# Positive correlations between pre- and post-copulatory sexual traits in warblers

K. Supriya[1,2], Trevor D. Price[3] , Melissah Rowe[4,5]

[1] Committee on Evolutionary Biology, University of Chicago, Chicago, Illinois 60637

[2]E-mail: ksupriya@uchicago.edu

[3] Department of Ecology and Evolution, University of Chicago, Chicago, Illinois 60637

[4] Natural History Museum, University of Oslo, 0318 Oslo, Norway

[5] Centre for Ecological and Evolutionary Synthesis, Department of Biosciences, University of Oslo, 0316 Oslo, Norway

# Abstract

Theoretical models predict that investment in pre-copulatory and post-copulatory sexually selected traits should trade-off. At the macroevolutionary scale, the majority of studies to date have focused on male weaponry as the target of pre-copulatory sexual selection, but the trade-off should equally apply to traits used to attract females, such as bird song and plumage. We studied the Old World leaf warblers (Phylloscopidae), a group of socially monogamous songbirds that experience relatively high levels of sperm competition. We examined the relationships between song duration and number of elements in the song with sperm length across 21 species, and between the same song variables and combined testes mass in a subset of these species (n=10). Across species, these song variables and testes mass/sperm length are generally positively correlated, albeit not statistically significantly so or with borderline significance. In contrast to theory, we found no evidence for negative associations between pre- and post-copulatory traits. We argue that this is a consequence of males of some species investing more into overall fertilization success (i.e. the sum of pre- and post-copulatory sexual selection) than males of other species, and high fertilization success is achieved through investment into both mate attraction and sperm competition.


Keywords: *Phylloscopus*, sexual selection, sperm competition, song duration

## Introduction

Male reproductive success is a product of pre- and post-copulatory episodes of sexual selection. That is, a male's fitness depends on traits that influence his mating success, such as ornaments and armaments, as well as traits that influence his success in fertilizing ova when in competition with sperm from other males (Parker 1998, Kvarnemo and Simmons 2013). Theoretical models predict that investment into traits that influence mating success should limit investment into ejaculate traits that affect fertilization success, and vice versa (Parker 1998, Parker et al. 2013). Recently, an increasing number of studies have focused on understanding covariance between pre- and post-copulatory sexually selected traits across species (i.e. macroevolutionary patterns of trait variation). To date, the majority of these studies have focused on pre-copulatory traits linked to aggressive male interactions, notably weaponry and body size (Lüpold et al. 2014, Simmons et al. 2017), but the theoretical predictions should equally apply to any pre-copulatory trait that requires costly investment, such as displays, ornaments, and vocalizations. Similarly, investment in post-copulatory traits has also typically focused on a limited set of traits, and especially testes size (Lüpold et al. 2014, Simmons et al. 2017). This is because larger testes produce more sperm (Amann 1970, Møller 1988), thereby providing a numerical advantage under conditions of sperm competition and increasing male competitive fertilization success (Parker 1982). In contrast, ejaculate features such as sperm size and quality have been less frequently considered, with few exceptions such as Lüpold et al. (2015). Such traits, however, may influence fertilization success and, given that producing competitive ejaculates is likely to incur costs, are predicted to influence pre-copulatory trait investment.

We investigated the across-species covariance between pre- and post-copulatory traits using 21 species of Old World leaf warblers (Family Phylloscopidae), a group of small, socially monogamous songbirds that experience moderate to relatively high levels of sperm competition. Across species, extra-pair paternity varies from 20%-45% (Supriya et al. 2016), and both male-male competition and female choice are important processes in this group (Marchetti 1998, Forstmeier et al. 2002). Thus, both pre- and post-copulatory episodes of sexual selection are likely to be important in this system and may shape patterns of trait diversification across species.

