TR (1994) Ejaculate size in zebra finches *Taeniopygia guttata* and a method for obtaining ejaculates from passerine birds. Ibis 136:97-106

Pitcher TE, Rodd FH, Rowe L (2007) Sexual colouration and sperm traits in guppies. J Fish Biol 70:165-177

Pitnick S, Hosken DJ, Birkhead TR (2009) Sperm Diversity. In: Birkhead TR, Hosken DJ, Pitnick S (eds) Sperm Biology: An Evolutionary Perspective. Elsevier, London, pp 69-149

Pizzari T, Cornwallis CK, Froman D (2007) Social competitiveness associated with rapid fluctuations in sperm quality in male fowl. Proc R Soc Lond B 274:853-860

Samour JH, Smith CA, Moore HD, Markham JA (1986) Semen collection and spermatozoa characteristics in budgerigars (*Melopsittacus undulatus*). Vet Rec 118:397-399

Sivinski J (1980) Sexual selection and insect sperm. Fla Entomol 63:99-111

Snook RR (2005) Sperm in competition: not playing by the numbers. Trends Ecol Evol 20:46-53

Stoltz JA, Neff BD (2006) Sperm competition in a fish with external fertilization: the contribution of sperm number, speed and length. J Evol Biol 19:1873-1881

Thornhill R (1983) Cryptic female choice and its implications in the scorpionfly *Harpobittacus nigriceps*. Am Nat 122:765-788

Urbach D, Bittner D, Lenz TL, Bernet D, Wahli T, Wedekind C (2007) Sperm velocity in an alpine whitefish: effects of age, size, condition, fluctuating asymmetry and gonad abnormalities. J Fish Biol 71:672-683

Ward PI (1998) Intraspecific variation in sperm size characters. Heredity 80:655-659

Tables

Table 1 Results of the principal components analysis on the sperm motility measures for *A. phoeniceus*. The factor loadings of PC1 and PC2 are listed, together with the variance explained by each component and the cumulative variance explained. The higher loadings for each PC are highlighted in bold. The results of the other three species were comparable, with PC1 explaining 54.0% of the variance in *X. xanthocephalus*, 67.6% in *Q. quiscula*, and 61.7% in *M. ater*

Variable	PC1	PC2
Curvilinear velocity	**0.77**	0.63
Average-path velocity	**0.82**	0.55
Straight-line velocity	**0.97**	0.05
Straightness	0.08	**0.96**
Linearity	0.55	**0.81**
Variance explained (%)	50.3	45.6
Total variance explained (%)		95.9

Table 2 Statistics of the inter-specific associations between morphological sperm traits and relative testes size (i.e. combined testes mass [CTM] corrected for body mass), all variables log-transformed; *N*=38 species. Effect sizes are reported as the partial correlation coefficients *r* with the lower (LCL) and upper 95% confidence limits (UCL)

					Effect size		
Traits	**Slope**	***t***	***P***	***λ***	***r***	**LCL**	**UCL**
Head length							
CTM	0.01	0.65	0.522	$<0.001^{0.04;1.0}$	0.11	-0.22	0.41
Body mass	-0.04	-1.98	0.056		-0.32	-0.56	0.01
Midpiece length							
CTM	0.22	2.86	0.007	$<0.001^{<0.001;1.0}$	0.44	0.13	0.64
Body mass	-0.20	-2.14	0.039		-0.34	-0.58	-0.02
Flagellum length							
CTM	0.16	2.77	0.009	$<0.001^{<0.001;1.0}$	0.43	0.12	0.64
Body mass	-0.16	-2.21	0.034		-0.35	-0.59	-0.03
Total length							
CTM	0.14	2.70	0.011	$<0.001^{<0.001;1.0}$	0.42	0.11	0.63
Body mass	-0.14	-2.24	0.031		-0.36	-0.59	-0.03

Table 3 Statistics of the unweighted and weighted inter-specific associations between the variation (expressed by intra-specific coefficients of variation, CV) in morphological sperm traits and relative testes size (combined testes mass [CTM] corrected for body mass); *N*=15 species

