# GEOGRAPHIC VARIATION IN SONG AND GENETICS IN THE TOWNSEND'S WARBLER (SETOPHAGA TOWNSENDI)

by

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#### Abstract

Bird song is shaped by both genetic and cultural processes, presenting a particularly tractable system for understanding the connection between communication and reproductive barriers that drive diversification. Song dialects-or differences in song structure across geographic space—can act as signposts of cultural differences between populations, which may also indicate genetic differences in the population. The Townsend's Warbler (Setophaga townsendi) is a species with geographic variation in mitochondrial genetic signatures. I investigate if concurrent variation occurs in song and nuclear genetic signatures, by describing the geographic variation of song and nuclear DNA across Townsend's Warbler range. I quantify distinct song types and patterns across Townsend's Warbler populations, along with patterns of genetic variation across their range using genotype-by-sequencing data. If genetic and cultural processes are tightly linked, I expect song to vary between regions of genomic differentiation. I demonstrate that individuals have a repertoire of at least two distinct song typesconsistent with the two-song-type pattern common in Parulid warblers-but find this pattern is less evident in a range-wide comparison. There is extensive variability in Type I song, whereas Type II song is more stereotyped across the range. There is little distinct grouping that coincides between song and the isolation-by-distance pattern in the nuclear DNA. A mismatch between song and genetic patterns suggests that song divergence is not predicted solely by genotype. By characterizing the within-species variation in song and genetic structure, we can expand our understanding of the dynamic interplay between cultural traits and population structuring.

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## Lay Summary

Bird song is shaped by both learning and genetic components, which allows us to examine how communication influences genetic population structure. When birds form dialects—songs that vary between populations—these have the potential to prevent interbreeding between these populations. The Townsend's Warbler (*Setophaga townsendi*) species has previous evidence for geographic structure in genetic variation, which allows us to test for dialects between populations. In this study, I surveyed the geographic structure of both songs and nuclear DNA patterns, to compare how they change across the Townsend's Warbler range. I find evidence that individuals have two song types, likely used in different social contexts. I find that one song type is more stereotyped than the other song type across their range. DNA patterns, but both song and genes suggest there are no barriers to breeding in this species.

### Preface

The research question and study design for this thesis were conceived by myself in collaboration with my supervisor, Dr. Darren Irwin. I conducted all of the field work, collecting song recordings and blood samples. Additional song recordings came from Macauley Library at the Cornell Lab of Ornithology and the Xeno-canto Foundation and Naturalis Biodiversity Center (Xeno-canto.org). A full list of recording credits can be found in the appendix. Additional blood samples came from the Burke Museum of Natural History in Seattle, Washington. A full list of the samples utilized from the Burke Museum can be found in the appendix. The genotype-by-sequencing library preparation was carried out in collaboration with Silu Wang, Ph.D candidate in the University of British Columbia Zoology Department. I conducted all analyses and writing of this thesis, with assistance and feedback from Dr. Irwin.

Animal Care approval (Canadian Council on Animal Care/ National Institutional Animal User Training Certificate number 8425-17F) was obtained for this work. Bird banding and scientific permits were obtained from Environment and Climate Change Canada (10746 M).

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## **List of Abbreviations**

DFA: Discriminant Function Analysis DFT: Discrete Fourier transformation GBS: Genotype-by-sequencing IBD: Isolation-by-Distance PCA: Principal Component Analysis SNP: Single Nucleotide Polymorphism

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## **Chapter 1: Introduction**

Mating signals can diverge, thus altering how species recognize one another (West-Eberhard 1983). Signal divergence can result in premating isolation, which has the potential to lead to speciation (Grant and Grant 1997). Bird song is a widely studied characteristic that has the potential to act as a premating barrier. In many avian orders, songs and calls are genetically hardwired, and song divergence has been used to justify splitting species (Traylor 1979; Kroodsma 1989;Isler *et al.* 1998; Leger and Mountjoy 2003).However, in several groups—certain hummingbirds, parrots, and oscine song birds—song is a learned characteristic, meaning a bird must learn the sounds to produce from a tutor and practice to match the sound it has learned (Kroodsma 2004; Lemon 1975). Because song is learned from other individuals in the population, there can be mistakes and modifications over time and space, which can be thought of as cultural evolution (Lynch 1996).

