IBIS
international journal of avian science
Ibis (2016), **158**, 155–166

Variation in sperm morphology among Afrotropical sunbirds

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Birds show considerable variation in sperm morphology. Closely related species and subspecies can show diagnostic differences in sperm size. There is also variation in sperm size among males within a population, and recent evidence from passerine birds suggests that the coefficient of inter-male variation in sperm length is negatively associated with the level of sperm competition. Here we examined patterns of inter- and intra-specific variation in sperm length in 12 species of sunbird (Nectariniidae) from Nigeria and Cameroon, a group for which such information is extremely limited. We found significant variation among species in sperm total length, with mean values ranging from 74 μm to 116 μm, placing these species within the short to medium sperm length range for passerine birds. Most of this variation was explained by the length of the midpiece, which contains the fused mitochondria and is an important structure for sperm energetics. Relative midpiece length was negatively correlated with the coefficient of inter-male variation in sperm total length across species, suggesting that sperm competition may have selected for greater midpiece length in this group. We also mapped sperm lengths onto a time-calibrated phylogeny and found support for a phylogenetic signal in all sperm length components, except head length. A test of various evolutionary or tree transformation models gave strongest support for the Brownian motion model for all sperm components, i.e. divergences were best predicted by the phylogenetic distance between lineages. The coefficients of inter-male variation in sperm total length indicate that sperm competition is high but variable among sunbird species, as is the case with passerine birds at large.

Keywords: comparative analysis, Nectariniidae, phylogenetic signal, sperm competition, sperm size.

Across the animal kingdom, spermatozoa vary remarkably in size, shape and behaviour (Cohen 1977, Pitnick *et al.* 2009, Pizzari & Parker 2009). In passerine birds, sperm length varies from approximately 40 μm to nearly 300 μm (Pitnick *et al.* 2009, Lifjeld *et al.* 2010, Immler *et al.* 2011). Given that the primary role of sperm is to fertilize ova, a highly conserved function, the evolutionary diversification of sperm form is surprising and the factors generating this diversity are poorly understood (Snook 2005, Pitnick *et al.* 2009). However, it is generally thought that genetic drift, mode of fertilization and postcopulatory sexual selection, i.e. sperm competition (Parker 1970) and cryptic female choice (Eberhard 1996), drive evolutionary changes in sperm

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phenotypes (Franzén 1970, Snook 2005, Pitnick *et al.* 2009).

There is comparative evidence from a range of taxonomic groups that sperm length tends to increase with sperm competition, for example in birds (Briskie *et al.* 1997, Kleven *et al.* 2009), insects (Morrow & Gage 2000), fish (Balshine *et al.* 2001) and mammals (Gomendio & Roldan 1991, Tourmente *et al.* 2011), although with some exceptions to this pattern (e.g. Gage & Freckleton 2003, Immler & Birkhead 2007). It is suggested that the evolution of longer sperm is driven by their ability to swim faster (Gomendio & Roldan 1991), live longer (Parker 1993, 1998) or displace shorter sperm from female sperm storage sites (Miller & Pitnick 2002, Lüpold *et al.* 2012). In passerine birds, increased sperm size is associated with a disproportionate increase in the size of the midpiece (Lüpold *et al.* 2009), which contains a single fused mitochondrion wrapped helically around the flagellum (Koehler 1995). A longer midpiece contains more adenosine triphosphate (Rowe *et al.* 2013), demonstrating the importance of this structure for sperm energetics. Sperm length in passerine birds is also positively correlated with the length of the sperm storage tubules in females (Briskie & Montgomerie 1992, Kleven *et al.* 2009). Briskie *et al.* (1997) hypothesized that longer sperm storage tubules enable female control over how sperm are used in fertilization. There is also a strong phylogenetic signal in the association between sperm length and sperm competition (Immler & Birkhead 2007, Kleven *et al.* 2009, Lifjeld *et al.* 2010, Immler *et al.* 2011), which suggests that the role of sperm competition in sperm length evolution varies across the passerine phylogeny.

More recently, studies have shown that increased levels of sperm competition are associated with reduced inter- and intra-male variation in sperm length in passerine birds (Calhim *et al.* 2007, Immler *et al.* 2008, Kleven *et al.* 2008, Lifjeld *et al.* 2010) and also in insects (Fitzpatrick & Baer 2011). Reduced variation in sperm length among males within a population suggests stronger stabilizing selection around an optimum length for high performance across different female environments (Calhim *et al.* 2007, Kleven *et al.* 2008, Lifjeld *et al.* 2010). In a comparative analysis, Lifjeld *et al.* (2010) showed that the coefficient of inter-male variation (CV_{bm}) in sperm length explained as much as 65% of the variation in extra-pair paternity rates among 24 passerine species. As there was no phylogenetic signal in this association, Lifjeld *et al.* (2010) proposed that the CV_{bm} metric could be used as a proxy for extra-pair paternity, and therefore sperm competition, in passerine birds. There is also a negative relationship between the coefficient of intra-male variation (CV_{wm}) in sperm length and measures of sperm competition (Immler *et al.* 2008, Lifjeld *et al.* 2010). Reduced variation in sperm length within a male or an ejaculate should imply a stronger developmental stability or quality control in spermatogenesis.

