Sperm head morphology is associated with sperm swimming speed: a comparative study of songbirds using electron microscopy

Abstract

Sperm exhibit extraordinary levels of morphological diversification across the animal kingdom. In songbirds, sperm have a helically shaped head incorporating a distinct acrosomal membrane or 'helical keel', the form and extent of which varies across species. The functional significance of this helical shape, however, remains unknown. Using scanning electron microscopy, we quantified inter- and intra-specific variation in sperm head morphology across 36 songbird species (Passeriformes: Passerida). Using phylogenetic comparative methods, we investigated the relationship between sperm head morphology and both sperm swimming speed and the frequency of extra-pair young (EPY). We found that species whose sperm had a relatively more pronounced helical form (i.e. long acrosome, short nucleus, wide helical membrane, and a more pronounced waveform along the sperm head 'core') had faster-swimming sperm. We found no evidence of a relationship between inter-specific variation in sperm head morphology and EPY, although we did find that among- and within-male variation in sperm head traits were negatively correlated with EPY. Applying principles of fluid mechanics, we discuss how the helical form of the sperm head may influence swimming speed, and suggest that further studies considering aspects of sperm morphology beyond sperm length are needed to improve our understanding of sperm structure-function relationships.

Keywords*:* passerine, structure-function relationships, scanning electron microscopy, sexual selection, sperm competition, sperm shape

Introduction

Despite their universal role as fertilizers of ova, sperm exhibit extraordinary levels of morphological diversity across the animal kingdom (Pitnick et al. 2009). Three factors are thought to explain the majority of the observed diversity in sperm form: (1) phylogeny (Jamieson 1987a; Supriya et al. 2016), (2) fertilization mode (i.e. internal vs. external fertilisation; Jamieson 1987b), and (3) post-copulatory sexual selection (i.e. sperm competition and cryptic female choice; Snook 2005; Pitnick et al. 2009; Pizzari and Parker 2009; Simmons and Fitzpatrick 2012). In particular, a considerable amount of recent research has highlighted the role of post-copulatory sexual selection in driving evolutionary change in sperm traits. For example, numerous comparative studies in a range of taxa have documented an association between sperm length and sperm competition risk or female reproductive tract morphology (see Pizzari and Parker 2009 and Pitnick et al. 2009 for reviews). To date, the majority of the research on sperm morphology has been descriptive (e.g. Retzius 1909, Aire et al. 2017) or, more recently, focused on studies of sperm length (i.e. total sperm length and length of the various sperm components). Sperm diversity, however, encompasses aspects of morphology beyond simple linear measures of length that may have adaptive and functional significance. A clear example of this is the variation in sperm head shape observed in murine rodents. In this group, the sperm head is characterized by an apical hook that varies across species in both size and curvature (Breed 2004), the function of which appears to be to increase sperm swimming speed when sperm cooperate and form 'sperm trains' (Moore et al. 2002). Research on sperm shape, however, is scarce, and as a consequence our understanding of sperm morphological variation remains limited.

Passerine birds represent a taxonomic group exhibiting considerable inter-specific variation in sperm morphology, with total sperm length ranging from 43 to 292 μm (Pitnick et

al. 2009). In this group, total sperm length is correlated with both the level of sperm competition and the size of female sperm storage tubules (Briskie et al. 1997; Lüpold et al. 2009a; Kleven et al. 2009), and increased rates of evolutionary divergence in sperm length in passerines has been shown to be associated with post-copulatory sexual selection (Rowe et al. 2015a). Post-copulatory sexual selection is also negatively correlated with intra-specific variation in sperm size, such that species under intense selection exhibit reduced among- and within-male variation in sperm length (Calhim et al. 2007; Kleven et al. 2008; Immler et al. 2008; Lifjeld et al. 2010). The adaptive and functional significance of sperm size variation in birds has received considerable attention in recent years. In the zebra finch (*Taeniopygia guttata*), the subset of sperm reaching the site of fertilization are characterized by low morphological variation relative to the sperm in the ejaculate as a whole (Hemmings et al. 2016) and longer sperm fertilize more eggs under competitive mating conditions (Bennison et al. 2015). More generally, sperm length is thought to be an important determinant of sperm swimming speed, an idea with some empirical support at both intra- and inter-specific level in passerine birds (Lüpold et al. 2009; Bennison et al. 2016; but see Kleven et al. 2009 and Cramer et al. 2015). In turn, sperm swimming speed is associated with paternity success in a range of taxa (Pizzari and Parker 2009) including birds (e.g. chickens (Birkhead et al. 1999) and ducks (Denk et al. 2005)). Thus, variation in sperm morphology can have important consequences for sperm performance and male fitness.

Interestingly, in passerine birds, selection appears to act in a similar manner on the length of the sperm midpiece and flagellum, while sperm head length shows a different evolutionary response to selection (Immler et al. 2011; Rowe et al. 2015a). Sperm head length has been suggested to be an evolutionarily conserved trait in birds (Jamieson 2007b; Rowe et al. 2015a), though several intra-specific studies find that head length is variable among males and between populations (e.g. Schmoll and Kleven 2011; Hogner et al. 2013; Støstad et al.

2016). Sperm head length also appears to have a different relationship with swimming speed than sperm midpiece and flagellum length. Specifically, Lüpold et al. (2009) found that sperm head length was negatively related to sperm velocity; a finding that is putatively explained by biomechanics of cell movement at low Reynolds environment, where a longer or more elongate sperm head is predicted to increase drag (Humphries et al. 2008). To date, however, studies in passerines primarily only consider straight head length, and thus neglect other aspects of sperm head morphological variation, reflected in traits such as head width and shape (see Rowe et al. 2015b for an exception).

