

Sperm as a speciation phenotype in promiscuous songbirds

Jan T. Lifjeld¹, Emily R. A. Cramer¹, Erica H. Leder^{1,2,3}, Kjetil Lysne Voje¹

¹Department of Research and Collections, Natural History Museum, University of Oslo, Oslo, Norway

²Department of Marine Sciences, University of Gothenburg, Tjärnö Marine Laboratory, Strömstad, Sweden

³Department of Biology, University of Turku, Turku, Finland

Corresponding author: Department of Research and Collections, Natural History Museum, University of Oslo, P.O. Box 1172 Blindern, 0318 Oslo, Norway.

Email: j.t.lifjeld@nhm.uio.no

Abstract

Sperm morphology varies considerably among species. Sperm traits may contribute to speciation if they diverge fast in allopatry and cause conspecific sperm precedence upon secondary contact. However, their role in driving prezygotic isolation has been poorly investigated. Here we test the hypothesis that, early in the speciation process, female promiscuity promotes a reduction in overlap in sperm length distributions among songbird populations. We assembled a data set of 20 pairs of populations with known sperm length distributions, a published estimate of divergence time, and an index of female promiscuity derived from extrapair paternity rates or relative testis size. We found that sperm length distributions diverged more rapidly in more promiscuous species. Faster divergence between sperm length distributions was caused by the lower variance in the trait in more promiscuous species, and not by faster divergence of the mean sperm lengths. The reduced variance is presumably due to stronger stabilizing selection on sperm length mediated by sperm competition. If divergent sperm length optima in allopatry causes conspecific sperm precedence in sympatry, which remains to be shown empirically, female promiscuity may promote prezygotic isolation, and rapid speciation in songbirds.

Keywords: sexual selection, speciation, prezygotic isolation, extrapair paternity, evolutionary rate, divergence time

Introduction

Recently diverged species often differ in multiple phenotypic traits. While some of these traits may have diverged after speciation was completed, others may have arisen early in the speciation process. If such rapid divergences also affect mating or fertilization patterns, they may lead to reproductive isolation and thus speciation (Coyne & Orr, 2004; Howard, 1999; Manier et al., 2013; Marshall et al., 2002). Traits “whose divergence contributes, directly or indirectly, to a reduction of gene flow during speciation” have been termed speciation phenotypes (Shaw & Mullen, 2011). Identifying such traits responsible for reproductive isolation is a central goal in speciation research (Coyne & Orr, 2004; Marie Curie Speciation Network, 2012; Price, 2008).

Inferring speciation phenotypes from multiple post-speciation trait divergences is not an easy task because divergence may arise via different processes and at different stages of the speciation process. Speciation phenotypes must, by definition, arise early in the speciation process, before reproductive isolation is complete, i.e., between populations of the same species or between related species with gene flow. Speciation phenotypes are also more likely to evolve by selection than drift because reproductive isolation requires selection against hybridization to counteract the homogenizing effects of gene flow upon secondary contact (Servedio et al., 2011; Thibert-Plante & Gavrillets, 2013). The traits that constitute the speciation phenotype may not be obvious after

speciation is complete, for example, if other traits diverge more substantially at a later stage (Garlovsky et al., 2024; Marie Curie Speciation Network, 2012; Shaw & Mullen, 2011). It is therefore important to know how fast potential speciation phenotypes evolve and diverge at the initial stages of the speciation process.

It is common to study phenotypic divergence among taxa by assessing differences in trait means, but the trait variance may also be important to consider in the context of speciation phenotypes. Divergence increases, and overlap is reduced, when the distance between means increases and when the variance in the trait becomes smaller (Figure 1). Trait variance may vary considerably among species and populations, depending on the strength of selection acting on the trait. For example, the population variance in sperm length is commonly reduced at higher levels of sperm competition (Fitzpatrick & Baer, 2011; Kleven et al., 2008; Varea-Sánchez et al., 2014). For a speciation phenotype, trait overlap is essential because it determines how well the trait can separate members of each group in a mating or fertilization context. The importance of reduced overlap between distributions has long been appreciated in studies on ecological speciation and the importance of niche breadth (Weissing et al., 2011) but has been relatively neglected in studies of sexually selected speciation phenotypes.

In passerine birds (order Passeriformes), the most speciose order of birds, between-species divergence in multiple traits

Received April 9, 2024; revisions received October 20, 2024; accepted October 29, 2024

Associate Editor: Marcus Kronforst; Handling Editor: Tim Connallon

© The Author(s) 2024. Published by Oxford University Press on behalf of The Society for the Study of Evolution (SSE).

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<https://creativecommons.org/licenses/by/4.0/>), which permits unrestricted reuse, distribution, and reproduction in any medium, provided the original work is properly cited.

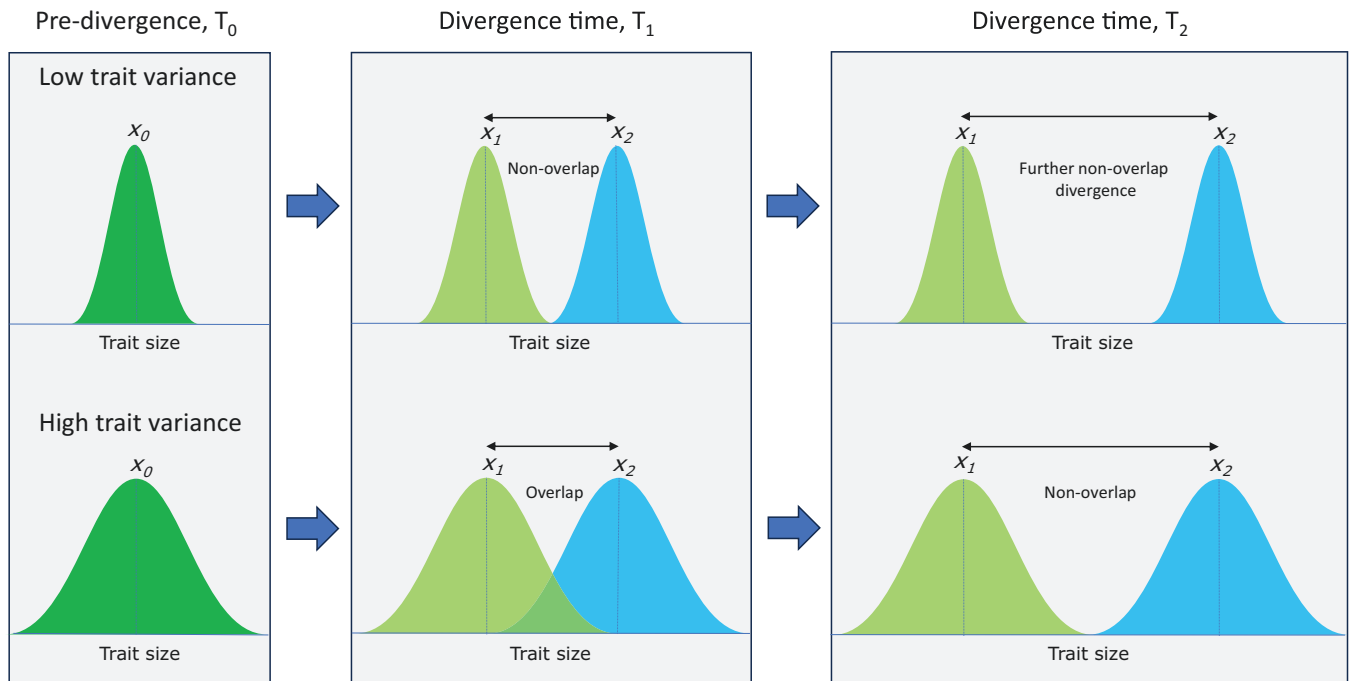


Figure 1. A graphical model of the rate of trait divergence as a function of trait variance. Two species with unequal trait variances split after time T_0 and diverge at the same rate in trait means as indicated by the length of arrowed lines above curves at two different time points, T_1 and T_2 . The low-variance species reaches nonoverlap between daughter populations (at divergence time T_1) before the high-variance species (at divergence time T_2). The model assumes no change in trait variance over time within each population.

