



Mapping seasonal migration in a songbird hybrid zone -- heritability, genetic correlations, and genomic patterns linked to speciation

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Seasonal migration is a widespread behavior relevant for adaptation and speciation, yet knowledge of its genetic basis is limited. We leveraged advances in tracking and sequencing technologies to bridge this gap in a well-characterized hybrid zone between songbirds that differ in migratory behavior. Migration requires the coordinated action of many traits, including orientation, timing, and wing morphology. We used genetic mapping to show these traits are highly heritable and genetically correlated, explaining how migration has evolved so rapidly in the past and suggesting future responses to climate change may be possible. Many of these traits mapped to the same genomic regions and small structural variants indicating the same, or tightly linked, genes underlie them. Analyses integrating transcriptomic data indicate cholinergic receptors could control multiple traits. Furthermore, analyses integrating genomic differentiation further suggested genes underlying migratory traits help maintain reproductive isolation in this hybrid zone.

seasonal migration | hybrid zone | genetic basis | songbirds | speciation

Seasonal migration is the yearly long-distance movement of individuals between their breeding and wintering grounds. This behavior likely evolved as an adaptive response to seasonally changing environments, allowing animals to track resources and avoid competition (1–3). Differences in migration likely also contribute to speciation. For example, hybrids between species with divergent migratory routes often take intermediate routes that take them over geographic barriers parental forms avoid, reducing their survival (4–7). It is widely held that migration has a strong genetic basis in many animal groups (8), but detailed knowledge of this topic remains limited.

Migration requires the coordinated action of multiple traits (9). Early experiments and quantitative genetic analyses suggested these traits are heritable and genetically correlated but were limited to a subset of species and traits (10–14). In addition, migratory traits were often quantified using proxies in the lab [e.g., in night-migrating songbirds the focus has been on fall timing and distance, using the date when nocturnal behavior is initiated in the lab and the number of nocturnal hours as proxies for timing and distance, respectively (12)]. The development of genomic resources has seen the addition of new species and work on the actual genetic variants that underlie migration but is rarely matched with more direct data on migration, restricting analytical approaches that can be applied to this question. For example, many studies rely on selection scans, estimating genomic differentiation between groups that are thought to differ in several migratory traits (15–17). Genomic differentiation can be generated by many processes (18, 19) and this approach does not tell us about individual migratory traits. We expanded existing work on migration genetics here, using advances in animal movement ecology to match genomic resources with one of the largest datasets of direct migration data available to date. We used a hybrid zone between two subspecies of Swainson's thrushes (coastal and inland).

The Swainson's thrush is well suited for work on the genetics of migration and allows expansion to migration's role in speciation. For example, considerable variation in migratory behavior has already been documented in this system, with direct tracking data showing the subspecies take different routes on migration, with coastal thrushes migrating along the west coast of North America to Mexico, Guatemala and Honduras, and inland thrushes migrating east across the Rocky Mountains through central North America to Columbia and Venezuela (20). Variation in additional migratory traits has also been documented [e.g., in timing (earlier in coastal) (20) and wing morphology (shorter, less pointed in coastal) (21)] and combined, differences in the migratory behavior of thrushes likely help maintain reproductive isolation in the system through selection against hybrids and premating barriers. For example, the hybrid zone between thrushes is narrow and population densities at its center are low suggesting some form of reproductive isolation exists between the subspecies [selection against hybrid $s = 0.19$ to 0.40 ; (22); 40% of birds

Significance

Seasonal migration is a behavioral response to seasonally changing environments that is exhibited by many taxa and has captured the attention of researchers and laypeople alike. A genetic basis for migration has been suggested for decades but detailed, genome-wide studies have only recently been permitted with advances in tracking and sequencing technology. We capitalized on these advances here, using a natural hybrid zone between songbirds and data on multiple migratory traits from hundreds of individuals to provide one of the most complete genetic characterizations of this behavior to date. Our results can be integrated in future studies to predict organismal ability to adapt to changes in the environment.

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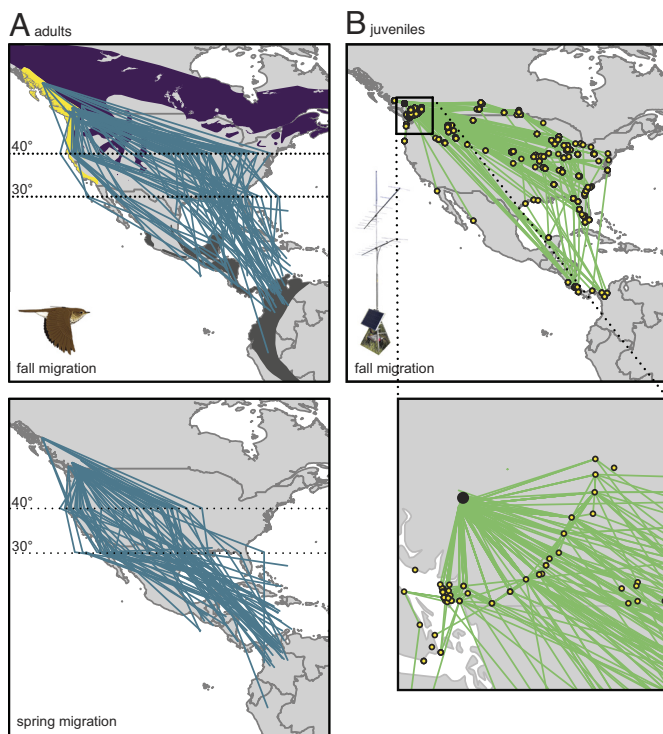


Fig. 1. Migratory tracks used in the present study. (A) Adult tracks (blue) derived from light-level geolocators and GPS tags. Summarized as the longitude at which birds pass 40° and 30° latitude. Breeding distribution of coastal (yellow) and inland (purple) subspecies shown in background along with winter grounds (gray). (B) Juvenile tracks (green) derived from Motus radio stations (yellow points). The *Inset* shows fence of stations established for the present study and location from which birds were released (black point).

at the center can be inferred to as hybrids (21)]. Hybrid thrushes have been shown to take intermediate (22) and ecologically inferior routes on migration, integrating stopover sites of lower habitat suitability and using routes that permit less movement (7). Differences in arrival time have also been documented in this system and could serve as premating barriers to gene flow (23). Beyond migration-related traits, differences in song and color have also been documented between thrushes and could contribute to reproductive isolation in the system (24, 25). Finally, it is important to note that considerable genomic work has already been conducted in the system, including genome scans identifying regions under selection (26), an early genome-wide association study (GWAS) connecting a large region to migratory orientation (27) and a recent transcriptomic analyses that identified genes differentially expressed between the subspecies and seasons (28).

For the quantification of migratory traits we relied on two technologies for tracking birds—archival tags that provide near-daily location estimates but must be retrieved limiting their use to adult males (the only age/sex class that can be reliably recaptured) and Motus (29), a network of automated radio stations. Motus stations are found throughout North America, and we established our own fence across the entire width of the Swainson's thrush hybrid zone (Fig. 1B). Birds that pass within a few kilometers of these stations are recorded allowing us to track large numbers of birds and any age/sex class as retrieval is not necessary. Of particular interest for work on the genetics of migration, we can track juveniles with Motus. Juveniles have not migrated before, learning navigational cues that could alter their behavior. Juvenile tracks presented here are the first for thrushes and have rarely been reported for songbirds more generally.

We began our analyses by estimating heritability for a series of migratory traits and testing for genetic correlations across them.

