The pace of mitochondrial molecular evolution varies with seasonal migration distance

Running head: Bird migration and molecular evolution

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Abstract

Animals that engage in long-distance seasonal migration experience strong selective pressures on their metabolic performance and life history, with potential consequences for molecular evolution. Species with slow life histories typically show lower rates of synonymous substitution (d_S) than "fast" species. Previous research suggests long-distance seasonal migrants have a slower life history strategy than short-distance migrants, raising the possibility that rates of molecular evolution may covary with migration distance. Additionally, long-distance migrants may face strong selection on metabolically important mitochondrial genes due to their long-distance flights. Using over 1000 mitochondrial genomes, we assessed the relationship between migration distance and mitochondrial molecular evolution in 39 boreal-breeding migratory bird species. We show that migration distance correlates negatively with d_S, suggesting that the slow life history associated with long-distance migration is reflected in rates of molecular evolution.

Mitochondrial genes in every study species exhibited evidence of purifying selection, but the strength of selection was greater in short-distance migrants, contrary to our predictions. This result may indicate effects of selection for cold tolerance on mitochondrial evolution among species overwintering at high latitudes. Our study demonstrates that the pervasive correlation between life history and molecular evolutionary rates exists in the context of differential adaptations to seasonality.

Keywords: Life History, Seasonal Migration, Molecular Evolution, d_S, Mitochondria

Introduction

Species' traits are the product of their genome and their environment, but in turn, traits and the environment also shape the molecular evolution of the genome. For example, metabolically demanding traits influence molecular evolution of mitochondrial genes (e.g. Shen et al. 2009; Chong and Mueller 2013; Strohm et al. 2015). More broadly, traits associated with the slow-fast continuum of life history (Stearns 1983) are correlated with rates of molecular evolution (Bromham 2020) such that life history evolution is thought to alter the pace of a lineage's molecular clock (Hwang and Green 2004; Moorjani et al. 2016). Environmental pressures associated with seasonality can influence life history (Varpe 2017) and metabolic demands (Weber 2009; Chen et al. 2018), suggesting that variation in adaptation to seasonality could have molecular evolutionary consequences. However, the linkages between molecular evolution and differential adaptations to seasonality are rarely explored.

In this study, we investigate how patterns of mitochondrial molecular evolution are related to variation in seasonal migration distance. Migratory animals survive harsh seasonal conditions on their breeding grounds by temporarily departing until conditions improve (Winger et al. 2019). Migration distance varies across species, ranging from short-distance movements within an ecoregion to hemisphere-crossing journeys. Long-distance seasonal migration requires high metabolic performance (Weber 2009), with potential implications for the dynamics of selection on the metabolically-important mitochondrial genes (Shen et al. 2009; Strohm et al. 2015). Migration distance has also been recognized as an important axis of life history variation (the balance between annual survival and reproduction) in birds (Greenberg 1980; Møller 2007; Bruderer and Salewski 2009; Winger and Pegan 2021). Migration distance may therefore also influence molecular evolutionary rates through effects on life history (Bromham 2020) that are

not directly associated with metabolic demands, but this relationship has not been assessed. Here, we assess how migration distance correlates with mitochondrial molecular evolution within the community of migratory birds breeding in the highly seasonal North American boreal region, and we test hypotheses regarding the roles of life history and metabolic adaptation in mediating a relationship between molecular evolution and seasonal migration.

Metabolic adaptation, life history, and mitochondrial molecular evolution

Reliance on locomotion (migration) for adaptation to seasonality may influence selection on mitochondrial genes, which play an important role in metabolism. Mitochondria typically experience purifying selection (i.e. selection that reduces genetic variation) because most mutations in these genes are deleterious to fitness (Nei et al. 2010; Nabholz et al. 2013; Popadin et al. 2013). Prior studies have shown that purifying selection tends to be stronger in the mitochondria of mobile animal species compared to less mobile relatives. This pattern has been demonstrated in comparisons between flighted and flightless birds (Shen et al. 2009) and insects (Mitterboeck et al. 2017; Chang et al. 2020), between migratory and nonmigratory fishes (Strohm et al. 2015), and between amphibians (Chong and Mueller 2013) and mollusks (Sun et al. 2017) with different locomotory modes. Within flighted birds, species with slow flight and those that rely on soaring (versus flapping) have been shown to experience relaxed mitochondrial purifying selection compared to faster-flying species (Shen et al. 2009; De Panis et al. 2021). Additionally, Montoya et al. (2022) recently demonstrated that flight habit, as represented by wing morphology, is associated with nonsynonymous mitochondrial evolutionary rate variation in a large clade of South American birds (Furnariidae). These studies suggest that mitochondrial genotype plays an especially important role in fitness for organisms that rely on high-energy

80 locomotion, including migratory birds. Metabolic demand may be strongest in long-distance
81 migrating species if these demands primarily arise from locomotion. However, species that breed
82 at high latitudes and migrate only short distances for the nonbreeding season may require
83 alternative metabolic adaptations for dealing with harsh seasonal conditions since their shorter
84 migrations do not allow them to fully escape cold, resource-depleted winters (Winger et al.
85 2019). The effect of variation in seasonal migration distance on the strength of mitochondrial
86 purifying selection is unknown.

87 A second and distinct way in which seasonal migration may influence molecular
88 evolution is through its relationship with life history and, consequently, molecular evolutionary
89 rate. The slow-fast continuum of life history is commonly characterized by "life-history traits"
90 that underly or correlate with differing rates of growth, survival, and reproduction (Read and
91 Harvey 1989; White et al. 2022). Within major lineages of plants, bacteria, vertebrates, and
92 invertebrates, species with "slow" life history (i.e., long generation time, low annual fecundity,
93 large size; Stearns 1983) also exhibit slower molecular substitution rate than "fast" species (i.e.,
94 those with shorter generation time, higher annual fecundity, and smaller size; Nabholz et al.
95 2008a; Smith and Donoghue 2008; Thomas et al. 2010; Weller and Wu 2015). Within migratory
96 birds breeding in the temperate zone, seasonal migration distance covaries with annual fecundity
97 and survival such that long-distance migrants show "slower" life history (i.e., higher annual
98 survival, lower annual fecundity) than short-distance migrants (Greenberg 1980; Bruderer and
99 Salewski 2009; Winger and Pegan 2021). As such, variation in migration distance across species
100 may affect molecular evolutionary rates because of its association with life history variation.
101 Specifically, the synonymous substitution rate "d_S" often correlates with the slow-fast life history
102 continuum (Nikolaev et al. 2007, Bromham et al. 2015, Hua et al. 2015; Table 1). Prior studies

103 suggest that life history may influence d_S through effects on DNA replication rate or selection for
104 mutation avoidance (reviewed in Bromham 2020), because d_S is thought to primarily reflect the
105 underlying mutation rate when synonymous mutations are selectively neutral (Kimura 1983; Nei
106 et al. 2010; Lanfear et al. 2014). Direct estimates of nuclear germline mutation rates support the
107 hypothesis that species-level variation in mutation rate correlates with life-history traits
108 (Bergeron et al. 2023).
109
110 *Predicting the relationship between seasonal migration distance and molecular evolution*
111 Long-distance migratory birds have been shown to exhibit a slower life history than
112 sympatric breeding short-distance migrants (Winger and Pegan 2021, Fig. 1). Thus, long-
113 distance migrants travel farther in each migratory trip than short-distance migrants and may also
114 require more trips per lifetime to achieve the level of lifetime fitness of short-distance migrants
115 (Møller 2007). Owing to the metabolic demands of migration and the importance of repeated
116 migration success for fitness in long-distance migrants, the migratory phenotypes of these
117 species are thought to be under strong variation-reducing natural selection (Conklin et al. 2017).
118 As such, we hypothesize that long-distance migrants exhibit both lower d_S (which could reflect
119 selection against mutation in the mitochondria; Hua et al. 2015) and stronger purifying selection
120 in their mitochondrial genes than short-distance migrants.
121 To test these hypotheses, we examined the relationship between migration distance and
122 rates of molecular evolution of the mitochondrial coding genes in a community of small-bodied
123 migratory songbirds breeding in the boreal forests of North America. The 39 co-distributed
124 species we studied are ideal for investigating the effects of migration distance on molecular
125 evolution because they vary greatly in migration distance (e.g., Fig. 1, Table S1), yet they

