

1 EARLY DIVERSIFICATION OF SPERM SIZE IN THE EVOLUTIONARY HISTORY OF  
2 THE OLD WORLD LEAF WARBLERS (PHYLLOSCOPIDAE)

3 Running title: Sperm diversification in leaf warblers.

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26 **Abstract**

27 Sperm morphological traits are highly variable among species and are commonly thought to  
28 evolve by postcopulatory sexual selection. However, little is known about the evolutionary  
29 dynamics of sperm morphology, and whether rates of evolutionary change are variable over time  
30 and among taxonomic groups. Here, we examine sperm morphology from 21 species of Old  
31 World leaf warblers (Phylloscopidae), a group of generally dull, sexually monochromatic birds,  
32 which are known to have high levels of extra-pair paternity. We found that sperm length differs  
33 markedly across species, spanning about 40% of the range observed across a larger selection of  
34 passerine birds. Furthermore, we found strong support for an “early-burst” model of trait  
35 evolution, implying that the majority of divergence in sperm length has occurred early in the  
36 evolutionary history of this clade with subsequent evolutionary stasis. This large early  
37 divergence matches the early divergence reported in ecological traits (i.e. body size and feeding  
38 behaviour). Our findings demonstrate that rates of evolution in sperm morphology can change  
39 over time in passerine taxa, and that evolutionary stasis in sperm traits can occur even in species  
40 exhibiting characteristics consistent with moderate to high levels of sperm competition. It  
41 remains a major challenge to identify the selection mechanisms and possible constraints  
42 responsible for these variable rates of sperm evolution.

43 **Keywords:** breeding synchrony, comparative methods, early burst, *Phylloscopus*, *Seicercus*,  
44 sexual selection, sperm competition, sperm morphology,

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## 47 **Introduction**

48 Sperm morphology shows enormous diversity at all levels of organization (Cohen, 1977; Pitnick  
49 *et al.*, 2009). Sperm traits, both morphological and functional, are thought to be largely shaped  
50 by sperm competition (Birkhead & Pizzari, 2002; Pizzari & Parker, 2009; Fitzpatrick & Lüpold,  
51 2014) as well as co-evolution with female reproductive traits (Miller & Pitnick, 2002; Snook,  
52 2005; Beese *et al.*, 2006). For example, several comparative studies have documented a  
53 relationship between sperm competition risk and sperm length (Gomendio & Roldan, 1991;  
54 Gage, 1994; Balshine *et al.*, 2001; Byrne *et al.*, 2003; Kleven *et al.*, 2009), and an association  
55 between reduced inter- and intra-male variation in sperm morphology and high levels of sperm  
56 competition (Breed *et al.*, 2007; Calhim *et al.*, 2007; Kleven *et al.*, 2008; Lifjeld *et al.*, 2010;  
57 Fitzpatrick & Baer, 2011; Varea-Sánchez *et al.*, 2014). Furthermore, sperm competition may  
58 favour faster swimming sperm (Fitzpatrick *et al.*, 2009; Kleven *et al.*, 2009) and some aspects of  
59 sperm size (i.e. sperm midpiece, flagellum length and flagellum: head length ratio) have been  
60 reported to positively correlate with sperm velocity (Humphries *et al.*, 2008; Lüpold *et al.*, 2009;  
61 Mossman *et al.*, 2009; Gomendio & Roldan, 2008; Fitzpatrick *et al.*, 2010; Malo *et al.*, 2006).  
62 Finally, across passerine bird species, sperm length and the length of female sperm storage  
63 tubules are positively correlated (Briskie *et al.*, 1997).

64 High levels of sperm competition have also been associated with rapid phenotypic  
65 divergence in sperm length (Rowe *et al.*, 2015). Sperm size evolution in birds is likely under  
66 some form of constraint, however, and hypothesized constraints include variation in the  
67 competitive benefits of sperm size trading off with competitive benefits of sperm numbers and  
68 sperm producing tissue (Calhim *et al.*, 2011; Immler *et al.*, 2011), female reproductive tract  
69 environment (Briskie *et al.*, 1997), and maintenance of sperm integrity (Hermosell *et al.*, 2013).

70 Various ecological factors, such as resource availability and breeding density, may also  
71 contribute to constraints on the evolution of sperm length via effects on overall male  
72 reproductive investment and strategies.

73 Phenotypic variation in traits among species reflects both current and past selective  
74 pressures. Recently developed phylogenetic comparative methods allow the investigation of the  
75 tempo and mode of evolution when fossil data are not available (Nee *et al.*, 1992; Butler & King,  
76 2004; Harmon *et al.*, 2010). These methods require information on trait values across  
77 contemporary taxa and a time-calibrated phylogenetic tree, and permit the evaluation of how  
78 traits have diversified over evolutionary time (Butler & King, 2004; Harmon *et al.*, 2010). The  
79 basic Brownian motion (BM) process models gradual accumulation of divergence over time in a  
80 stochastic manner, at a constant evolutionary rate (Felsenstein, 1988). Additional models include  
81 extra parameters; for example, trait evolution may be constrained such that traits evolve  
82 according to an Ornstein-Uhlenbeck (OU) process that incorporates a constant pull towards an  
83 optimum value (Hansen, 1997; Butler & King, 2004), or rates of evolution may accelerate or  
84 decelerate over time (early burst [EB] model, Harmon *et al.*, 2010).

85 In passerine birds, total sperm length varies considerably, ranging from approximately 40  
86  $\mu\text{m}$  to 290  $\mu\text{m}$ , and sperm morphological traits have been shown to have a high phylogenetic  
87 signal (Lifjeld *et al.*, 2010; Immler *et al.*, 2012). Strong phylogenetic signal is consistent with a  
88 Brownian Motion model of evolution but the few studies directly assessing this have found  
89 equivocal support for the BM model of sperm length evolution. Rowe *et al.* (2013) found  
90 evidence for gradual but directional evolution (i.e. a directional random walk model) in both total  
91 sperm length and length of the sperm midpiece in a study of 23 passerine species. Using data  
92 from 217 passerine birds, Immler *et al.* (2012) found significant departure from BM models but

93 no support for an Early Burst model for all three sperm traits examined (sperm head, midpiece  
94 and flagellum length). Finally, current evidence suggests that sperm head length follows a  
95 different evolutionary trajectory compared to midpiece and flagellum length and is best  
96 explained by an OU model of constrained evolution towards an optimal phenotype in head size  
97 (Immler *et al.*, 2012; Rowe *et al.*, 2015). Evolutionary diversification, however, may be  
98 idiosyncratic and group specific. Thus studies investigating the dynamics of sperm evolution  
99 may benefit from both large taxonomically broad approaches and smaller, clade-specific  
100 approaches.

101         The Old World leaf warblers (family Phylloscopidae) are a group of small insectivorous  
102 birds currently classified into two genera: *Phylloscopus* and *Seicercus* (Martens, 2010). These  
103 warblers breed throughout temperate and sub-tropical regions of continental Eurasia, with some  
104 species extending to parts of Africa and south-east Asian islands (Alström *et al.*, 2006), and often  
105 reach high abundances (Price & Jamdar, 1990; Ghosh-Harihar & Price, 2014). Although species  
106 vary extensively in song (Martens, 2010; Tietze *et al.*, 2015) they are socially monogamous and  
107 have sexually monomorphic, dull-coloured plumage (Price & Pavelka, 1996), characteristics that  
108 have often been considered to indicate low levels of sexual selection (Dunn *et al.*, 2001; Seddon  
109 *et al.*, 2013) and low rates of extra-pair paternity (Moller & Birkhead, 1994; Owens & Hartley,  
110 1998). Data on extra-pair paternity rates, however, imply that males in at least some of these  
111 species experience strong levels of sperm competition; based on seven study populations from  
112 four species, extra-pair paternity rate averages 35% (Table 1).

113         In this study, our primary goal was to examine the temporal dynamics of sperm evolution  
114 in the Old World leaf warblers, given the apparent high extra-pair paternity rates in some  
115 species. We compare the fit of various evolutionary models (BM, OU, EB) to sperm

116 morphological data from 21 species of these warblers, and show that large differences in sperm  
117 morphology arose early in the evolutionary history of this clade.

## 118 **Methods**

### 119 **General methods and sperm morphology**

120 We collected sperm samples from 21 species of Old World leaf warblers across multiple sites in  
121 India and Europe. Fieldwork was carried out in India by KS and TDP, and in Europe by TL, MR  
122 and JTL to coincide with the breeding season (i.e. April – June; see supplement S1 for details of  
123 sites and dates of sperm sample collection). We captured adult males using mist nets, both with  
124 and without song playback, and collected sperm samples via cloacal massage (Wolfson, 1952;  
125 Rowe & Pruett-Jones, 2011). Immediately after collection, sperm samples were mixed in a small  
126 amount (*c.* 20  $\mu$ L) of Phosphate Buffered Saline (PBS) solution and then fixed in 300  $\mu$ L of 5%  
127 Formaldehyde solution for later analysis. All sperm samples are vouchered in the bird collection  
128 at Natural History Museum, University of Oslo (<http://nhmo-birds.collectionexplorer.org/>) or at  
129 the Wildlife Institute of India.

130 We prepared slides for microscopy using 15  $\mu$ L of the fixed sperm sample. We then  
131 examined the slides using a light microscope and captured digital images of sperm at 160, 320 or  
132 400  $\times$  magnification based on the length of sperm, i.e. we used higher magnification for smaller  
133 sperm to accurately identify transitions between sperm components. We measured (to the nearest  
134 0.1  $\mu$ m) the following sperm traits: (1) head length, (2) midpiece length, (3) flagellum length and  
135 (4) total sperm length using digital image analysis software Leica IM50, Leica IM1000 or Leica  
136 Application Suite (see Laskemoen et al. (2013) for details of slide preparation and sperm  
137 measurement). We measured ten morphologically normal sperm for each male, from 1-42 males  
138 per species (see supplement S2). For each sperm trait, we used the means within individuals and

139 then calculated the mean for each species (see supplements S2 & S3). We noticed an unusual  
140 difference in sperm measurements between the two individual *Phylloscopus reguloides*, which  
141 were sourced from different locations (see supplement S1 & S3) representing distinct subspecies  
142 (Ali & Ripley, 1973). Therefore, instead of using the average values from these two males, we  
143 selected data from one individual for our analyses. We repeated the main analyses using data  
144 from the other individual, and found the results were similar (data not shown).

145 European samples were measured by TL at the Natural History Museum in Norway  
146 (NHMO) and KS measured the Indian samples at the Wildlife Institute of India. To standardize  
147 across observers, KS visited Norway prior to the work in India and measured a subset of  
148 previously measured sperm samples from a range of passerine species including fairy wrens,  
149 chaffinches and willow warblers. There was no bias in mean and variance of sperm  
150 measurements carried out by KS compared to the measurements carried out at NHMO  
151 (independent samples t-test for total sperm length measurements: mean (NHMO) = 73.50  $\mu\text{m}$ ;  
152 mean (KS) = 73.00  $\mu\text{m}$ ,  $t_{97,97} = 0.5758$ ,  $p = 0.566$ ,  $n = 50$  sperm; Fisher's test for variance in total  
153 sperm length measurements:  $F = 1.04$ ,  $p = 0.9$ , 95% CI = 0.6 -1.8,  $n = 50$ ). To assess inter-male  
154 variation in sperm length, we calculated the coefficient of variation in total sperm length (here  
155 termed CV<sub>am</sub>, previously referred to as CV<sub>bm</sub> in literature) among males for the 15 species  
156 from which we had sperm measurements from three or more males. Following Lifjeld *et al.*  
157 (2010), we corrected for bias in CV<sub>am</sub> due to small sample sizes using the formula  
158  $CV_{am} = SD/Mean \times 100 \times (1 + 1/4n)$ , where  $n$  is the sample size. We collected data on species body  
159 mass from the literature (Price *et al.*, 1997, 2014; Carrascal *et al.*, 2008). Data on mid-latitude of  
160 breeding range was taken from Price (2010) or calculated from maps available at [birdlife.org](http://birdlife.org).  
161 Finally, we collected data on combined testes mass (i.e. the sum of the left and right testis mass)

162 from published literature (Dunn *et al.*, 2001; Calhim & Birkhead, 2007) and museum collections  
163 (for ten of the 21 species). Data from museum collections were in the form of length and width  
164 dimensions of the testes, which were converted to mass using the formula given in Dunn *et al.*  
165 (2001) (see supplements S4, S5 & S6).