We continued with a series of GWAS, identifying specific genomic regions underlying each trait and using a meta-analytic approach to combine results across traits. We finished with two integrative analyses, overlapping GWAS results with estimates of genomic differentiation between parental populations of thrushes and results from previous transcriptomic work in the system (28) that identified genes differentially expressed between the seasons and subspecies. Our results are relevant for many fields, including the genetics of adaptation and speciation which has been dominated by work on morphological traits, as well as conservation since climate change is altering selective pressures on many behaviors like migration and estimates of heritability and genetic correlations across traits will help us predict organismal responses to these changes.

Results

Considerable Phenotypic Variation Documented in Migratory Traits. We fitted archival tags to 333 adult male Swainson's thrushes over 6 y and successfully downloaded data from 72 of these birds ([Dataset S3](#)). These birds exhibited considerable variation in several migratory traits (four related to orientation, two related to distance, and two related to timing; Figs. 1A and 2A). They left the breeding grounds between mid-August and early October, migrated an average of 6,500 km on fall migration and wintered between Mexico and Venezuela. They departed for spring migration anywhere from mid-March to the end of April. At their widest point, their routes spanned -122.246° to -77.131° of longitude (Fig. 2A).

We fitted 328 Motus radio tags to juvenile thrushes over 3 y and detected 261 of these birds ([Dataset S3](#)). We focused our analysis on fall migration (two traits related to orientation, and one related to timing; Figs. 1B and 2B) when we had the largest numbers of birds passing our fence of stations. They passed this fence between the end of August and beginning of October and exhibited similar variation to adults in their outward orientations, spanning -117.54° to -79.10° of longitude at the widest point. Interestingly, while the overall spread of orientations was similar to adults, there was a preponderance of birds exhibiting intermediate outward orientations in this age class (Fig. 2B). 36/182 birds were females, but we did not detect any differences between the sexes in the timing or orientation of migration (all P -values > 0.1).

Beyond the previously described traits related to the orientation, distance, and timing of migration, subsequent analyses also included data on wing morphology for all birds regardless of age class, including wing length (which ranged from 88 to 105 mm) and Kipp's distance [a measure of wing pointedness (30) that ranged from 22.9 to 35 mm, hereafter = "wing shape"; Fig. 2C). We observed a significant difference between sexes for both wing traits, with 61/433 females having shorter and rounder wings (P -values < 0.01).

Migratory Traits Exhibit Moderate to High Heritability. We genotyped all birds using reference genomes for both subspecies, low coverage whole genome resequencing data and a read-based algorithm for imputing missing genotypes (31) (see *Materials and Methods* for additional details). Estimates of ancestry and interspecific heterozygosity indicate this is a later generation hybrid zone with later generation hybrids and backcrosses ([SI Appendix, Fig. S7B](#)). In our first set of analyses, we used two approaches to examine how heritable each of the former migratory traits are (Fig. 2). First, we used bayesian sparse linear mixed models (BSLMMs) to estimate chip heritability [or the proportion of variation explained by additive genetic effects (PVE)] for each

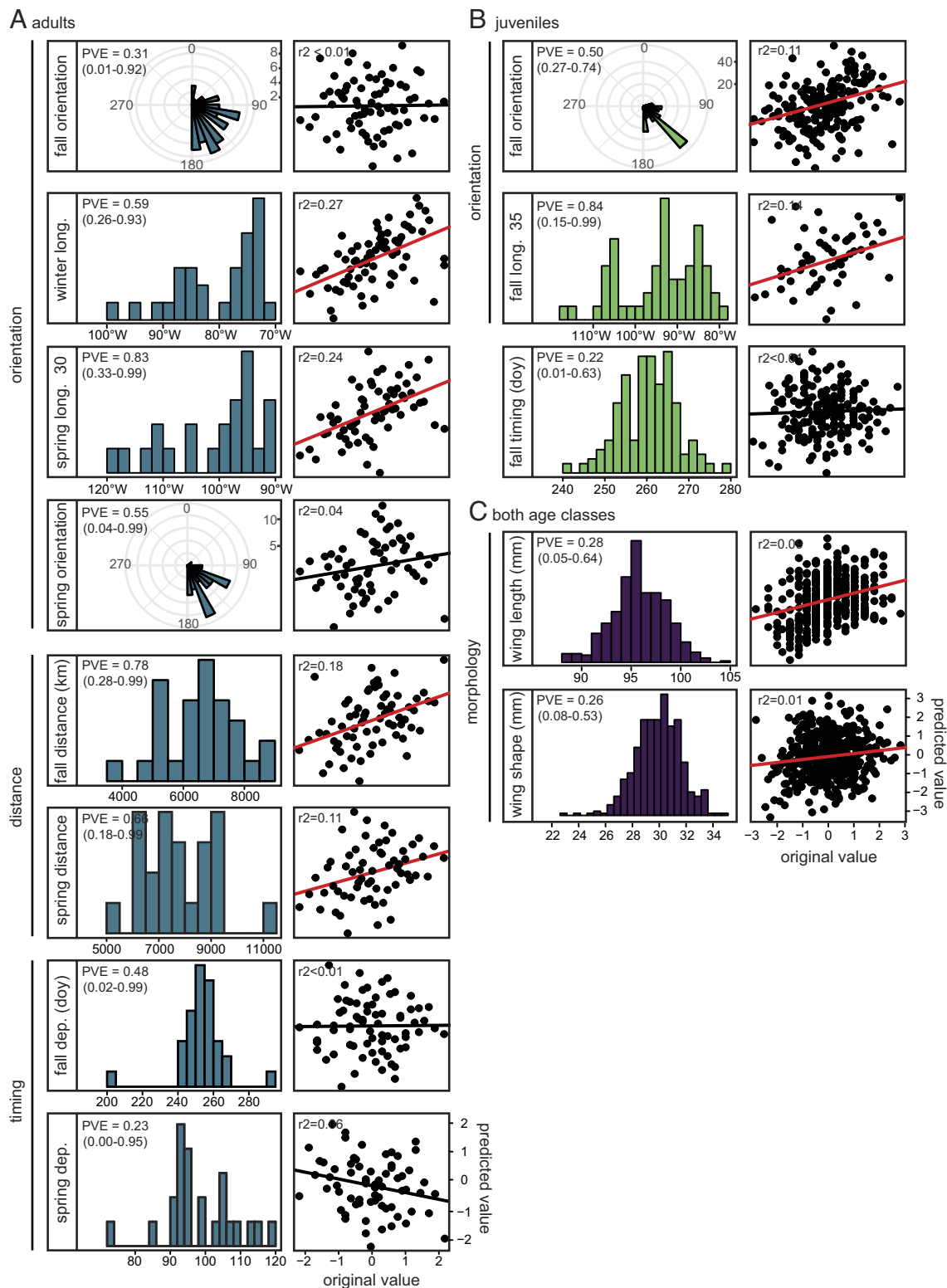


Fig. 2. Migratory traits included in the present study. (A) Traits estimated for adults from light-level geolocators and GPS tags, (B) traits estimated for juveniles from radio tags, and (C) morphological traits measured for both age classes. Each trait is represented by two plots, a histogram with PVE (proportion of phenotypic variation explained by genetic effects, or chip heritability) values and equal-tail probability intervals and a plot showing the relationship between original trait values and GEBVs (genomic estimated breeding values, or polygenic scores). Significant relationships in the later plots are indicated in red (all significant P -values < 0.01).

migratory trait. Starting with adults, estimates of PVE were moderate to high for orientation and distance variables (e.g., PVE for spring long. 30 = 0.83; PVE for fall distance = 0.78; Fig. 2A). Estimates of PVE were lower for timing variables (e.g., PVE for spring departure = 0.23; Fig. 2A). Transitioning to juveniles, we found a similar trend, with higher PVEs for orientation compared

to timing (e.g., PVE for fall orientation = 0.50 vs. fall timing = 0.21; Fig. 2B). Morphological variables exhibited similar values of PVE to timing variables (e.g., PVE for wing shape = 0.26; Fig. 2C).

