Sperm divergence in a passerine contact zone: indication of reinforcement at the gametic level

Abstract

Postcopulatory sexual selection may promote evolutionary diversification in sperm form, but the contribution of between-species divergence in sperm morphology to the origin of reproductive isolation and speciation remains little understood. To assess the possible role of sperm diversification in reproductive isolation, we studied sperm morphology in two closely related bird species, the common nightingale (*Luscinia megarhynchos*) and the thrush nightingale (*L. luscinia*), that hybridize in a secondary contact zone spanning Central and Eastern Europe. We found: (1) striking divergence between the species in total sperm length, accompanied by a difference in the length of the mitochondrial sperm component; (2) greater divergence between species in sperm morphology in sympatry than in allopatry, with evidence for character displacement in sperm head length detected in *L. megarhynchos*; (3) interspecific hybrids showing sperm with a length intermediate between the parental species, but no evidence for decreased sperm quality (the proportion of abnormal spermatozoa in ejaculates). Our results demonstrate that divergence in sperm morphology between the two nightingale species does not result in intrinsic postzygotic isolation, but may contribute to postcopulatory prezygotic isolation. This isolation could be strengthened in sympatry by reinforcement.

Key words: avian hybrid zone, *Luscinia*, nightingales, speciation, sperm size

Introduction

Understanding how reproductive isolation arises between incipient species remains a central goal in speciation research. Rapid diversification of sexual traits driven by sexual selection and sexual conflict is thought to play an important role in establishing reproductive isolation between species (Safran et al. 2013; Seddon et al. 2013). The morphology of male gametes (spermatozoa) may be an example of such sexual traits (Rowe et al. 2015). Spermatozoa exhibit remarkable variability in size and shape among species (Pitnick et al. 2009) and sometimes undergo rapid and substantial divergence even between closely related species or populations of the same species (e.g., Breed 1983; Landry et al. 2003; Pitnick et al. 2003; Hogner et al. 2013; Laskemoen et al. 2013a; Albrechtová et al. 2014). The diversity in spermatozoa (despite the common function of sperm cells to fertilize the ova) has been mostly attributed to postcopulatory sexual selection, including sperm competition and cryptic female choice (e.g., Snook 2005; Simmons and Fitzpatrick 2012; Rowe et al. 2015). The contribution of sperm divergence to the origin of reproductive isolation and speciation is, however, still not broadly understood.

Divergence in sperm traits between species can contribute to reproductive isolation in two ways. Firstly, it can cause postcopulatory prezygotic isolation, with heterospecific sperm having a reduced chance of fertilizing eggs compared to conspecific sperm. Such conspecific sperm precedence was thought to be important mainly in free spawning marine invertebrates, where the lack of complex courtship and mating behaviours should make the establishment of premating isolation difficult (e.g., Geyer and Palumbi 2005). However, examples of conspecific sperm precedence in *Drosophila* fruit flies and other insects (Gregory and Howard 1994; Wade and Johnson 1994) suggest that this form of reproductive isolation might also be important in terrestrial animals with internal fertilization. In vertebrates, postcopulatory prezygotic isolation is still understudied. Nevertheless, an example of conspecific sperm

precedence has been reported in mammals (Dean and Nachman 2009), and a recent study has shown that in a pair of songbird species, cryptic female preference for conspecific sperm could be involved in constituting reproductive barriers (Cramer et al. 2016).

Secondly, divergence in genes affecting production and maturation of spermatozoa may result in Dobzhansky-Muller incompatibilities (Coyne and Orr 2004) between these genes in the genomes of hybrid individuals, which will ultimately lead to sterility of hybrids, i.e. intrinsic postzygotic isolation. According to Haldane's Rule, hybrid sterility affects first the heterogametic sex (Haldane 1922). In birds, where males are the homogametic sex, hybrid male sterility usually evolves relatively slowly; complete loss of hybrid male fertility takes on the order of millions of years (Price and Bouvier 2002). A recent study on wild populations of two closely related flycatcher species, however, suggested that male hybrid sterility may also appear in species with little genetic divergence (Ålund et al. 2013).

An important goal of speciation research is to understand whether reproductive barriers separating the species evolved in allopatry, without the presence of interspecific gene flow, or after secondary contact in sympatry, in the presence of gene flow. Reinforcement is the process by which natural selection increases prezygotic reproductive isolation between species after secondary contact, because prezygotic isolation reduces the costs of maladaptive hybridization (Butlin 1987; Servedio 2004). Despite a long-lasting debate over whether reinforcement can occur (Coyne and Orr 2004), theoretical as well as empirical work during the last two decades provides clear support for its existence (e.g., Sætre et al. 1997; Rundle and Schluter 1998; Servedio and Noor 2003; Hoskin et al. 2005; Ortiz-Barrientos et al. 2009; Bímová et al. 2011). Almost all examples of reinforcement, however, concern premating behavioural isolation. By contrast, there is only a handful of well supported examples of reinforcement on the level of postcopulatory prezygotic (gametic) isolation (Grant 1966; Turner et al. 2010) of which only one is from animals (Matute 2010).

