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Asymmetries in reproductive anatomy: insights from promiscuous songbirds

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Short title: Avian reproductive tract asymmetry

Abstract

Directional asymmetry in gonad size is commonly observed in vertebrates and is particularly pronounced in birds, where the left testis is frequently larger than the right. The adaptive significance of directional asymmetry in testis size is poorly understood, and whether it extends beyond the testes (i.e. side-correspondent asymmetry along the reproductive tract) has rarely been considered. Using the Maluridae, a songbird family exhibiting variation in levels of sperm competition and directional testis asymmetry, yet similar in ecology and life-history, we investigated the relative roles of side-correspondence and sperm competition on male reproductive tract asymmetry at both inter- and intraspecific levels. We found some evidence for side-correspondent asymmetry. Additionally, sperm competition influenced directional asymmetry at each end of the reproductive tract: species experiencing higher levels of sperm competition had a relatively larger right testis and relatively more sperm in the right seminal glomerus. Within red-backed fairy-wrens (*Malurus melanocephalus*), auxiliary males had relatively more sperm in the left seminal glomerus in contrast to a right-bias asymmetry throughout the reproductive tract in breeding males. Since sperm numbers are important for competitive fertilization success, our results suggest that sperm competition shapes reproductive asymmetries beyond testis size, with likely functional consequences for male reproductive success.

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

33 **Introduction**

34 Directional asymmetry in the animal kingdom is a widespread phenomenon that can affect whole body
35 plans or the positioning, morphology or function of bilateral organs (reviewed in Palmer, 2004; 2009; Blum
36 & Ott, 2018). Asymmetries can be the result of natural selection processes, such as in the functionally
37 asymmetric human brain or the asymmetric shape of the ciliate *Paramecium* (reviewed in Blum & Ott,
38 2018). However, many cases of directional asymmetry are instead influenced by sexual selection, including
39 the species-specific chirality of snail shells (Davison *et al.*, 2005), the asymmetric size of the fighting claws
40 of fiddler crabs (e.g. Rosenberg, 2002) and **the non-random usage of the double** intromittent organ of
41 phallostethid fishes (e.g. Parenti, 1986) and reptiles (Tokarz, 1988; e.g. Shine, 2000). Directional
42 asymmetry in the size and/or shape (i.e. anterior-posterior elongation) of the testes is also widespread (e.g.
43 Romer, 1971; Yu, 1998), and is particularly common and often pronounced in birds (Romer, 1971; Lake,
44 1981; Calhim & Montgomerie, 2015). Avian gonads are internal and often show marked seasonal changes
45 in size (Lofts & Murton, 1973; Briskie & Montgomerie, 2007). Although considerable intra- (reviewed in
46 Calhim and Birkhead, 2009) and interspecific (e.g. Briskie & Montgomerie, 2007; Calhim & Montgomerie,
47 2015) variation in directional asymmetry in testis size and shape has been documented in birds, its
48 evolutionary causes and consequences remain largely unexplored.

49 The majority of birds have a larger left testis (i.e. left-biased directional testis size asymmetry; Calhim &
50 Montgomerie, 2015). This pattern is suggested to be a by-product of strong selection for the degeneration
51 of the right ovary and thus the occurrence of a **single left ovary and left oviduct** in female birds (Witschi,
52 1935). Alternatively, the restricted space in the body cavity (i.e. the packaging hypothesis) limits the
53 anterior-posterior growth of the right testis due to the presence of the liver (Witschi, 1935) and/or the
54 ventral-dorsal growth of the left testis due to the gizzard (TR Birkhead, pers. comm.). In fact, a more
55 elongated left testis (i.e. left-bias testis shape asymmetry) has been documented in taxa with larger gizzards
56 (Calhim & Montgomerie, 2015). Finally, the copulation bias hypothesis suggests that the function of the
57 larger left testis is to allow for greater sperm number insemination due to a match between left-bias
58 directional testis asymmetry and left-bias copulation direction (Petersen, Lombardo, & Power, 2001;
59 Nyland, Lombardo, & Thorpe, 2003; Delehanty & O'Hearn, 2005). Testing this hypothesis requires

quantitative data of copulation behavior across species that differ in the directional bias of testis asymmetry. However, such data are rare since copulatory behavior of wild birds is often difficult to observe. Moreover, this hypothesis relies on the untested assumption of side-correspondent asymmetry throughout the reproductive tract, such as in sperm producing tissue, size of male sperm storage structures or numbers of sperm therein.