126 otherwise share similar breeding habitat, population history, and body mass (Winger and Pegan
127 2021). This system allows us to test hypotheses about migration distance while minimizing
128 variation in other traits that could influence molecular evolution. We assessed effects of
129 migration distance on d_S (synonymous substitution rate) and d_N/d_S (purifying selection) in a
130 Bayesian phylogenetic framework (Lartillot and Poujol 2011) with full mitochondrial gene sets
131 we sequenced for 39 species. Further, we used population genetic datasets from all mitochondrial
132 genes that we generated for 30 of the species (for a total of 1008 samples used across all
133 analyses) to assess effects of migration distance on purifying selection at the population level.
134 Specifically, we assessed π_N/π_S, which is a population genetic summary statistic representing the
135 amount of nonsynonymous versus synonymous polymorphism within a population.
136
137 *Accounting for effects of N_e on substitution rates*
138 Molecular evolution is fundamentally influenced by effective population size (N_e), so it is
139 often difficult to determine whether links between traits and molecular evolutionary rates are
140 mediated by effects of traits on N_e versus other hypothesized mechanisms (e.g., Montoya et al.
141 2022). Therefore, we take advantage of our population-level datasets to directly test for effects of
142 N_e on molecular evolutionary rates and purifying selection, providing valuable context for the
143 interpretation of our results. Variation in N_e can cause variation in substitution rates because the
144 efficiency of natural selection in purging deleterious mutations is determined by the balance
145 between strength of selection and strength of drift, which is reflected in N_e (Ohta 1992).
146 Specifically, studies on empirical populations have demonstrated that populations with small N_e
147 typically show weaker purifying selection (i.e., higher d_N/d_S, e.g., Popadin et al. 2007, Leroy et
148 al. 2021; and higher π_N/π_S , e.g., Chen et al. 2017). Several recent studies found correlations

149 between traits associated with life history and genetic diversity, suggesting that species with
150 "slow" life histories often have low N_e (Romiguier et al. 2014; Brüniche-Olsen et al. 2021; De
151 Kort et al. 2021). There is also evidence that migratory behavior is predictive of population
152 genetic diversity, a parameter associated with N_e (García-Berro et al. 2023). It is therefore
153 important to assess whether molecular rate variation across species can alternatively be explained
154 by confounding variation in N_e.

155 Finally, we use estimates of N_e to test the assumption of neutral evolution at synonymous
156 sites, which is a fundamental assumption underlying the hypothesis that d_S reflects mutation rate
157 (Kimura 1983; Nei et al. 2010; Lanfear et al. 2014). If synonymous substitutions evolve
158 neutrally, we expect that d_S should not show a relationship with N_e because the processes that
159 lead to a relationship between N_e and substitution rate involve natural selection.

160

161 **Methods**

162 *Study system*

163 We focused on 39 species of migratory birds breeding in the North American boreal
164 forest, representing 11 families (Table S1). These are the same species for which a correlation
165 between migration distance and the slow-fast life history continuum—independent of body
166 size—has been demonstrated using data on annual fecundity and survivorship (Winger and
167 Pegan 2021). We focus our analyses on co-distributed populations of the eastern boreal belt of
168 North America (Omernik 1987, Fig. S1). Some species' breeding ranges extend into other
169 ecoregions (e.g., the mountain west or the temperate forests south of the boreal zone), but in
170 these cases, we only analyze samples from the boreal portion of the range to assess sympatric
171 populations. The species in the dataset exhibit broad variation in migration distance, with their

172 geographic range centroids shifting between 1048 km and 7600 km between the breeding and
173 non-breeding periods (Fig. 2, Table S1; Winger and Pegan 2021). These centroid shifts represent
174 migratory strategies ranging from short-distance movements within the temperate region to the
175 movement of an entire population across ocean and land barriers from North America to South
176 America. All species are less than 100 g in mass (range of mean mass across species is 6-87
177 grams; Table S1) and are broadly similar in habitat use. They are all territorial species with
178 socially monogamous breeding systems, which suggests that they probably do not vary
179 substantially in population sex ratio (which can affect N_e), although empirical sex ratio data is
180 not available for these species. Small songbirds are typically capable of breeding in their second
181 year, and this is true of all species in our study that have been assessed (Billerman et al. 2022).
182 Additionally, our study species share relatively similar demographic histories, with population
183 expansions estimated to have mostly occurred during the period of glacial retreat that preceded
184 the Last Glacial Maximum (~57,000 years before present; Kimmitt et al. 2023).
185
186 *Life history covariates: Migration distance and mass*
187 Direct measurements of migration distance of individuals are lacking for most of the species in
188 our system, so we used the distance between the centroid of a species' breeding range and the
189 centroid of its nonbreeding range to represent the migration distance of the species. Although the
190 distance between centroids does not represent individual variation in migration distance within a
191 species, this metric captures broad differences in migratory strategies between species. Our
192 method for calculating the distance between range centroids is described in detail in Winger and
193 Pegan (2021). We included mass as a covariate in our analyses because body mass and rates of
194 molecular evolution are often associated (Figuet et al. 2014; Nabholz et al. 2016), and the

195 relationships between survival and fecundity and migration distance demonstrated by Winger
196 and Pegan (2021) were recovered after accounting for variation in mass. We obtained mass data
197 from Dunning (2008) and Billerman et al. (2022).
198
199 *Sampling and DNA sequencing*
200 Our analysis of the relationship between migration distance and d_S requires one
201 mitochondrial genome for each species in the study, while analyses of N_e and π_N/π_S require
202 population-level sampling. For our analysis of d_S, we obtained whole mitochondrial genomes
203 from one individual of each of the 39 species in our study by sequencing DNA from tissue
204 samples associated with a museum specimen, as described below. These specimens were
205 collected during the breeding season from near the longitudinal center of the boreal forest
206 (Manitoba, Minnesota, or Michigan; Tables S1,S2). For two species (*Contopus cooperi* and
207 *Euphagus carolinus*), we used specimen-vouchered tissue samples of individuals salvaged
208 during migration in Michigan from collision mortalities.
209 For our population-level analyses, we generated a large dataset of 999 additional
210 mitochondrial genomes for 30 of the 39 species, building on a dataset of 19 species from
211 Kimmitt et al. (2023). Our larger dataset includes complete coding sequences for 8 to 49
212 individuals per species (mean 33 individuals per species; Table S1). These individuals were
213 sampled during the breeding season across a longitudinal transect of the boreal forest from
214 Alberta to the northeastern United States (Fig. S1, Table S2). Except for 24 blood samples from
215 New York state, all sequences we used came from frozen or ethanol-preserved tissue samples
216 associated with museum voucher specimens provided by several museum institutions (Table S2;
217 *Acknowledgments*).