Here we studied sperm morphology and its potential role in reproductive isolation in two closely related passerine birds, the common nightingale (*Luscinia megarhynchos*) and the thrush nightingale (*L. luscinia*). The sister species diverged 1.8 Mya (Storchová et al. 2010) and currently hybridize in a zone of secondary contact spanning Central and Eastern Europe (Becker 2007; Reifová et al. 2011a). The species are very similar morphologically as well as ecologically (Reif et al. 2015), although partial habitat segregation associated with bill size divergence has occurred after secondary contact in response to interspecific competition in sympatric populations (Reif et al. 2018; Sottas et al. 2018). Despite relatively strong assortative mating, the species occasionally hybridize in sympatry and produce viable hybrid progeny. It has been estimated that about 3-5% of sympatric individuals represent F_1 or early generation backcross hybrids (Becker 2007; Reifová et al. 2011b). Following Haldane's Rule, F_1 hybrid females are sterile, but F_1 hybrid males are fertile and might thus mediate gene flow between the species (Stadie 1991; Reifová et al. 2011a; Mořkovský et al. 2018). Estimated levels of interspecific gene flow (measured as the population migration rate, 2Nm) on autosomes are 0.763 from *L. megarhynchos* to *L. luscinia* and 0.081 in the opposite direction (Storchová *et al.* 2010).

In order to study the possible role of sperm divergence in reproductive isolation between species, we first looked at geographic patterns of sperm morphology to evaluate potential shifts in sperm morphology between allopatric and sympatric populations. We addressed the principal question whether there is higher divergence in sperm morphological traits in sympatric populations than in allopatric populations (character displacement), indicative of reinforcement processes acting during the postcopulatory prezygotic phase of mate choice. Second, we assessed the sperm morphology of interspecific hybrids. We evaluated the idea that hybrid individuals produce low quality ejaculates (see also Ålund et al. 2013)

which would indicate hybrid incompatibilities and lead to postzygotic reproductive isolation of the two species.

Material and Methods

Sampling: Data were collected in the Central European sympatric zone of the focal species (central Poland) and the adjacent allopatric regions (south-western Poland and the Czech Republic for common nightingales and north-eastern Poland for thrush nightingales), spanning almost 650 km (Fig. 1). Individual males were captured by a mist net or collapsible trap accompanied by playback of conspecific song. Sampling was performed at the beginning of the breeding season during May 2012 – 2016 and balanced with respect to the region (effect of region on sampling date: $F_{3,108} = 1.14$, $P = 0.34$; maximum difference in sampling date between regions was 1.58 ± 1.42 days between thrush nightingale sympatry and thrush nightingale allopatry). Each male was ringed and measured, and a blood sample was collected by brachial venipuncture, with blood stored in 96% ethanol for further genetic analysis. Sperm samples were obtained by a gentle massage of the cloacal protuberance (Wolfson 1952) and stored in 10% formalin. Eight retrapped birds provided two sperm samples each, but only one randomly chosen sample per individual was included in the analysis. In total, we analysed 117 males that provided sperm samples, of which five were later identified as hybrids (see below). The dataset thus consisted of 31 and 39 samples of *L. megarhynchos* (common nightingale, hereafter CN) from allopatry and sympatry, respectively; 16 and 26 samples of *L. luscinia* (thrush nightingale, hereafter TN) from allopatry and sympatry, respectively; and five interspecific hybrids from sympatry. All fieldwork procedures were approved by the Local Ethic Committee for Scientific Experiments on Animals in Poznan, Poland (permissions no. 27/2008 and 17/2015).

Analysis of sperm morphology: An aliquot of the fixed ejaculate was smeared onto a microscope slide and examined under a microscope. Twenty randomly chosen spermatozoa per male were photographed using a microscope BX51 (Olympus) at 200x total magnification. This magnification was used because nightingale sperm cells are typically beyond the field of view with 400x magnification utilised in many previous studies on avian species with shorter sperm (e.g., Opatová et al. 2016). The length of the sperm head (including acrosome), the midpiece and the tail of each sperm were later measured to the nearest 0.1 μm using QuickPhoto Industrial software (Olympus) following standard protocol (e.g., Knief et al. 2017). Total sperm length was calculated as the sum of these three components. For all measurements of sperm components, we focused on morphologically normal spermatozoa with the helical structure typical of passerine sperm, and excluded abnormal, immature and damaged cells from the analyses (for detailed description see also Opatová et al. 2016). All measurements were done by a single person (KO) to reduce observer error. In order to test the reliability of sperm measurements, one randomly selected sperm cell was measured twice in 30 randomly selected males representing both species, with repeated measures performed haphazardly, allowing various periods of time between measurements of the same cell and blind to previous measurements. Repeatabilities were calculated using the *rptR* package (Stoffel et al. 2017) with 95% confidence intervals based on lmm method and 1000 bootstrap samples for each of the three measured sperm components, i.e., head, midpiece and tail. The repeatability estimates (with 95% confidence intervals given in parentheses) were 0.94 (0.88 – 0.97) for head, 1.0 (0.99 – 1.0) for midpiece, and 0.99 (0.97 – 0.99) for tail, respectively.

