VOLUME 77, NUMBER 11, NOVEMBER 2023

EVOLUTION

INTERNATIONAL JOURNAL OF ORGANIC EVOLUTION





SOCIETY for the STUDY of EVOLUTION

Rapid sperm length divergence in a polygynandrous passerine: a mechanism of cryptic speciation?

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Abstract

When populations become geographically isolated, they begin to diverge in various traits and at variable rates. The dynamics of such trait divergences are relevant for understanding evolutionary processes such as local adaptation and speciation. Here we examine divergences in sperm and body structures in a polygynandrous songbird, the alpine accentor (*Prunella collaris*) between two allopatric high-altitude populations, in Morocco and Spain. The populations diverged around 82,000 years ago, as estimated with a coalescence-based phylogenetic analysis of genome-wide single-nucleotide polymorphisms. We found that birds in the two areas had nonoverlapping sperm lengths, which suggests adaptation to divergent female reproductive tract environments. Sperm length also showed an exceptionally low coefficient of among-male variation, a signal of strong stabilizing selection imposed by sperm competition. The evolutionary rate of sperm length was almost twice the rates for the most divergent morphological traits and more than three times higher than expected from literature data over a similar generational timescale. This rapid evolution of a key reproductive trait has implications for reproductive isolation and ultimately for speciation. Strong selection for different sperm length optima in allopatry predicts conspecific sperm precedence and disruptive selection in sympatry, hence a possible postcopulatory prezygotic barrier to gene flow.

Keywords: female promiscuity, local adaptation, reproductive isolation, sexual selection, speciation

Introduction

Many species show geographical variation in phenotype and a corresponding geographic structure of genomic variation (Avise, 2000; Zamudio et al., 2016). Gene flow dilutes these differences, while divergent selection pressures and drift sharpen them (Slatkin, 1987). The combination of reduced gene flow and strong divergent selection or drift will therefore promote population divergence in phenotypic traits. Such patterns are typically observed among allopatric populations, for example oceanic islands or montane "sky" islands, where gene flow is restricted by geographical barriers, populations are small, and selection pressures may differ locally (Coyne & Orr, 2004; Newton, 2003; Price, 2008). The magnitude of divergence will vary among traits depending on the strength and direction of selection shaping them. In addition to local adaptation, trait divergence can lead to reproductive isolation and speciation. This applies to traits involved in mate choice that make individuals mate with their own group (premating isolation) or traits that bias fertilization in favor of their own group in sperm competition (postcopulatory, prezygotic isolation). In addition, offspring resulting from crossbreeding can be inviable and have reduced fitness (postzygotic isolation) due to genomic incompatibilities. Reproductive isolation can, for obvious reasons, not be tested in the wild between allopatric populations but must be inferred from the divergence in traits that have a supposed function in mate choice or fertilization success (Coyne & Orr, 2004).

Birds are a well-studied group with numerous examples of geographic variation in phenotypic traits (Mayr, 1963; Newton, 2003; Price, 2008; Zink & Remsen, 1986). Populations will adapt to local environmental conditions through natural selection, but they can also diverge in sexually selected traits, such as plumage ornaments and coloration, courtship behavior, and vocalizations. Divergence in the latter traits could indicate premating isolation at an early phase of allopatric speciation. Little is known about geographic variation in gametic traits and their role in postcopulatory prezygotic isolation. This is puzzling, given the realization over the past decades that many pair-bonding species are sexually promiscuous (Brouwer & Griffith, 2019), which increases the opportunity for postcopulatory sexual selection (Cramer et al., 2020; Webster et al., 1995). If two populations have evolved divergent sperm traits that are under selection, it is an indication that sperm has adapted to different female environments, as the oviduct is the functional environment for sperm in species with internal fertilization.

Received June 10, 2023; revisions received July 29, 2023; accepted August 8, 2023

Associate Editor: Scott Taylor; ; Handling Editor: Miriam Zelditch

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When the populations meet again, a scenario of disruptive selection for two adaptive peaks could be expected, where sperm from the female's own population would have a fertilization advantage over sperm from the other population, that is, conspecific sperm precedence (Howard, 1999). The more males with which the females mate, the higher the chance of assortative fertilization such that hybridization will not occur. This mechanism assumes that females mate with more than one male, but it could also work under sexual monogamy if heterospecific sperm have reduced fertilization success. Divergent sperm traits could therefore indicate a prezygotic barrier to gene flow in promiscuous species and effectively become a "speciation phenotype" (Manier et al., 2013). The mechanism could work even if females mate with males at random (i.e., no premating isolation), but conspecific sperm precedence might select for premating isolation by reinforcement if there are significant costs associated with heterospecific or between-group copulations (Marshall et al., 2002).

Passerine birds show particularly high levels of female promiscuity as expressed by the frequency of extrapair paternity (Brouwer & Griffith, 2019; Griffith et al., 2002; Lifjeld et al., 2019). They are also highly diversified in sperm traits like sperm length (Omotoriogun et al., 2020). Sperm length is a trait that is correlated with, and probably adapts to, the length of female sperm storage tubules in birds (Briskie et al., 1997; Kleven et al., 2009). Divergent sperm length could then be a possible mechanism of postcopulatory, prezygotic isolation, as suggested for the highly promiscuous Ammospiza sparrows (Cramer et al., 2021). A comparative analysis of passerine species pairs showed that those with a high level of sperm competition diverge faster in sperm length than those with less sperm competition (Rowe et al., 2015). It is thus a plausible hypothesis that female promiscuity accelerates the evolution of sperm traits, which leads to a faster build-up of prezygotic isolation and hence rapid speciation. In a recent review of speciation processes in passerine birds, Fjeldså et al. (2020) suggested that factors associated with a mating system consisting of social monogamy with extrapair mating could have facilitated high speciation rates in the group of Passerides songbirds, which is the most species-rich infraorder of passerine birds. Passerides have comparably high rates of extrapair paternity (Lifield et al., 2019) and more diversified sperm lengths than other passerine groups (Marki, 2017; Omotoriogun et al., 2020). It is, however, important to keep in mind that macroevolutionary patterns of species differences do not necessarily reflect traits involved in speciation because divergent traits may evolve after speciation events. It is therefore essential to examine how rapidly traits considered important for reproductive isolation can evolve and to what extent they diverge among allopatric populations at the initial stages of a speciation process.