#### 1.1 Song as a Reproductive Barrier

Variation in song is influenced by both genetic and cultural forces. Genetic factors can constrain song variation by influencing morphology or learning capacity (Podos *et al.* 2004). On a local scale, bird populations seem to be able to modify and alter song (Kroodsma 2004). This alteration is thought to arise through imprecise song copying and rearrangement of song components (Marler 1960; Thorpe 1961). When an altered

song spreads through a population, this can be considered a cultural transmission (Lynch and Baker 1994). These cultural transmissions can have differential success in spreading between individuals from one generation to the next, resulting in certain song forms being more common than others (Lynch 1996). Song dialects form when there are geographic discontinuities in song structure across populations (Catchpole and Slater 2008). These song dialects act as signposts of cultural differences between populations, which may also indicate genetic differences in the population.

In some cases, these cultural differences in song can confer a fitness advantage for individuals in a population (Kroodsma 2004). If a song dialect acts as a signal for local adaptation, then a bird might be more likely to choose a mate within its own dialect group. In this case, song has the potential to act as a barrier to gene flow, thus genetic structure should reflect song structure (Marler and Tamura 1962; Nottebohm 1969). This increase in divergence may indicate that populations are in the process of speciation. Divergence in songs has been found to be linked with speciation in some species, such as Ficedula flycatchers, and antbirds (Myrmeciza spp.) (Haavie et al. 2004; Seddon and Tobias 2007). Additionally, population structure has been linked to geographic variation in song in several species of sparrows (MacDougall-Shackleton and MacDougall-Shackleton 2001; Danner et al. al. 2011; Lipshutz et al. 2017). In White-crowned Sparrows (Zonotrichia leucophrys), individuals respond more strongly to conspecific song, and song phenotype substantiates subspecies identity based on genetic analyses (MacDougall-Shackleton and MacDougall-Shackleton 2001; Lipshutz et al. 2017). A similar pattern has been observed in Rufous-collared Sparrows

(*Zonotrichia capensis*), where females respond more to local males than distant males and this pattern matches neutral genetic markers (Danner *et al.* 2011). In these cases, song learning appears to have driven divergence into dialects, and this divergence is echoed in genomic differentiation. In other instances, however, song learning enables interspecific sharing of songs.

Song learning can also act to enhance gene flow between populations (Helb *et al.* 1985). Song differences will not restrict gene flow between populations if males are able to modify songs or learn new songs, known as heterospecific copying (Payne 1981; Catchpole and Slater 2008). Heterospecific copying of song, in some cases, appears to be associated with increased hybridization between populations and species (Kenyon *et al.* 2011). For instance, a hybrid could learn the local song and successfully backcross in a population (Grant and Grant 1997). Additionally, there is a body of evidence that suggests that often genetic structure is not based on patterns of song variation (Nottebohm and Selander 1972; Handford and Nottebohm 1976; Fleisher and Rothstein 1988; Payne and Westneat 1988; Lougheed and Hanford 1992). In several cases the genetic structure is weaker than song structure, indicating interbreeding is less restricted than cultural exchange (Soha *et al.* 2004; Kenyon *et al.* 2016; Ortiz-Ramirez *et al.* 2016).

#### 1.2 Patterns of Song Variation

There are four general patterns for geographic song variation documented in the literature (Pitocchelli 2011). The first is that there is little to no variation among individuals. This pattern has been documented commonly in suboscine birds, for which song is innate, such as the Alder Flycatcher (*Empidonax alnorum*) (Lovell and Lein 2013). The second pattern observed is when there is high variability among individuals but no geographic structure. This is the case with several species of warblers, such as Common Yellowthroat (Geothlypis trichas) and MacGillivray's Warbler (Geothlypis tolmiei) (Borror 1967; Pitocchelli 1990, respectively). The third pattern is known as clinal variation, where the song changes gradually across the range of species. This has been documented in Greenish Warblers (*Phylloscopus trochiloides*) (Irwin 2000). The fourth pattern is when there is distinct geographic variation of song populations across a species' range and comes in two forms: dialects and regiolects (Pitochelli 2011). Dialect variation is when populations in close proximity have distinct song forms but have no physical barriers to interbreeding (Mundinger 1982). Regiolects are formed when populations have been separated by physical barriers or large geographic distances, therefore, individuals from different populations do not interbreed and develop distinct song forms (Mundinger 1982; Catchpole and Slater 2008).