The proportion of morphologically normal and abnormal spermatozoa in ejaculates was assessed under a 400x magnification BX51 Olympus light microscope (Olympus, Japan), with 100 sperm cells analysed per sample. Sperm were considered abnormal if they did not show the typical helical songbird head-shape or if they had broken or bent tails. Sperm abnormalities

were scored in the five hybrid individuals that provided sperm and in a subset of 40 pure individuals (20 CN and 20 TN) randomly chosen from the region of sympatry. All measurements and all scoring were done blind with respect to male species and region status and by the same person (KO) in order to reduce observer error.

Genotyping: All 70 sympatric males were genotyped using ddRAD sequencing (Peterson et al. 2012) to recognize interspecific hybrids. In addition, we genotyped 16 CN individuals and 18 TN individuals from allopatry to select species-diagnostic SNP markers. Genomic DNA was purified from each sample using the DNeasy TissueKit (Qiagen), according to the manufacturer's instructions. ddRAD sequencing followed the methods (including endonucleases and size-selection parameters) described in Piálek et al. (2019). Sequencing was performed on an Illumina HiSeq 2500 system (125 cycles P/E, v4 kit) in the EMBL Genomic Core Facility, Heidelberg, Germany.

Barcode sorting and quality filtering of raw reads were performed using process_radtags in Stacks v1.35 (Catchen et al. 2011). We discarded all reads of low quality, reads that contained ambiguous barcodes or restriction sites, and reads containing adapter sequence. The average number of retained reads per sample was 2,168,918 ± 534,306 (SD). To find homologous loci between individuals, the obtained paired reads were aligned onto the genome of *Ficedula albicollis* FicAlb1.5 (GenBank assembly GCA_000247815.2; https:// www.ncbi.nlm.nih.gov) using Bowtie 2 assembler (v2.2.4; Langmead and Salzberg 2012) and then processed in the ref_map pipeline implemented in Stacks. SNP variant calling was processed in the population component of Stacks (minimum number of individuals with the present locus, 0.5; minimum stack depth for each individual, 20) and resulted in 48,263 variable SNPs. The comparison of sequences from allopatric individuals revealed 1104 fixed SNPs between the two nightingale species.

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175 *Identification of interspecific hybrids:* To identify interspecific hybrids among sympatric
176 samples, we employed NewHybrids software (Anderson and Thompson 2002). This method
177 estimates the posterior probability that an individual falls into previously defined genotype
178 frequency categories. We defined fourteen possible categories including pure parental species
179 (pure CN and pure TN), first and second generation of intercrosses (F_1, F_2), and backcross
180 hybrids extending into fifth generation on both parental species (BC_1 – BC_5 on CN, and BC_1 –
181 BC_5 on TN). NewHybrids identifies hybrids based on the proportion of the genome that is
182 heterozygous or homozygous for alleles of one or the other species. For that reason, it is
183 important to ensure that SNP markers used in the analysis are more or less evenly distributed
184 across the genome. We therefore selected for this analysis 344 species-specific SNPs with a
185 minimum distance between each other of 1Mbp. The program was run with uniform priors for
186 π and θ and a burn-in period of 25,000 sweeps followed by 50,000 sweeps. Samples from
187 allopatric populations were specified as pure CN or TN using the z option. The program was
188 run three times with identical starting conditions, with the exception of the random number
189 seeds, to assess convergence. Independent runs converged to the same results. NewHybrids is
190 particularly suitable for identification of hybrids in species such as nightingales, where the
191 frequency of hybridization in natural populations is very low and female hybrids are sterile, so
192 that interbreeding between hybrids is very unlikely. Using this approach, we may have
193 misidentified hybrids of later than BC_5 generation as pure parental species. Identification of
194 such hybrids with less than 1 % of the genome coming from heterospecifics was, however,
195 beyond the scope of this study.
196

197 *Statistical analysis:* Analyses were performed in R 3.4.0 (R Core Team 2017). We focused on
198 three sperm traits selected *a priori*: the length of the sperm head, length of the midpiece and

total sperm length. The importance of these traits for sperm performance and function has been well documented. For example, the sperm head contacts the egg's perivitelline layer at fertilization (Karr et al. 2009), and its length correlates with other aspects of its shape which may affect the hydrodynamics of swimming (Støstad et al 2018). Midpiece length is thought to determine sperm swimming speed (Lüpold et al. 2009; Laskemoen et al. 2010; Knief et al. 2017) and ATP levels in birds (Rowe et al. 2013). Total sperm length may interact directly with the size of female sperm storage organs (Briskie and Montgomerie 1993) and contribute to the segregation of sperm in female SSTs (Hemmings and Birkhead 2017). Longer sperm also tend to be faster (Kleven et al. 2009; Knief et al. 2017). We also evaluated variation in tail length (the part of flagellum not wrapped by the midpiece), but the association with sperm performance remains unclear for this trait. Results of analyses involving tail length are provided in the Supporting Information section.