Here we report a study of various trait divergences between two allopatric populations of a promiscuous Passerides songbird, the alpine accentor (*Prunella collaris* Scopoli). The species has a patchy distribution in the high mountain ranges in the western Palearctic (Hatchwell & Christie, 2020), with presumably very little or no gene flow between isolated populations. We were especially interested in comparing sperm morphology in this species because it has a polygynandrous mating system where groups of males share copulatory access to a group of females and help them raise their broods (Davies et al., 1995; Nakamura, 1998a, 1998b). Sperm competition and mixed-paternity broods are thus the norm (Hartley et al., **1995; Heer, 2013).** We estimated the divergence time between the two study populations and calculated the evolutionary rate (*haldanes*) for divergence in various sperm and somatic morphological traits. We report that sperm length has evolved particularly fast in this mating system and discuss the implications for understanding the mechanisms of speciation in this and other species.

Methods

Study species

Prunella collaris belongs to a group of 12 species comprising the Prunellidae family (Gill et al., 2023). They are mostly distributed across mountain ranges of Asia, where the group presumably originated in the Himalayan region in the mid-Miocene, about 15-16 million years ago (mya) (Drovetski et al., 2013; Zang et al., 2023). Two species have expanded into the western Palearctic: P. collaris and the dunnock (P. modularis Linnaeus). Prunella collaris has a wide distribution from Japan in the east to Spain and Morocco in the west with nine recognized subspecies (Hatchwell & Christie, 2020). The nominate subspecies has a patchy distribution with small and isolated populations confined to the high mountain ranges in southern Europe and northwest Africa (Fig. 1). The species is considered Least Concern in the IUCN Red List (BirdLife International, 2021), but it is included as Near Threatened in the Spanish Red List since a range contraction is expected if the climate gets warmer (de Gabriel Hernando et al., 2021). Prunella modularis inhabits lowland forests and scrub areas up to the tree limit in most of its range and has a wide distribution in Europe (Hatchwell, 2005). The two species belong to two separate branches in the family that split about 7-9mya (Drovetski et al., 2013; Liu et al., 2017; Zang et al., 2023). The P. collaris branch has only one other extant species, the Altai accentor (P. himalayana Blyth), which diverged from the P. collaris lineage about 3-4 mya (Drovetski et al., 2013; Liu et al., 2017). The other 10 species in the family belong to the P. modularis branch and diversified during the last 4-6 my (Drovetski et al., 2013; Liu et al., 2017). Both P. collaris and P. modularis have a polygynandrous mating system (Davies et al., 1996), with a lack of sexual plumage dichromatism. The mating system of the other species in the family is poorly studied, but appears to be similar, and the sexes are monomorphic in all species (Winkler et al., 2020).

Field procedures

Fieldwork was conducted in June-July 2012-2015 in the Picos de Europa mountain range in northern Spain, at altitudes above 2,000 m, and in June–July 2013–2015 in the High Atlas, central Morocco, at altitudes above 3,000 m (Fig. 1). The required fieldwork permits for each year were issued by the Parque National de los Picos de Europa (Oviedo, Spain) and the Haut Commissariat aux Eaux et Forêts et à la Lutte Contre la Désertification (Rabat, Morocco). Birds were caught with mist nets in their territories. Adult birds were sexed based on the presence of a brood patch (females) or globular shaped cloacal protuberance (males). For each bird we measured the following morphological characters: right-wing chord (straightened and flattened), length of the right primary wing feathers and tail length using a ruler (precision ± 0.5 mm); right tarsus length and bill length using a digital caliper (precision ± 0.1 mm); and body mass using a digital weighing scale (precision ± 0.1 g). The length of primaries was used



Figure 1. The sampling locations (green dots) in Spain and Morocco and the breeding distribution (red areas) of the alpine accentor (*Prunella collaris*) in the western Mediterranean region (redrawn from BirdLife International, 2023).

to calculate three wing shape indices, isometric size, pointedness, and convexity, following the formulas in Lockwood et al. (1998), which are based on size-constrained component analysis. These indices rely on the length of individual primaries (P_1-P_8) , numbered ascendingly from the distal edge of the wing while omitting the vestigial-outermost primary feather. The three wing shape indices were calculated as follows:

Wing isometric size (*C*1); the index increases with increasing wing size, independently from its shape:

$$C1 = e^{\ln P_1^{0.209} \ln P_2^{0.200} \ln P_3^{0.198} \ln P_4^{0.195} \ln P_5^{0.192} \ln P_6^{0.192} \ln P_7^{0.204} \ln P_8^{0.210} - 10}$$

Wing pointedness (C2); increasing with decreasing wing pointedness:

 $C2 = e^{\ln P_1^{-3.49}} \ln P_2^{-1.816} \ln P_3^{-0.893} \ln P_4^{-0.003} \ln P_5^{0.829} \ln P_6^{1.351} \ln P_7^{1.661} \ln P_8^{2.363}$

Wing convexity (C3); increasing with increasing wing convexity:

 $C3 = e^{\ln P_1^{-6.231}} \ln P_2^{1.683} \ln P_3^{4.033} \ln P_4^{4.721} \ln P_5^{3.955} \ln P_6^{1.349} \ln P_7^{-3.185} \ln P_8^{-6.326} - 1$

The data set includes 28 males from the Spanish population and nine males from Morocco. We only compare males in this study since we had data for only two females in the Moroccan population. Divergence was quantified as Hedges' g (Hedges, 1981). This index expresses the difference between means in units of the pooled *SD* of the trait. For example, g = 1 means that the two populations differ by 1 *SD*. Sperm samples $(1-5 \ \mu$ l) were collected by cloacal massage (Laskemoen, Kleven, et al., 2013; Wolfson, 1952), diluted in a droplet $(10-20 \ \mu$ l) of PBS, and transferred to 5% phosphate-buffered formaldehyde solution for fixation and storage. Blood samples $(10-50 \ \mu$ l) were collected from each bird by venipuncture from the brachial or jugular vein and preserved in 96% ethanol.