#### 1.3 Song Structure in Parulid Warblers

Many species of wood-warblers (Parulidae) have at least two distinct song types, both sung by males, that are thought to serve two separate functions (Byers and Kroodsma 2009). In some species, these two song categories have different structure (e.g. Chestnut-sided Warbler [Setophaga pensylvanica; Byers 1995], Black-throated Green Warbler [S. virens; Spector 1992] and Blackburnian Warbler [S. fusca; Morse 1967]). In others, these two song categories are distinguished by the mode of delivery (Yellow Warbler [Setophaga petechia; Spector 1991], Grace's Warbler [S. graciae; Stacier 1989], Adelaide's Warbler [ S. adelaidae; Staicer 1996]). Thus, within woodwarblers, there are two general groups: "form-encoded"-where songs are distinguished based on song structure alone—and "performance-encoded"—where songs are distinguished into categories based on song structure and behavior (Spector 1992; Byers 1995). Type I songs tend to be sung from the centers of their territories, early in the breeding season. Byers and Kroodsma (2009) suggest that Type I songs are used to attract mates. Further, they posit that Type I songs are typically highly stereotypical across the range of a species, but geographic variation in this song type has been documented in Hermit Warblers (Setophaga occidentalis), Black-throated Gray Warblers (S. nigrens), American Redstarts (S. ruticilla), and Northern Parula (S. americana) (Janes and Ryker 2006; Morrison and Hardy 1983; Lemon et al. 1985; Moldenhauer 1992, respectively). Since type I songs are thought to serve an important function in female choice, there is potential for premating isolation based on dialects in

these species. In contrast, Type II songs tend to be sung on the peripheries of territories and are thought to mainly be used to defend territories against neighboring males. Type II songs have exhibited greater geographic variation than Type I songs in Blue-winged Warblers (*Vermivora cyanoptera*) and Chestnut-sided Warblers (*Setophaga pensylvanica*) (Gill and Murray 1972; Kroodsma 1981).

#### 1.4 Study Goals

Townsend's Warblers possess documented differences between coastal and interior populations in mitochondrial DNA (Krosby and Rowher 2009). This pattern suggests that this species may be a particularly tractable system to explore differences in song and the nuclear genome between these populations, to understand how culture and genetic population structure interact with one another within a species. In this study, I explore how songs vary across the Townsend's Warblers' range to test whether songs differ between coast and inland populations. I seek to identify the number of song types that Townsend's Warblers possess and to determine if there is geographic variation in either Type I or Type II song, or both. I examine whether this species has regiolects and whether there are boundaries to certain song dialects. I assess patterns of genetic variation in nuclear DNA to determine whether nuclear genomic signatures also differ between coast and inland. Finally, I compare song patterns to genetic patterns to determine if song and genes show closely concordant patterns of variation.

# Chapter 2: How do song and genetic structure vary across the Townsend's Warbler (*Setophaga townsendi*) range?

#### 2.1 Introduction

Bird song has the potential to play an important role as a premating barrier during the process of speciation. This is of particular interest in oscine passerines because song has both genetic and cultural components. Genetics can act to constrain the learning capacity or morphological capabilities, such that a species is limited in what sounds it can produce (Catchpole and Slater 2008). Oscine passerines can learn their song such that changes can arise within a species due to cultural and/or genetic mutations in different populations (Lynch 1996). When these cultural changes in song cause discrete differences across populations, these are referred to as dialects (Mundinger 1982). If a bird learns his song before dispersal and has reduced fitness after dispersal—because it is singing the nonlocal song—song dialect boundaries can represent dispersal boundaries (Toews 2017). This will reduce gene flow between populations, and genetic and cultural boundaries will coincide (Marler and Tamura 1962). If birds can disperse and learn the dialect of their new area, then there will be fewer restrictions to gene flow based on song dialects (Payne 1981; Catchpole and Slater 2008).