We evaluated geographic patterns in sperm morphology (including all 20 sperm cells measured per male, with male identity fitted as random effect) using a linear mixed effects model with the sperm trait of interest as a response variable. Models on pure species involved two categorical predictors (region of sampling: allopatry or sympatry; species: TN or CN) and the *species x region* interaction as fixed effects. A significant interaction term could indicate character displacement in the trait of interest in sympatric populations (Reifová et al. 2011b; see Supporting Information Fig. S1). Sampling date (1 = January 1st) and geographic position (latitude and longitude) were also fitted as covariates (fixed effects) in initial global models because previous studies have shown effects of both on sperm morphology in passerines (Lüpold et al. 2011, 2012; Cramer et al. 2013). Factor levels were coded as 0 and 1 (TN – 0, CN – 1; sympatry – 0, allopatry – 1) and all predictor variables were centred to enable interpretation of the main fixed effects without the need to remove the interaction term (Schielzeth 2010). Midpiece length and total sperm length were strongly correlated in both

224 species (Pearson's r_{1400} = 0.81 in CN and r_{840} = 0.84 in TN, respectively), and separate analyses
225 based on these traits provided qualitatively and quantitatively similar results (see below and
226 Fig. 2). Hence, in the main text we primarily present analyses based on total sperm length and
227 detailed results concerning the midpiece length are provided in the Supporting Information (see
228 below). In contrast, the correlation between sperm head length and sperm total length was much
229 weaker or absent (Pearson's r_{1400} = 0.24 in CN and r_{840} = 0.04 in TN, respectively), and this
230 was also reflected by a close correlation between the relative sperm head length (head length
231 divided by total sperm length) and sperm head length (Pearson's r_{1400} = 0.93 in CN and r_{840} =
232 0.86 in TN, respectively). We used the package *lme4* (Bates et al. 2015) for linear mixed effects
233 models with male identity incorporated as a random grouping variable. Within-individual
234 repeatabilites for sperm head, midpiece, tail and total sperm length were calculated separately
235 for each species without accounting for region of sampling and other covariates (intercept only
236 included), using the *rptR* package (Stoffel et al. 2017) with 95% confidence intervals based on
237 lmm method and 1000 bootstrap samples.
238 To check whether sperm morphology in hybrids differed from parental species, we used
239 all available samples from sympatry using the sperm trait of interest as dependent variable, and
240 species identity (CN, TN or hybrid) as the explanatory variable. To evaluate patterns in the
241 proportion of abnormal spermatozoa in ejaculates of hybrids and parental species, we
242 constructed a generalized linear effects model with family set first to "binomial" (logit), using
243 sperm identity (CN, TN or hybrid) as an explanatory variable and the number of abnormal
244 sperm and normal sperm in the ejaculate (grouped by cbind function) as the dependent variable,
245 using the glm function. To control for overdispersion in the model, however, we finally used a
246 quasibinomial approach and F statistics (Crawley 2012).
247 To test fixed effects, we always began with global (full) models and removed
248 nonsignificant interactions and then nonsignificant main effects in a backward stepwise

procedure (Harrison et al. 2018). Significances of explanatory fixed variables in initial models were based on the drop1 function in R and therefore based on the Type III sum of squares. Full models are reported along with the simplified version of the models (i.e., reduced models; Crawley 2012). To compare models, we also used likelihood-ratio tests and report on changes in the likelihood ratio (LRT in mixed models) or F statistics (in generalized linear models) between two models of interest, and associated change in degrees of freedom (ΔDf) and *P* values (Crawley 2012; Harrison et al. 2018). *Posthoc* tests were performed using the *multcomp* package and ghlt function (Hothorn et al. 2008). All tests were two-tailed.

Results

Identification of interspecific hybrids

From 70 sympatric individuals, 39 were identified as pure CN and 26 as pure TN in the NewHybrids analysis. Five individuals were identified as hybrids. Specifically, we identified two F_1 hybrids, two BC_1 hybrids on TN, and one BC_3 hybrid on TN. The posterior probabilities were in all cases higher than 95%. The remaining 31 pure CN males and 16 pure TN males were sampled in allopatry (see Fig. 1).

Sperm morphology of pure nightingale species in sympatry and allopatry

We measured 1400 sperm cells of pure CN and 840 sperm cells of pure TN males, representing 70 and 42 individuals, respectively. Within-individual repeatabilities were significant for all sperm traits of interest in both species (Supporting Information Table S1). Sperm morphology variation for both species in allopatry and sympatry is summarized in Fig. 2 and in Supporting Information Table S2.