## 166 **Phylogeny**

167 A phylogeny for the 21 species in our study, plus one outgroup (*Horornis diphone*, Cettiidae),  
168 was generated by D. Hooper (pers. comm.) (the nexus file of the phylogeny is attached as  
169 supplement S10) based on two mitochondrial genes and three nuclear genes downloaded from  
170 GenBank (Supplement S7; note, not all sequences were available for all species). We used  
171 BEAST v1.82 (Drummond *et al.*, 2012) to produce a time-calibrated tree using a secondary  
172 calibration for the root node derived from the 95% HPD confidence in the split between  
173 *Phylloscopidae* and *Cettiidae* (uniform distribution 14.5-17.93Ma) (Price *et al.*, 2014). The  
174 analysis was run for 50 million generations and was sampled every 5000 generations giving a  
175 total of 10,000 trees of which the first 10% were discarded as burnin (the XML file used as input  
176 in BEAST is attached as supplement S11). We used the program Tracer v1.6 (Rambaut &  
177 Drummond, 2007) to visualize sampling of the posterior distribution and run parameters in order  
178 to ensure models had reached stable sampling distributions (i.e. all Effective sample size values  
179 > 200). We then generated a maximum clade credibility tree (Supplementary Figure 1) from the  
180 resulting 9,000 trees, using Tree Annotator in BEAST (Drummond *et al.*, 2012). We used this  
181 tree in our analyses of correlated evolution between traits (e.g. in PGLS regressions) and  
182 phylogenetic signal, as well as for visualizing evolutionary change in trait values across the  
183 phylogeny.



184 To account for phylogenetic uncertainty in our comparison of different evolutionary models  
185 we extracted the last 100 trees from the posterior distribution in the BEAST analysis. For each  
186 evolutionary model, we computed the model likelihood on each of the 100 trees; the average  
187 model likelihood over the set of 100 trees accounts for phylogenetic uncertainty (see Price *et al.*,  
188 2014 for further details on both phylogeny construction methods and statistical analyses that  
189 account for phylogenetic uncertainty). The average model likelihoods were then converted into  
190 Akaike Information Criterion corrected for small sample size (AICc) values for statistical  
191 assessment using the formula:  $2k - 2\ln(L) + 2k(k+1)/(n-k-1)$  where  $k$  is the number of parameters  
192 in the model,  $n$  is the sample size and  $\ln(L)$  is the log-likelihood (Burnham & Anderson, 2002)

### 193 **Statistical Analysis**

194 All analyses were performed using R 2.15.0 (R Core Team, 2014). First, we compared CVam  
195 values for the Old World leaf warblers with CVam values for the other passerine species  
196 reported in Albrecht *et al.* (2013) using a two-tailed t-test. We also conducted a phylogenetically-  
197 controlled two-tailed independent samples t-test. For this analysis, we used the tree from Jetz *et*  
198 *al.* (2012) (1000 trees from birdtree.org using the Hackett sequenced species backbone and  
199 compiled into the maximum clade credibility tree using Tree Annotator in BEAST) to extract (1)  
200 a phylogeny for all 126 non-Phylloscopidae species in the Albrecht *et al.* (2013) data set and (2)  
201 a phylogeny for the 15 warbler species in our dataset with CVam data. We estimated 95%  
202 confidence limits on the ancestral state CVam value of the root node for each phylogeny using  
203 the R package APE, assuming a Brownian motion model of evolution (Paradis *et al.*, 2004).  
204 Following Wheatcroft and Price (2014), these confidence limits were manually converted into an  
205 unequal variance two-sample t-test to test for significant differences in ancestral state CVam  
206 between the two groups.

207 We calculated phylogenetic independent contrasts in total sperm length and body mass  
208 using the PIC function in APE (Paradis *et al.*, 2004) using the full 21 species dataset. We  
209 assigned contrasts to nodes on the phylogeny to visually identify those nodes at which changes in  
210 sperm length and body mass have been especially large (Garland, 1992; Richman & Price,  
211 1992). Next, we calculated Blomberg's  $k$  as a measure of phylogenetic lability using the R  
212 package Picante (Kembel *et al.*, 2010). We compared phylogenetic signal for the range of sperm  
213 traits and body mass:  $k > 1$  indicates that traits are more similar between related species than  
214 expected under Brownian motion evolution, whereas  $k < 1$  indicates high lability, at least in the  
215 tips of the tree (Blomberg *et al.*, 2003).

216 We investigated the dynamics of sperm phenotypic evolution in Old World leaf warblers  
217 by comparing the fit of three different evolutionary models to data on sperm length: Brownian  
218 motion (BM), a random-walk model with no constraint on phenotypes (Felsenstein, 1988);  
219 Ornstein-Uhlenbeck with a single-optimum (OU), which is a random walk model within a  
220 constrained trait space whereby trait values have a tendency to return to a central starting value  
221 (Hansen, 1997; Butler & King, 2004); and Early Burst (EB), in which the rate of trait evolution  
222 decreases exponentially through time (Harmon *et al.*, 2010). Models were run for each of the  
223 four sperm morphological traits (head, midpiece, flagellum and total sperm length), as well as for  
224 body mass. We compared models using the AICc values calculated from the 100 tree analysis;  
225 the model with the lowest AICc indicates the best fit, while  $\Delta AICc > 2$  indicates less plausible  
226 models (Burnham & Anderson, 2002). All methods are described in greater detail by Harmon *et*  
227 *al.* (2010), who also show that phylogenies with ten or more taxa are sufficient for these  
228 analyses. To perform the analyses, we used the R package Geiger (Harmon *et al.*, 2008). All trait  
229 values were log-transformed prior to analysis. For total sperm length, we visualized variation in

230 trait values over the evolutionary history of the group by constructing a traitgram using the R  
231 package phytools (Revell, 2012), which uses maximum-likelihood ancestral state reconstruction  
232 assuming Brownian motion (Revell, 2013) and incorporates uncertainty in ancestral state  
233 estimates.

234         To assess the relationship between total sperm length and body mass, we conducted a  
235 phylogenetic generalized least-squares (PGLS) regression using the maximum clade credibility  
236 tree. Additionally, since sperm competition levels have been hypothesized to be generally lower  
237 in tropical birds compared to temperate birds (Stutchbury & Morton, 1995, 2001), we tested for a  
238 relationship between (log) combined testes mass and mid-latitude, while controlling for (log)  
239 body mass by including it as a covariate in the model, using just the ten species for which we had  
240 testes data. We also tested for a relationship between mid-latitude and CVam, as these values  
241 have been used as a measure of sperm competition in previous studies (e.g. Albrecht *et al.*,  
242 2013). To avoid the statistical issues that may arise with the use of a ratio (CVam) (Fitzpatrick &  
243 Baer, 2011), we used multiple regression and included mean (log) sperm length as a covariate in  
244 the model (however, the correlation between sperm length and CVam is low:  $r = -0.04$ ,  $p = 0.89$ ;  
245 controlling for phylogeny:  $r = -0.11$ ,  $p = 0.71$ ). We repeated the multiple regression using  
246 standard deviation of (log) sperm length as the measure of variance instead of CVam and found  
247 similar results (Supplement S8). These analyses were performed using the R packages APE  
248 (Paradis *et al.*, 2004) and nlme (Pinheiro *et al.*, 2014). We repeated all of the analyses outlined  
249 above using raw species values for comparison, because in some models of evolution, raw  
250 species values give more appropriate Type I error rates than phylogenetically corrected  
251 approaches (Price, 1997).

## 252 **Results**

253 Total sperm length differed more than twofold across species of Old World Leaf Warblers (from  
254  $78.06\mu\text{m} \pm 0.46$  (mean  $\pm$  S.E.,  $n=5$ ) in *P. humei* up to  $183.33\mu\text{m} \pm 1.47$ ,  $n=8$  in *P. borealis*).  
255 Similarly, sperm midpiece and flagellum length were highly variable across species. Midpiece  
256 length showed slightly more than threefold variation in size ( $51.32\mu\text{m} \pm 0.65$  ( $n=5$ ) in *P. humei*  
257 to  $164.92\mu\text{m}$  ( $n=1$ ) in *Seicercus whistleri*), and flagellum length showed almost 2.7x variation in  
258 size ( $64.92\mu\text{m} \pm 0.58$  ( $n=5$ ) in *P. humei* to  $172.07\mu\text{m}$  ( $n=1$ ) in *Seicercus whistleri*) (data in  
259 supplement S2).

260 Values of the intraspecific (among-male) coefficient of variation in total sperm length  
261 (CV<sub>am</sub>) for 15 species of Old World leaf warblers for which we had data from three or more  
262 males were all relatively low (Table 2). Moreover, values for these warblers (mean  $\pm$  S.E. =  $1.58$   
263  $\pm 0.51$ , range: 0.43 -2.54) were significantly lower than those observed across 126 other  
264 passerine species listed in Albrecht *et al.* (2013) (mean  $\pm$  S.E. =  $2.73 \pm 0.09$ , range: 0.58 – 5.76;  
265 unequal variance two-tailed t-test:  $t_{30.41} = 7.18$ ,  $p < 0.001$ ; phylogenetic controlled unequal  
266 variance two-tailed t-test:  $t_{149.54} = 2.10$ ,  $p = 0.04$ ). When we applied a stricter criterion for  
267 including warbler species (i.e. only including the 12 species for which we had data from five or  
268 more males) our results were similar, with warblers generally exhibiting lower CV<sub>am</sub> values  
269 relative to other passerines (warbler mean  $\pm$  S.E. =  $1.72 \pm 0.42$ , range: 1.23 -2.54; unequal  
270 variance two-tailed t-test:  $t_{26.46} = -6.66$ ,  $p < 0.001$ ; phylogenetic controlled unequal variance two-  
271 tailed t-test:  $t_{147.26} = 1.74$ ,  $p = 0.08$ ).

272 Phylogenetic independent contrasts revealed four particularly large contrasts in total  
273 sperm length and body mass. Visualization of these contrasts on the phylogeny implied that the  
274 largest evolutionary changes in total sperm length happened early in the clade's radiation (Fig. 1,  
275 see also Figure S2 for a similar pattern in sperm head, midpiece and flagellum length). A strong

276 phylogenetic signal in sperm morphology is also indicated by values of Blomberg's  $k$  exceeding  
277 one and thus indicating related species are more similar than expected under a Brownian motion  
278 model of evolution (Blomberg's  $k$ : head length  $k = 2.47$ , midpiece length  $k = 1.77$ , flagellum  
279 length  $k = 1.82$ ; total length  $k = 1.72$ ; all  $p$  (randomization test)  $< 0.001$ ). The Early Burst model  
280 was the best-fit model for total sperm length and the length of all sperm components (head  
281 length, midpiece length and flagellum length), even when phylogenetic uncertainty was taken  
282 into consideration (Table 3). Visualisation of the evolution of total sperm length on a traitgram  
283 reflects these results by showing a large, early divergence in total length, though interestingly,  
284 some distantly related species exhibit convergence towards a similar sperm size (Fig. 2, see also  
285 Figure S3).