In contrast to temperate species, we know surprisingly little about mating systems in tropical birds (Macedo *et al.* 2008). Stutchbury and Morton (2001) hypothesized that sperm competition levels should generally be lower in tropical than in temperate birds but very few studies have actually tested this idea empirically (Stutchbury *et al.* 1998, Stutchbury & Morton 2001, Albrecht *et al.* 2013 are exceptions). Albrecht *et al.* (2013) found no difference in overall sperm competition levels between tropical and temperate passerine birds, using the sperm length CV_{bm} index. They also noted that tropical species are apparently as variable as temperate zone birds in terms of sperm competition levels, and mentioned specifically waxbills (Estrildidae) and sunbirds (Nectariniidae) as examples of families with low and high sperm competition levels, respectively. However, it is difficult to infer general patterns from just a few species; only three species of sunbird were included in that study. General descriptive information about sperm morphology is also largely lacking for tropical birds. Moreover, tropical birds are relatively less well studied in terms of systematics (Reddy 2014) and general biology (Macedo *et al.* 2008).

Here, we examine variation in sperm morphology in 12 species of sunbirds from West Africa (Nigeria and Cameroon). Sunbirds are generally small (*c.* 5–22 g), socially monogamous species exhibiting a territorial breeding system (Fry *et al.* 2000, Cheke *et al.* 2001, Riegert *et al.* 2014). Additionally, the majority of species are sexually dimorphic in both body size and plumage coloration: males are larger and exhibit colourful iridescent plumage patches (either year-round or seasonally), whereas females are generally drab (Fry *et al.* 2000, Borrow & Demey 2001, Cheke *et al.* 2001). The primary objectives of our study

were to describe sperm length variation in sunbirds at multiple levels of organization (i.e. among species and among and within males belonging to a single species) and test for signatures of phylogeny and sperm competition in the observed patterns of sperm morphological variation. We also tested for phenotypic correlates of sperm CV_{bm} as a proxy for sperm competition.

METHODS

Data collection and sampling procedure

In Nigeria, fieldwork was conducted at Amurum Forest Reserve, Jos (09°53′N, 08°59′E); Yankari Game Reserve, Bauchi (09°50′N, 10°30′E); Omo Forest Reserve, Ogun (06°51′N, 04°30′E); International Institute of Tropical Agriculture, Ibadan (07°30′N, 03°55′E) and Okomu National Park, Benin (06°33′N, 05°26′E). In Cameroon, we sampled birds along the slope of Mount Cameroon (04°15′N, 09°09′E) and in the vicinity of Laide Farm, Bamenda-Banso Highlands (06°05′N, 10°28′E). Birds were captured using mist-nets (in some instances with the assistance of song playback) during the breeding season (i.e. April to September in 2010–2013 in Nigeria and October to December in 2010–2012 in Cameroon). Sperm samples (*c.* 0.5–3 μL) were collected by cloacal massage (Wolfson 1952) and immediately diluted in a small volume of phosphate-buffered saline (*c.* 20 μL) and then fixed in 300 μL of 5% formaldehyde solution for later slide preparation. For each bird, a small blood sample (*c.* 10–50 μL) was collected from the brachial vein and preserved in 96% ethanol for later DNA extraction and DNA sequencing. We also fitted each bird with a uniquely numbered aluminium band (supplied by South African Bird Ringing Unit) to prevent resampling of individuals.

Sperm morphology

For each sample, a small aliquot (*c.* 15 μL) of formaldehyde-fixed sperm was applied to a glass slide and allowed to air-dry. Slides were then gently rinsed with distilled water and air-dried again. We captured high-magnification (160× or 320×) digital images of sperm using a Leica DFC420 camera mounted on a Leica DM6000 B digital light microscope (Leica Microsystem, Heerbruug, Switzerland). We used Leica Application Suite (version 2.6.0 R1) to measure (to the nearest ± 0.1 μm) the length of the sperm head, midpiece and tail (i.e. the section of the flagellum not entwined by the midpiece), from which we calculated flagellum length (as the sum of midpiece and tail length), sperm total length (as sum of head, midpiece and tail length) and the ratios of flagellum : head length, midpiece : flagellum length and midpiece : total length.

For each male, we measured 10 morphologically normal spermatozoa following the recommendation in Laskemoen *et al.* (2007). All sperm measurements were taken by one person (TCO) to avoid observer effects. We determined the repeatability of sperm measurements by measuring the same 15 sperm from a single individual twice, and found that measurements were highly repeatable (head: $r = 0.87$, $F_{14,15} = 14.75$, $P < 0.001$; midpiece: $r = 0.81$, $F_{14,15} = 9.76$, $P < 0.001$, tail: $r = 0.83$, $F_{14,15} = 10.94$, $P < 0.001$; Lessells & Boag 1987). For each sperm trait we used the means within individuals to calculate the mean for each species. For two species we had sperm samples from both Nigeria and Cameroon. There were no significant differences between countries in sperm length or components for either species, but we used the Nigerian data only (larger n) for our comparative analyses. Finally, we calculated CV_{wm} values of sperm total length for each individual and then used the mean of these values to calculate an average CV_{wm} for each species. Similarly, we calculated the CV_{bm} of sperm total length as: $CV_{bm} = (\text{sd/mean}) * 100 * (1 + (1/4n))$, which corrects for variation in sample size (n) (Sokal & Rohlf 1995).