There was a striking difference in total sperm length between the two nightingale species, with no overlap in size and with CN sperm being clearly longer than TN sperm (Fig.

2 and Table 1). Species identity (CN and TN, respectively) was the only significant predictor of total sperm length retained in the final simplified model (Table 1). There was no evidence for shifts in total sperm length with regard to sympatry or allopatry. A *posthoc* analysis, based on a mixed model involving four male categories (CN allopatric and sympatric, TN allopatric and sympatric), along with geographic coordinates and sampling date as covariates, confirmed the difference in sperm length between CN and TN and that there was no shift in sperm sizes between sympatry and allopatry in either species (Table 2). Midpiece length followed the same pattern (Fig. 2 and Supporting Information Tables S2 – S5). Tail length showed no variation with respect to region or species (Fig. 2 and Supporting material Table S3 and S5).

Differences between species in sperm head length were negligible when controlled for effects of all covariates in the full model (Table 1). However, the difference in sperm head sizes between species was apparent in sympatry, with sperm heads being shorter in CN than in TN (Fig. 2). The *region x species* interaction was retained in the final simplified model, along with its components and the date of sampling (Table 1). A *posthoc* analysis, based on a mixed model involving four male categories (CN allopatric and sympatric, TN allopatric and sympatric), along with geographic coordinates and sampling date as covariates, confirmed the shift in sperm head sizes in sympatry in CN but not in TN, resulting in the difference in sperm head length between species in sympatry (Table 2).

Sperm of hybrid individuals

The analysis involved 1400 sperm cells of five hybrid, 39 CN and 26 TN males, respectively. Male species identity (pure CN, pure TN or hybrid) determined total sperm length (comparison of models with and without the male species identity included: LRT = 44.54, ΔDf = 1, $P <$ 0.001). A *posthoc* analysis based on a mixed model involving male species identity revealed that the sperm length of hybrid males was intermediate in size between both parental species

(Fig. 2 and Table 3), and the same applied to midpiece length (Supporting Information Table S6). The effect of male species identity on sperm head length was also significant (LRT 44.54, $\Delta Df = 1$, $p < 0.001$) but *posthoc* analysis indicated that hybrids differed in sperm head length only from TN, having shorter heads (Table 3). The proportion of abnormal spermatozoa in hybrid individuals was low (2–27%, median 4%) and did not deviate from values obtained from 20 randomly chosen pure TN and 20 CN males in sympatry (ranging between 1 – 32% in both species with median being 4%; quasibinomial model, comparison of initial and null model: $F = 0.78$, $P = 0.46$, $\Delta Df = 2$; see also Supporting Information Table S7). This result should be treated with caution given that there were only five hybrid individuals involved in the analysis, and these were represented not only by F_1 hybrids, but also backcrosses (see above).

Discussion

In this study, we investigated sperm morphology in the secondary contact zone between two closely related nightingale species with incomplete reproductive isolation. We found that sperm sizes differed substantially between two species with the difference in sperm head length being particularly apparent in the region of sympatry. Interspecific hybrids had sperm of intermediate size between the two parental species, but were otherwise morphologically normal. Our results suggest the potential for sperm to act as a postcopulatory prezygotic barrier in this system, but provide no evidence that it contributes to an intrinsic postzygotic barrier.

Common nightingale sperm are longer and have longer midpieces but shorter heads than thrush nightingale sperm. The divergence between the two nightingale species in sperm length is similar to the average divergence in sperm length between species within other passerine genera (15% divergence in nightingales compared to 14.5% average divergence within genera, range 1.5% - 72.8%, based on data presented in Rowe et al. 2015). However,

the fact that there was no overlap in total sperm length between nightingale species is striking. Sperm length may be particularly evolutionarily labile in genus *Luscinia*, as the bluethroat *L. svecica*, also shows clear, albeit a bit lower, divergence in total sperm length among recently-diverged subspecies (Hogner et al. 2013). This may be caused by relatively high rates of extra-pair copulations leading to strong postcopulatory selection in these species (Johnsen and Lifjeld 2003; Landgraf et al. 2017; Janoušek et al. 2019). The difference between nightingale species in sperm morphology may imply that sperm functional traits, such as speed, also differ. Studies of other passerines show that swimming speed correlates positively with total sperm length (Kleven et al. 2009; Bennison et al. 2015; Rowe et al. 2015; Knief et al. 2017) and the length of the midpiece with its mitochondrial load (Lüpold et al. 2009; Rowe et al. 2013). Furthermore, swimming speed correlates negatively with the relative length of the sperm head in other passerine species (e.g., Lüpold et al. 2009), potentially due to increased drag of relatively long head (Humphries et al. 2008). Sperm head is proportionally shorter in CN than TN (data not shown) as a result of longer sperm cells in the former species. Sperm of common nightingales may therefore move faster than sperm of thrush nightingales. Differences in sperm morphology (and potentially also speed) could even result in asymmetric heterospecific fertilization advantage, whereby one species would be superior regardless of context (conspecific or heterospecific). This could explain why most F_1 hybrids in the nightingale hybrid zone come from mating of CN males with TN females (Vokurková et al. 2013) as well as stronger introgression from CN to TN (Storchová et al. 2010). Further research is, however, needed to evaluate this idea.