In Parulid warblers, songs could potentially diverge in several ways. Most Parulid warblers have two song types. Type I song is thought to be used in advertisement to females (Byers and Kroodsma 2008). Type II song is thought to be used in male-male competition and countersinging bouts between males. Song dialects could form in either of these song categories, and both have been demonstrated in other species (Type I dialects: Lemon *et al.* 1985; Janes and Ryker 2006; Type II dialects: Gill and Murray 1972; Kroodsma 1981).

#### 2.1.1 Townsend's Warbler Song

In Townsend's Warbler, only males produce songs (Wright *et al.* 1998). Early descriptions of song in this species noted two song types: one with clear, whistled notes and one with three to six "buzzy" notes (Wright *et al.* 1998). These qualitative field descriptions have classified the clear, whistled song as the Type I song, and it is typically sung from the centre of the male's territory (Wright *et al.* 1998). The buzzier song is classified as the Type II song, and it is typically sung during countersinging bouts (Wright *et al.* 1998). A recent study by Kenyon *et al.* (2017), however, did not reveal such distinct song type patterns. Instead, Townsend's Warblers seem to have 3 loosely defined song types.

Townsend's Warblers hybridize with both Hermit Warblers and Black-throated Green Warblers, where song may play a role in mate choice (Rowher and Wood 1998; Kenyon *et al.* 2017). Townsend's Warblers hybridize with Black-throated Green Warblers in

northeastern British Columbia (Toews *et al.* 2011). In allopatry, Black-throated Green Warblers have the two-song-type pattern characteristic of Parulid warblers, demonstrated with the songs of a population forming two distinct clusters in a principal components analysis (PCA, Morse 1967; Kenyon *et al.* 2017). In contrast, Townsend's Warbler populations in allopatry do not show such a clear pattern, with songs of a population forming three indistinct clusters along a PCA (Kenyon *et al.* 2017). In the hybrid zone, songs appear to be transmitted as discrete units of culture, with little blending between Black-throated Green and Townsend's Warbler songs (Kenyon *et al.* 2017). Additionally, song is only weakly associated with the genetic backgrounds of individuals in the hybrid zone, suggesting that song is not acting as a reproductive barrier between these species.

Townsend's Warblers hybridize with Hermit Warblers in Washington and Oregon (Rowher and Wood 1998; Rowher *et al.* 2001). In regions of hybridization between Hermit and Townsend's Warblers, species identity cannot be distinguished by song alone (Wright *et al.* 1998; Mikkelsen and Wang, pers. comm.).

#### 2.1.2 Evidence of Genetic Population Structure in Townsend's Warbler

The breeding range of Townsend's Warbler extends from Alaska to Oregon (Wright *et al.* 1998, Fig 1). They hybridize with Hermit Warblers where their ranges meet in Washington and Oregon. These two species are distinguished by plumage differences, primarily the extent of black on the crown, throat, and cheek patches, and extent of

yellow on the breast (Rowher and Wood 1998). Some Townsend's warblers that reside in the coastal edge of their range have mitochondrial haplotypes of Hermit Warblers. In contrast, interior populations of Townsend's Warblers do not possess traces of Hermit Warbler mitochondrial heritage (Krosby and Rohwer 2009). One possible explanation for this pattern is a secondary contact event. Under this scenario, Townsend's Warblers were isolated in a glacial refugium in the interior West and Hermit Warblers occupied the habitat along the Pacific coast (Rowher *et al.* 2001). A postglacial expansion of Townsend's Warblers towards the coast caused hybridization and mitochondrial capture of Hermit Warbler haplotypes (Krosby and Rowher 2009). Alternatively, this pattern could be caused by northward introgression of Hermit Warbler mitochondria, though the geographic structure of Hermit Warbler haplotypes suggests this is not the case.