Phylogeny

We sequenced the first part of the mitochondrial cytochrome oxidase I (COI) gene, which corresponds to the standard DNA barcode marker for animals (Hebert *et al.* 2003). Details of the DNA extraction, PCR and sequencing procedures are available as Supporting Information Appendix S1, Fig. S1 and Table S1. To complement these data, but from different individuals, sequences were collected from another mitochondrial gene (*NADH2*) and three nuclear introns (FGB5, MB2, TGFb2) using standard protocols (Kimball *et al.* 2009, Fuchs *et al.* 2012). All COI sequences are publicly available at the BOLD database (Ratnasingham & Hebert 2007) in the project folder BONSU. Data

for the remaining loci are available on GenBank (KU174652–KU174703); Supporting Information Table S2. COI sequences were trimmed to an even length, and all loci were aligned using MAFFT v. 7 (Katoh & Standley 2013), generating alignments for each locus of: COI – 654 bp, NADH2 – 1041 bp, FGB5 – 570 bp, MB2 – 749 bp, TGFb2 – 589 bp (total 3603 bp), for 12 sunbird species and a flowerpecker (Flame-crowned Flowerpecker *Dicaeum anthonyi*), a member of the sister family to the sunbirds (Johansson *et al.* 2008) used to root the phylogenetic analyses described below. Species nomenclature follows the International Ornithologists' Union (IOC) World Bird List (Gill & Donsker 2015). A cross-reference to names used by other checklists is presented in Supporting Information Table S3.

We estimated a maximum likelihood tree using the GTRGAMMA model of RAXML v. 8.1.24 (Stamatakis 2014) applied to the concatenated dataset using nine partitions (COI – codons 1, 2 & 3; NADH2 – codons 1, 2 & 3; FGB5, MB2, TGFb2). Analyses were conducted via the CIPRES Science Gateway supercomputer portal. To obtain a Bayesian tree and determine divergence times among species we used BEAST v. 1.8.2 (Drummond *et al.* 2012) and the mean rates of divergence and associated standard deviations reported by Lerner *et al.* (2011) for each of the two mtDNA genes analysed and two of the introns (FGB and TGFb2). The rates reported by Lerner *et al.* (2011) are derived from the sequence of lineage splits in Hawaiian Honeycreepers (Fringillidae), and were calibrated using the well-established dates of sequential uplift of the Hawaiian Archipelago. The BEAST analyses was run for 100 million generations with an HKY + G + I model of nucleotide substitution applied to each locus, a strict molecular clock enforced and a Yule process for the tree prior. We made use of TRACER v.1.6.0 (Rambaut *et al.* 2014) to check that the effective sample size of the underlying posterior distribution was large enough (ESS > 200) for meaningful estimation of parameters.

Sexual size dimorphism and plumage dichromatism

We collected data on male and female body mass, wing length and sexual dichromatism from the literature (Fry *et al.* 2000, Borrow & Demey 2001, Cheke *et al.* 2001, Cox *et al.* 2011). Sexual size dimorphism was estimated as the ratio of female : male body mass and female : male wing length, which we calculated using the mean values for each sex obtained from the literature. Next, we categorized plumage dichromatism as 0 or 1, with 0 representing species that were monochromatic or showed only minor differences between the sexes (i.e. less than 10% of plumage differed) and 1 representing species that showed complete differences in colour or pattern (Supporting Information Table S4). Additionally, we scored male plumage ornamentation as the number of separate and distinct colour patches in the male plumage, i.e. head, throat-chest-belly and nape-back-rump. All plumage traits were assessed using image plates in Cheke *et al.* (2001). Finally, based on literature (Fry *et al.* 2000, Cheke *et al.* 2001), all species were assumed to be socially monogamous, with the exception of the Olive Sunbird *Cyanomitra olivacea* and the Collared Sunbird *Hedydipna collaris*, which were classified as polygynous and polyandrous, respectively.

Data analysis

All analyses were performed using the statistical package R version 2.12.2 (R Development Core Team 2013). To improve data distributions, we log-transformed data prior to analysis. The ratios of midpiece : flagellum length, midpiece : total length, female : male body mass and female : male wing length were logit-transformed following the recommendation of Warton and Hui (2011). We tested for differences among species in sperm morphology (i.e. sperm total length and length of the various components) and CV_{wm} using ANOVA. To assess whether species differed in CV_{bm}, we tested for homogeneity of variance in sperm length using Levene's test. Next, for all sperm traits (i.e. head, midpiece, flagellum and total sperm length), we tested for the presence of a phylogenetic signal by calculating Blomberg's K (Blomberg *et al.* 2003), using the phylosig function in the 'phytools' package (Revell 2012): $K > 1$ indicates that traits are more similar between related species than expected under Brownian motion evolution, whereas $K < 1$ indicates high lability, at least at the tips of the tree (Blomberg *et al.* 2003). The presence of a phylogenetic signal was tested using a randomization test. We reconstructed the ancestral character state of sperm length using 'cont

Map' (Revell 2013). The mapping relies upon states estimated at internal nodes using maximum likelihood with 'fastAnc' and was plotted with 'contMap'.

