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Stefan Lupold
Department of Animal and Plant Sciences, University of Sheffield, s.luepold@sheffield.ac.uk

George M. Linz
USDA/APHIS/WS National Wildlife Research Center, george.m.linz@aphis.usda.gov

James W. Rivers
Department of Ecology, Evolution, and Marine Biology, University of California, Santa Barbara, California 93106, rivers@lifesci.ucsb.edu

David F. Westneat
Department of Biology, 101 Morgan Building, University of Kentucky, Lexington, Kentucky 40506, biodfw@uky.edu

Tim R. Birkhead
Department of Animal and Plant Sciences, University of Sheffield, t.r.birkhead@sheffield.ac.uk

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SPERM COMPETITION SELECTS BEYOND RELATIVE TESTES SIZE IN BIRDS

Stefan Lüpold,[1,2] George M. Linz,[3,4] James W. Rivers,[5,6] David F. Westneat,[7,8] and Tim R. Birkhead[1,9]

[1]*Department of Animal and Plant Sciences, University of Sheffield, Western Bank, Sheffield, S10 2TN, United Kingdom*

[2]*E-mail: s.luepold@sheffield.ac.uk*

[3]*USDA/APHIS/WS National Wildlife Research Center, 2110 Miriam Circle, Bismarck, North Dakota 58501*

[4]*E-mail: george.m.linz@aphis.usda.gov*

[5]*Department of Ecology, Evolution, and Marine Biology, University of California, Santa Barbara, California 93106*

[6]*E-mail: rivers@lifesci.ucsb.edu*

[7]*Department of Biology, 101 Morgan Building, University of Kentucky, Lexington, Kentucky 40506*

[8]*E-mail: biodfw@uky.edu*

[9]*E-mail: t.r.birkhead@sheffield.ac.uk*

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Sperm morphology varies considerably across taxa, and postcopulatory sexual selection is thought to be one of the main forces responsible for this diversity. Several studies have investigated the effects of the variation in sperm design on sperm function, but the consequences of variation in sperm design on testis morphology have been overlooked. Testes size or architecture may determine the size of the sperm they produce, and selection for longer sperm may require concomitant adaptations in the testes. Relative testes size differs greatly between species and is often used as an index of sperm competition, but little is known about whether larger testes have more sperm-producing tissue or produce sperm at a faster rate. Using a comparative approach in New World Blackbirds (Icteridae), we found (1) a strong link between testis histology and sperm length, suggesting selection on testis architecture through selection on sperm size, and (2) that species under intense sperm competition had a greater proportion of sperm-producing tissue within their testes. These results support the prediction that sperm competition fosters adaptations in reproductive organs that extend beyond testes size, and raise questions about the trade-offs influencing reproductive investment.

KEY WORDS: **Icteridae, New World Blackbirds, seminiferous tissue, sperm competition, sperm size, testis architecture.**

Sperm size and shape varies markedly across the animal kingdom (e.g., Cohen 1977; Jamieson 2007; Pitnick et al. 2009). In addition to the mode of fertilization and phylogenetic effects (e.g., Franzén 1970), postcopulatory sexual selection, comprising sperm competition (Parker 1970) and cryptic female choice (Miller and Pitnick 2002; Snook 2005), is thought to be one of the main selective forces responsible for this variation (e.g., Gage 1994; LaMunyon and Ward 1999; Anderson and Dixson 2002; Immler and Birkhead 2007).

Studies seeking to explain the variation in sperm morphology have generally focused on sperm function, such as sperm velocity (e.g., Gomendio and Roldan 1991; Malo et al. 2006). Faster sperm are more likely to fertilize eggs in the context of sperm competition (e.g., Moore and Akhondi 1996; Birkhead et al. 1999; Froman et al. 1999; Gage et al. 2004; Malo et al. 2005) and theoretical models suggest that sperm velocity may be determined by sperm traits (Katz et al. 1989; Cardullo and Baltz 1991). Based on these models, several studies have tested whether sperm competition intensity influences the size of sperm midpiece and flagellum, or total sperm length. Whereas sperm competition appears to be positively associated with sperm design in some taxa (Gage 1994; Balshine et al. 2001; Anderson

and Dixson 2002; Byrne et al. 2003), in others there is either a negative or no relationship (Gage and Freckleton 2003; Immler and Birkhead 2007), indicating that there is no universal pattern across taxa.

Another possibility is that sperm size is selected for by coevolution with sperm-storage structures within the female reproductive tract, for which there is evidence in various taxa (e.g., Dybas and Dybas 1981; Briskie and Montgomerie 1992; Pitnick and Markow 1994; Miller and Pitnick 2002; Simmons and Kotiaho 2007). Postcopulatory sexual selection can therefore affect sperm design in different ways, resulting in enormous variation in sperm size and shape but often inconsistent patterns across different taxonomic groups.

An unexplored implication of selection on sperm design, however, is whether or not the testes show associated variation. Although in many invertebrates spermatogenesis occurs in testicular follicles (Blum 1970), the sperm-producing structures of vertebrates are the seminiferous tubules: long, highly convoluted tubules within the testes (Huber 1916; Lake 1957; Gier and Marion 1970). In their cross-section, seminiferous tubules consist of a central lumen and a ring of seminiferous epithelium, in which sperm develop from the periphery toward the central lumen before being passed into the efferent ducts (e.g., Courot et al. 1970; Aire 2007b). Because the elongated spermatids and spermatozoa are directed radially within the tubules, with the sperm tails directed toward the lumen (e.g., Aire 2007b), we might expect (particularly in passerine birds in which sperm are fairly rigid) that longer sperm require a thicker epithelium and wider tubules, resulting in positive covariation between seminiferous tubule diameter and sperm length, across species. However, whether testes show such adaptations in response to selection for longer sperm has not previously been assessed.

By contrast, it has been known for over half a century and demonstrated across various taxa that larger testes tend to produce more sperm per unit of time than smaller testes (Willett and Ohms 1957; Amann 1970; de Reviers and Williams 1984; Schärer et al. 2004), although among *Drosophila* species those with relatively large testes produce fewer but longer sperm than others (Pitnick and Markow 1994; Pitnick 1996). Because the outcome of sperm competition is largely determined by sperm numbers (e.g., Martin et al. 1974; Martin and Dziuk 1977; Parker 1982), selection favors larger testes relative to body size both between (Harcourt et al. 1981; Kenagy and Trombulak 1986; Kusano et al. 1991; Gage 1994; Hosken 1997) and within species (e.g., Hosken and Ward 2001), and relative testes size is often used as an indirect measure of sperm competition risk (Møller 1991; Briskie and Montgomerie 1992; Dunn et al. 2001; Pitcher et al. 2005; Calhim and Birkhead 2007).

Although larger testes produce more sperm per unit time, the daily sperm production (DSP) rate of a testis may depend not only on the amount of seminiferous tissue, but also on (1) the number of sperm produced per unit of seminiferous tissue and (2) the speed at which individual sperm cells are produced (Amann 1970, 1981; further details in Discussion). It is unknown whether the higher sperm production rate of larger testes is higher because they contain more or denser sperm-producing tissue, because individual sperm cells develop faster, or a combination of these factors. In primates, the number of sperm produced simultaneously per unit of parenchyma (i.e., spermatogenic efficiency; e.g., Wistuba et al. 2003) does not differ between species relative to their levels of sperm competition (Wistuba et al. 2003; Luetjens et al. 2005). Similarly, the duration of the spermatogenic cycle, which dictates the speed of sperm production (Amann 1970), also appears to be independent of sperm competition in primates, although based on only a few species (J. Wistuba, pers. comm.). If the cycle length is also unrelated to sperm competition, this, together with the lack of an association between sperm competition and spermatogenic efficiency, suggests that it may be the amount of sperm-producing tissue that best accounts for the variation in the DSP rate reported across primates (e.g., Amann et al. 1976; Amann and Howards 1980).