We obtained high-depth mitochondrial genomes captured as a byproduct from low-coverage whole genome sequencing, as described in detail in Kimmitt et al. (2023). Briefly, sequencing libraries were prepared using a modified Illumina Nextera library preparation protocol (Schweizer et al. 2021) and sequenced on HiSeq or NovaSeq machines using services provided by Novogene and the University of Michigan Advanced Genomics Core. We used NOVOPlasty v4.3.1 (Dierckxsens et al. 2016) to assemble mitochondrial contigs, specifying a target genome size of 20-30 kb and using a k-mer of 21. We provided NOVOPlasty with a conspecific mitochondrial seed sequence (Table S1) for each species. We annotated the contigs built by NOVOPlasty using Geneious Prime 2020.2.2 (https://www.geneious.com) with copies of mitochondrial genes from GenBank (Table S1). Whenever applicable in the filtering and analysis steps described below, we used options specifying the vertebrate mitochondrial code.

Our initial dataset across all species contained mitochondrial sequences from 1229 total individuals. To ensure data quality, we used BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to check species identity and we removed samples with evidence of species misidentification, chimerism, or introgression from related species (14 samples removed). We aligned and translated sequences with the R package DECIPHER v2.18.1 (Wright 2016), and we visually inspected each alignment, ensuring that sequences contained no premature stop codons or other alignment issues. We used DECIPHER to remove partial stop codons and the untranslated C in the ND3 sequence of woodpecker (Picidae) species (Mindell et al. 1998). As our population analyses require complete data matrices, we excluded individuals with incomplete datasets (those with assemblies that were missing genes and/or with ambiguous base calls; 202 samples removed). We removed five individuals during population structure analysis, described below. This data filtering resulted in 1008 complete mitochondrial coding sequences: 999 individuals

241 across 30 species used in the population genomic analyses plus one sequence for each of the 9
242 additional species we used only in the interspecific Coevol analyses. We concatenated the 13
243 mitochondrial coding sequences for analysis. The full list of samples, including those removed
244 from the analyses, can be found in Table S2.
245
246 *Estimating θ as a proxy for N_e*
247 We used θ as a proxy for effective population size (N_e). N_e can be calculated based on θ
248 and mutation rate (Watterson 1975, Nabholz et al. 2008b; Table 1), but accurate estimates of
249 mitochondrial mutation rate are lacking for most non-model organisms. Accordingly, many
250 empirical studies interested in N_e focus on genetic diversity, which is thought to reflect the
251 harmonic mean of N_e over time and which does not require mutation rate information to calculate
252 (e.g., Ellegren and Galtier 2016; Hague and Routman 2016). We hereafter use the genetic
253 diversity parameter θ as a proxy for N_e. We used LAMARC v2.1.10 (Kuhner 2006) to estimate θ
254 for each species. LAMARC estimates θ in a maximum likelihood framework using information
255 about the intervals between coalescence events from sampled genealogies, which the program
256 generates from population sequence data (Felsenstein 1992; Kuhner et al. 1995; Kuhner 2006).
257 We imported our population-level full mitochondrial coding sequence data into LAMARC after
258 converting our concatenated fasta files into the phylip format for each species. We used the
259 program's likelihood-based method in 10 initial chains (samples = 500, discard = 1000, interval
260 = 20) and 2 final chains (samples = 10,000, discard = 1000, interval = 20). We used the F84
261 model of molecular evolution, and we provided a separate transition/transversion ratio for each
262 species using values we calculated from population mitochondrial coding sequence datasets
263 using the R package 'spider' (Table S1; Brown et al. 2012). All other input parameters were left

at their default values. We examined the output for each species to check for chain convergence, and we ran two replicate chains for each species to make sure they produced consistent results. For five species (*Leiothlypis ruficapilla, Setophaga castanea, Setophaga coronata, Setophaga fusca,* and *Vireo olivaceus*), we repeated LAMARC for 25 initial chains instead of 10 to improve convergence and used the values from these longer runs.

Estimation of θ can be biased by purifying selection, and the magnitude of this bias may vary across species due to differences in purifying selection and sample size (Subramanian 2016). To evaluate whether these biases influence our results, we compared θ to π_S, or nucleotide diversity at synonymous polymorphisms, which is not biased by purifying selection assuming that synonymous sites are evolving neutrally. We estimated π_S from each species using the python package egglib v3.1.0 (De Mita and Siol 2012) and calculated Pearson's correlation coefficient between θ and π_S. We also repeated Coevol models (described below) with each proxy of N_e to assess whether the choice of proxy influences our results.

Population Structure

Our population-level analyses (estimation of θ and π_N/π_S) assume no geographic population genetic structure within the samples used. To check this assumption, we calculated mitochondrial genetic distance between all individuals within each species using "nei.dist()" from the R package poppr v2.9.3 (Kamvar et al. 2014) and created a neighbor-joining tree with "nj()" from the R package ape v5.6-2 (Paradis and Schleip 2019). We identified and removed 4 individuals from *Regulus satrapa* and one individual from *Oporornis agilis,* all from Alberta in the far western part of our sampling area, that were genetically distinct from all other samples in

their respective species. Otherwise, there was little evidence of geographic genetic structure in the mitochondrial genome in these species.

Estimating d_S and d_N/d_S and their correlations with traits associated with life history

We used Coevol v1.6 (Lartillot and Poujol 2011) to evaluate associations between migration distance and molecular evolutionary rates using a single representative of each species. Coevol uses a Bayesian phylogenetic framework to estimate d_S and d_N/d_S and to simultaneously measure the relationship between these traits and covariates of interest (migration distance, mass, and θ). We included mass to account for the expected relationship between mass and molecular rates (Nabholz et al. 2016). Models with mass also provide a useful point of comparison, allowing us to ask whether migration distance correlates with d_S and d_N/d_S to the same extent as (or more or less than) this well-studied life-history trait. Similarly, including θ in the models allows us to assess whether variation in N_e accounts for differences in molecular evolutionary rates.

We provided Coevol with one complete concatenated mitochondrial coding sequence from each species and a phylogenetic tree (Fig. 2) we generated with data from birdtree.org (Jetz et al. 2012) as described in Pegan and Winger (2020). In brief, we sampled 2000 trees comprising all North American bird species from the Jetz et al. dataset, and we used the python package "DendroPy" (Sukumaran and Holder 2010) to generate a consensus tree. We then trimmed this tree to include only the 39 species used in this study. Importantly, Coevol uses the phylogenetic tree for topological information but estimates relative branching times from the sequence data (Lartillot and Poujol 2021). Coevol also does not require prior information about mutation rates. We investigated the potential effects of phylogenetic tree topology on our results

by sampling 10 random marginal trees from the original tree dataset (trimmed to include only relevant species) and re-running Coevol on each tree, which we found to produce consistent results (Table S3).

We created two data subsets for Coevol models: one subset contained all species in the study and included mass and migration distance as covariates. The other subset included the 30 species for which we had population-level data available; for these, we included θ as a covariate in addition to mass and migration distance. We also repeated these analyses using π_S as a proxy for N_e instead of θ. For each data subset, we ran Coevol four times: two repeated analyses with the option "dnds" (estimating d_S; models 1 and 2, Table 2) and two with "dsom" (estimating d_N/d_S; models 3 and 4, Table 2). We let each analysis run for approximately 20000 steps and examined the resulting trace files to ensure convergence and evaluate estimated sample sizes (ESS). All models converged, and all parameters had ESS > 300. We removed the first 500 steps of each analysis and thinned the posterior sample to retain every 10^{th} step to reduce autocorrelation. Replicate analyses produced highly similar estimates, and the values we report here represent the mean value of estimates made by each replicate. We present full Coevol model output in Tables S4-S6.

The method implemented in the Coevol software estimates correlation coefficients between substitution rates and each covariate, as well as partial correlation coefficients (which hold constant the effects of other covariates in the model). Each correlation or partial correlation coefficient is accompanied by a posterior probability. In the case of Coevol, posterior probabilities near 0 indicate strong support for a negative relationship, while posterior probabilities near 1 indicate strong support for a positive relationship (Lartillot and Poujol 2021).

