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1   # Asymmetries in reproductive anatomy: insights from promiscuous songbirds

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13   **Short title:** Avian reproductive tract asymmetry

14   **Abstract**

15   Directional asymmetry in gonad size is commonly observed in vertebrates and is particularly pronounced

16   in birds, where the left testis is frequently larger than the right. The adaptive significance of directional

17   asymmetry in testis size is poorly understood, and whether it extends beyond the testes (i.e. side-

18   correspondent asymmetry along the reproductive tract) has rarely been considered. Using the Maluridae, a

19   songbird family exhibiting variation in levels of sperm competition and directional testis asymmetry, yet

20   similar in ecology and life-history, we investigated the relative roles of side-correspondence and sperm

21   competition on male reproductive tract asymmetry at both inter- and intraspecific levels. We found some

22   evidence for side-correspondent asymmetry. Additionally, sperm competition influenced directional

23   asymmetry at each end of the reproductive tract: species experiencing higher levels of sperm competition

24   had a relatively larger right testis and relatively more sperm in the right seminal glomerus. Within red-

25   backed fairy-wrens (*Malurus melanocephalus*), auxiliary males had relatively more sperm in the left

26   seminal glomerus in contrast to a right-bias asymmetry throughout the reproductive tract in breeding males.

27   Since sperm numbers are important for competitive fertilization success, our results suggest that sperm

28   competition shapes reproductive asymmetries beyond testis size, with likely functional consequences for

29   male reproductive success.

30

31   **Keywords:** sperm competition, testis size, Maluridae, reproductive evolution

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Introduction

Directional asymmetry in the animal kingdom is a widespread phenomenon that can affect whole body plans or the positioning, morphology or function of bilateral organs (reviewed in Palmer, 2004; 2009; Blum & Ott, 2018). Asymmetries can be the result of natural selection processes, such as in the functionally asymmetric human brain or the asymmetric shape of the ciliate *Paramecium* (reviewed in Blum & Ott, 2018). However, many cases of directional asymmetry are instead influenced by sexual selection, including the species-specific chirality of snail shells (Davison *et al.*, 2005), the asymmetric size of the fighting claws of fiddler crabs (e.g. Rosenberg, 2002) and the non-random usage of the double intromittent organ of phallostethid fishes (e.g. Parenti, 1986) and reptiles (Tokarz, 1988; e.g. Shine, 2000). Directional asymmetry in the size and/or shape (i.e. anterior-posterior elongation) of the testes is also widespread (e.g. Romer, 1971; Yu, 1998), and is particularly common and often pronounced in birds (Romer, 1971; Lake, 1981; Calhim & Montgomerie, 2015). Avian gonads are internal and often show marked seasonal changes in size (Lofts & Murton, 1973; Briskie & Montgomerie, 2007). Although considerable intra- (reviewed in Calhim and Birkhead, 2009) and interspecific (e.g. Briskie & Montgomerie, 2007; Calhim & Montgomerie, 2015) variation in directional asymmetry in testis size and shape has been documented in birds, its evolutionary causes and consequences remain largely unexplored.

The majority of birds have a larger left testis (i.e. left-biased directional testis size asymmetry; Calhim & Montgomerie, 2015). This pattern is suggested to be a by-product of strong selection for the degeneration of the right ovary and thus the occurrence of a single left ovary and left oviduct in female birds (Witschi, 1935). Alternatively, the restricted space in the body cavity (i.e. the packaging hypothesis) limits the anterior-posterior growth of the right testis due to the presence of the liver (Witschi, 1935) and/or the ventral-dorsal growth of the left testis due to the gizzard (TR Birkhead, pers. comm.). In fact, a more elongated left testis (i.e. left-bias testis shape asymmetry) has been documented in taxa with larger gizzards (Calhim & Montgomerie, 2015). Finally, the copulation bias hypothesis suggests that the function of the larger left testis is to allow for greater sperm number insemination due to a match between left-bias directional testis asymmetry and left-bias copulation direction (Petersen, Lombardo, & Power, 2001; Nyland, Lombardo, & Thorpe, 2003; Delehanty & O'Hearn, 2005). Testing this hypothesis requires

60   quantitative data of copulation behavior across species that differ in the directional bias of testis asymmetry.

61   However, such data are rare since copulatory behavior of wild birds is often difficult to observe. Moreover,

62   this hypothesis relies on the untested assumption of side-correspondent asymmetry throughout the

63   reproductive tract, such as in sperm producing tissue, size of male sperm storage structures or numbers of

64   sperm therein.

65   Surprisingly, the idea of whether or not asymmetry patterns across species extend beyond the testes to other

66   components of the male reproductive tract is largely unexplored. Limited empirical evidence of asymmetry

67   at the distal end of the reproductive tract comes from the Struthioniformes, Tinamiformes and

68   Anseriformes: these groups have a phallus that tends to bend to the left when erect (King, 1989),

69   corroborating the correlated evolution with female reproductive tract asymmetry (Witschi, 1935). In

70   contrast, there is little information concerning asymmetry of lower reproductive structures in Passeriformes

71   (but see Laskemoen *et al.*, 2008 for a intraspecific study). In passerines, the distal end of each ductus

72   deferens coils to form the seminal glomerus, structures that undergo seasonal changes in size in parallel

73   with the testes, sometimes forming a pronounced sexually dimorphic feature – the cloacal protuberance

74   (Wolfson, 1954; 1960; Lake, 1981). Thus, passerines offer an ideal group in which to investigate side-

75   correspondent correlations between testis asymmetry and asymmetries in lower portions of the male

76   reproductive tract.

77   Sperm competition is a key evolutionary force shaping variation in several primary sexual traits, including

78   testis size and testis histology, sperm morphology, and sperm quality in wide variety of taxa (e.g. Simmons

79   & Fitzpatrick, 2012; Lüpold & Pitnick, 2018), including birds (Briskie & Montgomerie, 2007; Calhim &

80   Birkhead, 2007; Lüpold *et al.*, 2009a,b; Rowe & Pruett-Jones, 2011). In a comparative study covering 67

81   bird families, higher levels of sperm competition (i.e. relative testes mass) were associated with decreased

82   asymmetry in testes (Calhim & Montgomerie, 2015). This relationship, however, explained just 6% of the

83   variation and was only statistically significant in taxa exhibiting left-biased directional asymmetry.

84   Importantly, while broad comparative studies can be extremely informative, the large variation in selection

85   pressures across such taxonomically diverse levels (i.e. different orders/families), as well as errors

86   associated with diverse sources of data, can mask important biological patterns. Thus, while the sperm

87   competition hypothesis has received some support, there is much scope for additional studies.

88   In passerines, the degree and direction of directional testis asymmetry also varies within-species (reviewed

89   in Calhim & Birkhead, 2009), and the former has been found to positively correlate with both age (e.g.

90   Birkhead *et al.*, 1997; Merilä & Sheldon, 1999; Graves, 2004) and the expression of male secondary sexual

91   characters (Møller, 1994). Møller (1994) suggested that the more symmetrical testes in lower quality males

92   reflected the compensatory growth of the typically smaller testis to compensate for the stunted growth and

93   thus assumed (anatomical or physiological) malfunction of the typically larger testis (Domm & Juhn, 1927;

94   Calhim & Birkhead, 2009). This pattern, however, was not observed in subsequent studies (reviewed in

95   Calhim & Birkhead, 2009).

96   Due to potentially diverging evolutionary histories and selection pressures across avian families (Calhim

97   & Montgomerie, 2015), a useful alternative approach is to examine primary reproductive trait asymmetry

98   variation within a limited taxonomic group exhibiting similar life-histories and ecology. The monophyletic

99   family Maluridae, which shows considerable variation in sperm competition levels and in directional testis

100   asymmetry, is emerging as a model system to study the evolution of avian mating systems and sperm

101   competition (Cockburn *et al.*, 2013; Rowe & Pruett-Jones, 2013). Malurids are small (*c.* 5–40 grams),

102   insectivorous passerines, and species tend to be similar to each other in general morphology and life-history

103   traits (e.g. long-lived, non-migratory, dome nests). Additionally, all species are known, or believed, to

104   exhibit cooperative breeding with sons and sometimes daughters remaining on their natal territory to assist

105   parents in raising subsequent offspring (Rowley & Russell, 1997). Finally, malurids exhibit considerable

106   variation in potential sexual selection pressures and the intensity of sperm competition: despite being

107   socially monogamous, relative testes size varies ten-fold across species (c. 0.44 – 4.45% of male body

108   mass; Rowe & Pruett-Jones, 2013) and the occurrence of extra-pair paternity (EPP) ranges from 6% to 95%

109   of broods (Mulder *et al.*, 1994; Kingma *et al.*, 2009; Rowe & Pruett-Jones, 2013). Within the Maluridae,

110   the fairy-wrens (*Malurus* sp.) have received the most attention to date, likely due to their ease of study and

111   strong sexual selection pressures, suggested by their wide range in EPP rates and pronounced sexual

112   dimorphism. This genus also shows pronounced plumage divergence among species (Johnson, Price, &

113   Pruett-Jones, 2013), and in some cases variation in male plumage coloration within a species. The red-

114   backed fairy-wren is a clear example of a malurid with intraspecific variation in plumage colour (Karubian,

115   2002), and high levels of EPP (c. 63% of broods contain extra-pair young; Webster, Varian, & Karubian,

116   2008). Breeding male plumage types are associated with differences in male mating effort and behaviors:

117   red/black males, which are more attractive to females (Karubian, 2002), invest in EPP seeking behaviors,

118   whereas brown males invest in physical and acoustic mate guarding (defensive role in paternity; Karubian,

119   2002; Dowling & Webster, 2017). Consequently, males are likely to face different sperm competition

120   levels: cuckolding red/black males in an offensive role for paternity, whereas brown males can behaviorally

121   prevent (e.g. mate guard) or reduce (e.g. frequent copulations) sperm competition in a defensive role.