often confounds attempts to discern the traits that were the driving force of speciation. Passerine birds are highly differentiated in morphology, ecology and behavior, and have radiated into a wide range of niches and habitats (Fjelds  et al., 2020b). They also show striking diversity in sexual traits, like plumage color, behavioral displays, and song (Schmitt & Edwards, 2022). The latter traits are often involved in mate choice and could potentially be drivers of premating isolation (Price, 2008), though there is no firm empirical evidence for causality (Fjelds  et al., 2020b). Less attention has been devoted to gametic traits, which in theory could affect fertilization patterns after mating and thus drive speciation through postcopulatory prezygotic isolation (Coyne & Orr, 2004; Garlovsky et al., 2024; Griffith, 2010; Manier et al., 2013; Wade et al., 1994). Passerine birds have filiform sperm but with distinct differences in shape and ultrastructure among the major clades (Jamieson, 2007; McFarlane, 1963). The infraorder Passerides is characterized by an elongated midpiece that is coiled around the flagellum and species-specific sperm lengths that span a much broader range than in other passerine clades (Durrant et al., 2020; Lifjeld, 2019; Omotoriogun et al., 2020). Christidis et al. (2020) hypothesized that the high diversification rate in the Passerides radiation, and especially the “higher songbirds” (parvorder Passerida), is linked to sperm differentiation and a socially monogamous mating system with high levels of sperm competition. The hypothesis is supported by a comparative study showing that passerine species with higher sperm competition diverge faster in mean sperm length (Rowe et al., 2015a). Note that sperm competition is a consequence of female mating with multiple males, and we, therefore, prefer to use the term “female promiscuity” to characterize the behavior.

Here, we explore the idea that sperm length may act as a speciation phenotype in songbird species with high female

promiscuity. Some recent studies of promiscuous songbirds have suggested that divergent sperm lengths may cause prezygotic isolation in hybrid zones (Albrecht et al., 2019; Cramer et al., 2021; Poignet et al., 2022), though conspecific sperm precedence (Howard, 1999) has not yet been demonstrated based on this trait. As required for a speciation phenotype, there is evidence for selection on sperm length in songbirds, particularly in species with high female promiscuity. Comparative studies show that the among-male variation in mean sperm length decreases when female promiscuity increases, which is interpreted as a consequence of increased stabilizing selection for an optimal sperm length (Calhim et al., 2007; Kleven et al., 2008; Lifjeld et al., 2019). In addition, sperm length shows strong coevolution with the length of female sperm storage tubules in songbirds (Briskie et al., 1997; Kleven et al., 2009), which is part of the selective environment shaping sperm traits in internal fertilizers. This coevolutionary pattern suggests that the fit between the sperm and the female sperm storage tubules impacts paternity success (Cramer et al., 2023; Higginson et al., 2012; L pold & Pitnick, 2018; Miller & Pitnick, 2002). If sperm length and female sperm storage tubule length codiverge in allopatry, it might lead to conspecific sperm precedence in sympatry. Higher female promiscuity is expected to enhance such a conspecific sperm precedence pattern by allowing more females access to sperm from conspecific males.

As outlined above, another requirement for speciation phenotypes is that they diverge rapidly, early in the speciation process. This idea has not been tested for sperm length in songbirds. However, some evidence of population divergence in sperm length is already known from songbirds. In barn swallows *Hirundo rustica* (Laskemoen et al., 2013), bluethroats *Luscinia svecica* (Hogner et al., 2013), long-tailed finches *Poephila acuticauda* (Rowe et al., 2015b), and African

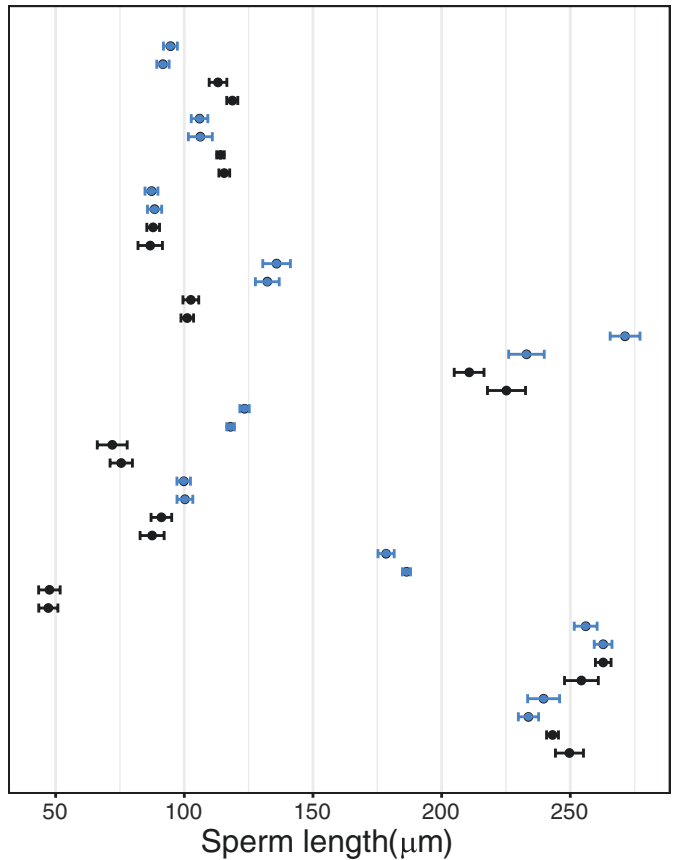
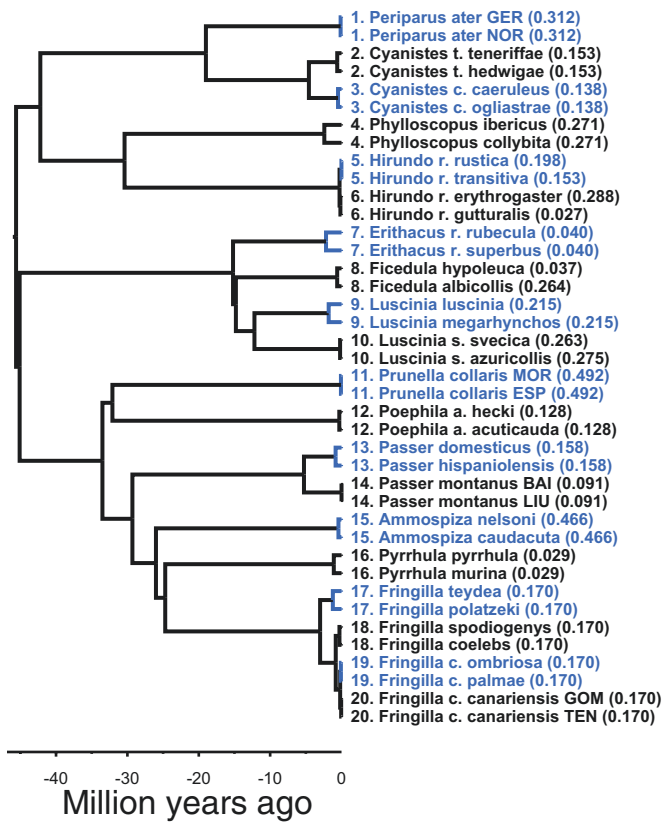