Surprisingly, the idea of whether or not asymmetry patterns across species extend beyond the testes to other components of the male reproductive tract is largely unexplored. Limited empirical evidence of asymmetry at the distal end of the reproductive tract comes from the Struthioniformes, Tinamiformes and Anseriformes: these groups have a phallus that tends to bend to the left when erect (King, 1989), corroborating the correlated evolution with female reproductive tract asymmetry (Witschi, 1935). In contrast, there is little information concerning asymmetry of lower reproductive structures in Passeriformes (but see Laskemoen *et al.*, 2008 for an intraspecific study). In passerines, the distal end of each ductus deferens coils to form the seminal glomerus, structures that undergo seasonal changes in size in parallel with the testes, sometimes forming a pronounced sexually dimorphic feature – the cloacal protuberance (Wolfson, 1954; 1960; Lake, 1981). Thus, passerines offer an ideal group in which to investigate side-correspondent correlations between testis asymmetry and asymmetries in lower portions of the male reproductive tract.

Sperm competition is a key evolutionary force shaping variation in several primary sexual traits, including testis size and testis histology, sperm morphology, and sperm quality in wide variety of taxa (e.g. Simmons & Fitzpatrick, 2012; Lüpold & Pitnick, 2018), including birds (Briskie & Montgomerie, 2007; Calhim & Birkhead, 2007; Lüpold *et al.*, 2009a,b; Rowe & Pruett-Jones, 2011). In a comparative study covering 67 bird families, higher levels of sperm competition (i.e. relative testes mass) were associated with decreased asymmetry in testes (Calhim & Montgomerie, 2015). This relationship, however, explained just 6% of the variation and was only statistically significant in taxa exhibiting left-biased directional asymmetry. Importantly, while broad comparative studies can be extremely informative, the large variation in selection pressures across such taxonomically diverse levels (i.e. different orders/families), as well as errors

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3 86 associated with diverse sources of data, can mask important biological patterns. Thus, while the sperm
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5 87 competition hypothesis has received some support, there is much scope for additional studies.
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9 88 In passerines, the degree and direction of directional testis asymmetry also varies within-species (reviewed
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11 89 in Calhim & Birkhead, 2009), and the former has been found to positively correlate with both age (e.g.
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13 90 Birkhead *et al.*, 1997; Merilä & Sheldon, 1999; Graves, 2004) and the expression of male secondary sexual
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15 91 characters (Møller, 1994). Møller (1994) suggested that the more symmetrical testes in lower quality males
16
17 92 reflected the compensatory growth of the typically smaller testis to compensate for the stunted growth and
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19 93 thus assumed (anatomical or physiological) malfunction of the typically larger testis (Domm & Juhn, 1927;
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21 94 Calhim & Birkhead, 2009). This pattern, however, was not observed in subsequent studies (reviewed in
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23 95 Calhim & Birkhead, 2009).
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27 96 Due to potentially diverging evolutionary histories and selection pressures across avian families (Calhim
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29 97 & Montgomerie, 2015), a useful alternative approach is to examine primary reproductive trait asymmetry
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31 98 variation within a limited taxonomic group exhibiting similar life-histories and ecology. The monophyletic
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33 99 family Maluridae, which shows considerable variation in sperm competition levels and in directional testis
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35 100 asymmetry, is emerging as a model system to study the evolution of avian mating systems and sperm
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37 101 competition (Cockburn *et al.*, 2013; Rowe & Pruett-Jones, 2013). Malurids are **small (c. 5–40 grams)**,
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39 102 insectivorous passerines, and species tend to be similar to each other in general morphology and life-history
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41 103 traits (e.g. long-lived, non-migratory, dome nests). Additionally, all species are known, or believed, to
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43 104 exhibit cooperative breeding with sons and sometimes daughters remaining on their natal territory to assist
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45 105 parents in raising subsequent offspring (Rowley & Russell, 1997). **Finally, malurids exhibit** considerable
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47 106 variation in potential sexual selection pressures and the intensity of sperm competition: despite being
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49 107 socially monogamous, relative testes size varies ten-fold across species (c. 0.44 – 4.45% of male body
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51 108 mass; Rowe & Pruett-Jones, 2013) and the occurrence of extra-pair paternity (EPP) ranges from 6% to 95%
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53 109 of broods (Mulder *et al.*, 1994; Kingma *et al.*, 2009; Rowe & Pruett-Jones, 2013). Within the Maluridae,
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55 110 the fairy-wrens (*Malurus* sp.) have received the most attention to date, likely due to their ease of study and
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57 111 strong sexual selection pressures, suggested by their wide range in EPP rates and pronounced sexual
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dimorphism. This genus also shows pronounced plumage divergence among species (Johnson, Price, & Pruett-Jones, 2013), and in some cases variation in male plumage coloration within a species. The red-backed fairy-wren is a clear example of a malurid with intraspecific variation in plumage colour (Karubian, 2002), and high levels of EPP (c. 63% of broods contain extra-pair young; Webster, Varian, & Karubian, 2008). Breeding male plumage types are associated with differences in male mating effort and behaviors: red/black males, which are more attractive to females (Karubian, 2002), invest in EPP seeking behaviors, whereas brown males invest in physical and acoustic mate guarding (defensive role in paternity; Karubian, 2002; Dowling & Webster, 2017). Consequently, males are likely to face different sperm competition levels: cuckolding red/black males in an offensive role for paternity, whereas brown males can behaviorally prevent (e.g. mate guard) or reduce (e.g. frequent copulations) sperm competition in a defensive role. Finally, helper males gain some limited paternity (Webster *et al.*, 2008), always in offensive role. The male types also differ in investment in gonads, as red/black males tend to have relatively larger testes compared to brown males, and more so compared to helper males (Rowe *et al.*, 2010). Therefore, this species is the ideal complement to the interspecific analyses, allowing the corresponding assessment at the intraspecific level.