	Unweighted				*Weighted*		
Traits	**Slope**	***t***	***P***	***λ***	**Slope**	***t***	***P***
CV of head length							
CTM	-1.64	-3.5	0.005	$0.57^{0.05;0.10}$	-2.01	-3.71	0.003
Body mass	0.30	2.63	0.023		0.35	3.18	0.009
CV of midpiece length							
CTM	-2.61	-2.19	0.049	$<0.001^{0.08;1.0}$	-2.90	-2.36	0.036
Body mass	0.37	1.38	0.193		0.16	0.62	0.548
CV of flagellum length							
CTM	-1.91	-2.25	0.044	$<0.001^{0.002;1.0}$	-2.18	-2.42	0.032
Body mass	0.22	1.15	0.273		0.06	0.30	0.769
CV of total length							
CTM	-1.84	-2.17	0.051	$<0.001^{<0.001;1.0}$	-2.18	-2.43	0.032
Body mass	0.16	0.82	0.428		-0.02	-0.11	0.917

Figures

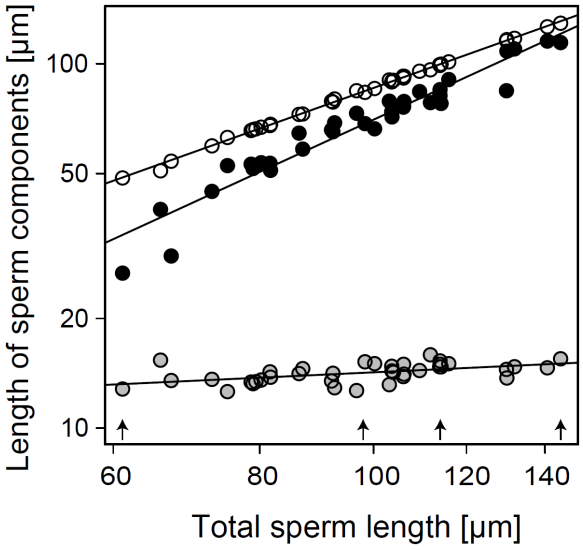


Fig. 1 Relationships of sperm head (grey circles), midpiece (solid black circles) and flagellum length (open circles) with total sperm length. The arrows at the bottom of the graph refer to the four species used for intra-specific analyses of sperm velocity (from left to right: *Molothrus ater*, sperm length=61 µm; *Quiscalus quiscula*, 98 µm; *Xanthocephalus xanthocephalus*, 114 µm; *Agelaius phoeniceus*, 145 µm). All slopes deviate significantly from 0 and from one another (for statistics see text)

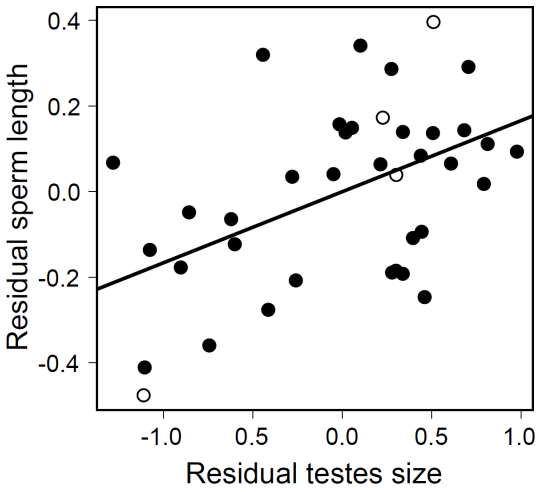


Fig. 2 Partial residual plot showing the inter-specific association of total sperm length with combined testes mass (both corrected for body mass) across 38 species of Icteridae (r=0.42, P=0.01). Each point indicates a species, and the 4 open circles depict the species used for intra-specific analyses of sperm velocity (from top to bottom: *A. phoeniceus, X. xanthocephalus, Q. quiscula, M. ater*).