Figure 2. Overview of the 20 population pairs with their dated phylogenetic relatedness and their sperm lengths (mean \pm 1 SD). Female promiscuity estimates (proportion of extrapair young) are given in brackets behind each taxon name. Capital letters behind species names indicate country or locality. The population data with source references are given in [Supplementary Table S1](#).

blue tits *Cyanistes teneriffae* (Gohli et al., 2015), sperm length varies significantly among subspecies. Even within the same subspecies, there can be substantial geographic variation in sperm length (Lifjeld et al., 2023; Schmoll & Kleven, 2011; Yang et al., 2020).

Here we present a comparative analysis of Passerida songbirds to examine the rate of divergence in sperm length distributions between populations and how it is influenced by female promiscuity. We assembled a data set of 20 population pairs, spanning the speciation continuum from recently diverged allopatric populations of the same subspecies to closely related species that maintain some gene flow in hybrid zones. For all 20 pairs, we had estimates of their sperm length distributions, their divergence time, and a score of female promiscuity.

Materials and methods

Selection of population pairs

Populations were selected for analysis based on the following four criteria: (a) taxonomic rank, i.e., we considered only populations and/or subspecies of the same species, species that have recently been elevated from subspecies to species rank, or congeneric species with known hybrid zones, (b) presence of data on sperm length, requiring a minimum of six males per population; (c) availability of a published estimate of divergence time based on a molecular clock model, and (d)

availability of an estimate of female promiscuity in the focal study populations or for the species complex.

Our final data set contained 20 pairs of populations, ranging from geographically isolated populations of the same subspecies (i.e., where genetic dating estimates imply that there has been some reduction in gene flow among the populations) to hybridizing congeneric species. In cases where multiple populations within one species met our criteria, we chose the population with the largest sample size of sperm measurements. An overview of the data set of the 20 population pairs is illustrated in [Figure 2](#), with their phylogenetic relatedness, divergence time, sperm length distributions and the index of female promiscuity indicated. The data for each population are summarized in [Supplementary Table S1](#) with their references. Taxonomy follows the IOC World Bird List ver 14.1 (Gill et al., 2024).

Sperm length divergence

Sperm length data were extracted from raw data in published studies or from the database of the Avian Sperm Collection at the Natural History Museum, University of Oslo (Lifjeld, 2019). In some cases, data from published studies were extended with additional unpublished data from the same study populations available in the museum’s database. As a standard, ten or more sperm cells were measured by light microscopy from formalin-fixed ejaculates collected via cloacal massage to calculate the mean sperm length for each male (Grønstøl et al., 2023). Mean values were log-transformed

(natural logarithms) to make downstream analyses scale-independent. From these log-transformed means, we calculated the population mean and standard deviation. Since the standard deviation tends to be underestimated at small sample sizes (N), we adjusted the standard deviation with the correction factor $(1 + 1/4N)$, following Sokal & Rohlf (1981).

Divergence in sperm length was quantified using Hedges' g (Hedges, 1981). This metric expresses the difference between means divided by the pooled standard deviation of the two populations. The pooled standard deviation was calculated by the formula:

$$\text{Pooled standard deviation} = \sqrt{\frac{(N_A - 1) \cdot S_A^2 + (N_B - 1) \cdot S_B^2}{N_A + N_B - 2}}$$

where S_A and S_B denote standard deviation in population A and population B, respectively, and N_A and N_B their sample sizes. Hedges' g thus increases when the population means are farther apart and when the variance in the trait is reduced. A potential source of bias in this metric is that unequal variances and unequal sample sizes may affect the pooled standard deviation estimate (Safran et al., 2012). We consider this potential error rather small and negligible in comparison to the large differences in sperm length variance that we observed among species pairs in our data set. Nonetheless, we also calculated the overlap coefficient for two normal distributions with unequal variances (Inman & Bradley, 1989), using the mean and adjusted standard deviation for each population only. This coefficient ranges between 0 (no overlap) and 1 (complete overlap) and expresses the area under both curves as a proportion of the sum of areas under each of the two curves.

Divergence time

For each population pair, we used a published estimate of divergence time that was based on a time-calibrated phylogeny of either mitochondrial DNA, nuclear DNA, or both, and scaled in years or number of generations. For translation between the two-time units, we used a recent generation length database for all bird species (Bird et al., 2020). Maximum divergence time among the 20 population pairs was 2.41 million years ago (mya). We used the number of generations as our measure of divergence time since a generational time scale is more appropriate for the comparison of an evolutionary process among species with different generation lengths (Gingerich, 2001).

Female promiscuity

Most songbird females are socially mated to one male during a breeding event, but there is much variation among species in their tendency to copulate with extrapair mates. This behavior can be verified by molecular paternity analysis of offspring, and in more promiscuous species males also typically have larger testes (Møller & Briskie, 1995). Here we use the proportion of extrapair young (EPY) in the population as our index for female promiscuity. We preferentially used paternity studies from the same study population from which the sperm length data originated. In the case of multiple paternity studies from the same population, we calculated the proportion of EPY from the total number of offspring genotyped. When no paternity data were available from the same population, we used the species-level estimate of EPY recommended by Brouwer & Griffith (2019: Table S5). Two species pairs in our

data set do not have regular monandrous pair bonds and thus "EPY" is ambiguous. For the highly promiscuous *Ammospiza* sparrows with no obvious pair bonds, we conservatively considered the male who sired most offspring in a brood to be the pair male, and offspring not sired by him to be EPY. For the polygynandrous *Prunella collaris*, we assigned offspring not sired by the alpha (dominant) male as EPY (Lifjeld et al., 2023).

For three pairs in which no paternity studies were available from either population or species (i.e., blue chaffinches *Fringilla teydea* and *F. polatzeki*, chiffchaffs *Phylloscopus collybita* and *P. ibericus*, and bullfinches *Pyrrhula pyrrhula* and *P. murina*), we estimated an EPY rate from relative testes mass. These estimates were derived from a linear regression between EPY rates and relative testes mass for 66 Passerides songbird species ($F_{1,64} = 12.3$, $R^2 = 0.16$, $p < .001$). Relative testes mass was here expressed as the residual from a regression line between the combined testes mass and body mass (logged [ln] values in g) calculated from 160 Passerida songbird species, using a Phylogenetic Generalized Least Squares (PGLS) model. The testes and body mass data for the 160 species were compiled and averaged from three published data sets (Calhim & Birkhead, 2007; Calhim & Montgomerie, 2015; Rowe et al., 2015a). Their phylogenetic relationships were taken from a consensus tree derived from BirdTree.org (Jetz et al., 2012). Testes mass data were not available for all 20 population pairs, so using this metric for all pairs was not possible.