#### 2.1.3 Study Goals

Given the known differences between populations in mitochondrial signatures, in this study I use the Townsend's Warbler as a system to understand the relationship between song variation and genetic variation. By understanding how song and genetics relate in this species, we can understand more deeply how these factors interact in hybrid zones between the Townsend's Warbler and its sister species, the Hermit Warbler and the Black-throated Green Warbler. Further, this study addresses the complex interactions of learning and genetics to create communication signals in birds. I investigate the Townsend's Warbler song to describe Type I and Type II song diversity

and geographic variation. Then, I compare these geographic patterns to genetic structure, to look for evidence of premating barriers in either Type I or Type II song. In order to describe song types, I observe the repertoires of several individual birds. I seek to demonstrate the number of distinct song types of an individual and, more broadly, across the species. Once song types have been identified, I examine if dialect or regiolect structure exists in either Type I or Type II songs. I examine population structure using 1.1 million Single Nucleotide Polymorphisms (SNPs), to determine nuclear DNA variation across Townsend's Warbler range. Further, I test the connection between song dialects and the genetic traits differentiating Townsend's Warbler populations, to determine how closely song matches genetic relationships. If there is a link between genetic differences between coastal and interior populations. Alternatively, if song and genetics are uncoupled, then there may be little to no geographic structuring of song dialects or song structuring will differ from genetic structure.

#### 2.2 Methods

#### 2.2.1 Sampling

I conducted field work at 30 locations across British Columbia in May to July of 2017 (Fig. 1). Song recordings were collected from singing males using a Marantz PMD660 and an Audio-Technica 815a Shotgun microphone. At least five males were recorded singing at each location. The GPS coordinates of each sample were taken at locations of each recording. Typical recordings of focal individuals were eight to ten minutes long and consisted of ten to forty songs. Birds usually did not switch between song types within a single recording period, so most recordings only include one song type. Later in the season, a playback of song recordings was used to encourage birds to sing. The playback consisted of three songs from different regions of the Townsend's Warbler range. I recorded a total number of 180 singing individuals in the 2017 field season. Recordings were supplemented by 39 recordings from Xeno-Canto and 34 recordings from Macauley Library at the Cornell Lab of Ornithology (See Appendix B.1 and B.2 for credits).

After recording, some birds were captured in mist nets, after being attracted using a playback recording. Each individual was banded with an aluminum leg band with a unique number and colored leg bands in a unique combination for future field identification by sight. A 20  $\mu$ L blood sample was extracted from the brachial vein of the individual and stored in lysis buffer. Each bird was aged into either "Second Year" or

"After Second Year" using feather wear on primary coverts and rectrices and median covert pattern (Pyle 1957). They were sexed based on plumage characteristics, primarily by throat colour and colour of streaking on the head (Pyle 1957). Cloacal protuberance and brood patches were scored to assess breeding condition. The GPS coordinates for each sample used for DNA analysis were taken at the location where each bird was captured. Blood samples were taken from 104 birds in the 2017 field season (Fig. 1). These samples were supplemented with 82 samples from the Burke Museum of Natural History and Culture, University of Washington, Seattle (See Appendix B.3 for ID numbers).

### 2.2.2 Recording Analysis

The songs of 253 birds were used in this analysis (Fig. 1). Songs were analyzed using Raven Pro 1.4 and warbleR (Araya-Salas and Smith-Vidaurre 2017). For each bird, songs were characterized into "types" based on visual similarity. Three songs of each unique "type" in a bird's repertoire were randomly selected for measurement. Spectrograms were visualized in Raven Pro 1.4 using Hann spectrogram windows with 512 samples, discrete Fourier transform (DFT) size of 512 samples, hop size of 5.6 ms, sampling frequency of 44.1 kHz, and a time resolution of 11.6 ms. Twenty variables were measured manually from each song (Fig. 2): total number of notes, number of unique notes, duration, minimum and maximum frequencies, bandwidth, and aggregate entropy of the whole song and part 1 and 2 of the song. Aggregate entropy represents

the amount of disorder in a sound signal. Higher entropy values indicate greater fluctuation in frequency, whereas a pure tone will have an entropy score of zero. To verify that aggregate entropy was not affected by noise in the recording, I calculated the signal-to-noise ratios (SNR) with warbleR (Araya-Salas and Smith-Vidaurre 2017) for each song and plotted the residuals (Fig. 3). The SNR is calculated by comparing the mean amplitude of the song over the mean amplitude of the background noise, which is measured before and after the song. A smaller SNR value indicates there is greater background noise. If aggregate entropy were affected by the noise in a recording, then aggregate entropy would be negatively associated with the SNR. Mean and standard deviation of each variable was calculated for the three songs of each type.