The fit of five evolutionary models for the diversification of sperm length and sperm components in the time-calibrated phylogeny was compared against a null model of Brownian motion, using the fitContinuous function in the 'geiger' package (Harmon *et al.* 2008). These models were: Lambda – phenotypic divergence covaries with phylogenetic distance, but allows for variable evolutionary rates; Delta – the evolutionary rate accelerates or decelerates over time; Kappa – evolutionary change occurs mainly at speciation events, and is not proportional to branch length; Ornstein–Uhlenbeck – a random walk within a constrained trait space, where traits tend to converge towards a single value; and Early Burst – an early burst of trait diversification followed by reduced evolutionary rates (or stasis). Models were compared using the Akaike information criterion corrected for small sample size (AICc); the model with the lowest AICc value indicates the best-fit model. We also calculated Akaike weights for all models and used both ΔAICc and Akaike weight values to assess model support. Values of $\Delta AICc < 2$ from the best supported model are indicative of substantial support for the model (Burnham & Anderson, 2004). For further details about the application of these models in another African passerine group, see Omotoriogun *et al.* (2016).

We performed phylogenetic generalized least-squares (PGLS) regressions using the package 'caper' (Orme *et al.* 2012) to examine the relationships among sperm traits and the relationships between sperm traits and CV_{bm}. For these latter models, separate models were run for each sperm trait. Similarly, we used PGLS regressions to determine whether measures of either sexual size dimorphism or sexual dichromatism predict sperm length CV_{bm} (i.e. sperm competition) in sunbirds. This approach accounts for the statistical non-independence of data points due to shared ancestry of species (Pagel 1999, Freckleton *et al.* 2002). PGLS also allows for the estimation (via maximum likelihood) of the phylogenetic scaling parameter λ ($\lambda = 0$ indicates phylogenetic independence, $\lambda = 1$ indicates phylogenetic dependence). The empirical λ value was tested against $\lambda = 1$ and $\lambda = 0$ using a likelihood ratio test. Finally, we compared levels of CV_{bm} in sunbirds with those of other passerine birds using a two-sample *t*-test. For this analysis, CV_{bm} values for other species were extracted from Albrecht *et al.* (2013).

RESULTS

Sperm samples were analysed from a total of 189 males from 12 species belonging to five genera (Table 1, Supporting Information Table S5). Sperm total length ranged from 74 μm in the Northern Double-collared Sunbird *Cinnyris reichenowi* to 116 μm in the Scarlet-chested Sunbird *Chalcomitra senegalensis*, and differed significantly among species ($F_{11,177} = 903.33$, $P < 0.0001$; Table 1). The variation in sperm total length among species was largely explained by variation in midpiece and flagellum length, whereas head length was short in all species (range 12–14 μm; Table 1, Fig. 1). However, all sperm components varied significantly among species ($P < 0.0001$; Table 1).

The phylogeny suggests that some sunbird genera are not monophyletic; for example, the six *Cinnyris* species were spread across the entire phylogeny (Fig. 2; see also the same phylogeny in Supporting Information Fig. S2 annotated with the 95% highest probability density estimates for each node and rooted with the outgroup taxon), a result also supported with much greater taxon sampling (R. C. K. Bowie unpubl. data). Using

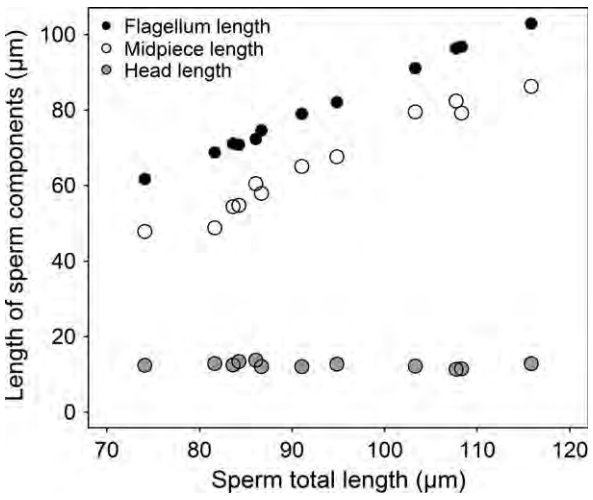

Figure 1. Relationship between sperm total length and sperm head, midpiece and flagellum length among sunbirds ($n = 12$ species). Data points represent species means.

Table 1. Descriptive statistics (mean ± sd) of sperm traits for 12 species of sunbird with tests of species differences (ANOVA). Lengths are given in micrometres; coefficients of intra-male (CV_{wm}) and inter-male (CV_{bm}) variation in sperm total length are given in per cent.

