1   **Running head:** *Sperm morphology in sunbirds*

2

3   **Variation in sperm morphology variation among Afrotropical sunbirds**

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27   Birds show considerable variation in sperm morphology. Closely related species and subspecies can

28   show diagnostic differences in sperm size. There is also variation in sperm size among males within a

29   population, and recent evidence from passerine birds suggests that the coefficient of inter-male

30   variation in sperm length is negatively associated with the level of sperm competition. Here we

31   examined patterns of inter- and intra-specific variation in sperm length in 12 species of sunbird

32   (Nectariniidae) from Nigeria and Cameroon, a group for which such information is extremely limited.

33   We found significant variation among species in sperm total length, with mean values ranging from 74

34   µm to 116 µm, placing these species within the short to medium sperm length range for passerine

35   birds. Most of this variation was explained by the length of the midpiece, which contains the fused

36   mitochondria and is an important structure for sperm energetics. Relative midpiece length was

37   negatively correlated with the coefficient of inter-male variation in sperm total length across species,

38   suggesting that sperm competition may have selected for greater midpiece length in this group. We

39   also mapped sperm lengths onto a time-calibrated phylogeny and found support for a phylogenetic

40   signal in all sperm length components, except head length. A test of various evolutionary or tree

41   transformation models gave strongest support for the Brownian motion model for all sperm

42   components, i.e. divergences were best predicted by the phylogenetic distance between lineages. The

43   coefficients of inter-male variation in sperm total length indicate that sperm competition is high, but

44   variable among sunbird species, similar to passerine birds at large.

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46   **Key words:** comparative analysis, phylogenetic signal, sperm size, sperm competition, Nectariniidae

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53   Across the animal kingdom, spermatozoa vary remarkably in size, shape and behaviour (Cohen 1977,

54   Pitnick *et al.* 2009, Pizzari & Parker 2009). In passerine birds, sperm length varies from approximately

55   40 µm to nearly 300 µm (Pitnick *et al.* 2009, Lifjeld *et al.* 2010, Immler *et al.* 2011). Given that the

56   primary role of sperm is to fertilize ova, a highly conserved function, the evolutionary diversification

57   of sperm form is surprising and the factors generating this diversity are poorly understood (Snook

58   2005, Pitnick *et al.* 2009). However, it is generally thought that genetic drift, mode of fertilization and

59   postcopulatory sexual selection, i.e. sperm competition (Parker 1970) and cryptic female choice

60   (Eberhard 1996), drive evolutionary changes in sperm phenotypes (Franzén 1970, Snook 2005, Pitnick

61   *et al.* 2009).

62   There is comparative evidence from a range of taxonomic groups that sperm length tends to

63   increase with sperm competition, for examples in birds (Briskie *et al.* 1997, Kleven *et al.* 2009),

64   insects (Morrow & Gage 2000), fish (Balshine *et al.* 2001) and mammals (Gomendio & Roldan 1991,

65   Tourmente *et al.* 2011), although with some exceptions to this pattern (e.g. Gage & Freckleton 2003,

66   Immler & Birkhead 2007). It is suggested that the evolution of longer sperm is driven by their ability

67   to swim faster (Gomendio & Roldan 1991), live longer (Parker 1993, Parker 1998) or displace shorter

68   sperm from female sperm storage sites (Miller & Pitnick 2002, Lüpold *et al.* 2012). In passerine birds,

69   increased sperm size is associated with a disproportionate increase in the size of the midpiece (Lüpold

70   *et al.* 2009), which contains a single fused mitochondrion wrapped helically around the flagellum

71   (Koehler 1995). A longer midpiece contains more adenosine triphosphate (Rowe *et al.* 2013), thus

72   demonstrating the importance of this structure for sperm energetics. Sperm length in passerine birds is

73   also positively correlated with the length of the sperm storage tubules in females (Briskie &

74   Montgomerie 1992, Kleven *et al.* 2009). Briskie *et al.* (1997) hypothesized that longer sperm storage

75   tubules enable female control over how sperm are used in fertilization. There is also a strong

76   phylogenetic signal in the association between sperm length and sperm competition (Immler &

77   Birkhead 2007, Kleven *et al.* 2009, Lifjeld *et al.* 2010, Immler *et al.* 2012), which suggests that the

78   role of sperm competition in sperm length evolution varies across the passerine phylogeny.

79   More recently, studies have shown that increased levels of sperm competition are associated

80   with reduced inter- and intra-male variation in sperm length in passerine birds (Calhim *et al.* 2007,

81   Immler *et al.* 2008, Kleven *et al.* 2008, Lifjeld *et al.* 2010), and also in insects (Fitzpatrick & Baer

82   2011). Reduced variation in sperm length among males within a population suggests stronger

83   stabilizing selection around an optimum length for high performance across different female

84   environments (Calhim *et al.* 2007, Kleven *et al.* 2008, Lifjeld *et al.* 2010). In a comparative analysis,

85   Lifjeld *et al.* (2010) showed that the coefficient of inter-male variation (CV_{bm}) in sperm length

86   explained as much as 65% of the variation in extra-pair paternity rates among 24 passerine species.

87   Since there was no phylogenetic signal in this association, Lifjeld *et al.* (2010) proposed that the CV_{bm}

88   metric could be used as a proxy for extra-pair paternity, and therefore sperm competition, in passerine

89   birds. There is also a negative relationship between the coefficient of intra-male variation (CV_{wm}) in

90   sperm length and measures of sperm competition (Immler *et al.* 2008, Lifjeld *et al.* 2010). Reduced

91   variation in sperm length within a male or an ejaculate should imply a stronger developmental stability

92   or quality control in spermatogenesis.

93   In contrast to temperate species, we know surprisingly little about mating systems in tropical

94   birds (Macedo *et al.* 2008). Stutchbury and Morton (2001) hypothesized that sperm competition levels

95   should be generally lower in tropical than in temperate birds, but very few studies have actually tested

96   this idea empirically (see Stutchbury *et al.* 1998, Stutchbury & Morton 2001, Albrecht *et al.* 2013 for

97   exceptions). Albrecht *et al.* (2013) found no difference in overall sperm competition levels between

98   tropical and temperate passerine birds, using the sperm length CV_{bm} index. They also noted that

99   tropical species are apparently as variable as temperate zone birds in sperm competition levels, and

100   mentioned specifically waxbills (Estrildidae) and sunbirds (Nectariniidae) as examples of families

101   with low and high sperm competition levels, respectively. However, it is difficult to infer general

102   patterns from just a few species; only three species of sunbirds were included in that study. General

103   descriptive information about sperm morphology is also largely lacking for tropical birds. Moreover,

104 tropical birds are relatively less well studied in terms of systematics (Reddy 2014) and general biology

105 (Macedo *et al.* 2008).

106   Here, we examine variation in sperm morphology in 12 species of sunbirds from West Africa

107 (Nigeria and Cameroon). Sunbirds are generally small (*c.* 5–22 g), socially monogamous species

108 exhibiting a territorial breeding system (Fry *et al.* 2000, Cheke *et al.* 2001, Riegert *et al.* 2014).