122   Finally, helper males gain some limited paternity (Webster *et al.*, 2008), always in offensive role. The male

123   types also differ in investment in gonads, as red/black males tend to have relatively larger testes compared

124   to brown males, and more so compared to helper males (Rowe *et al.*, 2010). Therefore, this species is the

125   ideal complement to the interspecific analyses, allowing the corresponding assessment at the intraspecific

126   level.

127   Here, we investigate the degree of asymmetry across primary reproductive traits in male birds, using

128   malurids as a case study. Our main aim is to assess the roles of side-correspondent asymmetry and sperm

129   competition at both intra- and interspecific scales (Fig. 1A). This way, we can investigate the idea that

130   patterns of asymmetry may incorporate aspects of male reproductive anatomy beyond testis asymmetry,

131   and thus extend our knowledge of primary sexual trait asymmetry in studies of reproductive investment in

132   animals.

Materials and Methods

134   **Data collection**

135   *Field methods*

136   Populations of ten species of Maluridae were studied at several sites across Australia between 2004-2006

137   and in 2011: superb fairy-wrens (*M. cyaneus cyanochlamys*) at Murray River National Park, South

138   Australia (140˚32'E, 34˚20'S); splendid (*M. splendens melanotus*), variegated (*M. lamberti assimilis*), and

139   white-winged (*M. leucopterus leuconotus*) fairy-wrens at Brookfield Conservation Park, South Australia

140   (139˚29'E, 34˚20'S); blue-breasted fairy-wrens (*M. pulcherrimu*s) at Lincoln National Park, South Australia

141   (135˚52'E, 34˚52'S); red-backed fairy-wrens (*M. melanocephalus*) at Moomin Reservoir and Kalinvale

142   Farm, near Herberton, Queensland (145º23'E, 17º23'S); red-winged fairy-wrens (*M. elegans*) near

143   Manjimup, Western Australia (116º08'E, 34º15'S); lovely fairy-wrens (*M. amabilis*) near Julatten,

144   Queenland (145º20'E, 16º38'S); southern emu-wrens (*Stipiturus malachurus malachurus*) near Smith's

145   Lake, New South Wales (152º28'E, 32º22'S); and striated grasswrens (*Amytornis striatus striatus*) at

146   Pooginook and Cooltong Conservation Parks, near Berri, South Australia (140˚35'E, 34˚16'S).

147   Adult birds were trapped using mist nets and song playback. Upon capture, birds were weighed (± 0.1 g)

148   using a Pesola spring balance and then euthanised in accordance with animal ethics approvals, and we

149   dissected out the testes and seminal glomera. Only males in peak breeding condition, indicated by

150   behavioral, morphological or physiological characteristics (e.g. breeding plumage, enlarged cloacal

151   protuberance, active spermatogenesis) were included in the study.

152   *Reproductive tract data*

153   We measured the length, width and fresh mass (± 0.01 g) of each testis and fresh mass of each seminal

154   glomerus separately. Testes were then fixed in 6–10% neutral buffered formalin for 24 hours before being

155   stored in 70% ethanol. To determine the total number of sperm stored in each seminal glomerus, sperm

156   were flushed from each glomerus separately into a known volume of buffer (phosphate buffered saline in

157   2011, otherwise Lago Formulation Avian Semen Extender, Hygieia Biological Laboratories, USA), and

158   sperm numbers were quantified using a Makler counting chamber (Irvine Scientific, USA). We replicated

159   the counts using separate aliquots of the diluted sperm sample (Nakagawa & Schielzeth, 2010), and

160   calculated an average sperm count based on the two replicates. All samples were mixed thoroughly before

161   counting. We also quantified the relative amount of spermatogenic tissue in each testis using standard

162   histological techniques and image analysis (Rowe & Pruett-Jones, 2011). Briefly, for each of four non-

163   sequential sections, we measured the proportion of seminiferous tubule tissue (relative to interstitial tissue)

164   using Image-J software and calculated the proportion of tissue per testis (i.e. left and right testis separately)

165   by averaging the values from the four sections.

Asymmetry measures

167   Asymmetry measures for all reproductive traits were calculated as a scale-free index (Palmer & Strobeck,

168   2003) by taking the natural log from the ratio of the left-side measurement by the right-side measurement.

169   For example, testis size (i.e. mass) asymmetry (TA) = natural log (left testis mass [g]/right testis mass [g]).

170   We also calculated asymmetry index for seminal glomerus mass (SGA), seminal glomerus sperm numbers

171   (SGSNA), and proportion of sperm producing tissue in the testis (SPTA). Testis shape asymmetry

172   (TAshape) is also a scale-free index that reflects differences in the degree of elongation in the anterior-

173   posterior plane that was calculated as the difference between natural log (left testis length/right testis length)

174   and natural log (left testis width/right testis width). The absolute value of these scale-free indices reflects

175   the degree (i.e. the difference between the two sides) and their sign determines the bias (i.e. which side is

176   larger or longer), with positive and negative values corresponding to left and right bias, respectively.

177   Species-specific asymmetry indices were obtained by averaging asymmetry indices from all individuals

178   belonging to a species. The latter calculation led to some discrepancies between our data and the values

179   presented in Calhim and Montgomerie (2015). In that study, species-specific asymmetry indices were

180   calculated from species-level mean left and right side testis sizes, thus those values ignored intraspecific-

181   level variation. We present results using both approaches and show that they do not qualitatively affect our

182   results (Table 1 and Supplementary Table S1).

Gizzard size

184   Gizzard size data for five species: short-tailed grasswren (*Amytornis merrotsyi merrotsyi*), blue-breasted

185   fairy-wren, variegated fairy-wren, white-winged fairy-wren, and superb fairy-wren (subspecies *leggei*)

186   were obtained from the South Australian Museum. Gizzard volume was calculated from the available linear

187   dimensions of preserved specimens, assuming an ellipsoid shape: volume=$4/3*\pi*(\text{length}/2)*(\text{width}/2)^2$. In

188   our use of these data, we assumed the differences in gizzard size across subspecies in the superb fairy-wren

189   to be negligible. Similarly, in the absence of gizzard size data for the striated grasswren, the short-tailed

190 grasswren was used. Though we fully acknowledge the potential error associated with these data, we

191 believe it is an acceptable compromise, since until recently these two taxa were classified as the same

192 subspecies and they share similar ecology and mating system (Christidis, 1999).

Statistical analysis

194 Interspecific patterns were analyzed taking into account the phylogenetic relationships among species,

195 which accounts for the expectation that closer relatives will be more similar to each other due to shared

196 evolutionary history (Felsenstein, 1985; Harvey & Pagel, 1991). We used a generalized least-squares

197 approach within a phylogenetic framework ('pgls' function) using the packages 'caper' (Orme *et al.*, 2013)

198 and 'ape' (Paradis, Claude, & Strimmer, 2004) in R v. 3.5.2 (R Foundation for Statistical Computing, 2012).

199 We used the molecular phylogeny of Driskell *et al.* (2011), choosing this phylogeny over other available

200 phylogenies (e.g. Gardner *et al.*, 2010; Lee, Joseph, & Edwards, 2012) because of the availability of data

201 on branch lengths and because the main difference between available phylogenies concerns the positioning

202 of the purple-crowned fairy-wren, which is not included in our study. As well as intercept and slopes, this

203 analysis produces the phylogenetic scaling parameter λ (an index between 0 and 1) and its 95% confidence

204 level (CL). This parameter λ represents the degree of phylogenetic dependency in correlations among traits:

205 if $\lambda=1$, traits covary in direct proportion to their shared evolutionary history; while if $\lambda=0$, (i.e. trait

206 coevolution is independent of phylogeny; Freckleton, Harvey & Pagel, 2002).

207 We were interested in testing and comparing non-exclusive yet alternative evolutionary paths to explain

208 interspecific patterns of asymmetry using four aspects of male reproductive tract morphology: testis mass,

209 proportion of sperm producing tissue, seminal glomerus mass, and number of sperm stored therein. We

210 investigated the associations between up and downstream traits (Fig. 1A paths 1–5, i.e. side-

211 correspondence), and the role of sperm competition (Fig. 1A paths 6–9). The strength of sperm competition

212 was estimated as relative testes mass, which we modelled by including both combined testes mass and body

213 mass as covariates in our models. This is a commonly used index in avian comparative studies (e.g. Calhim

214 *et al.,* 2007; Lüpold *et al.,* 2009a), including Maluridae (e.g. Rowe & Pruett-Jones, 2013). In order to

215 directly compare path strengths (i.e. different models), we standardized all variables (i.e. subtracted the

216 mean and divided by the standard deviation) prior to analyses. This way, model coefficients correspond to

217 standardized effect sizes, reflecting estimated unit change in the dependent variable per unit change in the

218 independent variable, where the unit is standard deviations.

219 The role of gizzard size on testis size and shape asymmetry was also tested using pgls analyses as described

220 above. However, since these relationships are not part of the path analyses in Fig. 1, standardized

221 coefficients were not required. Instead, we mean-centered the predictor variables so that the intercept

222 estimate reflects the asymmetry value predicted at the average gizzard size.