We choose to examine among species variation in vocalizations as our pre-copulatory trait of interest. Though limited, a few studies have considered vocal characteristics or the anatomy underlying sound production in examinations of the relationship between pre- and post-copulatory traits (Dunn et al. 2015, Charlton and Reby 2016). In the Old World warblers, male song is the focus of both female choice and male-male competition (Marchetti 1998, Mahler and Gil 2009) and in two species song duration and performance measures are linked to female choice of extra-pair males (Forstmeier et al. 2002, Gil et al. 2007). In birds more generally, many aspects of song (e.g. song duration) are linked to female mate choice in both lab (Wasserman and Cigliano 1991, Caro et al. 2010) and field settings (Martín-Vivaldi et al. 1999, Woodgate et al. 2012). It also appears that longer songs are generally costly to produce (Oberweger and Goller 2001, Gil and Gahr 2002). Moreover, food supplementation and/or nutritional enrichment increases song output in adults (Thomas 1999, Casagrande et al. 2014, Yamada and Soma 2016), and males with longer songs can incur costs due to high social aggression from other males (*sensu* the cost of a "badge" Vehrencamp 2000; Lattin and Ritchison 2009, see Linhart et al. 2012 for an example in *Phylloscopus*). Thus males singing longer songs appear to allocate more resources to traits linked to pre-copulatory success relative to those singing shorter songs. In birds, an alternative pre-copulatory trait of interest is plumage dichromatism (Dale et al. 2015, Dunn et al. 2015). We chose not to assess sexual dichromatism, however, because these warblers are sexually monochromatic (Price et al. 2000), and thus plumage dichromatism is less likely to reflect pre-copulatory sexual selection compared to vocal traits in this group (but see (Marchetti 1998) for an example of male colour patches influencing mating success in one *Phylloscopus* species).

We used total sperm length as our post-copulatory trait. Though few comparative studies have considered sperm length (see Lupold et al. 2015 for an exception), it is widely held that sperm length is shaped by sperm competition in a range of taxa (Pitnick et al. 2009), including birds (Immler et al. 2011). Moreover, sperm size has been linked to male reproductive success in wild, free-living passerines (Laskemoen et al. 2010, Calhim et al. 2011), and, in the zebra finch (*Taeniopygia guttata*), sperm competition experiments have shown that sperm length influences fertilization success, with males possessing longer sperm siring a significantly greater proportion of embryos relative to males with short sperm (Bennison et al. 2015). Producing longer sperm is also considered costlier than producing a shorter sperm (Pitnick et al. 1995, Ramm and Stockley 2010, Godwin et al. 2017). Thus sperm length reflects a sexual trait that is both costly and the product of post-copulatory sexual selection, and as such theoretical models predict that sperm length will trade-off with energy invested in pre-copulatory sexually selected traits. In addition, for a subset of species, we examined variation in testes mass as a measure of post-copulatory investment in sperm production. In birds, relative testes mass is positively correlated with rates of extra-pair paternity (Moller and Briskie 1995), and has been widely used as an index of sperm competition in across-species comparisons (e.g. Pitcher et al. 2005, Rowe et al. 2015).

## Methods

### Data collection

We gathered data on song and sperm for 21 species of Old World leaf warblers. We focused on two specific, clearly defined song traits, total number of elements in the song, where element is defined as a continuous sound trace on the spectrogram (hence this measure is equivalent to the number of gaps plus 1), and song duration. Song data are from Mahler and Gil (2009), with the exception of 4 species in our dataset that were not covered in that study. We measured traits for these 4 species using songs downloaded from xeno-canto.org or provided by P. Alström (personal communication). All song measurements were made using Raven lite (Charif et al. 2006). We used 5 songs per male from three males of each species and used the average of these measures for our analysis; such sample sizes are sufficient given the high repeatability of song traits within species in these warblers (Mahler and Gil 2009) (Supplementary data S1).

Data on sperm length and testes mass were taken from Supriya et al. (2016). Sperm midpiece, flagellum, and total length were highly correlated (all pairwise $r = 0.99$, $p < 0.0001$) and for all analyses we used total sperm length. Analyses using midpiece length, however, returned similar results (data not shown). For one species, *Phylloscopus reguloides*, data were available from two distinct populations (one individual per population). As sperm length for these two populations differed considerably, we chose not to use an average value from these individuals, but instead used data from one individual in all our analyses. We repeated our analyses using data from the other individual, and the results were qualitatively similar (results not shown).

We included two other variables in our analysis: mid-latitude of breeding range and body mass. Body mass data was collected from the literature (Price et al. 1997, 2014, Carrascal et al. 2008). Mid-latitude was included because song duration has been reported to increase with latitude in these warblers (Irwin 2000, Singh and Price 2015). Mid-latitudes were taken from Price (2010) or estimated from maps available at birdlife.org.