Species	Country	Head length	Midpiece length	Flagellum length	Total length	CV_{wm} (total length)	CV_{bm} (total length)
Chalcomitra senegalensis (n = 66)	Nigeria	12.83 ± 0.55	86.07 ± 3.10	102.97 ± 2.37	115.62 ± 2.83	1.61 ± 0.47	2.03
Cinnyris bouvieri (n = 7)	Cameroon	12.05 ± 0.47	65.06 ± 1.62	79.00 ± 1.86	91.05 ± 1.88	2.01 ± 0.55	2.13
Cinnyris coccinigastrus (n = 1)	Nigeria	12.21	79.52	91.09	103.30	1.55	
Cinnyris cupreus (n = 7)	Nigeria	12.08 ± 0.25	57.90 ± 2.21	74.61 ± 1.64	86.69 ± 1.75	1.65 ± 0.30	2.09
Cinnyris reichenowi (n = 16)	Cameroon	12.42 ± 0.48	47.78 ± 2.14	61.69 ± 1.59	74.11 ± 1.71	2.32 ± 1.38	2.35
Cinnyris ursulae (n = 1)	Cameroon	13.44	54.74	70.81	84.26	1.19	
Cinnyris venustus (n = 4)	Nigeria	12.73 ± 0.26	67.57 ± 2.68	82.11 ± 1.70	94.84 ± 1.82	2.17 ± 1.07	2.04
Cyanomitra olivacea (n = 49)	Nigeria	13.80 ± 0.47	60.46 ± 1.42	72.28 ± 1.37	86.08 ± 1.27	1.54 ± 0.42	1.49
Cyanomitra olivacea (n = 16)[a]	Cameroon	13.80 ± 0.42	60.37 ± 1.51	72.61 ± 1.53	86.41 ± 1.54	1.49 ± 0.30	1.78
Cyanomitra oritis (n = 18)	Cameroon	12.91 ± 0.63	48.74 ± 2.82	68.75 ± 2.59	81.66 ± 1.91	2.50 ± 1.75	3.50
Cyanomitra verticalis (n = 9)	Nigeria	12.59 ± 1.08	54.37 ± 2.67	71.12 ± 2.91	83.62 ± 2.77	1.62 ± 0.59	3.41
Deleornis fraseri (n = 3)	Nigeria	11.50 ± 0.47	79.18 ± 1.25	96.80 ± 2.36	108.30 ± 2.72	1.62 ± 0.63	
Deleornis fraseri (n = 2)[a]	Cameroon	12.47 ± 0.58	83.54 ± 3.24	100.32 ± 3.30	112.78 ± 2.72	1.68 ± 0.22	
Hedydipna collaris (n = 8)	Nigeria	11.35 ± 0.55	82.39 ± 1.39	96.37 ± 1.63	107.71 ± 1.69	1.69 ± 0.19	1.62
ANOVA		$F_{11,177}$ = 23.13 P < 0.0001	$F_{11,177}$ = 741.16 P < 0.0001	$F_{11,177}$ = 959.18 P < 0.0001	$F_{11,177}$ = 903.33 P < 0.0001	$F_{11,177}$ = 2.92 P = 0.002	

[a]Measurements of sperm traits for these populations were not included in the ANOVA test and comparative (PGLS) analysis.

PGLS regressions that controlled for the phylogeny, head length was not correlated with midpiece length ($\beta = -0.02 \pm 0.02$ se, $t = -1.59$, $P = 0.14$, $\lambda = 0^{1.00;\ 0.12}$), flagellum length ($\beta = -0.03 \pm 0.02$ se, $t = -1.69$, $P = 0.12$, $\lambda = 0^{1.00;\ 0.01}$) or sperm length ($\beta = -0.02 \pm 0.02$ se, $t = -1.50$, $P = 0.16$, $\lambda = 0^{1.00;\ 0.11}$) in the 12 species. Furthermore, sperm total length was not associated with male body mass ($\beta = 0.33 \pm 1.08$ se, $t = 0.31$, $P = 0.766$, $\lambda = 1^{0.27;\ 1.00}$).

Four species had sperm lengths > 100 μm (Table 1), and as they all belong to different genera, there was no strong genus-specific differentiation in sperm lengths among our study species. However, when we mapped the sperm lengths onto the phylogeny derived from two mitochondrial genes and three nuclear introns (Fig. 2), there was a trend for closely related species to have similar sperm lengths. Hence, there was also a significant phylogenetic signal in sperm length as estimated by Blomberg's K (Table 2). Sperm midpiece and flagellum length, which are strongly

Figure 2. Reconstruction of sperm length along branches and nodes of the phylogeny of 12 sunbird species. The legend shows the colour range from red (short sperm) to blue (long sperm), and a scale for the branch-lengths in million years (mya). The phylogeny is based on a Bayesian tree constructed from five concatenated genes and rooted with the Flame-crowned Flowerpecker *Dicaeum anthonyi* (for details see Methods); the mean divergence times with 95% highest probability density estimates are available in Supporting Information Fig. S2.

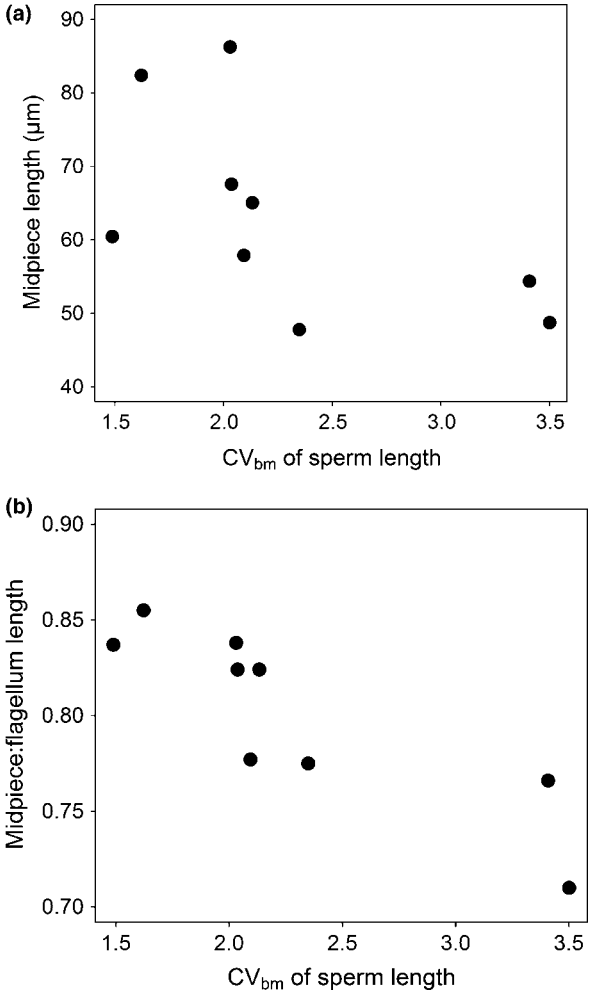

Figure 3. The relationship between the coefficient of inter-male variation of sperm total length (CV_{bm}) and (a) midpiece length, and (b) sperm midpiece : flagellum ratio in sunbirds (n = 9 species). Data points represent species means (see also Table 4).

intercorrelated with sperm total length, also showed a significant phylogenetic signal, but sperm head length did not (Table 2).