109 Additionally, the majority of species are sexually dimorphic in both body size and plumage coloration:

110 males are larger and exhibit colourful iridescent plumage patches (either year round or seasonally),

111 whereas females are generally drab (Fry *et al.* 2000, , Borrow & Demey 2001, Cheke *et al.* 2001). The

112 primary objectives of our study were to describe sperm length variation in sunbirds at multiple levels

113 of organisation (i.e. among species and among and within males belonging to a single species) and test

114 for signatures of phylogeny and sperm competition in the observed patterns of sperm morphological

115 variation. We also tested for phenotypic correlates of sperm CV_{bm} as a proxy for sperm competition.

116

117 **METHODS**

118 **Data collection and sampling procedure**

119 In Nigeria, fieldwork was conducted at Amurum Forest Reserve, Jos (09° 53′ N, 08° 59′ E); Yankari

120 Game Reserve, Bauchi (09° 50′ N, 10° 30′ E); Omo Forest Reserve, Ogun (06° 51′ N, 4° 30′E);

121 International Institute of Tropical Agriculture, Ibadan (07° 30′ N, 03° 55′ E) and Okomu National Park,

122 Benin (06° 33′ N, 05° 26′). In Cameroon, we sampled birds along the slope of Mount Cameroon (04°

123 15′ N, 09°09′ E) and in the vicinity of Laide Farm, Bamenda-Banso Highlands (06° 05′ N, 10° 28′ E).

124 Birds were captured using mist-nets (in some instances with the assistance of song playback) during

125 the breeding season (i.e. April to September in 2010–2013 in Nigeria and October to December in

126 2010–2012 in Cameroon). Sperm samples (*c.* 0.5–3 µl) were collected by cloacal massage (Wolfson

127 1952) and immediately diluted in a small volume of phosphate-buffered saline (*c.* 20 µl) and then

128 fixed in 300 µl of 5% formaldehyde solution for later slide preparation. For each bird, a small blood

129 sample (*c.* 10–50 µl) was collected from the brachial vein and preserved in 96% ethanol for later DNA

130   extraction and DNA sequencing. We also fitted each bird with a uniquely numbered aluminium band

131   (supplied by South African Bird Ringing Unit) to prevent resampling of individuals.

132

133   **Sperm morphology**

134   For each sample, a small aliquot (*c*. 15 µl) of formaldehyde-fixed sperm was applied to a glass slide

135   and allowed to air-dry. Slides were then gently rinsed with distilled water and air-dried again. We

136   captured high magnification (160× or 320×) digital images of sperm using a Leica DFC420 camera

137   mounted on a Leica DM6000 B digital light microscope (Leica Microsystem, Heerbruug, Switzerland).

138   We used Leica Application Suite (version 2.6.0 R1) to measure (to the nearest $\pm$ 0.1 µm) the length of

139   the sperm head, midpiece and tail (i.e. the section of the flagellum not entwined by the midpiece),

140   from which we calculated flagellum length (as the sum of midpiece and tail length), sperm total length

141   (as sum of head, midpiece and tail length) and the ratios of flagellum:head length, midpiece:flagellum

142   length and midpiece:total length.

143   For each male, we measured 10 morphologically normal spermatozoa following the recommendation

144   in Laskemoen *et al.* (2007). All sperm measurements were taken by one person (TCO) to avoid

145   observer effects. We determined the repeatability of sperm measurements by measuring the same 15

146   sperm from a single individual twice, and found that measurements were highly repeatable (head: $r =$

147   0.87, $F_{14, 15} = 14.75$, $P < 0.001$; midpiece: $r = 0.81$, $F_{14, 15} = 9.76$, $P < 0.001$, tail: $r = 0.83$, $F_{14, 15} =$

148   10.94, $P < 0.001$; Lessells & Boag 1987). For each sperm trait we used the means within individuals to

149   calculate the mean for each species. For two species we had sperm samples from both Nigeria and

150   Cameroon. There were no significant differences between countries in sperm length or components for

151   either species, but we used the Nigerian data only (larger *n*) for our comparative analyses. Finally, we

152   calculated CV_{wm} values of sperm total length for each individual and then used the mean of these

153   values to calculate an average CV_{wm} for each species. Similarly, we calculated the CV_{bm} of sperm total

154   length following the formula $CV_{bm} = (SD/ Mean) * 100 * (1+ (1/4n))$, which corrects for variation in

155   sample size (*n*) (Sokal & Rohlf 1995). **Phylogeny**

156   We sequenced the first part of the mitochondrial cytochrome oxidase I (COI) gene, which corresponds

157   to the standard DNA barcode marker for animals (Hebert *et al.* 2003). Details of the DNA extraction,

158   PCR and sequencing procedures are available as Appendix S1, Fig S1 and Table S1. To complement

159   these data, but from different individuals, sequences were collected from another mitochondrial gene

160   (NADH2) and three nuclear introns (FGB5, MB2, TGFb2) using standard protocols (Kimball *et al.*

161   2009, Fuchs *et al.* 2012). All COI sequences are publicly available at the BOLD database

162   (Ratnasingham & Hebert 2007) in the project folder BONSU. Data for the remaining loci are available

163   on GenBank (xxxxxx-xxxxxx) Table S2. COI sequences were trimmed to an even length, and all loci

164   were aligned using MAFFT v. 7 (Katoh & Standley 2013), generating alignments for each locus of:

165   COI: 654 base pairs, NADH2: 1041 bp, FGB5: 570 bp; MB2: 749 bp, TGFb2: 589 bp – Total 3603

166   bp); for 12 sunbird species and a flowerpecker (Flame-crowned Flowerpecker *Dicaeum anthonyi*), a

167   member of the sister family to the sunbirds (Johansson *et al.* 2008) used to root the phylogenetic

168   analyses described below. Species nomenclature follows the International Ornithologists' Union (IOC)

169   World Bird List (Gill & Donsker 2015). A cross reference to names used by other checklists is

170   presented in Table S3.

171   We estimated a maximum likelihood tree using the GTRGAMMA model of RaxML v. 8.1.24

172   (Stamatakis 2014) applied to the concatenated dataset using nine partitions (COI – codons 1, 2 & 3;

173   NADH2 – codons 1, 2 & 3; FGB5, MB2, TGFb2). Analyses were conducted via the CIPRES Science

174   Gateway supercomputer portal. To obtain a Bayesian tree and determine divergence times among

175   species we made use of BEAST v 1.8.2 (Drummond *et al.* 2012) and the mean rates of divergence and

176   associated standard deviations reported by Lerner *et al.* (2011) for each of the two mtDNA genes

177   analysed and two of the introns (FGB and TGFb2). The rates reported by Lerner *et al.* (2011) are

178   derived from the sequence of lineage splits in Hawaiian Honeycreepers (Fringillidae), and were

179   calibrated using the well-established dates of sequential uplift of the Hawaiian Archipelago. The

180   BEAST analyses was run for 100 million generations with a HKY+G+I model of nucleotide

181   substitution applied to each locus, a strict molecular clock enforced and a Yule process for the tree

182 prior. We made use of Tracer v.1.6.0 (Rambaut *et al.* 2014) to check that the effective sample size of

183 the underlying posterior distribution was large enough (ESS > 200) for meaningful estimation of

184 parameters.