286 As for sperm length, body mass also showed a strong phylogenetic signal (Blomberg's  $k$   
287  $= 1.14$ ,  $p$  (randomization test)  $< 0.001$ ), with large contrasts near the base of the phylogeny (Fig.  
288 1). In the analysis of body mass, the best-fit model was the BM model. However, the EB model  
289 also received considerable support ( $\Delta AICc = 1.5$ ; Table 3) and is thus a plausible model to  
290 describe evolution of body mass in this subset of species. We also found a weak positive trend in  
291 the association between body mass and total sperm length in the Old World leaf warblers ( $n=21$ ,  
292 slope  $\pm$  S.E  $= 0.65 \pm 0.285$ ,  $r = 0.46$ ,  $t_{19} = 2.268$ ,  $p = 0.035$ ; PGLS slope  $\pm$  S.E  $= 0.35 \pm 0.249$ ,  $t_{19}$   
293  $= 1.409$ ,  $p = 0.18$ ; Fig. 3). This is counter to the negative association between mass and sperm  
294 length in the data set of 126 species (excluding Old World leaf warblers) taken from Albrecht *et*  
295 *al.* (2013) (slope  $\pm$  S.E  $= -0.090 \pm 0.08$ ,  $t_{124} = -1.189$ ,  $p = 0.24$ , PGLS slope  $\pm$  S.E  $= -0.063 \pm$   
296  $0.026$ ,  $t_{124} = -2.443$ ,  $p = 0.016$ ; Fig 3).

297 Combined testes mass varied between 0.01-0.21g (mean  $\pm$  S.E.  $= 0.09 \pm 0.02$ ) and relative  
298 testes mass (i.e. combined testes mass as % body mass) ranged from 0.11- 4.12 (mean  $\pm$  S.E.  $=$

299 1.15 ± 0.4) in the subset of ten species for which we had testes mass data. We did not find any  
300 relationship between combined testes mass and mid-latitude (with body mass as a covariate) in  
301 the (partial regression coefficients:  $n=10$ , slope ± S.E. =  $0.049 \pm 0.047$ ,  $t_7 = 1.048$ ,  $p = 0.329$ ,  
302 PGLS slope ± S.E. =  $0.079 \pm 0.07$ ,  $t_7 = 1.124$ ,  $p = 0.298$ ). In contrast, we found a weak positive  
303 association between CVam and mid-latitude, when including mean sperm length as a covariate  
304 (partial regression coefficients:  $n=15$ , slope ± S.E. =  $0.028 \pm 0.01$ ,  $t_{12} = 2.8$ ,  $p = 0.016$ , PGLS  
305 slope ± S.E. =  $0.018 \pm 0.01$ ,  $t_{12} = 1.91$ ,  $p = 0.08$ ). When we included only those species with  
306 sperm data from five or more males, this relationship, while still positive, was not significant  
307 (partial regression coefficients:  $n=12$ , slope ± S.E. =  $0.017 \pm 0.012$ ,  $t_9 = 1.38$ ,  $p = 0.2$ , PGLS  
308 slope ± S.E. =  $0.012 \pm 0.01$ ,  $t_9 = 1.192$ ,  $p = 0.26$ ).

## 309 Discussion

310 Sperm morphology is highly variable across species, and it is widely held that postcopulatory  
311 sexual selection is an important driver of evolutionary change in sperm traits. We found large  
312 differences in sperm morphology among species of Old World leaf warblers. In fact, inter-  
313 specific variation in sperm length among species of Old World leaf warblers spanned more than  
314 40% of the range exhibited in a much larger selection of species across the infraorder Passerida  
315 ( $n = 203$  species in the dataset of Immler *et al.*, 2012), which itself contains about 35% of all bird  
316 species. Similarly, variation in sperm midpiece length in these warblers was considerable and  
317 spanned more than 40% of the full range observed across currently investigated Passerida  
318 species (Immler *et al.*, 2012).

319 We also found that, within species, there was relatively low among-male variability in  
320 total sperm length (CVam) compared to those observed across a sample of 126 other passerine  
321 species which suggests that, within these warbler species, males produce sperm that are highly

322 uniform in length. Interestingly, our finding of generally low CV<sub>am</sub> values in Old World leaf  
323 warblers despite the striking variation among species in total sperm length may indicate an  
324 underlying relationship between sperm size and sperm quality control during spermatogenesis  
325 (Ramm & Schärer, 2014), though further investigation of testes architecture and the kinetics of  
326 sperm production would be necessary to draw any firm conclusions. Nonetheless, regardless of  
327 how variation in sperm length originates, low intraspecific variation in total sperm length among  
328 males has been associated with high rates of female multiple mating (i.e. extra-pair paternity,  
329 polyandry) and/or relatively large testes mass in a range of taxa including birds, mammals and  
330 insects (Calhim *et al.*, 2007; Kleven *et al.*, 2008; Lifjeld *et al.*, 2010; Fitzpatrick & Baer, 2011;  
331 Laskemoen *et al.*, 2013; Varea-Sánchez *et al.*, 2014), and has been widely attributed to  
332 stabilizing selection on sperm morphology imposed by strong sperm competition, but see  
333 Morrow and Gage (2001) for additional explanations.

334         The average relative testes mass (combined testes mass as % body mass) in passerines is  
335  $1.58 \pm 0.11\%$  (Rowe *et al.*, 2015) with a relative testes mass of 1% corresponding to about  
336 17.6% extra-pair young according to a linear model (Pitcher *et al.*, 2005). Species with relative  
337 testes mass greater than 1% have been shown to experience moderate to high levels of sperm  
338 competition. For example, the relative testes mass of the Bluethroat *Luscinia svecica* is 1.02 and  
339 percentage of extra-pair young is 26.3% (Johnsen & Lifjeld, 2003), while the corresponding  
340 figures for the Yellowhammer *Emberiza citrinella* are 1.1 and 37.4% (Sundberg & Dixon, 1996)  
341 (see supplement S9 for data on other species). Five of the ten Phylloscopid species for which we  
342 have testes data have relative testes mass greater than or equal to 1%, ranging from 1- 4.12%  
343 (Supplement S4). These five species, as well as the four species for which we have extra-pair  
344 paternity data, are not restricted to one part of the phylogeny (Table 1, Supplement S4, Figure 1)

345 and can therefore be considered somewhat representative of the Old world leaf warblers more  
346 generally. We acknowledge that testes data, especially those calculated using testes dimensions  
347 from museum data could contain errors and should be interpreted with caution (Calhim &  
348 Birkhead, 2007). However, the relative testes mass values for these warblers together with the  
349 observed high extra-pair paternity rates in those species that have been studied (Table 1) and the  
350 low intraspecific variation in sperm length, imply that many Old World leaf warbler species  
351 exhibit characteristics consistent with moderate to high levels of sperm competition, and thus  
352 suggest that sperm competition is likely to be an important evolutionary force in this group.  
353 Thus, our findings are consistent with previous work suggesting that high sperm competition  
354 drives interspecific divergence in sperm morphology in passerine birds (Rowe *et al.*, 2015).

355 We found support for the Early Burst model of morphological change in sperm length in  
356 the Old World leaf warblers. These results imply that rapid diversification in sperm length  
357 occurred early in the history of this clade and rates then decreased significantly with time (i.e.  
358 rapid evolution followed by relative stasis). These findings contrast with the patterns of sperm  
359 evolution documented in a heterogeneous dataset of 217 passerine species extending further back  
360 in time, in which there was no evidence for EB model of trait evolution (Immler *et al.*, 2012).  
361 Early Bursts in trait evolution have been demonstrated to be statistically difficult to detect (Slater  
362 & Pennell, 2013) implying a strong signal for Early Burst in sperm size evolution in Old World  
363 leaf warblers. Our results contrast with putative patterns of recent rapid evolution in sperm size  
364 in bluethroats *Luscinia svecica* (Hogner *et al.*, 2013) and African blue tits *Cyanistes teneriffae*  
365 (Gohli *et al.*, 2015), as well as, in this study, between two subspecies of *Phylloscopus reguloides*  
366 (based on one individual from each, see Table S1) and *P. maculipennis* and *P. pulcher* (see Fig.  
367 1). Interestingly, we also find some convergence in sperm size between distantly related species



368 among these warblers (Fig. 2), which we suggest may be due to rapid divergence in sperm length  
369 between closely related species under conditions of sperm competition (*sensu* Rowe et al. 2015)  
370 within a finite trait space (i.e. less related species exhibit similar sperm lengths simply as a  
371 consequence of divergence between closely related species given that there is an upper and lower  
372 limit to sperm length). Together, these results imply that the dynamics of sperm size evolution  
373 are variable across taxonomic groups and can include rapid periods of evolution interspersed  
374 with long periods of stasis, even under conditions of sperm competition.

375         Just as sperm length appears to have undergone early rapid diversification, large changes  
376 in body size and feeding method appear to have occurred early in the evolutionary history of the  
377 Old World leaf warblers (Richman & Price, 1992; Price, 2010; Ghosh-Harihar, 2014, see also  
378 Table 3 for support of EB evolution of body mass in this study). Concordant patterns of  
379 evolution between body size and sperm size are reflected in a positive association between body  
380 size and total sperm length in these species, which is not typical of the Passerida as a whole (Fig.  
381 3). We suggest that movement into new ecological niches may have driven rapid divergent  
382 evolution of sperm length mediated by selection under conditions of sperm competition (*sensu*  
383 *lato*). Briskie *et al.* (1997) argued that longer sperm evolve as a correlated response to longer  
384 sperm storage tubules, and that longer sperm storage tubules may make it easier for the female to  
385 control which sperm are used for fertilizations. Thus it is plausible that changes in female body  
386 size placed new selection pressures on sperm traits.

387         In the Phylloscopidae, early divergences in sperm length were generally followed by a  
388 long period of relative stasis in sperm length evolution. One explanation for the slowdown in  
389 trait divergence is based on ecological factors, which may impose constraints on the evolution of  
390 sperm length via effects on overall male reproductive investment and strategies. For example,

391 resource availability and migration schedules can affect strategies of investment into  
392 reproduction versus survival (Tuomi *et al.*, 1983; Reznick, 1985; Parker *et al.*, 2013), with  
393 increased investment in survival constraining further investment in sperm traits, such as sperm  
394 size. A second explanation for recent stasis in sperm length is that investment continues to be  
395 evolutionarily labile, but that lability is reflected in other sperm traits important to male  
396 fertilization success and not sperm length. Perhaps most likely is recent selection for sperm  
397 numbers, which are a major determinant of male fertilization success under a competitive mating  
398 scenario in birds and in a range of other taxa (Martin *et al.*, 1974; Parker, 1982; Boschetto *et al.*,  
399 2011).