The amount of sperm-producing tissue in a single testis can vary (1) solely in the overall size of the testis, (2) in the proportion of seminiferous tissue relative to somatic tissue within the testis, or (3) both combined. Compared to the ample evidence for an increase in relative testes size (i.e., combined testes mass [CTM] corrected for body mass) with sperm competition, the proportions of testicular components in relation to sperm competition remain unexplored. The testes consist of an outer capsule around the parenchyma, which is generally rather thin (Lake 1971) but differs considerably in thickness across species (Aire 2007a). Additionally, the parenchyma consists largely of the seminiferous tubules and the interstitial tissue, with the latter containing blood vessels, Leydig cells, lymphatic space, and conjunctive tissue (e.g., Lofts and Murton 1973; Tae et al. 2005). Finally, the seminiferous tubules themselves consist of seminiferous epithelium and a lumen of variable size. Sperm are produced only in the seminiferous epithelium. Hence, testes of the same size may differ considerably in the actual amount of sperm-producing tissue. In a comparative study of six rodent species, the volumetric proportion of seminiferous tubules ranged between 33% and 92% (Russell et al. 1990). An even more extreme situation appears to exist in another rodent, the capybara (*Hydrochoerus hydrochaeris*), in which the proportion of seminiferous tissue may be less than 10% (Moreira et al. 1997). These examples indicate that testes size alone may not be the most meaningful index of the amount of sperm-producing tissue. Hence, it must be established whether sperm competition influences the proportion of sperm-producing tissue in the testes. Such information is lacking, particularly in birds, in which to date information on testicular

architecture is largely restricted to a few domestic species (see Aire 2007a).

The aims of our study were to test whether sperm size was associated with the size of the seminiferous tubules and whether testis design was related to the level of sperm competition, using the New World Blackbirds (Icteridae) as our main study group and the Old World Warblers (Sylviidae) for a comparison between families. These two families show different selection on sperm length, with sperm length increasing with sperm competition in the Icteridae (Lüpold et al., unpubl. ms.) but decreasing in the Sylviidae (Immler and Birkhead 2007). Using the between-family comparison, we tested whether our results were independent of the direction of selection on sperm size.

Methods

STUDY SPECIES

The New World Blackbirds comprise over 100 species that vary considerably in their mating systems (e.g., Jaramillo and Burke 1999). Their phylogeny is well established, allowing us to control for phylogenetic effects (species and phylogeny in Supporting Fig. S1A). Moreover, sperm length across the icterid species studied so far ranges between 61 and 145 μm and is positively associated with sperm competition risk (Lüpold et al., unpubl. ms.). To test whether our results were independent of the direction of selection on sperm size, we compared the Icteridae with the Sylviidae, a family in which sperm length decreases with increasing sperm competition risk (Immler and Birkhead 2007; species and phylogeny in Supporting Fig. S1B). We hypothesized that if sperm length is linked to the size of the seminiferous tubules, tubule size should also be positively associated with sperm competition in the Icteridae, but negatively related in the Sylviidae. Conversely, selection for more sperm (or more seminiferous tissue) in species under intense sperm competition should result in an increasing amount of seminiferous tissue relative to testes size in both taxa, irrespective of the direction of the correlation between sperm size and sperm competition.

SAMPLE COLLECTION

From the Icterids, we used freshly fixed testes from birds collected in breeding condition in North America and South America as part of museum collection, management programmes, or research projects. All birds were collected under license of the respective museums and other institutions and, within the United States, under an additional collective the U.S. Fish and Wildlife Service scientific collection/export permit. We also obtained four species from a formalin-fixed museum collection. In total, we used 1–12 (mean = 3) males from each of 21 different species of Icterids. For the comparison with the Sylviidae, we included two males from each of seven sylviid species that had previously been collected (e.g., Immler and Birkhead 2007).

After dissection, the testes and one or both seminal glomera (i.e., sperm-storage organs at the end of the deferent duct) were fixed in 10% formalin. Although other fixatives may yield better fixation for histological analyses, the general use of formalin by museums meant that all specimens were fixed in the same way by the various collectors. Before histological preparation, we measured the length, width, and height of both testes from each male to the nearest 0.1 mm using callipers and weighed them to the nearest 0.001 g using a Mettler AT261 digital balance (Mettler, Greifensee, Switzerland).

We retrieved sperm from the distal region of the seminal glomera of the dissected specimens and collected additional samples from live birds either through cloacal massage (e.g., Burrows and Quinn 1937; Samour et al. 1986), using model females (Pellatt and Birkhead 1994), or from fecal samples (Immler and Birkhead 2005). This resulted in sperm samples of 34 species, including the 21 species from which we also had testes for histology.

TESTICULAR HISTOLOGY

Depending on testes size, we prepared either the entire testes or parts thereof. We dehydrated the samples with an ascending series of alcohols and embedded them in paraffin. Subsequently, we took four to five sections (5-μm thick) across different parts of the testes, with at least 300 μm between sections, and stained them with haematoxylin and eosin.

We captured digital images of the testis sections, using a Spot Insight QE camera (Diagnostic Instruments, Inc., Sterling Heights, MI) mounted onto a Leitz Laborlux S microscope (Vila Nova de Famalicão, Portugal), with a resolution of 0.82 pixels/μm. We took measurements across all testicular sections from each pair of testes, to the nearest 2 px (i.e., 2.4 μm). In the Icteridae, we selected from each testis 40–50 tubule cross-sections that were approximately round (i.e., sectioned perpendicular to the tubule), and 20–30 cross-sections per testis in the Sylviidae due to less material available. For each tubule cross-section we took the following measurements: (1) length and width; (2) area (tracing the circumference of tubules); and (3) the height of the seminiferous epithelium (see Supporting Fig. S2).

To calculate the proportions of the different testis components, we captured the maximum image size viewed under the microscope (1600 × 1200 px, corresponding to 2.86 mm^2 of the original sample) and deducted any areas that were not part of the parenchyma (e.g., capsule, empty spaces around the specimen, or occasional artifacts of the specimen). Within the remaining parenchyma area, we measured all tubule cross-section areas as above, but this time irrespective of their shape, and the area covered by the lumen within each tubule. The seminiferous tissue was simply the difference between total tubule area and the lumen area. We then summed all areas of seminiferous tissue and lumen, respectively, and assigned all tissue between the tubules

(i.e., difference between the total parenchyma area and the total area covered by seminiferous tissue or lumen) to the interstitial tissue. Finally, we calculated the proportions of seminiferous tissue, lumen, and interstitial tissue relative to the total parenchyma area. For further information on the different testis components and their measurements see the Supporting Figure S2.

For the proportions of the above testis components relative to total testis volume, we first measured the height of the testicular capsule in several independent locations of our histological sections and calculated mean capsule height. Subsequently, we computed the testis volume (V_T) as the volume of a regular ellipsoid, using the measured testis length, width, and height, and the volume of the parenchyma (V_P; i.e., all tissue surrounded by the testicular capsule) again as a regular ellipsoid with the same measurements as for V_T, but each shortened by 2× (mean capsule height). Assuming equal proportions of the different testis components throughout the parenchyma, we computed the proportions relative to overall testes size as $p_i \times V_P/V_T$, where p_i is the mean proportion of the corresponding testis component as measured from our images. For the volumetric proportion of the testicular capsule we used $(V_T - V_P)/V_T$. We calculated all these values for each testis before combining the values for the two testes within individual males and finally calculating the species mean from all males within a species.

SPERM LENGTH AND SPERM COMPETITION

From each male, we measured the length of 5–10 morphologically normal and undamaged sperm from digital images with a resolution of 8.5 or 13.6 px/μm, depending on sperm size, and again with an accuracy of 2 px. We then used mean sperm length of each male to calculate mean sperm length for each species.

As an index of sperm competition risk, we used relative testes size by including CTM and body mass (both log-transformed) as independent variables in our analyses, which is preferable to the use of residuals from a regression between the two variables (e.g., García-Berthou 2001). All our results of relative testes size thus include log (body mass) as a covariate, but for simplicity and because the actual results of the covariate are not important in our study, we report only the results of the partial correlation of the corrected CTM, referred to as rCTM (relative CTM).

For five species, for which we had testes from only one to two males in our collection, we compared our measures on overall testes size with data from the literature and from two museum databases (i.e., Field Museum Chicago and Smithsonian National Museum of Natural History), using only data from birds that were likely to be in breeding condition and have fully developed testes given their geographic location, date of collection, and the range of testes sizes in the database. Careful use of testes measures is important to determine levels of sperm competition based on relative testes size (Calhim and Birkhead 2007).