The previously described PVE values suggest many migratory traits are heritable in the Swainson's thrushes. Nevertheless,

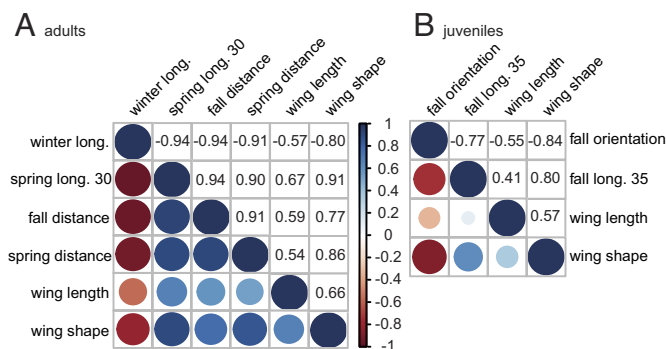


Fig. 3. Genetic correlations. Correlations estimated for (A) adults from light-level geolocators and GPS tags and (B) juveniles from radio tags. The same results are shown above and below the diagonal [with the strength of correlations indicated by size and color (below) or numerical values (above)].

equal-tail probabilities intervals (ETPI; equivalent to parametric CIs) around estimates of PVE for some traits were quite wide (e.g., fall departure, Fig. 2A), indicating that we had limited precision when estimating PVE for some traits (Fig. 2). Accordingly, we conducted a complementary analysis obtaining genomic estimated breeding values (GEBVs or polygenic scores) for each trait. GEBVs are expected trait values for an individual based on the additive effects of their genes and should predict an individual's actual trait value if the trait is heritable (32). In accordance with the magnitude of PVE values reported above, we had strong predictive power for orientation and distance traits (e.g., correlation coefficient [r] for winter longitude = 0.52; r for fall distance = 0.42, corresponding to 27% and 18% of variation in observed GEBVs explained by the predictions; Fig. 2A) and low predictive power for timing and morphological traits (e.g., r for fall departure in adults = 0.013; r for wing shape = 0.13 corresponding to 0.01% and 1.9% of the variation explained; Fig. 2A and B).

We limited all subsequent analyses to traits with significant relationships between the expected/predicted and actual trait values [four of the adult traits (winter longitude, spring long. 30, fall distance and spring distance), two juvenile traits (fall orientation and fall long. 35) and both morphological traits (wing length and wing shape), Fig. 2], as we cannot be sure that we have captured genetic variation underlying the remaining traits with our genomic dataset.

Migratory Traits Are Strongly Genetically Correlated. We used GEBVs to estimate genetic correlations across traits [i.e., standardized G-matrices (32, 33)]. These correlations were quite high, especially between orientation and distance traits (e.g., winter longitude and spring long. 30, $r = -0.94$; winter longitude and fall distance, $r = -0.94$; Fig. 3A). The direction of the correlations we documented corresponded with expectations based on differences between the subspecies. For example, winter longitude was negatively correlated with most traits. Lower longitudes correspond with more western (i.e., coastal) wintering grounds. Previous data from the system shows coastal birds migrate shorter distances, have shorter and less pointed wings and orient in more western directions (20, 21). We documented similar patterns for juveniles, with the highest genetic correlation between fall orientation and wing shape ($r = -0.85$; Fig. 3B).

Migratory Traits Map to a Small Number of Shared Regions, Including Structural Variants. We transition here to examine local patterns of genetic variation, identifying specific loci associated with migratory traits. We used LMMs for our first set of analyses (34). Consistent with elevated PVEs and strong genetic correlations across migratory traits, several SNPs showed

strong associations with these traits and were shared across traits. For example, SNPs at the center of chromosome 3 showed strong associations with four traits (winter longitude, spring long. 30, fall and spring distance; *SI Appendix*, Fig. S1). A similar pattern was documented on chromosome 12, with SNPs at the start of this chromosome showing strong associations with both winter longitude and fall distance (*SI Appendix*, Fig. S1).

Most of the SNPs with strong associations occurred in large blocks, showing similar patterns with SNPs across large regions of the genome. This pattern is exemplified with the results for wing length (Fig. 4A), where there is a large block of association that spans the region between 28 and 96 Mb on chromosome 1. This pattern is reminiscent of findings for large structural variants (SVs) like inversions. Future work using long-read data is needed to evaluate this suggestion more directly. We describe a preliminary analysis below.

Coastal and inland thrushes differ in wing length (shorter in coastal), and we have reference genomes for both subspecies. An alignment of these reference genomes failed to identify any large SVs spanning the region between 28 and 96 Mb (*SI Appendix*, Fig. S3A). Instead, many small SVs appeared to occur in this region (*SI Appendix*, Fig. S3B and C). This finding was supported by the use of short-read data from multiple individuals. For example, we used short-read data to call SVs in two datasets: 1) between parental populations adjacent to the hybrid zone and 2) between long and short-winged birds within the hybrid zone (*Materials and Methods* and *SI Appendix*, *Supporting Information Text*). We found an accumulation of small SVs in the region of strong association for wing length on chromosome 1 (Fig. 4B). We also constructed local PCAs across this region; as expected with SVs, individuals were clustered into three groups within the target region (i.e., individuals are homozygous for either pure form or heterozygous across most SNPs of this region; 35). This clustering

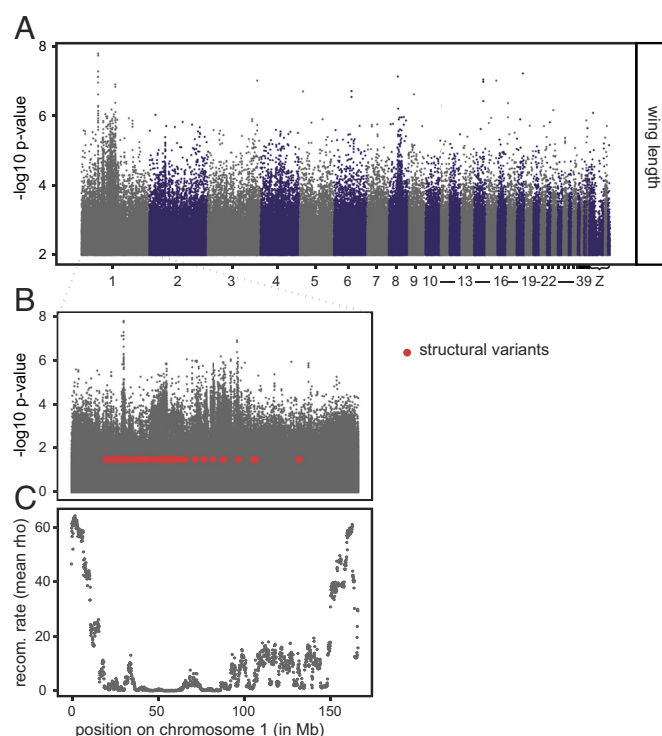


Fig. 4. Results from GWAS for wing length. (A) Manhattan plot shows patterns of association across the genome. (B) Inset highlights patterns for chromosome 1. Red points indicate the location of structural variants that distinguish the subspecies and long- vs. short-winged hybrids. (C) Recombination rate calculated in 100 kb windows shown below Manhattan plot in Inset.

was not evident outside the target region (*SI Appendix, Fig. S4*). And finally, this region also showed a dramatic reduction in recombination rate (Fig. 4C). Functional annotation of variants in this region on chromosome 1 showed most SNPs lie in intronic and intergenic regions (34.9% and 23%, respectively). A small number of variants lie in up- or downstream regions of genes (2.6% and 3.5%, respectively) and exonic regions (0.8%). Two of these exonic SNPs cause nonsynonymous (missense) mutations and are located in *SLC6A19* (Solute Carrier Family 6 Member 19) and *ITPRIP* (Inositol 1,4,5-Triphosphate Receptor Interacting Protein).