400 Our review of extra-pair paternity studies (Table 1), combined with data on relative testes  
401 mass (Supplement S4) and intraspecific variation in sperm length among males (CV<sub>am</sub>) suggests  
402 that moderate to high levels of sperm competition characterize many of the Old World leaf  
403 warblers, despite the striking similarity of species in morphology and plumage. A range of  
404 hypotheses have been proposed to explain interspecific variation in extra-pair paternity rates in  
405 birds (Møller & Ninni, 1998; Griffith *et al.*, 2002; Westneat & Stewart, 2003), and information  
406 from these warblers may help shed light on these alternatives. Two long-standing ecological  
407 hypotheses are that high breeding density and high breeding synchrony promote extra-pair  
408 copulations (Dunn *et al.*, 1994; Westneat & Sherman, 1997; Bennett & Owens, 2002; Stewart *et*  
409 *al.*, 2009). Breeding density has been a relatively good predictor of inter-population variation in  
410 extra-pair paternity within a species, whereas breeding synchrony has been a better predictor in  
411 comparisons among species (Stutchbury & Morton, 1995; Griffith *et al.*, 2002; Mayer &  
412 Pasinelli, 2013). Both breeding density and synchrony may be high in the Old World leaf  
413 warblers. Warbler species are often among the most common birds in the community (Price &

414 Jamdar, 1990; Ghosh-Harihar & Price, 2014). For instance, at some sites in the Western  
415 Himalayas, about 40% of the bird population consists of these warblers and densities of some  
416 species are up to 4 pairs/ha (Price *et al.*, 1990; Price, 1991). There is also some evidence of  
417 relatively high breeding synchrony (Bjørnstad & Lifjeld, 1997; Marchetti, 1998).

418 Breeding density and synchrony have been reported to vary with latitude (MacArthur,  
419 1964; Ricklefs, 1966; Wyndham, 1986; Macedo *et al.*, 2008), and latitude has been used as a  
420 proxy for breeding synchrony, with greater synchrony at higher latitudes. We found a trend  
421 towards higher values of CV<sub>am</sub> in species distributed at higher latitudes. Given the negative  
422 relationship between CV<sub>am</sub> and female multiple mating and/or relative testes size (a common  
423 proxy for the strength of sperm competition in a range of species) observed in a range of taxa  
424 (Calhim *et al.*, 2007; Kleven *et al.*, 2008; Lifjeld *et al.*, 2010; Fitzpatrick & Baer, 2011;  
425 Laskemoen *et al.*, 2013; Varea-Sánchez *et al.*, 2014) these results may offer some insight into the  
426 relationship between latitude and sperm competition risk. Specifically, our results offer  
427 provisional evidence that sperm competition is higher in species closer to the equator, which is  
428 opposite to previous expectations (Stutchbury & Morton, 1995; Spottiswoode & Møller, 2004).  
429 However, we would hesitate to interpret this as higher sperm competition in less synchronously  
430 breeding species, as latitude is correlated with a range of ecological variables including resources  
431 and climate (Martin, 1996; Weatherhead & Yezerinac, 1998). Indeed, most of the species we  
432 studied are migratory (Ali & Ripley, 1973; Katti & Price, 2003): the southern species are  
433 elevational migrants in the Himalayas and may be as equally synchronous as species at higher  
434 latitudes. Regardless of the underlying causes of the relationship between latitude and putative  
435 levels of sperm competition our findings provide an interesting contrast to previous studies,  
436 which have reported a positive association or no relationship between these variables (Stutchbury

437 & Morton, 1995; Pitcher & Stutchbury, 1998; Spottiswoode & Møller, 2004; Macedo *et al.*,  
438 2008; Albrecht *et al.*, 2013).

439         In conclusion, our results implicate postcopulatory sexual selection in driving exceptional  
440 diversity in sperm length among Old World leaf warblers, but show that most of this diversity  
441 accumulated early in the history of the group. Sexual dichromatism is commonly used as an  
442 index of the strength of sexual selection in birds (Owens *et al.*, 1999; Seddon *et al.*, 2013), as  
443 well as a range of other taxa (Misof, 2002; Stuart-Fox & Owens, 2003; Wagner *et al.*, 2012).  
444 This is because comparative studies have revealed correlations between the degree of  
445 dichromatism and other indices of sexual selection such as mating system (Dunn *et al.*, 2001)  
446 and rates of extra-pair paternity (Moller & Birkhead, 1994; Owens & Hartley, 1998). Though  
447 these correlations suggest there should be little precopulatory sexual selection in Old World leaf  
448 warblers, several studies of these warblers have presented evidence for precopulatory sexual  
449 selection through female choice based on territory and song traits of males (Marchetti, 1998; Gil  
450 & Slater, 2000; Gil *et al.*, 2007). Thus, taken together with the current study, it is possible that  
451 sexual selection is acting at both pre and postcopulatory stages in these dull, sexually  
452 monochromatic species, and we have shown here that these warblers differ significantly in a  
453 cryptic, sexually selected trait - sperm length.

454

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749 **Table 1.** Estimated extra-pair paternity rates in Old World leaf warblers.

<b>Species</b>	<b>EPY (%)</b>	<b>Study site</b>	<b>Reference</b>
<i>Phylloscopus trochilus</i>	33.0	Norway	(Bjørnstad & Lifjeld, 1997)
<i>Phylloscopus trochilus</i>	28.0	Sweden	(Fridolfsson <i>et al.</i> , 1997)
<i>Phylloscopus trochilus</i>	23.5	Scotland	(Gil <i>et al.</i> , 2007)
<i>Phylloscopus trochiloides</i>	42.0	India	(Scordato, 2012)
<i>Phylloscopus trochiloides</i>	31.0	Kyrgyzstan	(Scordato, 2012)
<i>Phylloscopus humei</i>	44.0	India	(Scordato <i>et al.</i> , 2012)
<i>Phylloscopus fuscatus</i>	45.0	Russia	(Forstmeier <i>et al.</i> , 2002)

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770 **Table 2.** Adjusted CVam (among-male coefficient of variation in total sperm length) values for  
 771 15 species of Old World leaf warblers; only species with three or more males measured were  
 772 included in calculations of CVam.

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Species	CVam	N
<i>Phylloscopus borealis</i>	2.19	8
<i>Phylloscopus canariensis</i>	1.38	30
<i>Phylloscopus collybita</i>	1.38	15
<i>Phylloscopus ibericus</i>	1.32	15
<i>Phylloscopus trochilus</i>	2.04	42
<i>Phylloscopus sibilatrix</i>	2.54	10
<i>Phylloscopus occipitalis</i>	2.02	20
<i>Phylloscopus humei</i>	1.23	5
<i>Phylloscopus chloronotus</i>	1.77	8
<i>Phylloscopus xanthoschistos</i>	1.7	7
<i>Phylloscopus bonelli</i>	1.25	8
<i>Phylloscopus sindianus</i>	1.77	12
<i>Phylloscopus maculipennis</i>	1.36	3
<i>Phylloscopus pulcher</i>	1.34	4
<i>Seicercus affinis</i>	0.43	3

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**Table 3.**  $\Delta$  AICc scores (AICc – AICc score for best-fit model) and Akaike (AICc) weights showing support for evolutionary models. For each trait, the model with the lowest AICc value (i.e  $\Delta$  AICc = 0) is considered the best-fitting model (bold with \*). The parameters estimated by the models are:  $\sigma^2$  = net rate of trait evolution or the initial rate of evolution in the EB model,  $\alpha$  = evolutionary constraint parameter in the OU model moving trait values back to the optimum, and  $r$  = change in rate of trait evolution through time in the EB model. AICc was computed after first averaging the likelihood over 100 trees sampled from the Bayesian phylogenetic analysis

	Brownian motion (BM)			Ornstein-Uhlenbeck (OU)				Early Burst (EB)			
	$\sigma^2$	$\Delta$ AICc	AICc weight	$\sigma^2$	$\alpha$	$\Delta$ AICc	AICc weight	$\sigma^2$	$r$	$\Delta$ AICc	AICc weight
Sperm head length	0.001	2.280	0.228	0.001	0	5.035	0.058	<b>0.006</b>	<b>-0.233</b>	<b>0*</b>	<b>0.714</b>
Sperm midpiece length	0.005	2.632	0.201	0.005	0	5.379	0.051	<b>0.032</b>	<b>-0.224</b>	<b>0*</b>	<b>0.748</b>
Sperm flagellum length	0.003	3.513	0.142	0.003	0	6.258	0.036	<b>0.027</b>	<b>-0.246</b>	<b>0*</b>	<b>0.822</b>
Total sperm length	0.003	2.964	0.176	0.003	0	5.708	0.048	<b>0.022</b>	<b>-0.241</b>	<b>0*</b>	<b>0.776</b>
Body mass	<b>0.002</b>	<b>0*</b>	<b>0.579</b>	0.002	0	2.745	0.147	0.007	-0.124	1.503	0.273

### Figure legends

**Figure 1** Maximum clade credibility phylogeny of the 21 Old World leaf warblers included in our study, see supplementary Fig.1 for bootstrapped nodal support values on the phylogeny. Most large contrasts (indicated by warmer colours at nodes) in sperm size and body mass are at the base of the phylogeny. Time scale is in millions of years.

**Figure 2** Traitgram of total sperm length in Old World leaf warblers shows large early divergence in sperm size followed by stasis of sperm length divergence between more recent clades at the tips.

**Figure 3** Relationship between sperm size and male body mass. Total sperm length is positively associated to body mass in Old World leaf warblers (red dots), but not in other Passerida (black dots). Both variables were log-transformed before plotting. Ordinary least-square regression lines (solid) and phylogenetic generalized least square regression lines (dotted) shown: red for warblers and black for other Passerida.

### Supplementary documents

**Supplement S1** Sampling dates and locations for Old World leaf warbler males used in the analyses with accession numbers for Natural History Museum Oslo (NHMO).

**Supplement S2** Summary of sperm morphology traits (head length, midpiece length, flagellum length and total length) for 21 species of Old World leaf warblers along with coefficient of variation in total sperm length within and among males, the values for the latter adjusted for small sample sizes are also shown.

**Supplement S3** Sperm morphology measurements (head length, midpiece length, flagellum length and total length) for all the warbler males included in this study.

**Supplement S4** Combined testes mass data along with the sources for the data and calculations of the % body mass made accounted for by testes mass for ten of the Old World leaf warbler species included in this study.

**Supplement S5** Testes dimensions data for seven Old World leaf warbler species from the Field Museum of Natural History and California Academy of Sciences along with the locations and dates of data collection.

**Supplement S6** Calculations of combined testes mass (CTM, sum of left and right testes mass) from the testes dimensions for seven Old World leaf warbler species.

**Supplement S7** Genbank accession numbers for the genes used to reconstruct the Old World leaf warbler phylogeny used in our study.

**Supplement S8** Results of a phylogenetic generalized least squares analysis (PGLS) of standard deviation of (log) total sperm length against mid-latitude and (log) total sperm length, showing results similar to those obtained using CVam as the dependent variable.

**Supplement S9** Data on combined testes mass and extra-pair paternity for passerine bird species other than the Old World leaf warblers.

**Supplement S10** Phylogeny of 21 species of Old World leaf warblers included in our study with one outgroup species in nexus file format.