The sperm head of passerine birds (with the exception of two species of bullfinch, Birkhead et al. 2006; Lifjeld et al. 2013) is characterized by a twisting helical core that includes the acrosome and the nucleus (see Fig 1). The acrosome is at the anterior of the head and contains enzymes which allow the sperm to penetrate the perivitelline layer of the ovum (Nishio and Matsuda 2017), while the nucleus contains the densely compacted DNA. In songbirds, the acrosome has a helical membrane (also known as a helical keel) that spirals around the cell core, and recent evidence suggests that the direction of this helical spiral is sinistral (counterclockwise; Schilthuizen et al. 2017). Together, the helical core and helical membrane give songbird sperm a distinct spiral or corkscrew shape, and there appears to be substantial variation in this shape across species (Jamieson 2007a). However, the evolutionary drivers of this variation and the adaptive and functional significance of the distinctive helical form of songbird sperm remain unknown.

Here, we address this lack of knowledge by examining inter- and intra-specific variation in sperm head morphology, using data for 36 songbird species (Passeriformes: Passerida) obtained from high-resolution scanning electron microscopy (SEM) images of sperm cells. Additionally, using phylogenetic comparative methods, we investigate covariance between sperm head morphology and sperm swimming speed, as well as

covariance between inter- and intra-specific variation in sperm head morphology and the level of sperm competition, as expressed by the frequency of extra-pair young.

Methods

Sperm morphology

We utilized sperm samples from 36 songbird species taken from the avian sperm collection at the Natural History Museum in Oslo, Norway (NHMO). Species were selected to represent a broad range of songbird families and a representative range of total sperm lengths observed across birds more generally (43-292 µm, Pitnick et al. 2009). We also preferentially chose species for which data on rates of extra-pair young were available (for a full list of species, sampling locations, and associated data see Table S1). We randomly selected individuals from the available samples, aiming for 8 males per species ($n_8 = 12$, $n_7 = 10$, $n_6 = 8$, $n_5 = 4$, $n_4 = 1$, $n_3 = 1$). All samples in the NHMO sperm collection were collected from males in breeding condition using cloacal massage (Wolfson 1952; Kucera and Heidinger 2018), and upon collection all samples were fixed and stored in 5% buffered formaldehyde solution. All sampling was conducted in adherence to ethical guidelines for the use of animals in research and with permission from all relevant local authorities.

We assessed sperm morphology using data collected with both light microscopy and high-resolution scanning electron microscopy (SEM). Light microscopy data included values of total sperm length and sperm head length available in the avian sperm collection database at NHMO. All light microscopy measures were obtained using digital image analysis. Briefly, images were captured using a digital camera (DFC420, Leica Microsystems, Heerbrugg, Switzerland) mounted on a digital light microscope (DM6000 B, Leica Microsystems) set to

160× or 320× magnification and total sperm length and sperm head length (± 0.1 μm) were measured using Leica Application Suite (version 2.6.0 R1). This system automatically calibrates images according to the magnification settings, and thus no further image processing is necessary. For each individual, data were taken from 10 morphologically normal sperm and were used to calculate trait means for each male. In turn, these values were used to calculate average values for each species (see Table S1 for data).

Next, we used SEM to obtain digital images of sperm head morphology. Individuals used for SEM measurements were the same males for which we obtained light microscopy data, with the exception of the Spanish sparrow (*Passer hispaniolensis*), where males were sampled in different years. We prepared samples for SEM following Lifjeld et al. (2013). Briefly, formaldehyde-fixed sperm were attached to glass coverslips precoated with poly-lysine and dehydrated using a graded ethanol series, before being critical point dried (BAL-TEC CPD 030 Critical Point Dryer). Coverslips were then mounted on SEM stubs using carbon tape and sputter coated with 4–6 nm platinum (Cressington 308R). Samples were examined and digital images recorded using a Hitachi S-4800 field emission scanning electron microscope operated at 5.0 kV and at variable magnification (7000 - 11000× depending on the species). For each male, we aimed to image 10 randomly chosen, morphologically normal sperm heads; which were defined as a cell with no outer signs of damage on the cell surface. Moreover, only sperm cells positioned horizontally were imaged. Ten cells were sufficient to estimate mean trait values (Fig. S1). We imaged an average of 9.5 cells per male (range: 5 – 30, median: 10). We also took one SEM image of a whole sperm cell for each species (n = 33), for comparison with total sperm length measurements from light microscopy. For sample sizes and species means, see Table S2; for raw data, see Table S3.

We used digital image analysis (ImageJ 1.50i, Schneider et al. 2012) to obtain the following measures of sperm head morphology from SEM images (see Fig. 1 for illustration and Table S4 for definitions): 1) acrosome centreline (*ACL*), 2) nucleus centreline (*NCL*), 3) acrosome straight length (*ASL*); 4) nucleus straight length (*NSL*); 5) diameter of the nucleus (*ND*); 6) diameter of the acrosome (*AD*), and 7) helical membrane width (*HMW*). In all instances, measurements were calibrated using the scale bar in each image. From these measurements, we also calculated 8) nucleus volume (*NV*), 9) acrosome volume (*ACV)*, 10) head volume (*HV*), and 11) head length (*HL*). Finally, in some species the core of the sperm head (i.e. the acrosome and nucleus, not including the helical membrane) has a pronounced waveform, whereas in other species the core appears to be relatively straight. We therefore assessed variation in the waveform of the core of the sperm head as a measure of variation in sperm shape by calculating 12) sperm head "waveform" (*WAV*), the ratio of straight length to centreline length (*ASL+NSL*)/(*ACL+NCL*), which approximates a sinusoidal waveform of varying amplitude. For a subset of sperm cells (n = 159), selected to cover a range of *WAV* values, we measured *WAV* twice, and repeatability analysis (Nakagawa and Schielzeth 2010) showed that data for single sperm cells was significantly repeatable ($r = 0.94$, $p < 0.0001$), as were species mean values ($r = 0.98$, $p < 0.0001$).