The tests of various evolutionary models supported a Brownian motion model of evolution for sperm total length and all components (all ΔAICc = 0.00 and all ΔAICc weights > 0.35; Table 3). This implies that trait divergences did not deviate consistently from a random walk and were best predicted by the genetic distance between species or lineages.

The inter-male variance in sperm total length differed significantly among sunbird species (Levene's test: $F_{11,177} = 2.518$, $P = 0.006$). The

Table 2. Test of phylogenetic signal in sperm traits among sunbirds.

Sperm traits	*K*	Blomberg's *K* *P* (randomization)
Head length	0.881	0.762
Midpiece length	1.263	0.012
Flagellum length	1.238	0.019
Total length	1.231	0.013

n = 12 species using Blomberg's K (P-values for randomization test).

CV_{bm} in sperm length ranged from 1.49 to 3.50 for the nine species for which the metric was calculated (i.e. $n > 3$; Table 1), with an average of 2.30 ± 0.71 sd. The CV_{bm} values for sunbirds did not differ significantly (t-test: $t_{131} = -1.17$, $P = 0.88$) from other passerine birds (i.e. 124 species in Albrecht *et al.* 2013, Table S1). There was no association between sperm CV_{bm} and sexual size dimorphism (female : male body mass and wing length). Similarly, sperm CV_{bm} was not associated with either sexual dichromatism or male plumage ornamentation (Supporting Information Table S6). Furthermore, sperm CV_{bm} was not associated with sperm total length or any of its components, or with the flagellum : head ratio (Table 3). However, the sperm CV_{bm} value was inversely correlated with relative midpiece length, and also a tendency in the same direction for absolute midpiece length (Fig. 3, Table 4). The intra-male variation (CV_{wm}) in sperm length differed significantly among species and was generally quite low (range 1.19–2.50; Table 1) but there was no correlation between intra-male (CV_{wm}) and inter-male (CV_{bm}) variation in sperm lengths across species ($\beta = 0.23 \pm 0.17$ se, $t = 1.32$, $P = 0.23$, $\lambda = 0^{1.00;\ 0.20}$, $n = 9$).

DISCUSSION

We show how the length of sperm cells and their main structural components vary among and within 12 species of sunbird from West Africa. This is the first comparative analysis of sperm morphology from this family of birds (Nectariniidae), which encompasses altogether 143 species in Africa and the Oriental region (Gill & Donsker 2015). Our results show significant variation in mean sperm total length among the species, within the range 74–116 μm (Table 1). Immler *et al.*

Table 3. Tests of various evolutionary models for sperm length diversification in 12 species of sunbirds using the fitContinuous function in the 'geiger' package (Harmon *et al.* 2008). For each sperm trait, the model with the lowest AICc value (i.e. ΔAICc = 0) is considered the best-fitting model (bold type with *). The parameters estimated by the models are: σ^2 = net rate of trait evolution in Brownian motion model or the initial rate of evolution in the Early Burst model; λ = extent to which the phylogeny predicts covariance among traits for species; δ = compares the contribution of early vs. late trait evolution across a phylogeny; κ = evolutionary change in trait associated with speciation events along the branch-length in the Kappa models; α = evolutionary constraint parameter in the Ornstein–Uhlenbeck model moving trait values back to the optimum; r = change in rate of trait evolution through time in the Early Burst model.

		Length of sperm traits			
Model	Parameter	Head	Midpiece	Flagellum	Total sperm
Brownian motion	ΔAICc	**0.000***	**0.000***	**0.000***	**0.000***
	AICc weight	0.461	0.494	0.529	0.545
	σ^2	0.0001	0.0053	0.0033	0.0024
Lambda	ΔAICc	2.703	3.929	3.929	3.929
	AICc weight	0.119	0.0692	0.0742	0.0764
	λ	< 0.0001	1.0000	1.0000	1.0000
	σ^2	0.0004	0.0053	0.0033	0.0024
Delta	ΔAICc	2.772	3.616	3.532	3.585
	AICc weight	0.115	0.0809	0.0905	0.0908
	δ	2.99	0.5103	0.4657	0.4873
	σ^2	0.0004	0.0072	0.0047	0.0033
Kappa	ΔAICc	3.9286	3.9286	3.9286	3.9286
	AICc weight	0.1195	0.0692	0.0742	0.0765
	κ	1.0000	1.0000	1.0000	1.0000
	σ^2	0.0006	0.0053	0.0033	0.0024
Ornstein–Uhlenbeck	ΔAICc	2.703	3.929	3.929	3.929
	AICc weight	0.1195	0.0692	0.0742	0.0765
	α	20.978	< 0.0001	< 0.0001	< 0.0001
	σ^2	0.1275	0.0053	0.0034	0.0024
Early Burst	ΔAICc	3.9285	1.6374	2.4233	2.7988
	AICc weight	0.0647	0.2177	0.1575	0.1345
	r	0.00	−0.6980	−0.5032	−0.4294
	σ^2	0.00057	0.0782	0.0252	0.0140

(2011) listed sperm lengths for 196 passerine species in the range 41.8–284.8 μm. Sunbirds therefore have sperm length within the short-to-medium range for passerine birds.