## Statistical analysis

All variables except mid-latitude were log-transformed before analysis. Correlations among variables are given in Table 1; note song duration and total number of elements were only moderately correlated (r = 0.6), therefore we conducted our analyses using both song duration and total number of elements separately. We controlled for phylogeny in our analyses based on the tree reported in Supriya et al. (2016; see Fig. 1). Because we do not expect any particular direction of causality between song and sperm traits, we calculated phylogenetic partial correlations between total sperm length and each of the song variables, while controlling for body mass and mid-latitude using the approach laid out in Lüpold et al. (2015). Briefly, we obtained phylogenetic independent contrasts for all variables using the R package APE (Paradis et al. 2004). Next, we constructed a matrix of pairwise correlations between contrasts of song traits, sperm length, body mass and mid-latitude using the formula given in Crawley (2012) and used the R package CORPCOR (Schäfer et al. 2014) to convert the correlation matrix into the partial correlation matrix. We assigned a p-value to the partial correlations from multiple regressions using phylogenetic generalized least squares using the R package CAPER with no transformations of branch length (Orme et al. 2013). In order to visually interpret the correlations, we assigned phylogenetic independent contrasts to nodes on the phylogeny to identify those nodes at which changes in trait values have been especially large (Richman and Price 1992). Since the strength of correlation between traits may change over the course of the evolutionary history of a clade (Revell and Collar 2009), this approach is a better way to visualize correlation between traits than simple plots of contrast values. Finally, because differences in the evolutionary lability of traits can affect the strength of correlated evolution between them, we calculated Blomberg's k as an estimate of the phylogenetic signal in the song traits, sperm length and testes mass (Blomberg et al. 2003), using the R package Picante (Kembel et al. 2010). Values of $k < 1$ indicate high trait lability, that is, closely related species tend to differ in trait values and large contrasts are near the tips of the phylogeny, whereas values > 1 indicate related species tend to be highly similar in trait values and thus large contrasts occur near the base of the phylogeny. The R code we used for analyses and figures is available as a supplement to this paper.

## Results

Visualization of the phylogenetic independent contrasts in total sperm length and number of song elements showed large contrasts at the base of the phylogeny for both traits, with some further large contrasts in number of song elements among the tips of the tree (Fig. 1a). After controlling for phylogeny, the correlation between sperm length and number of song elements was $r = 0.41$, $P = 0.06$ ($N = 21$) and the partial correlation (i.e. correlation controlling for body mass and latitude) was $r = 0.36$, $P = 0.13$ (Fig 1b). Corresponding values for the correlation between sperm length and song duration was $r = 0.18$, $P = 0.4$, while values for the partial correlation controlling for body mass and latitude was $r = 0.07$, $P = 0.8$ (Fig. 1c).

After controlling for phylogeny, the correlation between testes mass and number of song elements was $r = 0.02$, $P = 0.95$ ($N = 10$) and the partial correlation (i.e. the correlation controlling for mass and latitude) was $r = -0.096$, $P = 0.82$ (Fig. 2a). Corresponding values for the correlation between testes mass and song duration were $r = 0.54$, $P = 0.1$, while the partial correlation controlling for mass and latitude was $r = 0.56$, $P = 0.15$ (Fig. 2b)

Blomberg's k values for the two song traits were less than 1 ($N = 21$ species, total number of song elements, $k = 0.79$, $p = 0.04$; song duration $k = 0.71$, $p= 0.06$; all p-values for randomization tests against $k = 1$), implying that song has high phylogenetic lability. In contrast, values of Blomberg's k indicate a strong phylogenetic signal in total sperm length (total length $k = 1.72$; $p < 0.001$). Testes mass did not show the same phylogenetic conservatism as sperm length (testes mass k= 0.67, p= 0.53 (N = 10 species); for the same subset of 10 species: total sperm length k= 1.1, p= 0.06; song duration k= 0.66, p= 0.53).