We used a principal component analysis (PCA) on six of the sperm head traits (*ACL*, *NCL*, *HL*, *HMW*, *AD*, and *ND*) to reduce the number of linear measurement parameters to a limited number of synthetic variables. We choose not to include measures of waveform or head volume in the PCA as these variables are already composite variables and indicate aspects of sperm head morphology that we considered interesting in their own right. Specifically, waveform describes the shape of the core of the sperm head irrespective of the contribution of the helical membrane, while head volume gives an indication of overall sperm head size. To ensure relatedness between species was taken into account in the PCA analysis

171 (Revell 2009), we performed a phylogenetic PCA using the phyl.pca function in the phytools
172 package (Revell 2012), with a covariance matrix to preserve variance, and optimizing lambda
173 using Maximum Likelihood. As there have been some concerns regarding the use of
174 phylogenetic PCA (Uyeda et al. 2015), we also performed a standard PCA (Table S5); this
175 produced similar results in all analyses (Table S6). The phylogenetic PCA generated six
176 principal components, and the first three of these explained 98% of the variation in sperm
177 head morphology (Table 1). The first PC (PC1) explained 83% of variation (Table 1),
178 whereas the other PCs explained relatively small amounts of variation and lacked a clear
179 biological interpretation. We therefore used PC1 for our analyses as a single index of sperm
180 head morphology.

181 To examine intra-specific variation in sperm head morphology, we calculated the
182 coefficient of variation (among- and within-male) corrected for small sample size as CV_{adj} =
183 ((1+1/4n)x((SD/mean)x100)) for all sperm head traits (hereafter referred to as CVam and
184 CVwm). For CVwm we calculated species mean values for use in analyses. Following Immler
185 et al. (2008), we used a PCA approach to summarize variation in both CVam and CVwm
186 using the CV values for the same six sperm head traits used in our analysis of inter-specific
187 variation (*ACL*, *NCL*, *HL*, *HMW*, *AD*, and *ND*, see above). We then used the first principal
188 component (PC1) from each PCA as an index of 1) CVam and 2) CVwm in sperm head
189 morphology, hereafter referred to as 1) CVam-PC1 and 2) CVwm-PC1. All six traits loaded
190 positively on both CVam-PC1 and CVwm-PC1. In most cases, loadings were moderate to
191 strong, such that we interpret higher values for PC1 representing an increase in variation in
192 sperm head traits for both CVam and CVwm. For further details on these PCA analyses, see
193 Table S7.

194 We found that the measurements from light microscopy and SEM were closely
195 correlated (Pearson's correlations: r = 0.90 and r = 0.99, for head length and total sperm

length respectively, both $p < 0.001$), although measurements obtained via SEM were consistently shorter (on average by 11% for sperm heads and 8% for total lengths). This disparity could be due to blur in the low-resolution light microscope. Nonetheless, the strong correlations allowed us to use SEM measurements for sperm head traits whilst using light microscopy data for total sperm length (which is preferable due to the number of cells measured) in all subsequent analyses.

Sperm swimming speed

We obtained data on sperm swimming speed for 33 species in our dataset from the sperm collection database at NHMO. Data on sperm swimming speed was obtained using computer assisted sperm analysis (CASA; HTM-CEROS sperm tracker, CEROS version 12, Hamilton Thorne Research) following standard methods (for further details, see Lifjeld et al. 2013). In all instances, sperm swimming speed was assessed at 40 °C in Dulbecco's Modified Eagle Medium (D-MEM). We used curvilinear velocity (VCL) as our measure of sperm swimming speed. However, VCL, VSL, and VAP were all strongly correlated (all $r > 0.83$, all $p < 0.001$), and analyses using VSL or VAP returned similar results (data not shown). In addition to the data from the NHMO database, we also obtained data for the Spanish sparrow and the collared flycatcher (*Ficedula albicollis*) from the literature (Cramer et al. 2015; Cramer et al. 2016b). These two studies used phosphate-buffered saline (PBS) as a medium instead of D-MEM. We therefore repeated all relevant analyses with medium as a covariate. These models returned similar results (medium was a non-significant covariate in all analyses), and thus we consider the effect of medium in this study to be negligible.

Index of sperm competition

We used the rate of extra-pair young (EPY, the total number of young sired by extra-pair males divided by the total number of young sampled) as our primary index of sperm competition level. EPY is considered the most direct measure of sperm competition available as the occurrence of EPY necessarily indicates some level of sperm competition, with increasing rates of EPY suggesting higher levels of sperm competition (Møller and Briskie 1995; Calhim et al. 2007). These data were obtained from the literature for a total of 32 species (see list of references in Table S1). We repeated our analysis using two additional estimates of sperm competition level: 1) Relative testes mass, a commonly used proxy for the level of sperm competition (Møller and Briskie 1995; Pitcher et al. 2005), estimated by including both (ln-transformed) combined testes mass and body mass as independent variables in statistical models. Data on testes mass and body mass (n = 33) were taken from Møller (1991) and Rowe et al. (2015a). 2) The coefficient of among-male variation in total sperm length (CVam, see above), using data (n = 36) from the sperm collection database at NHMO. Analyses using these alternative estimates returned similar results as our primary analysis (see Table S8).

Phylogeny

We downloaded DNA sequences for all 36 species from GenBank, plus the satin bowerbird (*Ptilonorhynchus violaceus*) which was used as an outgroup, using two mitochondrial (ND2 and Cytb) and four nuclear genes (Myo2, ODC, GAPDH, and RAG1). We were able to find sequences for all six genes for a majority of the species ($n_6 = 21$, $n_5 = 7$, $n_4 = 6$, $n_3 = 1$, $n_2 = 2$). Sequences were aligned for each gene using SeaView (Gouy et al. 2009).

To produce an ultrametric tree, we used a Bayesian approach in the software BEAST v1.8.4 (Drummond et al. 2012). We generated a concatenated alignment of all six genes

totaling 6926 base pairs. The data was partitioned by gene and we unlinked rate heterogeneity, base frequencies and substitution rates across the six partitions. We applied the most appropriate model of nucleotide evolution to each partition as determined by jModelTest2 (Darriba et al. 2012) following the Bayesian Information Criterion (BIC): GTR+G+I for ND2, HKY+G+I for CytB, HKY+G for Myo2, ODC and GADPH, and TN93+G for RAG1. We applied a relaxed uncorrelated lognormal distribution for the molecular clock model and assumed a birth-death speciation process for the tree prior. To calibrate the tree we used a secondary calibration from Claramunt and Cracraft (2015) to date the divergence of the satin bowerbird from the remainder of taxa in our dataset, using normally distributed prior (mean = 45.698, sd = 2). We ran Markov Chain Monte Carlo (MCMC) chains for 50 million generations sampling every 5000th generation. Tracer v1.6 (Rambaut et al. 2015) was used to assess convergence diagnostics and we removed the first 5 million generations as burn-in. A maximum clade credibility tree was constructed using TreeAnnotator v1.8.4 (Drummond et al. 2012), and the tree was visualized using the package ggtree (Yu et al. 2017).