185

186 **Sexual size dimorphism and plumage dichromatism**

187 We collected data on male and female body mass, wing length and sexual dichromatism from the

188 literature (Fry *et al.* 2000, Cheke *et al.* 2001, Borrow & Demey 2001, Cox *et al.* 2011). Sexual size

189 dimorphism was estimated as the ratio of female body mass:male body mass and female wing

190 length:male wing length, which we calculated using the mean values for each sex obtained from the

191 literature. Next, we categorized plumage dichromatism as 0 or 1, with 0 representing species that were

192 monochromatic or showed only minor differences between the sexes (i.e. less than 10% of plumage

193 differed) and 1 representing species that showed complete differences in colour or pattern (Table S4).

194 Additionally, we scored male plumage ornamentation as the number of separate and distinct colour

195 patches in the male plumage, i.e. head, throat-chest-belly and nape-back-rump. All plumage traits were

196 assessed using image plates in Cheke *et al.* (2001). Finally, based on literature (Frey *et al.* 2000,

197 Cheke *et al.* 2001), all species were assumed to be socially monogamous with the exception of the

198 Olive Sunbird *Cyanomitra olivacea* and the Collared Sunbird *Hedydipna collaris,* which were

199 classified as polygynous and polyandrous, respectively.

200

201 **Data analysis**

202 All analyses were performed using the statistical package R version 2.12.2 (R Development Core

203 Team 2013). To improve data distributions, we log-transformed data prior to analysis. The ratios of

204 midpiece:flagellum length, midpiece:total length, female:male body mass and female:male wing

205 length were logit transformed following recommendation of Warton and Hui (2011). We tested for

206 differences among species in sperm morphology (i.e. sperm total length and length of the various

207 components) and CV_{wm} using ANOVA. To assess whether species differed in CV_{bm}, we tested for

208   homogeneity of variance in sperm length using Levene's test. Next, for all sperm traits (i.e. head,

209   midpiece, flagellum and total sperm length), we tested for the presence of a phylogenetic signal by

210   calculating Blomberg's K (Blomberg *et al.* 2003), using the phylosig function in the 'phytools'

211   package (Revell 2012): $K > 1$ indicates that traits are more similar between related species than

212   expected under Brownian motion evolution, whereas $K < 1$ indicates high lability, at least at the tips of

213   the tree (Blomberg *et al.* 2003). The presence of a phylogenetic signal was tested using a

214   randomization test. We reconstructed the ancestral character state of sperm length using 'contMap'

215   (Revell 2013). The mapping relies upon states estimated at internal nodes using maximum likelihood

216   with 'fastAnc' and was plotted with 'contMap'.

217   The fit of five evolutionary models for the diversification of sperm length and sperm

218   components in the time-calibrated phylogeny were compared against a null model of Brownian motion,

219   using the fitContinuous function in the 'geiger' package (Harmon *et al.* 2008). These models were

220   Lambda: phenotypic divergence covaries with phylogenetic distance, but allows for variable

221   evolutionary rates; Delta: the evolutionary rate accelerates or decelerates over time; Kappa:

222   evolutionary change occurs mainly at speciation events, and is not proportional to branch length;

223   Ornstein-Uhlenbeck: a random walk within a constrained trait space, where traits tend to converge

224   towards a single value; and Early Burst: an early burst of trait diversification followed by reduced

225   evolutionary rates (or stasis). Models were compared using the Akaike Information Criterion corrected

226   for small sample size (AICc); the model with the lowest AICc value indicates the best-fit model. We

227   also calculated Akaike weights for all models and used both ΔAICc and Akaike weights values to

228   assess model support. Values of ΔAICc ≤2 are indicative of substantial support for the model

229   (Burnham & Anderson, 2004). For further details about the application of these models in another

230   African passerine group, see Omotoriogun *et al.* (2016).

231   We performed phylogenetic generalized least-squares (PGLS) regressions using the package

232   'caper' (Orme *et al.* 2012), to examine the relationships among sperm traits and the relationships

233   between sperm traits and CV_{bm}. For these latter models, separate models were run for each sperm trait.

234   Similarly, we used PGLS regressions to determine whether measures of either sexual size dimorphism

235   or sexual dichromatism predict sperm length CV_{bm} (i.e. sperm competition) in sunbirds. This approach

236   accounts for the statistical non-independence of data points due to shared ancestry of species (Pagel

237   1999, Freckleton *et al.* 2002). PGLS also allow for the estimation (via maximum likelihood) of the

238   phylogenetic scaling parameter λ ($\lambda = 0$ indicate phylogenetic independence, whereas $\lambda = 1$ indicates

239   phylogenetic dependence): superscript values in the reported results likelihood ratio tests for empirical

240   λ value against $\lambda = 1$ and $\lambda = 0$, respectively. Finally, we compared levels of CV_{bm} in sunbirds to those

241   of other passerine birds using a two-sample *t*-test. For this analysis, CV_{bm} values for other species

242   were extracted from Albrecht *et al*. (2013).

243

244   **RESULTS**

245   Sperm samples were analysed from a total of 189 males from 12 species belonging to five

246   genera (Table 1, Table S5). Sperm total length ranged from 74 µm in the Northern Double-collared

247   Sunbird *Cinnyris reichenowi* to 116 µm in the Scarlet-chested Sunbird *Chalcomitra senegalensis*, and

248   differed significantly among species ($F_{11, 177} = 903.33$, $P < 0.0001$; Table 1). The variation in sperm

249   total length among species was largely explained by variation in midpiece and flagellum length, while

250   head length was short in all species (range 12–14 µm; Table 1, Fig. 1). However, all sperm

251   components varied significantly among species ($P < 0.0001$; Table 1). Across the 12 species, head

252   length was not correlated with midpiece length ($\beta = -0.02 \pm 0.02$ SE, $t = -1.59$, $P = 0.14$, $\lambda = 0^{1.00; \, 0.12}$),

253   flagellum length ($\beta = -0.03 \pm 0.02$ SE, $t = -1.69$, $P = 0.12$, $\lambda = 0^{1.00; \, 0.01}$) or sperm length ($\beta = -0.02 \pm$

254   0.02 SE, $t = -1.50$, $P = 0.16$, $\lambda = 0^{1.00; \, 0.11}$). Furthermore, sperm total length was not associated with

255   male body mass ($\beta = 0.33 \pm 1.08$ SE, $t = 0.31$, $P = 0.766$, $\lambda = 1^{0.27; \, 1.00}$). The tests are PGLS regressions

256   controlling for the phylogeny depicted in Fig. 2.

257   Four species had sperm lengths > 100 µm (Table 1), and since they all belong to different

258   genera, there was no strong genus-specific differentiation in sperm lengths among our study species.