Sperm traits and measurements

Sperm samples were prepared for light microscopy using the protocol described in Cramer et al. (2021) and Grønstøl et al. (2023). Briefly, we photographed sperm cells at 320× in a Leica DM6000B microscope with a digital camera (Leica DFC420) and measured the length of sperm segments using Leica Application Suite software v4.13. We measured the length of the sperm head, midpiece, and tail (i.e., the midpiece-free end of the flagellum). The sum of the three measurements gives the total sperm length, and the sum of the midpiece and tail equals the flagellum length. We measured 10 sperm cells per male and used the mean value for each segment. We also calculated the coefficient of variation of sperm lengths among the 10 sperm cells from the same male (CV_{wm}) and among males (CV_{am}) using their mean values with the correction factor of (1 + 1/4N) for variable sample sizes, since

CV is generally underestimated at low sample sizes (Sokal & Rohlf, 1981).

Sperm length CV_{am} is negatively correlated with the risk of sperm competition measured as the frequency of extrapair young (EPY) in socially monogamous passerines (Calhim et al., 2007; Kleven et al., 2008; Lifjeld et al., 2010, 2019). It is generally assumed that the association reflects a causal relationship where sperm competition acts as a force of stabilizing selection that reduces the trait variance around an optimum value. For an estimation of sperm competition risk in P. collaris, we examined paternity data in two studies (Hartley et al., 1995; Heer, 2013) and assumed that the number of offspring not sired by the alpha male in the polygvnandrous groups would be equivalent to EPY in a socially monogamous pair. Hartley et al. (1995) found that 41 of 110 (37.3%) young were not sired by the alpha male, and Heer (2013) reported 44 of 72 (61.1%) young. We used the average percentage of the two studies (= 49.2%) as our estimate of sperm competition risk.

Calculation of evolutionary rates

The evolutionary rate of a trait can be measured as the amount of change over a time period in a population or lineage, or as the divergence between two populations or lineages over the time since their last common ancestor (divergence time). The two rates are equivalent. We calculated the divergence rates in *haldanes* according to Gingerich (2001) using logged measurements (natural logarithms). The *haldane* is calculated as the difference between the population means expressed in standard deviations (cf. Hedges' g), divided by the number of generations since their last common ancestor. We calculated the number of generations by dividing the estimated time of divergence (see below) by a generation length of 2.68 years for *P. collaris* (Bird et al., 2020).

Genomic analysis

We used a whole-genome sequencing approach to estimate the time of divergence between the two *P. collaris* populations. We sequenced the whole genomes of seven individuals: three males from the Spanish population, three males from the Moroccan population, and a male *P. modularis* from Norway. The sequences for a male *P. himalayana* were downloaded from the short-read archive of NCBI (https://www. ncbi.nlm.nih.gov/sra) with accession numbers SRR9946605, SRR9946606, and SRR9946608 (Feng et al., 2020).

DNA was extracted with the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's protocol. DNA library preparation and sequencing (150 bp, paired end) on an Illumina Novaseq SP flowcell were performed by the Norwegian Sequencing Centre at Oslo University Hospital. The sequencing output yielded between 76 and 132 million read pairs per individual, while the downloaded *P. himalayana* had 180 million read pairs, corresponding to greater than 20x sequencing depth for all individuals. Adapter removal and quality trimming were performed with *cutadapt* v.3.5 with a quality threshold of 20 and a minimum length threshold of 90 (Martin, 2011). Reads from one Moroccan individual were used to assemble a draft genome for *P. collaris* using MaSuRCA v. 4.0.8 (Zimin et al., 2013).

Reads were mapped to the *P. collaris* genome using Bowtie2 v. 2.4.5 (Langmead & Salzberg, 2012) and converted to sorted binary files using SAMtools v. 1.15 (Danecek et al., 2021; Li et al., 2009). The *mpileup* function implemented in BCFtools

v. 1.15.1 (Danecek et al., 2021) was used for SNP calling with a threshold of 10 for minimum mapping quality to obtain genotype likelihoods, and the *call* function with the consensus caller option (-c) was used to call the genotypes. Genotypes were further filtered by retaining only loci where the locus was a bi-allelic SNP, the coverage depth was between 100 and 300 over all individuals, the SNP was more than 20 bases away from an indel, had a depth of eight reads or greater for an individual, had a genotype quality greater than 10, and was present in all individuals. Both BCFtools and VCFtools v. 1.1.16 (Danecek et al., 2011) were used for filtering. This filtering retained 24,070,775 SNPs scored in all individuals. These SNPs were further pruned by keeping only one SNP (the first SNP) every 2,000 bases, leaving 473,945 SNPs.

Estimation of divergence time with SNP data

We performed a Bayesian phylogenetic analysis on the SNP data using SNAPP v. 1.6.1 (Bryant et al., 2012), an add-on package for the program BEAST2 v.2.7.3 (Bouckaert et al., 2019). The program calculates the probability of a species tree by mathematically integrating over all possible gene trees from the individual SNP markers. Branch lengths can be converted to a time scale by specifying a time constraint on one or more divergence events in the tree following Stange et al. (2018). We used a divergence time of 7.3 mya as a calibration point for the split between *P. modularis* and *P. collaris* (Drovetski et al., 2013; Zang et al., 2023) and defined it through a lognormal prior distribution with a mean of 7.3 mya and a standard deviation of 0.138.