To take a deeper qualitative look at song types and dialect structures, songs were visualized in a spectrogram and sorted based on visual similarity. Songs recorded for each individual were classified into song "types" based on previous classifications (Wright *et al.* 1998) and field observations. In the field, I heard individual birds shifting between two song types. The clear whistled song was broadcasted loudly from branches as the male moved about his territory. The "buzzier" song was often sung more quietly than the "whistley clear" song and used during countersinging bouts between two males—which I observed approximately ten times. Anecdotally, males were more responsive and came closer to the net in response to the buzzier song during playbacks than the clear song. Based on these observations, I classified the clear song as the Type I song (i.e., used more in female attraction), and the buzzy song as the Type II song (i.e., used more in territorial defense). There was a tremendous diversity of song structure in the Type I songs across the range. Occasionally songs

lacked a second part to the song or songs would have similar beginnings but different endings. Because of this variation in structure, Type I songs were first classified into categories based on visual similarity of part 1 of the song [A-F], to reflect similarity in this part of the structure among songs with different endings. Then, they were placed into subcategories based on visual similarity of the whole song [1, 2 or 3].

In order to examine individual repertoires and differences between Type I and Type II songs, three birds were recorded singing both Type I and Type II songs. For these birds, every song in the recording was measured with the same 20 variables and visualized in a spectrogram.

#### 2.2.3 Data Analysis for Song

I ran principal component analyses (PCA) using the package pcaMethods (Stacklies *et al.* 2007) in R v3.4.3 (R Development Team 2017) to examine song patterns. The PCA was scaled by the unit variance of the measurements to standardize variance between variables. For songs that did not have all components, I used singular value decomposition (SVD) —which estimates missing values by constructing a linear combination of the four most significant eigenvalues (approximately 20% of the eigenvalues)—to impute missing values (Troyanskaya *et al.* 2001). The purpose of PCA is to find axes that explain the most covariance within the data and plot points based on those axes, rather than discriminating between groups. To identify how many song types exist in this species, I ran one PCA on all songs and plotted the mean PC scores

of the three songs of each type. The scores of 75 individuals' mean Type I songs and 75 individuals' mean Type II songs were used for this analysis, in order to avoid skewing variation because of uneven sample sizes. Songs were designated into types based on aforementioned field observations. Type-I-singing individuals used in this analysis were randomly selected from the pool of individuals recorded singing Type I songs. I performed a two-sample t-test on PC1and PC2 to determine if the mean scores for Type I and Type II differ significantly.

To compare Type I and Type II songs within an individual, I ran a PCA on all songs of that individual and plotted PC scores 1 and 2. Then, I visualized every song for a given individual using the R package warbleR (Araya-Salas and Smith-Vidaurre 2017) to identify the number of song types qualitatively, as well.

To further determine whether songs differed based on song type identity, I conducted a discriminant function analysis (DFA) using MASS in R v3.4.3 (R Development Team 2017). A DFA creates a linear combination of variables to maximize differences between groups based on a predefined grouping variable, in this case, my visual classification into Type I or Type II song. To compare between Type I and Type II songs, I averaged the values of each of the three songs for a bird for only variables from part 1 and part 2 of the song (i.e. Maximum frequency of Part 1, Length of Part 2, etc). Using all 20 variables was not possible because they were too highly correlated, so I removed variables that looked at the whole song (i.e. Maximum frequency of song) because that information is encapsulated within the variables from Parts 1 and 2 of the song. Then I ran a DFA on mean values for each bird, omitting individuals with missing variables. The scores of 68 individuals' mean Type I songs and 72 individuals' mean

Type II songs were used for this analysis. I ran a two-sample t-test on the linear discriminant scores to determine if the groups are significantly different. I tested the accuracy of group assignment by calculating the resubstitution error and using a jackknife method. Resubstitution error calculates how well songs are classified when all samples are used to develop the DFA. A jackknife method systematically removes each song from the analysis and calculates a prediction. Then, I calculated the proportion of assignments that were correct across all analyses. The jackknife method is a more robust measurement than the resubstitution error method.