223 For the intraspecific patterns in reproductive asymmetry, we repeated the same analyses as for the

224 interspecies patterns (Fig. 1A). Phylogenetically controlled analyses were not needed, however, as these

225 data were from a single species – the red-backed fairy-wren. Instead, we used general linear models and

226 model plots to determine if the model assumptions were met. Importantly, this approach allowed us to

227 identify statistical outliers, which were then removed and the analyses repeated. We present the results with

228 the complete dataset, since (i) no qualitative differences were found between models with and without

229 outliers, (ii) close inspection of these 'outliers' showed no biological reason for their removal, and (iii)

230 samples sizes were already small. In these analyses, we used the reproductive morph of the male as the

231 index of sperm competition. Specifically, we categorized males into three morphs based on plumage

232 coloration and behavior: breeding males (red/black vs. brown) and non-breeding helper males. Plumage

233 color was determined by scoring the percentage of the male's body that was red/black versus brown

234 following Karubian (2002) and Lindsay *et al.* (2009). In general, and in the case of individuals utilized in

235 this study, these categories were discrete and unmistakable (Webster *et al.*, 2008). The helper male morph

236 was set as the reference level in all statistical analyses, except those that included asymmetry in proportion

237 of sperm producing tissue in the testis (SPTA), as we had no data for individuals in this group.

Ethical statement

239 All work was undertaken with approval from the appropriate agencies, including the University of Chicago

240 Animal Care and Use Committee (#71453), the Department of Environment and Heritage (South Australia)

241 Wildlife Ethics Committee (Project# 13/2004; Scientific Permit Q24832; AW licence# 142), the Director-

242 General of New South Wales Department of Primary Industries Animal Care and Ethics Committee

243 (06/3846; NSW NPWS scientific licence# S12048), the Department of Environment and Conservation

244 (Western Australia; Regulation 17 licence# SF008305; Regulation 4# CE003378), James Cook University

245 Animal Ethics Review Committee (A1004 and A1691), the Environmental Protection Agency (EPA) of

246 Queensland, and the Department of Environment and Resource Management (Queensland; Scientific

247 Purposes Permit# WISP09844011). Finally, export of samples from Australia was approved by the

248 Australian Government Department of Environment and Heritage (WT2005-10120, WT2006–10958 and

249 WT2012–10) and in accordance with Nagoya protocols.

250 ## Results

251 **Overview of reproductive tract asymmetry**

252 There was considerable inter- and intraspecific variation in asymmetry in all traits except the proportion of

253 sperm producing tissue (see Supplementary Tables S2, S3). The southern emu-wren showed the largest

254 intraspecific range in testis size asymmetry (-0.56 – 0.47) and in proportion of sperm producing tissue (-

255 0.0118 – 0.0130). This species also showed considerable variation in other asymmetry measures, making

256 it the most variable species in our dataset. The least variable species on average across all asymmetry

257 measures was the superb fairy-wren.

258 **Interspecific patterns**

259 We found that a larger right testis was associated with a larger investment in the right seminal glomerus

260 tissue (path 1/Table 1 and Fig. 1B; Fig. 2A). No other side-correspondent asymmetry paths were

261 statistically significant, although the trend was towards positive associations (paths 2–5/Table 1 and Fig.

262 1B). We also found a significant negative association between relative testis size and testis size asymmetry

263 (path 6/Table 1 and Fig. 1B; Fig. 2B). Note that sperm competition explains two thirds of the variation in

264 testis size asymmetry within the Maluridae (*cf.* 6% across 67 avian families; see Calhim & Montgomerie

265 2015) In addition, there was a trend towards asymmetry in the number of sperm stored in the seminal

266 glomerus (path 8/Table 1 and Fig. 1B; Fig. 2C). Thus, as the level of sperm competition increased,

267 asymmetry in testes size and seminal glomera sperm numbers shifted from left-biased to strongly right-

268 biased.

269 In contrast, we found no significant relationship between gizzard volume and either testis size or shape

270 asymmetry, once body mass was accounted for (Table 2). However, in our limited sample, there was a trend

271 for larger gizzards to be associated with a relatively larger right testis (Supplementary Fig. S1A) and more

272 elongated left testis (Supplementary Fig. S1B). However, this effect is likely due to a single species (i.e.

273 variegated fairy-wren) and as such these findings should be interpreted with caution.

274 **Intraspecific patterns**

275 We found no evidence of side-correspondent asymmetry in red-backed fairy-wrens (paths 1–5/Table 3).

276 However, we found that both types of breeding male showed, on average, symmetric sperm numbers in

277 their seminal glomera, while helpers exhibited considerable left bias (Table 3, Fig. 3, Supplementary Table

278 S3). Helpers also had significantly lower total number of stored sperm in the seminal glomera (helper mean

279 $\pm$ SD sperm count x10^6: 26.18 $\pm$ 15.98) relative to brown breeders (108.62 $\pm$ 78.11; linear model estimate

280 mean $\pm$ s.e.=82.44 $\pm$ 33.84, t=2.44, p=0.030), although not significantly lower relative to red/black breeders

281 (69.72 $\pm$ 29.83; estimate=43.54 $\pm$ 33.84, t=1.29, p=0.22). Although time of sampling had no effect on

282 breeding male asymmetry in numbers of sperm stored across species (Supplementary Table S4 and

283 Supplementary Fig. S2A), the helper vs. breeder morphs differences within the red-backed fairy-wren are

284 only present later in day (Supplementary Table S5 and Supplementary Fig. S2B).

Discussion

286 Directional asymmetry in testis size is widespread in birds, and varies from the strong left-side bias of the

287 Glaucous-winged gull (*Larus glaucescens*) to the extreme right-side bias in the African black coucal

288 (*Centropus grilii*), in which males lack the left testis entirely; though left bias in testis size is the common

289 pattern observed across bird species (c. 75% of cases; Calhim & Montgomerie, 2015). Here, we report

290 considerable interspecific variation in the direction and degree of testis size and shape asymmetry within a

291   single avian family, the Maluridae. Moreover, we found, for the first time, that asymmetry in reproductive

292   tissues extends beyond testes size to include asymmetry in seminal glomera mass and the number of sperm

293   stored therein. In contrast, we found no evidence for asymmetry in the amount of sperm producing tissue

294   contained within the testis.

295   We observed considerable intraspecific variability among species in the degree of asymmetry in testis size

296   and shape, in seminal glomera size, and in the number of sperm stored therein. Broadly speaking, the

297   southern emu-wren and superb fairy-wren were, respectively, the most and the least variable species.

298   Concurrently, the southern emu-wren has the lowest rates of extra-pair paternity and relative testes mass,

299   while the superb fairy-wren has the highest rates of extra-pair paternity reported for the species in the

300   current study (Rowe & Pruett-Jones, 2013). Though further work is clearly needed before firm conclusions

301   can be drawn, this pattern may suggest that the intense selection imposed via sperm competition in the

302   superb fairy-wren may reduce variation among males in a population such that all males are closer to a

303   species-specific 'optimum' investment in sperm production. Such a pattern would be similar to that

304   observed for sperm morphology, for which the level of sperm competition is associated with reduced

305   intermale variation in sperm size and shape in birds (Calhim, Immler, & Birkhead, 2007; Kleven *et al.*,

306   2008; Støstad *et al.*, 2018) and other taxa (e.g. Fitzpatrick & Baer, 2011).

307   Across species, we found that covariance between testis size asymmetry and relative testes mass (i.e. our

308   proxy of sperm competition) shifted from being left-biased in species with relatively low relative testes

309   mass to right-biased in species with high relative testes mass. In a larger comparative study of passerine

310   birds, Calhim and Montgomerie (2015) found that higher sperm competition was associated with more

311   symmetry in testis size, although this pattern was only statistically significant among taxa with a larger left

312   testis. Relative testes size, sperm production, and levels of extra-pair paternity in Malurids are among the

313   highest reported in passerine birds (Tuttle & Pruett-Jones, 2004; Rowe & Pruett-Jones, 2006; 2011; *cf.*

314   Griffith, Owens, & Thuman, 2002; Rowe & Pruett-Jones, 2013). Thus, our findings provide an interesting

315   contrast to previous work examining the role of sperm competition in shaping testes asymmetry across taxa.

316   Specifically, our study suggests that some malurid species may respond to the intense selective pressure of

317 sperm competition by moving beyond testis size symmetry to right-biased asymmetry. Perhaps in small

318 passerines with relatively large gizzards (c. 4% of body mass for Maluridae; passerine gizzard mass index:

319 mean±s.e.= 3.04±0.17, n=22; Calhim & Montogomerie 2015) and strong selective pressure for larger

320 combined testes size, more testicular tissue can only be achieved by growing the less space-constrained

321 right testis. In fact, the degree of right-bias in testis size asymmetry values in malurids are among the most

322 extreme values reported for birds (*cf.* Calhim & Montgomerie, 2015). It may therefore be particularly

323 interesting to investigate if the same pattern occurs in other right-bias testis asymmetry clades with high

324 rates of extra-pair paternity, such as Hirundinidae or Fringillidae, and whether these clades also have a

325 relatively large gizzard.