Statistical analysis

We used mean values for each species for our analysis. All sperm morphology values (including CV values) and swimming speed values were log transformed (natural log) to meet the requirements of the statistical models, whereas EPY was logit transformed. All statistical analyses were done in R v 3.3.3 (R Core Team 2017). First, we used Pearson's correlations to investigate the relationships among sperm head morphology traits, as well as among sperm head traits and total sperm length. We then explored how phylogeny influences sperm head morphology by calculating Blomberg's K (Blomberg et al. 2003) as a measure of

phylogenetic signal using the phylosig function of the package phytools (Revell 2012). Additionally, to visually identify the nodes in the phylogeny at which the largest changes in sperm head morphology have occurred, we calculated phylogenetic independent contrasts of PC1 using the PIC function in the ape package (Paradis et al. 2004) and plotted them on the phylogeny.

To determine the relationship between sperm head morphology and sperm swimming speed, we used Phylogenetic Generalized Least Squares (PGLS) analyses to account for the phylogenetic non-independence of species data (Pagel 1999; Freckleton et al. 2002). The PGLS model uses a maximum likelihood framework to assess the amount of expected covariance between species based on their shared evolutionary history and uses this information to control for the influence of phylogenetic relationships among the study taxa (Symonds and Blomberg 2014). Quadratic terms of all predictor variables were initially included in all models to explore non-linear associations in the data, but were removed if not significant ($p > 0.1$). Three separate PGLS models were constructed used sperm swimming speed (VCL) as a response variable, where the predictor variables were 1) our index of sperm head morphology (PC1), 2) waveform (*WAV*), and 3) head volume (*HV*). Total sperm length was used as a covariate in all three models to account for effects of allometry and to separate the effects of total sperm length and sperm head morphology. A fourth PGLS model was used to explore the relationship between VCL and total sperm length. Multicollinearity did not appear to confound our results; variance inflation factors (VIF) were below 3 for all combinations of predictors, which is well below the recommended threshold value of 10 (Kleinbaum et al. 2007). We also used PGLS models to explore the relationship between total sperm length and PC1, cell waveform and PC1, and head volume and PC1.

Next, we assessed the relationship between inter-specific variation in sperm head morphology and EPY using another set of PGLS models. In these models EPY was set as the

predictor variable in each case, while 1) our index of sperm head morphology (PC1), 2) waveform (*WAV*), and 3) head volume (*HV*) were response variables in three separate analyses. We also assessed the relationship between EPY and total sperm length to allow comparison with previous studies. We repeated these analyses using our two alternative estimates of sperm competition - relative testes mass (i.e. combined testes mass and body mass included as independent variables in the models) and among-male variation in total sperm length (CVam) - as predictor variables in the PGLS models.

Finally, to investigate the relationship between the level of sperm competition and intra-specific variation in sperm head morphology, we performed six separate PGLS analyses. EPY was the predictor variable in all six models, while indicators of among-male variation (CVam-PC1, CVam of waveform, and CVam of head volume) were the response variables in the three first models, and indicators of within-male variation (CVwm-PC1, CVwm of waveform, CVwm of head volume) were the response variables in the other three models. All PGLS analyses were performed using the pgls function of the caper package (Orme 2013), and model assumptions were checked through visual analysis of model plots obtained using the plot(model) function.

Results

Sperm morphology and relationships between sperm traits

Using measurements from SEM images, we observed a moderate level of variation in sperm head length across the 36 songbird species, with mean head length ranging from 8.32 to 14.45 µm (Table S2). This is in contrast to the more than six-fold variation observed in total sperm length (43.3 µm to 282.1 µm, Table S2). Sperm head length was positively correlated with

316 total sperm length ($r = 0.71$, $p < 0.001$, Fig. 2a). Moreover, as total sperm length increased
317 there was a marked change in the relationship between acrosome and nucleus lengths:
318 acrosome length increased with total sperm length ($r = 0.84$, $p < 0.001$), whereas nucleus
319 length decreased ($r = -0.74$, $p < 0.001$, Fig. 2a). Sperm cell diameter also varied with total
320 sperm length. Specifically, both nucleus diameter and acrosome diameter were positively
321 associated with sperm length (nucleus: $r = 0.79$, $p < 0.001$; acrosome: $r = 0.79$, $p < 0.001$; Fig.
322 2b). An interesting consequence of the associations between total sperm length and nucleus
323 length and diameter was that nucleus volume varied little across the 36 species (~2 μm^3),
324 albeit with an overall significant, positive correlation with total sperm length ($r = 0.59$, $p <$
325 0.001, Fig. 2c). In contrast, acrosome volume varied considerably across species, though it
326 was also positively associated with total sperm length ($r = 0.81$, $p < 0.001$; Fig. 2c). Longer
327 sperm also had larger helical membranes ($r = 0.85$, $p < 0.001$, Fig. 2d) and a more pronounced
328 waveform ($r = 0.36$, $p = 0.03$, Fig. 2e). Finally, nearly all combinations of sperm head traits
329 were strongly correlated with one another (see Table S9), with correlation coefficients ranging
330 from 0.36 to 0.99 (all $p < 0.05$); with the exception of waveform and head length ($r = 0.30$, p
331 $= 0.06$) and waveform and nucleus volume ($r = 0.30$, $p = 0.06$).

