The Linnean Society of London BIOLOGICAL Journal of the Linnean Society

Biological Journal of the Linnean Society, 2015, ••, ••–••. With 3 figures.

Sperm size evolution in African greenbuls (Passeriformes: Pycnonotidae)

TAIWO C. OMOTORIOGUN[1,2]*, TOMAS ALBRECHT[3,4], DAVID HOŘÁK[5], TERJE LASKEMOEN[1], ULF OTTOSSON[2], MELISSAH ROWE[1,6], ONDŘEJ SEDLÁČEK[5] and JAN T. LIFJELD[1]

[1]*Natural History Museum, University of Oslo, Oslo, Norway*
[2]*AP Leventis Ornithological Research Institute, University of Jos, Jos, Nigeria*
[3]*Institute of Vertebrate Biology, Academy of Sciences of the Czech Republic, Brno, Czech Republic*
[4]*Department of Zoology, Faculty of Science, Charles University in Prague, Prague, Czech Republic*
[5]*Department of Ecology, Faculty of Science, Charles University in Prague, Prague, Czech Republic*
[6]*Centre for Ecological and Evolutionary Synthesis, Department of Biosciences, University of Oslo, Oslo, Norway*

Received 18 May 2015; revised 15 July 2015; accepted for publication 16 July 2015

Sperm morphology is highly diversified across the animal kingdom and recent comparative evidence from passerine birds suggests that postcopulatory sexual selection is a significant driver of sperm evolution. In the present study, we describe sperm size variation among 20 species of African greenbuls and one bulbul (Passeriformes: Pycnonotidae) and analyze the evolutionary differentiation of sperm size within a phylogenetic framework. We found significant interspecific variation in sperm size; with some genera exhibiting relatively long sperm (e.g. *Eurillas*) and others exhibiting short sperm head lengths (e.g. *Phyllastrephus*). However, our results suggest that contemporary levels of sperm competition are unlikely to explain sperm diversification within this clade: the coefficients of inter-male variation (CV_{bm}) in sperm length were generally high, suggesting relatively low and homogeneous rates of extra-pair paternity. Finally, in a comparison of six evolutionary or tree transformation models, we found support for both the Kappa (evolutionary change primarily at nodes) and Lambda (lineage-specific evolutionary rates along branches) models in the evolutionary trajectories of sperm size among species. We therefore conclude that African greenbuls have more variable rates of sperm size evolution than expected from a neutral model of genetic drift. Understanding the evolutionary dynamics of sperm diversification remains a future challenge. © 2015 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2015, **00**, 000–000.

ADDITIONAL KEYWORDS: competition – diversification – evolutionary rate – phylogenetic signal – Pycnonotidae.

INTRODUCTION

Across the animal kingdom, sperm cells are highly diversified in size, shape, and structure (Cohen, 1977; Pitnick, Hosken & Birkhead, 2009). There is a strong phylogenetic signal in this diversity, such that sperm traits can be informative in systematics and taxonomy (Jamieson, Ausio & Justin, 1995). Nevertheless, it remains unclear why sperm cells have diversified to such a great extent given their common function of locating and fertilizing ova. It is presumed that this diversity either reflects the outcome of genetic drift over evolutionary time scales or is driven by selection. Sperm must perform in an environment that can exert various selection pressures on them. For birds, which are internal fertilizers, this environment is the female oviduct. Here, sperm need to cross various biochemical, physiological, morphological, and behavioural barriers to their successful insemination, storage, migration, and eventually fertilization of the egg (Birkhead, Møller & Sutherland, 1993; Pitnick *et al.*, 2009). These challenges presented by the female reproductive tract can vary across species, as can the level of sperm

*Corresponding author. E-mail: t.c.omotoriogun@nhm.uio.no

competition. Sperm competition arises when sperm from two or more males compete for fertilization of the same ova (Parker, 1970). Differences in sperm competitiveness among males can therefore create the opportunity for postcopulatory sexual selection, which may lead to evolutionary changes in sperm traits. Moreover, there is a theoretical possibility for female mate preferences in postcopulatory sexual selection (i.e. cryptic female choice; Eberhard, 1996; Snook, 2005). One possible way to look for signatures of selection is to perform comparative analyses of sperm differentiation within a phylogenetic framework. If sperm evolve purely by random drift (Brownian motion; BM), divergences between taxa or lineages are expected to be proportional to the phylogenetic distance between them (Pagel, 1997; Blomberg, Garland & Ives, 2003). Deviations from such a covariance pattern might suggest variable rates of evolutionary change, either among lineages or for different time periods in the evolutionary history of a group. In the present study, we apply this approach to the study of sperm length evolution in a group of passerine birds with a well-resolved, time-calibrated phylogeny.

The order Passeriformes is the largest avian order, encompassing a majority of all extant species (Gill & Donsker, 2015). Passerine birds have a unique sperm morphology characterized by an enlarged and pointed acrosome on a helically shaped head and an elongated midpiece coiled around the flagellum to form a mitochondrial helix (Humphreys, 1972; Koehler, 1995; Jamieson, 2006). Flagellum length appears considerably more variable among passerines relative to any other avian order, and especially so within the Passerida parvorder (*sensu* Sibley & Ahlquist, 1990) of oscine songbirds (Jamieson, 2006). Here, members of each of the three larger superfamilies, Sylvioidea, Muscicapoidea and Passeroidea, display the maximum range of interspecific sperm length variation known for birds, approximately 40 μm to 300 μm (for lists of species-specific sperm lengths, see Pitnick *et al.*, 2009; Lifjeld *et al.*, 2010; Immler *et al.*, 2011; Immler, Gonzalez-Voyer & Birkhead, 2012). Passerines also appear to have higher levels of sperm competition relative to the other avian orders, although there is still considerable variation among species (Westneat & Sherman, 1997; Griffith, Owens & Thuman, 2002). Recent comparative studies have revealed three general patterns that link sperm length variation to the level of sperm competition in passerines.

First, there is general trend that longer sperm have evolved in taxa with high sperm competition (Briskie, Montgomerie & Birkhead, 1997; Kleven *et al.*, 2009; Immler *et al.*, 2011). A similar pattern is observed for other animal groups, including insects (Morrow & Gage, 2000), fish (Balshine *et al.*, 2001), and mammals (Gomendio & Roldan, 1991; Tourmente, Gomendio & Roldan, 2011; but see also Gage & Freckleton, 2003). In birds, however, the relationship does not appear to be linear and there are many species with high sperm competition that exhibit relatively short sperm (Immler & Birkhead, 2007; Immler *et al.*, 2011). Second, pairs of closely-related species with high sperm competition have more divergent sperm lengths than those with low sperm competition (Rowe *et al.*, 2015). This indicates that the rate of evolutionary change in sperm length is higher in species with more sperm competition, and also suggests that changes may go in either direction and not always towards longer sperm. Finally, there is a strong negative association between the level of sperm competition and the variation in sperm length among males in a population (Calhim, Immler & Birkhead, 2007; Kleven *et al.*, 2008; Lifjeld *et al.*, 2010; Laskemoen *et al.*, 2013). This is consistent with a model of stabilizing selection where males with sperm sizes around the population mean are predicted to be more successful in sperm competition. Thus, sperm competition appears to be a strong force of stabilizing selection, which, over evolutionary time scales, causes rapid evolution and diversification in sperm length. Stabilizing selection causing trait divergence may appear paradoxical, although this is not the case. Stabilizing selection with a moving adaptive peak is a well-recognized process of evolutionary change (Estes & Arnold, 2007).