259   However, when we mapped the sperm lengths onto the phylogeny derived from two mitochondrial

genes and three nuclear introns (Fig. 2; see also the same phylogeny in Fig. S2 annotated with the 95%

highest probability density estimates for each node and rooted with the outgroup taxon), there was a

trend that closely related species tended to have similar sperm lengths. Hence, there was also a

significant phylogenetic signal in sperm length as estimated by Blomberg's K (Table 2). The

phylogeny suggests that some sunbird genera are not monophyletic, e.g. the six *Cinnyris* species were

spread across the entire phylogeny (Fig. 2), a result also supported with much greater taxon sampling

(R.C.K. Bowie unpubl. data). Sperm midpiece and flagellum length, which are strongly 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 consistently deviate from a random walk and was 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 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 (Table S6).

Furthermore, sperm CV_{bm} was not associated with sperm total length or any of its components, nor 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 were

generally quite low (range 1.19–2.50; Table 1), but there was no correlation between intra-male (CV_{wm})

285 and inter-male (CV_{bm}) variation in sperm lengths across species ($\beta = 0.23 \pm 0.17$ SE, $t = 1.32$, $P = 0.23$,

286 $\lambda = 0^{1.00.00;\ 0.20}$, $n = 9$).

287

288 **DISCUSSION**

289 We have shown here how the length of sperm cells and their main structural components vary among

290 and within 12 species of sunbird from West Africa. This is the first comparative analysis of sperm

291 morphology from this family of birds (Nectariniidae), which encompasses altogether 143 species in

292 Africa and the Oriental region (Gill & Donsker 2015). Our results show significant variation in mean

293 sperm total length among the species, within the range of 74–116 µm (Table 1). Immler *et al.* (2011)

294 listed sperm lengths for 196 passerine species in the range of 41.8–284.8 µm. Sunbirds therefore have

295 sperm length within the short-to-medium range for passerine birds.

296 We found evidence of a phylogenetic signal in the differentiation of sperm length among

297 species (Table 2), which implies that species tend to differ more in sperm size the more distantly

298 related they are in the phylogeny. There was also a significant phylogenetic signal in sperm midpiece

299 and flagellum length, which constitute the larger parts of the sperm. We were not able to detect any

300 significant deviation from a Brownian model of sperm evolution. This result stands in contrast to a

301 recent study on sperm evolution in African greenbuls (Omotoriogun *et al.* 2016), that found evidence

302 of lineage-specific rates of evolution in sperm length and generally more rapid differentiation around

303 speciation events than along the branches in the phylogeny. It must be emphasized; however, that our

304 sample of 12 sunbird species represents less than 10% of the total number of species in the family, so

305 it is possible that a larger data set, with more statistical power and better resolution at the deeper nodes

306 in the phylogeny, would detect other patterns of sperm evolution. At present, there is no clear theory

307 for why the rate of sperm evolution should vary among groups of passerine birds.

308 Sperm heads were generally short and varied much less than other sperm components.

309 There was also no significant phylogenetic signal in sperm head length variation. There is a general

310 trend among passerine birds that sperm head length is evolutionary conserved and varies within a

311 rather narrow size range compared to the vast variation in midpiece and flagellum lengths (Jamieson

312 2006, Rowe *et al.* 2015). The head consists of the acrosome, which is functionally important in the

313 fertilization process, and the nucleus, containing the haploid genome, which is normally densely

314 packed (Jamieson 2006). Assuming drag is kept to the 'ideal' minimum level for swimming, the

315 evolution of much longer flagella in some species could technically allow for an increase in head size,

316 so there may be additional reasons for the conservation of short head lengths (Humphries *et al.* 2008).

317   Our phylogeny suggests that some of the currently accepted taxonomic genera of sunbirds

318 are not monophyletic. Recently, Lauron *et al.* (2015) noted the same pattern in a study of coevolution

319 between malaria parasites and their sunbird hosts. Thus, there is clearly a need for more

320 comprehensive studies of the sunbird phylogeny and an improved taxonomy.

321   Generally, passerine birds have higher rates of extra-pair paternity, i.e. more sperm

322 competition, than other orders of birds, but the level of sperm competition is also variable among

323 passerine species (Westneat & Sherman 1997, Griffith *et al.* 2002). The sperm length CV_{bm} metric

324 carries information about the level of sperm competition (Calhim *et al.* 2007, Lifjeld *et al.* 2010), and

325 it has recently been applied in several comparative analyses of sperm competition in passerine birds

326 (Albrecht *et al.* 2013, Rowe *et al.* 2013, Gohli *et al.* 2013). Using the formula given in Lifjeld *et al.*

327 (2010, Fig. 2), the minimum (1.49) and maximum (3.50) CV_{bm}-values observed for the sunbirds

328 correspond to estimated frequencies of 39% and 7% extra-pair young, respectively, thus indicating a

329 considerable span in the level of sperm competition. The average CV_{bm}-value of 2.30 calculated from

330 nine sunbird species corresponds to a frequency of about 20% extra-pair young, which is slightly

331 higher than the average for passerine birds based on molecular paternity studies (Griffith *et al.* 2002).

332 The three sunbird species reported in Albrecht *et al.* (2013) had a mean CV_{bm} of 2.58 (range 2.26 –

333 2.76). Paternity studies from sunbirds are however limited. We are only aware of the study by

334 Zilbermann *et al.* (1999) who found that 23% of young in the Palestine Sunbird *Cinnyris oseus* were

335 sired by extra-pair males, which makes a good match with our estimate. Extra-pair copulation

336 behaviour is also reported from the Purple-rumped Sunbird *Leptocoma zeylomica* (Lamba 1978), and

337 there are also observations of cloaca-pecking in sunbirds (Cheke *et al.* 2001), which may indicate

338 multiple mating by females (cf. Davies 1984). CV_{bm} values were lowest (and sperm competition levels

339 presumably highest) for the two species that do not exhibit the typical socially monogamous mating

340 system, i.e. the Olive Sunbird and the Collared Sunbird, which are considered socially polygynous and

341 polyandrous, respectively (Cheke *et al.* 2001, Fry *et al.* 2000). Overall, it seems likely that sunbirds

342 are characterized by mating systems where sperm competition is common, but that the level of sperm

343 competition may vary with the social mating system. Sunbirds also tend to be sexually dimorphic in

344 both size and plumage, but we found no significant associations between the CV_{bm} index and measures

345 of sexual size dimorphism or sexual dichromatism in our sample of species.

346   Although sperm size evolution in sunbirds to a large degree seems to mirror the

347 phylogenetic relationships among species, we found one strong correlation with sperm competition

348 that may suggest a role of selection. Relative midpiece size was greater in species with more sperm

349 competition (i.e. lower CV_{bm}; Fig. 3). It is therefore possible that sperm competition favours the

350 evolution of longer midpieces, with a higher mitochondrial loading of the sperm, which is important in

351 sperm energetics (Rowe *et al.* 2013). Because the midpiece is wrapped around the flagellum, the

352 flagellum needs to be as long as, or longer, than the midpiece for reasons of structural support.