Here, we investigate the degree of asymmetry across primary reproductive traits in male birds, using malurids as a case study. Our main aim is to assess the roles of side-correspondent asymmetry and sperm competition at both intra- and interspecific scales (Fig. 1A). This way, we can investigate the idea that patterns of asymmetry may incorporate aspects of male reproductive anatomy beyond testis asymmetry, and thus extend our knowledge of primary sexual trait asymmetry in studies of reproductive investment in animals.

Materials and Methods

Data collection

Field methods

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3 136 Populations of ten species of Maluridae were studied at several sites across Australia between 2004–2006
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5 137 and in 2011: superb fairy-wrens (*M. cyaneus cyanochlamys*) at Murray River National Park, South
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7 138 Australia (140°32'E, 34°20'S); splendid (*M. splendens melanotus*), variegated (*M. lamberti assimilis*), and
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9 139 white-winged (*M. leucopterus leuconotus*) fairy-wrens at Brookfield Conservation Park, South Australia
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11 140 (139°29'E, 34°20'S); blue-breasted fairy-wrens (*M. pulcherrimus*) at Lincoln National Park, South Australia
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13 141 (135°52'E, 34°52'S); red-backed fairy-wrens (*M. melanocephalus*) at Moomin Reservoir and Kalinvale
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15 142 Farm, near Herberton, Queensland (145°23'E, 17°23'S); red-winged fairy-wrens (*M. elegans*) near
16
17 143 Manjimup, Western Australia (116°08'E, 34°15'S); lovely fairy-wrens (*M. amabilis*) near Julatten,
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19 144 Queensland (145°20'E, 16°38'S); southern emu-wrens (*Stipiturus malachurus malachurus*) near Smith's
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21 145 Lake, New South Wales (152°28'E, 32°22'S); and striated grasswrens (*Amytornis striatus striatus*) at
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23 146 Pooginook and Cooltong Conservation Parks, near Berri, South Australia (140°35'E, 34°16'S).
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28 147 Adult birds were trapped using mist nets and song playback. Upon capture, birds were weighed (± 0.1 g)
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30 148 using a Pesola spring balance and then euthanised in accordance with animal ethics approvals, and we
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32 149 dissected out the testes and seminal glomera. Only males in peak breeding condition, indicated by
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34 150 behavioral, morphological or physiological characteristics (e.g. breeding plumage, enlarged cloacal
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36 151 protuberance, active spermatogenesis) were included in the study.
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40 152 *Reproductive tract data*
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42 153 We measured the length, width and fresh mass (± 0.01 g) of each testis and fresh mass of each seminal
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44 154 glomerus separately. Testes were then fixed in 6–10% neutral buffered formalin for 24 hours before being
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46 155 stored in 70% ethanol. To determine the total number of sperm stored in each seminal glomerus, sperm
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48 156 were flushed from each glomerus separately into a known volume of buffer (phosphate buffered saline in
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50 157 2011, otherwise Lago Formulation Avian Semen Extender, Hygieia Biological Laboratories, USA), and
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52 158 sperm numbers were quantified using a Makler counting chamber (Irvine Scientific, USA). We replicated
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54 159 the counts using separate aliquots of the diluted sperm sample (Nakagawa & Schielzeth, 2010), and
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56 160 calculated an average sperm count based on the two replicates. All samples were mixed thoroughly before
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58 161 counting. We also quantified the relative amount of spermatogenic tissue in each testis using standard
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60 162 histological techniques and image analysis (Rowe & Pruett-Jones, 2011). Briefly, for each of four non-

sequential sections, we measured the proportion of seminiferous tubule tissue (relative to interstitial tissue) using Image-J software and calculated the proportion of tissue per testis (i.e. left and right testis separately) by averaging the values from the four sections.