332 The strong correlations between sperm head traits further justified the use of a PCA to
333 obtain an index of sperm head morphology. PC1 loaded strongly with all variables (Table 1);
334 nucleus length loaded negatively on PC1, whereas all other traits loaded positively, with a
335 particularly strong loading for membrane width. We therefore interpret PC1 as indicating
336 sperm with a longer total head length, a long and wide acrosome, a short and wide nucleus,
337 and a wide helical membrane. As such, we consider PC1 as describing, at least in part, the
338 characteristic helical form of sperm head morphology, with high values of PC1 reflecting
339 sperm heads with a strong helical form as a result of a more distinct helical membrane, while
340 low PC1 values reflect a shallower helical sperm head form with little or no membrane (see

Fig. 3 for examples). Finally, PC1 was positively correlated with total sperm length ($t = 9.54$, $p < 0.001$, $r = 0.85$ (0.75 – 0.91), $\lambda = 1.0^{<0.001, 1}$, Fig. 4) cell waveform ($t = 2.49$, $p = 0.02$, $r = 0.39$ (0.07 – 0.61), $\lambda = 0.85^{0.01, 0.09}$), and head volume ($t = 8.01$, $p < 0.001$, $r = 0.81$ (0.67 - 0.88), $\lambda = 1.0^{<0.001, 1}$). See Fig. S2 for example images of all 36 species.

Phylogenetic variation and signal in sperm head morphology

Using our index of sperm head morphology (PC1), we observed considerable variation across the phylogeny of the 36 species (Fig. 5), although sperm head morphology was more variable among families than within families (ANOVA, $F_{17} = 4.98$, $p < 0.001$). Species that branch off early in our phylogeny (e.g. nuthatch (*Sitta europaea*), goldcrest (*Regulus regulus*)) had low values for PC1, though species with low values were also found within larger superfamilies where other species had relatively high values of PC1 (e.g. blackcap (*Sylvia atricapilla*)). The nodes at which there have been large changes in sperm head morphology were found both near the tips of the tree and deeper in the lineages, suggesting that divergence in these traits has occurred at several points throughout the phylogeny rather than, for example, at a single divergence event early in the clade's evolutionary history.

We found support for statistically significant phylogenetic signal in most sperm head morphology traits, though the degree of phylogenetic dependency was variable (Table 2). There was a strong phylogenetic signal in most sperm head morphology traits (as indicated by values of Blomberg's K exceeding 1 (Blomberg et al. 2003), Table 2), suggesting that related species are more similar in many head morphology traits than expected under a BM model of evolution. In contrast, sperm head length and all volume traits showed Blomberg's K values less than 1 (Table 2). This suggests that these traits exhibit a relatively weak phylogenetic signal (i.e. the phylogenetic signal is lower than that expected under a BM process), which

365 may be because of relatively low levels of trait variation spread more evenly throughout the
366 phylogeny.

367

368 **Correlations between sperm head morphology and sperm swimming speed**

369 Sperm swimming speed (VCL) was significantly correlated with our index of sperm head
370 morphology (PC1) in a non-linear relationship (Table 3, Fig. 6). Sperm swimming speed was
371 also positively, but linearly, correlated with sperm head shape (i.e. waveform of the core of
372 the sperm head, Table 3). Finally, sperm swimming speed was positively and significantly
373 associated with sperm head volume in a non-linear manner (Table 3). In contrast, sperm
374 swimming speed was not associated with total sperm length in our dataset; neither when
375 assessed as a covariate in a model with PC1 nor when examined in a univariate model (Table
376 3).

377

378 **Correlations between sperm head morphology and the rate of extra-pair young**

379 When exploring relationships at the inter-specific level, we found no associations between
380 any of the sperm head traits (PC1, waveform, or head volume) and EPY across songbird
381 species (all $p > 0.1$, Table 4). Similarly, total sperm length was not related to EPY (Table 4).
382 Analyses using alternate indicators of sperm competition level (i.e. relative testes mass and
383 total sperm length CVam) returned similar results, with two exceptions: 1) total sperm length
384 was positively correlated with both relative testes mass and CVam ($p < 0.05$ for both
385 analyses), and 2) head volume was moderately correlated with relative testes mass ($r = 0.38$, p
386 $= 0.03$). See Table S8 for full model results.

In contrast, we found support for an association between intra-specific variation in sperm head morphology and EPY. Specifically, considering among-male variation in sperm head morphology (CVam), we found a significant negative correlation between CVam-PC1 and EPY (Table 5, Fig. 7a), as well as between the CVam of waveform and EPY (Table 5), whereas CVam of head volume and EPY were not correlated (Table 5). Similarly, considering within-male variation in sperm head morphology (CVwm), we found a significant negative association between CVwm-PC1 and EPY (Table 5, Fig. 7b). EPY was not correlated with either CVwm of waveform or CVwm of head volume, although all correlations showed a negative trend (Table 5). Finally, when analyzing relationships between EPY and the CV of head traits separately rather than in a PCA, variation in acrosome length was especially strongly correlated with EPY (Table S10).

Discussion

Using high resolution images obtained with SEM, we show that sperm head morphology (i.e. size and shape) is highly variable across songbird species. More specifically, sperm head morphology incorporates variation in a number of correlated traits, including acrosome and nucleus length and diameter, the extent of the acrosomal helical membrane, and the waveform of the sperm head core. In combination, these morphological traits contribute to the overall helical form of the sperm head, and species vary from a relatively straight sperm head with a shallow helical form and a narrow acrosomal membrane to a strongly helical sperm head, often with a prominent helical membrane.

The majority of the sperm head morphology traits we examined were correlated with total sperm length. As such, longer sperm have larger heads and a more pronounced helical form and acrosomal membrane, and thus head morphology appears to contribute to overall

411 cell size. The exception to this pattern was the negative relationship between total sperm
412 length and the length of the nucleus. Nucleus length was also negatively related to nucleus
413 width; the consequence of which seems to be a relatively uniform nucleus volume across
414 songbird species. In birds, genome size is relatively conserved compared to other vertebrate
415 groups (Tiersch and Wachtel 1991; Gregory 2018). Thus, the low variation in nucleus volume
416 observed in this study may indicate that DNA packaging and the efficiency of sperm
417 chromatin condensation is relatively consistent across songbird species. One interesting
418 possibility is that the low variation in nucleus size, together with the negative correlation
419 between nucleus and acrosome lengths, explains general patterns of sperm head (i.e. acrosome
420 + nucleus) length evolution, which previous research has suggested to be evolutionarily
421 constrained (Rowe et al. 2015a).