353 Selection for a longer midpiece will therefore as a consequence also imply selection for a longer

354 flagellum, and hence a longer sperm. The correlation between sperm competition and relative

355 midpiece size is therefore consistent with a trend among certain passerine groups, that sperm

356 competition favours the evolution of longer sperm with a longer midpiece (Briskie *et al.* 1997, Kleven

357 *et al.* 2009, Lifjeld *et al.* 2010, but see Immler & Birkhead 2007).

358   In conclusion, our study highlights a considerable inter-specific variation in mean sperm

359 length and its variance across a sample of 12 sunbird species. The variation in sperm length reflects to

360 a large extent the phylogenetic relationships among species. Differences in sperm length can therefore

361 be explained by a neutral model of genetic drift, but there is also some indication that sperm

362 competition drives the evolution of longer sperm through selection for a longer midpiece. We also

363   found relatively low coefficients of inter-male variation in sperm length, which suggests that sperm

364   competition is common in this group of birds.

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381   **REFERENCES**

382   **Albrecht, T., Kleven, O., Kreisinger, J., Laskemoen, T., Omotoriogun, T.C., Ottosson, U., Reif, J.,**
383   **Sedláček, O., Hořák, D., Robertson, R.J. & Lifjeld, J.T.** 2013. Sperm competition in tropical
384   versus temperate zone birds. *Proc. R. Soc. Lond. B* **280:** 20122434.
385   **Balshine, S., Leach, B.J., Neat, F., Werner, N.Y. & Montgomerie, R.** 2001. Sperm size of African
386   cichlids in relation to sperm competition. *Behav. Ecol.* **12:** 726–731.
387   **Blomberg, S.P., Garland, T. & Ives, A.R.** 2003. Testing for phylogenetic signal in comparative data:
388   behavioral traits are more labile. *Evolution* **57:** 717–745.
389   **Borrow, N. & Demey, R.** 2001. *The Birds of Western Africa*. London, UK: Christopher Helm.
390   **Briskie, J.V. & Montgomerie, R.** 1992. Sperm size and sperm competition in birds. *Proc. R. Soc. Lond.*
391   *B* **247:** 89–95.
392   **Briskie , J.V., Montgomerie, R. & Birkhead, T.R.** 1997. The evolution of sperm size in birds. *Evolution*
393   **51:** 937–945.

394   **Calhim, S., Immler, S. & Birkhead, T.R.** 2007. Postcopulatory sexual selection is associated with
395   reduced variation in sperm morphology. *PLoS One 2: e413*.

396   **Cheke, R.A., Mann, C.F. & Allen, R.** 2001. *A Guide to the Sunbirds, Flowerpeckers, Spiderhunters and*
397   *Sugarbirds of the World,* New Haven: Yale University Press.

398   **Cohen, J.** 1977. *Reproduction*. London: Butterworths.

399   **Cox, D.T.C., Brandt, M.J., McGregor, R., Ottosson, U., Stevens, M.C. & Cresswell, W.** 2011. Patterns
400   of seasonal and yearly mass variation in West African tropical savannah birds. *Ibis* **153:** 672–
401   683.

402   **Davies, N.B.** 1984. Polyandry, cloaca-pecking and sperm competition in dunnocks. *Nature* **302:** 334–
403   336.

404   **Drummond, A.J., Suchard, M.A., Xie, D. & Rambaut, A.J.** 2012. Bayesian phylogenetics with BEAUti
405   and BEAST 1.7. *Mol. Biol. Evol.* **29**: 1969–1973.

406   **Eberhard, W.G.** 1996. *Female Control: Sexual Selection by Cryptic Female Choice.* Princeton, New
407   Jersey: Princeton University Press.

408   **Fitzpatrick, J.L. & Baer, B.** 2011. Polyandry reduces sperm length variation in social insects. *Evolution*
409   **65**: 3006–3012.

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

412   **Freckleton, R.P., Harvey, P.H. & Pagel, M.** 2002. Phylogenetic analysis and comparative data: a test
413   and review of evidence. *Am. Nat.* **160:** 712–726.

414   **Fry, C.H., Keith, S. & Urban, E.K.** 2000. *The Birds of Africa.* London: Academic Press.

415   **Fuchs, J., Irestedt, M., Fjeldså, J., Couloux, A., Pasquet, E. & Bowie, R.C.K.** 2012. Molecular
416   phylogeny of African bush-shrikes and allies: tracing the biogeographic history of an
417   explosive radiation of corvoid birds. *Mol. Phylogenet. Evol.* **64:** 93–105.

418   **Gage, M.J.G. & Freckleton, R.P.** 2003. Relative testes size and sperm morphometry across mammals:
419   no evidence for an association between sperm competition and sperm length. *Proc. R. Soc.*
420   *Lond. B* **270:** 625–632.

421   **Gill, F. & Donsker, D.** 2015. IOC World Bird List (Version 5.3). Available at: www.worldbirdnames.org
422   (accessed 27 September 2015).

423   **Gohli, J., Anmarkrud, J.A., Johnsen, A., Kleven, O., Borge, T. & Lifjeld, J.T.** 2012. Female promiscuity
424   is positively associated with neutral and selected genetic diversity in passerine birds.
425   *Evolution* **67**: 1406–1419.

426   **Gomendio, M. & Roldan, E.R.S.** 1991. Sperm competition influences sperm size in mammals. *Proc. R.*
427   *Soc. Lond. B* **243:** 181–185.

428   **Griffith, S.C., Owens, I.P.F. & Thuman, K.A.** 2002. Extra pair paternity in birds: a review of
429   interspecific variation and adaptive function. *Mol. Ecol.* **11:** 2195–2212.

430   **Harmon, L.J., Weir, J.T., Brock, C.D., Glor, R.E. & Challenger, W.** 2008. GEIGER: investigating
431   evolutionary radiations. *Bioinformatics* **24:** 129–131.

432   **Hebert, P., Ratnasingham, S. & deWaard, J.** 2003. Barcoding animal life: cytochrome c oxidase
433   subunit 1 divergences among closely related species. *Proc. R. Lond. B* **270:** S96–99.

434   **Humphries, S., Evans, J.P. & Simmons, L.W.** 2008. Sperm competition: linking form to function. *BMC*
435   *Evol. Biol.* **8:** 319.

436   **Immler, S. & Birkhead, T.** 2007. Sperm competition and sperm midpiece size: no consistent pattern
437   in passerine birds. *Proc. R. Soc. Lond. B* **274:** 561–568.

438   **Immler, S., Calhim, S. & Birkhead, T.R.** 2008. Increased postcopulatory sexual selection reduces the
439   intramale variation in sperm design. *Evolution* **62:** 1538–1543.

440   **Immler, S., Gonzalez-Voyer, A. & Birkhead. T.R**.2012. Distinct evolutionary patterns of
441   morphometric sperm traits in passerine birds*. Proc. R. Soc. Lond. B* **279:** 4174–4182.