The script prep_snapp.rb of Stange et al. (2018) was used to prepare the XML format input file for SNAPP (Stange et al., 2018) with further pruning to keep only 100,000 SNPs of the 473,945 SNPs. The Markov-chain Monte Carlo (MCMC) process was run in three replicates with a chain length of 100 million and results were stored every 25,000 iterations. Since the populations of *P. collaris* are restricted to disjunct high alpine areas while *P. himalayana* and *P. modularis* have much wider and continuous distributions, we assumed that the population sizes were not equal and thus allowed variable population sizes in the model for divergence by using the parameters. We did so by modifying the XML file and adding SNAPPs "GammaMover" and "RateMixer" operators to allow changes in each branch's population size over the course of the MCMC.

The SNAPP log files were checked in Tracer v 1.7.2 (Rambaut et al., 2014), ensuring that the effective samples sizes of all model parameters were >200. The log and tree files from the three replicate SNAPP runs were combined in LogCombiner, a utility program within BEAST2 (Bouckaert et al., 2019). Node ages (mean height) and credible intervals (95% highest posterior density [HPD]) were estimated together with the maximum clade credibility tree in TreeAnnotator v 1.7.4 (Drummond et al., 2012) and visualized in FigTree v 1.4.4 (Rambaut, 2017).

Phylogenetic analysis of full mitogenomes

We reconstructed the full mitogenomes from the short-read sequences using MitoBim (Hahn et al., 2013) using the mitogenome of *P. himalayana* (GenBank acc. NC_053082.1) as a bait sequence. This sequence stems from the same voucher specimen as the one used in the SNP analysis (Feng et al., 2020). Sequences were aligned with MUSCLE (Edgar, 2004) in MEGA11 (Tamura et al., 2021) using default settings.

Trait	High Atlas, Morocco $(N = 9)$	Picos de Europa, Spain (N=28)	Test of difference between means	Test of difference between variances	Divergence (Hedges' g)	Evolutionary rate (<i>baldanes</i>)
Sperm traits						
Head length (µm)	13.87 ± 0.66	13.22 ± 0.50	$t_{35} = 3.11, P = 0.004$	F-ratio = 1.76, p = 0.26	1.19	3.83×10^{-5}
Midpiece length (µm)	90.29 ± 2.31	88.17 ± 1.07	$t_{9.12} = 2.66, P = 0.026$	F-ratio = 4.69, p = 0.002	1.46	4.70×10^{-5}
Flagellum length (µm)	109.54 ± 1.50	104.76 ± 1.31	$t_{35} = 9.20, P < 0.001$	F-ratio = 1.30, p = 0.57	3.52	1.13×10^{-4}
Total sperm length (µm)	123.41 ± 1.83	117.98 ± 1.50	$t_{35} = 8.93, P < 0.001$	F-ratio = 1.49, p = 0.42	3.42	1.10×10^{-4}
CV_{wm} total length (%) ^a	1.25 ± 0.24	1.21 ± 0.44	$t_{35} = 0.21, P = 0.83$	F-ratio = 0.30, p = 0.078	0.08	6.95×10^{-6}
CV_{am} total length (%) ^a	1.53	1.29				
Somatic traits						
Body mass (g)	39.89 ± 2.46	41.48 ± 2.08	$t_{35} = -1.91, P = 0.065$	F-ratio = 1.40, p = 0.48	0.73	2.43×10^{-5}
Bill length (mm)	19.27 ± 0.86	18.70 ± 0.73	$t_{35} = 1.92, P = 0.063$	F-ratio = 1.39, p = 0.49	0.74	2.40×10^{-5}
Tarsus length (mm)	25.31 ± 0.40	26.29 ± 0.76	$t_{35} = -3.66, P < 0.001$	F-ratio = 0.27, p = 0.062	1.40	4.57×10^{-5}
Tail length (mm)	70.39 ± 2.23	65.96 ± 2.18	$t_{35} = 5.27, P < 0.001$	F-ratio = 1.05, p = 0.85	2.02	6.45×10^{-5}
Wing length (mm)	106.11 ± 2.32	104.91 ± 3.63	$t_{35} = 0.93, P = 0.36$	F-ratio = 0.41, p = 0.36	0.36	1.20×10^{-5}
Wing shape ^b						
Isometric size (C1)	1.68 ± 0.15	1.46 ± 0.16	$t_{35} = 3.65, P < 0.001$	F-ratio = 0.86, p = 0.88	1.40	4.33×10^{-5}
Pointedness (C2) ^c	2.31 ± 0.06	2.44 ± 0.05	$t_{35} = -6.24, P < 0.001$	F-ratio = 0.68, p = 0.58	2.39	7.76×10^{-5}
Convexity (C3)	1.51 ± 0.06	1.52 ± 0.11	$t_{28,37} = -0.37, P = 0.71$	F-ratio = 0.24, p = 0.044	0.10	2.29×10^{-6}

Table 1. Divergences in sperm and somatic morphological traits of male alpine accentors *Prunella collaris* between Morocco and Spain. Ten sperm cells were measured per male. Mean values are given ±1 SD.

^a Adjusted for variation in sample size by the formula CV = *SD*/mean × 100 × (1 + 1/4N). ^b For calculation of the three indices, see Methods. ^c A lower value means a more pointed wing.

Mitochondrial divergence times were then estimated with BEAST2 (Bouckaert et al., 2019) using the same constraints of 7.3 mya divergence time for the split between *P. modularis* and *P. collaris*. We applied the GTR model of sequence evolution, a strict-clock model, and a Yule tree prior, and ran two replicate analyses, each with an MCMC chain length of 10 million iterations. Posterior tree distributions were analyzed with Tracer and visualized in FigTree as described above.

Results

Moroccan and Spanish *P. collaris* were diverged in several morphological traits (Table 1): Moroccan birds had shorter tarsi, longer tails, larger (isometric size), and more pointed wings than the Spanish birds. There was also a trend of Moroccan birds having lower body mass and longer bills, but these differences only approached statistical significance.