Asymmetry measures

Asymmetry measures for all reproductive traits were calculated as a scale-free index (Palmer & Strobeck, 2003) by taking the natural log from the ratio of the left-side measurement by the right-side measurement. For example, testis size (i.e. mass) asymmetry (TA) = natural log (left testis mass [g]/right testis mass [g]). We also calculated asymmetry index for seminal glomerus mass (SGA), seminal glomerus sperm numbers (SGSNA), and proportion of sperm producing tissue in the testis (SPTA). Testis shape asymmetry (TAshape) is also a scale-free index that reflects differences in the degree of elongation in the anterior-posterior plane that was calculated as the difference between natural log (left testis length/right testis length) and natural log (left testis width/right testis width). The absolute value of these scale-free indices reflects the degree (i.e. the difference between the two sides) and their sign determines the bias (i.e. which side is larger or longer), with positive and negative values corresponding to left and right bias, respectively. Species-specific asymmetry indices were obtained by averaging asymmetry indices from all individuals belonging to a species. The latter calculation led to some discrepancies between our data and the values presented in Calhim and Montgomerie (2015). In that study, species-specific asymmetry indices were calculated from species-level mean left and right side testis sizes, thus those values ignored intraspecific-level variation. We present results using both approaches and show that they do not qualitatively affect our results (Table 1 and Supplementary Table S1).

Gizzard size

Gizzard size data for five species: short-tailed grasswren (*Amytornis merrotsyi merrotsyi*), blue-breasted fairy-wren, variegated fairy-wren, white-winged fairy-wren, and superb fairy-wren (subspecies *leggei*) were obtained from the South Australian Museum. Gizzard volume was calculated from the available linear dimensions of preserved specimens, assuming an ellipsoid shape: $\text{volume} = 4/3 * \pi * (\text{length}/2) * (\text{width}/2)^2$. In our use of these data, we assumed the differences in gizzard size across subspecies in the superb fairy-wren to be negligible. Similarly, in the absence of gizzard size data for the striated grasswren, the short-tailed

grasswren was used. Though we fully acknowledge the potential error associated with these data, we believe it is an acceptable compromise, since until recently these two taxa were classified as the same subspecies and they share similar ecology and mating system (Christidis, 1999).

Statistical analysis

Interspecific patterns were analyzed taking into account the phylogenetic relationships among species, which accounts for the expectation that closer relatives will be more similar to each other due to shared evolutionary history (Felsenstein, 1985; Harvey & Pagel, 1991). We used a generalized least-squares approach within a phylogenetic framework (‘pgls’ function) using the packages ‘caper’ (Orme *et al.*, 2013) and ‘ape’ (Paradis, Claude, & Strimmer, 2004) in R v. 3.5.2 (R Foundation for Statistical Computing, 2012). We used the molecular phylogeny of Driskell *et al.* (2011), choosing this phylogeny over other available phylogenies (e.g. Gardner *et al.*, 2010; Lee, Joseph, & Edwards, 2012) because of the availability of data on branch lengths and because the main difference between available phylogenies concerns the positioning of the purple-crowned fairy-wren, which is not included in our study. As well as intercept and slopes, this analysis produces the phylogenetic scaling parameter λ (an index between 0 and 1) and its 95% confidence level (CL). This parameter λ represents the degree of phylogenetic dependency in correlations among traits: if $\lambda=1$, traits covary in direct proportion to their shared evolutionary history; while if $\lambda=0$, (i.e. trait coevolution is independent of phylogeny; Freckleton, Harvey & Pagel, 2002).

We were interested in testing and comparing non-exclusive yet alternative evolutionary paths to explain interspecific patterns of asymmetry using four aspects of male reproductive tract morphology: testis mass, proportion of sperm producing tissue, seminal glomerus mass, and number of sperm stored therein. We investigated the associations between up and downstream traits (Fig. 1A paths 1–5, i.e. side-correspondence), and the role of sperm competition (Fig. 1A paths 6–9). The strength of sperm competition was estimated as relative testes mass, which we modelled by including both combined testes mass and body mass as covariates in our models. This is a commonly used index in avian comparative studies (e.g. Calhim *et al.*, 2007; Lüpold *et al.*, 2009a), including Maluridae (e.g. Rowe & Pruett-Jones, 2013). In order to directly compare path strengths (i.e. different models), we standardized all variables (i.e. subtracted the

mean and divided by the standard deviation) prior to analyses. This way, model coefficients correspond to standardized effect sizes, reflecting estimated unit change in the dependent variable per unit change in the independent variable, where the unit is standard deviations.

The role of gizzard size on testis size and shape asymmetry was also tested using pgls analyses as described above. However, since these relationships are not part of the path analyses in Fig. 1, standardized coefficients were not required. Instead, we mean-centered the predictor variables so that the intercept estimate reflects the asymmetry value predicted at the average gizzard size.