π_N/π_S

π_N/π_S is measured by comparing polymorphisms among individuals within a species rather than by comparing between species in a phylogenetic framework (and thus cannot be estimated by Coevol). We estimated π_N/π_S from each species with population-level fasta alignments, using the python package egglib v3.1.0 (De Mita and Siol 2012) to create a "CodingDiversity" class with attributes describing nucleotide diversity at codons with synonymous or nonsynonymous polymorphisms. Predictions about the effect of purifying selection on polymorphisms are more complex than predictions about substitution rates because within-population variation can be purged by strong directional selective sweeps in addition to purifying selection (Kryazhimskiy and Plotkin 2008). We predict a negative relationship between migration distance and the π_N/π_S ratio, indicating stronger selection (directional or purifying) on mitochondrial function in long-distance migrants. We used linear modeling to test for an effect of migration distance, mass, and θ on π_N/π_S (Tables S7, S8). Prior to linear modeling, we centered and scaled our predictors using the function "standardize" from the R package "robustHD" (Alfons 2021) with the mean value of each predictor as the center. We used a similar linear modeling approach to test whether θ exhibits a relationship with mass or migration distance to ensure that apparent relationships between these traits and molecular rates are not confounded by correlation with θ.

For each response variable (θ and π_N/π_S; Tables S7, S8), we first created a model with all covariates of interest. We then used the function "phylosig()" from the R package phytools v0.7-70 (Revell 2010) to test for phylogenetic signal in the model's residuals (Revell 2012). For both response variables, the estimate of λ (phylogenetic signal) was low (< 0.2), and the p-value for evidence of phylogenetic signal was > 0.8, so we proceeded with linear modeling rather than

using models with phylogenetic covariance matrices. For each response variable, we created a null (intercept-only) model with no predictors and models with all possible combinations of our predictors of interest, and we used the function "model.sel()" from the R package MuMIn v1.43.17 (Bartón 2019) to compare the models' AICc.

Results

For each model, we report correlation coefficients between traits of interest (migration distance, mass, or θ) and molecular evolutionary rates (d_S or d_N/d_S) and assess their strength based on posterior probabilities (*pp*), which are close to 0 in the case of a strong negative correlation and close to 1 in the case of a strong positive correlation. We also report partial correlation coefficients and their posterior probabilities, which indicate the relationship between variables of interest after accounting for the effects of all other covariates.

The Pearson correlation coefficient between θ and π_S was high (0.77; $p < 0.0001$), suggesting that these two variables are consistent proxies of N_e. We found that results of Coevol models with θ as a covariate were consistent with results of models using π_S, so we conclude that results of analyses with θ are not driven by biases in the estimation of θ. We hereafter focus on models using θ, and full results of Coevol models using π_S instead of θ are presented in Table S6.

Correlations between migration distance and molecular evolutionary rates (d_S and d_N/d_S)

Our analyses show that migration distance negatively correlates with d_S across the 39 species we studied, consistent with our initial predictions (Fig. 2, Fig. S2). For Coevol models with the full species set, the correlation coefficient relating migration distance to d_S was -0.39

with a posterior probability (*pp*) of 0.018, indicating strong support for a negative relationship. The partial correlation coefficient (which accounts for mass) between migration distance and d_S was -0.47 (*pp* = 0.0090).

We did not detect evidence of a relationship between migration distance and d_N/d_S (correlation coefficient = 0.096, *pp* = 0.63). The partial correlation coefficient (accounting for mass) between migration distance and d_N/d_S indicated that this relationship was not well supported (partial correlation coefficient = 0.26, *pp* = 0.82).

Results from the Coevol models of the subset of 30 species for which we had estimates of θ were consistent with results produced by the full subset (39 species) models, although support for the correlation between d_S and migration distance was slightly weaker. In the model estimating d_S, migration distance had a correlation coefficient of -0.43 (*pp* = 0.02) and a partial correlation coefficient of -0.31 (*pp* = 0.11). In the model estimating d_N/d_S, we did not find support for a relationship with migration distance, as this variable had a correlation coefficient of -0.15 (*pp* = 0.32) with d_N/d_S and a partial correlation coefficient of -0.010 (*pp* = 0.52) with d_N/d_S.

Correlations between mass and molecular evolutionary rates (d_S and d_N/d_S)

Coevol models with the full species set support the expected negative relationship between mass and d_S (correlation coefficient = -0.28, *pp* = 0.065; Fig. 2). This relationship weakens when effects of migration distance are accounted for (i.e., with partial correlation coefficient = -0.18, *pp* = 0.20). We did not find a strong correlation between mass and d_N/d_S (correlation coefficient = -0.25, *pp* = 0.19; partial correlation coefficient = -0.072, *pp* = 0.41). In models of d_S with the subset of 30 species that included θ as a predictor, mass had a correlation coefficient of -0.17 (*pp* = 0.21) and a partial correlation coefficient (which controls for the

401 effects of migration distance) of -0.23 (pp = 0.15). In models of d_N/d_S from this subset, mass had
402 a correlation coefficient of 0.16 (pp = 0.7) and a partial correlation coefficient of 0.26 (pp =
403 0.84).
404
405 *The influence of N_e on molecular evolutionary rates*
406 In models using the subset of 30 species with population-level data, we did not find
407 evidence for a correlation between θ and d_S (correlation coefficient = -0.23, pp = 0.15; partial
408 correlation coefficient = -0.12, pp = 0.67). This result is consistent with neutral evolution of
409 synonymous sites among the species we studied. By contrast, we found strong support for the
410 nearly neutral theory's predicted negative relationship (Ohta 1992; Popadin et al. 2007; Leroy et
411 al. 2021) between θ and d_N/d_S (correlation coefficient = -0.60, pp = 0.025; partial correlation
412 coefficient = -0.57, pp = 0.031; Fig. 3), indicating stronger purifying selection in species with
413 higher N_e.
414
415 *Linear modeling of π_N/π_S*
416 In comparison of AICc, the highest-ranked model of π_N/π_S showed a strongly supported
417 negative relationship between θ and π_N/π_S (Fig. 4, Table S7, model weight 0.55), as predicted if
418 purifying selection is stronger in species with higher N_e. Compared to a model with θ alone, a
419 model with both θ and migration distance shows an increase in multiple r^2 from 0.15 to 0.28 and
420 a decrease in AICc by more than two units, suggesting the inclusion of migration distance
421 improves model fit. However, contrary to our prediction, migration distance has a weak positive
422 relationship with π_N/π_S (Fig. 4). The estimated coefficient relating θ and π_N/π_S in the best-fit
423 model is -0.027 (std error = 0.01) and the estimated effect of migration distance from the best-fit

model is 0.022 (std error = 0.01). Model comparison did not support the inclusion of mass as a predictor of π_N/π_S (Table S7).

N_e does not confound patterns of rate correlations

We used linear modeling to test whether migration distance or mass show a relationship with θ, our proxy of N_e. We did not find strong evidence that mass or migration distance are correlated with θ among the 30 species we studied. The null model for θ (an intercept-only model with no predictors) showed the lowest AICc, suggesting that the addition of mass and migration distance as predictors did not improve model fit (Table S8, model weight 0.45). However, the model with migration distance as a predictor was within 2 AICc units of the null model and showed a model weight of 0.30, indicating considerable model uncertainty. The estimated effect of migration distance on θ was positive but had a negligible effect size in the second-best model (estimate = 0.0017, std error = 0.0013 model multiple $r^2 = 0.054$).