Moroccan birds had longer sperm than the Spanish birds with no overlap between the two sample distributions (Figure 2). All sperm segments were also significantly longer in Moroccan than in Spanish birds (Table 1). The divergence estimates, expressed as Hedges' g, were largest for flagellum length and total sperm length and larger for these than for any of the somatic traits examined. Since the flagellum makes up about 89% of the total sperm length, the two measures are strongly intercorrelated and should be considered the same trait statistically. Hedges' g is influenced both by the difference between averages and the variance of the trait. It increases when variance is reduced and the overlap in trait distributions becomes smaller. For total sperm length, the coefficient of variation (CV_{am}) was relatively low in both populations, both when compared with other morphological traits (SD as a percentage of the mean, see values in Table 1) and when compared with other Passerides songbirds (Figure 3). A low sperm length CV is typically found in species with a high risk of sperm competition, as indicated by the proportion of extrapair young (EPY) in socially monogamous species (Figure 3). The position of the



Figure 2. Box plot of total sperm length in *Prunella collaris* in the two study populations. Circles indicate the mean sperm length of individual males based on the measurements of ten sperm cells. The colored boxes indicate the interquartile range (IQR), with the central line depicting the median and the whiskers extending to $1.5 \times IQR$.

two P. collaris populations at the lower right in the scatterplot (Figure 3) suggests that the low sperm length CV is associated with the high risk of sperm competition in their polygynandrous mating system. Our field observations of color-banded birds revealed that females in the Spanish population copulated or solicited copulations with more than one male within the same sexual interaction event more frequently (34%, N = 206) than females in Morocco (16%, N = 31; χ^2_1 = 3.97, p = .046). These events were observations at different sites or at different times, but the same males or females could be involved at multiple events. In Spain, up to three males could be involved per event and in Morocco never more than two. This could indicate a higher intensity of sperm competition in the Spanish than in the Moroccan population, consistent with a lower sperm CV_{am} in Spain, although the difference in sperm total length variance between the two populations was not statistically significant (Table 1).

The phylogenetic analysis of 100,000 SNPs among the three *Prunella* species gave a species tree (Figure 4) with a divergence time of 82 kya (95% HPD 59-106 kya) between the two P. collaris populations and 0.93 mya (95% HPD 0.68-1.20 mya) for the split between the P. himalayana and P. collaris lineages. The analysis also revealed a much lower level of nucleotide diversity (theta) in the P. collaris populations compared with P. himalayana and P. modularis, and lower diversity in Moroccan than in Spanish P. collaris (cf. the *theta* values in Figure 4). This is consistent with the pattern of small and isolated mountain populations in Morocco and Spain with little or no gene flow between them. The theta value is the scaled product between the mutation rate and the effective population size (Wakeley, 2009) and can be used to estimate the effective population size (N_{i}) by the formula $N_e = theta/(4 \times r/n_e)$, where r is the mutation rate estimate and n_{a} is the number of generations per million years. We



smoothing curve.

Figure 3. Scatterplot of the coefficient of variation in sperm length among males (CV_{am}) and the frequency of extrapair young (EPY) in socially monogamous Passerides songbird species. Values for 58 species (black dots) were taken from Lifjeld et al. (2019, Supplementary Table S3). The CV_{am} values of the two *P. collaris* populations are indicated with red-filled dots and plotted at an EPY frequency that considers young not sired by the alpha male as being equivalent to EPY in socially monogamous species (see text). The blue line is a locally weighted



Figure 4. A time-calibrated species tree of the two study populations of alpine accentor *Prunella collaris* together with the Altai accentor *P. himalayana* and the dunnock *P. modularis*. The tree was derived from 100,000 SNPs from three individuals in each of the two *P. collaris* populations and one individual in each of *P. modularis* and *P. himalayana*, using the SNAPP program and a time calibration point of 7.3 mya for the split between the *P. modularis* and the *P. himalayana/collaris* lineage (Drovetski et al. 2013; Zang et al. 2023). The numbers at the nodes indicate the estimated node age and the numbers above the branches indicate the nucleotide diversity, *theta*. The blue bars at each node depict the 95% highest probability density of the node age estimates from 10,803 trees produced from the SNAPP iterations.

estimated N_e to 32,000 individuals in the Moroccan population, 88,000 individuals in the Spanish population, 390,000 individuals in *P. himalayana*, and 199,000 individuals in *P. modularis*. A similar pattern of population differences in the level of genetic diversity is also evident when comparing individual heterozygosity across all 24 million SNPs (Table 2). Observed heterozygosity (H_0) per individual was lower in the two *P. collaris* populations than in *P. himalayana* and *P. modularis*, and significantly lower in the Moroccan *P. collaris* than in the Spanish *P. collaris* (two-sample *t*-test: $t_4 =$ -16.6, p < .001).

We also assembled the full mitochondrial genomes from the short-read sequences and constructed a time-calibrated gene tree using BEAST2 with the same calibration point as in the SNAPP tree (7.3 mya for the split between *P. modularis* and *P. collaris* lineage). The analysis revealed that the mitogenomes of the two *P. collaris* populations have not yet been sorted (Figure 5). Their coalescence time was estimated to be 0.844 mya, which is much earlier than the estimated time since last common ancestor based on the SNP data (Figure 4). The coalescence time for the *P. himalayana* and *P. collaris* mitogenomes was estimated to be 4.0 mya.

With the estimated divergence time of 82 kya for the two *P. collaris* populations (Figure 4), we can then calculate the evolutionary rate for the divergent morphological traits (Table 1). The *haldanes* currency expresses evolutionary rates as the change in units of standard deviations per generation (Gingerich, 2001). With a generation time of 2.68 years, the divergence time spans an estimate of 30,654 generations. The evolutionary rate of total sperm length was estimated to be 1.10×10^{-4} *haldanes*. This rate was almost twice the rate for the most divergent somatic traits (tail length, tarsus length, and wing pointedness; Table 1).