We documented a strong pattern of association on chromosome 5 for one trait: fall orientation in juveniles (Fig. 5A). Several strongly associated SNPs occur in close proximity to one another in this region (Fig. 5B). It is 430 kb long and encompasses a single gene—*SGCZ* (Sarcoglycan Zeta). We ran a principal component analysis (PCA) with genotypes from SNPs in this gene and found that individuals clustered into three distinct groups on PC1 and PC2 (Fig. 5C). A linear model showed that both PC1 and PC2 are correlated with fall orientation (Fig. 5D and *SI Appendix, Fig. S5*). Strong associations in this region are not recovered in any of the other juvenile traits, but several adult traits show strong associations in the same region on chromosome 5, including spring long, 30 and spring distance. This is also the region identified for orientation in a previously published GWAS (27) but much smaller (30 Mb vs. 430 kb here). Functional annotation of variants in this region showed that all of the variants lie in intronic or noncoding transcript regions.

Despite clear regions of strong association for most of the migratory traits we studied, none of these individual SNPs reached statistical significance after controlling for multiple tests. Nevertheless, strong genetic correlations and consistent local patterns of genomic association suggest most of these traits are controlled by the same (or tightly linked) set of variants. We leveraged this fact to conduct multitrait GWAS, combining GWAS summary statistics across multiple traits, providing more statistical power than single-trait GWAS (36). We ran separate analyses for adults and juveniles and found several chromosomes that showed strong patterns of association in adults, including regions on chromosomes 1, 2, 3, 4, 5, 6, 12, and Z (Fig. 6A). In the multitrait GWAS for juveniles, we found strong patterns of association on chromosomes 1 and 8 (Fig. 6B).

After adjustment for multiple tests, 188 SNPs were significantly associated with migration in the former analyses (184 for adults and four for juveniles; Holm–Bonferroni adjustment, P -value < 0.05). We identified linkage blocks around these SNPs (i.e., areas where nearby SNPs showed strong $[r > 0.5]$ linkage with causal SNPs) and extracted a total of 192 genes from these blocks (*Dataset S1*). A GO analysis using ontologies for these genes pulled out three ontologies, one related to nicotinic receptor activity [GO:0022848, including *CHRNA4* (Cholinergic Receptor Nicotinic Beta 4 Subunit), *CHRNA5*, and *CHRNA3* (Cholinergic Receptor Nicotinic Alpha 5 and 3 Subunit)] and two related to thrombin receptor signaling and activity [GO:0015057 and GO:0070493, including *F2R* (Coagulation Factor II Thrombin Receptor), *F2RL2* (F2R Like 2) and *F2RL1* (F2R Like Trypsin Receptor 1)]. Outside Swainson's thrushes, four other studies have used genomic data to identify genes linked to migration in songbirds [European blackcaps (37); *Vermivora* warblers (38); common quails (39); and willow warblers (40)]. Combined, 1,784 genes were identified in these studies. Only 2/192 genes [*CNTN5* (Contactin 5) and *MACIR* (Macrophage Immunometabolism Regulator)] from the present study overlapped with this list.

Genomic Loci Associated with Migration Are under Divergent Selection. Recall, seasonal migration likely helps maintain reproductive isolation between coastal and inland thrushes. Accordingly, we might expect to find evidence for divergent selection at loci underlying migration in this system. We evaluated this connection by obtaining whole genome resequencing data from birds adjacent to the hybrid zone and estimating genomic differentiation between the subspecies. We used a modified version of F_{ST} that helps control for the effects of linked selection [ΔF_{ST} (37, 41, 42)]. If differences in migration are helping maintain reproductive isolation between thrushes, loci underlying this trait should be in areas of elevated ΔF_{ST} . In support of this prediction, permutation tests showed that significant SNPs from our multitrait GWAS analyses fell in windows with significantly higher ΔF_{ST} than background levels (permutation test with 10,000 random samples, P -value < 0.001 ; *SI Appendix, Fig. S9*).

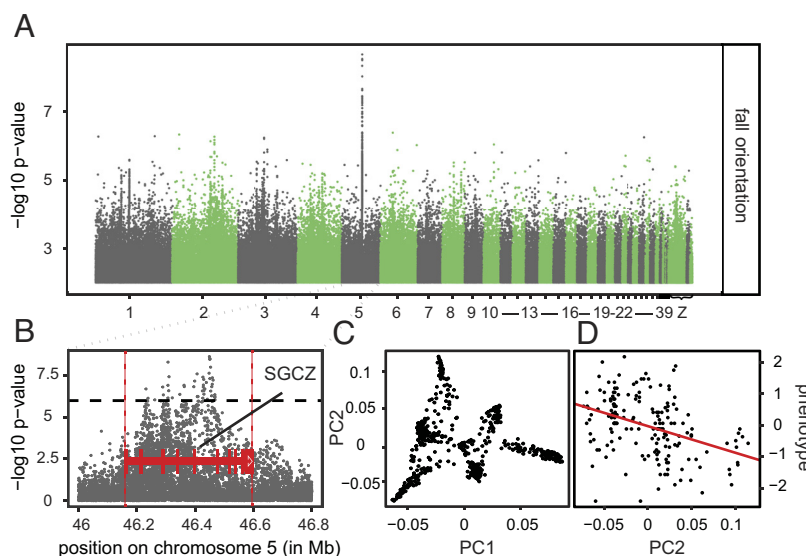


Fig. 5. Results from GWAS for fall orientation with juvenile birds. (A) Manhattan plot shows patterns of association across the genome. (B) Highlights patterns for chromosome 5. The location of *SGCZ* is shown in red (exons indicated by hashes). (C) Results from a PCA using genotypes from SNPs within this gene are also shown. (D) Both PCs are significantly correlated with fall orientation (original phenotypic values normal quantile transformed; results for PC1 shown in *SI Appendix, Fig. S5*).