326 Closer examination of the interspecific patterns provides some interesting contrasts. First, white-winged

327 fairy-wrens have both the largest relative testes size and the strongest right-bias in testes asymmetry. This

328 species exhibits a somewhat atypical 'clan' mating system (Rowley & Russell, 1995), in which younger

329 males hold territories and are socially pair-bonded to a female, but exhibit female-like, brown plumage

330 (Pruett-Jones, unpublished data). Superimposed on these individual territories is the territory of a single

331 male in full nuptial plumage (i.e. blue/white plumage), and this male interacts with pairs occupying

332 individual territories within his 'clan' (Rowley & Russell, 1995). Although this complicates our

333 understanding of extra-pair mating in this species, the occurrence of female multiple mating is undoubtedly

334 high in this species and thus strong selection imposed via sperm competition may explain the extreme

335 values we observed. Moreover, while the splendid and superb fairy-wren have right-bias in testes

336 asymmetry and high rates of extra-pair paternity (42-72% extra-pair offspring; Rowe & Pruett-Jones, 2013),

337 the red-winged fairy-wren shows left-bias asymmetry in testes mass despite comparably high rates of extra-

338 pair paternity (57% extra-pair offspring; Brouwer *et al.* 2011; Rowe & Pruett-Jones, 2013). Similarly,

339 relative testes mass is not particularly high in that species, which may suggest that selection on increasing

340 sperm numbers (i.e. through increases in tissue devoted to sperm production) is not especially strong in the

341 red-winged fairy-wren relative to that experienced by other high extra-pair paternity species; instead,

342 additional traits may be more closely linked to male fertilization success (e.g. sperm length), though such

343   ideas remain to be tested. Nonetheless, overall our findings offer further support for the idea that sperm

344   competition can shape testes asymmetry in passerine birds.

345   Our results also shed light on the other hypotheses relating to interspecific variation in avian testis

346   asymmetry. First, we found no asymmetry in the proportion of sperm producing tissue in the testes at either

347   the inter- or intraspecific level. Second, in all individuals both the left and right testes appeared to be in a

348   state of full spermatogenesis (stage 6 *sensu* Scott & Middleton, 1968). Together, this suggests that the two

349   testes are functionally equivalent, which contradicts the premise of the compensation hypothesis (Møller,

350   1994), namely that one testis is malfunctioning. The packaging hypothesis suggests that asymmetry in male

351   gonads reflects space constraints within the body cavity due to the positioning of organs such as the liver

352   (Witschi, 1935) and the gizzard (TR Birkhead, pers. comm.). We found that species with a relatively large

353   gizzard tended to have a more elongated and relatively smaller left testis. Although we acknowledge that

354   our results should be interpreted with extreme caution, these findings are consistent with the packaging

355   hypothesis and suggest that further studies investigating the role of space constraints in shaping testes

356   asymmetry would help elucidate the evolutionary causes of gonad asymmetry in birds. Our findings also

357   provide limited support for the key assumption behind the copulatory bias hypothesis (Delehanty &

358   O'Hearn, 2005), where a strong side-correspondence in reproductive tract asymmetry is expected. We found

359   a significant interspecific positive association between testis and seminal glomerus size directional

360   asymmetries, but this relationship did not extend to the proportion of sperm producing tissue in the testis

361   or to sperm numbers stored in the seminal glomerus.

362   We found little evidence for a role of sperm competition in explaining intraspecific variation of

363   reproductive organ asymmetry. Specifically, asymmetry in reproductive organs did not differ between the

364   male reproductive phenotypes in the red-backed fairy-wren, with the exception of the number of sperm

365   stored in the seminal glomerus. In this instance, helpers exhibited strong left-bias in the number of stored

366   sperm, which stands in stark contrast to values observed in brown and red/black breeders. In general,

367   helpers appear to invest relatively little in sperm production relative to breeders in the red-backed fairy-

368   wren (Rowe *et al.*, 2010) and, in this study, helpers had considerably fewer sperm in storage (1/3–1/4 of

369   breeder values). Helpers also appear to obtain few mating opportunities relative to breeders, as helpers sire

370   a low proportion of the extra-pair young in a population (Webster *et al.*, 2008). However, why this should

371   translate into left-bias asymmetry in numbers of stored sperm is unclear. One could speculate that the few

372   copulations helpers achieve are right-sided (in agreement with the copulation bias hypothesis for a species

373   with right biased testis size asymmetry), and their overall low sperm production did not replenish the right

374   seminal glomerus. The observation that helper vs. breeder differences are only clear in individuals sampled

375   later in the day further supports this hypothesis. However, given our small sample size for helper males, it

376   is plausible that this result constitutes a type I error, though the magnitude of the difference between helpers

377   and breeders might suggest otherwise.

378   The major contribution of our study is that we show that patterns of asymmetry in reproductive organs

379   extend beyond testes asymmetry to incorporate multiple aspects of male reproductive anatomy and

380   physiology. Most notably, we found that species exhibiting a larger right testis also possessed a larger

381   seminal glomerus on the right side, which is consistent with findings at the intraspecific level in the

382   bluethroat (*Luscinia svecica*; Laskemoen *et al.*, 2008) and indicates the size of the seminal glomerus is a

383   function of testis size. Moreover, in addition to predicting a relatively larger right testis, high levels of

384   sperm competition tended to be positively associated with greater number of sperm stored in the right

385   seminal glomerus. In birds, sperm numbers are an important determinant of male fertilization success under

386   competitive mating conditions (Martin *et al.*, 1974; Birkhead, 1998). Additional sperm phenotypic traits,

387   such as sperm size (Bennison *et al.*, 2015) or sperm motile performance (Pizzari *et al.*, 2008), also influence

388   male fertilization success in birds. However, recent theoretical and empirical work suggests that selection

389   imposed through post-copulatory processes (e.g. sperm competition and cryptic female choice) favors

390   increasing sperm numbers rather that sperm size in passerine birds, especially when the risk of sperm

391   competition is very high (Immler *et al.*, 2011). Given that rates of extra-pair paternity are extremely high

392   in many of the malurids studied here, our results suggest that sperm competition can shape testes asymmetry

393   in the Maluridae with consequences for functional traits (i.e. sperm quantity) that influence the outcome of

394   sperm competition and, ultimately, male reproductive success.

395   In conclusion, we show that directional asymmetry in male reproductive organs can incorporate multiple

396   aspects of the male reproductive tract. By utilizing a taxonomically restricted set of species, we aimed to

397   reduce potential sources of evolutionary variation in testes asymmetry to a primary variable of interest –

398   the level of sperm competition. This approach allowed us to discover influential patterns in the asymmetry

399   of reproductive traits. Specifically, our findings suggest that sperm competition can shape reproductive

400   asymmetries in the Maluridae with likely functional consequences for male reproductive success, which in

401   turn suggests that extremely high sperm competition may push directional asymmetry of testes size towards

402   a right-bias in testis mass.

Declaration of interest

404   The authors declare no competing interests.

Acknowledgments

References

412   Bennison C, Hemmings N, Slate J, Birkhead T. 2015. Long sperm fertilize more eggs in a bird.
413   *Proceedings of the Royal Society B: Biological Sciences* 282: 20141897.

414   Birkhead TR. 1998. Sperm competition in birds. *Reviews of Reproduction* 3: 123–129.

415   Birkhead TR, Buchanan KL, Devoogd TJ, Pellatt EJ, Szekely T, Catchpole CK. 1997. Song, sperm
416   quality and testes asymmetry in the sedge warbler. *Animal Behaviour* 53: 965–971.

417   Blum M, Ott T. 2018. Animal left–right asymmetry. *Current Biology* 28: R301–R304.

418 Briskie JV, Montgomerie R. 2007. Testis size, sperm size and sperm competition. In: Jamieson B, ed.
419 *Reproductive biology and phylogeny of birds. Part A. Phylogeny, morphology, hormones, fertilization.*
420 Enfield, NH: Science Publishers, 513–551.

421 Brouwer, L, van de Pol, M, Atema, E, Cockburn, A. 2011. Strategic promiscuity helps avoid inbreeding
422 at multiple levels in a cooperative breeder where both sexes are philopatric. *Molecular Ecology* 20: 4796–
423 4807.

424 Calhim S, Birkhead TR. 2007. Testes size in birds: quality versus quantity - assumptions, errors, and
425 estimates. *Behavioral Ecology* 18: 271–275.

426 Calhim S, Birkhead TR. 2009. Intraspecific variation in testis asymmetry in birds: evidence for naturally
427 occurring compensation. *Proceedings of the Royal Society B: Biological Sciences* 276: 2279–2284.

428 Calhim S, Montgomerie R. 2015. Testis asymmetry in birds: the influences of sexual and natural
429 selection. *Journal of Avian Biology* 45: 175–185.

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

432 Christidis L. 1999. Evolution and biogeography of the Australian grasswrens, *Amytornis* (Aves :
433 Maluridae): biochemical perspectives. *Australian Journal Of Zoology* 47: 113.

434 Cockburn A, Brouwer L, Double MC, Margraf N, Van De Pol M. 2013. Evolutionary origins and
435 persistence of infidelity in *Malurus*: the least faithful birds. *Emu* 113: 208–217.

436 Davison A, Chiba S, Barton NH, Clarke B. 2005. Speciation and gene flow between snails of opposite
437 chirality (R Butlin, Ed.). *PLoS Biology* 3: e282–13.

438 Delehanty DJ, O'Hearn PP. 2005. Behavioral and morphological asymmetries in chukar *Alectoris chukar*
439 copulation. *Journal of Avian Biology* 36: 276–279.

440 Domm LV, Juhn M. 1927. Compensatory hypertrophy of the testes in brown leghorns. *Biological Bulletin*
441 52: 458–473.