We are aware that using relative testes size as an index of sperm competition in a study like this may be problematic due to potential circularity, particularly in analyses of proportions of testicular components relative to sperm competition. However, due to the lack of information on genetic mating systems (e.g., extra-pair paternity, EPP) in most species or inconsistent definitions of EPP (i.e., proportion of extra-pair young per nest, parent, or population, or proportion of nests containing at least one extra-pair young), we addressed the issue of circularity in two different ways: First, we established across the species, for which data on percentage of extra-pair young are available, whether EPP covaried with rCTM. Using data from *Molothrus ater* (EPP = 4.7%; Alderson et al. 1999), *Dolichonyx oryzivorus* (EPP = 14.6%; Bollinger and Gavin 1991), *Agelaius phoeniceus* (EPP = 28.3%, the mean from three studies; Gibbs et al. 1990; Westneat 1993; Gray 1996), *Icterus bullockii* (EPP = 32%; Richardson and Burke 1999), and *Quiscalus mexicanus* (EPP = 37%; Johnson et al. 2000a), EPP (arcsine transformed) increased significantly with rCTM ($n = 5$; partial $r = 0.97$, $t = 5.40$, $P = 0.03$).

Second, we obtained information on breeding density and social mating system of the icterid species from the literature (mostly from Webster (1992) and Jaramillo and Burke (1999); see online Supporting Fig. S1A). We classified the species into three different categories of breeding density (dispersed, aggregated, and colonial; following Webster 1992), excluding the four cowbird (*Molothrus*) species from analyses of breeding density because as brood parasites they do not build nests. In comparative studies, dispersed breeders have both lower rates of extra-pair fertilizations (e.g., Westneat and Sherman 1997) and relatively smaller testes than birds in denser nesting populations (e.g., Pitcher et al. 2005), thus indicating different levels of sperm competition risk. For the social mating system, we classified the species into five categories: (1) monogamous, (2) largely monogamous (i.e., very low or occasional polygyny), (3) polygynous, (4) lekking/promiscuous, and (5) cooperative. None of the species of our study is considered polyandrous, hence we omitted this category. Due to the relatively small dataset for the number of categories and with only one cooperative breeder and two promiscuous species, we distinguished only between (largely) monogamous (1–2) and polygamous (3–5) in our analyses. Although both breeding density and social mating system are fairly crude measures of sperm competition (e.g., EPP is not considered), they provided somewhat independent estimates of sperm competition risk to rCTM. We thus applied the two categorical measures of sperm competition to further address the main objectives of our study, circumventing the potential circularity caused by the use of rCTM.

STATISTICAL ANALYSES

We conducted statistical analyses using the statistical package R version 2.6.1 (R Foundation for Statistical Computing 2007),

and transformed nonnormal data distributions appropriately to meet the parametric requirements of the statistical models (see below). For the comparison between the Icteridae and Sylviidae, we performed the main analyses independently in both families and were mostly interested in the direction of the slopes. The inverse relationship of sperm length with sperm competition in the Sylviids compared to the Icterids allowed us to establish whether adaptations were associated with the selection on sperm length.

To control for phylogenetic effects, we constructed a phylogenetic tree by combining several published subsets of the icterid phylogeny (details in Supporting Fig. S1A). For the Sylviidae, we used the phylogeny provided by Immler and Birkhead (2007). We then accounted for statistical nonindependence of datapoints by shared ancestry of species (Felsenstein 1985; Harvey and Pagel 1991) using a generalized least-squares (GLS) approach in a phylogenetic framework (Pagel 1999; Freckleton et al. 2002). This approach allows the estimation of the phylogenetic scaling parameter λ, with values of λ close to 0 indicating phylogenetic independence, and λ close to 1 indicating a complete phylogenetic association of the traits. We used likelihood-ratio tests to establish whether the model with the maximum-likelihood value of λ differed from models with values of $\lambda = 1$ or 0, respectively. Superscripts following the λ estimates denote significance levels of these likelihood-ratio tests (first superscript: against $\lambda = 1$; second superscript: against $\lambda = 0$). We will report these results together with the effect size r (i.e., partial correlation coefficient) and 95% noncentral confidence intervals that we calculated from the t-values of the GLS models, following the equations in Nakagawa and Cuthill (2007). Confidence intervals excluding zero indicate statistical significance at the α level of 0.05 (Smithson 2003; Nakagawa and Cuthill 2007). However, for factorial predictors such as mating system or breeding density, we used η^2 (eta squared) to estimate the strength of the associations, with η^2 being the proportion of the total variance attributable to the effect in question (Fisher 1925; Olejnik and Algina 2003).

Results

The testes from all species were densely packed with seminiferous tissue, which comprised 88–96% of the testicular volume, but there were still considerable differences in testis morphology between species, both in the size of individual testis components and in their proportions (Table 1).

TESTIS HISTOLOGY AND SPERM SIZE

Seminiferous tubule size, as measured by their cross-section area, was independent of absolute testes size ($n = 21$; $t = 1.19$, $P = 0.25$, $\lambda < 0.0001^{<0.01;1.0}$; r (95%CI) = 0.27 (−0.19 to 0.60)), but covaried positively with relative testes size ($n = 21$; $t = 3.14$, $P = 0.006$, $r = 0.61$ (0.21 to 0.79), $\lambda < 0.0001^{0.02;1.0}$; Fig. 1A), using GLS models corrected for phylogeny. The association between tubule size and rCTM, however, was likely to be mediated by sperm length, because after including sperm length in the model, tubule size was positively related with sperm length but no longer correlated with rCTM ($n = 21$; sperm length: $t = 3.41$, $P = 0.005$, $r = 0.70$ (0.27 to 0.86); rCTM: $t = 0.45$, $P = 0.66$, $r = 0.13$ (–0.40 to 0.57); $\lambda < 0.0001^{0.02;1.0}$). To test whether these results were confounded by the multicollinearity between the three predictor variables (i.e., sperm length, CTM, and body mass), we calculated the variance inflation factors (VIF; Marquardt 1970; Fox 2002) for all predictors and found no evidence for a severe impact of collinearity (all VIF values between 2.0 and 3.6; i.e., well below the threshold of 10 suggested by Marquardt 1970 or Kleinbaum et al. 1998).

We obtained similar results between tubule size, sperm length, and sperm competition using social mating system or breeding density in the GLS model instead of rCTM. As with rCTM, both these sperm competition indices had no significant effect on tubule size, but sperm length had a very strong effect (results in Supporting Table S1). That is, cross-section area (hence also the diameter) of the seminiferous tubules appears to be primarily linked to sperm length and not sperm competition.

The height of the seminiferous epithelium within the tubules increased with both tubule cross-section area ($n = 21$; $t = 10.14$, $P < 0.0001$, $r = 0.92$ (0.83 to 0.96), $\lambda = 0.88^{0.68;0.22}$) and sperm length ($n = 21$; $t = 6.56$, $P < 0.0001$, $r = 0.84$ (0.65 to 0.91), $\lambda = 1.00^{1.0;0.36}$). In contrast, the area of the lumen within the tubules was independent of tubule size ($n = 21$; $t = -0.07$, $P = 0.94$, $r = -0.02$ (−0.43 to 0.41), $\lambda = 0.33^{0.03;0.27}$) and sperm length ($n = 21$; $t = -0.80$, $P = 0.43$, $r = -0.19$ (−0.55 to 0.27), $\lambda = 0.26^{0.02;0.31}$).

To test whether the driving force of the tubule characteristics is sperm competition or sperm length, we conducted identical analyses in the Sylviidae. Given our results from the Icteridae, we

Table 1. Descriptive statistics of testis morphology in the Icteridae (n=21 species).