442 **Immler, S., Pitnick, S., Parker, G.A., Durrant, K.L., Lüpold, S., Calhim, S. & Birkhead, T.R.** 2011.
443   Resolving variation in the reproductive tradeoff between sperm size and number. *Proc. Natl*
444   *Acad. Sci. USA* **108:** 5325–5330.
445 **Jamieson, B.G.M.** 2006. Avian spermatozoa: structure and phylogeny. In Jamieson, B.G.M. (ed.)
446   *Reproductive Biology and Phylogeny of Aves:* 249–511. Enfield, NH: Science Publishers Inc.
447 **Johansson, U.S., Fjeldså, J. and Bowie, R.C.K.** 2008. Phylogenetic relationships within Passerida
448   (Aves: Passeriformes): a review and a new molecular phylogeny based on three nuclear
449   intron markers. *Mol. Phylogenet. Evol.* **48:** 858–876.
450 **Katoh, K. & Standley, D.M.** 2013. MAFFT multiple sequence alignment software version 7:
451   improvements in performance and usability. *Mol. Biol. Evol.* **30:** 772–780.
452 **Kimball, R.T., Braun, E.L., Barker, F.K., Bowie, R.C.K., Braun, M.J., Chojnowski, J.L., Hackett, S.J.,**
453   **Han, K-L., Harshman, J., Heimer-Torres, V., Holznagel, W., Huddleston, C.J. Marks, B.D.,**
454   **Miglia, K.J., Moore, W.S., Reddy, S., Sheldon, F.H., Smith, J.V., Witt, C.C. & Yuri, T.** 2009. A
455   well-tested set of primers to amplify regions spread across the avian genome. *Mol.*
456   *Phylogenet. Evol*. **50:** 654–660.
457 **Kleven, O., Fossøy, F., Laskemoen, T., Robertson, R.J., Rudolfsen, G. & Lifjeld, J.T.** 2009.
458   Comparative evidence for the evolution of sperm swimming speed by sperm competition
459   and female sperm storage duration in passerine birds. *Evolution* **63:** 2466–2473.
460 **Kleven, O., Laskemoen, T., Fossøy, F., Robertson, R.J. & Lifjeld, J.T.** 2008. Intraspecific variation in
461   sperm length is negatively related to sperm competition in passerine birds. *Evolution* **62:**
462   494–499.
463 **Koehler, L.D.** 1995. Diversity of avian spermatozoa ultrastructure with emphasis on the members of
464   ther order Passeriformes. In Jamieson, B.G.M., Ausio, J. & Justine, J.-L. (eds) *Advances in*
465   *Spermatozoal Phylogeny and Taxonomy:* 437–444. Paris: Muséum National d'Histoire
466   Naturelle.
467 **Lamba, B.S.** 1978. Nidification of some common Indian birds 13: the Purple-rumped Sunbird,
468   *Nectarinia zeylonica* Linnaeus. *Indian J. For.* **1:** 329–344.
469 **Laskemoen, T., Kleven, O., Fossøy, F. & Lifjeld, J.T.** 2007. Intraspecific variation in sperm length in
470   two passerine species, the Bluethroat *Luscinia svecica* and the Willow Warbler *Phylloscopus*
471   *trochilus. Orn. Fenn.* **84:** 131–139.
472 **Lauron, E.J., Loiseau, C., Bowie, R.C.K., Spicer, G.S., Smith, T.B., Melo, M. & Sehgal, R.N.M.** 2015.
473   Coevolutionary patterns and diversification of avian malaria parasites in African sunbirds
474   (Family Nectariniidae). *Parasitology* **142**: 635–647.
475 **Lerner, H.R.L., Meyer, M., James, H. F., Hofreiter, M. & Fleischer, R. C. 2011.** Multilocus resolution
476   of phylogeny and timescale in the extant adaptive radiation of Hawaiian honeycreepers. *Curr.*
477   *Biol*. **21**: 1838–1844.
478 **Lessells, C.M. & Boag, P.T.** 1987. Unrepeatable repeatabilities: a common mistake. *Auk* **104:** 116–
479   121.
480 **Lifjeld, J.T., Laskemoen, T., Kleven, O., Albrecht, T. & Robertson, R.J.** 2010. Sperm length variation
481   as a predictor of extrapair paternity in passerine birds. *PLoS ONE* **5**: e13456.
482 **Lüpold, S., Calhim, S., Immler, S. & Birkhead, T.R.** 2009. Sperm morphology and sperm velocity in
483   passerine birds. *Proc. R. Soc. Lond. B* **276:** 1175–1181.
484 **Lüpold, S., Manier, M.K., Berben, K.S., Smith, K.J., Daley, B.D., Buckley, S.H., Belote, J.M. & Pitnick,**
485   **S.** 2012. How multivariate ejaculate traits determine competitive fertilization success in
486   *Drosophila melanogaster*. *Curr. Biol.* **22:** 1667–1672.
487 **Miller, G.T. & Pitnick, S.** 2002. Sperm-female coevolution in *Drosophila*. *Science* **298:** 1230–1233.
488 **Macedo, R.H., Karubian, J. & Webster, M.S.** 2008. Extrapair paternity and sexual selection in socially
489   monogamous birds: are tropical birds different? *Auk* **125:** 769–777.