442 Dowling J, Webster MS. 2017. Working with what you've got: unattractive males show greater mate-
443 guarding effort in a duetting songbird. *Biology Letters* 13: 20160682.

444 Driskell AC, Norman JA, Pruett-Jones S, Mangall E, Sonsthagen S, Christidis L. 2011. A multigene
445 phylogeny examining evolutionary and ecological relationships in the Australo-Papuan wrens of the
446 subfamily Malurinae (Aves). *Molecular Phylogenetics and Evolution* 60: 480–485.

447 Felsenstein J. 1985. Phylogenies and the comparative method. *American Naturalist* 125: 1–15.

448 Fitzpatrick JL, Baer B. 2011. Polyandry reduces sperm length variation in social insects. *Evolution* 65:
449 3006–3012.

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

452 Gardner JL, Trueman JWH, Ebert D, Joseph L, Magrath RD. 2010. Phylogeny and evolution of the
453 Meliphagoidea, the largest radiation of Australasian songbirds. *Molecular Phylogenetics And Evolution*
454 55: 1087–1102.

455 Graves GR. 2004. Testicular volume and asymmetry are age-dependent in black-throated blue warblers
456 (*Dendroica caerulescens*). *Auk* 121: 473–485.

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

459 Harvey PH, Pagel MD. 1991. *The comparative method in evolutionary biology*. Oxford University Press.

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

463 Johnson AE, Price JJ, Pruett-Jones S. 2013. Different modes of evolution in males and females generate
464 dichromatism in fairy-wrens (Maluridae). *Ecology and evolution* 3: 3030–3046.

465 Karubian J. 2002. Costs and benefits of variable breeding plumage in the red-backed fairy-wren.
466 *Evolution* 56: 1673–1682.

467 King AS. 1989. Phallus. In: King AS, McLelland J, eds. *Form and Function in Birds*. New York:
468 Academic Press, 459.

469 Kingma SA, Hall ML, Segelbacher G, Peters A. 2009. Radical loss of an extreme extra-pair mating
470 system. *BMC ecology* 9: 15.

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

473 Lake PE. 1981. Male genital organs. In: King AS, McLelland J, eds. *Form and Function in Birds*. New
474 York: Academic Press.

475 Laskemoen T, Fossoy F, Rudolfsen G, Lifjeld JT. 2008. Age-related variation in primary sexual
476 characters in a passerine with male age-related fertilization success, the bluethroat *Luscinia svecica*.
477 *Journal of Avian Biology* 39: 322–328.

478 Lee JY, Joseph L, Edwards SV. 2012. A species tree for the Australo-Papuan Fairy-wrens and allies
479 (Aves: Maluridae). *Systematic Biology* 61: 253–271.

480 Lindsay WR, Webster MS, Varian CW, Schwabl H. 2009. Plumage colour acquisition and behaviour are
481 associated with androgens in a phenotypically plastic tropical bird. *Animal Behaviour* 77: 1525–1532.

482 Lofts B, Murton RK. 1973. Reproduction in birds. In: DS F, JR K, KC P, eds. *Avian Biology*. Academic
483 Press, 1–107.

484 Lüpold S, Pitnick S. 2018. Sperm form and function: what do we know about the role of sexual selection?
485 *Reproduction*: REP–17–0536.

486 Lüpold S, Linz GM, Rivers JW, Westneat DF, Birkhead TR. 2009a. Sperm competition selects beyond
487 relative testes size in birds. *Evolution* 63: 391–402.

488 Lüpold S, Calhim S, Immler S, Birkhead TR. 2009b. Sperm morphology and sperm velocity in passerine
489 birds. *Proceedings of the Royal Society B: Biological Sciences* 276: 1175–1181.

490 Martin PA, Reimers TJ, Lodge JR, Dziuk PJ. 1974. Effect of ratios and numbers of spermatozoa mixed
491 from 2 males on proportions of offspring. *Journal of Reproduction And Fertility* 39: 251–258.

492 Merilä J, Sheldon BC. 1999. Testis size variation in the greenfinch *Carduelis chloris*: relevance for some
493 recent models of sexual selection. *Behavioral Ecology and Sociobiology* 45: 115–123.

494 Mulder R, Dunn P, Cockburn A, Lazenbycohen K, Howell M. 1994. Helpers liberate female fairy-wrens
495 from constrains on extra-pair mate choice. *Proceedings of the Royal Society B: Biological Sciences* 255:
496 223–229.

497 Møller A. 1994. Directional selection of directional asymmetry - Testes size and secondary characters in
498 birds. *Proceedings of the Royal Society B: Biological Sciences* 258: 147–151.

499 Nakagawa S, Schielzeth H. 2010. Repeatability for Gaussian and non-Gaussian data: a practical guide for
500 biologists. *Biological Reviews of The Cambridge Philosophical Society* 85: 935–956.

501 Nyland KB, Lombardo MP, Thorpe PA. 2003. Left-sided directional bias of cloacal contacts during house
502 sparrow copulations. *The Wilson Bulletin* 115: 470–473.

503 Orme D, Freckleton R, Thomas G, Fritz S, Petzoldt T, Isaac N, Pearse W. 2013. caper: Comparative
504 analyses of phylogenetics and evolution in R.

505 Palmer AR. 2004. Symmetry breaking and the evolution of development. *Science* 306: 828–833.

506 Palmer AR. 2009. Animal asymmetry. *Current Biology* 19: R473–7.

507 Palmer RA, Strobeck C. 2003. Fluctuating asymmetry analyses revisited. In: Polak M, ed. *Developmental
508 Instability*. Oxford University Press, 279–319.

509 Paradis E, Claude J, Strimmer K. 2004. APE: Analyses of phylogenetics and evolution in R language.
510 *Bioinformatics* 20: 289–290.

511 Parenti LR. 1986. Bilateral asymmetry in phallostethid fishes (Atherinomorpha) with description of a new
512 species from Sarawak. *Proceedings of the Californian Academy of Sciences* 44: 225–236.

513 Petersen A, Lombardo M, Power H. 2001. Left-sided directional bias of cloacal contacts during tree
514 swallow copulations. *Animal Behaviour* 62: 739–741.

515 Pizzari T, Worley K, Burke T, Froman DP. 2008. Sperm competition dynamics: ejaculate fertilising
516 efficiency changes differentially with time. *BMC Evolutionary Biology* 8: 332.

517 Romer AS. 1971. *The Vertebrate Body*. Philadelphia: Saunders.

518 Rosenberg MS. 2002. Fiddler crab claw shape variation: a geometric morphometric analysis across the
519 genus *Uca* (Crustacea: Brachyura: Ocypodidae). *Biological Journal of The Linnean Society* 75: 147–162.

520 Rowe M, Pruett-Jones S. 2006. Reproductive biology and sperm competition in Australian fairy-wrens.
521 *Avian and Poultry Biology Reviews* 17: 21–37.

522 Rowe M, Pruett-Jones S. 2011. Sperm competition selects for sperm quantity and quality in the
523 Australian Maluridae. *PLoS ONE* 6: e15720.

524 Rowe M, Pruett-Jones S. 2013. Extra-pair paternity, sperm competition and their evolutionary
525 consequences in the Maluridae. *Emu* 113: 218–231.

526 Rowe M, Swaddle JP, Pruett-Jones S, Webster MS. 2010. Plumage coloration, ejaculate quality and
527 reproductive phenotype in the red-backed fairy-wren. *Animal Behaviour* 79: 1239–1246.

528 Rowley I, Russell E. 1995. The breeding biology of the white-winged fairy-wren *Malurus leucopterus-
529 leuconotus* in a Western-Australian coastal heathland. *Emu* 95: 175–184.

530   Rowley I, Russell EM. 1997. Fairy-wrens and grasswrens: Maluridae. In: Perrins C, Bock WJ, Kikkawa
531   J, eds. *Bird Families of the World*. Oxford University Press.

532   Scott DM, Middleton ALA. 1968. The annual testicular cycle of the brown-headed cowbird (*Molothrus
533   ater*). *Canadian Journal of Zoology* 46: 77–87.

534   Shine R. 2000. Are snakes right-handed? Asymmetry in hemipenis size and usage in gartersnakes
535   (*Thamnophis sirtalis*). *Behavioral Ecology* 11: 411–415.

536   Simmons LW, Fitzpatrick JL. 2012. Sperm wars and the evolution of male fertility. *Reproduction* 144:
537   519–534.

538   Støstad HN, Johnsen A, Lifjeld JT, Rowe M. 2018. Sperm head morphology is associated with sperm
539   swimming speed: A comparative study of songbirds using electron microscopy. *Evolution* 49: 233–15.

540   Tokarz RR. 1988. Copulatory behaviour of the lizard *Anolis sagrei*: alternation of hemipenis use. *Animal
541   Behaviour* 36: 1518–1524.

542   Tuttle EM, Pruett-Jones S. 2004. Estimates of extreme sperm production: morphological and
543   experimental evidence from reproductively promiscuous fairy-wrens (*Malurus*). *Animal Behaviour* 68:
544   541–550.

545   Webster MS, Varian CW, Karubian J. 2008. Plumage color and reproduction in the red-backed fairy-
546   wren: Why be a dull breeder? *Behavioral Ecology* 19: 517–524.

547   Witschi E. 1935. Origin of asymmetry in the reproductive system of birds. *American Journal of Anatomy*
548   56: 119–141.

549   Wolfson A. 1954. Sperm storage at lower-than-body temperature outside the body cavity in some
550   passerine birds. *Science* 120: 68–71.