**Supplement S11** XML file used as input in BEAST to reconstruct the phylogeny used in this study.

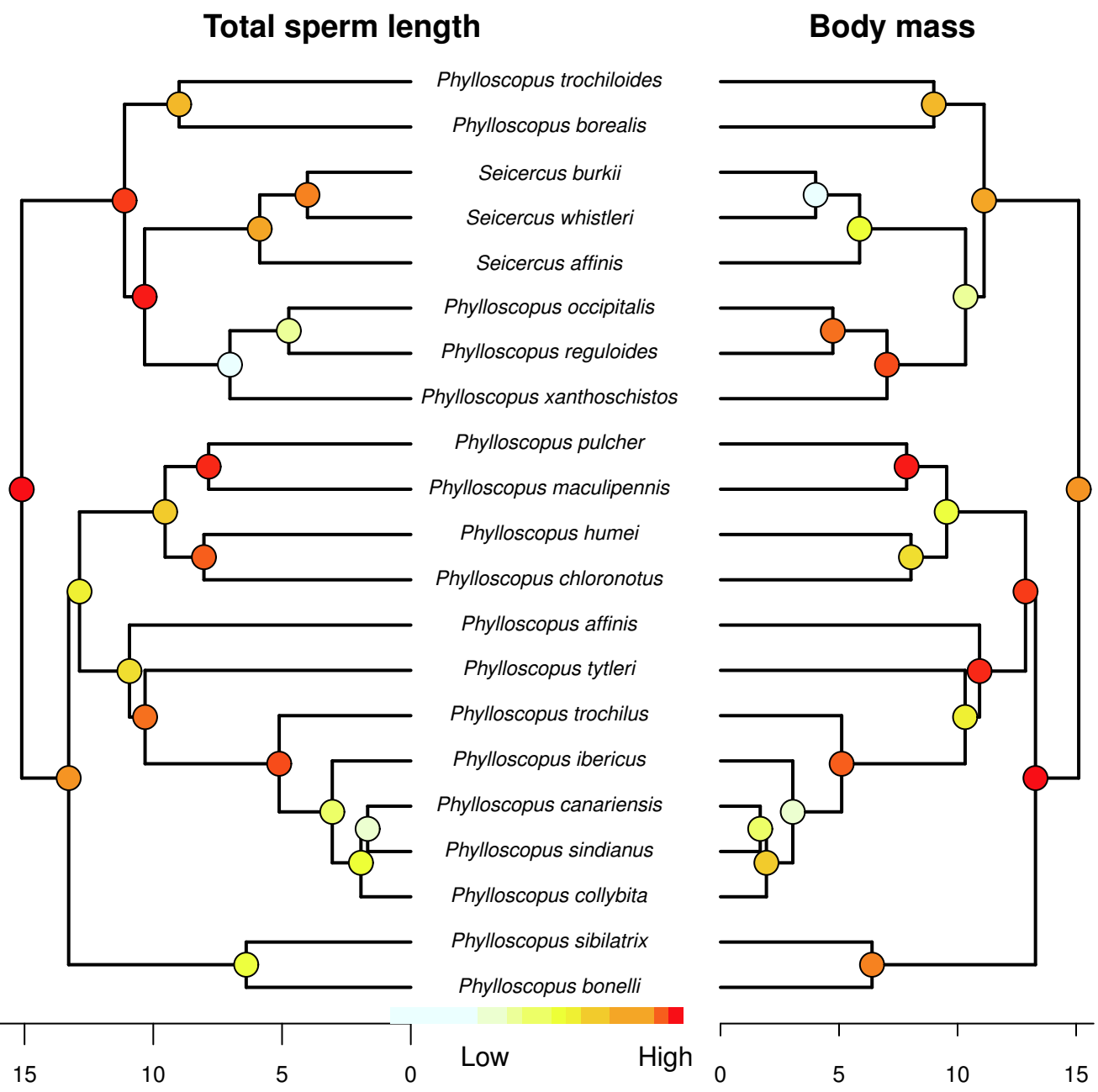
**Supplementary figures:**

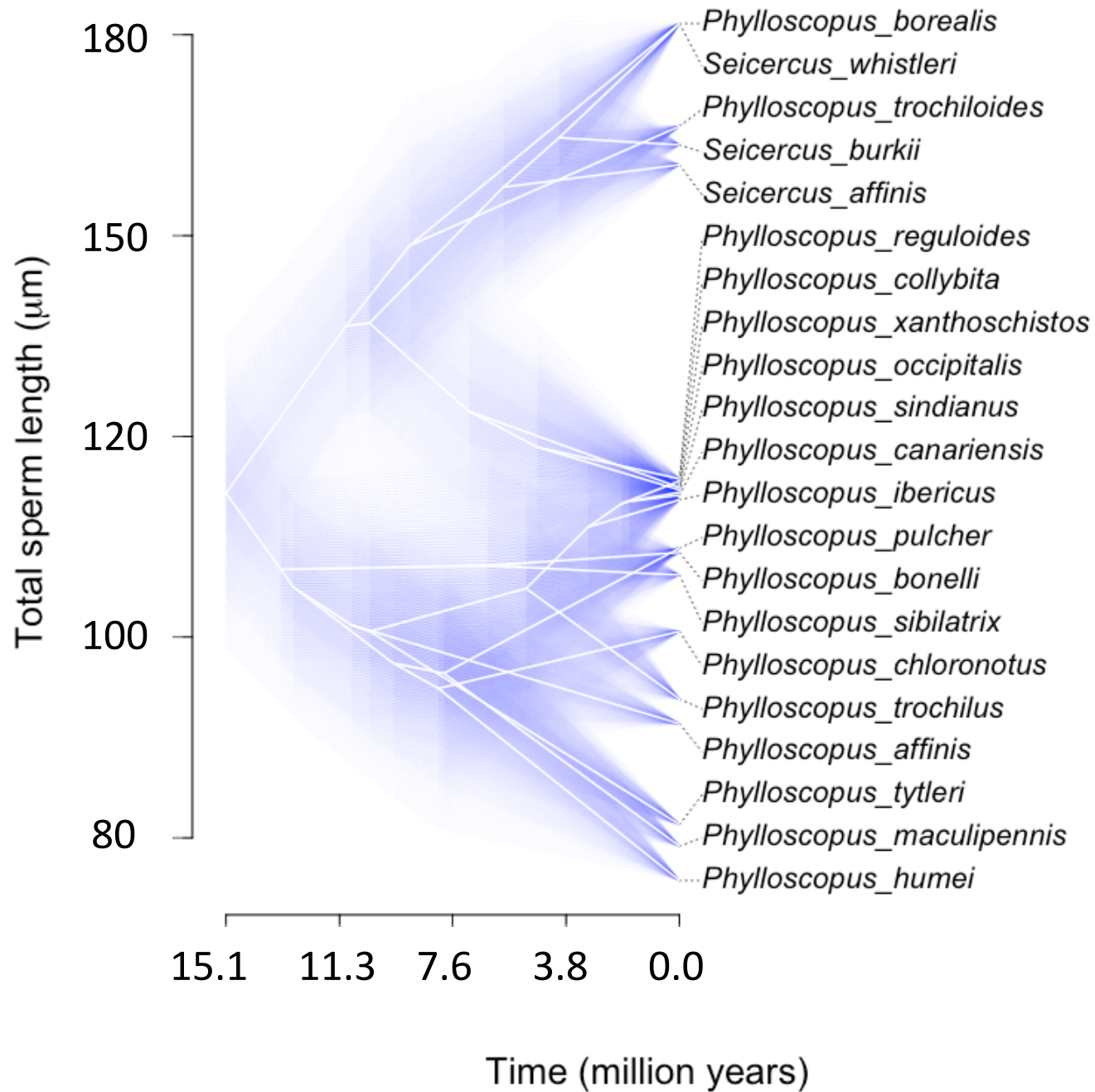
**Figure S1** Maximum likelihood phylogeny of 21 species of Old World leaf warblers included in our study showing the bootstrap support values at each node.

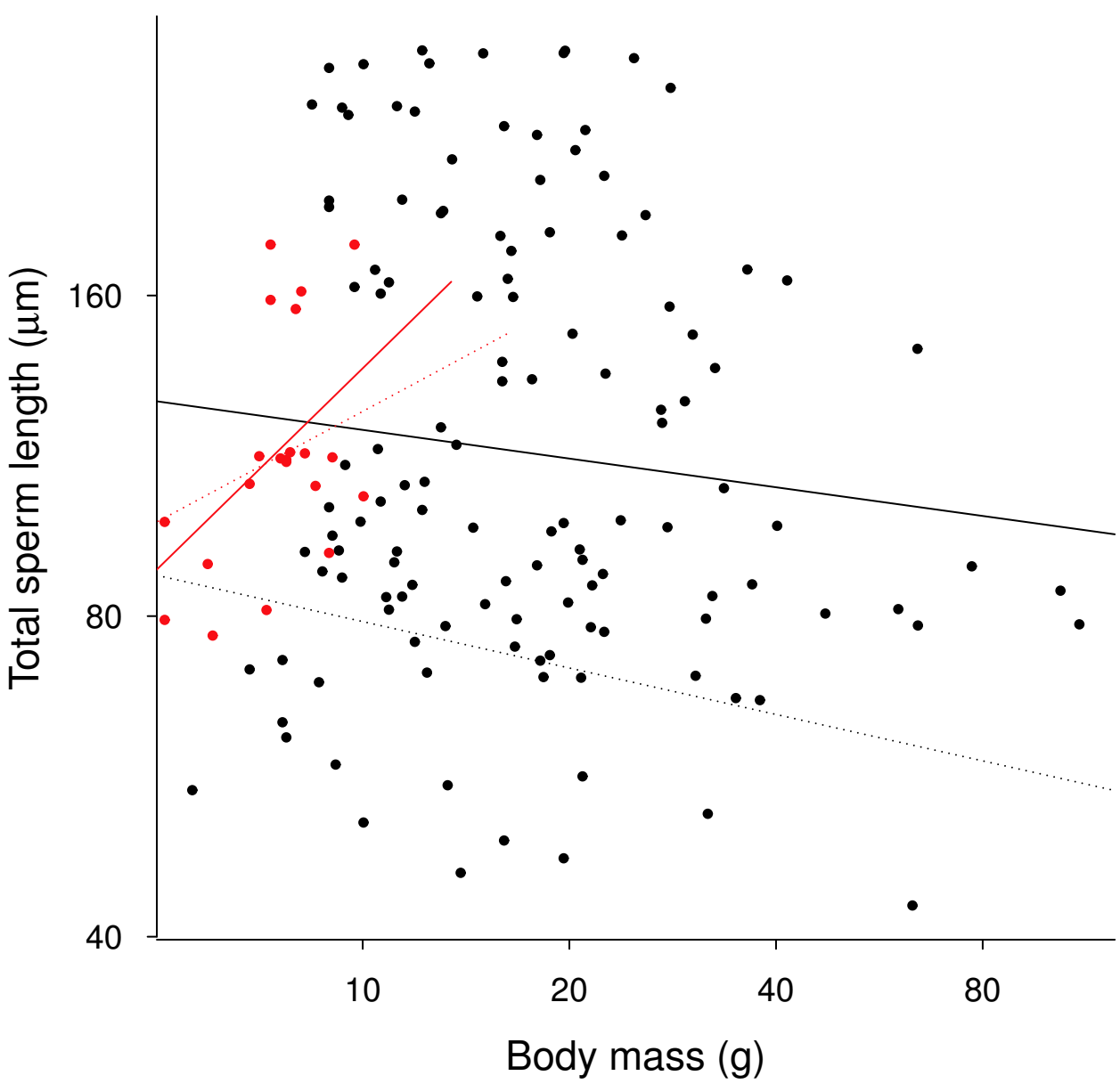
**Figure S2** Larger contrasts (indicated by warmer colours at nodes) in sperm head length, midpiece length and flagellum length are at the base of the phylogeny, smaller contrasts (indicated by cooler colours) are towards the tips of the phylogeny.

**Figure S3** Traitgram of sperm head length, midpiece length and flagellum length in Old World leaf warblers shows large early divergence in sperm size followed by stasis of sperm length divergence between more recent clades at the tips.









Dear Dr. Mank

Thank you for giving us the opportunity to resubmit this manuscript, which we have revised given the helpful comments from you and the reviewer. Below we indicate how we have addressed the reviewer's concerns.