422 Our results revealed a positive association between sperm head morphology and sperm
423 swimming speed. Specifically, species with relatively large sperm with a strong helical form,
424 a more pronounced waveform along the cell core, and a more pronounced helical membrane
425 had faster swimming sperm; although this relationship was non-linear for both PC1 and head
426 volume. These findings offer an interesting contrast to a previous study showing a negative
427 relationship between sperm head length and sperm swimming speed in passerine birds
428 (Lüpold et al. 2009a). In that study, the reported negative effect of head size on sperm
429 swimming speed was attributed to drag forces generated by the head (Lüpold et al. 2009a); for
430 sperm, the amount of drag produced by the head is predicted to be proportional to head size
431 (e.g. surface area; Humphries et al. 2008). While the rationale in Humphries et al. (2008) is
432 correct, the sperm form considered there was spherical/spheroid, and thus these assumptions
433 may not accurately reflect the biomechanics of helically shaped sperm. Thus, while we
434 recommend that *in vitro* studies of sperm motion are interpreted with some caution (see
435 below), our findings suggest that, in addition to the effect of total sperm length on swimming

436 speed (Lüpold et al. 2009a; Mossman et al. 2009; Laskemoen et al. 2010), sperm head
437 morphology may influence sperm performance in songbirds.

438 When attempting to understand structure-function relationships in sperm, it is
439 important to remember that the hydrodynamic environment in which sperm cells operate is
440 very different to that experienced by large organisms and objects (e.g. fish). Specifically,
441 because of the relatively small size and slow speed of sperm, they operate at low Reynolds
442 number (defined as the ratio of inertia to the viscous force, Purcell 1977). A low Reynolds
443 number means that the flow of fluid around an object is dominated by viscous forces (Purcell
444 1977), while inertial forces are effectively absent. Importantly, under such conditions,
445 translation (directional movement) and rotation are linearly coupled, which simply means that
446 for a rotating helical shape, the rotational movement will result in forward movement.
447 Passerine sperm swim by rapidly rotating about the longitudinal axis (i.e. 'twist-drill' motility,
448 Humphreys, 1972; Vernon and Woolley 1999). As such, the characteristic helical shape of the
449 sperm head in passerines is predicted to generate forward propulsion and thus increase
450 swimming speed, despite a relatively large surface area that might otherwise only contribute
451 to drag. Moreover, a more pronounced helical shape is likely to experience greater rotational
452 force and thus swim faster relative to a straighter, rod-like sperm cell, an idea supported by
453 experimental studies in bacteria; in *Helicobacter pylori*, wild-type strains characterized by a
454 helical cell shape swam 8-13% faster than mutant strains exhibiting a straighter cell shape
455 (Martínez et al. 2015). This propulsive effect of a helical sperm shape contrasts with a
456 spherical/spheroid sperm, which would instead create viscous drag and thus limit swimming
457 speed (Humphries et al. 2008). However, we note that the non-linear relationship we observed
458 between sperm swimming speed and aspects of sperm size (i.e. PC1 and head volume) might
459 indicate that drag forces become relevant for the largest cells.

460 Unfortunately, the mechanism for generation of rotation about the axis in passerine
461 sperm is unclear (Vernon and Woolley 1999). However, if the flagellum of the sperm cell
462 executes a helical wave to create thrust (such as a low amplitude, long wavelength helical
463 wave proposed by Vernon and Woolley 1999), this will cause the head to rotate, which in turn
464 can influence sperm swimming speed. Alternatively, any mechanism generating torque, but
465 not thrust, would generate forward movement simply due to the overall helical shape of sperm
466 in passerines (i.e. the helical head and mitochondrial helix). Regardless of the mechanism
467 generating rotation, both our empirical data and the principles of biomechanics suggest that
468 the helical shape of passerine sperm can contribute to sperm swimming speed in songbirds.

469 In addition to understanding the biomechanics of movement at low Reynolds numbers,
470 an important consideration is that the movement of sperm is influenced by the physical
471 environment of the female reproductive tract (Lüpold and Pitnick 2018), and most studies of
472 sperm function, including ours, are based on *in vitro* experiments. Yet, as Lüpold and Pitnick
473 (2018) emphasize, studies investigating the difference between sperm behavior *in vivo* and *in*
474 *vitro* are distinctly lacking from the literature. This is certainly the case for birds, and while a
475 few studies have examined the effect of female fluids on sperm performance (e.g. Møller et
476 al. 2008; Cramer et al. 2016a), we currently have almost no knowledge of how avian sperm
477 behave under biologically realistic conditions. Nonetheless, it is likely that a number of
478 factors, such as the architecture of the oviduct, the viscosity of the fluidic environment, and
479 molecular interactions with the female reproductive tract tissue and fluids, influence avian
480 sperm function. Avian sperm have indeed been shown to modify their swimming behavior
481 when in close proximity to surfaces (the 'wall effect', Woolley 2003) and in response to
482 changes in fluid viscosity (Vernon and Woolley 1999). However, these studies were
483 conducted *in vitro,* and as such it is unknown how the rotational movement (and thus the
484 speed) of the helical passerine sperm is affected by these factors *in vivo*.

485 Sperm swimming speed is positively associated with the strength of sperm
486 competition in a range of taxa (Simmons and Fitzpatrick 2012), including passerine birds
487 (Kleven et al. 2009). Given this, and the relationship between head morphology and
488 swimming speed in our dataset, it is somewhat surprising that we did not find a relationship
489 between sperm head morphology and our indicators of sperm competition. However, while
490 previous studies have found a positive association between total sperm length and sperm
491 competition level (Briskie et al. 1997; Kleven et al. 2009; Lüpold et al. 2009a), our findings
492 are consistent with reports that sperm head length and the level of sperm competition are
493 uncorrelated in passerines (Lüpold et al. 2009a; Lüpold et al. 2009b). We did, however, find a
494 negative association between EPY and both among- and within-male variation in sperm head
495 morphology (i.e. CVam and CVwm). These results are consistent with previous findings of a
496 negative relationship between intra-specific variation in sperm length and sperm competition
497 level in passerine birds (Calhim et al. 2007; Kleven et al. 2008; Immler et al. 2008 Lifjeld et
498 al. 2010), though our results demonstrate that such a pattern also applies specifically to sperm
499 head morphology. As such, our work contributes to a body of research suggesting that post-
500 copulatory sexual selection can act as a stabilizing evolutionary force favoring the production
501 of what might be an 'optimal' sperm size and shape.