Data analysis

We used PGLS regression to investigate the effects of divergence time and the level of female promiscuity on the pairwise divergence in sperm length (Hedges' g). A time-calibrated phylogeny of the 20 population pairs (considering each pair as a tip) was constructed by using the topology and node ages for species given in the OneZoom bird tree at BirdTree.org (Jetz et al., 2012). For intraspecific nodes and certain shallow intrageneric nodes, we used node ages estimated in more detailed studies, viz. Lombardo et al. (2022) for the split between the two *Hirundo rustica* pairs, Valente et al. (2020) for the split between the two *Cyanistes* pairs and the split between blue chaffinches and the three other *Fringilla* pairs, and Recuerda et al. (2021) for the nodes among the three *Fringilla* pairs. Variables were \log_{10} - or logit-transformed to achieve normality of residuals.

All statistical analyses were carried out using R v 4.4.1 (R Core Team, 2024). The R packages tidyverse v 2.0.0 (Wickham et al., 2019), ape v 5.0 (Paradis & Schliep, 2019), nlme v 3.1.164 (Pinheiro et al., 2023), AICcmodavg v 2.3.3 (Mazerolle, 2023), and ggpubr v 0.6.0 (Kassambara, 2023), were used for general data processing, phylogeny construction, PGLS regressions, model selection and result visualizations. Raw data and R scripts used in the analyses can be accessed through Dryad at <https://doi.org/doi:10.5061/dryad.w0vt4b8zd>.

Results

Across the 20 population pairs, sperm length divergence spanned from almost zero in the *Pyrrhula* bullfinches (Hedges' $g = 0.09$, overlap coefficient = 0.952) to virtually nonoverlapping distributions between the two *Luscinia* nightingales (Hedges' $g = 6.11$, overlap coefficient = 0.003;

Figure 3). Hedges' g and the overlap coefficient were strongly correlated, but Hedges' g gives a better representation of the distance between distributions when the overlap coefficient approaches zero (Figure 3).

Sperm length divergence (Hedges' g) would in general be expected to increase with divergence time under a Brownian motion model. We also predicted that sperm length should diverge faster in species with higher female promiscuity. We therefore tested for the effect of female promiscuity in a PGLS with divergence time as a copredictor. We found that female promiscuity had a significant and independent effect on sperm length divergence, whereas the effect of divergence time was not significant (Table 1: Model 1). Female promiscuity also had a statistically significant effect in a univariate PGLS model, whereas divergence time did not (Table 1: models 2 and 3; see also Figure 4A and B for visualization of the relationships). The phylogenetic signal was weak in all models (Pagel's λ close to zero). A model selection approach of the three regression models using the AIC_c criterion revealed that the model with female promiscuity as the sole predictor (model 3) provided the best model fit (lowest AIC_c ; Table 1). Results were almost identical when divergence time was expressed in years instead of generations (Supplementary Table S2).

It should be noted that trait divergence divided by divergence time is equivalent to the evolutionary rate of a trait. A common measure of evolutionary rate is the *haldane* metric, which is defined as the change in Hedges' g per generation (Gingerich, 2001). When we controlled for the number of generations in the PGLS regression of Hedges' g , female promiscuity was a significant predictor of sperm length divergence (Table 1: model 1). It demonstrates that female promiscuity affects the evolutionary rate of sperm length as quantified in *haldanes*. In other words, species with higher female promiscuity diverge faster in sperm length.

The Hedges' g metric is a ratio, i.e., the difference between means divided by the pooled standard deviation. It is therefore of interest to examine whether the significant effect of female promiscuity on sperm length divergence is associated with the numerator or the denominator of this ratio, or with both. We found that female promiscuity had a significant effect only on the denominator (Table 1: models 4 and 5; illustrated in Figure 4C and D). That is, more promiscuous species had smaller standard deviations, but similar divergence in mean values, than low-promiscuity species. Thus, populations with high levels of female promiscuity diverge faster in sperm length mainly because they have reduced variation in the trait.

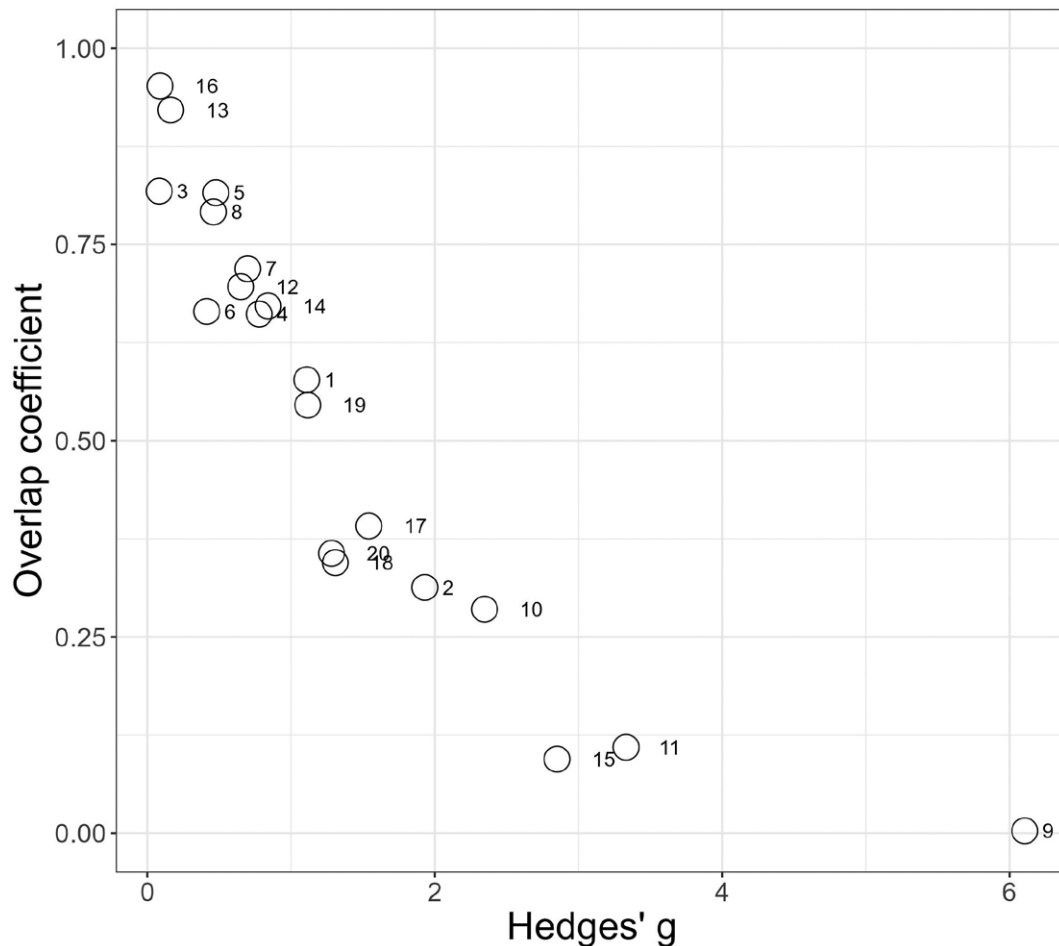


Figure 3. The relationship between Hedges' g (difference in means divided by the pooled standard deviation) and the overlap coefficient calculated from sperm length distributions in 20 pairs of songbird populations. The two variables were strongly negatively correlated (Pearson $r = -0.915$, \log_{10} -transformed Hedges' g). Numbers at each data point refer to the pair numbers in Figure 2.