We found evidence of a phylogenetic signal in the differentiation of sperm length among species (Table 2), which implies that species tend to differ more in sperm size, the more distantly related they are in the phylogeny. There was also a significant phylogenetic signal in sperm midpiece and flagellum length, which constitute the larger parts of the sperm. We were not able to detect any significant deviation from a Brownian model of sperm evolution. This result stands in contrast to a recent study on sperm evolution in African greenbuls (Omotoriogun *et al.* 2016), which found evidence of lineage-specific rates of evolution in sperm length and generally more rapid differentiation around speciation events than along the branches in the phylogeny. It must be emphasized, however, that our sample of 12 sunbird species represents less than 10% of the total number of species in the family, so it is possible that a larger dataset, with more statistical power and better resolution at the deeper nodes in the phylogeny, would detect other patterns of sperm evolution. At present, there is no clear theory for why the rate of sperm evolution should vary among groups of passerine birds.

Sperm heads were generally short and varied much less than other sperm components. There was also no significant phylogenetic signal in sperm head length variation. There is a general trend among passerine birds that sperm head length is evolutionarily conserved and varies within a rather narrow size range compared with the vast variation in midpiece and flagellum lengths (Jamieson 2006, Rowe *et al.* 2015). The

head consists of the acrosome, which is functionally important in the fertilization process, and the nucleus, containing the haploid genome, which is normally densely packed (Jamieson 2006). Assuming drag is kept to the 'ideal' minimum level for swimming, the evolution of much longer flagella in some species could technically allow for an increase in head size, so there may be additional reasons for the conservation of short head lengths (Humphries *et al.* 2008).

Generally, passerine birds have higher rates of extra-pair paternity, i.e. more sperm competition, than other orders of birds, but the level of sperm competition is also variable among passerine species (Westneat & Sherman 1997, Griffith *et al.* 2002). The sperm length CV_{bm} metric carries information about the level of sperm competition (Calhim *et al.* 2007, Lifjeld *et al.* 2010), and it has recently been applied in several comparative analyses of sperm competition in passerine birds (Albrecht *et al.* 2013, Gohli *et al.* 2013, Rowe *et al.* 2013). Using the formula given in Lifjeld *et al.* (2010) (Fig. 2), the minimum (1.49) and maximum (3.50) CV_{bm} values observed for the sunbirds correspond to estimated frequencies of 39 and 7% extra-pair young, respectively, thus indicating a considerable span in the level of sperm competition. The average CV_{bm} value of 2.30 calculated from nine sunbird species corresponds to a frequency of about 20% extra-pair young, which is slightly higher than the average for passerine birds based on molecular paternity studies (Griffith *et al.* 2002). The three sunbird species reported in Albrecht *et al.* (2013) had a mean CV_{bm} of 2.58 (range 2.26–2.76). Paternity studies from sunbirds are, however, limited: we are only aware of the study by Zilberman *et al.* (1999), who found that 23% of young in the Palestine Sunbird *Cinnyris oseus* were sired by extra-pair males, which makes a good match with our estimate. Extra-pair copulation behaviour is also reported from the Purple-rumped Sunbird *Leptocoma zeylomica* (Lamba 1978), and there are also observations of cloaca-pecking in sunbirds (Cheke *et al.* 2001), which may indicate multiple mating by females (Davies 1984). CV_{bm} values were lowest (and sperm competition levels presumably highest) for the two species that do not exhibit the typical socially monogamous mating system, i.e. the Olive Sunbird and the Collared Sunbird, which are considered socially polygynous and polyandrous, respectively (Fry *et al.* 2000, Cheke *et al.* 2001).

Table 4. Regression analysis controlling for phylogeny (PGLS) between the sperm length CV_{bm} index (predictor) and sperm size traits in sunbirds (n = 9 species). The model including the maximum-likelihood values or lambda (λ) value was compared against λ = 1 and λ = 0, with superscripts following the λ values indicating the probability (P) of likelihood-ratio of sperm trait (first position, against λ = 0; second position, against λ = 1).

Sperm traits	$\beta \pm$ se	t	P	λ
Head	0.05 ± 0.36	0.14	0.89	$0^{1.00;\ 0.19}$
Midpiece	−11.36 ± 5.89	−1.92	0.09	$0^{1.00;\ 0.51}$
Flagellum	−8.64 ± 6.32	−1.37	0.21	$0^{1.00;\ 0.60}$
Total length	−8.59 ± 6.22	−1.38	0.21	$0^{1.00;\ 0.50}$
Flagellum : head	−0.75 ± 0.59	−1.27	0.24	$0^{1.00;\ 0.47}$
Midpiece : flagellum	−0.34 ± 0.08	−4.58	0.003	$0^{1.00;\ 0.24}$
Midpiece : total length	−0.28 ± 0.08	−3.33	0.013	$0^{1.00;\ 0.35}$

Overall, it seems likely that sunbirds are characterized by mating systems in which sperm competition is common, but that the level of sperm competition may vary with the social mating system. Sunbirds also tend to be sexually dimorphic in both size and plumage, but we found no significant associations between the CV_{bm} index and measures of sexual size dimorphism or sexual dichromatism in our sample of species.