Interspecific differences in sperm morphology, and/or the correlated differences in sperm function, could cause decreased fertilization success by heterospecific sperm, via several pathways. Not only could relative and absolute sizes of sperm components determine sperm speed (see above, also Støstad et al. 2018), but female birds store sperm in specialized organs

(sperm storage tubules), and in general, the sperm cell is half the length of the tubule (Briskie and Montgomerie 1993). If females' sperm storage tubules differ in length between the nightingales as dramatically as sperm length differs, there might be differential ejaculate storage and utilization biased towards conspecific spermatozoa (also Pitnick et al. 2003). Recently, passerines have been shown to have a mechanism to discriminate sperm by their total length and speed via differential sperm storage in female SSTs (Hemmings and Birkhead 2017). Additionally, the sperm head and acrosome are responsible for the first contact between the sperm and perivitelline layer of the egg (Karr et al. 2009), and as such, biochemical and mechanical aspects of the head and acrosome could impact the ability of heterospecific sperm to fertilize an egg. Sperm head length *per se* may be less important in this context, but length correlates with other structural measures of the sperm head (Støstad et al. 2018), and head morphology correlates with functional traits such as proper DNA condensation (Carrell and Liu 2001) and ability to penetrate perivitelline layer (Saadi et al. 2013) in disease states in mammals. Sperm head length may therefore evolve due to selection on correlated traits. While the differences we observed in sperm head length between nightingale species in sympatry were small compared to differences for other sperm components, head length is generally less variable across passerine species (minimum – maximum and average CV for species studied by Rowe et al. 2015: head 10.16 – 24.58, 15.8; midpiece 1.53 – 250.77, 81.6; flagellum 29.11 – 261.69, 59.0; see also Støstad et al. 2018). The apparent evolutionary conservation of head length across species may indicate that the small change we observe has substantial consequences for heterospecific fertilization success. It is worth noting, however, that within individual repeatabilities of all evaluated sperm traits, including head, were similar in both species of nightingales (Supporting Information Table S1).

The clear shift in common nightingale sperm head length between allopatry and sympatry, making head length (and thus sperm morphology in general) even more

differentiated between species in regions where they co-occur, may indicate that postcopulatory prezygotic reinforcement has shaped sperm morphology in a manner similar to that suggested for the interaction of sperm velocity with the female environment in *Ficedula* flycatchers (Cramer et al. 2016). The pattern in nightingale sperm is consistent with evolution via reinforcement for several reasons. Changes in sperm head length could reduce levels of hybridization and gene flow by allowing for the preferential use of conspecific sperm (see above). There is strong selection against hybridization in these species, as hybrid females are sterile (Stadie 1991; Reifová et al. 2011a; Mořkovský et al. 2018). Differences in sperm head length and other sperm components are likely genetically determined (also see Birkhead et al. 2005), and repeatability within individuals sampled multiple times seems to be moderate to high in birds (e.g., Lüpold et al. 2012; Laskemoen et al. 2013b). We do not have comparable data based on repeated sampling of individuals over time, but within-male repeatability in sperm dimensions was significant in both nightingale species for all sperm components evaluated. Though sperm head length increased across the season, indicating some seasonal plasticity (as has been shown in some other songbirds for several sperm components; e.g., Lüpold et al. 2011; Cramer et al. 2013), sampling date was balanced in all regions and could not explain the difference in common nightingale sperm head length between sympatry and allopatry. Similarly, the potential presence of undetected later generation backcross hybrids in sympatric populations should not account for the observed shifts in sperm morphology between sympatry and allopatry. In fact, if introgression influenced sperm morphology, we would expect increased convergence rather than divergence in sympatry. Finally, on-going interspecific gene flow (Storchová et al. 2010; Mořkovský et al. 2018) and the presence of backcross hybrids in sympatric populations show that speciation is not yet complete between these taxa. Demonstrating incomplete isolation in the model system is important when considering reinforcement (Butlin 1987). Although all of these observations are consistent with

399 the view that increased divergence in sperm head length in nightingales might represent a case
400 of reinforcement at the gametic level, further research involving sampling more sympatric as
401 well as allopatric localities and demonstrating that increased divergence in sperm head length
402 results in stronger postcopulatory prezygotic isolation will be needed to provide definite
403 evidence for reinforcement.