In the present study, we analyzed variation in sperm length among 20 species of African greenbuls and one species of bulbul, all belonging to the Pycnonotidae family, which is part of the Sylvioidea clade, with larks, swallows, and several families of warblers and babblers as their closest relatives (Fregin *et al.*, 2012). The Pycnonotidae consists of two major clades: the African greenbul radiation and the Asian bulbul radiation (Pasquet *et al.*, 2001; Moyle & Marks, 2006). The African bulbuls (*Pycnonotus*) belong to the Asian radiation and have more recently colonized Africa. The African greenbuls consist of approximately 60 species from 13 genera (Gill & Donsker, 2015). The phylogeny of the group is now well resolved and the revised classification reflects monophyletic genera (Johansson *et al.*, 2007; Jetz *et al.*, 2012). Our study species represent six genera of greenbuls from Western Africa, for which there is almost no information available concerning sperm morphology; as indeed is the case for most African birds.

The main aim of the present study was to examine how sperm size has diversified over the evolutionary history of our study species and to test how well various evolutionary models might explain the contemporary interspecific variation in sperm total length

and length of sperm components (i.e. head, midpiece, and flagellum lengths). We mapped species' sperm lengths onto an ultrametric tree constructed from the most comprehensive multilocus phylogenies available (Jetz *et al.*, 2012), supplemented with some of our own sequences of a mitochondrial gene, and tested the fit of a range of evolutionary or tree transformation models. We also quantified intraspecific variation in sperm total length as a proxy for extra-pair paternity, aiming to test for a possible signal of sperm competition in the diversification of sperm size.

MATERIAL AND METHODS

Study Species

African greenbuls are characteristically cryptic, olive–green to brown, medium-sized (approximately 13–26 cm) birds occurring in the understory and canopies of Afrotropical forests. They are largely frugivorous. The sexes show plumage monomorphism, whereas size dimorphism exists in some species and, in these instances, males are larger than females (Keith, Urban & Fry, 1992). The mating system is predominantly monogamy (Fry, Keith & Urban, 2000), with the exception of *Eurillas latirostris*, which has been classified as a lekking species (Brosset, 1982). We collected data from six greenbul genera: *Eurillas* (five species), *Phyllastrephus* (six species), *Criniger* (three species), *Bleda* (three species), *Arizelocichla* (two species), and *Chlorocichla* (one species). These species are mainly distributed in the lowland rainforest, although *Phyllastrephus* and *Arizelocichla* greenbuls occur in montane forests where they apear to have radiated quite recently (Fjeldså *et al.*, 2007). In addition to the 20 species of greenbul, we included one species of bulbul, *Pycnonotus barbatus*. This species is common and widely distributed in various habitats in Africa.

Data Collection and Sampling Procedure

We captured birds using mist-nets during the breeding season in 2010–2013 in Nigeria and Cameroon. Sampling in Nigeria was conducted at a range of sites, including Amurum Forest Reserve, Jos (09°53′ N, 08°59′ E), Omo Forest Reserve, Ogun (06°51′ N, 4°30′ E), International Institute of Tropical Agriculture (IITA), Ibadan (07°30′ N, 03°55′ E), and Okomu National Park, Benin (06°33′ N, 05°26′E). In Cameroon, birds were sampled in the vicinity of Laide Farm, Bamenda-Banso Highlands (06°05′ N, 10°28′ E) and in Mt Cameroon National Park (04°15′ N, 09°09′E).

Sperm samples were obtained by cloacal massage (Wolfson, 1952), whereby the exuded semen of 0.5–3 μL was collected with a 10-μL capillary tube, diluted in a small volume (approximately 20 μL) of phosphate-buffered saline and then fixed in 300 μL of 5% formaldehyde solution for subsequent slide preparation. We also collected a blood sample from the brachial vein for DNA extraction and sequencing of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene as part of an ongoing effort to build a DNA barcode library for West-African birds (Hebert, Ratnasingham & deWaard, 2003). Birds were fitted with uniquely numbered aluminium band (Safring) to prevent resampling of individuals. For all our study species, body mass information was taken from Fry *et al.* (2000).

Sperm Morphology

A small aliquot (approximately 15 μL) from each formaldehyde-fixed sperm sample was applied onto a microscope glass slide and allowed to air-dry. We then gently rinsed slides with distilled water and air-dried them again. Next, high magnification (× 160 or × 320) digital images of sperm cells were taken using a DFC420 camera mounted on a DM6000 B digital light microscope (Leica Microsystems). The LAS, version 2.6.0 R1 (Leica Microsystems) was used to measure (± 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 (sum of midpiece and tail length), sperm total length (sum of head and midpiece and tail length), and the ratios of midpiece : flagellum length, flagellum : head length, and midpiece : sperm total length. We measured 10 morphologically intact spermatozoa for each male (i.e. no head damage or broken tail) in accordance with the recommendations of Laskemoen *et al.* (2007). Sperm measurements were highly repeatable for head, midpiece, and tail (all $r > 80\%$; all $P < 0.001$).

We calculated the coefficient of intra-male (CV_{wm}) and inter-male (CV_{bm}) variation in sperm total length using the formula, CV = (SD/mean) × 100. For the CV_{bm} metric, we corrected for sample size (N) variation using CV_{bm} = (SD/mean) × 100 [1 + (1/4N)], as recommended in Sokal & Rohlf (1995).

Species Phylogeny

The phylogeny for our study species was obtained from BirdTree.org (Jetz *et al.*, 2012), which comprises publicly available molecular sequence data for a wide range of avian species. We downloaded 1000 phylogenetic trees (Hackett backbone) for 18 of our 21 study species and summarized these trees onto a

single maximum clade credibility tree using median node heights at 0.5 posterior probability limits in TREEANNOTATOR, version 1.6.2 (Rambaut & Drummond, 2009). We then manually coded the three remaining species (i.e. those with missing sequence data from Jetz *et al.*, 2012) into the maximum clade credibility tree (i.e. at the middle branch length of their sister taxon) based on literature sources for *Phyllastrephus poliocephalus* (Zuccon & Ericson, 2010) and a mitochondrial gene tree (COI) derived from our study individuals for both *Phyllastrephus baumanni* and *Chlorocichla simplex*.

To obtain this COI tree, we sequenced the first part of the COI gene, between 650 and 750 bp in length corresponding to the standard DNA barcode marker for animals (Hebert *et al.*, 2003; see the Supporting information, Table S1, Appendix S1). Sequences are available in the folder BONGR at the BOLD database (Ratnasingham & Hebert, 2007). We first aligned sequences using CLUSTALW in MEGA, version 6.06 (Tamura *et al.*, 2013) and then applied the Kimura two-parameter model to construct a maximum likelihood tree with branch length at 10 000 bootstrap iterations. Species nomenclature is based on the IOC World Bird List (Gill & Donsker, 2015).

Statistical analysis

All analyses were performed with R, version 2.15.2 (R Development Core Team, 2013). We applied log-transformations to improve distributions for all sperm traits prior to analysis, with the exception of the ratios of sperm midpiece : flagellum length and sperm midpiece : total length, which were logit-transformed in accordance with the recommendations of Warton & Hui (2011). We used analysis of variance to test for differences in sperm traits (i.e. the length of sperm head, midpiece, flagellum, total length, and CV_{wm}) among species, and tested for differences in the CV_{bm} of total sperm length using homogeneity of variance tests (Levene's test).