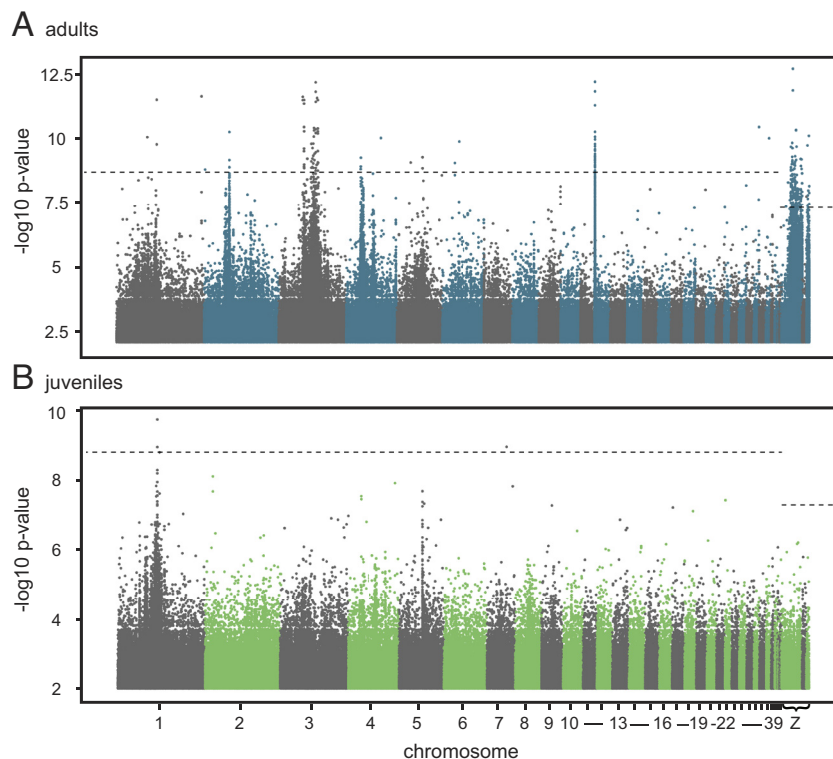


Fig. 6. Results from multitrait GWAS summarizing results from individual-trait GWAS. Associations for (A) adults and (B) juveniles are shown. The dashed line indicates significance ($\alpha < 0.05$ after FDR correction) and was calculated separately for the Z chromosome.

Integration with Transcriptomic Data Solidifies Connection with Cholinergic Receptors. To help narrow down the list of genes connected to migration and speciation in our system and begin gaining insight into the functional consequences genetic variants could have on the final migratory phenotype, we looked for associations between our genomic results and a recent transcriptomic analysis (28; *SI Appendix, Supporting Information Text*). Birds were held in captivity for the former transcriptomic analysis (28) and genes differentially expressed between the seasons (i.e., environment), subspecies (i.e., genotype), and at the intersection between these two levels [i.e., genes with genotype $\times$ environment (G $\times$ E) patterns of differential expression] were identified in five brain regions. We are particularly interested in overlap with the G $\times$ E genes as they likely underlie differences in migration that help maintain subspecific differences between thrushes. Seven of 497 G $\times$ E genes were among the genes identified in our multitrait GWAS analysis (*Dataset S2*; *CNTN5*, *CHRNA5*, *CHRNA4*, *ARHGEF28*, *DDX4*, *IQGAP2*, and *TLE1*). This is a larger number than expected by chance (4.9 genes would be expected by chance; there are 19,432 genes in our annotation, 192 of these 19,432 genes were identified in our multi-GWAS analysis and there are 497 G $\times$ E genes [or “draws”; $(192/19432) \times 497 = 4.9$ genes]). *CHRNA5* and *CHRNA4* are of particular interest as they are also located in an area of elevated genomic differentiation on chromosome 12 (Fig. 7A). *CHRNA5* was overexpressed in the hypothalamus during the migratory period in the inland subspecies and *CHRNA4* was overexpressed in Cluster N during the nonmigratory period in the coastal subspecies (Fig. 7C).

Discussion

Seasonal migration is a taxonomically widespread, complex behavior that allows species to maximize their fitness in seasonal environments, demonstrates considerable evolutionary flexibility, and contributes to speciation (5, 43). We integrated advances from

movement ecology and genomics to provide one of the most complete genetic characterizations of this behavior to date. Overlap with estimates of genomic differentiation and results from previous transcriptomic analysis allowed us to gain additional insight into the molecular basis of speciation and of migration more generally. This kind of interdisciplinary work will be important for future songbird conservation.

Most of the migratory traits we measured showed moderate to high levels of heritability and genetic correlations across them were strong and in line with expectations based on known differences

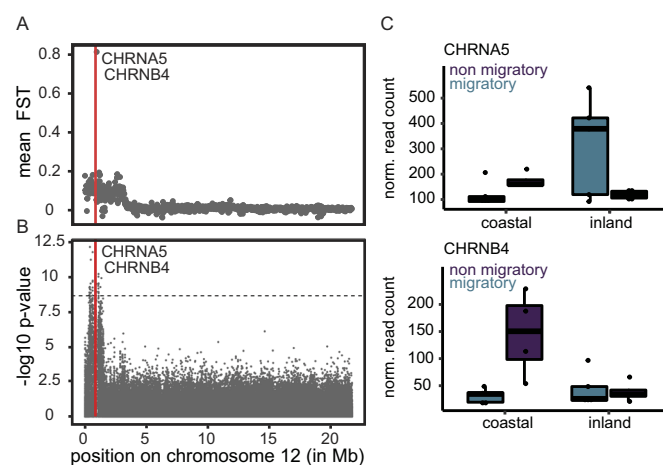


Fig. 7. Connecting GWAS results with estimates of genomic differentiation and previous transcriptomic analysis. (A) Estimates of F_{ST} and (B) associations from multitrait GWAS along chromosome 12 (GWAS estimates also shown in Fig. 6A). Two genes (*CHRNA5* and *CHRNA4*) are in an area of elevated F_{ST} and association at the start of this chromosome. (C) Results from transcriptomic analysis showing these two genes are differentially expressed between the subspecies and seasons. *CHRNA5* was differentially expressed between the subspecies but only during the migratory period. *CHRNA4* was differential expressed between the subspecies but only during the nonmigratory period.

between subspecies. Seasonal migration can evolve quickly in both the lab and natural settings (44–47). Our results suggest that high levels of heritability and genetic correlations could facilitate these rapid responses. There is considerable interest in how migrants will respond to climate change, with some concern that long-distance migrants like the Swainson's thrush may lack the ability to respond to this threat [e.g., shorten their migratory routes and advance arrival times on breeding grounds (48–50)]. Our results suggest that the Swainson's thrush might possess the genetic variation required for these changes and genetic correlations across migratory traits could reinforce their response to selection. Previous authors estimated heritability for migratory traits using tools from quantitative genetics (e.g., correlations between siblings) and came to similar conclusions (12). They focused on a limited set of species (mostly from Europe) and traits (mostly for fall migration and quantified using proxies). We built on this foundational work using a species from North America, a different set of traits, and approach that can be readily applied to additional systems.

Estimates of heritability were not high for all of the traits measured; of particular interest, heritability was lower for all timing traits, and we had less predictive power for these traits. This finding suggests that timing may be more flexible (i.e., environmentally mediated) than other migratory traits and could be related to the fact that while timing depends in large part on changes in photoperiod, it can also be modified by additional, more variable features of the environment [e.g., weather conditions on route, temperature, and precipitation (51, 52)]. This may not be the case for other migratory traits (although more direct data on exactly which variables affect each trait and their contributions are needed). In addition, increased flexibility in timing may be favored by selection as it could allow birds to adjust migratory departures to maximize their individual energy stores, flying conditions, and migratory pace (53, 54). This flexibility could be particularly relevant for adults in the spring, when birds that reach their breeding sites efficiently, at the right time (related to resource availability) and in the best body condition possible, will likely experience an increase in their fitness. It will be of great interest to conduct comparable work with epigenetic data, testing whether epigenetic markers (which likely mediate responses to environmental cues) explain a greater proportion of the variation in migratory timing than the genetic markers employed here. Note, lower estimates of heritability for timing do not necessarily mean that migration as a whole cannot respond to selection as the estimates we documented are still considerable and genetic correlations across traits could still facilitate changes.