For the intraspecific patterns in reproductive asymmetry, we repeated the same analyses as for the interspecies patterns (Fig. 1A). Phylogenetically controlled analyses were not needed, however, as these data were from a single species – the red-backed fairy-wren. Instead, we used general linear models and model plots to determine if the model assumptions were met. Importantly, this approach allowed us to identify statistical outliers, which were then removed and the analyses repeated. We present the results with the complete dataset, since (i) no qualitative differences were found between models with and without outliers, (ii) close inspection of these ‘outliers’ showed no biological reason for their removal, and (iii) samples sizes were already small. **In these analyses, we used the reproductive morph of the male as the index of sperm competition.** Specifically, we categorized males into three morphs based on plumage coloration and behavior: breeding males (red/black vs. brown) and non-breeding helper males. Plumage color was determined by scoring the percentage of the male’s body that was red/black versus brown **following Karubian (2002) and Lindsay *et al.* (2009).** In general, and in the case of individuals utilized in this study, these categories were discrete and unmistakable (Webster *et al.*, 2008). The helper male morph was set as the reference level in all statistical analyses, except those that included asymmetry in proportion of sperm producing tissue in the testis (SPTA), as we had no data for individuals in this group.

Ethical statement

All work was undertaken with approval from the appropriate agencies, including the University of Chicago Animal Care and Use Committee (#71453), the Department of Environment and Heritage (South Australia) Wildlife Ethics Committee (Project# 13/2004; Scientific Permit Q24832; AW licence# 142), the Director-

General of New South Wales Department of Primary Industries Animal Care and Ethics Committee (06/3846; NSW NPWS scientific licence# S12048), the Department of Environment and Conservation (Western Australia; Regulation 17 licence# SF008305; Regulation 4# CE003378), James Cook University Animal Ethics Review Committee (A1004 and A1691), the Environmental Protection Agency (EPA) of Queensland, and the Department of Environment and Resource Management (Queensland; Scientific Purposes Permit# WISP09844011). Finally, export of samples from Australia was approved by the Australian Government Department of Environment and Heritage (WT2005-10120, WT2006–10958 and WT2012–10) and in accordance with Nagoya protocols.

Results

Overview of reproductive tract asymmetry

There was considerable inter- and intraspecific variation in asymmetry in all traits except the proportion of sperm producing tissue (see Supplementary Tables S2, S3). The southern emu-wren showed the largest intraspecific range in testis size asymmetry (-0.56 – 0.47) and in proportion of sperm producing tissue (-0.0118 – 0.0130). This species also showed considerable variation in other asymmetry measures, making it the most variable species in our dataset. The least variable species on average across all asymmetry measures was the superb fairy-wren.

Interspecific patterns

We found that a larger right testis was associated with a larger investment in the right seminal glomerus tissue (path 1/Table 1 and Fig. 1B; Fig. 2A). No other side-correspondent asymmetry paths were statistically significant, although the trend was towards positive associations (paths 2–5/Table 1 and Fig. 1B). We also found a significant negative association between relative testis size and testis size asymmetry (path 6/Table 1 and Fig. 1B; Fig. 2B). Note that sperm competition explains two thirds of the variation in testis size asymmetry within the Maluridae (cf. 6% across 67 avian families; see Calhim & Montgomerie 2015) In addition, there was a trend towards asymmetry in the number of sperm stored in the seminal glomerus (path 8/Table 1 and Fig. 1B; Fig. 2C). Thus, as the level of sperm competition increased,

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

In contrast, we found no significant relationship between gizzard volume and either testis size or shape asymmetry, once body mass was accounted for (Table 2). However, in our limited sample, there was a trend for larger gizzards to be associated with a relatively larger right testis (Supplementary Fig. S1A) and more elongated left testis (Supplementary Fig. S1B). However, this effect is likely due to a single species (i.e. variegated fairy-wren) and as such these findings should be interpreted with caution.

Intraspecific patterns

We found no evidence of side-correspondent asymmetry in red-backed fairy-wrens (paths 1–5/Table 3). However, we found that both types of breeding male showed, on average, symmetric sperm numbers in their seminal glomera, while helpers exhibited considerable left bias (Table 3, Fig. 3, Supplementary Table S3). Helpers also had significantly lower total number of stored sperm in the seminal glomera (helper mean $\pm$ SD sperm count $\times 10^6$: 26.18 ± 15.98) relative to brown breeders (108.62 ± 78.11 ; linear model estimate mean $\pm$ s.e. = 82.44 ± 33.84 , $t=2.44$, $p=0.030$), although not significantly lower relative to red/black breeders (69.72 ± 29.83 ; estimate = 43.54 ± 33.84 , $t=1.29$, $p=0.22$). Although time of sampling had no effect on breeding male asymmetry in numbers of sperm stored across species (Supplementary Table S4 and Supplementary Fig. S2A), the helper vs. breeder morphs differences within the red-backed fairy-wren are only present later in day (Supplementary Table S5 and Supplementary Fig. S2B).