We performed phylogenetic generalized least-squares (PGLS) regressions to examine associations among sperm traits, and also to test whether sperm size was associated with male body mass. Separate models were run for each sperm trait. The PGLS approach accounts for the statistical non-independence of data points as a result of common ancestry of species (Pagel, 1999; Freckleton, Harvey & Pagel, 2002) and allows the estimation (via maximum likelihood) of the phylogenetic scaling parameter lambda (λ): λ values = 0 indicate phylogenetic independence, whereas values = 1 indicate phylogenetic dependence. We tested the likelihood ratio of λ value against $\lambda = 1$ and $\lambda = 0$. PGLS regressions were performed using the package 'caper' (Orme *et al.*, 2012).

To quantify the phylogenetic signal in sperm traits, we calculated Pagel's λ (Pagel, 1999) and Blomberg's K (Blomberg *et al.*, 2003) using the package 'phytools' (Revell, 2012). Log-likelihood ratio tests were used to determine whether estimated maximum likelihood values for λ differed from 0 (i.e. no phylogenetic signal), whereas, for Blomberg's K, we used the randomization test to determine whether traits exhibited a phylogenetic signal (i.e. $K > 0$). Values of K can exceed 1, in which case they indicate more similarity among related taxa than expected under a BM model of trait evolution. We used these two measures (i.e. Pagel's λ and Blomberg's K) because they are not identical measures of phylogenetic signal; rather, λ measures the strength of the phenotypic–genotypic covariance assuming BM ($\lambda = 1$ equals BM), whereas K reflects the partitioning of trait variance among and within clades: high K implies more variance among clades (i.e. deeper in the phylogeny), whereas low K means more variance among the terminal branches. In addition, we mapped sperm size evolution on the phylogeny using the contMap function in 'phytools' (Revell, 2013). This method allows for the visualization of contemporary trait values, as well as their constructed phenotypic values at internal nodes in the tree. We visualized trait variation for both sperm total length and sperm head length separately because of the different evolutionary trajectories of these traits (Immler *et al.*, 2011; Rowe *et al.*, 2015). Additionally, we visualized ancestral trait values for sperm total length using a traitgram using the function 'phenogram', and then extended this to incorporate uncertainty in the reconstructed ancestral trait values using the function fancyTree in the 'phytools' package (Revell, 2013).

Finally, we used the fitContinuous function in the 'geiger' package (Harmon *et al.*, 2008) to compare the fit of five tree transformation models against a null model of BM (i.e. sperm divergence is perfectly predicted by the phylogenetic distance). The models were (1) Lambda: phenotypic divergence covaries with phylogenetic distance but allows for variable evolutionary rates; (2) Delta: the evolutionary rate accelerates or decelerates over time; (3) Kappa: evolutionary change occurs at speciation events but is not proportional to branch length; (4) Ornstein–Uhlenbeck (OU): a random walk within a constrained trait space, where traits tend to converge towards a single value; and (5) Early Burst (EB): an early burst of trait diversification followed by reduced evolutionary rates (or stasis). These models provide an estimation of the net rate of evolution (σ^2) for the trait in question. For models departing from a simple BM

process, several additional parameters that describe the evolutionary trajectory of a trait are also estimated. The Lambda model estimates the parameter λ, which describes the extent to which phylogeny predicts covariance among trait for species. The Delta model estimates the parameter δ, which compares the contributions of early versus late evolution across a phylogeny; $\delta = 1$ indicates gradual evolution, $0 < \delta < 1$ indicates most trait evolution is near the base of the tree, and $\delta > 1$ indicates most trait evolution occurs near the tips of the tree. The Kappa model estimates the parameter κ, where $\kappa = 1$ indicate gradual evolution across the phylogeny, $\kappa = 0$ implies a punctuated model of evolution with evolutionary change associated with speciation events, $0 < \kappa < 1$ indicates more trait evolution than expected on shorter branches and thus more stasis on longer branches, and $\kappa > 1$ indicates more trait evolution than expected on longer branches. The OU model includes the parameter α, which reflects the evolutionary constraint on trait evolution or the 'attraction' towards a single optimal phenotypic value and, as α approaches 0, the model collapses to a BM model. Finally, in the Early Burst model, the additional parameter is r, which indicates the change in rate of trait evolution through time; when $r = 0$, the model collapses to a pure BM model in which σ^2 is constant.

We compared models 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 weights values to assess model support. Value of $\Delta AICc \leq 2$ indicates substantially supported models, whereas those in which $4 \leq \Delta AICc \leq 7$ indicates less plausible models (Burnham & Anderson, 2004). We analyzed the evolution of head length, midpiece length, flagellum length, and total sperm length separately.

RESULTS

Sperm total length ranged from 70 μm in *P. baumanni* to 117 μm in *Eurillas curvirostris* (Table 1; see also Supporting information, Table S2). All sperm traits showed significant variation among species (Table 1), although values for sperm head length varied within a narrow range (11–16 μm). By contrast, sperm total length was highly variable and most of this variation was explained by the length of the flagellum (Fig. 1,T 1,Table). The coiled midpiece was typically elongated and extended two-thirds or more along the length of the flagellum (Fig. 1).

Sperm head length showed significant negative association with midpiece length ($\beta = -0.03 \pm 0.01$ SE, $t = -5.24$, $P < 0.001$, $\lambda = 1^{0.005;\ 1.00}$) but not with flagellum length ($\beta = 0.02 \pm 0.02$ SE, $t = 1.22$, $P = 0.24$, $\lambda = 0.80^{0.002;\ 0.006}$) among species (see PGLS regression among sperm traits in the Supporting information, Table S3). Sperm total length was not significantly associated with male body mass ($\beta = -0.54 \pm 0.39$ SE, $t = -1.37$, $P = 0.19$, $\lambda = 0.70^{0.05;\ <0.001}$). There was significant heterogeneity of variances among species for sperm midpiece length (Levene's test: $F_{20,145} = 1.80$, $P = 0.03$) but not for flagellum length ($F_{20,145} = 1.08$, $P = 0.38$) or total sperm length (Levene's test: $F_{20,145} = 0.91$, $P = 0.57$). The homogeneity of variances for total sperm length implies that the corresponding coefficients of variation in male sperm lengths (i.e. CV_{bm}) (Table 1) did not vary significantly among species. Because the sperm length CV_{bm} metric is negatively correlated with the rate of extra-pair paternity in passerine birds (Calhim, Immler & Birkhead, 2007; Immler, Calhim & Birkhead, 2008; Kleven *et al.*, 2008; Lifjeld *et al.*, 2010), these results suggest that there is little or no variation among the study species in extra-pair paternity. The mean $\pm$ SD sperm total length CV_{bm} value for the 12 species for which sperm length was measured for >3 males, was 2.82 ± 0.89 (range 1.61–4.23) (Table 1).