Although sperm size evolution in sunbirds to a large degree seems to mirror the phylogenetic relationships among species, we found one strong correlation with sperm competition that may suggest a role of selection. Relative midpiece size was greater in species with more sperm competition (i.e. lower CV_{bm}; Fig. 3). It is therefore possible that sperm competition favours the evolution of longer midpieces, with a higher mitochondrial loading of the sperm, which is important in sperm energetics (Rowe *et al.* 2013). Because the midpiece is wrapped around the flagellum, the flagellum needs to be as long as or longer than the midpiece for reasons of structural support. Selection for a longer midpiece will therefore as a consequence also imply selection for a longer flagellum, and hence a longer sperm. The correlation between sperm competition and relative midpiece size is therefore consistent with a trend among certain passerine groups that sperm competition favours the evolution of longer sperm with a longer midpiece (Briskie *et al.* 1997, Kleven *et al.* 2009, Lifjeld *et al.* 2010, but see Immler & Birkhead 2007).

In conclusion, our study highlights a considerable inter-specific variation in mean sperm length and its variance across a sample of 12 sunbird species. The variation in sperm length reflects to a large extent the phylogenetic relationships among species. Differences in sperm length can therefore be explained by a neutral model of genetic drift, but there is also some indication that sperm competition drives the evolution of longer sperm through selection for a longer midpiece. We also found relatively low coefficients of inter-male variation in sperm length, which suggests that sperm competition is common in this group of birds. Our phylogeny also suggests that some of the currently accepted taxonomic genera of sunbirds are not monophyletic. Recently, Lauron *et al.* (2015) noted the same pattern in a study of coevolution between malaria parasites and their sunbird hosts. Thus, there is clearly a need for more comprehensive studies of the sunbird phylogeny and an improved taxonomy.

This study was sponsored by the Research Council of Norway (project no. 196554/V40 to J.T.L.), Norwegian State Educational Loan Fund (PhD scholarship to T.C.O.), the Czech Science Foundation (Project no. 14-36098G), International Foundation for Science (grant no. TJ/32343 to T.C.O.) and the United States National Science Foundation (DEB-1120356 & DEB-1441652 to R.C.K.B.). We thank the A.P. Leventis Ornithological Research Institute, University of Jos, for providing vehicles for our field work in Nigeria. The Nigeria National Park Service, Mt Cameroon National Park authorities, and International Research and Training Centre, Yaoundé, Buea University granted permission to access field sites. Talatu Tende, Onoja Joseph, Taiye A. Adeyanju and Chima Nwaogu helped during our field work in Nigeria. Gunnhild Marthinsen, Silje Hogner and Lars Erik Johannessen assisted during laboratory analysis. We thank the Field Museum of Natural History and the Louisiana State University Museum of Natural Science, the University of Michigan Museum of Natural History, and Thomas Smith and Kevin Njabo for loan of tissue samples. Thanks to two anonymous referees and Rebecca Kimball for their helpful comments. This is contribution no. 104 from the A.P. Leventis Ornithological Research Institute.

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Received 14 June 2014;
revision accepted 1 November 2015.
Associate Editor: Martin Collinson.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Detail of DNA extraction, PCR, sequencing of the mitochondrial COI gene and phylogeny construction of sunbirds.

Fig. S1. A maximum likelihood tree of 15 species of sunbirds based on the mitochondrial COI gene.

Fig. S2. A Bayesian tree based on the concatenated sequences from two mitochondrial genes (*COI* and *NADH2*) and three nuclear introns (FGB5, MB2, TGFb2) and with 95% highest probability densities (HPD) estimated around each mean divergence time for each node.

Table S1. Voucher information of the samples used for sequencing the mitochondrial COI gene.

Table S2. Voucher information of the samples used for sequencing the mitochondrial NADH2 gene and three nuclear introns (FGB5, MB2, TGFb2).

Table S3. The common and species names of sunbirds according to the IOC World Bird List, and with cross reference to Taxonomy in Flux, BirdLife International and Internet Bird Collection checklists.

Table S4. Plumage categories used in the analysis testing for association between inter-male coefficient of variation of sperm length (CV_{bm}) and plumage dichromatism in sunbirds ($n = 9$ species). Sexual dichromatism scored as monochromatic (0) or dichromatic (1). Male plumage ornamentation scored as the number of distinct colour patches on the male plumage. Scores were based on plate illustrations of adult birds in Cheke *et al.* (2001).

Table S5. Detail of individual male sperm morphology data analysed for 12 species of sunbird. Length (μm) of sperm head, midpiece, flagellum and total are based on the average of 10 spermatozoa measured per individual. The CV_{wm} is intra-male coefficient of variation of sperm total length.

Table S6. Regression analysis controlling for phylogeny (PGLS) between inter-male coefficient of variation of sperm length and sexual size dimorphism, and sexual dichromatism in sunbirds ($n = 9$ species). The model including the maximum-likelihood of lambda (λ) value was compared against the models including $\lambda = 1$ and $\lambda = 0$, and superscripts following the λ values indicate probability (P) of likelihood-ratio of indices of sexual size dimorphism or plumage dichromatism (first position, against $\lambda = 0$; second position, against $\lambda = 1$).