Beyond migratory timing, the strong genetic correlations we documented across traits suggest they are controlled by the same (or tightly linked) set of genes (i.e., that genetically we are dealing with multiple phenotypic aspects of one trait). We found some indication that SVs could be helping link genes underlying migration, as genomic regions associated with most traits spanned large regions (e.g., average LD block >40 kb) and the elevated region of association for wing length coincided with high densities of SVs [although most avian macrochromosomes show elevations in recombination rates toward telomeres and thus some of the pattern documented on chromosome 1 (Fig. 4B) likely also derives from this pattern; (55)]. SVs are thought to shelter coadapted alleles that underlie complex traits like migration (56–58) and SVs like inversions have been connected with migratory traits in other species [e.g., willow warbler (15, 59) and common quail (39)]. Unlike the previously published cases, variation in the migratory behavior of thrushes does not appear to be connected to one or two large SVs but many small SVs

that together reduce recombination and facilitate coadaptation. Note, inversions in both the willow warbler and common quail occur on chromosome 1. This is not the same molecule designated as chromosome 1 in the Swainson's thrush genome (chromosome 1 in the Swainson's thrush corresponds with chromosome 2 in the other two species).

Other genes identified in our study may serve as master regulators, controlling multiple migratory traits, the expression of multiple genes, and helping explain genetic correlations. For example, genes encoding nicotinic acetylcholine receptors (nAChRs) emerged as strong candidates in our study. Not only was a GO category connected to genes that encode subunits of nAChRs enriched in our list of candidates, but two of these genes (*CHRNA5* and *CHRNA4*) were also located in an area of elevated genomic differentiation between the subspecies and were differentially expressed between the subspecies and seasons in a previous transcriptomic study (28). The connection with differential expression suggests the genetic variants we identified here act as cis-regulatory changes influencing genes that encode nAChRs. nAChRs form ligand-gated ion channels on the surface of postsynaptic cells and are activated by the neurotransmitter acetylcholine. Ion fluxes at these receptors change the membrane potential of postsynaptic cells, mediating the transfer of information across the synapse. Acetylcholine plays an essential role in the modulation of complex behaviors, especially those that respond adaptively to environmental and metabolic conditions [vs. ongoing stimuli that do not require immediate action (60)]. This kind of adaptive response is undoubtedly important for migratory traits that respond readily to environmental cues. Cholinergic receptors are known to affect sleep (61), food intake (62), and spatial cognitive processes that are important for navigation (63–66). Each one of these behaviors is essential for migration; songbirds become nocturnal and hyperphagic during migration and likely acquire navigational cues on migration that facilitate subsequent trips. Future work using pharmacological manipulations could probe the connection between nAChRs and migration in thrushes further.

SGCZ may also serve as a master regulator that controls multiple migratory traits. This gene occurs on chromosome 5 and SNPs within it showed strong associations with fall orientation in juveniles. The same region showed an association with several adult traits (e.g., spring long, 30 and spring distance) and was in the region connected to orientation in a previous GWAS with thrushes (27). We were able to narrow this region down from 30 Mb to a single gene in the present study. *SGCZ* is part of the sarcoglycan complex, a group of six N-glycosylated transmembrane proteins that work with other members of the dystrophin–glycoprotein complex (DGC) to link the actin cytoskeleton to the extracellular matrix. This complex is critical for maintaining the structural integrity of muscle fibers and the stability of neuromuscular synapses (67). Neuromuscular synapses modulate the connection between motor nerves and muscle (skeletal/smooth/cardiac). Muscle integrity and neuromuscular control are integral for diverse physiological processes in the body and might be especially important during energetically demanding tasks like long-distance endurance flights birds perform on migration. *SGCZ* has also been associated with additional processes important for migration, including muscle cell development (68), regulation of neural connections and memory-like processes (69), abdominal fat deposition (70), and differences in sleep and wake cycles (71). The functional effects of SNPs in and around *SGCZ* are currently unknown, as none of the SNPs were protein coding and this gene was not differentially expressed between the seasons or subspecies in our previous transcriptomic work. This does not preclude the potential for the SNPs we identified here to affect gene regulation;

for example, differential expression may be limited to a tissue we did not assay in our previous work (e.g., muscle) and it is possible these SNPs generate alternate isoforms (vs. affect overall levels of expression). Short-reads from our previous transcriptomic analysis are not sufficient to reconstruct these isoforms but future work using long-reads could evaluate this suggestion.

Differences in seasonal migration likely help maintain reproductive isolation between thrushes, with hybrids taking intermediate and ecologically inferior routes to parental forms on migration (7, 22). Our results support this hypothesis, with genes associated with migration occurring in areas of elevated genomic differentiation. There are several hypotheses behind how variation in genomic differentiation arises (18, 19, 72). In the case of Swainson's thrushes and these migratory genes, we believe it is a combination of divergent selection in allopatry and gene flow upon secondary contact. Specifically, coastal and inland birds were likely isolated in different refugia during glacial advances of the Pleistocene (73). Selective pressure for migration would have been low during this time but increased as the glaciers receded, and subspecies started expanding their ranges northward. Contemporary migration routes match these colonization routes. Several features of these routes differ [e.g., in the distance, timing, and location of resources (20, 43, 74)] and thus each subspecies likely fixed alternate alleles as they increased their propensity to migrate along them. Upon secondary contact, genomic regions around these genes likely remained distinct and help maintain reproductive isolation. Other genomic regions that did not harbor loci important for adaptation were homogenized by gene flow.

As a final note, we would like to connect our findings to the conservation of migratory songbirds. The future of many songbird species will rely on their ability to modify their migratory behavior (48, 49, 75). This may be especially true in the western North American flyway where Swainson's thrushes are found, as this flyway has relatively narrow corridors shaped by extreme topography, complex weather, and habitats (76). Accordingly, it is critical to incorporate evolutionary processes into management programs for migratory songbirds, addressing the full lifecycle conservation needs of seasonal migrants. We have laid important groundwork for this integration here, combining flyway-wide tracking of birds from both sexes, multiple age cohorts, and across seasons with genomic characterizations of migration to understand how genetic mechanisms facilitate individual-level changes in migration phenology and speciation at scales that affect entire migration systems. Future work integrating our results into demo-genetic models (77) that are projected onto future climate change scenarios could help predict coming population trends and even evaluate different management plans. We hope this kind of interdisciplinary approach will ultimately help minimize biodiversity loss with climate change.

Materials and Methods

All sampling was performed in accordance with relevant guidelines and regulations, including permits obtained from Environment and Climate Change Canada (10921 and 10921 A), the State of Alaska - Department of Fish and Game (20-1134; 21-117; 22-083), the Washington Department of Fish and Wildlife (20-104; 21-076; 22-061), United States Department of Interior (24199), United States Fish and Wildlife Service (MB65923D), and the United States Department of Agriculture (USDA - 137701).

Phenotypic Data Collection and Analysis. We used mist nets and audio recordings of Swainson's thrush song to capture birds and fit tracking devices (archival tags for adults and radio transmitters for juveniles) using leg-loop harnesses (78) made from braided nylon cord. We took morphometric measurements for all birds in this study, including measurements of wing length and pointedness (Kipp's distance, the distance between the longest primary to first

secondary feather; 30) as they have previously been identified as an adaptation to long-distance flight (30, 79). We also took blood samples for all birds from the brachial vein (~25 μ L) for later genomic analyses.