Trait	Mean	Range
Absolute measurements		
Combined testes mass (g)	0.83	0.11–3.36
Testicular capsule height (μm)	23.2	16.0–35.0
Seminiferous tubule cross-section ($\times 10^3$ μm^2)	82.1	41.1–125.7
Epithelium height (μm)	118.5	86.1–157.8
Volumetric proportions		
Seminiferous tissue (%)	90.6	87.8–95.7
Interstitial tissue (%)	4.4	1.7–9.4
Lumen (%)	3.1	0.9–6.8
Testicular capsule (%)	1.9	1.0–3.6

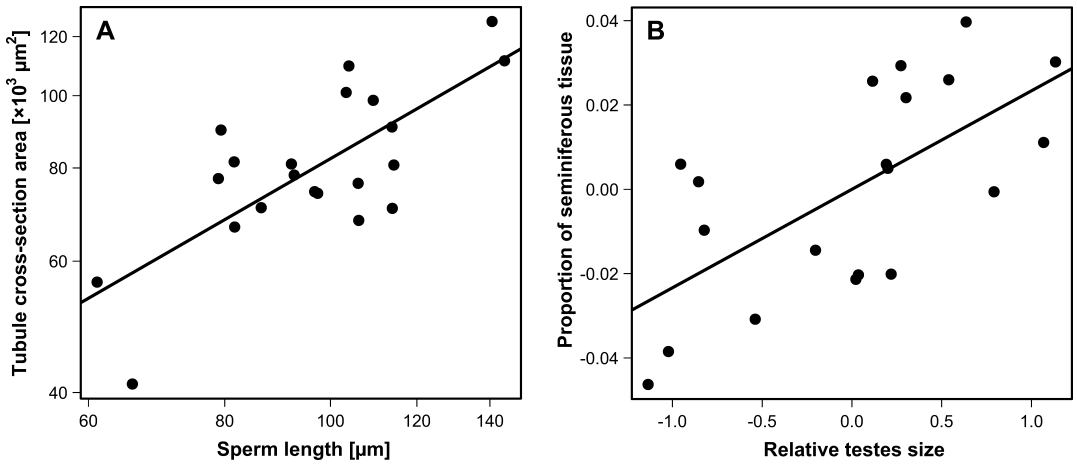

Figure 1. **Interspecific relationships across the Icteridae (A) between the cross-section area of the seminiferous tubules and sperm length ($r = 0.82$, $P < 0.0001$), and (B) between the proportion of sperm-producing tissue and relative testes size (shown as a partial residual plot; $r = 0.65$, $P = 0.003$). Each datapoint represents a species. For further statistical details see text and Table 2, respectively.**

hypothesized that Sylviidae under high sperm competition risk (and with short sperm) would have smaller tubules than species under low risk (with longer sperm). Across the seven sylviid species analyzed, both tubule cross-section area and height of the epithelium decreased with increasing rCTM, although not statistically significant in both cases: tubule area, $t = -1.90$, $P = 0.13$, $r = -0.74$ (-0.93 to 0.34), $\lambda = 0.82^{0.56;0.15}$; epithelium, $t = -2.99$, $P = 0.04$, $r = -0.87$ (-0.96 to -0.05), $\lambda = 1.0^{1.0;0.06}$. The direct links between the two tubule measures and sperm length were not significant, but both associations were positive as predicted (tubule area: $t = 1.15$, $P = 0.30$, $r = 0.50$ (-0.45 to 0.85), $\lambda = 1.0^{1.0;0.58}$; epithelium: $t = 1.29$, $P = 0.25$, $r = 0.54$ (-0.42 to 0.86), $\lambda = 0.77^{0.76;0.65}$). These results, combined with those of the Icteridae, suggest that the cross-section area of the seminiferous tubules is not proportional to testes size but is more likely linked to sperm length.

PROPORTION OF TESTIS COMPONENTS AND SPERM COMPETITION RISK

We found a positive relationship between the proportion of seminiferous tissue and the level of sperm competition as measured by rCTM (Table 2; Fig. 1B). There was also a trend for a

Table 2. **Associations between the proportion of different testis components and absolute or relative testes size across Icteridae (n=20). Body mass was included as a covariate in all analyses of relative testes size and was significant (all P<0.048) except for lumen (P=0.53).**

Traits	Slope	t	P	λ	Effect size		
					r	LCL	UCL
Absolute testes size							
Seminiferous tissue	0.009	1.54	0.14	$<0.0001^{0.09;1.0}$	0.349	−0.123	0.652
Interstitial tissue	0.165	1.32	0.20	$<0.0001^{0.01;1.0}$	0.304	−0.170	0.626
Lumen	0.010	0.09	0.93	$0.943^{0.69;0.06}$	0.021	−0.414	0.444
Testicular capsule	−0.288	−6.98	<0.001	$<0.0001^{<0.01;1.0}$	−0.861	−0.924	−0.686
Relative testes size							
Seminiferous tissue	0.021	3.42	0.003	$<0.0001^{0.08;1.0}$	0.649	0.263	0.817
Interstitial tissue	−0.272	−1.92	0.07	$0.014^{0.07;0.68}$	−0.433	−0.704	0.043
Lumen	−0.068	−0.48	0.64	$0.889^{0.63;0.08}$	−0.119	−0.521	0.350
Testicular capsule	−0.352	−6.32	<0.001	$<0.0001^{<0.01;1.0}$	−0.845	−0.917	−0.642

The model including the maximum-likelihood value of λ was compared against the models including λ=1 and 0, and superscripts following the λ estimates indicate significance levels of the likelihood-ratio tests (first position: against λ=1; second position: against λ=0).

Effect size is presented as the partial correlation coefficient r, along with the noncentral 95% confidence intervals (LCL, lower confidence limit; UCL, upper confidence limit). Confidence intervals that do not cross zero are statistically significant at the α level of 0.05. For details on the calculation see Nakagawa and Cuthill (2007) and references therein.

There was no significant impact by the collinearity of testes and body mass as the two predictor variables (VIF=1.90).

negative association between the proportion of interstitial tissue and rCTM whereas the proportion of the lumen was independent of sperm competition (Table 2). None of these proportions were significantly correlated with absolute testes size (Table 2). The proportion of capsular tissue was negatively correlated with both relative and absolute testes size because the height of the capsule increased only slightly compared to testes size, such that small testes tended to have relatively thicker capsule than large testes.

With social mating system and breeding density as predictors of sperm competition, polygamous species had a higher proportion of sperm-producing tissue than monogamous species ($F_{1,18} = 4.43$, $P = 0.05$, $\eta^2 = 0.20$; $\lambda < 0.0001^{1.0;1.0}$), and the same was true for species with higher breeding density than dispersed breeders ($F_{2,14} = 7.11$, $P = 0.007$, $\eta^2 = 0.50$; $\lambda < 0.0001^{0.06;1.0}$). These results were thus consistent with the one obtained with rCTM as the sperm competition index.

Because in the Icteridae, species under intense sperm competition produce longer sperm, the higher proportion of seminiferous tissue within their testes could simply be due to the larger tubules and thicker epithelium in response to the longer sperm. To test whether the increase in the amount of seminiferous tissue per testis with sperm competition was a spurious effect or adaptive, we again compared the icterid results with those from the Sylviidae. We predicted that to maximize sperm production, Sylviids under intense sperm competition should also have a greater proportion of seminiferous tissue than species under low competition, irrespective of tubule size.

As predicted, in the Sylviidae, the proportion of seminiferous tissue increased with sperm competition ($t = 2.93$, $P = 0.04$, $r = 0.86$ (-0.07 to 0.96), $\lambda < 0.0001^{0.18;1.0}$) and the proportion of interstitial tissue decreased ($t = -3.27$, $P = 0.03$, $r = -0.88$ (-0.96 to -0.02), $\lambda = 1.0^{1.0;0.46}$) although both tubule size and epithelium height tended to decrease with sperm competition (see above). These results clearly show that the proportion of seminiferous tissue is not driven simply by the size of the tubules. In both families independently, species under intense sperm competition have testes with more densely packed sperm-producing tissue, irrespective of sperm or tubule size.

Discussion

Our results revealed a strong association between the size of the seminiferous tubules within the testes and the size of sperm produced by these tubules, suggesting that selection on sperm design entails adaptations in the testes. We also found that species under intense sperm competition have a higher proportion of sperm-producing tissue within their testes, indicating that the rate of sperm production may increase disproportionately with testes size.