Mapping sperm total length onto the phylogenetic tree (Figs 2A, 3), we found that the majority of species ($N = 12$), with representatives from all genera except *Eurillas*, exhibited total sperm length within a relatively narrow range of 79 to 89 μm, which is close to the estimated ancestral value for sperm total length (84 μm) for the group (Fig. 3). The *Eurillas* had consistently longer sperm (103–117 μm) than all other genera. Within this genus, the sister species *Eurillas ansorgei* and *Eurillas gracilis* appear to have diverged fairly rapidly in total sperm length (Fig. 2A, Table 1). The genus *Phyllastrephus* is characterized by a short sperm head (Fig. 2B); values ranged from 11.8 μm to 12.5 μm, which was not overlapping with the other genera (13.4–15.5 μm) (Table 1). In three genera, *Phyllastrephus*, *Criniger*, and *Arizelocichla*, single species have evolved considerably shorter sperm total lengths than their congeners (i.e. approximately 70 μm). Finally, the genus *Criniger* appeared to show rapid divergence in sperm total length (Figs 2A, 3; Table 1), especially in the sister species *Criniger barbatus* and *Criniger chloronotus*. Sample sizes were admittedly quite low but, assuming that their intraspecific variances in sperm length are similar to those of the other greenbuls, the data do suggest this clade may have undergone very rapid sperm evolution.

When we tested for a phylogenetic signal in the sperm sizes, we found an interesting contrast between the results for Pagel's λ and Blomberg's K

Table 1. Sperm morphology of 20 greenbuls and one bulbul species showing the mean ± SD of sperm head, midpiece, flagellum, and total length (μm); included are intra-male coefficient of variation (CV_{wm}) and inter-male coefficient of variation of sperm length (CV_{bm}) and an analysis of variance (ANOVA) test of difference between species

Species	Country	Head length	Midpiece length	Flagellum length	Total length	CV_{wm} (total length)	CV_{bm} (total length)
Phyllastrephus poensis (*N* = 3)	Cameroon	12.50 ± 0.26	58.09 ± 2.08	67.29 ± 1.11	79.79 ± 0.98	2.06 ± 0.17	
Phyllastrephus baumanni (*N* = 2)	Nigeria	11.08 ± 0.44	51.22 ± 1.22	58.63 ± 1.19	69.71 ± 0.74	1.76 ± 0.55	
Phyllastrephus albigularis (*N* = 17)	Nigeria	11.88 ± 0.41	63.66 ± 2.33	73.31 ± 1.99	85.19 ± 2.13	1.98 ± 0.51	2.53
Phyllastrephus xavieri (*N* = 4)	Cameroon	11.82 ± 0.47	66.63 ± 0.97	76.27 ± 3.12	88.09 ± 3.51	1.75 ± 0.42	4.23
Phyllastrephus icterinus (*N* = 5)	Nigeria	12.03 ± 0.63	65.65 ± 2.47	76.67 ± 0.97	88.70 ± 1.36	2.04 ± 0.46	1.61
Phyllastrephus icterinus (*N* = 4)*	Cameroon	11.88 ± 0.38	64.36 ± 1.82	73.74 ± 1.73	85.62 ± 1.66	2.06 ± 0.36	2.06
Phyllastrephus poliocephalus (*N* = 1)	Cameroon	12.41	60.64	68.62	81.03	1.77	
Criniger calarus (*N* = 3)	Cameroon	14.21 ± 0.75	36.75 ± 7.54	56.18 ± 2.30	70.39 ± 3.00	1.91 ± 0.91	
Criniger calarus (*N* = 1)*	Nigeria	15.33	44.64	56.95	72.28	1.90	
Criniger barbatus (*N* = 1)	Nigeria	14.51	78.59	87.71	102.22	1.96	
Criniger chloronotus (*N* = 1)	Cameroon	15.49	51.82	71.37	86.87	3.24	
Eurillas ansorgei (*N* = 1)	Cameroon	13.40	82.52	94.99	108.39	2.70	
Eurillas gracilis (*N* = 1)	Nigeria	14.65	91.28	101.80	116.45	2.27	
Eurillas curvirostris (*N* = 7)	Nigeria	15.64 ± 1.08	91.07 ± 1.05	101.53 ± 3.02	117.18 ± 3.67	1.75 ± 0.61	3.24
Eurillas curvirostris (*N* = 2)*	Cameroon	15.27	90.64	98.25	113.52	1.40	
Eurillas virens (*N* = 31)	Nigeria	14.69 ± 0.70	79.38 ± 3.36	90.13 ± 3.48	104.81 ± 3.52	1.90 ± 0.64	3.38
Eurillas virens (*N* = 1)*	Cameroon	15.40	75.18	87.99	103.40	2.32	
Eurillas latirostris (*N* = 26)	Nigeria	14.43 ± 0.68	85.90 ± 3.09	95.42 ± 3.47	109.85 ± 3.26	1.76 ± 0.64	3.00
Bleda syndactylus (*N* = 1)	Nigeria	15.17	58.02	66.03	81.21	2.21	
Bleda syndactylus (*N* = 1)*	Cameroon	14.95	59.57	71.14	86.09	3.08	
Bleda canicapillus (*N* = 24)	Nigeria	14.39 ± 0.58	62.47 ± 1.24	71.86 ± 2.32	86.26 ± 2.22	2.01 ± 0.62	2.61
Bleda notatus (*N* = 4)	Cameroon	14.51 ± 1.16	63.61 ± 1.58	72.45 ± 2.30	86.97 ± 2.07	1.70 ± 0.42	2.38
Chlorocichla simplex (*N* = 2)	Nigeria	14.07 ± 0.68	53.07 ± 0.15	64.71 ± 1.69	78.79 ± 2.37	2.26 ± 0.03	
Arizelocichla montana (*N* = 7)	Cameroon	13.73 ± 0.44	61.79 ± 2.49	72.82 ± 1.29	86.55 ± 1.68	2.02 ± 0.66	2.01
Arizelocichla tephrolaema (*N* = 5)	Cameroon	13.54 ± 0.60	45.59 ± 1.46	56.90 ± 2.11	70.45 ± 2.41	3.27 ± 0.89	3.60
Pycnonotus barbatus (*N* = 20)	Nigeria	13.47 ± 0.64	59.82 ± 2.01	70.21 ± 2.88	83.68 ± 3.11	2.37 ± 0.68	3.76
ANOVA		$F_{20,145} = 22.61$ $P < 0.0001$	$F_{20,145} = 194.70$ $P < 0.0001$	$F_{20,145} = 156.40$ $P < 0.0001$	$F_{20,145} = 164.60$ $P < 0.0001$	$F_{20,145} = .08$ $P = 0.007$	

*Sperm morphology were not used in ANOVA and phylogenetic generalized least-squares (PGLS) analysis.

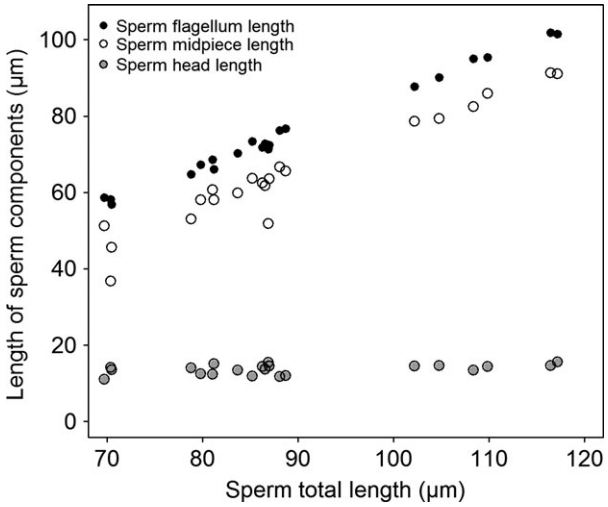

Figure 1. Relationship between sperm total length and sperm head, midpiece, and flagellum length among greenbuls including one bulbul ($N = 21$ species). Each data point represents the species mean for each sperm trait.