Table 1. Phylogenetic general least squares (PGLS) models of sperm length divergence measures in 20 population pairs of Passerida songbirds. H = Hedges' g , SD = standard deviation. Divergence time was expressed in numbers of generations, and promiscuity as the proportions of EPY. All variables were \log_{10} -transformed, except for promiscuity which was logit-transformed. The results of model selection using the Akaike Information Criterion (AIC_c) are indicated for models 1–3, with model 3 as the best model (lowest AIC_c and $\Delta AIC_c > 2$ for the other models).

Model	Dependent variable	Predictor	Coefficient	SE	t -Value	P	AIC_c	ΔAIC_c
1	Sperm length divergence (H)	Intercept	0.738	0.518	1.425	0.172	40.66	6.35
		Divergence time	-0.037	0.106	-0.389	0.703		
		Female promiscuity	0.370	0.106	3.492	0.003		
		Residual SE = 0.427, total DF = 20, residual DF = 17, $\lambda = 0.299$						
2	Sperm length divergence (H)	Intercept	0.017	0.582	0.029	0.977	42.67	8.36
		Divergence time	-0.033	0.116	-0.279	0.783		
		Residual SE = 0.499, total DF = 20, residual DF = 18, $\lambda = -0.185$						
3	Sperm length divergence (H)	Intercept	0.548	0.204	2.691	0.015	34.31	0.00
		Female promiscuity	0.368	0.104	3.523	0.002		
		Residual SE = 0.413, total DF = 20, residual DF = 18, $\lambda = 0.260$						
4	Difference in mean sperm length	Intercept	-1.328	0.200	-6.635	<0.001		
		Promiscuity	0.165	0.104	1.586	0.130		
		Residual SE = 0.406, total DF = 20, residual DF = 18, $\lambda = 0.231$						
5	Pooled SD of sperm length	Intercept	-1.870	0.068	-27.632	<0.001		
		Promiscuity	-0.202	0.036	-5.672	<0.001		
		Residual SE = 0.137, total DF = 20, residual DF = 18, $\lambda = 0.194$						

Discussion

Sperm length divergence

Our analyses confirm that sperm length diverges faster in songbird species with higher levels of female promiscuity. The pattern arises primarily because promiscuous species have less variation in sperm lengths, and not because the mean sperm lengths differentiate faster. Thus, trait variation is here a key parameter affecting the overlap between diverging populations. This result matches the graphical model illustrated in Figure 1, where two species diverge at the same rate in trait means, but the species with the lower trait variance (and higher promiscuity) reaches nonoverlap faster.

Rowe et al. (2015a) found that the divergence rate of mean sperm length increased significantly with sperm competition risk in passerines. We found little or no support for such an effect in our data (Table 1: model 4; Figure 4C). The lack of congruence can be due to several factors. Most species pairs in the Rowe et al. (2015) study were older splits (≈ 5 –10 million years), and the study included several species outside the Passerida clade and used a different proxy for female promiscuity (relative testes size only). It therefore remains unclear whether female promiscuity increases the evolutionary rate of mean sperm length at the intraspecific level. Nevertheless, our two studies agree that female promiscuity has an overall accelerating effect on sperm length divergence in passerine birds, and the present study indicates that at least the reduced variance has a significant impact within the speciation continuum.

Selection of sperm length

Why does the variance in sperm length become reduced in more promiscuous species? The pattern seems to be a common phenomenon among internal fertilizers and is reported from various groups like social insects (Fitzpatrick & Baer, 2011), sharks (Rowley et al., 2019) and rodents (Varea-Sánchez et al., 2014),

in addition to songbirds. A widely accepted explanation is that sperm length is adapted to the size of sperm storage compartments in females and that stabilizing selection for an optimal length strengthens when females copulate with multiple males (Lüpold & Pitnick, 2018). The best-matching sperm length in each female environment will have a competitive advantage (lock-and-key principle), and the most successful males will be those with a sperm length that has the highest overall fertilization success integrated over multiple female environments. Intraspecific variation in the length of sperm-storage tubules is documented in several passerine birds (Briskie, 1993, 1994; Briskie & Birkhead, 1993). A modeling approach demonstrated how female promiscuity leads to stabilizing selection on sperm length when females vary in the length of their sperm-storage tubules relative to the magnitude of variation in sperm lengths among males (Cramer et al., 2023). Any changes in the female tubule length distribution in the population, whether caused by random drift or selection, will then be rapidly followed by corresponding changes in the sperm length distribution in highly promiscuous species. Selection of female tubule length may be more relaxed if fertilization of the ova is not compromised by any sperm-tubule length mismatch, and thus tubule length may be more likely to drift. When females are sexually monogamous or less promiscuous, selection for an optimal sperm length will be weaker, among-male variation larger, and the response to shifts in female tubule lengths slower. It may seem paradoxical that female promiscuity facilitates rapid divergence in a trait under stabilizing selection, but the essence here is that strong selection for an optimal trait size facilitates rapid adaptation of the trait in response to changes in its selective environment. While this sperm-female coevolution remains to be confirmed empirically in birds, there is experimental evidence from insects that sperm length can evolve rapidly in response to manipulations of the level of female promiscuity (Godwin et al., 2017) and the length of female sperm storage organs (Miller & Pitnick, 2002).