Discussion and Conclusions

Some novel results emerged from our analyses. First, the time-calibrated species tree estimated a more recent split between the P. collaris and P. himalayana (<1 mya) than previous estimates (3–4 mya) based on mitogenomes (Zang et al., 2023) or a few mitochondrial and nuclear markers (Drovetski et al., 2013; Liu et al., 2017). Still, the divergence of the two focal populations, both belonging to the nominate subspecies, was surprisingly old (late Pleistocene) given the lack of population separation in mitochondrial markers and their relatively close geographic proximity within the species distribution across the Palearctic region. Second, the two populations show substantial divergence in several morphological traits shaped by natural selection and in sperm length. The latter divergence is presumably driven by strong postcopulatory sexual selection in the polygynandrous mating system of this species. Third, the rapid sperm evolution points to a cryptic diversification process that has implications for reproductive isolation and hence speciation. In the following, we discuss these three points in more detail.

Divergence times

Our estimate of the most recent common ancestor of the Moroccan and Spanish *P. collaris* populations was 82 kya. It must be emphasized that this is an estimate that is sensitive to the underlying assumptions. Our approach, which used the multispecies coalescent model, assumes the absence of gene flow and that SNP alleles are selectively neutral. Furthermore, we used a calibration point of 7.3 mya for the *P. modularis–P. collaris* split, following Drovetski et al. (2013) and Zang et al. (2023). If we instead follow Liu et al. (2017) and use their estimate of 9 mya as a calibration point, the divergence time

Table 2. Frequency of heterozygous SNPs in the sequenced individuals in the two populations of alpine accentor (*Prunella collaris*) and the singleindividuals of Altai accentor (*P. himalayana*) and dunnock (*P. modularis*). The total number of SNPs scored across all individuals was 24,070,775.Observed heterozygosity is the proportion of heterozygous SNPs of the total number of SNPs. Individuals are given by their accession numbers in theNHMO Bird Collection (*P. collaris* and *P. modularis*) or in the B10K project (*P. himalayana*).

Bird ID	Species	Location	No. of heterozygous SNPs	Observed heterozygosity H_{o}
NHMO-BI-36528	P. collaris	Morocco	914,422	0.03799
NHMO-BI-69704	P. collaris	Morocco	887,102	0.03685
NHMO-BI-84998	P. collaris	Morocco	954,795	0.03967
NHMO-BI-32025	P. collaris	Spain	1,245,288	0.05173
NHMO-BI-69709	P. collaris	Spain	1,260,617	0.05237
NHMO-BI-69718	P. collaris	Spain	1,253,671	0.05208
B10K-DU-013-18	P. himalayana	Mongolia	4,214,106	0.17507
NHMO-BI-106704	P. modularis	Norway	2,609,264	0.10840



Figure 5. A time-calibrated gene tree for the mitochondrial genomes of the six alpine accentors *Prunella collaris* in Morocco and Spain together with a mitochondrial genome of an Altai accentor *P. himalayana* and a dunnock *P. modularis*. The tree is maximum clade credibility tree and estimates the mean coalescence time at each node with 95% highest probability densities depicted as blue bars.

for the *P. collaris* populations increases to 101 kya. Even with these different constraints, it is safe to conclude that the two populations began to diverge at the early stage of the last major glaciation, the Würmian, which started 115 kya and ended 12 kya. During this period, the Alps, the Atlas, and other mountain ranges in the Mediterranean region were glaciated (Hughes & Woodward, 2017), and the lowlands between the mountain ranges in Europe were characterized by steppe tundra, or the "mammoth steppe." This biome extended at times widely across the entire Palearctic. *Prunella*

collaris was part of this community as revealed by several Würmian fossils from the lowlands in SW Europe (Tyrberg, 1991). It suggests that the current disjunct and small-range populations in the high mountains of the Mediterranean region are relict populations from a larger and more continuous range during the Würmian glacials (Tyrberg, 1991). Our SNP-based divergence time corroborates this view of vicariance. Furthermore, the relatively low but different effective population sizes of the two *P. collaris* populations indicate that they are isolated in terms of current gene flow,

though we cannot exclude the possibility of episodes of gene flow, perhaps from more nearby populations, after they started to diverge. The overlap in mitochondrial haplotypes (Fig. 5) could theoretically be explained by recent gene flow, but incomplete lineage sorting is an alternative explanation and seems more likely in this case. We therefore conclude that the two populations seem to have evolved in allopatry as a result of vicariance since the early Würmian epoch and that there is no indication of recent dispersal across the Mediterranean.

The Prunellidae family has been described as an adaptive radiation that has evolved through allopatric speciation followed by a long waiting time until diverged species can coexist in sympatry (Drovetski et al., 2013; Liu et al., 2017). The argument is based on the observation that most sympatric species represent old splits and that more recent clades contain only allopatric species, although there is some disagreement about the time estimates in the two studies. Drovetski et al. (2013) found a minimum divergence time of 0.91 mya for a sympatric species pair (P. fulvescens Severtsov and P. koslowi Przevalski), whereas Liu et al. (2017) estimated the same split to 2.5 mya. The two studies also reported much older divergence time for the P. collaris-P. himalayana split, respectively, 3.04 mya (Drovetski et al., 2013) and 4.1 mya (Liu et al., 2017). We found that the latter species pair, which are clearly sympatric in parts of their range, diverged only 0.93 mya (Fig. 4). These discrepancies are presumably due to the different methodological approaches used. Gene trees reflect the coalescence times of single markers, which may be much older than the actual start of the speciation process. Our study suggests that the speciation process can be completed much faster than indicated by these previous studies. Although sympatry is the ultimate test of completed speciation, allopatric species can still be good species and the long waiting time until sympatry is reached could have other causes, like constraints on dispersal.