551   Wolfson A. 1960. The ejaculate and the nature of coition in some passerine birds. *Ibis* 102: 124–125.

552   Yu Z. 1998. Asymmetrical testicular weights in mammals, birds, reptiles and amphibia. *International
553   Journal of Andrology* 21: 53–55.

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556 **Figure Legends**

557 **Figure 1.** Schematic representation of the direct associations between side-correspondent development

558 (paths 1–5) or sperm competition (paths 6–9) and four primary sexual trait asymmetries along the male

559 reproductive tract: testis size asymmetry (TA), seminal glomerus size asymmetry (SGA), seminal glomerus

560 sperm number asymmetry (SGSNA), and proportion of sperm producing tissue asymmetry (SPTA). Sperm

561 competition was measured as relative testes size (combined testes mass, controlling for body mass). A:

562 General schematic representation; B: Patterns observed at the interspecific level. Arrow thickness denotes

563 standardized effect size r and the style the direction of the association (solid and dashed for positive and

564 negative, respectively). See Table 1 for complete statistical output.

565 **Figure 2.** Scatterplots (mean ± s.e.) representing the three statistically significant and/or strongest

566 (standardized effect size r> |0.66|) interspecific associations (see Figure 1). (A) positive relationship

567 between testis size asymmetry and seminal glomerus size asymmetry (path 1); (B) negative association

568 between relative testes size and testis size asymmetry (path 6); and (C) negative association between

569 relative testes size and seminal glomerus sperm number asymmetry (path 8). Here, unlike in the analysis,

570 relative testes size is represented as the residuals of a phylogenetically controlled linear model of testes

571 mass on body mass, using standardized variables. Asymmetry values are scale-free indices (see main text

572 for details), with the absolute value reflecting the degree of difference and the sign denoting the directional

573 bias (positive and negative for left- or right-bias, respectively). Species codes: 1=lovely fairy-wren (n=4),

574 2=superb fairy-wren (n=6), 3=red-winged fairy-wren (n=6), 4=variegated fairy-wren (n=5), 5=white-

575 winged fairy-wren (n=6), 6=red-backed fairy-wren (n=20), 7=blue-breasted fairy-wren (n=5), 8=splendid

576 fairy-wren (n=6), 9=southern emu-wren (n=6), 10=striated grasswren (n=6).

577 **Figure 3.** Differences in the asymmetry in the number of sperm in the seminal glomerus (mean ± s.e.)

578 between the three male morphs in the red-backed fairy-wren.

579

580 **Tables**

581 **Table 1.** Comparison of alternative paths to explain interspecific variation in asymmetry along the

582 reproductive tract in malurids.

pathway*	model	coefficient§	estimate	s.e.	t	p	ML lambda	R²	n
Side-correspondent development									
1	**SGA ~ TA**	intercept	-0.011	0.235	-0.05	0.96	0.00	0.50	10
		TAsize	0.700	0.247	2.84	**0.02**			
2	SGSNA ~ SGA	intercept	0.462	1.235	0.37	0.72	0.98	0.03	10
		SGA	0.137	0.299	0.46	0.66			
3	SGSNA ~ TA	intercept	0.023	0.318	0.07	0.95	0.00	0.10	10
		TAsize	0.314	0.333	0.94	0.37			
4	SGSNA ~ SPTA	intercept	-0.061	0.345	-0.18	0.87	0.00	0.30	8
		tissueA	0.590	0.373	1.58	0.16			
5	SPTA ~ TA	intercept	0.091	0.386	0.24	0.82	0.00	0.07	8
		TAsize	0.306	0.447	0.69	0.52			
Sperm competition									
6	**TA ~ BM + CTM**	intercept	-0.014	0.214	-0.07	0.95	0.00	0.65	10
		BM	0.382	0.241	1.59	0.16			
		CTM	-0.862	0.241	-3.58	**0.01**			
7	SGA ~ BM + CTM	intercept	-0.019	0.32	-0.06	0.96	0.00	0.19	10
		BM	0.158	0.36	0.44	0.67			
		CTM	-0.465	0.36	-1.29	0.24			
8	**SGSNA ~ BM + CTM**	intercept	0.013	0.27	0.05	0.96	0.00	0.43	10
		BM	0.472	0.303	1.56	0.16			
		CTM	-0.662	0.303	-2.19	**0.06**			
9	SPTA ~ BM + CTM	intercept	0.066	0.328	0.20	0.85	0.00	0.38	8
		BM	0.337	0.329	1.02	0.35			
		CTM	-0.598	0.351	-1.70	0.15			

* Pathway numbers correspond to those outlined in Figure 1.
§ Coefficient estimates denote standardized effect size r, with statistically significant (or marginally non-significant) effects shown in bold text. TA=testis size asymmetry, SGA=seminal glomerus size asymmetry, SGSNA=seminal glomerus sperm number asymmetry, SPTA=proportion of sperm producing tissue asymmetry, BM=body mass, CTM=combined testes mass, ML lambda=maximum likelihood estimated lambda.

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585 **Table 2.** Relationships between relative gizzard size and testis asymmetry in size and shape in malurids,

586 controlling for phylogeny.

| model	coefficient*	estimate	s.e.	t	p	ML lambda	R^2	n
TA ~ BM + GV	intercept	-0.053	0.024	-2.22	0.16	0.00	0.79	5
	BM	0.048	0.018	2.72	0.11			
	GV	-0.920	0.368	-2.50	0.13			
TA shape ~ BM + GV	intercept	0.008	0.021	0.39	0.73	0.00	0.93	5
	BM	-0.017	0.015	-1.12	0.38			
	GV	0.826	0.321	2.57	0.12			

* Coefficient estimates denote standardized effect size r, with statistically significant (or marginally non-significant) effects shown in bold text. TA=testis size asymmetry, TA shape=testis shape asymmetry, BM=body mass, GV=gizzard volume, ML lambda=maximum likelihood estimated lambda.

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589   **Table 3.** Comparison of alternative paths to explain intraspecific variation in asymmetry along the

590   reproductive tract in red-backed fairy-wrens.

pathway*	model	coefficient§	estimate	s.e.	t	p	R^2	n
Side-correspondent development								
1	SGA ~ TA	intercept	-0.002	0.259	-0.01	0.99	0.002	16
		TA	0.042	0.237	0.18	0.86		
2	SGSNA ~ SGA	intercept	0.000	0.231	0.00	1.00	0.20	16
		SGA	0.452	0.238	1.90	0.08		
3	SGSNA ~ TA	intercept	0.004	0.258	0.02	0.99	0.01	16
		TA	-0.088	0.237	-0.37	0.71		
4	SGSNA ~ SPTA	intercept	-0.504	0.088	-5.73	**0.01**	0.04	5
		SPTA	0.034	0.098	0.34	0.75		
5	SPTA ~ TA	intercept	-0.047	0.500	-0.09	0.93	0.09	5
		TA	-0.251	0.464	-0.54	0.63		
Sperm competition								
6	TA ~ male morph	intercept (helper)	-0.015	0.063	-0.24	0.81	0.07	4
		vs brown breeder	-0.077	0.076	-1.01	0.32		9
		vs red/black breeder	-0.015	0.074	-0.21	0.84		11
7	SGA ~ male morph	intercept (helper)	-0.061	0.126	-0.49	0.63	0.02	4
		vs brown breeder	-0.092	0.162	-0.56	0.58		6
		vs red/black breeder	-0.054	0.162	-0.33	0.75		6
8	**SGSNA ~ male morph**	intercept (helper)	2.157	0.426	5.07	**<0.001**	0.63	4
		vs brown breeder	-2.304	0.55	-4.19	**0.001**		6
		vs red/black breeder	-2.277	0.55	-4.14	**0.001**		6
9	SPTA ~ male morph	intercept (brown breeder)	0.001	0.001	1.63	0.20	0.42	2
		vs red/black breeder	-0.001	0.001	-1.46	0.24		3

* Pathway numbers correspond to those outlined in Figure 1.
§ Coefficient estimates for paths 1–5 denote standardized effect size r and those for paths 6–9 are in the original units. Statistically significant (or marginally non-significant) effects are shown in bold text. TA=testis size asymmetry, SGA=seminal glomerus size asymmetry, SGSNA=seminal glomerus sperm number asymmetry, SPTA=proportion of sperm producing tissue asymmetry, BM=body mass, CTM=combined testes mass.