Discussion

Seasonal migration distance correlates with mitochondrial d_S

We examined the relationship between life history and patterns of mitochondrial sequence evolution within North American boreal birds. These species occupy a region where strong seasonality demands specialized adaptations that carry life history tradeoffs (Varpe 2017; Winger and Pegan 2021). Our results implicate the life-history axis of seasonal migration distance as a novel correlate of mitochondrial synonymous substitution rate (d_S). Previous work demonstrates that, even after accounting for body size, long-distance migrants in this system

447 have slower life history strategies than short-distance migrants, showing higher annual adult
448 survival and lower fecundity (Winger and Pegan 2021). Here, we find that the slow life history
449 of long-distance migrants is accompanied by a slower rate of neutral molecular evolution in the
450 mitochondria of these species compared with that of shorter-migrating species in the region.
451 Indeed, among the 39 species we studied, the correlation between migration distance and d_S is
452 stronger than the correlation between mass and d_S, which is notable given that the relationship
453 between mass and substitution rate has been documented in previous work (Nabholz et al. 2016).
454 As such, we suggest that the association between migration distance and the slow-fast life history
455 continuum extends to effects on d_S.
456
457 *What evolutionary processes link migration distance with mitochondrial d_S?*
458 Substitution rates are fundamentally influenced by mutation rate, which provides new
459 molecular variants with potential to become substitutions, and by natural selection, which
460 influences whether variants are fixed as substitutions or lost. The correlation between migration
461 distance and d_S therefore reflects one or both processes. d_S is often treated as a proxy for
462 mutation rate alone based on the assumption that natural selection does not operate on
463 synonymous sites (Nei et al. 2010), but in some cases, synonymous sites are known to evolve
464 non-neutrally (Chamary et al. 2006; Künstner et al. 2011; Wei et al. 2014; Wynn and Christensen
465 2015). If synonymous sites are not evolving neutrally, nearly neutral theory suggests that the
466 relationship between d_S and migration distance could be explained by larger N_e in long-distance
467 migrants (Ohta 1992). We tested the key assumption that synonymous sites evolve neutrally by
468 assessing the relationship between d_S and our proxy for N_e (θ) (Table S4). We found no
469 correlation, suggesting that synonymous sites are indeed evolving neutrally in our system. We

also found no correlation between θ and migration distance (Table S8). Together, these results suggest that that variation in d_S among species with different migration distances is not well explained by variation in natural selection or effective population size. Rather, we suggest that the negative relationship between migration distance and d_S may reflect a negative relationship between migration distance and mutation rate.

Why might long-distance migrants have a lower mitochondrial mutation rate?

We predicted that migration distance would correlate with d_S because of its relationship with the slow-fast continuum of life history in these species independent of body size (Winger and Pegan 2021). In turn, a species' position on the slow-fast life history continuum is hypothesized to affect mutation rate (Bromham 2020). There are several potential mechanisms to explain the link between life history and mutation rate, and the relative importance of each is not clear (Bromham 2020). The "copy error effect" hypothesis suggests that the explanation is related to generation time, assuming that "fast" species with short generation times and young age at first reproduction experience higher rates of germline replication (and thus replication-induced mutation) than species with "slow" life histories (Li et al. 1996; Thomas et al. 2010; Lehtonen and Lanfear 2014).

However, recent studies comparing cell division rates with directly-measured mutation rates suggest that replication-induced copy errors may not be the only driver of differences in mutation rate between lineages (Wu et al. 2020; Wang et al. 2022). The "mutation avoidance" hypothesis offers another non-exclusive explanation for lower d_S in organisms with slow life history based on higher mutation costs in longer-lived species (Bromham 2020). Under this hypothesis, organisms with slow life history are predicted to have adaptations that reduce the

493 introduction of mutations from DNA damage or DNA replication and repair processes (Galtier et
494 al. 2009; Tian et al. 2019; Zhang et al. 2021; Cagan et al. 2022). Long-distance migrants may be
495 especially sensitive to the costs of mitochondrial mutation, which may cause mitochondrial
496 senescence (Galtier et al. 2009; Hua et al. 2015), because of the high physical performance
497 demanded by their migratory behavior across their entire lifespans (Møller 2007; Conklin et al.
498 2017). Further research is necessary to understand what processes contribute to the apparent
499 reduction of mutation rate in species at the slow end of the slow-fast continuum of life history.
500 Another possible link between migration distance and mutation rate is oxidative damage
501 from metabolism, which is recognized as a potential source of mutation rate variation (Martin
502 and Palumbi 1993, Gillooly et al. 2005, Berv and Field 2018; but see Lanfear et al. 2007, Galtier
503 et al. 2009). Thus, a potential explanation for our results—lower mitochondrial d_S in long-
504 distance migrants—is that long-distance migrants incur less metabolically-induced DNA damage
505 than short-distance migrants. This explanation is initially surprising in light of studies showing
506 that migratory birds experience oxidative damage from endurance flight (Jenni-Eiermann et al.
507 2014; Skrip and McWilliams 2016). However, we suggest that three plausible and non-exclusive
508 scenarios could lead to lower metabolically-induced DNA damage in long-distance compared to
509 short-distance migrants. First, long-distance migrants may have better adaptations for flight
510 efficiency (Weber 2009; Elowe et al. 2023), reducing the oxidative damage they experience per
511 mile traveled. Second, the mutation avoidance hypothesis predicts that long-distance migrants
512 may have more efficient DNA repair mechanisms than short-distance migrants, which could
513 reduce metabolically-induced mutation rates even when long-distance flight does induce high
514 oxidative stress. Last, short-distance migrants in our boreal study system may experience greater
515 oxidative damage arising from their increased need for winter cold tolerance than long-distance

migrants that winter in the tropics. The mitochondria also play an important role in the metabolic challenge of maintaining homeostasis during cold weather and resource shortages (Bicudo et al. 2001; Chen et al. 2018). Short-distance boreal migrants likely face more of these kinds of challenges than long-distance migrants during migration and winter (Winger and Pegan 2021). Despite the view that long-distance migration is an extreme performance challenge, its alternative—spending the winter within the temperate zone—is also an extreme metabolic challenge for small-bodied homoeothermic endotherms that do not hibernate (Dawson and Yacoe 1983; Winger et al. 2019). Further investigation of the comparative metabolic challenges faced by short versus long-distance boreal migrants is needed to clarify whether and how migration distance influences metabolically-induced mutation in the mitochondria.

Purifying selection is not stronger in long-distance migrants

Whereas evolutionary rate at synonymous sites (d_S) may primarily reflect mutation rate, evolution at nonsynonymous sites is expected to strongly reflect natural selection because nonsynonymous mutations alter the amino acid sequence of a gene's protein product. We found that the ratio of nonsynonymous to synonymous substitutions (d_N/d_S) among our species is universally much less than 1 (Fig. 3), indicating that the mitochondrial genes we studied are under purifying selection in all species in the system. We similarly found low ratios of nonsynonymous to synonymous polymorphisms within each population (π_N/π_S; Fig. 4), which is also consistent with purifying selection. Moreover, both d_N/d_S and the π_N/π_S ratio are strongly correlated with θ, our proxy for N_e (Fig. 3, 4), as expected under nearly neutral theory (Ohta 1992). A nuance of our results is that d_N/d_S reflects the accumulation of substitutions across the entire history of a lineage, whereas population parameters such as θ and π_N/π_S may be more

strongly influenced by recent demographic processes. However, that we and others (e.g., Popadin et al. 2007; Leroy et al. 2021) find empirical evidence for the relationship between θ and d_N/d_S predicted by nearly neutral theory, despite this potential mismatch in evolutionary timescales, suggests that similar demographic processes may shape empirical estimates of genetic diversity and molecular evolutionary rates.