TESTIS HISTOLOGY AND SPERM SIZE

It is increasingly well established that the fertilization environment, including the morphology of the female reproductive tract and the risk of sperm competition, generates selection on sperm design (Briskie and Montgomerie 1992; García-González and Simmons 2007; Immler and Birkhead 2007), but the precise nature of such selection is poorly understood. Regardless of the mechanisms that exert selection on sperm design, we show here that changes in sperm size also involve adaptations of the testes. Our results revealed a strong positive relationship between sperm length and the size (or diameter) of the sperm-producing tubules, indicating that in vertebrates, in which spermatogenesis occurs radially within the seminiferous tubules, longer sperm require wider tubules. We showed that across species, the cross-section area of these tubules was not simply proportional to the overall size of the testes but appeared to be driven solely by the size of the sperm manufactured within them, and we provide two lines of evidence for this. First, tubule size was independent of absolute testes size across species, and after including sperm length in the analysis of tubule size and relative testes size, sperm length was the only significant predictor of tubule size. Second, in the Icteridae, where sperm size increases with relative testes size (Lüpold et al., unpubl. ms.), both the tubule size and seminiferous epithelium height also increased. In contrast, in the Sylviidae, in which sperm length is inversely related with relative testes size (Immler and Birkhead 2007), both these measures decreased. The underlying selection mechanism is probably that tubule size determines the length of the sperm they produce and if postcopulatory sexual selection favors longer sperm, species under intense selection evolve wider tubules to accommodate their longer sperm. Likewise, if selection favors shorter sperm as in the Sylviidae, tubule size decreases accordingly to optimize the use of the space within the testes (also see following section).

TESTIS HISTOLOGY AND SPERM NUMBER

In addition to the testicular response to selection on sperm size, our data suggest that increasing levels of sperm competition also select for more sperm-producing tissue. Because the number of sperm transferred during copulation is among the most important factors determining the outcome of sperm competition (Martin et al. 1974; Parker 1982), species under high levels of sperm competition should maximize their sperm output as expressed by the DSP rate. DSP is the number of sperm produced per testis per day (e.g., Amann 1970, 1981), and its calculation requires knowledge of the following three factors: (1) the total amount of sperm-producing tissue (or parenchyma), (2) the number of spermatids produced simultaneously per unit of sperm-producing tissue (or parenchyma; spermatogenic efficiency), and (3) the length of the spermatogenic cycle, which determines the speed at which individual sperm cells are generated and allows the derivation of

the time divisor (t) for the calculation of DSP (DSP = $a \times b/t$; Amann 1970, 1981). Each of these three factors can potentially contribute to an increase in DSP and will be discussed.

The most obvious way to increase the amount of sperm-producing tissue would be to grow larger testes, and indeed promiscuous species have larger testes relative to their body size than monogamous species (e.g., Harcourt et al. 1981; Kenagy and Trombulak 1986; Kusano et al. 1991; Hosken 1997). However, as we show here, promiscuous species also have a significantly higher proportion of seminiferous tissue within their testes. One reason for this could be that spatial constraints within the body cavity prevent enlargement of the testes beyond a certain limit. Such effects may exist particularly in birds, in which space within the body cavity may be restricted, with one testis (usually the left) located between the spine and the solid gizzard. Moreover, flying animals such as birds are restricted in the amount of tissue they can carry during flight (e.g., Wright and Cuthill 1989), which is also reflected by the fact that the testes are typically enlarged only during a brief period of time each year (Wright and Wright 1944; Selander and Hauser 1965; Partecke et al. 2004). A trade-off between investment in gonad size and flight ability was also recently documented in insects (Saglam et al. 2008). However, Schultz (1938) obtained similar results to our study, finding a positive relationship between relative testes size and the proportion of sperm-producing tissue across six primate species, although in primates, the testes are scrotal and hence spatial constraints may be less important. Overall, there is increasing evidence that the selective pressure to maximize sperm production goes well beyond enlarging overall testes size. Such a trend has also been shown by two recent intraspecific studies. In the capybara, subordinate males have a higher proportion of seminiferous tissue than dominant males, probably as an adaptation to their higher risk of sperm competition (López et al. 2008). Similarly, male house mice (*Mus musculus domesticus*) kept under highly competitive conditions produce more sperm, but without increasing testes size, than males under lower sperm competition risk (Ramm and Stockley 2008). All these results highlight the need to look beyond overall testes size to understand the links and trade-offs between sperm size, sperm number, and sperm competition.

Variation in the proportion of sperm-producing tissue, within and between species, raises the question of the reliability of relative testes size as an index of sperm competition. Relative testes size is thought to reflect DSP rate (Fig. 2), but inherently assumes equal density of sperm-producing tissue and equal spermatogenic rate per unit tissue, across individuals within species or across species. The spermatogenic rate may be independent of sperm competition, at least in some taxa such as primates (J. Wistuba, pers. comm.), but the present study and those mentioned above, show that the quantity of seminiferous tissue in the testes increases disproportionately with relative testes size. This means that in species under intense sperm competition, testes size will underestimate the intensity of postcopulatory sexual selection. This effect is unlikely to be important for the birds used in this study because in all cases the seminiferous tissue accounted for 88–96% of testes volume. However, for certain mammals, in which the proportion of seminiferous tissue also increases with relative testes size, but across a much greater range (primates: 48–74%, Schultz 1938; and rodents: 33–93%, Russell et al. 1990, respectively), DSP is likely to increase far more rapidly with increasing relative testes size than implied by testes size alone (Fig. 2). In general, the slope between DSP and relative testes size is likely

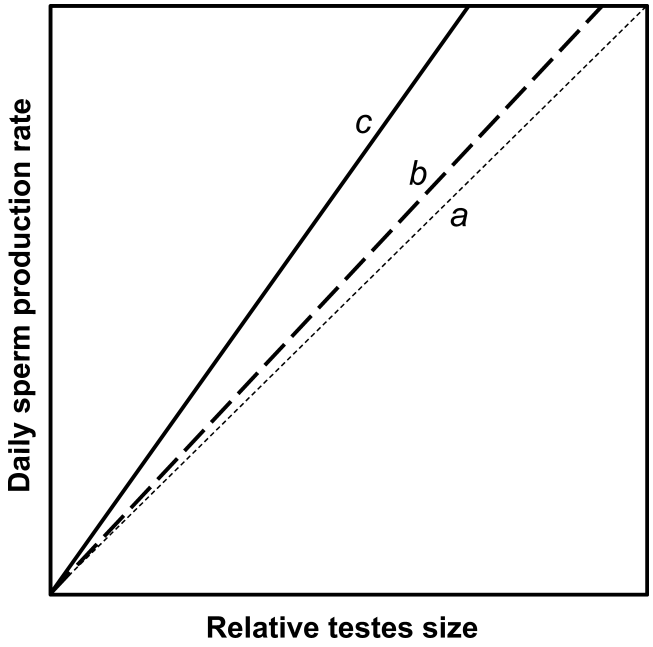

Figure 2. **Hypothetical interspecific relationships between relative testes size and daily sperm production rate as measured by the amount of actual sperm-producing tissue, showing that as the level of sperm competition increases, relative testes size as an index of sperm competition increasingly underestimates sperm production rate. The thin dotted line (a) depicts the assumed relationship between overall testes size and sperm production. The thick lines (b and c) represent hypothetical slopes across two different taxa (differing in the range of the proportion of seminiferous tissue between species) after correcting relative testes size for the proportion of seminiferous tissue. As long as the proportion of seminiferous tissue is very high and fairly consistent across species (e.g., Icteridae), the deviation from the assumed slope is only small (long-dashed line b). However, if the variation (or range) in the proportion of seminiferous tissue increases between species, with species under intense sperm competition having far denser testes than species under low sperm competition, the deviation from the assumed slope increases as represented by the solid line (c; e.g., rodents). In the latter case, a correction for the amount of sperm-producing tissue would be advised, whereas for line b, the results may not change significantly after such a correction.**

to increase with the variation in the proportions of seminiferous tissue between the individuals or species examined, particularly if the quantity of seminiferous tissue increases disproportionately with relative testes size. This systematic "error," together with variation in the quality of EPP data and testes size measures, may also explain why relative testes size often explains only relatively little of the variation in EPP in comparative studies (e.g., Møller and Briskie 1995; Calhim and Birkhead 2007).

We therefore suggest that the actual quantity (e.g., mass or volume) of seminiferous tissue is likely to be a more accurate index of DSP than overall testes size, as it accounts for the variation in the proportion of seminiferous tissue. The mass (or volume) of seminiferous tissue can be calculated by multiplying overall testes mass (or volume) by the proportion of seminiferous tissue (determined by microscopy as described in this study or by point counts of histological preparations; e.g., Russell et al. 1990).