While the two study populations, both belonging to the nominate subspecies, have not yet sorted in mitochondrial DNA, it is interesting to note that other subspecies of *P. collaris* show strong divergence in mitochondrial DNA. The BOLD database of DNA barcode sequences from the COI gene (Ratnasingham & Hebert, 2007) identifies three barcode clusters (BINs) for *P. collaris*, which are sequence clusters that closely approximate species-level divergences (Ratnasingham & Hebert, 2013). These BINs are geographically separated and correspond roughly to Europe, the Middle East, and central to eastern Asia. *Prunella collaris* thus seems to contain some deep divergences that might be as old as some of the more recent species divergences in the family. A comprehensive analysis of the phylogeography, species limits, and taxonomy of the *P. collaris* complex is therefore warranted.

Trait divergences

Moroccan *P. collaris* had longer tails, larger and more pointed wings, and shorter tarsi than their Spanish counterparts. These differences point to different ecological and behavioral adaptations in the two populations. From the general theory of functional anatomy and morphology in birds, we know that a long tail is typical for species that need high maneuverability to feed aerially or move in cluttered habitats. It also maintains stability and generates lift at a slow speed (Thomas & Balmford, 1995). Longer and more pointed wings are typical for fast fliers and long-distance migrants (Mönkkönen, 1995; Norberg, 1995; Pennycuick, 2008). The same pattern is also observed within species with a gradient in migratory distance (e.g., Bensch et al., 1999; Matyjasiak et al., 2022). In alpine species, there is a general increase in wing length and a decrease in body mass at high altitudes, which could be viewed as adaptations to altitude itself (Sander & Chamberlain, 2020). A longer tarsus implies a longer step length and potentially faster movement on the ground, while a shorter tarsus gives more stability for birds that use their feet mostly for perching or sitting (Zeffer et al., 2003). Prunella collaris lives in open habitats and forages mostly on the ground (Hatchwell & Christie, 2020). It is a sedentary species but may migrate short distances to lower areas during the winter (Hatchwell, 2005). Whether there are behavioral differences between populations regarding locomotion patterns in the air or on the ground has not been studied, but the observed morphological divergences would predict more ground-dwelling behavior in Spain and more flight-dependent behavior in Morocco. The differences might also be associated with altitude, as the Moroccan birds were breeding at a higher elevation than the Spanish birds.

Sperm length is a trait that is much differentiated among passerine species (Omotoriogun et al., 2020) with several examples of significant intraspecific variation among geographical subspecies (Gohli et al., 2015; Hogner et al., 2013; Laskemoen, Albrecht, et al., 2013). Thus, sperm length is a trait that seems to evolve fast in songbirds. Nevertheless, the strong population divergence between the two P. collaris populations in sperm length seems surprisingly fast, given the lack of divergence in traditional phylogenetic markers, like mitochondrial DNA, and the fact that the populations belong to the same taxonomic subspecies with no obvious geographical differences in plumage. Our calculation of evolutionary rates shows that sperm length has diverged twice as fast as the most divergent body structures in this case (Table 1). We can also compare our observed evolutionary rates with published morphology data. Gingerich (2001) presented a database of evolutionary rates for different time scales, from experimental studies over a few generations to paleontological rates over thousands to millions of generations. The expected evolutionary rate for a time span of 30,654 generations, which we estimated for the P. collaris populations, is 3.14×10^{-5} haldanes (calculated from the formula in his Figure 8D). The evolutionary rate for sperm length in P. collaris is 3.5 times higher than this (cf. Table 1). We can therefore conclude that sperm length has evolved fast, both in comparison to other morphological traits in this species and to morphological traits in general.

Sperm length and reproductive isolation

The rapid evolution of sperm length in *P. collaris* can hardly be explained by extrinsic, environmental factors. The functional environment for sperm is the female reproductive tract, where sperm need to perform and compete for fertilizing the ovum. There are three general arguments for why sperm length evolves by postcopulatory sexual selection. First, there is a clear coevolutionary association between sperm length and the length of the female sperm storage tubules in birds (Briskie et al., 1997; Kleven et al., 2009). These tubules can store one to three layers of sperm stacked end to end, depending on the species (Briskie & Montgomerie, 1993). A path analysis suggested that sperm length has evolved in response to the risk of sperm competition, but only indirectly via an increase in the length of female sperm storage tubules (Briskie et al., 1997). Evidence from a similar sexual coevolution in *Drosophila* fruit flies strongly suggests that the traits are genetically linked and that sperm traits quickly respond to changes in the female reproductive tract (reviewed in Lüpold & Pitnick, 2018). A pattern of positive covariation between the length of sperm and the length of sperm storage compartments across species has been documented in many other insect groups (e.g., Dybas & Dybas, 1981; Minder et al., 2005; Presgraves et al., 1999). A recent simulation model of cryptic female choice (Kustra & Alonzo, 2023) concluded that a genetic correlation between female preference and the sperm trait will arise in response to the risk of sperm competition and that the evolution of the sperm trait will lag behind the evolution of the female preference trait.

Second, the coefficient of among-male variation in sperm length is negatively related to the risk of sperm competition (Calhim et al., 2007; Lifjeld et al., 2010, 2019). The same relationship is documented for populations of the same species with different levels of extrapair paternity (Laskemoen, Albrecht, et al., 2013). Low among-male variation in sperm length is hardly an effect of low effective population size, as several population comparisons have revealed relatively high levels of sperm length variation in island populations or subspecies with a restricted range (e.g., Gohli et al., 2015; Hogner et al., 2013; Laskemoen, Albrecht, et al., 2013; Lifjeld et al., 2013). Instead, the common interpretation of this negative relationship is that sperm competition is a force of stabilizing selection that favors sperm around an optimal length and disfavors the extremes. A theoretical modelling approach has also confirmed that female promiscuity leads to stabilizing selection on sperm length and that selection becomes stronger when among-female variation in storage organs is lower than the among-male variation in sperm length (Cramer et al., 2023). Any changes in the female environment will then be rapidly followed by an evolutionary response in sperm length under high sperm competition risk. Third, there is comparative evidence that sperm length diverges faster in passerine birds when the sperm competition risk is high (Rowe et al., 2015).