(Table 2). Pagel's λ indicated a significant phylogenetic signal for all traits, except midpiece length, which showed no significance ($P = 0.203$). However, all Blomberg's K-values were low and nonsignificant for all traits. Because Blomberg's K is sensitive to variation among terminal branches, the putative rapid divergence between the two *Criniger* sister species may have had a large influence on signal strength in our dataset. When we removed *C. barbatus* from the test, the values for Blomberg's K exceeded 1.3 and revealed a significant phylogenetic signal (likelihood ratio test, all $P < 0.003$) for all sperm component lengths and their ratios.

The tests of five different models for sperm traits' evolution suggest that evolutionary trajectories in sperm total length, flagellum, and head length were often best explained by the Kappa model (ΔAICc = 0) (Table 3), For flagellum length and sperm total length, the Lambda model also had reasonable support (Table 3). For midpiece length, the Lambda model had the best support but the Kappa and OU models also had reasonable support (Table 3). Finally, the evolutionary trajectory of sperm head length was best explained by the Kappa model. The other models (BM, Delta, and EB) assume that the evolutionary rate changes over time within lineages in various ways, and they all received no support for the evolution of sperm traits.

DISCUSSION

In the present study, we analyzed sperm size diversification in a group of endemic African passerines: the greenbuls. Very little information exists on sperm morphology for this group (two species included in Albrecht *et al.*, 2013) (see Supporting information,

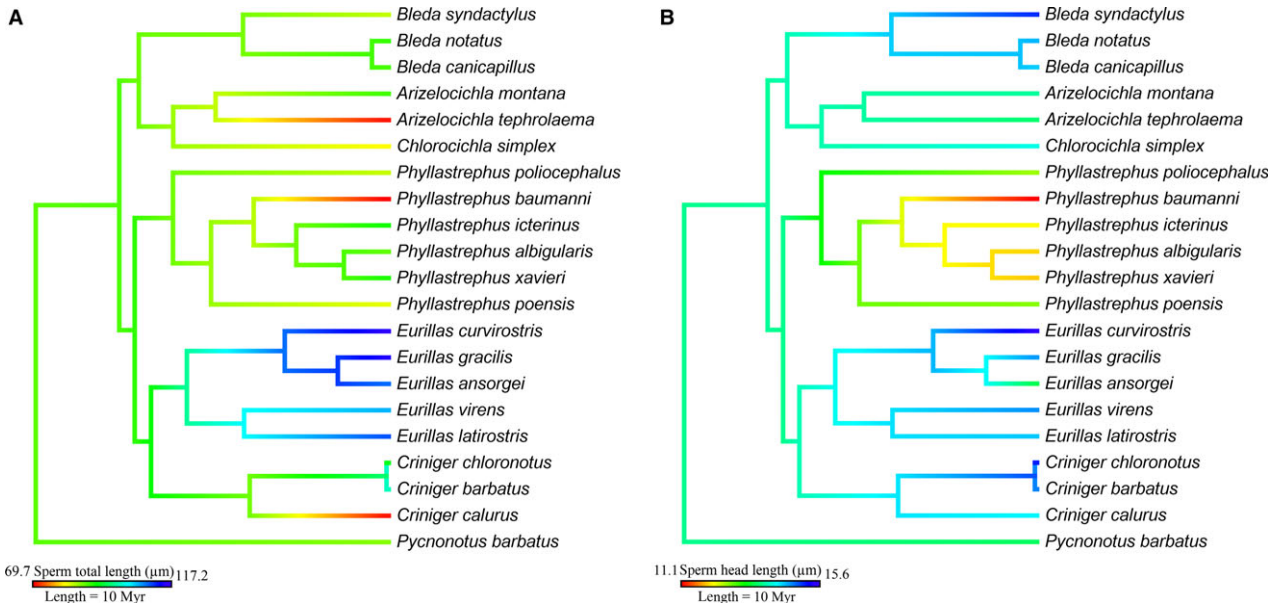

Figure 2. Ancestral character estimation and variation in (A) sperm total length and (B) sperm head length along the branches and nodes of the phylogeny of 20 study species of greenbul and one bulbul. Numbers on the scale bars represent the range of sperm total length and sperm head length, respectively, for the species. The scale bar for colours also indicates the scale for branch lengths in million years (Myr).

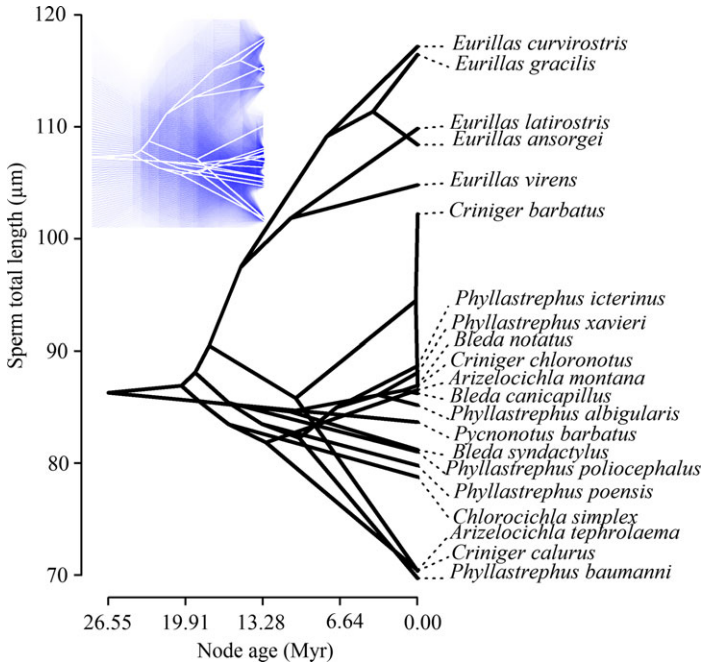

Figure 3. Traitgram showing the projection of the greenbul phylogeny into a space defined by sperm total length (μm) (*y*-axis) and node age [i.e. time since divergence from the root (*x*-axis]. The vertical position of nodes and branches are computed via ancestral character estimation using maximum likelihood. The embedded images indictates uncertainty through increasing transparency of the plotted blue lines around the point estimates, with the entire range showing the 95% confidence interval.

Table 2. Phylogenetic signal in sperm traits among 20 species of greenbul and one bulbul using Pagel's λ and Blomberg's *K* with *P*-values

	Pagel's λ		Blomberg's *K*	
Sperm traits	λ	*P* (likelihood ratio test)	*K*	*P* (randomization)
Head length	0.889	< 0.001	0.464	0.105
Midpiece length	0.588	0.203	0.091	0.769
Flagellum length	0.804	0.017	0.203	0.441
Total length	0.833	0.013	0.246	0.362

Table S1); thus, the descriptive data on sperm morphology reported in the present study contribute to the general knowledge base for the individual species, and also fill a gap in our broader understanding of how sperm morphology varies among clades in the passerine phylogeny. More importantly, through the use of the analysis of evolutionary trajectories of sperm size diversification in a phylogenetic time-calibrated framework, the results indicate lineage-specific rates of sperm evolution in this group. The diversity of sperm sizes among contemporary species therefore appears not only to be a result of neutral evolution by genetic drift, but also suggests a role for selection and constraints. Below, we discuss these perspectives in more detail.