An even more accurate index of DSP can be obtained from the same data by also incorporating seminiferous tubule size. Because longer sperm are produced by wider tubules, at least in passerine birds, and sperm length may also be related to germ cell size within the epithelium, the proportion of a tubular cross-section used to produce an individual sperm cell is likely to be fairly constant across different tubule sizes. We have not quantified the number of sperm cells, but preliminary data indicate that in average-sized tubule cross-sections for each icterid species of this study there are between 30 and 35 sperm bundles. In contrast, for a given testicular size, the length of the convoluted seminiferous tubule is traded off against its width (or cross-section area). A longer (and narrower) tubule could thus produce more sperm simultaneously (i.e., same spermatogenic stage) than a short tubule, thus increasing the sperm production rate. The length of the tubule L can be calculated by dividing the product of the parenchymal volume V and the proportion p of the tubular components (seminiferous tissue + lumen) in the parenchyma by the tubular cross-section area A, that is $L = V \times p/A$ (Johnson and Neaves 1981). This approach indirectly estimates the amount of testicular tissue per sperm. Hence, in addition to improving the accuracy of our estimates of DSP it also facilitates the study of the trade-offs between sperm size and number. Currently there are insufficient data on DSP to allow us to assess how much additional information on testicular architecture will increase the accuracy of our estimates of DSP, but we encourage researchers to examine testis histology in relation to DSP and levels of sperm competition across a range of taxa. More data will allow us to critically reassess whether we need to add a correction factor to testes size to obtain a better indicator of DSP and sperm competition.

Finally, DSP is also influenced by the kinetics of cell division, typically measured by the spermatogenic cycle length (e.g., Leblond and Clermont 1952; Amann and Lambiase 1969). Evaluation of this cycle length involves intratesticular injection of a labeling substance, such as tritiated thymidine or 5-bromodeoxyuridine (BrdU), and dissection of birds at different periods after injection to follow the progression of germ cells through the cycle of the seminiferous epithelium (e.g., Amann and Lambiase 1969; Noirault et al. 2006). Very few studies have conducted such analyses, with a strong bias toward domestic and laboratory species. The spermatogenic cycle length in mammals varies considerably between species (Amann and Lambiase 1969; Amann et al. 1976; Berndtson 1977; Johnson et al. 2000b), and in primates at least, appears to be independent of sperm competition (J. Wistuba, pers. comm.). It remains unresolved whether this is also the case in other taxa. In birds, information on the spermatogenic cycle is restricted to a few domestic species (de Reviers 1968; Marchand et al. 1977; Lin et al. 1990; Noirault et al. 2006). Consequently, we know very little about the variation in temporal aspects of spermatogenesis, which also prevents reliable calculations of DSP. Overall, we therefore know little about the factors determining the duration of spermatogenesis, but it seems intuitive that long sperm should take longer to produce than short sperm, and indeed there is clear evidence for this in *Drosophila* (Lindsley and Tokuyasu 1980; Hennig and Kremer 1990; Pitnick 1996; Schärer et al. 2008). If this is also the case in birds, it may explain why across the Icterids, species under intense sperm competition, which also produce longer sperm, have relatively more sperm-producing tissue than other species. High sperm competition species could boost sperm production without further elevating the rate of spermatogenesis. If the same holds for the Sylviidae, warblers under strong sperm competition may constitute an example of maximizing seminiferous tissue and spermatogenic rate by producing shorter sperm. These results indicate a possible proximate basis for the trade-off between sperm size and number predicted by theoretical models (e.g., Parker 1982, 1993) and shown empirically in *Drosophila* (e.g., Pitnick 1996). It appears that long sperm may not only be costly energetically or as a result of a longer generation time, but also because they require more space within the testes, thus restricting the number of sperm produced. More data on spermatogenesis across a range of species would help us better understand the links between sperm production, testis histology, and mating systems.

CONCLUSIONS

We found that postcopulatory sexual selection appears to select on testis morphology in two different ways: (1) through selection on sperm length, and (2) through selection for more sperm-producing tissue. The latter finding not only leads to relatively larger testes but also to a greater proportion of sperm-producing tissue within the testes. These results indicate that the link between the testes and their sperm is more complex than a simple testes size–sperm number relationship. For a comprehensive understanding of these effects, we need additional inter- and detailed intraspecific

studies, for example, using species with high levels of sperm-size variation or by manipulating sperm competition between males to assess its influence on the testes.

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LITERATURE CITED

Aire, T. A. 2007a. Anatomy of the testis and male reproductive tract. Pp. 37–113 *in* B. G. M. Jamieson, ed. Reproductive biology and phylogeny of birds. Science Publishers, Enfield, New Hampshire.

——— 2007b. Spermatogenesis and testicular cycles. Pp. 279–347 *in* B. G. M. Jamieson, ed. Reproductive biology and phylogeny of birds. Science Publishers, Enfield, New Hampshire.

Alderson, G. W., H. L. Gibbs, and S. G. Sealy. 1999. Determining the reproductive behaviour of individual brown-headed cowbirds using microsatellite DNA markers. Anim. Behav. 58:895–905.

Amann, R. P. 1970. Sperm production rates. Pp. 433–482 *in* A. P. Johnson, W. R. Gomes, and N. L. Vandemark, eds. The testis. Academic Press, New York.

———. 1981. A critical review of methods for evaluation of spermatogenesis from seminal characteristics. J. Androl. 2:37–58.

Amann, R. P., and S. S. Howards. 1980. Daily spermatozoal production and epididymal spermatozoal reserves of the human male. J. Urol. 124:211–215.

Amann, R. P., and J. T. Lambiase, Jr. 1969. The male rabbit. III. Determination of daily sperm production by means of testicular homogenates. J. Anim. Sci. 28:369–374.

Amann, R. P., L. Johnson, D. L. J. Thompson, and B. W. Pickett. 1976. Daily spermatozoal production, epididymal spermatozoal reserves and transit time of spermatozoa through the epididymis of the rhesus monkey. Biol. Reprod. 15:586–592.

Anderson, M. J., and A. F. Dixson. 2002. Motility and the midpiece in primates. Nature 416:496.

Balshine, S., B. J. Leach, F. Neat, N. Y. Werner, and R. Montgomerie. 2001. Sperm size of African cichlids in relation to sperm competition. Behav. Ecol. 12:726–731.

Berndtson, W. E. 1977. Methods for quantifying mammalian spermatogenesis: a review. J. Anim. Sci. 44:818–833.

Birkhead, T. R., J. G. Martínez, T. Burke, and D. P. Froman. 1999. Sperm mobility determines the outcome of sperm competition in the domestic fowl. Proc. R. Soc. Lond. B 266:1759–1764.

Blum, M. A. 1970. Invertebrate testes. Pp. 393–438 *in* A. D. Johnson, W. R. Gomes, and N. L. Vandemark, eds. The testis. Academic Press, New York.

Bollinger, E. K., and T. A. Gavin. 1991. Patterns of extra-pair fertilizations in bobolinks. Behav. Ecol. Sociobiol. 29:1–7.

Briskie, J. V., and R. Montgomerie. 1992. Sperm size and sperm competition in birds. Proc. R. Soc. Lond. B 247:89–95.

Burrows, W. H., and J. P. Quinn. 1937. The collection of spermatozoa from domestic fowl and turkey. Poult. Sci. 16:19–24.

Byrne, P. G., L. W. Simmons, and J. D. Roberts. 2003. Sperm competition and the evolution of gamete morphology in frogs. Proc. R. Soc. Lond. B 270:2079–2086.

Calhim, S., and T. R. Birkhead. 2007. Testes size in birds: quality versus quantity—assumptions, errors, and estimates. Behav. Ecol. 18:271–275.

Cardullo, R. A., and J. M. Baltz. 1991. Metabolic regulation in mammalian sperm: mitochondrial volume determines sperm length and flagellar beat frequency. Cell Motil. Cytoskelet. 19:180–188.

Cohen, J. 1977. Reproduction. Butterworths, London, UK.

Courot, M., M.-T. Hochereau-de Reviers, and R. Ortavant. 1970. Spermatogenesis. Pp. 339–432 *in* A. D. Johnson, W. R. Gomes, and N. L. Vandemark, eds. The testis. Academic Press, New York.

de Reviers, M. 1968. Détermination de la durée des processus spermatogeniques chez le coq à l'aide de la thymidine tritiée. VIe Congrès International de la Reproduction Animale et l'Insememination Artificielle, Paris:183–185.

de Reviers, M., and J. B. Williams. 1984. Testis development and production of spermatozoa in the cockerel (*Gallus domesticus*). Pp. 183–202 *in* F. J. Cunningham, P. E. Lake, and D. Hewitt, eds. Reproductive biology of poultry. British Poultry Science, Harlow.