404 Despite marked differences in sperm morphology between the two nightingale species
405 and their relatively old divergence (1.8 Mya, Storchová et al. 2010), F_1 and early generation
406 backcross hybrids did not suffer from increased proportions of abnormal sperm. This result is
407 consistent with observed fertility of hybrid males in experimental crosses (Stadie 1991) and
408 explains why backcross hybrids are still present in the nightingale contact zone. Our limited
409 number of hybrids sampled, however, does not allow us to evaluate a possible polymorphism
410 in hybrid male sterility in the sympatric population. Similarly, hybrid males between house
411 sparrows (*Passer domesticus*) and Spanish sparrows (*P. hispaniolensis*) show little evidence of
412 reduced sperm functionality (Cramer et al. 2015). In contrast, hybrid males between pied and
413 collared flycatchers, which belong to the same family as nightingales (Muscicapidae), show
414 complete sterility with either no sperm or no normal sperm in their ejaculates (Ålund et al.
415 2013). Given the much more recent origin of the two flycatcher species (less than 0.5 Mya;
416 Backström et al. 2013), postzygotic isolation appears to evolve at highly variable rates among
417 different passerine species pairs, even when those species pairs are closely related. Although
418 divergence in sperm morphology does not seem to result in hybrid male sterility (intrinsic
419 postzygotic isolation), it may strengthen extrinsic postzygotic isolation in a similar way as
420 postcopulatory prezygotic isolation. If interspecific hybrids with intermediate sperm
421 morphology have reduced chances of fertilizing females of either of the parental species,
422 through mechanisms explained above, it may reduce the likelihood of backcrossing and thus
423 the levels of gene flow between the species.

The finding that hybrid male sperm were intermediate in size between the common and thrush nightingales seems to be consistent with the idea that sperm length is genetically determined, at least to some extent. Genes affecting sperm (and other genes with male-biased expression) are expected to be located predominantly on the avian Z chromosome (Ellegren 2011), and, indeed, recent empirical studies show that the Z chromosome affects phenotypic variation in sperm cell length in zebra finches (*Taeniopygia guttata*, e.g., Kim et al. 2017; Knief et al. 2017). Since the Z chromosome plays a major role in nightingale speciation (Storchová et al. 2010), it would be interesting to test whether possible postcopulatory prezygotic isolation associated with divergent sperm morphology between the two nightingale species could be linked to the Z chromosome.

It has been argued that reproductive barriers acting later in the reproductive process are less important to the speciation process than barriers acting earlier (Coyne and Orr 2004). The argument is that any form of precopulatory isolation, if evolved, will prevent heterospecific copulation as well as the origin of interspecific hybrids and thus reduce the possible importance of postcopulatory barriers. In nightingales, precopulatory isolation is currently relatively strong, which may reduce the importance of possible postcopulatory prezygotic isolation caused by sperm divergence. However, precopulatory isolation is to a large degree caused by segregation of species habitats, which has evolved only after secondary contact in response to interspecific competition (Reif et al. 2018; Sottas et al. 2018). It is thus possible that postcopulatory isolation associated with sperm divergence represented an important barrier to gene flow in early phases of secondary contact when precopulatory barriers were relatively weak. In addition, similar to the precopulatory isolation, the postcopulatory isolation might have been strengthened after secondary contact to reduce the costs of hybridization producing sterile hybrid females.

Conclusions

Our study demonstrates clear divergence in sperm sizes between two closely related passerine species, which still hybridize in nature and show incomplete postzygotic reproductive isolation. In addition, based on our analysis of sperm head length variation in nightingales in sympatry and allopatry, we report on one of the first examples of a sperm morphology shift in a vertebrate contact zone (but see Naretto et al. 2016), potentially indicative of reinforcement at the postcopulatory prezygotic level. The understanding of whether and how sperm head morphology affects fertilization success and/or contributes to conspecific sperm precedence in common nightingales will require further study, also involving sperm competition experiments with captive populations (reviewed in Howard et al. 2009; also Bennison et al. 2015; Hemmings and Birkhead 2017). Although our data are limited, the absence of dramatic changes in sperm quality in F_1 hybrid males suggests that sperm divergence in nightingales does not necessarily result in intrinsic postzygotic isolation as has been demonstrated for example in pied and collared flycatchers (Ålund et al. 2013). This is consistent with the view that complete intrinsic postzygotic isolation usually arises slowly in birds and might play a relatively small role in speciation compared to other barriers (Rabosky and Matute 2013).

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Table 1. Initial (global) and reduced (final) linear mixed effects models testing for associations between sperm morphology traits and region, species and their interaction. Species was coded as thrush nightingale = 0 and common nightingale = 1; region (geographic origin of samples) coded as sympatry = 0 and allopatry = 1. All explanatory variables were centred in order to enable the main effect estimates to be properly interpreted without the need to remove the interaction term from the model. Male identity (n = 112) was included as a random grouping variable. Provided are significances for predictors based on drop1 function and Type III Sum of squares (controlled for effects of other predictors in the model). Significance of the main effects were not tested when involved in interactions. Geographic position (latitude and longitude) as well as sampling date were included as covariates. Initial global models and reduced models for midpiece length and tail length are provided in Supporting Information files. The predictors retained in final reduced models are highlighted in bold. See Methods for further information about model simplification procedures.