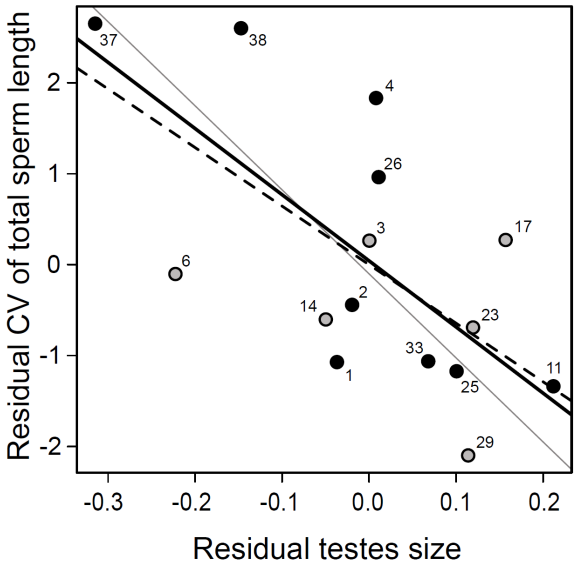


Fig. 3 Partial residual plot showing the negative association between the degree of intra-specific variation (CV) in total sperm length and combined testes mass, both corrected for body mass. Each point represents a species (*N*=15; black points: species with ≥10 males; grey points: species with 6–9 males). The thick lines are the regression lines across all 15 species (solid: weighted for sample size, *r*=-0.59, *P*=0.032; dashed: unweighted, *r*=-0.55, *P*=0.051), and the thin line depicts the (weighted) regression line of the analysis restricted to the minimum adequate sample sizes within species (i.e. black points only: *N*=9, *r*=-0.88, *P*=0.007). All statistics are controlled for phylogeny, and the numbers refer to the species in the online Supplementary Table S1

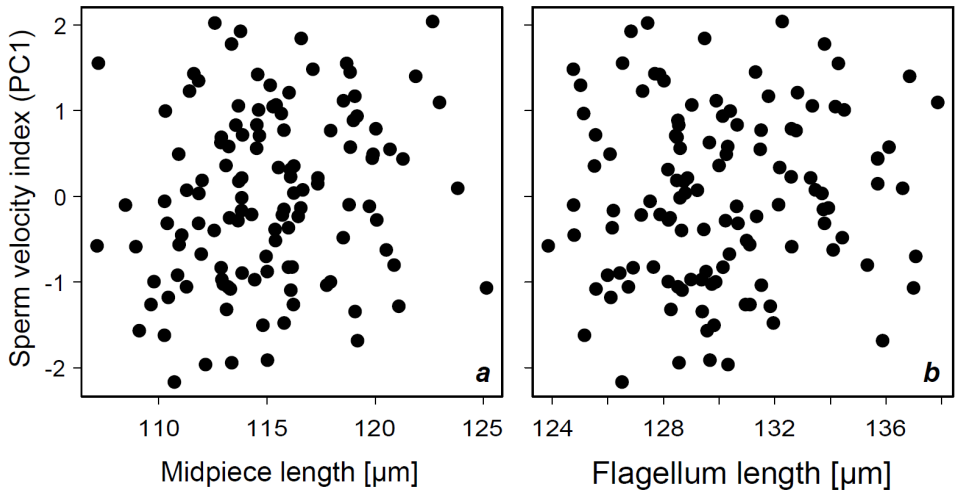


Fig. 4 Two examples showing no significant relationship between the sperm velocity (PC1 of sperm velocity) and (*a*) midpiece length (r=0.17, P=0.06) and (*b*) flagellum length (r=0.08, P=0.39) in 120 different male red-winged blackbirds (*A. phoeniceus*)