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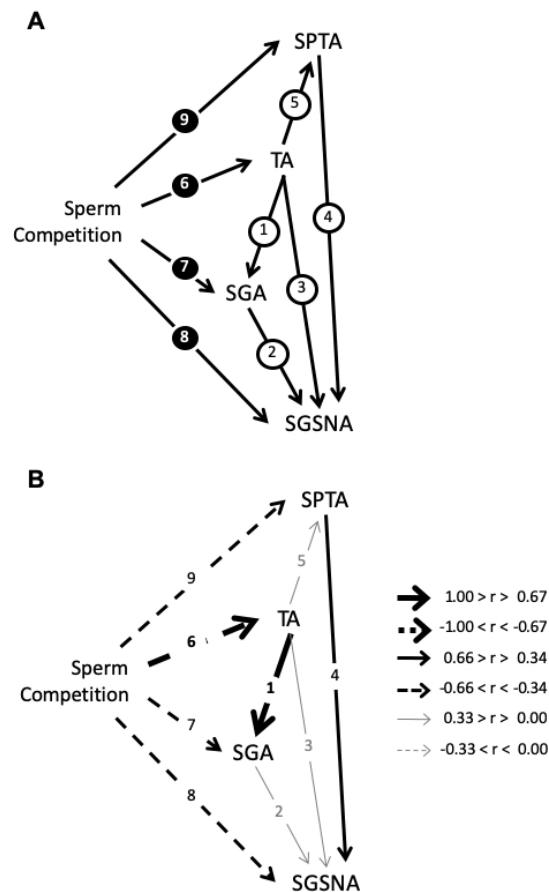
Schematic representation of the direct associations between side-correspondent development (paths 1–5) or sperm competition (paths 6–9) and four primary sexual trait asymmetries along the male reproductive tract: testis size asymmetry (TA), seminal glomerus size asymmetry (SGA), seminal glomerus sperm number asymmetry (SGSNA), and proportion of sperm producing tissue asymmetry (SPTA). Sperm competition was measured as relative testes size (combined testes mass, controlling for body mass). A: General schematic representation; B: Patterns observed at the interspecific level. Arrow thickness denotes standardized effect size r and the style the direction of the association (solid and dashed for positive and negative, respectively). See Table 1 for complete statistical output.

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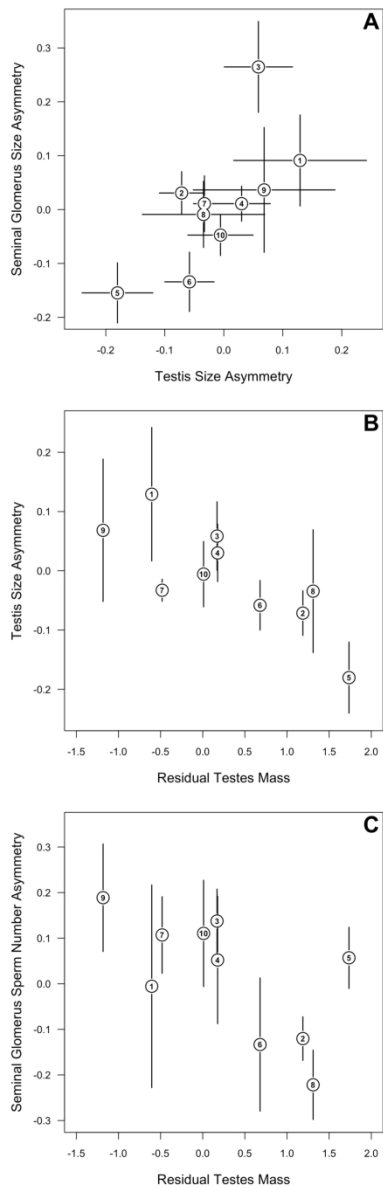
Scatterplots (mean ± s.e.) representing the three statistically significant and/or strongest (standardized effect size r> |0.66|) interspecific associations (see Figure 1). (A) positive relationship between testis size asymmetry and seminal glomerus size asymmetry (path 1); (B) negative association between relative testes size and testis size asymmetry (path 6); and (C) negative association between relative testes size and seminal glomerus sperm number asymmetry (path 8). Here, unlike in the analysis, relative testes size is represented as the residuals of a phylogenetically controlled linear model of testes mass on body mass, using standardized variables. Asymmetry values are scale-free indices (see main text for details), with the absolute value reflecting the degree of difference and the sign denoting the directional bias (positive and negative for left- or right-bias, respectively). Species codes: 1=lovely fairy-wren (n=4), 2=superb fairy-wren (n=6), 3=red-winged fairy-wren (n=6), 4=variegated fairy-wren (n=5), 5=white-winged fairy-wren (n=6), 6=red-backed fairy-wren (n=20), 7=blue-breasted fairy-wren (n=5), 8=splendid fairy-wren (n=6), 9=southern emu-wren (n=6), 10=striated grasswren (n=6).

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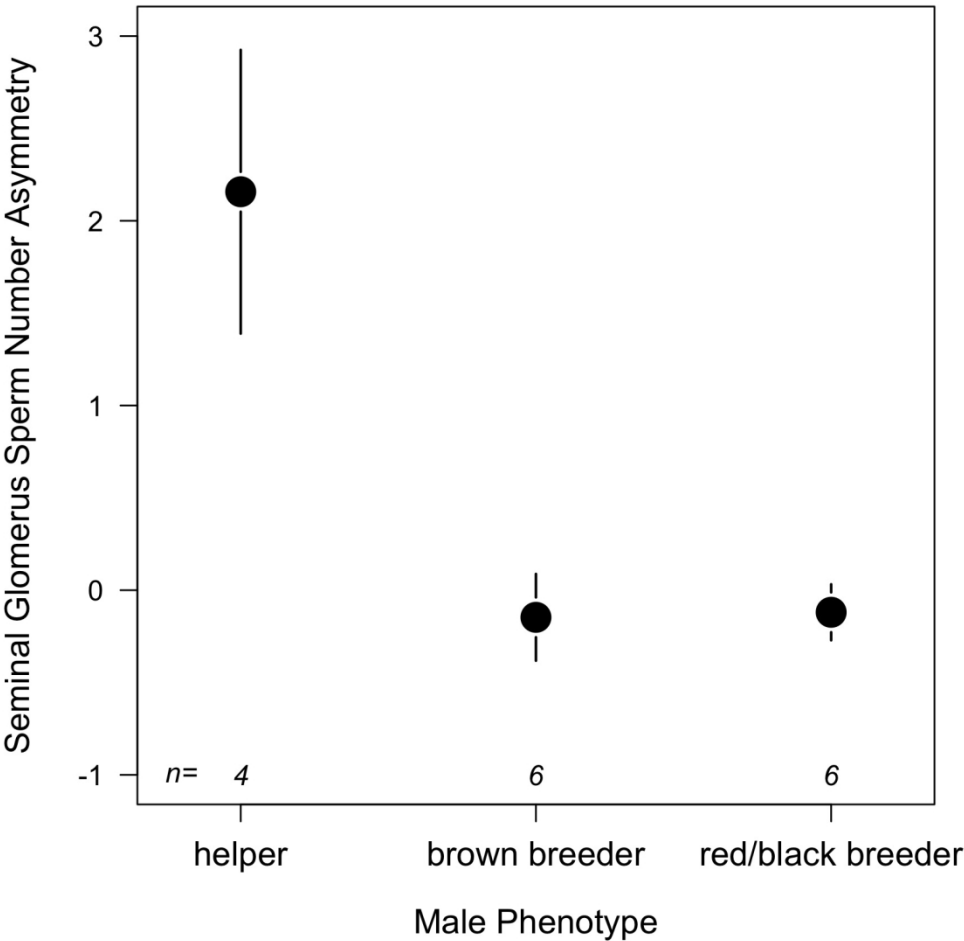
Differences in the asymmetry in the number of sperm in the seminal glomerus (mean ± s.e.) between the three male morphs in the red-backed fairy-wren.

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Asymmetries in reproductive anatomy: insights from promiscuous songbirds

Supplementary Table S1. Comparison of alternative paths to explain interspecific variation in asymmetry along the reproductive tract in malurids. Repeat of the analyses presented in Table1 using species-specific testis asymmetry indices calculated from species-specific left and right testis mean mass and length values.

pathway*	model	coefficient§	estimate	s.e.	t	p	ML lambda	R^2	n
Side-correspondent development									
1	**SGA ~ TA**	intercept	-0.014	0.237	-0.06	0.954	0.00	0.491	10
		TAsize	0.690	0.248	2.777	**0.024**			
2	SGSNA ~ SGA	intercept	0.612	1.141	0.536	0.606	1.00	0.327	10
		SGA	0.453	0.230	1.971	0.084			
3	SGSNA ~ TA	intercept	0.024	0.272	0.089	0.931	0.00	0.353	10
		TAsize	0.595	0.285	2.09	0.070			
4	SGSNA ~ SPTA	intercept	0.451	1.217	0.37	0.724	1.00	0.154	8
		tissueA	0.307	0.294	1.044	0.337			
5	SPTA ~ TA	intercept	0.072	0.387	0.187	0.858	0.00	0.051	8
		TAsize	0.247	0.433	0.57	0.589			
Sperm competition (relative testes mass)									
6	**TA ~ BM + CTM**	intercept	-0.01	0.199	-0.048	0.963	0.00	0.697	10
		BM	0.368	0.223	1.649	0.143			
		CTM	-0.898	0.224	-4.016	**0.005**			
7	SGA ~ BM + CTM	intercept	-0.019	0.328	-0.058	0.956	0.00	0.153	10
		BM	0.216	0.368	0.587	0.576			
		CTM	-0.409	0.368	-1.11	0.304			
8	**SGSNA ~ BM + CTM**	intercept	0.015	0.258	0.059	0.955	0.00	0.493	10
		BM	0.487	0.289	1.685	0.136			
		CTM	-0.721	0.29	-2.49	**0.042**			
9	SPTA ~ BM + CTM	intercept	0.06	0.35	0.173	0.87	0.00	0.296	8
		BM	0.288	0.351	0.822	0.448			
		CTM	-0.53	0.374	-1.418	0.215			

* Pathway numbers correspond to those outlined in Figure 1.
§ Coefficient estimates denote standardized effect size r, with statistically significant (or marginally non-significant) effects shown in bold text. TA=testis size asymmetry, SGA=seminal glomerus size asymmetry, SGSNA=seminal glomerus sperm number asymmetry, SPTA=proportion of sperm producing tissue asymmetry, BM=body mass, CTM=combined testes mass, ML lambda=maximum likelihood estimated lambda.