Adult tracking—archival tags. We fitted archival tags to adult male birds over several breeding seasons (June 2010 to 2013, 2019 to 2021). Most work was conducted at the center of the hybrid zone ($n = 294$), in Hope (49.385, -121.316) and Pemberton (50.264, -122.867; British Columbia, Canada), Hyder (55.950, -130.039; Alaska, USA), and Cle Elum (47.323, -121.099, Washington, USA). A small subset of parental forms were also included ($n = 39$); they were tracked in the first year of fieldwork from Porpoise Bay (49.506, -123.747), Pacific Spirit Park (49.277, -123.23), Kamloops (50.874, -120.689), and Kelowna (49.871, -120.941; British Columbia, Canada). We used two kinds of archival tags: light-level geolocators (LLGs, Intigeo-P65B1-11-NOT, Migrate Technology) and GPS tags (PinPoint-10-SOB tags, Lotek). LLGs record daily light intensities that are used to infer latitude and longitude and thereby document the bird's location over an entire year. GPS tags do not have large enough batteries to record daily data; instead, we programmed these devices to take GPS location coordinates every 6 d during the migratory periods (September–November and March–June) and every 30 d during the rest of the year. Parental forms ($n = 39$) and a subset of hybrids ($n = 100$) were tracked in previous studies (20, 22); the remaining hybrids ($n = 194$) were specifically tracked for this study and data from all birds ($n = 333$) were analyzed in the present study.

Adult tracking—light-level geolocator and GPS tag analysis. We analyzed data from 56 birds fitted with light-level geolocators (64 were recaptured; we were unable to download data from one and seven birds were no longer carrying their devices). We analyzed light data following methods described in Delmore et al. (80); for details see *SI Appendix, Supporting Information Text*. For GPS tags, we analyzed data from 16 birds (18 birds were recaptured but two tags only had data from a short time on the breeding grounds). We processed GPS data with the PinPoint Host (version 2.15.5.0) from Lotek and exported latitude, longitude, and date of the GPS fixes for each individual. GPS tags only record data at predetermined dates. Accordingly, there is some uncertainty in timing estimates from these tags. We ran downstream analyses with and without GPS tags and obtained similar results. We chose to include them in final analyses.

Adult tracking—migratory phenotypes. We considered four orientation traits for adults in this study: outward and return orientation at the breeding grounds (fall and spring orientation, respectively), the longitude of the wintering grounds (hereafter= "winter longitude"; first location where the bird stayed over 30 d after fall migration began), and the longitude at which birds passed 30° latitude (hereafter= "spring long. 30°") on spring migration. In order to estimate orientation at the breeding grounds, we generated a circle (300 km diameter; comparable to the orientation estimates of juvenile birds see below) around each release site and extracted the bearing from release site to the point where the birds crossed this circle. For distance variables, we estimated distance along the migratory path for fall and spring separately (e.g., fall distance = breeding grounds to wintering grounds). For timing variables, we used departure dates for fall (last day at the breeding grounds before the bird started migrating south) and spring (last day at the wintering grounds before the bird started migrating north) migration.

Juvenile tracking—radio transmitters. We fitted 328 radio transmitters to juvenile thrushes of both sexes (267 males and 61 females) in the hybrid zone ($n = 277$ in Pemberton [50.22035, -122.885]; 25 in Tatlayoko [51.65333, -124.405] and 25 in Bella Coola [52.36044, -126.743]) over several seasons in August/September (2019 to 2021). Sexes were determined using sequencing data (chromosome Z depth < 0.9 = female) and confirmed sexes using molecular markers (2550F/2718R) for birds with chromosome Z depth 0.85 to 0.99. We used two types of radio transmitters (NTQB2-3-2 and NTQB2-4-2; Lotek); both emit uniquely identifiable signals at 166.380 MHz frequency with burst intervals every 25 and 11 s respectively. These radio transmitters are detected by receiver stations of the Motus Wildlife Tracking System (<https://www.motus.org>). Motus is a collaborative research network of globally distributed receiver stations that use automated radio telemetry to document the movement of individuals fitted with radio transmitters emitting at the correct frequency. We used existing stations and established our own fence of stations across the hybrid zone of the Swainson's thrush from Vancouver (coastal range) to Kamloops (inland range; Fig. 1A and *SI Appendix, Fig. S7A*). This fence was originally comprised of 14 stations positioned ~20 km apart in order to ensure no birds could pass by without being detected. Birds were released north of the fence (Fig. 1B).

Juvenile tracking—radio transmitter analysis. We analyzed data from 261 birds fitted with radio tags after filtering (Dataset S3). In brief, we applied relaxed filtering to downloaded data (81) from the Motus website using the package *motus* in R and suggestions in the Motus R book (82) (<https://motuswts.github.io/motus/>). We excluded runs with run length 2 or less and any runs less than length 4 from hourBins which have more than 500 runs and where at least 95% of those runs have run length of 2 [filterByActivity(sql.motus, minLen = 2, maxLen = 4, maxRuns = 500, ratio = 0.95, return = "all," view = "alltagsGPS")]. We subsequently removed bad detections that fell outside of these parameters. In addition, each detection was confirmed manually (see *SI Appendix, Supporting Information Text* for details).

Juvenile tracking—migratory phenotypes. We considered two orientation traits for juveniles in this study: outward orientation from the breeding grounds (fall orientation) and the longitude at which the birds crossed 35 degrees latitude (hereafter: "fall long. 35") to evaluate variation in migratory tracks further south on fall migration. For estimating fall orientation, we generated a circle (300 km diameter) around the release site to determine initial orientation of the birds, using the bearing between the release site and the location within the 300 km diameter the tag was detected. We chose a 300 km diameter to include detections from our fence of receiver stations and used the *geosphere* package in R (83) to calculate bearings. For fall long. 35, we connected detection locations by straight lines in R using point-to-line function (https://rpubs.com/walkerke/points_to_line) and then extracted the longitude at which the birds crossed 35 degrees latitude on fall migration. Concerning fall timing for juveniles, we used the day that the birds passed our fence of receiver stations in British Columbia on their way south (hereafter: "fall timing") for this variable. We excluded data from spring migration for juveniles because detections were too low ($n = 16$), likely reflecting high mortality in particular for juvenile birds during their first migration (e.g., 56% survival for adults compared to 25% survival of juveniles; 84). Note, it is unlikely we are capturing local movements with these data as most birds were not local to our capture area; they were likely migrating south from breeding grounds further north by this time.

Genomic Data Collection.

Whole genome resequencing and genotype calling for tagged birds. DNA was extracted from blood for all individuals we had phenotypic data for ($n = 607$) using a standard phenol-chloroform protocol. Libraries were prepared following a modified protocol based on Picelli et al. (85) and Schumer et al. (86; *SI Appendix, Supporting Information Text*). Libraries were sequenced on the Illumina NovaSeq 6000 to 1 to 13× of coverage (median 3.55×).

The resulting sequence data were trimmed with trim galore (--clip_R1 15 --clip_R2 15 --three_prime_clip_R1 5 --three_prime_clip_R2 5; https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). We used two reference genomes to map sequences using the bwa mem algorithm (87), one for each subspecies of Swainson's thrush (*SI Appendix, Supporting Information Text*). We converted the resulting sam files to bam format with samtools (88), cleaned, sorted, added read groups, and marked duplicates using picardtools (<https://broadinstitute.github.io/picard/>). Samtools was used to remove reads that mapped at low quality and identify reads that mapped uniquely to both reference genomes (q30). Ngsutilj (<https://github.com/compgen-io/ngsutilj>) was then used to find the intersection of these reads and all subsequent analyses were limited to the former reads and their mapping position on the inland reference genome. We chose the inland reference genome because it is more contiguous and complete, although both references are of high quality (e.g., 96.8% vs. 94.2% of avian orthologs present in the inland vs. coastal reference genome following a BUSCO analysis).