Discussion

Directional asymmetry in testis size is widespread in birds, and varies from the strong left-side bias of the Glaucous-winged gull (*Larus glaucescens*) to the extreme right-side bias in the African black coucal (*Centropus grilii*), in which males lack the left testis entirely; though left bias in testis size is the common pattern observed across bird species (c. 75% of cases; Calhim & Montgomerie, 2015). Here, we report considerable interspecific variation in the direction and degree of testis size and shape asymmetry within a

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3 291 single avian family, the Maluridae. Moreover, we found, for the first time, that asymmetry in reproductive
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5 292 tissues extends beyond testes size to include asymmetry in seminal glomera mass and the number of sperm
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7 293 stored therein. In contrast, we found no evidence for asymmetry in the amount of sperm producing tissue
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9 294 contained within the testis.
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13 295 We observed considerable intraspecific variability among species in the degree of asymmetry in testis size
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15 296 and shape, in seminal glomera size, and in the number of sperm stored therein. Broadly speaking, the
16
17 297 southern emu-wren and superb fairy-wren were, respectively, the most and the least variable species.
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19 298 Concurrently, the southern emu-wren has the lowest rates of extra-pair paternity and relative testes mass,
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21 299 while the superb fairy-wren has the highest rates of extra-pair paternity reported for the species in the
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23 300 current study (Rowe & Pruett-Jones, 2013). Though further work is clearly needed before firm conclusions
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25 301 can be drawn, this pattern may suggest that the intense selection imposed via sperm competition in the
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27 302 superb fairy-wren may reduce variation among males in a population such that all males are closer to a
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29 303 species-specific ‘optimum’ investment in sperm production. Such a pattern would be similar to that
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31 304 observed for sperm morphology, for which the level of sperm competition is associated with reduced
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33 305 intermale variation in sperm size and shape in birds (Calhim, Immler, & Birkhead, 2007; Kleven *et al.*,
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35 306 2008; Støstad *et al.*, 2018) and other taxa (e.g. Fitzpatrick & Baer, 2011).
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40 307 Across species, we found that covariance between testis size asymmetry and relative testes mass (i.e. our
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42 308 proxy of sperm competition) shifted from being left-biased in species with relatively low relative testes
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44 309 mass to right-biased in species with high relative testes mass. In a larger comparative study of passerine
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46 310 birds, Calhim and Montgomerie (2015) found that higher sperm competition was associated with more
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48 311 symmetry in testis size, although this pattern was only statistically significant among taxa with a larger left
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50 312 testis. Relative testes size, sperm production, and levels of extra-pair paternity in Malurids are among the
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52 313 highest reported in passerine birds (Tuttle & Pruett-Jones, 2004; Rowe & Pruett-Jones, 2006; 2011; *cf.*
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54 314 Griffith, Owens, & Thuman, 2002; Rowe & Pruett-Jones, 2013). Thus, our findings provide an interesting
55
56 315 contrast to previous work examining the role of sperm competition in shaping testes asymmetry across taxa.
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59 316 Specifically, our study suggests that some malurid species may respond to the intense selective pressure of
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sperm competition by moving beyond testis size symmetry to right-biased asymmetry. Perhaps in small passerines with relatively large gizzards (c. 4% of body mass for Maluridae; passerine gizzard mass index: $\text{mean} \pm \text{s.e.} = 3.04 \pm 0.17$, $n=22$; Calhim & Montogomerie 2015) and strong selective pressure for larger combined testes size, more testicular tissue can only be achieved by growing the less space-constrained right testis. In fact, the degree of right-bias in testis size asymmetry values in malurids are among the most extreme values reported for birds (*cf.* Calhim & Montogomerie, 2015). It may therefore be particularly interesting to investigate if the same pattern occurs in other right-bias testis asymmetry clades with high rates of extra-pair paternity, such as Hirundinidae or Fringillidae, and whether these clades also have a relatively large gizzard.

Closer examination of the interspecific patterns provides some interesting contrasts. First, white-winged fairy-wrens have both the largest relative testes size and the strongest right-bias in testes asymmetry. This species exhibits a somewhat atypical 'clan' mating system (Rowley & Russell, 1995), in which younger males hold territories and are socially pair-bonded to a female, but exhibit female-like, brown plumage (Pruett-Jones, unpublished data). Superimposed on these individual territories is the territory of a single male in full nuptial plumage (i.e. blue/white plumage), and this male interacts with pairs occupying individual territories within his 'clan' (Rowley & Russell, 1995). Although this complicates our understanding of extra-pair mating in this species, the occurrence of female multiple mating is undoubtedly high in this species and thus strong selection imposed via sperm competition may explain the extreme values we observed. Moreover, while the splendid and superb fairy-wren have right-bias in testes asymmetry and high rates of extra-pair paternity (42-72% extra-pair offspring; Rowe & Pruett-Jones, 2013), the red-winged fairy-wren shows left-bias asymmetry in testes mass despite comparably high rates of extra-pair paternity (57% extra-pair offspring; Brouwer *et al.* 2011; Rowe & Pruett-Jones, 2013). Similarly, relative testes mass is not particularly high in that species, which may suggest that selection on increasing sperm numbers (i.e. through increases in tissue devoted to sperm production) is not especially strong in the red-winged fairy-wren relative to that experienced by other high extra-pair paternity species; instead, additional traits may be more closely linked to male fertilization success (e.g. sperm length), though such