502 As has been previously suggested (Rowe et al. 2015a), it is likely that the evolution of
503 sperm head morphology is influenced by factors other than (or in addition to) post-copulatory
504 sexual selection. The obvious candidate for investigation is the environment of the female
505 reproductive tract and the egg, both of which are likely to exhibit some degree of species-
506 specificity. The acrosome plays an important role in ovum recognition and penetration
507 (Nishio and Matsuda 2017), and so it is possible that this interaction exerts a selective force
508 on acrosome morphology that is decoupled from selection on swimming speed. However, our
509 understanding of the functional role of acrosome morphology in birds is limited. Thus, it is

clear that we need a greater understanding of both the environment of the female oviduct and sperm-egg interactions in birds, and indeed other internally fertilizing taxa, in order to more fully understand the functional significance of sperm variation.

Conclusion

In summary, we used high resolution microscopy to investigate sperm morphology in songbirds to a previously unquantified level of detail, and found a relationship between sperm head morphology and sperm performance. At the inter-specific level, we found no support for a relationship between sperm head morphology and our indicators of sperm competition, whereas our investigation of intra-specific variation revealed a negative association between sperm competition level (EPY) and among- and within-male variation in sperm head morphology. These findings indicate that aspects of sperm morphology beyond simple measures of sperm length are shaped by post-copulatory sexual selection and that such traits can influence sperm function in songbirds, and we suggest this may also be the case in other taxa exhibiting helical sperm cells. Moreover, our results highlight the challenges of applying knowledge of sperm locomotion generated in one taxon, to other taxa with markedly different sperm morphology, and as such we believe that investigations into sperm motion in taxa representing a broad range of sperm forms are warranted. Future studies should also aim to understand how the helical form of the sperm mitochondria in passerines might contribute to sperm motion. Finally, in addition to the importance of assessing sperm function under biologically realistic conditions (Lüpold and Pitnick 2018), our study suggests that a multi-dimensional approach to quantifying sperm morphology is necessary to improve our understanding of sperm evolution and sperm form-function relationships.

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Tables

Table 1. Loadings for the three main principal components (PCs) from a phylogenetic PCA analysis of sperm morphology traits from 36 songbird species, as well as the proportion of variance explained for each PC, and the cumulative proportion of variance explained. Three further PCs (which explained a smaller proportion of the variance, < 2% all PCs combined) are not shown.

	PC1	**PC2**	**PC3**
Acrosome length (*ACL*)	0.906	-0.152	0.380
Nucleus length (*NCL*)	-0.795	-0.590	-0.058
Head length (*HL*)	0.688	-0.542	0.454
Membrane width (*HMW*)	0.971	-0.097	-0.216
Acrosome diameter (*AD*)	0.813	0.244	0.376
Nucleus diameter (*ND*)	0.883	0.341	0.118
Proportion of variance	0.830	0.081	0.070
Cumulative proportion of variance explained	0.830	0.911	0.981

Table 2. Test of phylogenetic signal (Blomberg's K) in sperm head traits for 36 songbird species, with corresponding p-values.

Sperm trait	**Blomberg's K**	**p**
Acrosome length (*ACL*)	1.224	0.001
Nucleus length (*NCL*)	1.424	0.001
Head length (*HL*)	0.713	0.005
Membrane width (*HMW*)	1.642	0.001
Acrosome diameter (*AD*)	1.182	0.001
Nucleus diameter (*ND*)	1.439	0.001
Acrosome volume (*ACV*)	0.986	0.002
Nucleus volume (*NV*)	0.661	0.055
Head volume (*HV*)	0.943	0.002
Waveform (*WAV*)	1.065	0.001

Table 3. PGLS analysis of sperm swimming speed (VCL, μm/s) in relation to sperm head morphology traits across 35 songbird species. Effect sizes (partial *r*) and their noncentral 95% confidence intervals (LCL, lower confidence limit; UCL, upper confidence limit) were calculated for each response-predictor variable pair. The model including the maximum-likelihood value of λ was compared against the models including λ = 1 and λ = 0; superscripts following the λ estimates indicate significance levels of the likelihood-ratio tests (first position: against λ = 0; second position: against λ = 1). Significant relationships are presented in bold.

Response	**Predictor**	**df**	**t**	**p**	**Partial *r***	**LCL, UCL**	**λ**
Swimming speed	PC1	31	**4.200**	**0.0002**	**0.60**	**0.33, 0.75**	0.578 $^{0.14, 0.05}$
	$PC1^2$		**-3.902**	**0.0005**	**-0.57**	**-0.74, -0.29**	
	Total sperm length		-1.760	0.089	-0.30	-0.56, 0.05	
Swimming speed	Waveform	32	**2.793**	**0.009**	**0.44**	**0.12, 0.65**	0.962 $^{0.04, 0.53}$
	Total sperm length		1.432	0.162	0.25	-0.10, 0.52	
Swimming speed	Head volume	31	**2.309**	**0.028**	**0.38**	**0.04, 0.61**	0.615 $^{0.16, 0.03}$
	$Head\ volume^2$		**-3.324**	**0.002**	**-0.51**	**-0.70, -0.21**	
	Total sperm length		-0.436	0.666	-0.08	-0.40, 0.27	
Swimming speed	Total sperm length	33	1.608	0.117	0.27	-0.07, 0.53	0.968 $^{0.001, 0.64}$

Table 4. PGLS analysis of inter-specific variation in sperm head morphology traits in relation to the rate of extra-pair young (EPY) across 32 songbird species. Effect sizes (partial *r*) and their noncentral 95% confidence intervals (LCL, lower confidence limit; UCL, upper confidence limit) were calculated for each response-predictor variable pair. The model including the maximum-likelihood value of λ was compared against the models including λ = 1 and λ = 0; superscripts following the λ estimates indicate significance levels of the likelihood-ratio tests (first position: against λ = 0; second position: against λ = 1).