We used STITCH (Sequencing To Imputation Through Constructing Haplotypes; 31) to impute missing genotypes for individuals sequenced to low coverage. STITCH does not require a reference panel of high-quality haplotypes for imputation; it uses low coverage sequencing data from a set of individuals to model ancestral haplotypes in genomic regions. A hidden Markov model (HMM) is used to estimate haplotype probabilities for each individual. The HMM is updated with 40 iterations of expectation maximization (EM) before a final set of genotypes is assigned to each individual. We used bcftools to provide STITCH with an initial set of SNPs (89) (--min-BQ 20, --min-MQ 20, %QUAL>500, --skip-variants indels). We ran STITCH in blocks of 1 Mb (with buffer of 100 kb), initiating the program in the pseudoHaploid model with values of 80 and 500 for K (ancestral haplotypes)

and nGen (number of generations since population was founded). We switched to the more accurate diploid model after 36 EM for computational efficiency. Varying these parameters (K, nGen, and model EM) had qualitatively little effect on the resulting genotypes. We assessed the accuracy of imputation using a cross-validation approach (*SI Appendix, Supporting Information Text* and Fig. S6).

Genomic Data Analyses.

Heritability, genetic correlations, and GWAS. Estimates of heritability, genetic correlations, and associations between individual SNPs and each migratory trait were obtained using GEMMA (genome-wide efficient mixed-model association; version 0.98.1; (34)). All phenotypic measurements were quantile-normalized to allow comparison across traits. GEMMA accounts for population by incorporating a relatedness matrix for the included individuals. To avoid artifacts generated by linkage disequilibrium (LD) across the genome, we used an LD-pruned dataset to calculate the relatedness between individuals included in this analysis. We performed LD-pruning using PLINK (version 1.9b5; --geno 0.10 --indep-pairwise 200 20 0.2 --maf 0.05) and ran BSLMMs for each migratory trait. BSLMMs are a Bayesian approach to GWAS that fit all variants to a phenotype simultaneously (90). We used BSLMMs in GEMMA to estimate PVE (the proportion of variation explained by additive genetic effects, or chip heritability), GEBVs (genomic estimated breeding values, or polygenic scores), and genetic correlations (90). For additional information on BSLMMs see *SI Appendix, Supporting Information Text*.

We used LMMs in GEMMA to identify individual SNPs associated with each trait (34). We used default parameters and report *P*-values from Wald likelihood ratio tests. We included sex as a covariate for models run on morphological traits (wing length and wing shape). For additional information on LMMs, see *SI Appendix, Supporting Information Text*. Note, traits were not always quantified the same way in adults and juveniles (e.g., orientation further along the migratory path in adults was quantified as winter longitude, and in juveniles, this trait was quantified as fall longitude at 35 latitude and thus we may not expect patterns across age classes to be the same. We also quantified more traits in adults and the adults are a distinct sample compared to juveniles; only birds who survived migration are included and they will have learned navigational cues that could have affected the phenotypes we measured.

Results from genetic correlations and individual trait GWAS suggested many of the traits we mapped had a similar genetic basis (were controlled by the same variants). A joint analysis of correlated traits in a multitrait GWAS can increase statistical power for GWAS (36). Accordingly, we used this approach to summarize results across traits. We used the package *metaUSAT* (version: v1.17; 36) for this analysis, which uses Z-scores (=effect size/SE) from summary statistics from multiple single trait GWAS. We ran these analyses separately for adults and juveniles including all traits where we could predict phenotypic values in GEBVs (see above; the adult model included winter longitude, spring long. 30, fall and spring distance, wing length, and wing shape; the juvenile model included fall bearing, fall long. 35, wing length and wing shape).

We identified SNPs significantly associated (*P*-value < 0.05) with migration in both datasets after adjusting *P*-values for multiple testing using Holm-Bonferroni adjustment. We estimated LD (r^2) between these significant SNPs and the remaining SNPs on the same chromosome using vcftools (--geno-r2-positions). We considered SNPs with r^2 values > 0.5 to be in LD with causal SNPs and called these areas "linkage blocks" (see *SI Appendix, Supporting Information Text* for details). Genes within these LD blocks are considered candidates for migration. We ran GO analyses on these genes using the "goseq" package in R using ontologies for the Swainson's thrush (i.e., annotation for the inland subspecies genome from Feng et al. 2020; see *SI Appendix, Supporting Information Text* for details). We used SnpEff (version 5.1d; 91) to functionally annotate SNPs in a subset of genes; those in SGCZ on chromosome 5 (46000000–46800000) and those in genes on chromosome 1 (28792449–96508609).

Structural variants and recombination. Results from LMMs suggested that many of the SNPs we identified are in SVs. Future work using long-reads will be used to evaluate this suggestion more directly. In the present study, we focused on a region on chromosome 1 that showed many plateaus of strong association. We used two separate approaches to identify SVs on chromosome 1, one included both reference genomes and raw PacBio reads and another used short-read data (for details see *SI Appendix, Supporting Information Text*).

Methods for obtaining and analyzing high-coverage data from parental forms are described by Louder et al. (28). Briefly, capture, DNA extraction, trimming and

alignment were conducted as described above for low coverage data from tagged birds. The main difference was that whole genome resequencing libraries were prepared using Nextera (Illumina) DNA Flex Library Prep kits and sequenced on a NovaSeq 6000 (2×150 bp) to an average read depth of $18 \times$ (range 14 to $39 \times$).

SVs can reduce recombination in the genome. We used the high coverage, short-read data from parental populations described above to estimate recombination along chromosome 1 using LDhat (92). LDhat relies on population genetic data to estimate recombination rates, using a Bayesian reversible-jump Markov chain Monte Carlo (rjMCMC) to fit a model of recombination rate variation. We took aligned bam files and ran them through GATK's Best Practices to call SNPs, using the HaplotypeCaller algorithm in gvcf mode, Genomics DBImport and GenotypeGVCFs (93). The resulting SNPs were converted to the format required by LDhat using vcftools (-ldhat-geno). We used "interval" to generate a likelihood file, "rhomap" to estimate recombination rates (-its 1000000 -snp 2000 -bpen 5.0 -burn 2000 -exact). We summarized these estimates into windows of 100 kb (Fig. 4C).

Connection to reproductive isolation. We used the high-coverage, short-read data from parental populations described under "Structural variants and recombination" to estimate genomic differentiation between the subspecies while accounting for within-population variation (ΔF_{ST} ; see *SI Appendix, Supporting Information Text* for details). We used vcftools to estimate ΔF_{ST} in 25 kb windows (*SI Appendix, Fig. S8*) and a permutation test to compare these estimates at windows overlapping SNPs associated with migratory traits in our multi-GWAS ($n = 76$ windows on autosomes) to the rest of the genome, randomly selecting 76 windows from autosomes 10,000 times and comparing mean ΔF_{ST} in these windows to mean ΔF_{ST} in windows overlapping SNPs associated with migratory traits (*SI Appendix, Fig. S9*). If phenotype-associated regions are under divergent

selection (i.e., regions likely involved in reproductive isolation) they should have higher levels of ΔF_{ST} .

Data, Materials, and Software Availability. The raw sequencing data are available on the SRA under BioProject: [PRJNA979932](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA979932) (94). A github repository with basic bioinformatic pipeline is available here: https://github.com/HannahJusten/swth_wgs_gwas (95). All other data are included in the manuscript and/or supporting information.

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