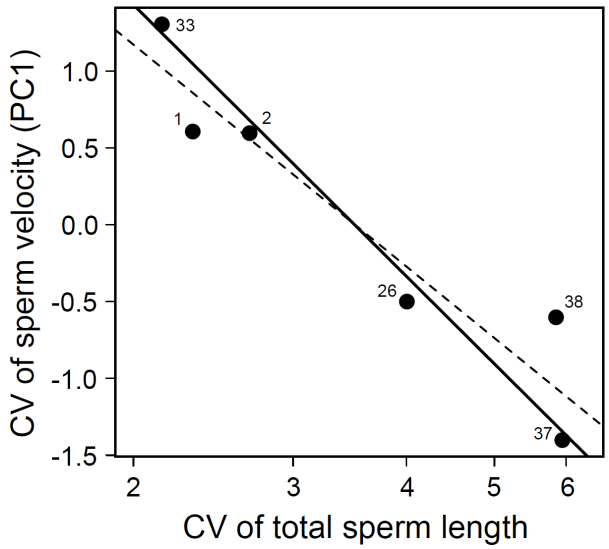
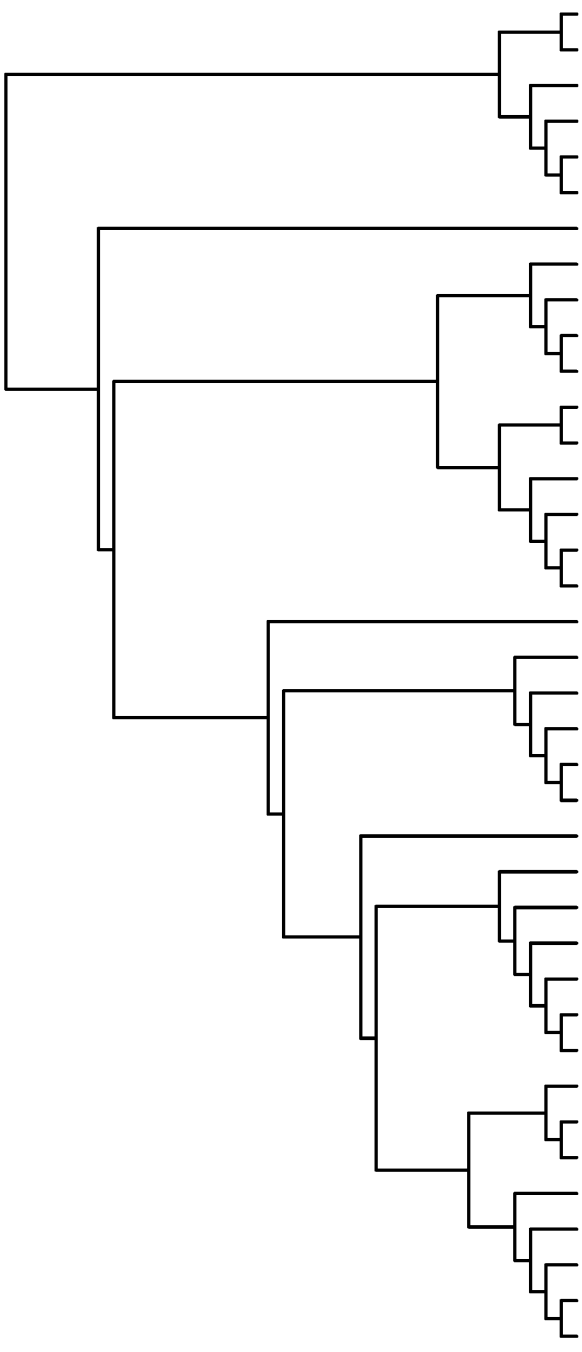


Fig. 5 Relationship between the index of overall variation in sperm velocity (intra-specific CV's of the 5 motility parameters combined in PCA) and the intra-specific coefficient of variation of total sperm length across 6 species of Icteridae with data from over 5 different males. The solid line is weighted for sample size (r=-0.96, P=0.004), the dashed line is unweighted (r=-0.97, P=0.003), and both statistics are controlled for phylogeny. The numbers refer to the species in the online Supplementary Table S1

Supplementary Material

Table S1 Consensus tree of the species used in this study, based on cytochrome-b and NADH dehydrogenase subunit 2 sequences. The numbers following each species name refer to the number of males included in the analyses. N_1: number of males used for sperm morphology (species marked with * comprise more than 5 males and were used for analyses of intra-specific variation); N_2: number of males used for intra-specific variation in sperm velocity (i.e. more than 5 males per species with motility data; species marked with ‡ were used for the intra-specific analyses between sperm design and velocity). These sample sizes were also used to weight the respective analyses (see Methods). The methods used for construction of the phylogeny are described below. Sperm and testes data are available from the corresponding author upon request.