## Discussion

In contrast to predictions from theory, we found no support for a negative correlation between the expression of pre- and post-copulatory sexual traits among Old World leaf warblers. In fact, across-species, species with longer songs and more song elements tended to have longer sperm and larger testes, with border-line significance in some correlations. Many empirical studies have reported positive correlations or a lack of correlation between pre- and post-copulatory traits across species (Lüpold et al. 2014, Simmons and Fitzpatrick 2016), including studies reporting positive covariance between testes mass and song (Greig et al. 2013) and positive or no correlation between testes mass and plumage characteristics (Dunn et al. 2001, Hegyi et al. 2008) in birds. We suggest that these positive correlations may arise because in some species males allocate more energy into reproduction than others, likely due to differences in life history and ecology, and when they do so they invest more energy into both obtaining matings and securing fertilizations subsequent to mating. Thus, variation in overall resource investment contributes importantly to variation among species in terms of the expression and interrelationship of pre- and post-copulatory sexually selected traits. As such, resource variation makes it difficult to use comparative studies to assess trade-offs between these traits, in much the same way that, within species, trade-offs between life-history can be obscured by the overall condition of individuals, with some males investing more in all stages of the life-history than others (Van Noordwijk and de Jong 1986, Price et al. 1993).

Variation in investment may arise for two distinct reasons. First, the intensity of sexual selection may differ across species, which can be caused by variation in life-history or ecological factors such as changes in operational sex ratio or breeding density (Emlen and Oring 1977, Kokko and Rankin 2006, Lüpold et al. 2017, Janicke and Morrow 2018). In this case, in some species males invest more into fertilization success, at costs to their survival, and in other species males are longer lived, and invest less into fertilization success at each reproductive bout. An alternative, but not necessarily mutually exclusive, explanation for variation in investment is that costs of increased investment in sexually selected traits may differ across environments. For example, some species may breed in locations with a large food flush leading to a reduction in the cost of traits.

In Old World Leaf Warblers, we suggest both factors may contribute to variation in total investment into sexual selection across species. Song duration correlates with latitude across warbler species (Mahler and Gil 2009, Singh and Price 2015), and one explanation for this is that costs of singing are reduced in the north (Irwin 2000, Singh and Price 2015). In addition, body size should correlate with investment into sexually selected traits, because it is reasonable to assume that a given absolute investment would be less costly for a larger species. Body size is positively correlated with total sperm length and both song duration and total number of song elements in our dataset (Table 1). However, including body size and latitude had only a small effect on the correlation between sperm length and number of elements, reducing significance from $P = 0.06$ to $P = 0.13$. We confirmed the small influence from a stepwise regression analysis in which mass and latitude dropped out before song elements (not shown). Hence we suggest that, beyond influences of latitude and body size, males of some species invest more in reproduction than others and this accounts for the positive correlations reported here; which is consistent with suggestions from a number of other studies reporting positive across-species correlations in pre- and post-copulatory traits (Simmons and Fitzpatrick 2016, Lüpold et al. 2017)

The positive correlations we detected in the current study were weak. This might indicate that the pre- and post-copulatory sexually selected traits we examined only partially reflect pre- and post-copulatory investment, resulting in considerable 'noise' in the data. Although there is evidence that the traits we investigated i.e. song and sperm length are important targets of pre- and post-copulatory sexual selection in warblers, other pre-copulatory traits such as territory quality, proportion of time spent on singing, colour of patches (Marchetti 1998) and post-copulatory traits such as sperm number and quality(Simmons and Fitzpatrick 2012, Fitzpatrick and Lüpold 2014) could contribute to differences in pre- and post-copulatory investment across species. Another reason for the weak correlation might be due to the difference in evolutionary lability of the traits we examined. While sperm length shows a strong phylogenetic signal, song is culturally transmitted and is an evolutionarily labile trait (Mahler and Gil 2009; this study). In fact, major differences in sperm length arose early in the evolutionary history of this group (Supriya et al. 2016) (see dark squares in Figure 1a). By contrast, some closely related species exhibit striking differences in song duration (see dark circles in Figure 1a). In some instances, large differences in song duration correspond to little evolutionary change in sperm length and are associated with latitude (e.g. *bonelli* and *sibilatrix*), which correlates with song complexity in birds more generally (Weir and Wheatcroft 2011, Singh and Price 2015).

Ultimately, our findings suggest that the assessment of trade-offs will require consideration of a wide range of traits that affect pre-copulatory and post-copulatory reproductive investment. Such an approach has been recommended in a recent review of evolutionary trade-offs between pre- and post-copulatory traits, which suggested taking into account life-history, ecological, and mating system variables (Simmons et al. 2017) and we echo that sentiment here. Our results support one general thesis of that paper, which is when multiple components of a life-history are considered, positive correlations between pre- and post-copulatory investment may arise because some species invest more into obtain fertilizations than others.