Response	**Predictor**	**df**	**t**	**p**	**Partial *r***	**LCL, UCL**	**λ**
PC1	EPY rate	30	1.051	0.302	0.19	-0.17, 0.48	$1.000^{< 0.001,\ 1}$
Waveform	EPY rate	30	-1.359	0.184	-0.24	-0.52, 0.12	$0.936^{< 0.001,\ 0.31}$
Head volume	EPY rate	30	1.139	0.264	0.20	-0.15, 0.49	$1.000^{< 0.001,\ 0.15}$
Total sperm length	EPY rate	30	1.595	0.121	0.28	-0.08, 0.55	$1.000^{0.02,\ 1}$

Table 5. PGLS analyses of the among-male (CVam) and within-male (CVwm) variation in sperm morphology traits for 32 songbird species in relation to extra-pair paternity (EPY). Effect sizes (partial *r*) and their noncentral 95% confidence intervals (LCL, lower confidence limit; UCL, upper confidence limit) were calculated for each response-predictor variable pair. The model including the maximum-likelihood value of λ was compared against the models including λ = 1 and λ = 0; superscripts following the λ estimates indicate significance levels of the likelihood-ratio tests (first position: against λ = 0; second position: against λ = 1). Significant relationships are presented in bold.

Response	**Predictor**	**df**	**t**	**p**	**Partial *r***	**LCL, UCL**	**λ**
CVam-PC1	EPY rate	30	**-3.322**	**0.002**	**-0.52**	**-0.70, -0.21**	1.000 $^{< 0.001,\ 1}$
CVam (waveform)	EPY rate	30	**-2.642**	**0.013**	**-0.43**	**-0.65, -0.10**	0.983 $^{< 0.001,\ 0.82}$
CVam (head volume)	EPY rate	30	-1.979	0.057	-0.34	-0.59, 0.01	0.000 $^{1,\ < 0.001}$
CVwm-PC1	EPY rate	30	**-2.783**	**0.009**	**-0.45**	**-0.66, -0.12**	0.000 $^{1,\ < 0.001}$
CVwm (waveform)	EPY rate	30	-1.670	0.105	-0.29	-0.56, 0.06	1.000 $^{< 0.001,\ 1}$
CVwm (head volume)	EPY rate	30	-0.103	0.919	-0.02	-0.35, 0.32	0.447 $^{0.26,\ 0.11}$

Figure legends

Figure 1. Sperm head morphology measurements (shown here on the sperm of the bluethroat (*Luscinia svecica*)). A) acrosome centreline (*ACL*), red line; B) nucleus centreline (*NCL*), yellow line; C) acrosome straight length (*ADL*), blue line; D) nucleus straight length (*NDL*), orange line; E) nucleus diameter (*ND*), lime green line; F) acrosome diameter (*AD*), dark green line; G) helical membrane width (*HMW*), purple line.

Figure 2. Correlations of total sperm length and a) length of sperm head components, b) diameter of sperm head components, c) volume of sperm head components, d) helical membrane width, and e) waveform, for 36 songbird species. All values are absolute values (not log transformed), unlike in the statistical analyses. Lines are simple regression lines.

Figure 3. Examples of sperm head cells from songbird species with a) the maximum value for PC1 (reed bunting (*Emberiza schoeniclus*), PC1 = 1.921), b) the closest to the median value for PC1 (willow warbler (*Phylloscopus trochilus*), PC1 = 0.138), c) the minimum value for PC1 (nuthatch, PC1 = -0.863). The top (left) white arrowhead indicates the acrosome/nucleus junction, whereas the lower (right) white arrowhead indicates the nucleus/midpiece junction. Images are taken with a scanning electron microscope, at magnifications of 7000 ×, 10000 × and 11000 ×, respectively. Images are not mirrored during processing, and the backgrounds have been modified to remove debris.

Figure 4. The relationship between total sperm length (μm) and sperm head morphology (PC1 values), for 36 songbird species. Increasing values of PC1 reflect a more helically

shaped sperm with a wider helical membrane (as indicated by silhouettes in this figure as well as in figures 5 and 6). A simple linear regression line is shown.

Figure 5. Phylogenetic variation in sperm head morphology across the 36 songbird species included in the study (plus the outgroup *Ptilonorhynchus violaceus*). Colors of the branches refer to each species' score for the index of sperm head morphology (PC1), whereas the size of the grey nodes reflect the phylogenetically independent contrasts (larger circles indicate a greater divergence in the trait). Note that internal branches are subjectively colored according to parsimony for aesthetic effect, whereas external branches (tips) are precisely colored. The phylogeny was constructed using two mitochondrial and four nuclear sequences from GenBank.

Figure 6. The relationship between sperm head morphology (PC1 scores) and sperm swimming velocity (VCL, μm/s) for 35 songbird species. The line is a regression line from a polynomial model of the two variables (not including total sperm length as a covariate or correcting for phylogeny).

Figure 7. The relationship between the rate of extra-pair young (EPY) and indicators of variation in sperm head morphology (PC1 scores) for a) among-male (CVam-PC1) and b) within-male (CVwm-PC1) variation, for 32 songbird species. Simple linear regression lines are shown.

Supplementary material

Files in this Data Supplement:

Supplementary File 1: Data from literature searches with references, data from light microscopy, and sample locations (Table S1); definitions of sperm head morphology traits (Table S4); supplementary statistical output (Table S5-S10); resampling graphs for sample size estimation (Figure S1); and images with examples of sperm cells from all 36 species (Figure S2).

Supplementary File 2: Sample sizes and species means from SEM measurements (Table S2); raw data from SEM measurements and individual accession numbers from the Natural History Museum sperm bank (Table S3).

Supplementary File 3: R script for the statistical analyses.