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3 343 ideas remain to be tested. Nonetheless, overall our findings offer further support for the idea that sperm
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5 344 competition can shape testes asymmetry in passerine birds.
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9 345 Our results also shed light on the other hypotheses relating to interspecific variation in avian testis
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11 346 asymmetry. First, we found no asymmetry in the proportion of sperm producing tissue in the testes at either
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13 347 the inter- or intraspecific level. Second, in all individuals both the left and right testes appeared to be in a
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15 348 state of full spermatogenesis (stage 6 *sensu* Scott & Middleton, 1968). Together, this suggests that the two
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17 349 testes are functionally equivalent, which contradicts the premise of the compensation hypothesis (Møller,
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19 350 1994), namely that one testis is malfunctioning. The packaging hypothesis suggests that asymmetry in male
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21 351 gonads reflects space constraints within the body cavity due to the positioning of organs such as the liver
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23 352 (Witschi, 1935) and the gizzard (TR Birkhead, pers. comm.). We found that species with a relatively large
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25 353 gizzard tended to have a more elongated and relatively smaller left testis. Although we acknowledge that
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27 354 our results should be interpreted with extreme caution, these findings are consistent with the packaging
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29 355 hypothesis and suggest that further studies investigating the role of space constraints in shaping testes
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31 356 asymmetry would help elucidate the evolutionary causes of gonad asymmetry in birds. Our findings also
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33 357 provide limited support for the key assumption behind the copulatory bias hypothesis (Delehanty &
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35 358 O'Hearn, 2005), where a strong side-correspondence in reproductive tract asymmetry is expected. We found
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37 359 a significant interspecific positive association between testis and seminal glomerus size directional
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39 360 asymmetries, but this relationship did not extend to the proportion of sperm producing tissue in the testis
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41 361 or to sperm numbers stored in the seminal glomerus.
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46 362 We found little evidence for a role of sperm competition in explaining intraspecific variation of
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48 363 reproductive organ asymmetry. Specifically, asymmetry in reproductive organs did not differ between the
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50 364 male reproductive phenotypes in the red-backed fairy-wren, with the exception of the number of sperm
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52 365 stored in the seminal glomerus. In this instance, helpers exhibited strong left-bias in the number of stored
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54 366 sperm, which stands in stark contrast to values observed in brown and red/black breeders. In general,
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56 367 helpers **appear to invest** relatively little in sperm production relative to breeders in the red-backed fairy-
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58 368 wren (Rowe *et al.*, 2010) and, in this study, helpers had considerably fewer sperm in storage (1/3–1/4 of
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breeder values). Helpers also appear to obtain few mating opportunities relative to breeders, as helpers sire a low proportion of the extra-pair young in a population (Webster *et al.*, 2008). However, why this should translate into left-bias asymmetry in numbers of stored sperm is unclear. One could speculate that the few copulations helpers achieve are right-sided (in agreement with the copulation bias hypothesis for a species with right biased testis size asymmetry), and their overall low sperm production did not replenish the right seminal glomerus. **The observation that helper vs. breeder differences are only clear in individuals sampled later in the day further supports this hypothesis.** However, given our small sample size for helper males, it is plausible that this result constitutes a type I error, though the magnitude of the difference between helpers and breeders might suggest otherwise.

The major contribution of our study is that we show that patterns of asymmetry in reproductive organs extend beyond testes asymmetry to incorporate multiple aspects of male reproductive anatomy and physiology. Most notably, we found that species exhibiting a larger right testis also possessed a larger seminal glomerus on the right side, which is consistent with findings at the intraspecific level in the bluethroat (*Luscinia svecica*; Laskemoen *et al.*, 2008) and indicates the size of the seminal glomerus is a function of testis size. Moreover, in addition to predicting a relatively larger right testis, high levels of sperm competition tended to be positively associated with greater number of sperm stored in the right seminal glomerus. In birds, sperm numbers are an important determinant of male fertilization success under competitive mating conditions (Martin *et al.*, 1974; Birkhead, 1998). Additional sperm phenotypic traits, such as sperm size (Bennison *et al.*, 2015) or sperm motile performance (Pizzari *et al.*, 2008), also influence male fertilization success in birds. However, recent theoretical and empirical work suggests that selection imposed through post-copulatory processes (e.g. sperm competition and cryptic female choice) favors increasing sperm numbers rather than sperm size in passerine birds, especially when the risk of sperm competition is very high (Immler *et al.*, 2011). Given that rates of extra-pair paternity are extremely high in many of the malurids studied here, our results suggest that sperm competition can shape testes asymmetry in the Maluridae with consequences for functional traits (i.e. sperm quantity) that influence the outcome of sperm competition and, ultimately, male reproductive success.