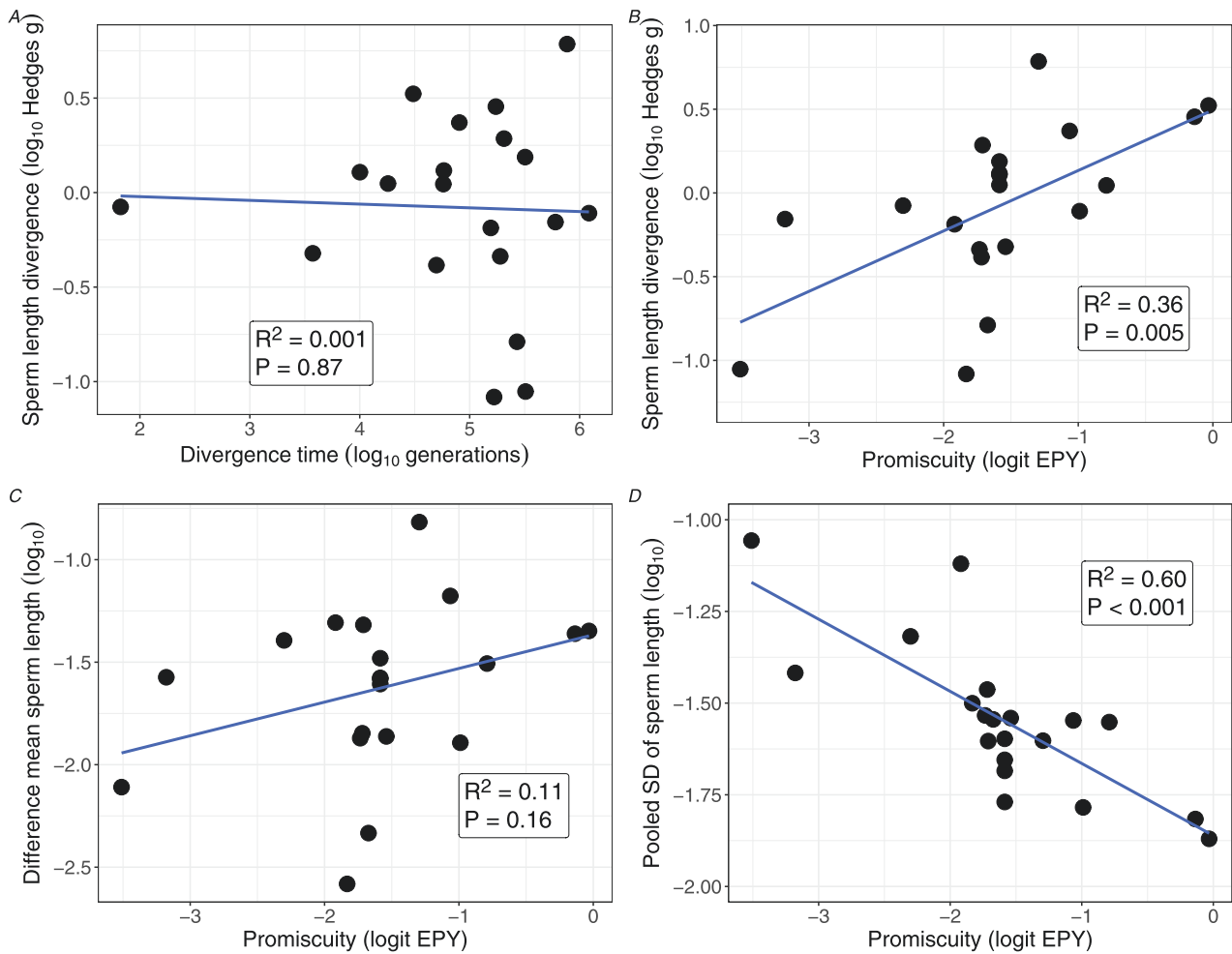


Figure 4. Sperm length divergence (Hedges’ g) in 20 population pairs of Passerida songbirds as a function of (A) divergence time in a number of generations and (B) female promiscuity. The two bottom panels show the relationship between female promiscuity and (C) the numerator of Hedges’ g (i.e., the population difference in mean sperm length) and (D) the denominator of Hedges’ g (i.e., the pooled standard deviation of sperm length). Lines are linear regressions not corrected for phylogeny ($\lambda = 0$). R^2 -values indicate the strength of the linear relationships. Note that variables were transformed as indicated on the axis labels.

Allopatric populations of songbirds with divergent sperm length distributions should therefore be under selection for different sperm length optima set by divergent female reproductive environments. If the populations gain secondary contact, promiscuous females may encounter and copulate with males of both groups. Then conspecific sperm precedence (Howard, 1999) and disruptive selection will be expected because sperm of intermediate lengths will generally fail in competition with sperm with a better fit to the female, i.e., sperm from males of her own group. The higher the level of female promiscuity, the higher the chances that females will receive sperm from a male of her own group that fertilizes her eggs. In this way, sperm length divergence could lead to prezygotic isolation and initiate cryptic speciation in promiscuous songbirds (Liffield et al., 2023). Sperm length is therefore a trait that may rapidly evolve in allopatry and cause assortative fertilization in sympatry. It thus meets the criteria for a “magic trait” in speciation (Gavrilets, 2004; Servedio et al., 2011), a concept that has been mainly used for phenotypic traits under divergent ecological selection. The problem with traits diverging in response to an environmental gradient is that they would be expected to converge when populations

meet again in the same environment unless they have already become mating cues that ensure assortative mating and prevent hybridization. Sperm traits may not fit as well with the concept of magic traits because the female oviduct is the selective environment for both the divergence process and for facilitating assortative fertilization. Females of the two groups represent two divergent environments that may drive disruptive selection on sperm length in a contact zone.

Sperm and speciation in songbirds

The ultimate sign of completed speciation is that species coexist without interbreeding, i.e., full pre-mating isolation. Since closely related species are often most divergent in traits involved in sexual communication and mate choice, it is often assumed that sexual selection is an important driver of speciation. However, thorough scrutiny of the empirical evidence has raised serious doubts about this assertion (Panhuis et al., 2001; Ritchie, 2007; Servedio & Boughman, 2017). There seems to be little or no evidence that sexual selection alone can lead to speciation (Servedio & Boughman, 2017), so it is more likely to work in combination with divergence in ecological traits. In passerine birds, the most prominent