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Table 1. Correlations among sperm and song traits in the Old World leaf warblers (N = 21, except for correlations involving testes mass, where N = 10). Phylogenetically corrected correlations (correlations between contrasts) are below the diagonal and raw correlations above. All variables except mid-latitude were log transformed. Significant correlations (P < 0.05) are in bold.

| Variables | Sperm length, µm | Total elements | Song duration, s | Body mass' g | Mid-latitude, ˚N | Combined testes mass, g |
|---|---|---|---|---|---|---|
| Sperm length, µm | | 0.39 | 0.13 | **0.46** | 0.01 | -0.53 |
| Total elements | *0.41* | | **0.63** | **0.6** | **0.48** | -0.05 |
| Song duration, s | *0.18* | ***0.67*** | | **0.61** | **0.67** | 0.53 |
| Body mass, g | *0.31* | ***0.57*** | ***0.64*** | | **0.56** | -0.006 |
| Mid-latitude, ˚N | *0.07* | ***0.51*** | ***0.73*** | ***0.58*** | | 0.26 |
| Combined testes mass, g | *-0.47* | *0.02* | *0.54* | *-0.02* | *0.24* | |

**Figure 1** (a) Phylogeny of the 21 Old World leaf warblers used in our study with 4 major clades indicated in green, blue, red and orange. Phylogenetically independent contrasts for total sperm length (circles) and number of song elements (squares) are shown at the nodes with darker shading indicating larger contrasts. Dark squares indicate divergence in sperm length arose primarily at the base of the phylogeny, while dark circles show divergence in song duration frequently occurred near the tips of the phylogeny

(b) Relationship between total sperm length and the number of song elements. Trend lines (reduced major axes) illustrates the relationship between song complexity and sperm length in the two most speciose groups. Phylogenetically corrected correlation across the entire clade (grey: N = 21, $P = 0.06$), and for the two larger clades with major axis trend lines indicated (blue: $P = 0.098$, red: $P = 0.064$). (c) Relationship between total sperm length and song duration. Phylogenetically corrected correlation across the entire clade (grey: N = 21, $P = 0.4$), and for the two larger clades with trend lines indicated (blue: $P = 0.38$, red: $P = 0.26$). All data were log-transformed before analysis.

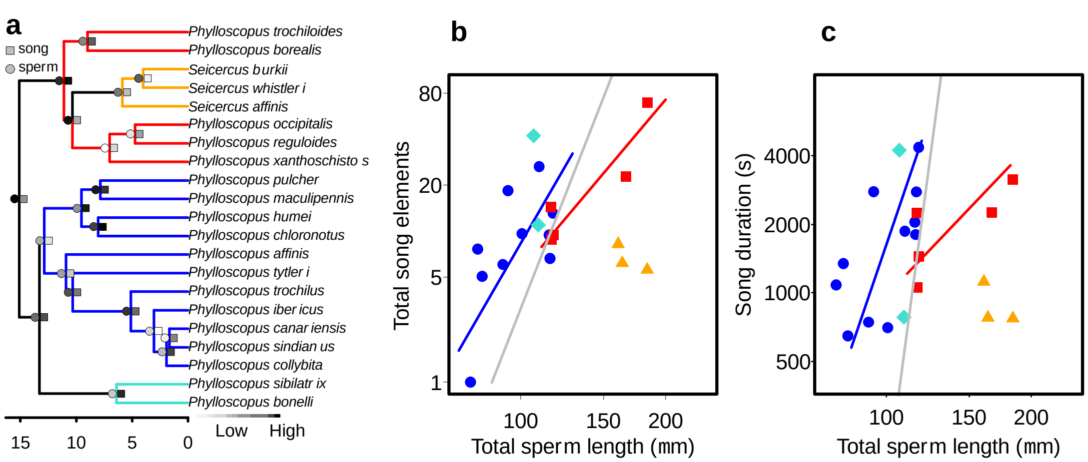

**Figure 2** Relationships between testes mass and song variables. Colour codes as in Figure 1. Combined testes mass refers to sum of left and right testis. All data were log-transformed before analysis.