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In conclusion, we show that directional asymmetry in male reproductive organs can incorporate multiple aspects of the male reproductive tract. By utilizing a taxonomically restricted set of species, we aimed to reduce potential sources of evolutionary variation in testes asymmetry to a primary variable of interest – the level of sperm competition. This approach allowed us to discover influential patterns in the asymmetry of reproductive traits. Specifically, our findings suggest that sperm competition can shape reproductive asymmetries in the Maluridae with likely functional consequences for male reproductive success, which in turn suggests that extremely high sperm competition may push directional asymmetry of testes size towards a right-bias in testis mass.

Declaration of interest

The authors declare no competing interests.

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Figure Legends

Figure 1. 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.

Figure 2. Scatterplots (mean $\pm$ 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 ($n=6$).

Figure 3. Differences in the asymmetry in the number of sperm in the seminal glomerus (mean $\pm$ s.e.) between the three male morphs in the red-backed fairy-wren.

Tables

Table 1. Comparison of alternative paths to explain interspecific variation in asymmetry along the reproductive tract in malurids.

pathway*	model	coefficient§	estimate	s.e.	t	p	ML lambda	R ²	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.

Table 2. Relationships between relative gizzard size and testis asymmetry in size and shape in malurids, 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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3 589 **Table 3.** Comparison of alternative paths to explain intraspecific variation in asymmetry along the
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5 590 reproductive tract in red-backed fairy-wrens.
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	pathway*	model	coefficient§	estimate	s.e.	t	p	R ²	n
10	Side-correspondent development								
11	1	SGA ~ TA	intercept	-0.002	0.259	-0.01	0.99	0.002	16
12			TA	0.042	0.237	0.18	0.86		
13	2	SGSNA ~ SGA	intercept	0.000	0.231	0.00	1.00	0.20	16
14			SGA	0.452	0.238	1.90	0.08		
15	3	SGSNA ~ TA	intercept	0.004	0.258	0.02	0.99	0.01	16
16			TA	-0.088	0.237	-0.37	0.71		
17	4	SGSNA ~ SPTA	intercept	-0.504	0.088	-5.73	0.01	0.04	5
18			SPTA	0.034	0.098	0.34	0.75		
19	5	SPTA ~ TA	intercept	-0.047	0.500	-0.09	0.93	0.09	5
20			TA	-0.251	0.464	-0.54	0.63		
21	Sperm competition								
22	6	TA ~ male morph	intercept (helper)	-0.015	0.063	-0.24	0.81	0.07	4
23			vs brown breeder	-0.077	0.076	-1.01	0.32	9	
24			vs red/black breeder	-0.015	0.074	-0.21	0.84	11	
25	7	SGA ~ male morph	intercept (helper)	-0.061	0.126	-0.49	0.63	0.02	4
26			vs brown breeder	-0.092	0.162	-0.56	0.58	6	
27			vs red/black breeder	-0.054	0.162	-0.33	0.75	6	
28	8	SGSNA ~ male morph	intercept (helper)	2.157	0.426	5.07	<0.001	0.63	4
29			vs brown breeder	-2.304	0.55	-4.19	0.001	6	
30			vs red/black breeder	-2.277	0.55	-4.14	0.001	6	
31	9	SPTA ~ male morph	intercept (brown breeder)	0.001	0.001	1.63	0.20	0.42	2
32			vs red/black breeder	-0.001	0.001	-1.46	0.24	3	

37 * Pathway numbers correspond to those outlined in Figure 1.
38 § 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
39 marginally non-significant) effects are shown in bold text. TA=testis size asymmetry, SGA=seminal glomerus size asymmetry, SGSNA=seminal
40 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.

190x275mm (96 x 96 DPI)

Scatterplots (mean $\pm$ 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 $\pm$ s.e.) between the three male morphs in the red-backed fairy-wren.

624x624mm (72 x 72 DPI)

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 Table 1 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 (cm ²)	
	mean	min	max	n	mean	min	max	n	mean	min	max	n	mean	min	max	n	mean	min	max	n	mean	n
Striated grasswren (<i>Amytornis striatus</i>)	-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 (<i>Malurus amabilis</i>)	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 (<i>Malurus cyaneus</i>)	-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 (<i>Malurus elegans</i>)	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 (<i>Malurus lamberti</i>)	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 (<i>Malurus leucopterus</i>)	-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 (<i>Malurus melanocephalus</i>)	-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 (<i>Malurus pulcherrimus</i>)	-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 (<i>Malurus splendens</i>)	-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 (<i>Stipiturus malachurus</i>)	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 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 (Type III SS)	(intercept)	2.47	1	8.09	0.019
	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

Supplementary Figure S1. Relationship between testis asymmetry in (A) size and (B) shape (mean $\pm$ s.e.) and gizzard volume (as a ratio to body mass) in five Maluridae species. Species codes follow those in Figure 2: 2=superb fairy-wren, 4=variegated fairy-wren, 5=white-winged fairy-wren, 7=blue-breasted fairy-wren, 10=striated grasswren.

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.