Dunn, P. O., L. A. Whittingham, and T. E. Pitcher. 2001. Mating systems, sperm competition, and the evolution of sexual dimorphism in birds. Evolution 55:161–175.

Dybas, L. K., and H. S. Dybas. 1981. Coadaptation and taxonomic differentiation of sperm and spermathecae in featherwing beetles. Evolution 35:168–174.

Felsenstein, J. 1985. Phylogenies and the comparative method. Am. Nat. 125:1–15.

Fisher, R. A. 1925. Statistical methods for research workers. Oliver & Boyd, London.

Fox, J. 2002. An R and S-Plus companion to applied regression. Sage, Thousands Oaks, CA.

Franzén, Å. 1970. Phylogenetic aspects of the morphology of spermatozoa and spermiogenesis. Pp. 29–45 *in* B. Baccetti, ed. Comparative spermatology. Academic Press, New York.

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

Froman, D. P., A. J. Feltmann, M. L. Rhoads, and J. D. Kirby. 1999. Sperm mobility: a primary determinant of fertility in the domestic fowl (*Gallus domesticus*). Biol. Reprod. 61:400–405.

Gage, M. J. G. 1994. Associations between body size, mating pattern, testis size and sperm lengths across butterflies. Proc. R. Soc. Lond. B 258:247–254.

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

Gage, M. J. G., C. P. Macfarlane, S. Yeates, R. G. Ward, J. B. Searle, and G. A. Parker. 2004. Spermatozoal traits and sperm competition in Atlantic salmon: relative sperm velocity is the primary determinant of fertilization success. Curr. Biol. 14:44–47.

García-Berthou, E. 2001. On the misuse of residuals in ecology: testing regression residuals vs. the analysis of covariance. J. Anim. Ecol. 70:708–711.

García-González, F., and L. W. Simmons. 2007. Shorter sperm confer higher competitive fertilization success. Evolution 61:816–824.

Gibbs, H. L., P. J. Weatherhead, P. T. Boag, B. N. White, L. M. Tabak, and D. J. Hoysak. 1990. Realized reproductive success of polygynous

red-winged blackbirds revealed by DNA markers. Science 250:1394–1397.

Gier, H. T., and G. B. Marion. 1970. Development of the mammalian testis. Pp. 1–45 *in* A. D. Johnson, W. R. Gomes, and N. L. Vandemark, eds. The testis. Academic Press, New York.

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

Gray, E. M. 1996. Female control of offspring paternity in a western population of red-winged blackbirds (*Agelaius phoeniceus*). Behav. Ecol. Sociobiol. 38:267–278.

Harcourt, A. H., P. H. Harvey, S. G. Larsen, and R. V. Short. 1981. Testis size, body weight and breeding system in primates. Nature 293:55–57.

Harvey, P. H., and M. D. Pagel. 1991. The comparative method in evolutionary biology. Oxford Univ. Press, Oxford, UK.

Hennig, W., and H. Kremer. 1990. Spermatogenesis of *Drosophila hydei*. Int. Rev. Cytol. 123:129–175.

Hosken, D. J. 1997. Sperm competition in bats. Proc. R. Soc. Lond. B 264:385–392.

Hosken, D. J., and P. I. Ward. 2001. Experimental evidence for testis size evolution via sperm competition. Ecol. Lett. 4:10–13.

Huber, G. C. 1916. A note on the morphology of the seminiferous tubules of birds. Anat. Rec. 11:177–180.

Immler, S., and T. R. Birkhead. 2005. A non-invasive method for obtaining spermatozoa from birds. Ibis 147:827–830.

———. 2007. Sperm competition and sperm midpiece size: no consistent pattern in passerine birds. Proc. R. Soc. Lond. B 274:561–568.

Jamieson, B. G. M. 2007. Avian spermatozoa: structure and phylogeny. Pp. 349–511 *in* B. G. M. Jamieson, ed. Reproductive biology and phylogeny of birds. Science Publishers, Enfield, New Hampshire.

Jaramillo, A., and P. Burke. 1999. New World Blackbirds: The Icterids. Helm, London.

Johnson, L., and W. B. Neaves. 1981. Age-related changes in the Leydig cell population, seminiferous tubules and sperm production in stallions. Biol. Reprod. 24:703–712.

Johnson, K., E. DuVal, M. Kielt, and C. Hughes. 2000a. Malemating strategies and the mating system of great-tailed grackles. Behav. Ecol. 11:132–141.

Johnson, L., D. D. Varner, M. E. Roberts, T. L. Smith, G. E. Keillor, and W. L. Scrutchfield. 2000b. Efficiency of spermatogenesis: a comparative approach. Anim. Reprod. Sci. 60:471–480.

Katz, D. F., E. Z. Drobnis, and J. W. Overstreet. 1989. Factors regulating mammalian sperm migration through the female reproductive tract and oocyte vestments. Gamete Res. 22:443–469.

Kenagy, G. J., and S. C. Trombulak. 1986. Size and function of mammalian testes in relation to body size. J. Mammal 67:1–22.

Kleinbaum, D. G., L. L. Kupper, and K. E. Muller. 1998. Applied Regression Analysis and Other Multivariable Methods. Duxbury Press, Pacific Grove.

Kusano, T., M. Toda, and K. Fukuyama. 1991. Testes size and breeding systems in Japanese anurans with special reference to large testes in the treefrog, *Rhacophorus arboreus* (Amphibia: Rhacophoridae). Behav. Ecol. Sociobiol. 29:27–31.

Lake, P. E. 1957. The male reproductive tract of the fowl. J. Anat. 91:116–129.

———. 1971. The male in reproduction. Pp. 1411–1447 *in* D. J. Bell, and B. M. Freeman, eds. Physiology and biochemistry of the domestic fowl. Academic Press, London.

LaMunyon, C. W., and S. Ward. 1999. Evolution of sperm size in nematodes: sperm competition favours larger sperm. Proc. R. Soc. Lond. B 266:263–267.

Leblond, C. P., and Y. Clermont. 1952. Definition of the stages of the cycle of the seminiferous epithelium in the rat. Ann. N. Y. Acad. Sci. 55:548–573.

Lin, M., R. C. Jones, and A. W. Blackshaw. 1990. The cycle of the seminiferous epithelium in the Japanese quail (*Coturnix coturnix japonica*) and estimation of its duration. J. Reprod. Fertil. 88:481–490.

Lindsley, D. L., and K. T. Tokuyasu. 1980. Spermatogenesis. Pp. 225–294 *in* M. Ashburner, and T. R. F. Wright, eds. The genetics and biology of *Drosophila*. Academic Press, London.

Lofts, B., and R. K. Murton. 1973. Reproduction in birds. Pp. 1–107 *in* D. S. Farner, and J. K. King, eds. Avian biology. Academic Press, New York.

López, M., M. G. Muñoz, and E. A. Herrera. 2008. Reproductive morphology of capybaras, *Hydrochoerus hydrochaeris* (Rodentia: Hystricognathi): no evidence for sperm competition? Mammal. Biol. 73:241–244.

Luetjens, C. M., G. F. Weinbauer, and J. Wistuba. 2005. Primate spermatogenesis: new insights into comparative testicular organisation, spermatogenic efficiency and endocrine control. Biol. Rev. 80:475–488.

Malo, A. F., J. J. Garde, A. J. Soler, A. J. Garcia, M. Gomendio, and E. R. S. Roldan. 2005. Male fertility in natural populations of red deer is determined by sperm velocity and the proportion of normal spermatozoa. Biol. Reprod. 72:822–829.

Malo, A. F., M. Gomendio, J. Garde, B. Lang-Lenton, A. J. Soler, and E. R. S. Roldan. 2006. Sperm design and sperm function. Biol. Lett. 2:246–249.

Marchand, C. R., L. Gomot, and M. de Reviers. 1977. Etude par autoradiographie et marquage à la thymidine tritiée de la durée de la spermatogénèse du canard de barbarie (*Cairina moschata* L.). Comptes Rendus des Séances de la Société de Biologie et de ses Filiales 171:927–931.

Marquardt, D. W. 1970. Generalized inverses, ridge regression, biased linear estimation, and nonlinear estimation. Technometrics 12:591–612.