Afrotropical birds are less well studied than birds in other regions of the world, particularly the temperate zones (Macedo, Karubian & Webster, 2008; Reddy, 2014). This general pattern also holds true for descriptive data on sperm morphology. Our study confirms that the African greenbuls exhibit the typical filiform passerine sperm with a corkscrew-shaped head and an elongated midpiece consisting of a mitochondrial helix coiled around most of the flagellum. An extended midpiece along a very long flagellum is typically seen in the Passerida group of the oscine passerines (Jamieson, 2006) to which the greenbuls belong. Within this group, sperm sizes for certain species can reach almost 300 μm. In the Hirundinidae family, which is closely related to the Pycnonotidae (Fregin *et al.*, 2012), sperm lengths can reach up to 240 μm, as exemplified by the tree swallow *Tachycineta bicolor* (Laskemoen *et al.*, 2010; Immler *et al.*, 2011). However, greenbul sperm are much shorter than this and lie within a relatively

Table 3. ΔAkaike information criterion corrected for small sample size (AICc) scores (AICc – AICc score for best-fit model) and AICc weights showing support for evolutionary models of sperm morphometrics in the Pycnonotidae

		Length of sperm traits			
Evolutionary models	Parameters	Head length	Midpiece length	Flagellum length	Total length
Brownian motion	ΔAICc	21.66	39.43	29.23	25.27
	AICc weight	< 0.0001	< 0.0001	< 0.0001	< 0.0001
	σ^2	0.0009	0.0280	0.0075	0.0048
Lambda	ΔAICc	6.45	**0.00***	2.32	2.01
	AICc weight	0.0365	0.4213	0.2357	0.2644
	λ	0.89	0.59	0.80	0.83
	σ^2	0.0003	0.0021	0.0012	0.0010
Delta	ΔAICc	18.08	30.15	21.92	18.68
	AICc weight	0.0001	< 0.0001	< 0.0001	< 0.0001
	δ	2.99	2.99	2.99	2.99
	σ^2	0.0004	0.0102	0.0029	0.0020
Kappa	ΔAICc	**0.00***	0.15	0.00*	0.00*
	AICc weight	0.9631	0.3913	0.7503	0.7231
	κ	0.00	< 0.0001	< 0.0001	< 0.0001
	σ^2	0.0015	0.0199	0.0086	0.0067
Ornstein–Uhlenbeck	ΔAICc	16.35	1.62	7.97	8.13
	AICc weight	0.0003	0.1874	0.0139	0.0124
	α	0.108	62.621	55.947	55.947
	σ^2	0.0015	6.3912	3.3466	2.6561
Early Burst	ΔAICc	24.45	42.22	32.03	28.06
	AICc weight	< 0.0001	< 0.0001	< 0.0001	< 0.0001
	r	0.00	0.00	0.00	0.00
	σ^2	0.0009	0.0280	0.0075	0.0049

For each sperm trait, the model with the lowest AICc value (i.e. ΔAICc = 0) is considered the best-fitting model, as indicated with an asterisk (*). Parameters estimated by the models: 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 phylogeny predicts covariance among trait for species; δ, comparing 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; *r*, change in rate of trait evolution through time in the Early Burst model. Details of model parameters are provided in the Material and methods.

narrow range of 70–120 μm. This is a quite common size range for many Passeridan taxa, including several families within the Sylvioidea superfamily that are closely related to the Pycnonotidae, such as Old World warblers, Sylviid babblers, larks, and long-tailed tits (see sperm lengths for the species listed in Lifjeld *et al.*, 2010; Immler *et al.*, 2011, 2012). Thus, the sperm of African greenbuls are of a size similar to their closest relatives, and they share the general pattern of a significant size variation among species.

Our results also show that the variance in sperm lengths among males in a population is rather homogeneous across species. Because sperm length variance (CV_{bm}) is negatively related to the frequency of extra-pair paternity (Calhim *et al.*, 2007; Lifjeld *et al.*, 2010; Laskemoen *et al.*, 2013), the homogeneous variances suggests that the level of sperm competition is not especially variable among our greenbul species. The mean CV_{bm} value for the group (2.87) gives an estimate of 14% extra-pair young when applying the formula given in Lifjeld *et al.* (2010) (Fig. 2), which is a quite moderate level for passerine birds (Griffith *et al.*, 2002). As far as we are aware, there are no published paternity studies from the Pycnonotidae. The lack of support for interspecific variation in the level of extra-pair paternity suggests there is little to no scope for detecting signatures of sperm competition in the evolution of sperm traits in this group (see PGLS regression between CV_{bm} and sperm traits in the Supporting information, Table S4).

The comparative analyses of sperm diversification within the greenbul phylogeny revealed a clear signature of phylogeny in which the magnitude of divergence between any two lineages is significantly influenced by the time since they split. However, this

pattern was not consistent with a BM model of neutral evolution because lineages did not have a constant rate of sperm evolution. There are several examples of variable divergence rates in the traitgram (Fig. 3), where single species or lineages (e.g. *Eurillas*) rapidly diverge from their relatives. These rapid divergences occurred for some lineages early in the evolutionary history of the group, as shown by the early increase in sperm length for the *Eurillas* greenbuls. Single species within the *Arizelocichla*, *Criniger*, and *Phyllastrephus* diverged from their congeners at the mid-age of the phylogeny and evolved shorter sperm. There is also a striking example of a recent and apparently rapid divergence in sperm length for the closely-related *Criniger barbatus* and *Criniger chloronotus*, which, in some earlier classifications (Howard & Moore, 1991), were considered conspecific subspecies. Taken together, these divergences leave a clear impression that sperm size can evolve fast in some lineages and be rather stable in others at a given point in time in the phylogeny.

We found that evolutionary diversification in sperm size in this group was best supported by the Kappa model, which suggests that most divergence in sperm size occurred shortly after the speciation event (the nodes) and evolution was proportionally faster in shorter branches, and so there was more stasis on longer branches. We also found reasonable support for the Lambda model in the evolutionary trajectories of sperm size. This model allows for variable rates of trait evolution among clades or lineages. A constant rate of evolution among lineages would be identical to the BM model. For the midpiece length, evolutionary trajectories were supported by multiple models: the Lambda, Kappa, and OU models received substantial support (all $\Delta AICc < 2$). Generally, there was no evidence that the diversification in sperm traits occurred predominantly early in the phylogeny (the Early Burst model) and/or that sperm lengths accelerated or decelerated within lineages (Delta model).

Compared to the midpiece, flagellum, and sperm total length where evolutionary trajectories were supported by two or more models, respectively, the evolution of sperm head length was only supported by the Kappa model. Generally, there is a consistent pattern in passerine birds that shows evolution of the sperm head differs fundamentally from the evolution of the flagellum or sperm total length (Immler *et al.*, 2011; Rowe *et al.*, 2015). For example, in a recent comparative analysis, Rowe *et al.* (2015) showed that sperm competition had a significant effect on the divergence rates in sperm total length for 36 pairs of passerine species, whereas sperm competition had no such influence on the divergence in sperm head length. The results also suggested sperm head size is evolutionarily constrained, whereas there was no evidence for such constraints on midpiece, flagellum or total sperm length (Rowe *et al.*, 2015).