Our results are consistent with the general finding that mitochondrial genes tend to experience strong purifying selection (Nabholz et al. 2013; Popadin et al. 2013). However, we did not find evidence supporting our prediction that long-distance migrants would show stronger purifying selection (i.e., lower d_N/d_S and π_N/π_S) than short-distance migrants. This finding may reflect the reality that all species in our system face generally strong mitochondrial purifying selection, such that the endurance flights of long-distance migrants do not incur much stronger selection than the level that exists among all the species we studied. Our results also imply that short-distance migrants in the boreal region do not experience *relaxed* purifying selection on mitochondrial genes compared to long-distance migrants. As noted above, short-distance boreal migrants contend with metabolic challenges associated with cold winter temperatures which may also exert selection on the mitochondria (Chen et al. 2018), as well as the metabolic demands of flight.

Migration distance and the costs of mitochondrial mutations

In this study, we based our predictions on several complementary hypotheses about the costs of mutation in species with slow life history and high demand for physiological performance, such as long-distance migrants. From the perspective of molecular evolution, the mutation avoidance hypothesis (Bromham 2020) and studies on the relationship between lifespan

562 and mutation rate (Nabholz et al. 2008a; Galtier et al. 2009; Tian et al. 2019; Zhang et al. 2021)
563 predict that phenotype-altering genetic variation is harmful enough to induce selection for
564 mutation avoidance in organisms with slow life history. From the perspective of population
565 biology, the hypothesis proposed by Conklin et al. (2017) predicts that "slow" species with high
566 performance demands experience a strong selective filter on phenotypic performance in early
567 life, reducing phenotypic variation in these populations. While Conklin et al. (2017) frame their
568 hypothesis around reduction of phenotypic variation, a similar prediction about reduction of
569 genetic variation emerges from a series of studies showing that mitochondrial purifying selection
570 is stronger in species with higher locomotory metabolic demands (Shen et al. 2009; Chong and
571 Mueller 2013; Strohm et al. 2015; Mitterboeck et al. 2017; Sun et al. 2017; Chang et al. 2020; De
572 Panis et al. 2021). Together, these hypotheses led us to predict that the costs of mitochondrial
573 mutation in long-distance migrants, which have slow life histories, would cause them to exhibit
574 slower mitochondrial mutation rates and stronger mitochondrial purifying selection than short-
575 distance migrants.

576 Our predictions were only partially supported. The negative relationship we found
577 between migration distance and d_S is consistent with lower mitochondrial mutation rate in long-
578 distance migrants, but we did not find evidence that these species experience stronger
579 mitochondrial purifying selection than do short-distance migrants. To reconcile these findings
580 and advance our understanding of how long-distance migration influences molecular
581 evolutionary dynamics, further research is needed on the relative metabolic demands of long-
582 distance flight versus cold tolerance and on the consequences of mitochondrial genetic variation
583 for migratory phenotype. Additionally, studying molecular rates across the nuclear genome will

584 help clarify which dynamics we report here are related to selection on the mitochondrial genome
585 and which reflect more general interactions between life history and molecular evolution.
586
587 *Conclusions: seasonal adaptation provides novel context for studying the links between life*
588 *history and molecular evolutionary rates*
589 Adaptation to seasonality entails life history tradeoffs (Varpe 2017). Organisms balance
590 these tradeoffs in different ways, creating variation in life history strategy within communities
591 that inhabit seasonal environments (e.g., Winger and Pegan 2021). Our study demonstrates that
592 life history variation related to seasonality can influence molecular evolutionary rates, which has
593 implications for the accurate reconstruction of evolutionary history (Berv and Field 2018; Shafir
594 et al. 2020; Ritchie et al. 2022). More broadly, communities adapted to seasonal habitats provide
595 an important context to investigate potential drivers of the relationship between life history and
596 molecular evolution. Co-distributed species show varying adaptations to seasonality—e.g., cold
597 tolerance, migration, hibernation—and they express these strategies to different degrees (Auteri
598 2022). Cold adaptations can influence biological processes hypothesized to be relevant for
599 germline replication rate or mutation rate (e.g., Wang et al. 2022), even among species that show
600 little variation in commonly-studied life history proxies such as body mass. Comparative studies
601 using seasonal communities can therefore allow us to draw new insights into how life history
602 tradeoffs affect mutation rate, one of the most fundamental processes in evolution.
603
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605 TMP and AAK; data analyzed by TMP and JSB; manuscript written by TMP and BMW;
606 manuscript revised by all authors.

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Data accessibility statement: Sequence data are available on GenBank. Accession numbers may be found in Table S2. Code and other associated data are available at Dryad DOI: 10.5061/dryad.cvdncjt99

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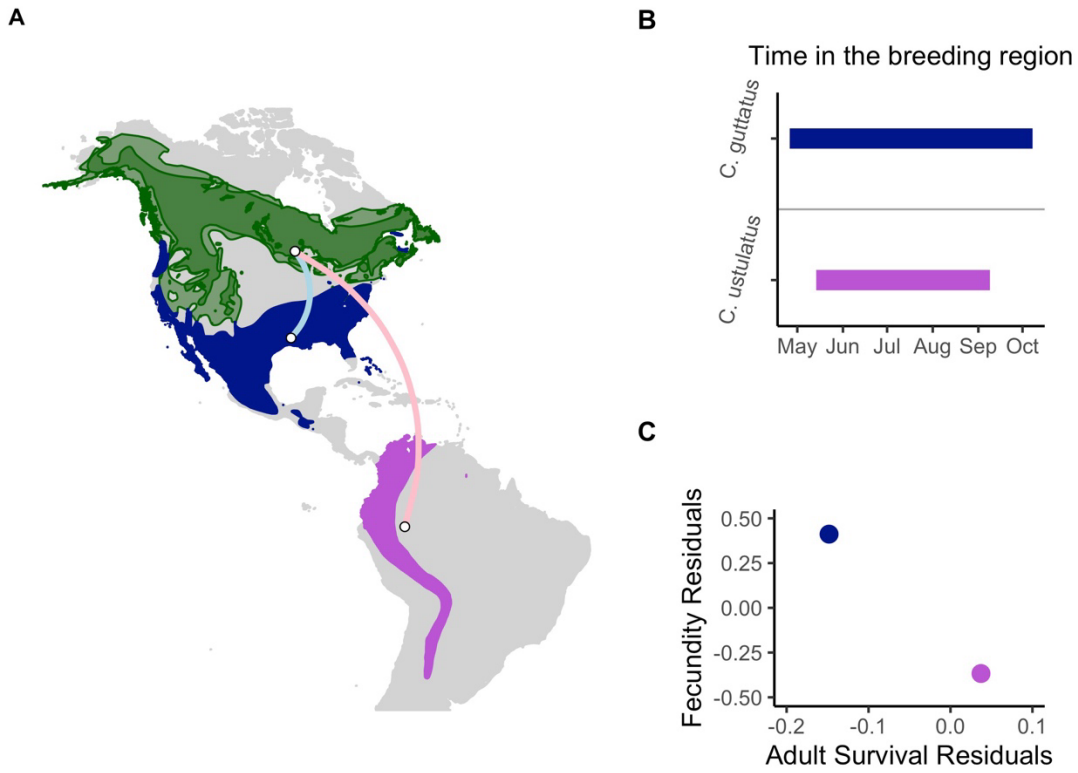

Figure 1. An example contrast between a shorter-distance migrant *Catharus guttatus* and a closely related longer-distance migrant *Catharus ustulatus swainsoni* illustrates the relationship between migration distance and life history in our study system. Both species have broadly overlapping breeding ranges (green), but *C. guttatus* (dark blue nonbreeding range) migrates a shorter distance (blue migratory route) than *C. u. swainsoni* (purple nonbreeding range, pink migratory route) (panel A). Accordingly, *C. guttatus* spends more time in its breeding range than *C. u. swainsoni* (panel B). With more time in the breeding range and the possibility of raising a second brood, the short-distance migrant has higher fecundity but lower adult survival—i.e., faster life history—than the long-distance migrant (panel C, showing model residuals from mass-corrected analysis of fecundity and survival). The short-distance migrant spends the winter in colder, more resource-depleted regions than the long-distance migrant.