	Species	N_1	N_2
1	*Dolichonyx oryzivorus*	19 *	13
2	*Xanthocephalus xanthocephalus*	28 *	24 ‡
3	*Sturnella neglecta*	6 *	
4	*S. magna*	15 *	
5	*S. loyca*	1	
6	*S. superciliaris*	7 *	
7	*Psarocolius montezuma*	1	
8	*Icterus parisorum*	1	
9	*I. nigrogularis*	2	
10	*I. bullockii*	1	
11	*I. galbula*	11 *	
12	*I. jamacaii*	1	
13	*I. mesomelas*	1	
14	*I. oberi*	6 *	
15	*I. cucullatus*	1	
16	*I. dominicensis*	1	
17	*I. spurius*	7 *	
18	*Amblycercus holosericeus*	2	
19	*Macroagelaius imthurni*	1	
20	*Gnorimopsar chopi*	2	
21	*Pseudoleistes virescens*	1	
22	*Chrysomus ruficapillus*	4	
23	*Agelaioides badius*	6 *	
24	*Dives atroviolacea*	1	
25	*Euphagus cyanocephalus*	8 *	
26	*Quiscalus quiscula*	31 *	20 ‡
27	*Q. lugubris*	1	
28	*Q. niger*	1	
29	*Q. major*	7 *	
30	*Q. mexicanus*	2	
31	*Agelaius xanthomus*	1	
32	*A. tricolor*	1	
33	*A. phoeniceus*	351 *	120 ‡
34	*Molothrus rufoaxillaris*	3	
35	*M. oryzivorus*	1	
36	*M. aeneus*	1	
37	*M. ater*	36 *	21 ‡
38	*M. bonariensis*	15 *	6

Tree construction

To reconstruct the phylogeny of the species used in this study, we used mitochondrial sequences of cytochrome-b and NADH dehydrogenase subunit 2 from GenBank, with *Vireo olivaceus* as an outgroup. We employed Bayesian Markov Chain Monte Carlo (MCMC) methods in the BayesPhylogenies package (Pagel and Meade 2004) to infer candidate trees. We ran two million MCMC generations, discarded the first one million generations as burn-in and sampled every 2,000th of the remaining generations, resulting in 500 candidate trees from which we constructed a 50% majority rule consensus tree using Mesquite v.2.5 (Maddison and Maddison 2008). No sequence data were available for *Sturnella loyca* and *Dives atroviolacea*. Based on the distribution and morphological similarity, we thus joined *S. loyca* with *S. superciliaris*, but with very short branch lengths to minimize the effect of these additional species on the overall phylogeny. For *D. atroviolacea*, which was the only species of this genus, we used the sequence data of its congeneric *D. warszewiczi*. The topology of the consensus tree is consistent with previously published subsets (e.g., Lanyon and Omland 1999, Omland et al. 1999, Johnson et al. 2000, Price and Lanyon 2004).

References

Johnson KP, Lanyon SM (2000) Evolutionary changes in color patches of blackbirds are associated with marsh nesting. Behav Ecol 11:515-519

Lanyon SM, Omland KE (1999) A molecular phylogeny of the blackbirds (Icteridae): five lineages revealed by cytochrome-b sequence data. Auk 116:629-639

Maddison WP, Maddison DR (2008) Mesquite: a modular system for evolutionary analysis. Version 2.5. (http://mesquiteproject.org)

Omland KE, Lanyon S, Fritz S (1999) A molecular phylogeny of the New World Orioles (*Icterus*): the importance of dense taxon sampling. Mol Phylogen Evol 12:224-239

Pagel M, Meade A (2004) A phylogenetic mixture model for detecting pattern-heterogeneity in gene sequence or character-state data. Syst Biol 53:571-581

Price JJ, Lanyon SM (2004) Patterns of song evolution and sexual selection in the oropendolas and caciques. Behav Ecol 15:485-497