The variable rate of sperm size evolution observed among greenbul species poses new questions about the mechanisms behind sperm diversification. The support for the Kappa model of evolution suggests that sperm size evolves particularly fast around speciation or splitting events. This might be explained by sperm divergences being accelerated by postcopulatory sexual selection at the early stages of speciation (e.g. by reinforcement). Our sperm data suggest that greenbuls have a mating system with sperm competition, although at moderate levels for passerine birds. However, sperm competition does not appear to be much variable among species, at least not among our contemporary study populations. Therefore, the variable rates of sperm evolution among lineages can hardly be explained by different levels of sperm competition in these lineages. Thus, we suggest that the variable rates of sperm size evolution must have other explanations than sperm competition *per se* and that determining what these factors might be remains a major challenge for future studies of sperm evolution.

ACKNOWLEDGEMENTS

We thank the Research Council of Norway (project number 196554/V40 to JTL), Norwegian State Educational Loan Fund (PhD scholarship to TCO); Czech Science Foundation (project no. 14-36098G to TA); and International Foundation for Science (grant number TJ/32343 to TCO) for financial support. Field work in Cameroon was covered by research permits issued by the Ministry of Research and Innovations (numbers 2011-000079, 2012-000075/MINRESI/B00/C00/C10/nye) and export permits were issued by the Ministry of Forest and Fauna (numbers 2013-1705, 2014-0104/PRS/MINFOF/SG/DFAP/SDVEF/SC. In Nigeria, the field work was conducted under the scheme of AP Leventis Ornithological Research Institute, University of Jos, which also provided a vehicle for fieldwork. We are also grateful to Deni Bown and Asiedu Robert who arranged for our access to the IITA forest; to Constance Eno Crossby, Taiye Adeyanju Adeniyi, Silje Hogner, and Lars Erik Johannessen for field assistance; and to Gunnhild Marthinsen for help in the laboratory. We thank two anonymous reviewers for their useful comments. This is contribution number 99 from the AP Leventis Ornithological Research Institute.

REFERENCES

Albrecht T, Kleven O, Kreisinger J, Laskemoen T, Omotoriogun TC, Ottosson U, Reif J, Sedláček O, Hořák D, Robertson RJ, Lifjeld JT. 2013. Sperm competition in tropical versus temperate zone birds. *Proceedings of the Royal Society of London Series B, Biological Sciences* **280:** 20122434.

Balshine S, Leach BJ, Neat F, Werner NY, Montgomerie R. 2001. Sperm size of African cichlids in relation to sperm competition. *Behavioral Ecology* **12:** 726–731.

Birkhead TR, Møller AP, Sutherland WJ. 1993. Why do females make it so difficult for males to fertilize their eggs? *Journal of Theoretical Biology* **161:** 51–60.

Blomberg SP, Garland T, Ives AR. 2003. Testing for phylogenetic signal in comparative data: behavioral traits are more labile. *Evolution* **57:** 717–745.

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

Brosset A. 1982. The social life of the African forest yellow-whiskered Greenbul *Andropadus latirostris*. *Zeitschrift für Tierpsychologie* **60:** 239–255.

Burnham KP, Anderson DR. 2004. Multimodel inference: understanding AIC and BIC in model selection. *Sociological Methods and Research* **33:** 261–304.

Calhim S, Immler S, Birkhead TR. 2007. Postcopulatory sexual selection is associated with reduced variation in sperm morphology. *PLoS ONE* **2:** e413.

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

Eberhard WG. 1996. *Female Control: Sexual Selection by Cryptic Female Choice*. Princeton, NJ: Princeton University Press.

Estes S, Arnold SJ. 2007. Resolving the paradox of stasis: models with stabilizing selection explain evolutionary divergence on all timescales. *American Naturalist* **169:** 227–244.

Fjeldså J, Johansson U, Lokugalappatti LGS, Bowie RK. 2007. Diversification of African greenbuls in space and time: linking ecological and historical processes. *Journal of Ornithology* **148:** 359–367.

Freckleton RP, Harvey PH, Pagel M. 2002. Phylogenetic analysis and comparative data: a test and review of evidence. *American Naturalist* **160:** 712–726.

Fregin S, Haase M, Olsson U, Alström P. 2012. New insights into family relationships within the avian superfamily Sylvioidea (Passeriformes) based on seven molecular markers. *BMC Evolutionary Biology* **12:** 157.

Fry CH, Keith S, Urban EK. 2000. *The Birds of Africa*. London: Academic Press.

Gage MJG, Freckleton RP. 2003. Relative testes size and sperm morphometry across mammals: no evidence for an association between sperm competition and sperm length. *Proceedings of the Royal Society of London Series B, Biological Sciences* **270:** 625–632.

Gill F, Donsker D. 2015. *IOC World Bird List version 4.2*. Available at: www.worldbirdnames.org.

Gomendio M, Roldan ERS. 1991. Sperm competition influences sperm size in mammals. *Proceedings of the Royal Society of London Series B, Biological Sciences* **243:** 181–185.

Griffith SC, Owens IPF, Thuman KA. 2002. Extra-pair paternity in birds: a review of interspecific variation and adaptive function. *Molecular Ecology* **11:** 2195–2212.

Harmon LJ, Weir JT, Brock CD, Glor RE, Challenger W. 2008. GEIGER: investigating evolutionary radiations. *Bioinformatics* **24:** 129–131.

Hebert PD, Ratnasingham S, deWaard JR. 2003. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London Series B, Biological Sciences* **270:** S96–S99.

Howard R, Moore A. 1991. *A Complete Checklist of Birds of the World*, 2nd edn. London: Academic Press.

Humphreys PN. 1972. Brief observation on the semen and spermatozoa of certain passerine and non-passerine birds. *Journal of Reproduction and Fertility* **29:** 327–336.

Immler S, Birkhead T. 2007. Sperm competition and sperm midpiece size: no consistent pattern in passerine birds. *Proceedings of the Royal Society of London Series B, Biological Sciences* **274:** 561–568.

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

Immler S, Pitnick S, Parker GA, Durrant KL, Lüpold S, Calhim S, Birkhead TR. 2011. Resolving variation in the reproductive tradeoff between sperm size and number. *Proceedings of the National Academy of Sciences of the United States of America* **108:**5325–5330.

Immler S, Gonzalez-Voyer A, Birkhead TR. 2012. Distinct evolutionary patterns of morphometric sperm traits in passerine birds. *Proceedings of the Royal Society of London Series B, Biological Sciences* **279:** 4174–4182.

Jamieson BGM. 2006. Avian spermatozoa: structure and phylogeny. In: Jamieson BGM, ed. *Reproductive Biology and Phylogeny of Aves*. Enfield, NH: Science Publishers Inc., 249–511.

Jamieson BGM, Ausio J, Justin J-L. 1995. *Advances in Spermatozoal Phylogeny and Taxonomy*. Paris: Museum National d'Histoire Naturelle.

Jetz W, Thomas GH, Joy JB, Hartmann K, Mooers AO. 2012. The global diversity of birds in space and time. *Nature* **491:** 444–448.