Prunella collaris has a polygynandrous mating system where sperm competition is the norm (Hatchwell & Christie, 2020). The divergent sperm length distributions suggest that there is a corresponding divergence in the female reproductive tracts, presumably in the length of sperm storage tubules, although little is currently known about intraspecific variation in this trait. This divergence has some important implications for speciation because strong conspecific sperm precedence will be expected in a scenario of contact between the two populations. When females in a hypothetical hybrid zone copulate with males of both groups with divergent sperm lengths, conspecific males will have an advantage in sperm competition and the pattern of assortative fertilization will strengthen with the extent of female promiscuity. Sperm length will then function as a barrier to hybridization and sperm competition will promote postcopulatory prezygotic isolation. In other words, sperm length can be seen as a potential "speciation phenotype" (sensu Manier et al., 2013) in this system. In theory, divergent sperm could be sufficient to drive such cryptic speciation, that is, without any corresponding divergence in phenotypic traits. Females will pay little or no cost of hybrid mating if promiscuity ensures fertilizations by males from their own group and avoids producing offspring with an intermediate phenotype and lower fitness. For males, on the

other hand, hybrid mating will entail costs of wasted parental care on nongenetic offspring. Thus, it has potential for the evolution of premating isolation by reinforcement (Marshall et al. 2002) causing character displacement in female traits driven by male mate choice. This will only be expected in polygynandrous systems where multiple males provide costly parental care. In the more common extrapair mating systems of socially monogamous species, extrapair males do not provide parental care and their cost of extrapair mating is therefore expected to be low. In these systems, cryptic speciation could be driven in a contact zone solely through the combination of female promiscuity and divergent sperm.

Allopatric speciation is considered a common mode of speciation in passerine birds (Fieldså et al., 2020). The traditional view is that populations diverge in response to environmental and ecological factors through local adaptation and niche evolution. Over time, they will also be expected to diverge in traits involved in mate choice (i.e., premating isolation). Such sexually selected traits are typically considered to be plumage colors, feather ornaments, and song, which are involved in precopulatory mate choice. Also naturally selected traits, like beak size, can become targets in mate choice as shown in Darwin's finches (Ratcliffe & Grant, 1983). However, geographical differentiation in reproductive traits may not necessarily lead to speciation. Song is a good example mainly because it is a learned trait in songbirds. In a zone of secondary contact, males may easily adopt the song of other groups, potentially leading to the erosion of divergence between the groups. Sperm length, on the other hand, is under genetic control and shows high between-season repeatability (Birkhead et al., 2005; Knief et al., 2017; Laskemoen, Kleven, et al., 2013; Míčková et al., 2023). Sperm traits have been largely ignored in the avian speciation literature, even though much research has been directed to sperm competition and mating system variation where the sperm cells themselves play a key role.

The rapid sperm evolution in *P. collaris* illustrates a potential for an alternative view of the driving factors in speciation processes in species with female promiscuity, like accentors. In their analysis of the speciation history of the Prunellidae family, Liu et al. (2017) interpreted the pattern of recently diverged species being largely allopatric and species coexisting in sympatry having diverged much earlier, purely in an ecological framework. That is, that species must evolve enough niche differentiation to be able to coexist in sympatry and that is a process that requires long "waiting time" until reproductive isolation has evolved. Our results allow for an alternative interpretation, that reproductive isolation evolves fast in such promiscuous species through rapid sperm evolution, and that allopatric populations can be "good species" long before they gain secondary contact. The long "waiting time" from completed speciation until sympatry can instead be explained by spatial constraints, like geographical barriers maintaining vicariance or low dispersal capacity. If our hypothesis holds true, postcopulatory prezygotic isolation can arise in promiscuous species an order of magnitude faster, that is, at the scale of 100,000 years, than the traditional view that speciation takes a million years or more (Newton, 2003; Price, 2008).

The role of sperm traits and sperm competition warrants further exploration in speciation research. The idea of cryptic speciation through postcopulatory sexual selection is not new. In insects, the mechanism of conspecific sperm precedence is well documented (Gregory & Howard, 1994; Price, 1997) and speciation rates are higher among polyandrous than among monandrous lineages (Arnqvist et al., 2000). We encourage more attention to such postcopulatory processes in birds. Passerides songbirds, with their diversified sperm phenotypes and promiscuous mating systems, are good candidates for further studies of a possible link between mating system and speciation (Christidis et al., 2020; Fjeldså et al., 2020) and sperm as speciation phenotypes (Manier et al. 2013). We particularly encourage the study of conspecific sperm precedence in hybrid zones where populations with diverged sperm traits meet and females copulate with multiple males.

Data availability

The paired-end sequencing reads are available on NCBI short-read archives under the BioProject ID PRJNA996629. The mitogenomes are available in GenBank (Accession numbers OR337894-900). Data sets with scripts are available at the Dryad data repository (DOI: 10.5061/ dryad.nk98sf7zg).

Author contributions

J.T.L. conceived and acquired funding for the study. M.d.G.H. and B.F.M. conducted the fieldwork, and M.d.G.H. conducted the wing shape analyses. G.G. conducted all sperm measurements and curated the data, J.A.A. assembled the mitogenomes, and M.M. conducted the BEAST analyses of mitogenomes and assisted with the SNAPP analyses. E.H.L. performed the bioinformatic analyses: assembled the genome, generated the SNP data set, and performed the SNAPP analyses for divergence times. J.T.L. wrote the manuscript with input from all authors.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

We thank Mohamed Dakki, Asmaâ Ouassou, and Mohamed Radi for help with acquiring the fieldwork permit in Morocco, Francisco de la Calzada, Isabel Roa, David Miguélez, Pablo Salinas, Alfonso Gómez, and Rubén González for their field assistance, Audun Schrøder-Nilssen for DNA extractions, Lars Erik Johannessen for making Figure 1, and Kjetil L. Voje for help with the evolutionary rate analyses. The computations were performed on resources provided by Sigma2—the national infrastructure for high-performance computing and data storage in Norway. The study was funded by two grants from the Research Council of Norway (grant nos. 196554 and 301592 to J.T.L.).

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