- Christidis, L., Marki, P. Z., & Fjeldså, J. (2020). Chapter 9. Infraorder passerides and the “higher” songbirds. In J. Fjeldså, L. Christidis, & P. G. P. Ericson (Eds.), *The largest Avain radiation. The evolution of perching birds, or the order Passeriformes* (pp. 169–183). Lynx Edicions.
- Coyne, J. A., & Orr, H. A. (2004). *Speciation*. Sinauer.
- Cramer, E. R. A., Grønstøl, G., Maxwell, L., Kovach, A. I., & Lifjeld, J. T. (2021). Sperm length divergence as a potential prezygotic barrier in a passerine hybrid zone. *Ecology and Evolution*, 11(14), 9489–9497. <https://doi.org/10.1002/ece3.7768>
- Cramer, E. R. A., Yilma, Z. B., & Lifjeld, J. T. (2023). Selection on sperm size in response to promiscuity and variation in female sperm storage organs. *Journal of Evolutionary Biology*, 36(1), 131–143. <https://doi.org/10.1111/jeb.14120>
- Durrant, K. L., Reader, T., & Symonds, M. R. E. (2020). Pre- and post-copulatory traits working in concert: Sexual dichromatism in passerines is associated with sperm morphology. *Biology Letters*, 16(1), 20190568. <https://doi.org/10.1098/rsbl.2019.0568>
- Fitzpatrick, J. L., & Baer, B. (2011). Polyandry reduces sperm length variation in social insects. *Evolution*, 65(10), 3006–3012. <https://doi.org/10.1111/j.1558-5646.2011.01343.x>
- Fjeldså, J., Alström, P., & Bowie, R. C. K. (2020a). Chapter 16. How new species evolve. In J. Fjeldså, L. Christidis, & P. G. P. Ericson (Eds.), *The largest Avain radiation. The evolution of perching birds, or the order Passeriformes* (pp. 327–337). Lynx Edicions.
- Fjeldså, J., Christidis, L., & Ericson, P. G. P. (2020b). *The largest avian radiation. The evolution of perching birds, or the order Passeriformes*. Lynx Edicions.
- Garlovsky, M. D., Whittington, E., Albrecht, T., Arenas-Castro, H., Castillo, D. M., Keais, G. L., & Weber, A. A. -T. (2024). Synthesis and scope of the role of postmating prezygotic isolation in speciation. *Cold Spring Harbor Perspectives in Biology*, 16(10), a041429. <https://doi.org/10.1101/cshperspect.a041429>
- Gavrilets, S. (2004). *Adaptive landscapes and the origin of species*. (Vol. 41). Princeton University Press.
- Gill, F., Donsker, D., & Rasmussen, P. (2024). *IOC world bird list (v14.1)*. <http://doi.org/10.14344/IOC.ML.13.1>
- Gingerich, P. D. (2001). Rates of evolution on the time scale of the evolutionary process. *Genetica*, 112–113(113), 127–144. <https://doi.org/10.1023/A:1013311015886>
- Godwin, J. L., Vasudeva, R., Michalczyk, L., Martin, O. Y., Lumley, A. J., Chapman, T., & Gage, M. J. G. (2017). Experimental evolution reveals that sperm competition intensity selects for longer, more costly sperm. *Evolution Letters*, 1(2), 102–113. <https://doi.org/10.1002/evl3.13>
- Gohli, J., Leder, E. H., Garcia-del-Rey, E., Johannessen, L. E., Johnsen, A., Laskemoen, T., & Lifjeld, J. T. (2015). The evolutionary history of *Afrocanarian blue tits* inferred from genomewide SNPs. *Molecular Ecology*, 24(1), 180–191. <https://doi.org/10.1111/mec.13008>
- Griffith, S. C. (2010). The role of multiple mating and extra-pair paternity in creating and reinforcing boundaries between species in birds. *Emu—Austral Ornithology*, 110(1), 1–9. <https://doi.org/10.1071/mu09057>
- Grønstøl, G., Danielsen, M., Cramer, E. R. A., Johannessen, L. E., Johnsen, A., Whittington, E., & Lifjeld, J. T. (2023). Effects of fixatives and storage duration on avian sperm morphology. *Journal of Ornithology*, 164(1), 171–181. <https://doi.org/10.1007/s10336-022-02015-x>
- Hedges, L. V. (1981). Distribution theory for Glass’s estimator of effect size and related estimators. *Journal of Educational Statistics*, 6(2), 107–128. <https://doi.org/10.2307/1164588>
- Higginson, D. M., Miller, K. B., Segraves, K. A., & Pitnick, S. (2012). Female reproductive tract form drives the evolution of complex sperm morphology. *Proceedings of the National Academy of Sciences of the United States of America*, 109(12), 4538–4543. <https://doi.org/10.1073/pnas.1111474109>
- Hogner, S., Laskemoen, T., Lifjeld, J. T., Pavel, V., Chutný, B., García, J., & Johnsen, A. (2013). Rapid sperm evolution in the bluethroat (*Luscinia svecica*) subspecies complex. *Behavioral Ecology and Sociobiology*, 67(8), 1205–1217. <https://doi.org/10.1007/s00265-013-1548-z>
- Howard, D. J. (1999). Conspecific sperm and pollen precedence and speciation. *Annual Review of Ecology and Systematics*, 30(1), 109–132. <https://doi.org/10.1146/annurev.ecolsys.30.1.109>
- Inman, H. F., & Bradley, E. L. (1989). The overlapping coefficient as a measure of agreement between probability distributions and point estimation of the overlap of two normal densities. *Communications in Statistics—Theory and Methods*, 18(10), 3851–3874. <https://doi.org/10.1080/03610928908830127>
- Jamieson, B. G. M. (2007). Avian spermatozoa: Structure and phylogeny. In B. G. M. Jamieson (Ed.), *Reproductive biology and phylogeny of birds. Part A* (pp. 349–511). Science Publishers Inc.
- Jetz, W., Thomas, G. H., Joy, J. B., Hartmann, K., & Mooers, A. O. (2012). The global diversity of birds in space and time. *Nature*, 491(7424), 444–448. <https://doi.org/10.1038/nature11631>
- Kassambara, A. (2023). “ggplot2” Based publication ready plots (Version 0.6.0). <https://rpkgs.datanovia.com/ggpubr/>
- Kleven, O., Fossoy, F., Laskemoen, T., Robertson, R. J., Rudolfsen, G., & Lifjeld, J. T. (2009). Comparative evidence for the evolution of sperm swimming speed by sperm competition and female sperm storage duration in passerine birds. *Evolution*, 63(9), 2466–2473. <https://doi.org/10.1111/j.1558-5646.2009.00725.x>
- Kleven, O., Laskemoen, T., Fossoy, F., Robertson, R. J., & Lifjeld, J. T. (2008). Intraspecific variation in sperm length is negatively related to sperm competition in passerine birds. *Evolution*, 62(2), 494–499. <https://doi.org/10.1111/j.1558-5646.2007.00287.x>
- Laskemoen, T., Albrecht, T., Bonisoli-Alquati, A., Cepak, J., de Lope, F., Hermosell, I. G., & Lifjeld, J. T. (2013). Variation in sperm morphology and sperm competition among barn swallow (*Hirundo rustica*) populations. *Behavioral Ecology and Sociobiology*, 67(2), 301–309. <https://doi.org/10.1007/s00265-012-1450-0>
- Lifjeld, J. T. (2019). The avian sperm collection in the Natural History Museum, University of Oslo. *Alauda*, 87(3), 93–101.
- Lifjeld, J. T., de Gabriel Hernando, M., Fuertes Marcos, B., Grønstøl, G., Anmarkrud, J. A., Matschiner, M., & Leder, E. H. (2023). Rapid sperm length divergence in a polygynandrous passerine: A mechanism of cryptic speciation? *Evolution*, 77(11), 2352–2364. <https://doi.org/10.1093/evolut/qp4d146>
- Lifjeld, J. T., Gohli, J., Albrecht, T., Garcia-del-Rey, E., Johannessen, L. E., Kleven, O., & Johnsen, A. (2019). Evolution of female promiscuity in Passerides songbirds. *BMC Evolutionary Biology*, 19(1), 169. <https://doi.org/10.1186/s12862-019-1493-1>
- Lombardo, G., Rambaldi Migliore, N., Colombo, G., Capodiferro, M. R., Formenti, G., Caprioli, M., & Torroni, A. (2022). The mitogenome relationships and phylogeography of barn swallows (*Hirundo rustica*). *Molecular Biology and Evolution*, 39(6), msac113. <https://doi.org/10.1093/molbev/msac113>
- Lorch, P. D., & Servedio, M. R. (2007). The evolution of conspecific gamete precedence and its effect on reinforcement. *Journal of Evolutionary Biology*, 20(3), 937–949. <https://doi.org/10.1111/j.1420-9101.2007.01306.x>
- Lüpold, S., & Pitnick, S. (2018). Sperm form and function: What do we know about the role of sexual selection? *Reproduction*, 155(5), R229–R243. <https://doi.org/10.1530/REP-17-0536>
- Manier, M. K., Lüpold, S., Belote, J. M., Starmer, W. T., Berben, K. S., Ala-Honkola, O., & Pitnick, S. (2013). Postcopulatory sexual selection generates speciation phenotypes in *Drosophila*. *Current Biology*, 23(19), 1853–1862. <https://doi.org/10.1016/j.cub.2013.07.086>
- Marie Curie Speciation Network. (2012). What do we need to know about speciation? *Trends in Ecology and Evolution*, 27(1), 27–39. <https://doi.org/10.1016/j.tree.2011.09.002>
- Marshall, J. L., Arnold, M. L., & Howard, D. J. (2002). Reinforcement: The road not taken. *Trends in Ecology and Evolution*, 17(12), 558–563. [https://doi.org/10.1016/s0169-5347\(02\)02636-8](https://doi.org/10.1016/s0169-5347(02)02636-8)
- Mazerolle, M. J. (2023). *Model selection and multilodel inference based on (Q)AIC(c)* (Version 2.3-3). <https://cran.r-project.org/package=AICcmovavg>