Johansson US, Fjeldsa J, Lokugalappatti LGS, Bowie RCK. 2007. A nuclear DNA phylogeny and proposed taxonomic revision of African greenbuls (Aves, Passseriformes, Pycnonotidae). *Zoologica Scripta* **36:**417–427.

Keith S, Urban EK, Fry CH. 1992. *The Birds of Africa*. London: Academic Press.

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

Kleven O, Fossoy F, Laskemoen T, Robertson RJ, Rudolfsen G, Lifjeld JT. 2009. Comparative evidence for the evolution of sperm swimming speed by sperm competition

and female sperm storage duration in passerine birds. *Evolution* **63:** 2466–2473.

Koehler LD. 1995. Diversity of avian spermatozoa ultrastructure with emphasis on the members of ther order Passeriformes. In: Jamieson BGM, Ausio J, Justine J-L, eds. *Advances in Spermatozoal Phylogeny and Taxonomy*. Paris: Muséum National d'Histoire Naturelle, 437–444.

Laskemoen T, Kleven O, Fossøy F, Lifjeld JT. 2007. Intraspecific variation in sperm length in two passerine species, the Bluethroat *Luscinia svecica* and the Willow Warbler *Phylloscopus trochilus*. *Ornis Fennica* **84:** 131–139.

Laskemoen T, Kleven O, Fossøy F, Robertson RJ, Rudolfsen G, Lifjeld JT. 2010. Sperm quantity and quality effects on fertilization success in a highly promiscuous passerine, the tree swallow *Tachycineta bicolor*. *Behavioral Ecology and Sociobiology* **64:** 1473–1483.

Laskemoen T, Albrecht T, Bonisoli-Alquati A, Cepak J, de Lope F, Hermosell I, Johannessen LE, Kleven O, Marzal A, Mousseau TA, Møller AP, Robertson RJ, Rudolfsen G, Saino N, Vortman Y, Lifjeld JT. 2013. Variation in sperm morphometry and sperm competition among barn swallow (*Hirundo rustica*) populations. *Behavioral Ecology and Sociobiology* **67:** 301–309.

Lifjeld JT, Laskemoen T, Kleven O, Albrecht T, Robertson RJ. 2010. Sperm length variation as a predictor of extra-pair paternity in passerine birds. *PLoS ONE* **5:** e13456.

Macedo RH, Karubian J, Webster MS. 2008. Extra-pair paternity and sexual selection in socially monogamous birds: are tropical birds different? *Auk* **125:** 769–777.

Morrow EH, Gage MJG. 2000. The evolution of sperm length in moths. *Proceedings of the Royal Society of London Series B, Biological Sciences* **267:** 307–313.

Moyle RG, Marks BD. 2006. Phylogenetic relationships of the bulbuls (Aves: Pycnonotidae) based on mitochondrial and nuclear DNA sequence data. *Molecular Phylogenetics and Evolution* **40:** 687–695.

Orme CDL, Freckleton RP, Thomas GH, Petzoldt T, Fritz SA, Isaac NJB, Pearse W. 2012. caper: comparative analyses of phylogenetics andevolution in R. *Methods in Ecology and Evolution* **3:** 145–151.

Pagel M. 1997. Infering evolutionary processes from phylogenies. *Zoologica Scripta* **26:** 331–348.

Pagel M. 1999. Inferring the historical patterns of biological evolution. *Nature* **401:** 877–884.

Parker GA. 1970. Sperm competition and its evolutionary consequenses in the insects. *Biological Reviews* **45:** 525–567.

Pasquet E, Han L-X, Khobket O, Cibois A. 2001. Towards a molecular systematics of the genus *Criniger*, and a preliminary phylogeny of the bulbuls (Aves, Passeriformes, Pycnonotidae). *Zoosystema* **23:** 857–863.

Pitnick S, Hosken DJ, Birkhead TR. 2009. Sperm morphological diversity. In: Birkhead TR, Hosken DJ, Pitnick S, eds. *Sperm Biology, An Evolutionary Perspective*. San Diego, CA: Academic Press, 69–149.

R Development Core Team. 2013. *R: a language and environment for statistical computing*. R Foundation for Statistical Computing. Available at: http://www.R-project.org.

Rambaut A, Drummond AJ. 2009. *TreeAnnotator, Version 1.5.4*. Available at: http://beast.bio.ed.ac.uk/TreeAnnotator

Ratnasingham S, Hebert PDN. 2007. BOLD: the Barcode of Life Data System. Availaible at: www.barcodinglife.org. *Molecular Ecology Notes* **7:** 355–364.

Reddy S. 2014. What's missing from avian global diversification analyses? *Molecular Phylogenetics and Evolution* **77:** 159–165.

Revell LJ. 2012. Phytools: phylogenetic tools for comparative biology (and other things). *Methods in Ecology and Evolution* **3:** 217–223.

Revell LJ. 2013. Two new graphical methods for mapping trait evolution on phylogenies. *Methods in Ecology and Evolution* **4:** 754–759.

Rowe M, Albrecht T, Cramer ERA, Johnsen A, Laskemoen T, Weir JT, Lifjeld JT. 2015. Postcopulatory sexual selection is associated with accelerated evolution of sperm morphology. *Evolution* **69:** 1044–1052.

Sibley CG, Ahlquist JE. 1990. *Phylogeny and Classification of Birds. A Study in Molecular Evolution*. New Haven, CT: Yale University Press.

Snook RR. 2005. Sperm in competition: not playing by the numbers. *Trends in Ecology and Evolution* **20:** 46–53.

Sokal RR, Rohlf FJ. 1995. *Biometry: the principles and practice of statistics in biological research*. New York, NY: Freeman.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* **30:** 2725–2729.

Tourmente M, Gomendio M, Roldan ERS. 2011. Sperm competition and the evolution of sperm design in mammals. *BMC Evolutionary Biology* **11:** 12.

Warton DI, Hui FKC. 2011. The arcsine is asinine: the analysis of proportions in ecology. *Ecology* **92:** 3–10.

Westneat DF, Sherman PW. 1997. Density and extra-pair fertilizations in birds: a comparative analysis. *Behavioral Ecology and Sociobiology* **41:** 205–215.

Wolfson A. 1952. The cloacal protuberance – a means for determining breeding condition in live malepasserines. *Bird-Banding* **23:** 159–165.

Zuccon D, Ericson PGP. 2010. The phylogenetic position of the Black-collared Bulbul *Neolestes torquatus*. *Ibis* **152:** 386–392.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Appendix S1. DNA extraction, cytochrome *c* oxidase subunit I (COI) gene sequencing, and phylogeny construction of greenbuls.
Figure S1. Maximum likelihood (Kimura two-parameter model, 10 000 bootstrap iterations) tree topology based on mitochondria (cytochrome *c* oxidase subunit I; COI) sequences from 61 individuals of 18 study species of greenbul.
Table S1. Detailed information of the 60 individuals of 21 species of greenbul used for the cytochrome *c* oxidase subunit I (COI) tree.
Table S2. Sperm morphology data for 167 individual males of 21 species of greenbul used in the analysis.
Table S3. Regression analysis controlling for phylogeny (phylogenetic generalized least-squares; PGLS) among sperm traits in 20 greenbuls and one bulbul species.
Table S4. Regression analysis controlling for phylogeny (phylogenetic generalized least-squares; PGLS) between sperm traits and sperm competition (sperm length CV_{bm}) among 10 greenbul and one bulbul species.