Global (full) model				**Reduced model**		
Response and predictor variables	**Estimate ± SE**	**F**	**p-value**	**Estimate ± SE**	**F**	**p-value**
Total sperm length						
Intercept	256.93 ± 0.57	-	-	256.93 ± 0.57	-	-
Sampling date	0.25 ± 0.15	2.88	0.09	-	-	-
latitude	3.86 ± 4.14	0.86	0.35	-	-	-

longitude	-1.52 ± 1.73	0.77	0.38	-	-	-
Region	3.53 ± 2.53	-	-	-	-	-
Species	38.17 ± 3.32	-	-	38.33 ± 1.20	1008.3	<0.001
Region x Species	-0.69 ± 7.25	0.01	0.92	-	-	-
Sperm head length						
Intercept	14.34 ± 0.05	-	-	14.34 ± 0.05	-	-
Sampling date	0.06 ± 0.01	25.86	< 0.001	0.05 ± 0.01	23.27	< 0.001
Latitude	0.34 ± 0.36	0.88	0.35	-	-	-
Longitude	0.05 ± 0.15	0.10	0.76	-	-	-
Region	0.45 ± 0.22	-	-	0.32 ± 0.10	-	-
Species	-0.31 ± 0.29	-	-	-0.80 ± 0.10	-	-
Region x Species	1.56 ± 0.62	6.12	0.015	0.50 ± 0.21	5.38	0.02

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694 **Table 2. Results of a *posthoc* analysis of variation in selected sperm traits in nightingales.** Analysis is based on a mixed linear model involving
695 four male categories (common nightingale allopatric and sympatric, thrush nightingale allopatric and sympatric) as predictors of variation in
696 selected sperm traits, along with geographic coordinates and sampling date as covariates. Male identity was included as random intercept. See the
697 main text for details on sample sizes. CNa – common nightingale in allopatry, CNs – common nightingale in sympatry, TNa – thrush nightingale
698 in allopatry, TNs – thrush nightingale in sympatry; p-value: ns – $p > 0.05$, * – $p < 0.05$, ** – $p < 0.01$, *** – $p < 0.001$

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	Comparison	**CNs-CNa**	**TNs-CNa**	**TNa-CNa**	**TNs-CNs**	**TNa-CNs**	**TNa-TNs**
Total sperm length	Estimate (SE)	-3.27 (3.73)	-41.74 (4.12)	-37.77 (7.55)	-38.47 (1.63)	-34.50 (5.53)	3.97 (5.50)
	z, p-value	-0.88, ns	-10.13, ***	-5.00, ***	-23.67, ***	-6.23, ***	0.72, ns
Sperm head length							
	Estimate (SE)	-1.03 (0.32)	-0.07 (0.35)	-0.59 (0.65)	0.96 (0.14)	0.43 (0.48)	-0.52 (0.48)
	z, p-value	-3.19, **	-0.20, ns	0.91, ns	6.82,***	0.76, ns	-1.10, ns

Table 3. Results of a *posthoc* analysis of variation in sperm traits in two nightingale species and their hybrids. Analysis is based on a mixed linear model involving three male categories (common nightingale sympatric, thrush nightingale sympatric, hybrid males) as predictors of variation in selected sperm traits in sympatry. Male identity was included as random intercept. See the main text for details on sample sizes. CN – common nightingale sympatry, TN – thrush nightingale in sympatry, H – hybrid males in sympatry; p-value: ns – $p > 0.05$, * – $p < 0.05$, ** – $p < 0.01$, *** – $p < 0.001$

	Comparison	**H-CN**	**H-TN**	**TN-CN**
Total sperm length	Estimate (SE)	-24.00 (2.93)	13.78 (3.00)	-37.79 (1.56)
	z, p-value	-8.20, ***	-4.58, ***	-24.23, ***
Sperm head length				
	Estimate (SE)	-0.10 (0.27)	-1.20 (0.28)	1.10 (0.15)
	z, p-value	-0.36, ns	4.28, ***	7.59, ***

FIGURE CAPTIONS

Figure 1. Map of localities where males of the thrush nightingale (*Luscinia luscinia,* blue triangles) and the common nightingale (*Luscinia megarhynchos,* red circles) were sampled. Light grey - allopatric range of *L. megarhynchos*, dark grey - allopatric range of *L. luscinia*, intermediate grey - range overlap of both species (i.e., sympatry). Species' ranges are redrawn from (Reifová et al. 2011b).

Figure 2. Sizes of sperm and its components in two nightingale species and their hybrids, in areas of allopatric and sympatric occurrence. In total, 2340 sperm cells were measured, 20 cells per male. The number of males measured in each region x species combination is provided in the main text. Blue – thrush nightingale; red – common nightingale; grey – hybrid individuals (both F_1 and backcrosses). See the main text for further details and associated statistics. Medians, quartiles, 1.5 interquartile range and outliers are presented.