490 **Morrow, E.H. & Gage, M.J.G.** 2000. The evolution of sperm length in moths. *Proc. R. Soc. Lond. B*
491   **267:** 307–313.
492 **Omotoriogun, T.C., Albrecht, T., Hořák, D., Laskemoen, T., Ottosson, U., Rowe, M., Sedláček, O. &**
493   **Lifjeld, J.T.** 2016. Sperm size evolution in African greenbuls (Passeriformes: Pycnonotidae).
494   *Biol. J. Linn. Soc.* **xx:** xxx–xxx. *(DOI: 10.1111/bij.12663)*
495 **Orme, C.D.L., Freckleton, R.P., Thomas, G.H., Petzoldt, T., Fritz, S.A., Isaac, N.J.B. & Pearse, W.** 2012
496   caper: comparative analyses of phylogenetics and evolution in R. *Methods Ecol. Evol.* **3:** 145–
497   151.
498 **Pagel, M.** 1999. Inferring the historical patterns of biological evolution. *Nature* **401:** 877–884.
499 **Parker, G.A.** 1970. Sperm competition and its evolutionary consequenses in the insects. *Biol. Rev.* **45:**
500   525–567.
501 **Parker, G.A.** 1993. Sperm competition games: sperm size and number under adult control. *Proc. R.*
502   *Soc. Lond. B* **253:** 245–254.
503 **Parker, G.A.** 1998. Sperm competititon and the evolution of ejaculates: towards a theory based. In
504   Birkhead, T.R. & Møller, A.P. (eds) *Sperm Competition and Sexual Selection*: 3–54. Academic
505   Press.
506 **Pitnick, S., Hosken, D.J. & Birkhead, T.R.** 2009. Sperm morphological diversity. In Birkhead, T.R.,
507   Hosken, D.J. & Pitnick, S. (eds) *Sperm Biology, an Evolutionary Perspective*: 69–149. San
508   Diego, CA: Academic Press.
509 **Pizzari, T. & Parker, G.A.** 2009. Sperm competition and sperm phenotype. In Birkhead, T.R., Hosken,
510   D.J. & Pitnick, S. (eds) *Sperm Biology, an Evolutionary Perspective*: 205–245. London: Elsevier.
511 **Ratnasingham, S. & Hebert , P. D. N.** 2007. BOLD: The Barcode of Life Data System
512   (www.barcodinglife.org). *Molecular Ecology Notes* **7:** 355–364.
513 **R Development Core Team.** 2013. *R: A Language and Environment for Statistical computing,* Vienna:
514   R Foundation for Statistical Computing. Available at: www.R-project.org.**Rambaut, A.,**
515   **Suchard, M.A., Xie, D. & Drummond, A.J.** 2014. Tracer v1.6, Available
516   at: http://beast.bio.ed.ac.uk/Tracer.
517 **Rambaut, A. & Drummond, A.J.** 2009. "TreeAnnotator v1. 6.2: MCMC output analysis.
518 **Reddy, S.** 2014. What's missing from avian global diversification analyses? *Mol. Phyl. Evol.* **77:** 159–
519   165.
520 **Revell, L.J.** 2012. Phytools: phylogenetic tools for comparative biology (and other things). *Methods*
521   *Ecol. Evol.* **3:** 217–223.
522 **Revell, L. J.** 2013. Two new graphical methods for mapping trait evolution on phylogenies. *Methods*
523   *Ecol. Evol.* **4:** 754–759.
524 **Riegert, J., Antczak, M., Fainová, D. & Blažková, P.** 2014. Group display in the socially monogamous
525   Northern Double-collared Sunbird *Cinnyris reichenowi*. *Behavioural Processes* **103:** 138–144.
526 **Rowe, M., Albrecht, T., Cramer, E.R.A., Johnsen, A., Laskemoen, T., Weir, J.T. & Lifjeld, J.T.** 2015.
527   Postcopulatory sexual selection is associated with accelerated evolution of sperm
528   morphology. *Evolution* **69:** 1044–1052.
529 **Rowe, M., Laskemoen, T., Johnsen, A. & Lifjeld, J.T.** 2013. Evolution of sperm structure and
530   energetics in passerine birds. *Proc. R. Soc. Lond. B* **280 (1753):** 20122616.
531 **Snook, R.R.** 2005. Sperm in competition: not playing by the numbers. *Trends Ecol. Evol.* **20:** 46–53.
532 **Sokal, R.R. & Rohlf, F.J.** 1995. *Biometry: the Principles and Practice of Statistics in Biological*
533   *Research*. New York , NY: Freeman.
534 **Stamatakis A.** 2014 RAxML version 8: a tool for phylogenetic analysis and post-analysis of large
535   phylogenies. *Bioinformatics*. **30:** 1312–1313.
536 **Stutchbury, B.J.M. & Morton, E.S.** 2001. *Behavioural Ecology of Tropical Birds*. San Diego, California:
537   Academic Press.

538 **Stutchbury, B.J.M., Morton, E.S. & Piper, W.H.** 1998. Extra-pair mating system of a synchronously
539   breeding tropical songbird. *J. Avian Biol.* **29:** 72–78.
540 **Stutchbury, B. & Morton, E.** 2008. Recent advances in the behavioral ecology of tropical birds. *The*
541   *Wilson Journal of Ornithology* **120:** 26–37.
542 **Tourmente, M., Gomendio, M. & Roldan, E.** 2011. Sperm competition and the evolution of sperm
543   design in mammals. *BMC Evolutionary Biology* **11**: 12.
544 **Warton, D.I. & Hui, F.K.C.** 2011. The arcsine is asinine: the analysis of proportions in ecology. *Ecology*
545   **92:** 3–10.
546 **Westneat, D.F. & Sherman, P.W.** 1997. Density and extra-pair fertilizations in birds: a comparative
547   analysis. *Behav. Evol. Sociobiol.* **41:** 205–215.
548 **Wolfson, A.** 1952. The cloacal protuberance - a means for determining breeding condition in live
549   male passerines. *Bird-Banding* **23:** 159–165.
550 **Zilberman, R., Moav, B. & Yom-Tov, Y.** 1999. Extra-pair paternity in the socially monogamous
551   Orange-tufted Sunbirds *Nectarinia osea osea*. *Israel Journal of Zoology* **45:** 407–421.

SUPPORTING INFORMATION

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

Appendix S1. Detail of DNA extraction, PCR, sequencing 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 name 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 $\lambda = 1$).

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

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* ($n = 16$)	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* ($n = 2$)	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		$F_{11, 177} = 23.13$ $P < 0.0001$	$F_{11, 177} = 741.16$ $P < 0.0001$	$F_{11, 177} = 959.18$ $P < 0.0001$	$F_{11, 177} = 903.33$ $P < 0.0001$	$F_{11,177} = 2.92$ $P = 0.002$	

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

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

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

Sperm traits	Blomberg's K	
	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

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 (boldface 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 versus 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 and r = change in rate of trait evolution through time in the Early Burst model.

Models	Parameters	Length of sperm traits			
		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	0.06924	0.0742	0.0765
	α	20.978	< 0.0001	< 0.0001	< 0.0001
	σ^2	0.1275	0.00530	0.00335	0.00239
Early Burst	ΔAICc	3.9285	1.63741	2.423338	2.798855
	AICc weight	0.0647	0.2177	0.1575	0.1345
	r	0.00	-0.6980	-0.50322	-0.42942
	σ^2	0.00057	0.07819	0.025182	0.01401

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 $\lambda = 1$ and $\lambda = 0$, with superscripts following the λ values indicating the probability (P) of likelihood-ratio of sperm trait (first position: against $\lambda = 0$; second position: against $\lambda = 1$).