Martin, P. A., and P. J. Dziuk. 1977. Assessment of relative fertility of males (cockerels and boars) by competitive mating. J. Reprod. Fertil. 49:323–329.

Martin, P. A., T. J. Reimers, J. R. Lodge, and P. J. Dziuk. 1974. Effect of ratios and numbers of spermatozoa mixed from two males on proportions of offspring. J. Reprod. Fertil. 39:251–258.

Miller, G. T., and S. Pitnick. 2002. Sperm-female coevolution in *Drosophila*. Science 298:1230–1233.

Møller, A. P. 1991. Sperm competition, sperm depletion, paternal care and relative testes size in birds. Am. Nat. 137:882–906.

Møller, A. P., and J. V. Briskie. 1995. Extra-pair paternity, sperm competition and the evolution of testis size in birds. Behav. Ecol. Sociobiol. 36:357–365.

Moore, H. D. M., and M. A. Akhondi. 1996. Fertilizing capacity of rat spermatozoa is correlated with decline in strait-line velocity measured by continuous computer-aided sperm analysis: epididymal rat spermatozoa from the proximal cauda have a greater fertilizing capacity *in vitro* than those from the distal cauda or vas deferens. J. Androl. 17:50–60.

Moreira, J. R., J. R. Clarke, and D. W. Macdonald. 1997. The testis of capybaras (*Hydrochoerus hydrochaeris*). J. Mammal 78:1096–1100.

Nakagawa, S., and I. C. Cuthill. 2007. Effect size, confidence interval and statistical significance: a practical guide for biologists. Biol. Rev. 82:591–605.

Noirault, J., J. P. Brillard, and M. R. Bakst. 2006. Spermatogenesis in the turkey (*Meleagris gallopavo*): quantitative approach in immature and adult males subjected to various photoperiods. Theriogenology 65:845–859.

Olejnik, S., and J. Algina. 2003. Generalized eta and omega squared statistics: measures of effect size for some common research designs. Psychol. Methods 8:434–447.

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

Parker, G. A. 1970. Sperm competition and its evolutionary consequences in the insects. Biol. Rev. 45:526–567.

———. 1982. Why are there so many tiny sperm? Sperm competition and the maintenance of 2 sexes. J. Theor. Biol. 96:281–294.

———. 1993. Sperm competition games: sperm size and sperm number under adult control. Proc. R. Soc. Lond. B 253:245–254.

Partecke, J., T. Van't Hof, and E. Gwinner. 2004. Differences in the timing of reproduction between urban and forest European blackbirds (*Turdus merula*): result of phenotypic flexibility or genetic differences? Proc. R. Soc. Lond. B 271:1995–2001.

Pellatt, E. J., and T. R. Birkhead. 1994. Ejaculate size in zebra finches *Taeniopygia guttata* and a method for obtaining ejaculates from passerine birds. Ibis 136:97–106.

Pitcher, T. E., P. O. Dunn, and L. A. Whittingham. 2005. Sperm competition and the evolution of testes size in birds. J. Evol. Biol. 18:557–567.

Pitnick, S. 1996. Investment in testes and the cost of making long sperm in *Drosophila*. Am. Nat. 148:57–80.

Pitnick, S., and T. A. Markow. 1994. Male gametic strategies: sperm size, testes size, and the allocation of ejaculate among successive mates by the sperm-limited fly *Drosophila pachea* and its relatives. Am. Nat. 143:785–819.

Pitnick, S., D. J. Hosken, and T. R. Birkhead. 2009. Sperm Diversity. Pp. 69–149 *in* T. R. Birkhead, D. J. Hosken, and S. Pitnick, eds. Sperm biology: an evolutionary perspective. Elsevier, London.

Ramm, S. A., and P. Stockley. 2008. Adaptive plasticity of mammalian sperm production in response to social experience. Proc. R. Soc. Lond. B. doi:10.1098/rspb.2008.1296.

Richardson, D. S., and T. Burke. 1999. Extra-pair paternity in relation to male age in Bullock's orioles. Mol. Ecol. 8:2115–2126.

Russell, L. D., H. P. Ren, I. S. Hikim, W. Schulze, and A. P. S. Hikim. 1990. A comparative study in twelve mammalian species of volume densities, volumes, and numerical densities of selected testis components, emphasizing those related to the Sertoli cell. Am. J. Anat. 188;21–30.

Saglam, I. K., D. A. Roff, and D. J. Fairbairn. 2008. Male sand crickets trade-off flight capability for reproductive potential. J. Evol. Biol. 21:997–1004.

Samour, J. H., C. A. Smith, H. D. Moore, and J. A. Markham. 1986. Semen collection and spermatozoa characteristics in budgerigars (*Melopsittacus undulatus*). Vet. Rec. 118:397–399.

Schärer, L., P. Ladurner, and R. M. Rieger. 2004. Bigger testes do work more: experimental evidence that testis size reflects testicular cell proliferation activity in the marine invertebrate, the free-living flatworm *Macrostomum* sp. Behav. Ecol. Sociobiol. 56:420–425.

Schärer, L., J.-L. Da Lage, and D. Joly. 2008. Evolution of testicular architecture in the Drosophilidae: a role for sperm length. BMC Evol. Biol. 8:143 doi:10.1186/1471–2148-8–143.

Schultz, A. H. 1938. The relative weight of the testes in primates. Anat. Rec. 72:387–394.

Selander, R. K., and R. J. Hauser. 1965. Gonadal and behavioral cycles in the great-tailed grackle. Condor 67:157–182.

Simmons, L. W., and J. S. Kotiaho. 2007. Quantitative genetic correlation between trait and preference supports a sexually selected sperm process. Proc. Natl. Acad. Sci. USA 104:16604–16608.

Smithson, M. 2003. Confidence intervals. Sage Publications, London.

Snook, R. R. 2005. Sperm in competition: not playing by the numbers. Trends Ecol. Evol. 20:46–53.

Tae, H. J., B. G. Jang, D. C. Ahn, E. Y. Choi, H. S. Kang, N. S. Kim, J. H. Lee, S. Y. Park, H. H. Yang, and I. S. Kim. 2005. Morphometric studies on the testis of Korean ring-necked pheasant (*Phasianus colchicus karpowi*) during the breeding and non-breeding seasons. Vet. Res. Commun. 29:629–643.

Webster, M. S. 1992. Sexual dimorphism, mating system and body size in New World blackbirds (Icterinae). Evolution 46:1621–1641.

Westneat, D. F. 1993. Polygyny and extrapair fertilizations in eastern red-winged blackbirds (*Agelaius phoeniceus*). Behav. Ecol. 4:49–60.

Westneat, D. F., and P. W. Sherman. 1997. Density and extra-pair fertilizations in birds: a comparative analysis. Behav. Ecol. Sociobiol. 41:205–215.

Willett, E. L., and J. I. Ohms. 1957. Measurement of testicular size and its relation to production of spermatozoa by bulls. J. Dairy. Sci. 40:1559–1569.

Wistuba, J., A. Schrod, B. Greve, J. K. Hodges, H. Aslam, G. F. Weinbauer, and C. M. Luetjens. 2003. Organization of seminiferous epithelium in primates: relationship to spermatogenic efficiency, phylogeny, and mating system. Biol. Reprod. 69:582–591.

Wright, J., and I. Cuthill. 1989. Manipulation of sex differences in parental care. Behav. Ecol. Sociobiol. 25:171–181.

Wright, P. L., and M. H. Wright. 1944. Reproductive cycle of the male red-winged blackbird. Condor 46:46–59.

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Supporting Information

The following supporting information is available for this article:

Figure S1. (A) Icteridae—Phylogeny of the Icteridae used in this study, along with their social mating system (SMS) and breeding density (BD). References for the different subsets of the phylogeny are listed below. (B) Sylvidae—Phylogeny of the Sylvidae used in this study (subset of the species used by Immler and Birkhead 2007)

Figure S2. Schematic illustration of the cross-section through a seminiferous tubule (and adjacent tubules), indicating the distinct testis components by the different shadings and the linear measurements taken by double arrows.

Table S1. Effects of sperm length and sperm competition on tubule cross-section area in the Icteridae, using social mating system and breeding density as indices of sperm competition (the latter excluding the four brood parasites).

Supporting Information may be found in the online version of this article.
(This link will take you to the article abstract).

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