914 Figure and data adapted from Winger and Pegan (2021). Our sampling for this study occurred
915 only within the eastern boreal belt (Fig. S1).
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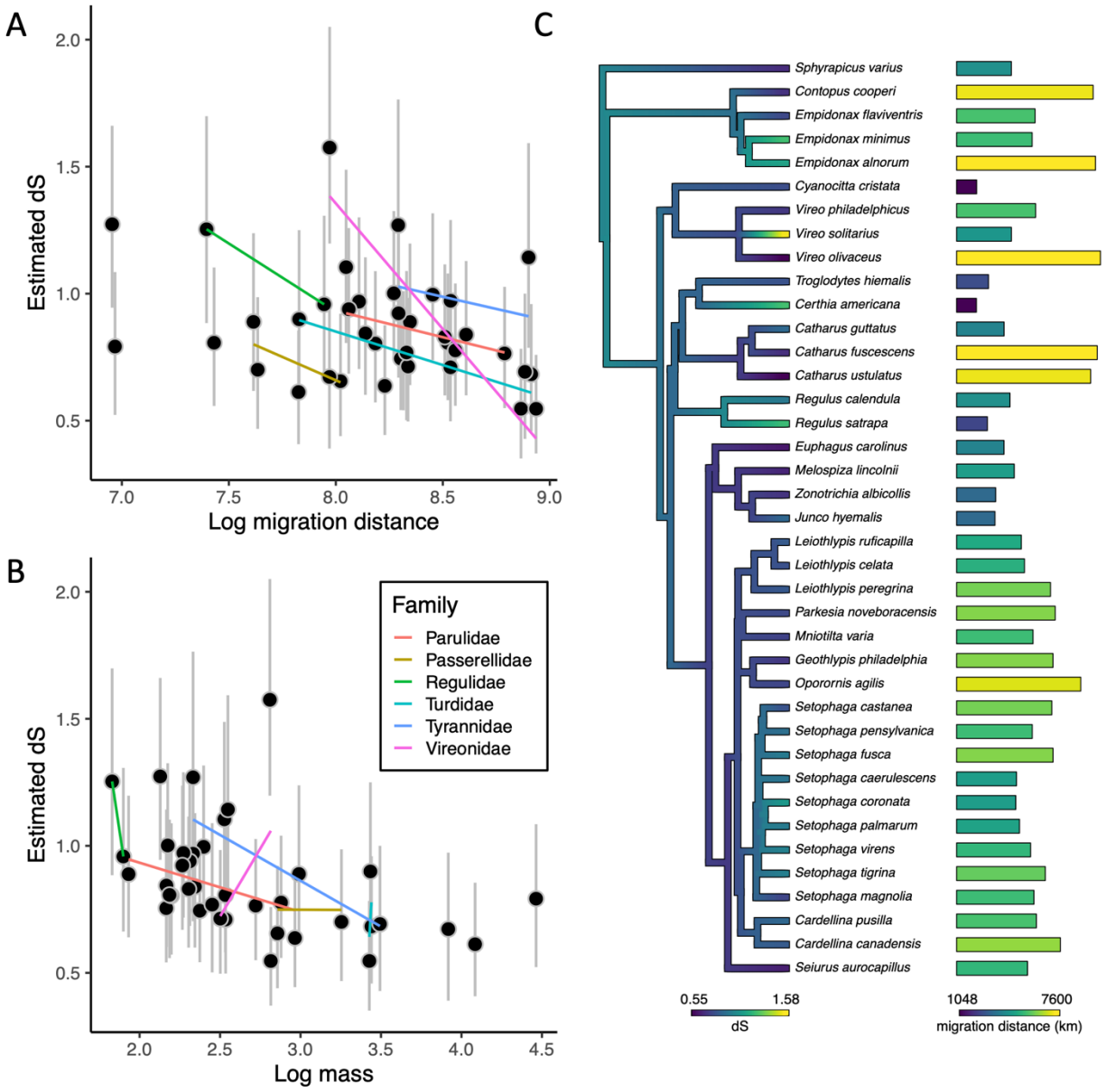
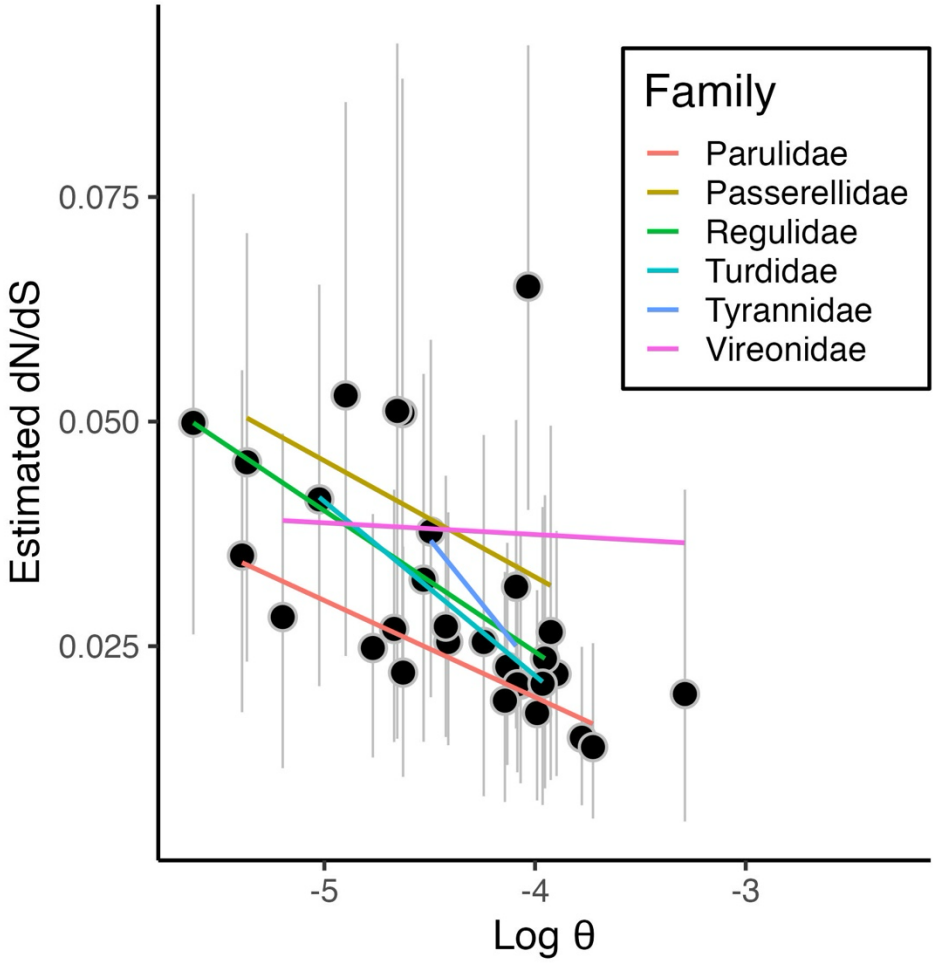


Figure 2. d_S versus traits associated with life history (A, B) and a phylogenetic tree showing d_S and migration distance for each species (C). In panels A and B, posterior mean tip estimates of d_S (black dots) from Coevol are shown compared to migration distance (A), and mass (B) from models using our full species set. Gray vertical bars indicate 95% credible intervals for each

estimate. These analyses reveal that both migration distance and mass have a negative relationship with d_S. Plotted lines use linear models to visualize the relationship between estimated tip d_S and a given covariate within each family of birds (when represented in our dataset by two or more species), demonstrating a consistently negative relationship between d_S and migration distance within and among major clades in our system. In panel C, the phylogenetic tree was created in phytools (Revell 2012) and is colored based on posterior mean tip and node estimates of d_S from Coevol.