Reviewer comments and our responses (in italics):

**Reviewer 1:**

For one I am not entirely sure, why the authors performed no direct test of an effect of the risk of sperm competition on sperm morphology by running regression analyses. I certainly value the extensive comparative methods applied in this study but the conclusions reached by the authors are a bit farfetched given that the most direct test of this hypothesis that it is the relatively high levels of sperm competition that drive the diversification in sperm morphology is missing.

*The only measure of sperm competition we have for the entire dataset is CVam, thus we are hesitant to make such a test a focus of the manuscript. Nonetheless, we agree that we should include this test of association in the paper, and we have done so. This analysis shows no relationship between sperm length and CVam (lines 243-244). However, we note that our results support the idea that sperm competition drives evolutionary diversification of sperm size in this group of birds, which is not the same thing as sperm competition exerting directional selection on sperm size (i.e. sperm evolving to be longer or shorter), which is what such a regression analysis would test.*

Furthermore, I wonder about the statistical power in this data set. A sample size of 21 species is rather modest for testing different evolutionary models. I therefore wonder how robust the support for significant EB models (and other parameters) is.

*We agree the data set is a little small (although a great deal of work went into collecting it). If sample sizes varied, and low sample size tests were not significant, this would be an issue. However, all models are evaluated on the same data, thus support for one model over the other appears to us to be strong statistical support, and indeed statistical significance with small samples perhaps implies greater biological significance than if sample sizes were larger. Moreover, previous work has shown that using trees with 10 or more species is good for testing evolutionary models, we refer to this in lines 225-227.*

Finally, I appreciate that the authors tested for differences in measurements between two observers. I wonder whether CV could also differ between the observers. It would be good to compare variation in the measurements between the two observers, not only the mean.

*That's a good point. We compared variation in measurements between the two observers and find no significant difference in variance of sperm measurements (lines 152-153).*

Minor comments by line number

114: replace “7” and “4” with “seven” and “four” (use words for numbers from zero to ten)

*Done (line numbers 111 & 112)*

142: “ten” instead of “10”

*Done (line number 137)*

230: “three” instead of “3”

*Done (line number 260)*

236: “five” instead of “5”

*Done (line number 266)*

261: see above – please check the entire ms for similar cases

*We have checked the rest of the manuscript and corrected all instances of similar cases.*

## **Reviewer 2:**

This study explores links between the evolution of sperm morphology and other morphological and ecological traits in a group of passerine birds, with a focus on between-male variation in sperm length traits. Patterns of variation in sperm length traits have recently been used in an increasing number of studies (mostly in birds), and several interesting correlates of this variation have been identified.

While I remain somewhat skeptical about the usefulness of these measures (a sentiment also expressed by the other reviewer), I feel that the publication of additional studies that explore these measures is timely and worthwhile, and will make a valuable contribution to the literature.

However, I would like to see a more careful discussion about the hidden assumptions behind these measures.

- 1) As recently pointed out (Ramm & Schärer 2014, *Biol. Rev.*, p. 881f) these variation measures currently do not explicitly consider what the null hypothesis for the CV should be when sperm length evolves. Does spermatogenesis have a constant error in regulating the rate of sperm length deposition (in which case longer sperm will turn out to be more 'accurate', i.e. have a lower CV), or does the spermatogenesis error scale linearly with sperm length (in which case CV would stay constant; arguably the notion that is tested here)?

*Based on reviewer comments on our previous drafts, we have shifted our focus from between-male variation in sperm length to evolution of sperm length and its relationship with ecological variables, specifically body mass. Consequently, we use CV values in our revised manuscript simply as supportive data to suggest these species experience moderate to high levels of sperm competition, and to discuss how sperm competition may relate to ecological variables such as breeding density and synchrony. Thus we feel that an extended discussion about CV measures, while interesting and timely, is generally outside the scope of the current paper.*

*Nonetheless, the reviewer brings up an interesting topic and we have referred to these ideas in our revised manuscript (lines 321-25). Moreover, we point out in the manuscript that in our dataset, we find no relationship between sperm length and CV<sub>am</sub> (lines 243-244). We have, however, kept this discussion point relatively brief, both because it is not in line with the main focus of our manuscript and because we feel that there are some additional ideas and concepts relevant to this topic that are not presented in Ramm and Schärer and expanding upon these in the current paper is not possible. Specifically, Ramm and Schärer suggest two contrasting scenarios are expected when asking how variation in sperm length is expected to change with mean sperm length. First, when there is no relationship between sperm length mean and variance (i.e. standard deviation), CV values are expected to decrease with increasing sperm length; suggesting sperm production is subject to a constant rate of quality control or a constant accuracy to regulate the rate and/or timing of tissue deposition involved in sperm elongation. Secondly, Ramm and Schärer suggest that when variation scales positively with sperm length, CV values are expected to remain constant; suggesting that sperm production quality control rate is a linear function of sperm size. Ultimately this suggests that longer sperm have reduced capacity for quality control or sperm production error checking (as variance increases with the mean, thus error checking reduces with mean sperm length). Under a positive mean-variance relationship, CV values would indeed remain constant. However, a negative linear relationship would suggest a different scenario, and can also be considered biologically plausible. Specifically, a negative linear relationship between mean and variance in sperm size would result in decrease in CV values with increasing sperm size. Furthermore, this would suggest that the error-checking rate increases with mean sperm length, which seems plausible given selection under conditions of sperm competition for both longer sperm and mechanisms that minimize sperm production errors (see Simmons and Fitzpatrick 2012, Rowe and Pruett-Jones 2011 for support of the idea that both sperm size and mechanisms that minimize sperm production errors are positively associated with sperm competition risk).*

*To discriminate amongst these scenarios it might be possible to look at the relationship between both mean and variance in sperm length (mean~standard deviation) and sperm length and CV<sub>am</sub> values. It is likely that the different scenarios would show different patterns (see Table below). We suggest it could be useful to identify taxonomic groups that show evidence of each of these scenarios and to perhaps investigate sperm production in these varying groups in an attempt to elucidate the origin of variation in sperm morphology within species.*

	<i>Relationship between mean-variance (std dev) of sperm size</i>	<i>Relationship between mean sperm length and CVam</i>	<i>Expectation for quality control in spermatogenesis</i>
i)	<i>No correlation</i>	<i>CV negatively correlated with sperm length</i>	<i>Selection for constant sperm accuracy or quality control</i>
ii)	<i>Positive linear relationship</i>	<i>No correlation</i>	<i>Sperm accuracy varies linearly with sperm size – but a negative relationship. Longer sperm have poorer quality control (and hence increased variance)</i>
iii)	<i>Negative linear relationship</i>	<i>CV negatively correlated with sperm length</i>	<i>Sperm accuracy varies positively with sperm size. Thus selection for both sperm size and greater sperm production accuracy</i>

*i) and ii) taken from Ramm and Schärer. iii) our suggestion*

*Interestingly, our dataset show no correlation between sperm mean and variance (though the slope estimate is negative: -0.32) and no correlation between sperm length and Cvam (though again the parameter estimate for this relationship is negative: -0.04). Thus, with the current data, we would be unable to state much about the origination of sperm variability. We suggest that a considerably larger data set would be necessary for such an analysis. Thus we think a discussion of the assumptions underlying the CV measure and those ideas presented in Ramm and Schärer requires a much more focused and extensive treatment than can be provided in the current manuscript.*

2) Linked to this, researchers often find significant between-male variation in sperm length traits within a species (see e.g. Morrow & Gage 2001, J. Zool.). However, if making an accurate and optimal sperm size is supposedly important, why does selection not remove that between male variation? The data available here should allow to shed some light on these questions.

*In the current paper, we actually find little variation between-males (i.e. CVam), with the majority species exhibiting CVam values < 2.2. Furthermore, we draw upon several recent studies (i.e. post 2001, when the Morrow and Gage paper was published) in a range of taxa (insects, mammals and birds) that suggests between-male variation (i.e. CVam) is in fact reduced under conditions of sperm competition (assessed as high rates of female multiple mating [i.e. extra-pair paternity, polyandry] and/or relatively large testes mass). Thus we think the questions posed by the reviewer have to some degree been*

*addressed in the literature, and thus do not warrant addressing with the current dataset given the relatively low number of species studied and lack of available data on EPP, relative testis mass or some other relevant data such as inbreeding or male condition from most of the species in the current study. Nonetheless, the reviewer's comment does highlight the need to include this citation in our revised text, and we now do so at lines 331-332.*

3) While I see that there is evidence for early divergence in sperm length traits, looking at figures 1 and 2 also suggest that there is considerable divergence late in the phylogeny, and also quite striking patterns convergence (e.g. two groups of three and four relatively distant species end up having near identical total sperm length). The latter is not really discussed at the moment.

*We agree that this is an interesting result and thank the reviewer for highlighting that it would be useful to discuss these results. We have now added some discussion of this in the paper (lines 282-83, lines 366-71). Specifically, we suggest that a possible explanation for this convergence is that high sperm competition is driving closely related taxa apart more rapidly, and thus less related species could become more similar, simply because of the divergence between more closely related species, given that there is perhaps a finite trait space in which selection can push the trait value.*

4) It might be worthwhile to explore more functional and mechanistic explanations for some of the measured traits. For example, given that sperm heads often mainly contain the highly condensed sperm nucleus, could one reasonably expect a correlation between genome size and sperm head size? Please also provide the equivalents of figures 1 and 2 for the other sperm length traits.

*We feel that discussing the functional and mechanistic explanations for the sperm traits is outside the scope of this paper. Moreover, our data are not necessarily suitable for testing the functional and mechanistic explanations of sperm length variation. For example, a correlation between genome size and head size, while very interesting, is not best handled by this dataset for at least two main reasons. First, genome size is not very variable in birds (Tiersch & Wachtel, 1991) and is available for only two *Phylloscopus* species in the Animal genome size database. Secondly, the head is made up of the nucleus and the acrosome, and there appears to be considerable variation in ratio of acrosome length to nucleus length (A:N ratio) and thus the relative proportion of total head length these two traits compromise (Jamieson 2007). For the *Phylloscopus* in this dataset, we do not know the relative lengths of these two components (i.e. acrosome and nucleus), but simply overall head length. Thus, for example, a species with a sperm head length of 12µm may have a nucleus length (which is arguably what you should be correlating genome size with) anywhere between 0.5 and 9 µm. Thus running a correlation just using head length is not a very good test of this idea.*

5) In the discussion it would be interesting to learn if data could be obtained for variation in female reproductive anatomy. Given that this is the environment in which the sperm



are functioning, it would be interesting to get some data on these aspects.

*We wholeheartedly agree with the reviewer that it would be interesting to have information on female reproductive anatomy. However, we simply don't have those data right now (moreover, such data are very hard to come by) and it is outside of the scope of this paper to discuss it. We also think that our results on sperm evolution are interesting in their own right and thus are not reliant on the inclusion of data on female anatomy.*

6) Are the species for which EPP rates were estimated likely to be representative of the whole group? It would be helpful if you could place the data on relative testis size and the EPP rates into context.

*We believe that the species for which EPP rates are estimated are generally representative of the whole group because they are not limited to one part of the phylogeny, but rather are distributed across multiple clades. To illustrate this, and similarly to show that relative testes mass data also likely represent the clade as a whole, we have now added this explanation to the discussion, lines 342-345, in our revised manuscript.*

7) What is the rationale for presenting both phylogenetically uncorrected and corrected analyses? Are you not confident about the accuracy of the phylogeny.