- McFarlane, R. W. (1963). The taxonomic significance of avian sperm. *Proceedings of the 13th International Ornithological Congress, 1*, 91–102.
- Miller, G. T., & Pitnick, S. (2002). Sperm-female coevolution in *Drosophila*. *Science*, 298(5596), 1230–1233. <https://doi.org/10.1126/science.1076968>
- Møller, A. P., & Briskie, J. V. (1995). Extra-pair paternity, sperm competition and the evolution of testis size in birds. *Behavioral Ecology and Sociobiology*, 36(5), 357–365. <https://doi.org/10.1007/BF00167797>
- Omotoriogun, T. C., Albrecht, T., Gohli, J., Hořák, D., Johannessen, L. E., Johnsen, A., & Lifjeld, J. T. (2020). Sperm length variation among Afrotropical songbirds reflects phylogeny rather than adaptations to the tropical environment. *Zoology*, 140, 125770. <https://doi.org/10.1016/j.zool.2020.125770>
- Panhuis, T. M., Butlin, R., Zuk, M., & Tregenza, T. (2001). Sexual selection and speciation. *Trends in Ecology and Evolution*, 16(7), 364–371. [https://doi.org/10.1016/s0169-5347\(01\)02160-7](https://doi.org/10.1016/s0169-5347(01)02160-7)
- Paradis, E., & Schliep, K. (2019). ape 5.0: An environment for modern phylogenetics and evolutionary analyses in {R}. *Bioinformatics*, 35(3), 526–528. <https://doi.org/10.1093/bioinformatics/bty633>
- Pinheiro, J. C., Bates, D. M., & Team, R. C. (2023). *nlme: Linear and nonlinear mixed effects models (Version R package version 3.1-164)*. <https://CRAN.R-project.org/package=nlme>
- Poignet, M., Baránková, L., Reif, J., Stopka, P., Stopková, R., Frolíková, M., & Reifová, R. (2022). Sperm morphology and performance in relation to postmating prezygotic isolation in two recently diverged passerine species. *Scientific Reports*, 12(1), 22275. <https://doi.org/10.1038/s41598-022-26101-5>
- Pomiankowski, A., & Møller, A. P. (1995). A resolution of the lek paradox. *Proceedings of the Royal Society B: Biological Sciences*, 260(1357), 21–29. <https://doi.org/10.1098/rspb.1995.0054>
- Price, T. (2008). *Speciation in birds*. Roberts & Co.
- R Core Team. (2024). R: A language and environment for statistical computing. <http://www.R-project.org>
- Recuerda, M., Carlos Illera, J., Blanco, G., Zardoya, R., & Milá, B. (2021). Sequential colonization of oceanic archipelagos led to a species-level radiation in the common chaffinch complex (Aves: *Fringilla coelebs*). *Molecular Phylogenetics and Evolution*, 164, 107291. <https://doi.org/10.1016/j.ympev.2021.107291>
- Ritchie, M. G. (2007). Sexual selection and speciation. *Annual Review of Ecology, Evolution, and Systematics*, 38(1), 79–102. <https://doi.org/10.1146/annurev.ecolsys.38.091206.095733>
- Rowe, M., Albrecht, T., Cramer, E. R. A., Johnsen, A., Laskemoen, T., Weir, J. T., & Lifjeld, J. T. (2015a). Postcopulatory sexual selection is associated with accelerated evolution of sperm morphology. *Evolution*, 69(4), 1044–1052. <https://doi.org/10.1111/evo.12620>
- Rowe, M., Griffith, S., Hofgaard, A., & Lifjeld, J. (2015b). Subspecific variation in sperm morphology and performance in the long-tailed finch (*Poephila acuticauda*). *Avian Research*, 6(1), 23. <https://doi.org/10.1186/s40657-015-0032-z>
- Rowley, A., Locatello, L., Kahrl, A., Rego, M., Boussard, A., Garza-Gisholt, E., & Fitzpatrick, J. L. (2019). Sexual selection and the evolution of sperm morphology in sharks. *Journal of Evolutionary Biology*, 32(10), 1027–1035. <https://doi.org/10.1111/jeb.13501>
- Safran, R., Flaxman, S., Kopp, M., Irwin, D. E., Briggs, D., Evans, M. R., & Uy, J. A. C. (2012). A robust new metric of phenotypic distance to estimate and compare multiple trait differences among populations. *Current Zoology*, 58(3), 426–439. <https://doi.org/10.1093/czoolo/58.3.426>
- Schmitt, C. J., & Edwards, S. V. (2022). Passerine birds. *Current Biology: CB*, 32(20), R1149–R1154. <https://doi.org/10.1016/j.cub.2022.08.061>
- Schmoll, T., & Kleven, O. (2011). Sperm dimensions differ between two coal tit *Parus ater* populations. *Journal of Ornithology*, 152(3), 515–520. <https://doi.org/10.1007/s10336-010-0603-z>
- Servedio, M. R., & Boughman, J. W. (2017). The role of sexual selection in local adaptation and speciation. *Annual Review of Ecology, Evolution, and Systematics*, 48(1), 85–109. <https://doi.org/10.1146/annurev-ecolsys-110316-022905>
- Servedio, M. R., Doorn, G. S. V., Kopp, M., Frame, A. M., & Nosil, P. (2011). Magic traits in speciation: ‘magic’ but not rare? *Trends in Ecology and Evolution*, 26(8), 389–397. <https://doi.org/10.1016/j.tree.2011.04.005>
- Shaw, K. L., & Mullen, S. P. (2011). Genes versus phenotypes in the study of speciation. *Genetica*, 139(5), 649–661. <https://doi.org/10.1007/s10709-011-9562-4>
- Sokal, R. R., & Rohlf, F. J. (1981). *Biometry*. W. H. Freeman & Co.
- Thibert-Plante, X., & Gavrilets, S. (2013). Evolution of mate choice and the so-called magic traits in ecological speciation. *Ecology Letters*, 16(8), 1004–1013. <https://doi.org/10.1111/ele.12131>
- Valente, L., Phillimore, A. B., Melo, M., Warren, B. H., Clegg, S. M., Havenstein, K., & Etienne, R. S. (2020). A simple dynamic model explains the diversity of island birds worldwide. *Nature*, 579, 92–96. <https://doi.org/10.1038/s41586-020-2022-5>
- Varea-Sánchez, M., Gómez Montoto, L., Tourmente, M., & Roldan, E. R. S. (2014). Postcopulatory sexual selection results in spermatozoa with more uniform head and flagellum sizes in rodents. *PLoS One*, 9(9), e108148. <https://doi.org/10.1371/journal.pone.0108148>
- Veen, T., Borge, T., Griffith, S. C., Sætre, G. -P., Bures, S., Gustafsson, L., & Sheldon, B. C. (2001). Hybridization and adaptive mate choice in flycatchers. *Nature London*, 411(3 May 2001), 45–50. <https://doi.org/10.1038/35075000>
- Wade, M. J., Patterson, H., Chang, N. W., & Johnson, N. A. (1994). Postcopulatory, prezygotic isolation in flour beetles. *Heredity*, 72 (Pt 2), 163–167. <https://doi.org/10.1038/hdy.1994.23>
- Weissing, F. J., Edelaar, P., & van Doorn, G. S. (2011). Adaptive speciation theory: A conceptual review. *Behavioral Ecology and Sociobiology*, 65(3), 461–480. <https://doi.org/10.1007/s00265-010-1125-7>
- Wickham, H., Averick, M., Bryan, J., Chang, W., McGowan, L. D. A., François, R., & Hester, J. (2019). Welcome to the Tidyverse. *Journal of Open Source Software*, 4(43), 1686. <https://doi.org/10.21105/joss.01686>
- Yang, Y., Zhang, H., Wang, S., Yang, W., Ding, J., & Zhang, Y. (2020). Variation in sperm morphology and performance in tree sparrow (*Passer montanus*) under long-term environmental heavy metal pollution. *Ecotoxicology and Environmental Safety*, 197, 110622. <https://doi.org/10.1016/j.ecoenv.2020.110622>