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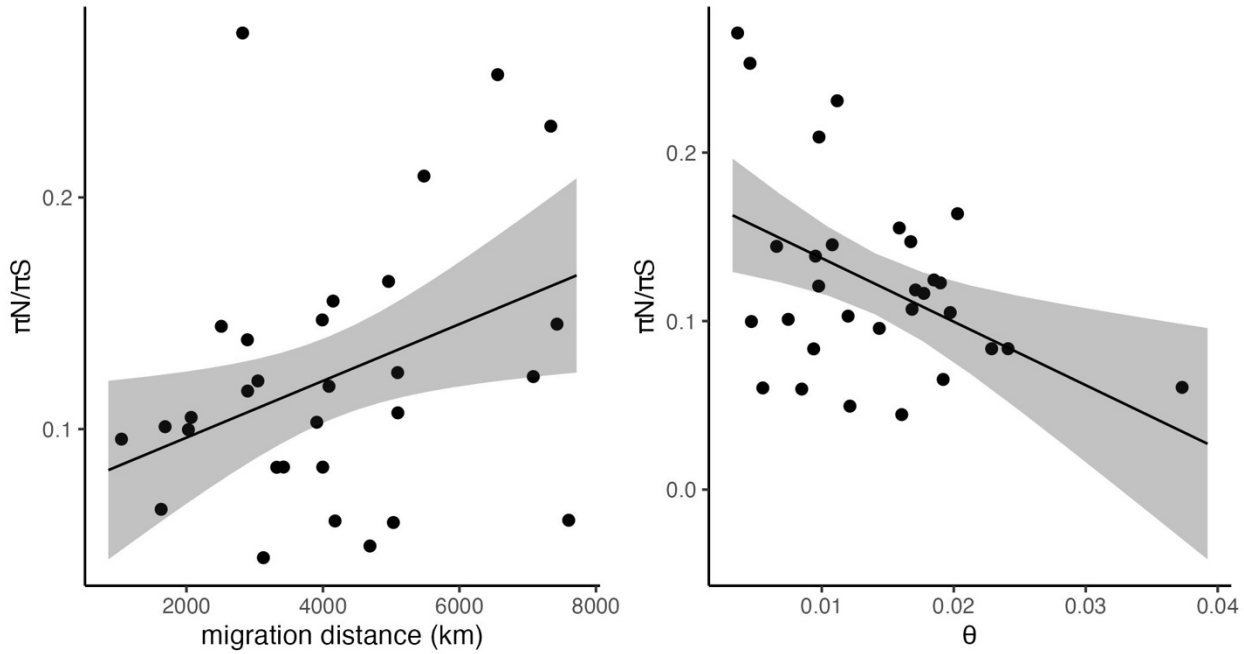
933

934 **Figure 3**. d_N/d_S versus θ. Posterior mean tip estimates (black dots) of d_N/d_S are shown compared
935 to θ from a Coevol model including species for which we could estimate θ. Gray vertical bars
936 indicate 95% credible intervals for each estimate. As in Fig. 2, plotted lines use linear models to
937 visualize the relationship between mean tip d_N/d_S and θ within each family of birds (when
938 represented in our dataset by two or more species), demonstrating a consistently negative
939 relationship between θ and d_N/d_S within and among major clades in our system.

940



941

942 **Figure 4**. The relationship between π_N/π_S and migration distance (left) and θ (right). π_N/π_S is
943 strongly influenced by θ, as expected if purifying selection removes more nonsynonymous
944 variation in species with larger N_e. π_N/π_S increases with migration distance, after accounting for
945 effects of θ. Regression lines and 95% confidence intervals show the marginal effect of each
946 variable as calculated by "ggpredict()" from the R package ggeffects v0.16.0 (Lüdecke 2018)
947 using the best-fit model, which included both predictors.

948

Table 1. Definitions of abbreviations for molecular substitution rates and population genetic parameters and predictions for their relationships with migration distance.

Concept	Abbr.	Description and assumptions	Predictions (this study)
Synonymous substitution rate	d_S	Assuming synonymous sites evolve neutrally, d_S primarily reflects μ (Nei et al. 2010; Lanfear et al. 2014)	Negative relationship between migration distance and d_S
Nonsynonymous substitution rate	d_N	Assuming nonsynonymous sites are generally deleterious, d_N is influenced by both μ and N_e (reviewed in Nei 2005)	NA
d_N/d_S ratio	d_N/d_S	Assuming nonsynonymous mutations are generally deleterious, d_N/d_S reflects strength of purifying selection on d_N while accounting for variation in μ. Low d_N/d_S = strong purifying selection. (Nei 2005; Kryazhimskiy and Plotkin 2008)	Negative relationship between θ and d_N/d_S, reflecting the influence of N_e on d_N/d_S. Negative relationship between migration distance and d_N/d_S, indicating positive relationship between migration distance and purifying selection strength.
Mutation rate	μ	May be influenced by life history; reviewed in (Bromham 2020)	NA, μ not measurable in our data
Effective population size	N_e	Defined as the ideal population size experiencing the same level of genetic drift as observed in the data (Waples 2022). Estimated in mitochondrial data as θ / μ. (Watterson 1975; Nabholz et al. 2008a)	NA, see θ
Theta	θ	Population genetic parameter representing genetic variation. Assuming low variation in μ, variation in θ primarily reflects variation in N_e	Negative relation between θ and d_N/d_S and between θ and $\pi N/\pi S$
Synonymous nucleotide diversity	π_S	Population genetic parameter representing population-level nucleotide diversity at synonymous sites.	NA
Nonsynonymous nucleotide diversity	π_N	Population genetic parameter representing population-level nucleotide diversity at synonymous sites.	NA
π_N/π_S ratio	π_N/π_S	Reduction of π_N compared to π_S is expected to reflect natural selection, but the relationship is more complex than with d_N/d_S	Negative relationship between migration distance and π_N/π_S, indicating positive relationship between migration distance and selection. Negative relationship between θ and π_N/π_S, indicating purifying selection on nonsynonymous polymorphisms.

Table 2. A summary of analyses. Models 1 and 2 use Coevol test our hypothesis that synonymous substitution rate (d_S) is influenced by migration distance, with mass and θ as additional covariates. Models 3 and 4 use the same approach with Coevol to estimate correlations between traits of interest and d_N/d_S. Models including θ use only 30 species because we did not have population-level data available to estimate θ for all 39 species. Coevol does not analyze molecular evolutionary parameters based on population-level data, so we used linear modeling to test whether traits of interest influence π_N/π_S (model 5). Finally, we also used linear modeling to test for potential confounding relationships between θ and life history-associated traits of interest (mass and migration distance; model 6).

		Data subset	**Method**
1	$d_S \sim$ migration distance + mass	full (39 species)	Coevol
2	$d_S \sim$ migration distance + mass + θ	theta (30 species)	Coevol
3	$d_N/d_S \sim$ migration distance + mass	full (39 species)	Coevol
4	$d_N/d_S \sim$ migration distance + mass + θ	theta (30 species)	Coevol
5	$\pi_N/\pi_S \sim$ migration distance + mass + θ	theta (30 species)	linear modeling
6	$\theta \sim$ migration distance + mass	theta (30 species)	linear modeling