*We are confident about the accuracy of our phylogeny, but one of us (Price 1997) has argued that there is great value in the non-phylogenetically controlled tests and presented reasonable evolutionary models where this is more appropriate, despite similarity of related species. We explain this in lines 247-250*

#### Minor Comments

line 51 I am not sure what you mean by 'levels of organization'

*We mean levels ranging from within-male to between phyla.*

line 154 Please clarify if these are based on samples that were measured twice independently by two people, in which case these should be paired tests here

*These are based on individuals within a range of species that were measured twice, independently by two people. These are not paired tests because even though both people measured sperm from the same males, the particular sperm cells measured would have differed between the observers in many cases.*

line 158 You are still using CVbm in your own supplementary files

*We thank the reviewer for pointing this oversight out, we have now changed CVbm to CVam in the supplement.*

line 159 You also give a values for a species with  $n=2$  in the supplementary files (also, please explain what CTM stands for in the supplementary files). Ideally, provide a explanatory text for each of the supplementary files.

*Only the species where we had data from three or more males were included in the analyses despite the CVam calculation from two males given for *Phylloscopus reguloides* in the supplement. To avoid confusion, we have now removed that from the supplement. We have now added brief explanatory text for each of the supplementary files at the end of our manuscript.*

line 170 Please clarify that this species is a Cettiidae

*Done*

line177 an XML file of what?

*The XML file is the BEAST formatted input file that contains sequence alignments for all the species, we have added a short explanation of this point in the revised text (lines 174-175).*

line 178 S8 is actually a different file

*We have now renamed this file to S11.*

line 180 what is ESS (it often stands for evolutionary stable strategy, but clearly not here)

*It stands for effective sample size. We have now spelt this out in full in the revised manuscript.*

line 361 should read Fig 3

*Done*

Table 3 Why not also present such an analyses for body size?

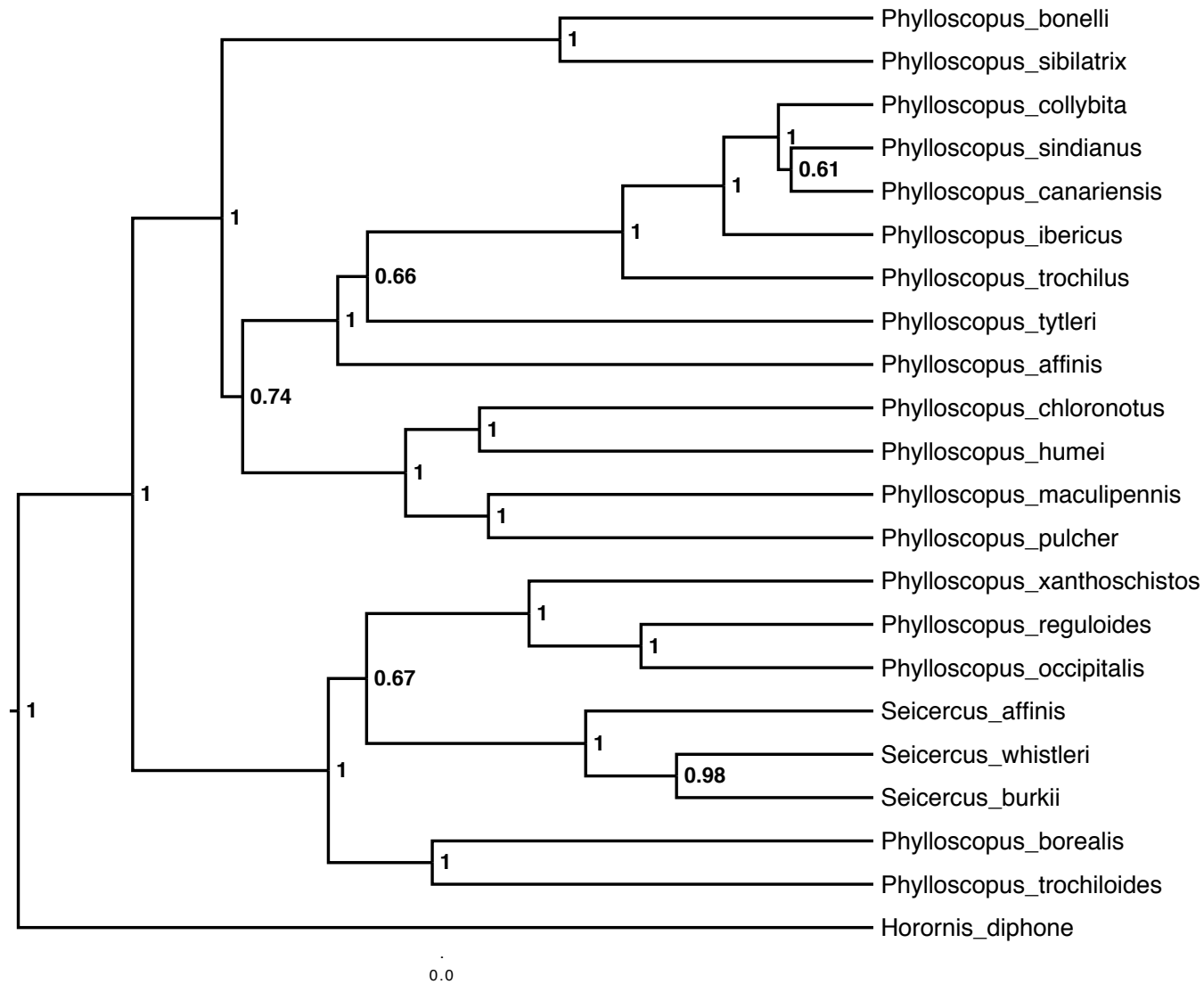
*We have now added it.*

Figure 1 Please state again what kind of a tree is depicted here and consider adding some information on nodal support values

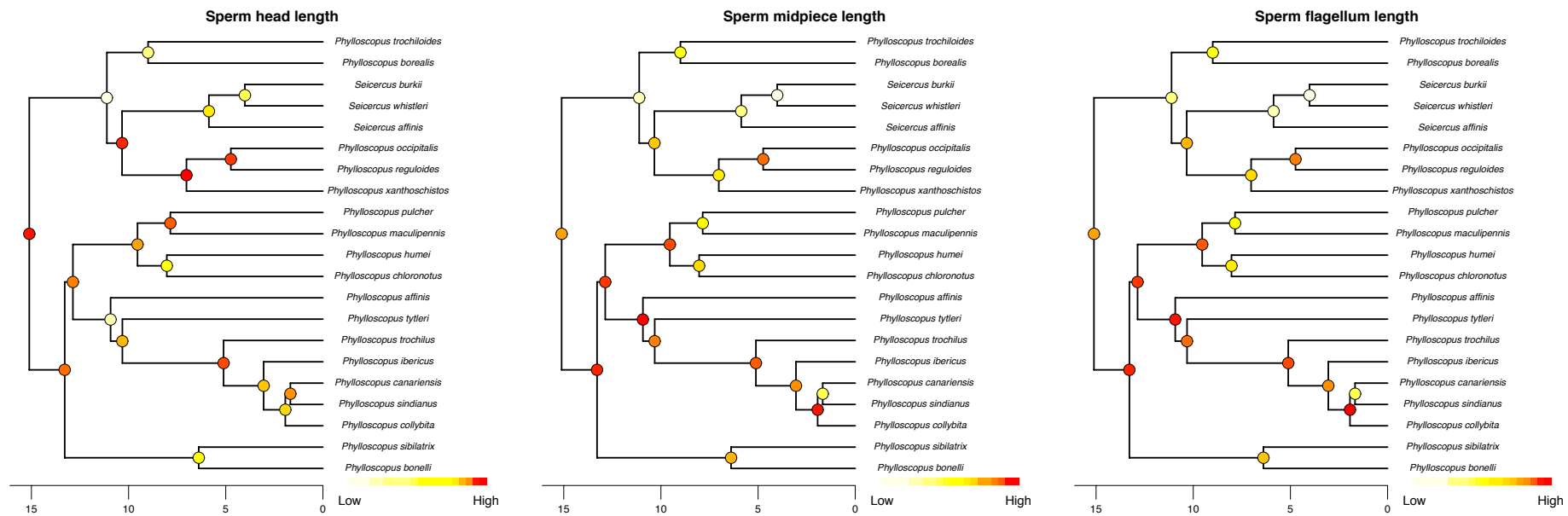
*Done, tree with nodal support values given in the supplement figures document.*

## **Literature cited**

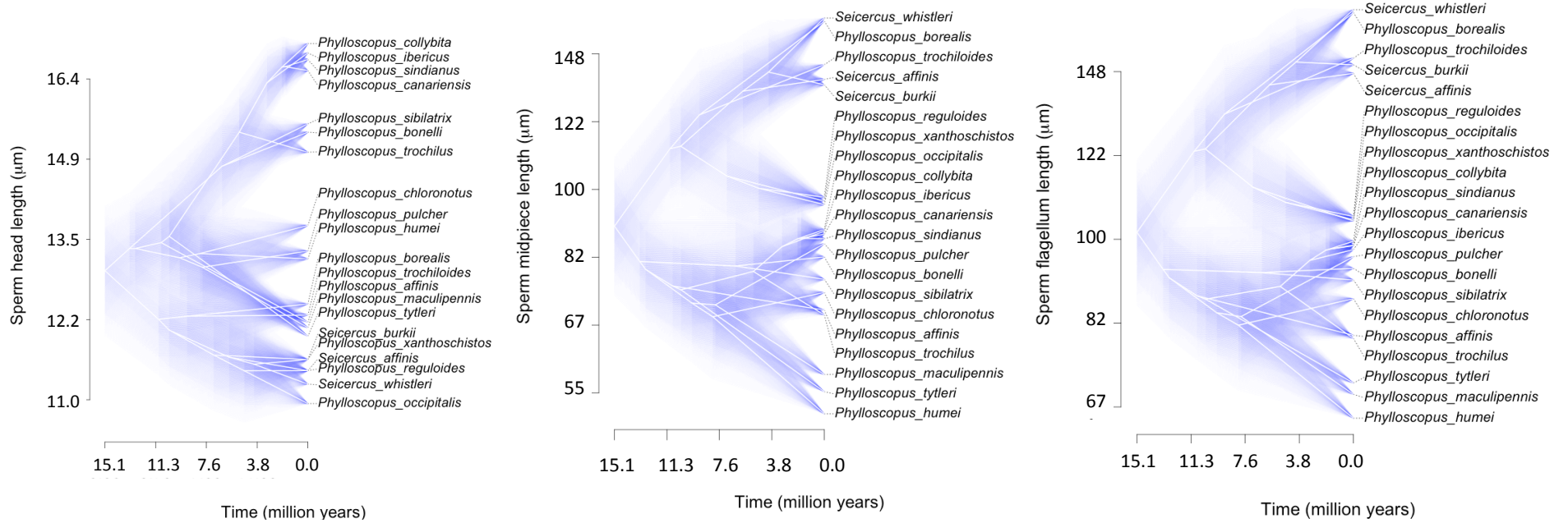
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**Figure S1** Maximum likelihood phylogeny of 21 species of Old World leaf warblers along with one outgroup species *Horornis diphone* (Family Cettidae) included in our study showing the bootstrap support values at each node.



**Figure S2** Larger contrasts (indicated by warmer colours at nodes) in sperm head length, midpiece length and flagellum length are at the base of the phylogeny, smaller contrasts (indicated by cooler colours) are towards the tips of the phylogeny.



**Figure S3** Traitgram of sperm head length, midpiece length and flagellum length in Old World leaf warblers shows large early divergence in sperm size followed by stasis of sperm length divergence between more recent clades at the tips.