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	$0^{1.00;\ 0.35}$

FIGURE LEGENDS

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

Figure 2. Reconstruction of sperm length in colour gradients 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). The length of the legend also provides 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 the Supplementary 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. For statistical tests, see Table 4.
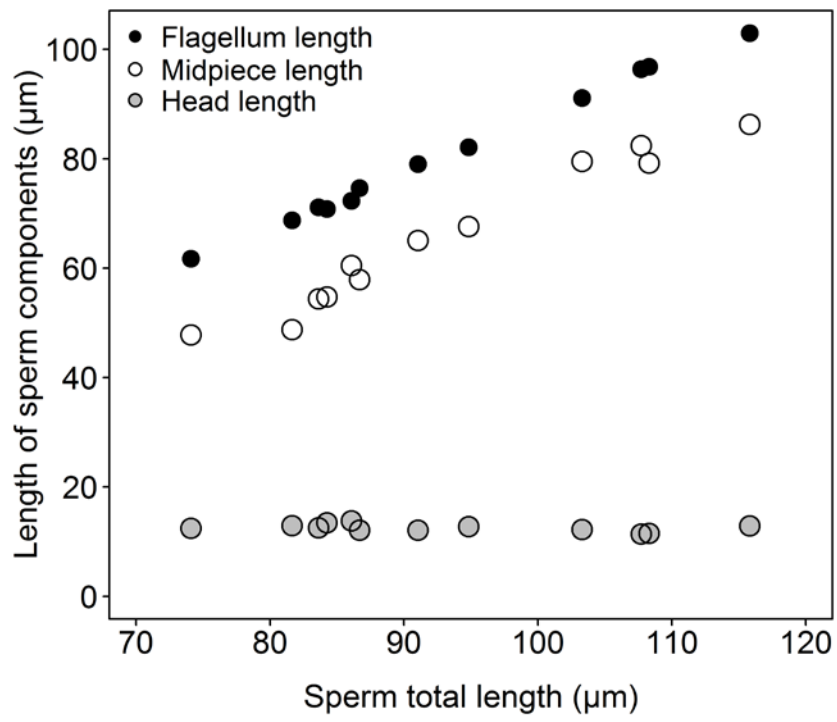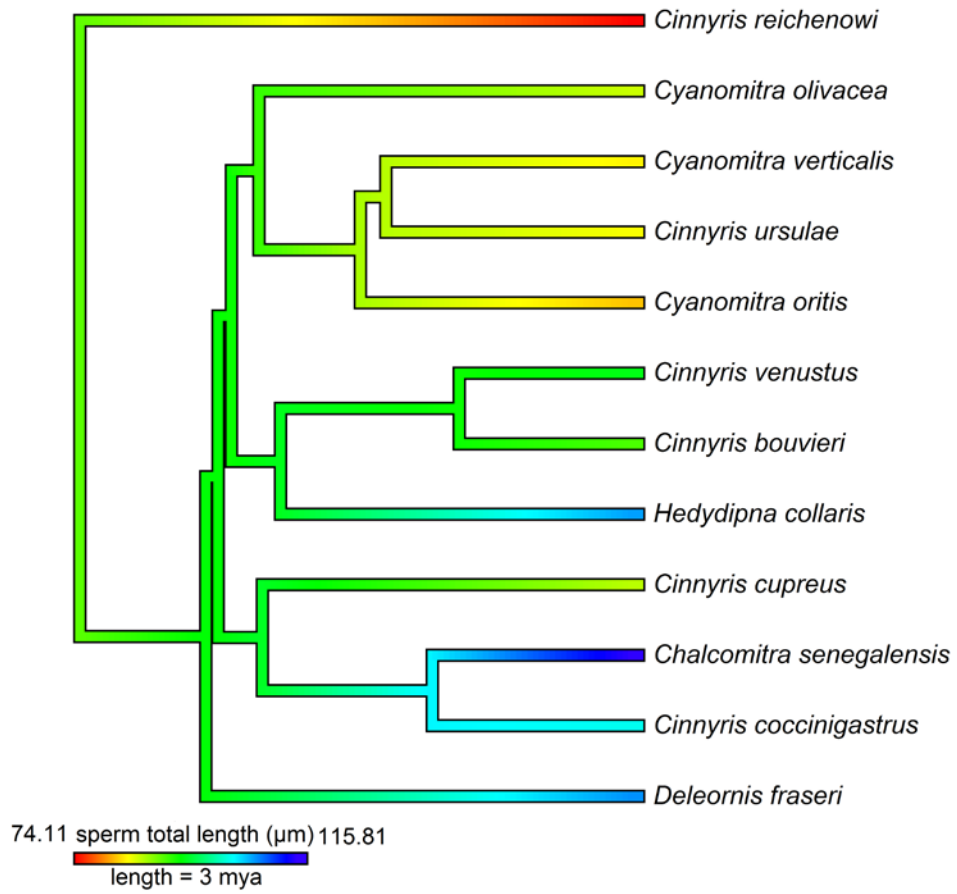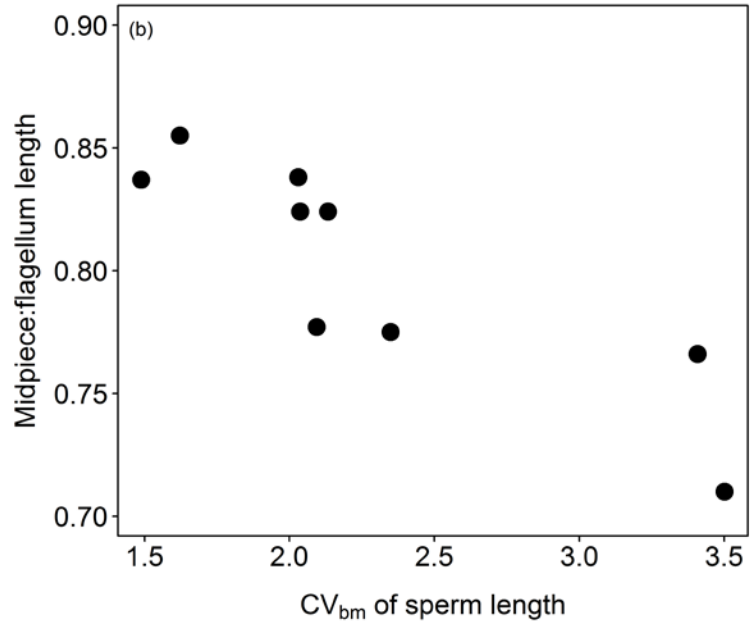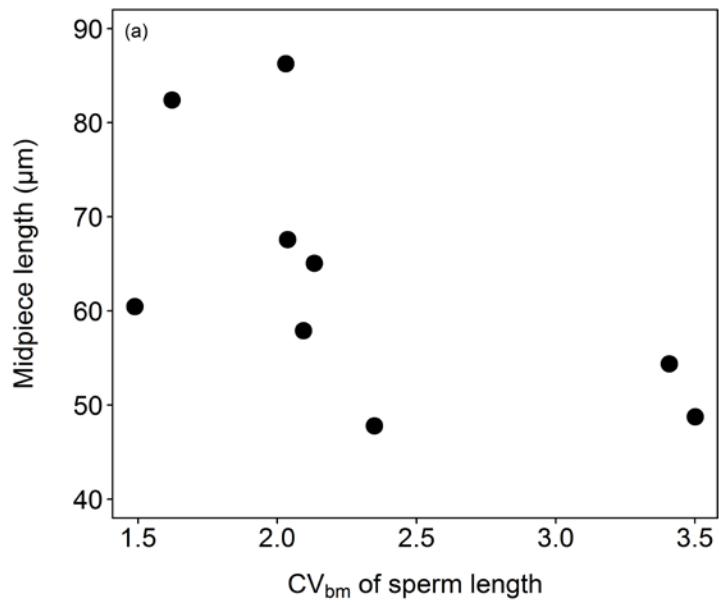
Figure 1

Figure 2

Figure 3