Asymmetries in reproductive anatomy: insights from promiscuous songbirds

Supplementary Table S2. Asymmetries in primary reproductive traits (mean, range and sample size) across 10 species of Maluridae. Asymmetry values are scale-free (see main text for details), with the absolute value reflecting the degree of difference and the sign denoting the directional bias (positive and negative for left- or right-bias, respectively). Note that red-backed fairy-wren data exclude helpers but include both red/black and brown male breeder morphs. Species-means asymmetry indices were calculated by averaging across individual asymmetry indices (cf. asymmetry indices calculated from species-means side-specific trait values as done in Calhim and Montgomerie 2015).

Species	Testis size asymmetry (TA)				Seminal glomerus size asymmetry (SGA)				Seminal glomerus sperm number asymmetry (SGSNA)				Proportion of sperm producing tissue asymmetry (SPTA)				Testis shape asymmetry				Gizzard volume (cm²)	
	mean	min	max	n	mean	min	max	n	mean	min	max	n	mean	min	max	n	mean	min	max	n	mean	n
Striated grasswren (*Amytornis striatus*)	-0.01	-0.18	0.23	6	-0.05	-0.20	0.13	5	0.11	-0.28	0.79	6	0.0011	-0.0039	0.004	3	0.20	0.07	0.34	6	0.809	2
Lovely fairy-wren (*Malurus amabilis*)	0.13	-0.09	0.66	4	0.09	-0.22	0.41	4	-0.01	-0.50	1.02	4	—	—	—	—	0.13	-0.04	0.29	4	—	—
Superb fairy-wren (*Malurus cyaneus*)	-0.07	-0.23	0.07	6	0.03	-0.13	0.17	6	-0.12	-0.29	0.07	6	-0.0013	-0.0024	0.000	6	-0.05	-0.12	0.05	5	0.353	3
Red-winged fairy-wren (*Malurus elegans*)	0.06	-0.18	0.34	6	0.26	0.05	0.79	6	0.14	-0.20	0.42	6	—	—	—	—	0.06	-0.11	0.35	5	—	—
Variegated fairy-wren (*Malurus lamberti*)	0.03	-0.13	0.24	5	0.01	-0.13	0.12	4	0.05	-0.33	0.82	5	0.0009	-0.0025	0.004	5	-0.14	-0.56	0.19	5	0.145	1
White-winged fairy-wren (*Malurus leucopterus*)	-0.18	-0.47	0.04	6	-0.15	-0.38	0.07	6	0.06	-0.19	0.38	6	-0.0003	-0.0015	0.001	4	0.05	-0.11	0.22	6	0.330	2
Red-backed fairy-wren (*Malurus melanocephalus*)	-0.06	-0.25	0.32	20	-0.13	-0.37	0.11	12	-0.13	-0.69	0.89	12	0.0004	-0.0011	0.002	5	0.05	-0.20	0.38	20	—	—
Blue-breasted fairy-wren (*Malurus pulcherrimus*)	-0.03	-0.13	0.03	5	0.01	-0.24	0.20	5	0.11	-0.16	0.42	5	0.0042	-0.0024	0.012	5	-0.02	-0.10	0.12	5	0.339	1
Splendid fairy-wren (*Malurus splendens*)	-0.03	-0.35	0.46	6	-0.01	-0.18	0.36	6	-0.22	-0.67	0.01	6	-0.0008	-0.0044	0.005	6	0.06	-0.09	0.38	6	—	—
Southern emu-wren (*Stipiturus malachurus*)	0.07	-0.56	0.47	6	0.04	-0.41	0.41	5	0.19	-0.45	0.51	5	0.0005	-0.0118	0.013	6	0.05	-0.10	0.21	6	—	—

Asymmetries in reproductive anatomy: insights from promiscuous songbirds

Supplementary Table S3. Asymmetries in primary reproductive traits (mean, range and sample size) across male morph in red-backed fairy-wrens. Asymmetry

12 values are scale-free (see main text for details), with the absolute value reflecting the degree of difference and the sign denoting the directional bias (positive and

negative for left- or right-bias, respectively).

Phenotype	Testis size asymmetry (TA)				Seminal glomerus size asymmetry (SGA)				Seminal glomerus sperm number asymmetry (SGSNA)				Proportion of sperm producing tissue asymmetry (SPTA)			
	mean	min	max	n	mean	min	max	n	mean	min	max	n	mean	min	max	n
Red/black breeder	-0.03	-0.25	0.32	11	-0.12	-0.37	0.11	6	-0.12	-0.68	0.39	6	-0.0002	-0.0011	0.0009	3
Brown breeder	-0.09	-0.20	0.02	9	-0.15	-0.27	0.00	6	-0.15	-0.69	0.89	6	0.0013	0.0004	0.0022	2
Helper	-0.02	-0.10	0.11	4	-0.06	-0.51	0.46	4	2.16	0.03	3.45	4	—	—	—	—

Asymmetries in reproductive anatomy: insights from promiscuous songbirds

Supplementary Table S4. Effect of time of sampling on the asymmetry of sperm numbers stored in the seminal glomerus across species. Results obtained using a PGLS analyses using standardized variables and a phylogeny where the different individuals are coded as polytomies at their corresponding species node (n=50 individuals of n=9 species).

model	coefficient§	estimate	s.e.	t	p	ML lambda	R^2	n
SGSNA ~ sampling time	intercept	-0.035	0.138	-0.255	0.800	0.00	<0.001	50
	time	0.040	0.155	0.258	0.798			

Supplementary Table S5. Effect of time of sampling on the asymmetry of sperm numbers stored in the seminal glomerus within red-backed fairy-wrens. Since the variable time had a bimodal distribution, we transformed it into a two-level factor ('early' = 6:00-10:00 and 'late'= 16:00-19:00,). We test for an interaction between sampling time and morph using a 2-way ANOVA with Type III sum of squares. The 'Anova' function in the R package 'car' was used for the latter.

	coefficient§	Sum of Squares	d.f.	F	p
Two-way ANOVA	(intercept)	2.47	1	8.09	0.019
(Type III SS)	**Morph**	4.15	2	6.78	**0.016**
	Time	3.42	1	11.16	**0.009**
	Morph*Time	3.13	2	5.12	**0.033**
	Residual	2.75	9		

Asymmetries in reproductive anatomy: insights from promiscuous songbirds

32   **Supplementary Figure S1.** Relationship between testis asymmetry in (A) size and (B) shape (mean ± s.e.)
and gizzard volume (as a ratio to body mass) in five Maluridae species. Species codes follow those in Figure
34   2: 2=superb fairy-wren, 4=variegated fairy-wren, 5=white-winged fairy-wren, 7=blue-breasted fairy-wren,
10=striated grasswren.
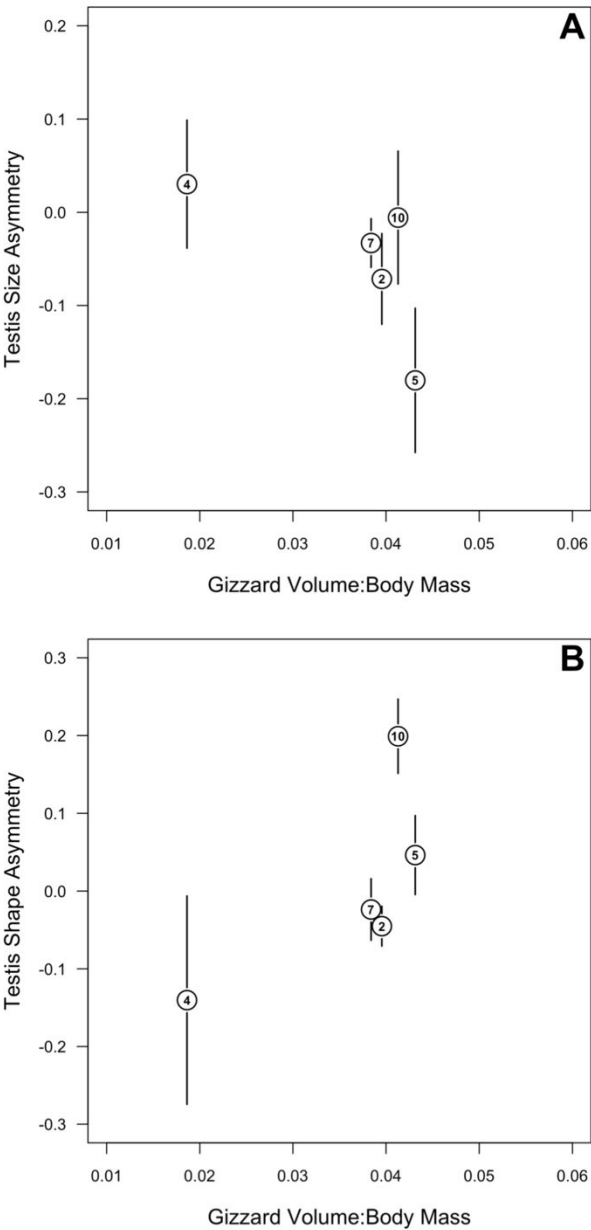
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Asymmetries in reproductive anatomy: insights from promiscuous songbirds

Supplementary Figure S2. Relationship between time of sampling and asymmetry in the number of sperm stored in the seminal glomera (SGSNA). (A) Interspecific patterns (breeders only) and (B) Intraspecific patterns in red-backed fairy-wrens.

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