PGLS of standard deviation of (log) total sperm length) against mid latitude and mean (log) total

	Slope	SE	t-value	p-value
mid latitude	0.018	0.007	2.674	0.0202
logTL	-0.323	0.745	-0.433	0.6724

sperm length



<b>common name</b>	<b>family</b>	<b>genus</b>	<b>species</b>	<b>subspecies</b>
Marsh warbler	Acrocephalidae	<i>Acrocephalus</i>	<i>palustris</i>	
Reed warbler	Acrocephalidae	<i>Acrocephalus</i>	<i>scirpaceus</i>	
Sedge warbler	Acrocephalidae	<i>Acrocephalus</i>	<i>schoenobaenus</i>	
Great reed warbler	Acrocephalidae	<i>Acrocephalus</i>	<i>arundinaceus</i>	
Long-tailed tit	Aegithalidae	<i>Aegithalos</i>	<i>caudatus</i>	
Red-winged blackbird	Icteridae	<i>Agelaius</i>	<i>phoeniceus</i>	
Meadow pipit	Motacillidae	<i>Anthus</i>	<i>pratensis</i>	
American goldfinch	Fringillidae	<i>Carduelis</i>	<i>tristis</i>	
White-throated dipper	Cinclidae	<i>Cinclus</i>	<i>cinclus</i>	
Eurasian blue tit	Paridae	<i>Cyanistes</i>	<i>caeruleus</i>	
Common house martin	Hirundinidae	<i>Delichon</i>	<i>urbicum</i>	
Chestnut-sided warbler	Parulidae	<i>Dendroica</i>	<i>pensylvanica</i>	
Yellow warbler	Parulidae	<i>Dendroica</i>	<i>petechia</i>	
Yellowhammer	Emberizidae	<i>Emberiza</i>	<i>citrinella</i>	
Reed bunting	Emberizidae	<i>Emberiza</i>	<i>schoeniclus</i>	
Least flycatcher	Tyrannidae	<i>Empidonax</i>	<i>minimus</i>	
European pied flycatcher	Muscicapidae	<i>Ficedula</i>	<i>hypoleuca</i>	
Collared flycatcher	Muscicapidae	<i>Ficedula</i>	<i>albicollis</i>	
Chaffinch	Fringillidae	<i>Fringilla</i>	<i>coelebs</i>	
Common yellowthroat	Parulidae	<i>Geothlypis</i>	<i>trichas</i>	
Barn swallow	Hirundinidae	<i>Hirundo</i>	<i>rustica</i>	
Wood thrush	Turdidae	<i>Hylocichla</i>	<i>mustelina</i>	
European crested tit	Paridae	<i>Lophophanes</i>	<i>cristatus</i>	
Bluethroat	Muscicapidae	<i>Luscinia</i>	<i>svecica</i>	<i>svecica</i>
Purple-crowned fairywren	Maluridae	<i>Malurus</i>	<i>coronatus</i>	<i>coronatus</i>
Red-winged fairywren	Maluridae	<i>Malurus</i>	<i>elegans</i>	
Red-backed fairywren	Maluridae	<i>Malurus</i>	<i>melanocephali</i>	<i>melanocephali</i>
Superb fairywren	Maluridae	<i>Malurus</i>	<i>cyaneus</i>	<i>cyanochlamys</i>
Splendid fairywren	Maluridae	<i>Malurus</i>	<i>splendens</i>	<i>melanotus</i>
Song sparrow	Emberizidae	<i>Melospiza</i>	<i>melodia</i>	
Swamp sparrow	Emberizidae	<i>Melospiza</i>	<i>georgiana</i>	
Northern wheatear	Muscicapidae	<i>Oenanthe</i>	<i>oenanthe</i>	
Great tit	Paridae	<i>Parus</i>	<i>major</i>	
Tree sparrow	Passeridae	<i>Passer</i>	<i>montanus</i>	
House sparrow	Passeridae	<i>Passer</i>	<i>domesticus</i>	
Savannah sparrow	Emberizidae	<i>Passerculus</i>	<i>sandwichensis</i>	
Indigo bunting	Cardinalidae	<i>Passerina</i>	<i>cyanea</i>	
Coal tit	Paridae	<i>Periparus</i>	<i>ater</i>	
Common redstart	Muscicapidae	<i>Phoenicurus</i>	<i>phoenicurus</i>	
Willow warbler	Phylloscopidae	<i>Phylloscopus</i>	<i>trochilus</i>	

Scarlet tanager	Thraupidae	<i>Piranga</i>	<i>olivacea</i>	
Willow tit	Paridae	<i>Poecile</i>	<i>montanus</i>	
Black-capped chickadee	Paridae	<i>Poecile</i>	<i>atricapillus</i>	
Sand martin	Hirundinidae	<i>Riparia</i>	<i>riparia</i>	
Eastern phoebe	Tyrannidae	<i>Sayornis</i>	<i>phoebe</i>	
Ovenbird	Parulidae	<i>Seiurus</i>	<i>aurocapilla</i>	
European serin	Fringillidae	<i>Serinus</i>	<i>serinus</i>	
American redstart	Parulidae	<i>Setophaga</i>	<i>ruticilla</i>	
Eastern bluebird	Turdidae	<i>Sialia</i>	<i>sialis</i>	
Southern emu-wren	Maluridae	<i>Stipiturus</i>	<i>malachurus</i>	<i>malachurus</i>
European starling	Sturnidae	<i>Sturnus</i>	<i>vulgaris</i>	
Eurasian blackcap	Sylviidae	<i>Sylvia</i>	<i>atricapilla</i>	
Tree swallow	Hirundinidae	<i>Tachycineta</i>	<i>bicolor</i>	
Zebra finch	Estrildidae	<i>Taeniopygia</i>	<i>guttata</i>	
House wren	Troglodytidae	<i>Troglodytes</i>	<i>aedon</i>	
Winter wren	Troglodytidae	<i>Troglodytes</i>	<i>troglodytes</i>	
Redwing	Turdidae	<i>Turdus</i>	<i>iliacus</i>	
American robin	Turdidae	<i>Turdus</i>	<i>migratorius</i>	
Common blackbird	Turdidae	<i>Turdus</i>	<i>merula</i>	
Golden-winged warbler	Parulidae	<i>Vermivora</i>	<i>chrysoptera</i>	

\*When the EPY data was from 2 or more citations, we calculated a value based on the total number of relative testes mass expressed as combined testes mass as a percentage of total body mass (CTM)

epy*	n young	epp*	n broods	references	patdy_mass (BM)	s_mass (CTM)
0.031	131	0.091	33	Leisler & Wink	12.0	0.140
0.065	186	0.154	52	Davies et al 20	12.0	0.150
0.078	344		77	Langefors et al	12.1	0.170
0.098	194	0.104	48	Leisler et al 20	27.2	0.46
0.024	288	0.158	38	Hatchwell et al	8.2	0.030
0.256	617			Weatherhead	66.0	0.920
0.270	178	0.513	39	P Nadvornik 20	18.0	0.320
0.143	70	0.267	15	Gissing et al 19	12.8	0.230
0.016	185	0.050	40	Øigarden et al	65.4	0.009
0.110	263	0.405	42	Krokene et al 2	12.1	0.300
0.192	73	0.350	20	Whittingham &	17.0	0.200
0.474	95	0.606	33	Byers et al 200	9.6	0.080
0.366	355	0.589	90	Yezerinac et al	9.6	0.170
0.374	123	0.688	32	Sundberg & Di	27.3	0.300
0.295	332	0.542	72	Kleven & Lifjeld	18.7	0.640
0.337	89	0.565	23	Tarof et al 200	10.7	0.190
0.044	135	0.148	27	Lifjeld et al 199	12.0	0.040
0.178	624	0.377	106	Sheldon & Elle	10.3	0.04
0.170	47	0.231	13	Sheldon & Bur	22.5	0.440
0.203	423	0.458	120	Thusius et al 20	10.5	0.210
0.288	917	0.481	210	Kleven et al 20	16.2	0.330
0.205	263	0.356	87	Evans et al 200	47.4	0.368
0.110	136	0.300	20	Lens et al 1997	10.7	0.004
0.263	1568	0.495	305	Johnsen & Lifjeld	16.8	0.171
0.044	227	0.058	104	Kingma et al. 20	11.4	0.05
0.570	261	0.700	118	Brouwer et al.	9.77	0.2
0.544	460	0.663	199	Baldassarre an	7.57	0.21
0.720	618	0.920	157	Dunn and Cock	8.93	0.29
0.422	386	0.554	159	Webster et al.	9.45	0.3
0.051	117	0.240	25	E.A. MacDougall	21.0	0.310
0.209	350	0.416	113	Olsen et al 200	18.0	0.690
0.110	73	0.294	17	Currie et al 199	26	0.71
0.085	82	0.308	13	Johannessen e	19.0	0.127
0.091	265	0.240	75	Cordero et al 2	22.0	0.310
0.124	955	0.265	309	Wetton & Park	24.2	0.420
0.405	571	0.602	161	Freeman-Galla	18.9	0.490
0.349	63	0.480	25	Westneat 1990	14.9	0.560
0.317	3143	0.717	431	Schmoll et al 2	9.1	0.130
0.020	253	0.105	38	Kleven et al 20	14.0	0.100
0.330	109	0.500	20	Bjørnstad & Lif	8.8	0.107

0.167	54	0.294	17 Klatt et al 2008	29	0.51
0.106	273	0.350	40 Rytönen et al	19.0	0.110
0.118	710	0.313	115 Otter et al 199	12.7	0.270
0.174	350	0.374	139 Alves & Bryant	14.0	0.240
0.118	76	0.200	20 Conrad et al 19	19.8	0.034
0.278	158	0.462	52 PK Roberts 200	18.9	0.200
0.094	139	0.191	47 Hoi-Leitner et	11.2	0.15
0.234	239	0.427	75 Reudink 2008 (	8.6	0.280
0.084	83	0.238	21 Meek et al 199	31.0	0.265
0.120	50	0.150	27 Maguire and M	7.32	0.045
0.168	196	0.438	48 Loyau et al 200	82.1	4.34
0.148	81		Gernot Segelba	17.0	0.800
0.475	867	0.825	166 Stapleton et al	20.1	0.640
0.017	299	0.050	80 Griffith et al. 2	12	0.05
0.166	1466	0.350	283 Forsman et al	11.1	0.085
0.163	153	0.379	29 Brommer et al	9.0	0.120
0.132	342	0.240	75 Asklund 2008 (	60.4	0.767
0.481	187	0.719	64 Rowe and Wea	74.6	0.780
0.186	322		J. Rutkowska p	76	1.01
0.313	240	0.556	54 Vallender et al	8.7	0.120

er of offspring from both papers, and not just an average of the two values, in the papers. Similar approach (1/BM \*100)

**RTM§**

1.166666667

1.25

1.404958678

1.691176471

0.365853659

1.393939394

1.777777778

1.796875

0.013700306

2.479338843

1.176470588

0.833333333

1.770833333

1.098901099

3.422459893

1.775700935

0.333333333

0.388349515

1.955555556

2

2.037037037

0.776371308

0.037383178

1.018463371

0.438596491

2.047082907

2.774108322

3.247480403

3.174603175

1.476190476

3.833333333

2.730769231

0.668421053

1.409090909

1.73553719

2.592592593

3.758389262

1.428571429

0.714285714

1.221461187

1.75862069  
0.578947368  
2.125984252  
1.714285714  
0.171717172  
1.058201058  
1.339285714  
3.255813953  
0.85483871  
0.614754098  
5.286236297  
4.705882353  
3.184079602  
0.416666667  
0.765765766  
1.333333333  
1.270709079  
1.045576408  
1.328947368  
1.379310345

ch was used for calculation of EPP i.e. number of broods with extra-pair young