Mean and coefficient of variation of all variables were calculated for each Type I and Type II songs. Coefficient of variation was used instead of standard deviation because it is independent of the unit of measurement, so it enables comparison between variables with differing units. All variables except Maximum frequency of song and Length of Part 1 were not-normally distributed. I performed a Mann-Whitney U test to compare the mean of each variable between Type I and Type II song.

To examine if there are dialect groups across the Townsend's Warbler range, I ran a PCA on all Type I songs and plotted mean PC scores of the three songs of each type by location and region where the bird song was collected. Regions were chosen based on geographic proximity of the locations where birds were sampled and are as follows: Oregon, Washington, Montana, Idaho, Vancouver Coast and Mountains region, Vancouver Gulf Islands, Okanagan, Kootenay, Cariboo-Chilcotin, Northern BC, Alaska. The scores of 199 individuals were used for this analysis. I ran a separate PCA using the same method on all Type II songs to determine if there is dialect structure in this song type. There were 75 individuals used in this analysis. An ANOVA between regions

was run on PC1 loadings for Type I and Type I PCAs conducted, to identify if regions are distinct. A Tukey-Kramer test was used to determine which regions were significantly different from each other along PC1 and PC2.

In order to examine how songs differ between coastal and interior populations, I used the package sp (Pebesma and Bivand 2005) in R v3.4.3 (DevelopmentTeam 2017). I created a line along the outer edge of the coast line from Washington to Alaska (Fig 4). Then, I calculated the shortest distance from each point to the coastline. I plotted song variables against the distance to the coast. To minimize differences across latitudes, I plotted song scores against distance from the coast along a Southern and Northern transect as well. For the Northern transect, latitude was limited to between 54° and 57° North. For the Southern transect, latitude was limited to between 48° and 50° North. I examined the song divergence between coastal and interior populations because of the apparent genetic differentiation between the coast and interior suggested by the mitochondrial DNA (Krosby and Rowher 2009). A bivariate analysis was run to test the association between song and geography.

#### 2.2.4 Genotype-By-Sequencing Library Preparation

Genetic backgrounds for 186 individuals across the Townsend's Warbler range were estimated using a modified genotype-by-sequencing (GBS) approach (Elshire *et al.* 2011; Alcaide *et al.* 2014). DNA was extracted from blood samples using a standard phenol-chloroform protocol.
Library preparation was conducted by combining 2.4 ng of common adaptor (6 µl at 0.4 ng/ µl; sequences: 5'-AGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG-3' and 5'-CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTTGCA-3'), 2.4 ng of barcode adaptor (6 µl at 0.4 ng/ µl; sequences: 5'-

ACACTCTTTCCCTACACGACGCTCTTCCGATCTxxxxTGCA-3' and 5'-

xxxxAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT-3'), and approximately 100 ng of genomic DNA (5 ng at 20 ng/ $\mu$ L) for each individual on a 96-well plate. I added 1  $\mu$ L of New England Biolabs High-Fidelity Pstl and 2 µL of New England Biolabs CutSmart Buffer for a final volume of 20 µL. This solution was incubated for 2 hours at 37°C. The Pstl enzyme cut sites at the nucleotide sequence CTGCAG. After the restriction digestion, I added 1.6 µL of New England Biolabs T4 Ligase, 5 µL of New England Biolabs 10X Buffer, and 23.4 µL of ultrapure water. Samples were then incubated for 1 hour at 22°C to ligate adaptors and DNA fragments, then at 65° C for 10 minutes to deactivate the enzyme. Fifteen  $\mu$ L of ligation product was cleaned with 23  $\mu$ L of Beckman-Coulter AMPure XP beads. DNA binds to the beads, and when placed on a magnetic plate, cling to the walls of each well. The remaining waste liquid was removed, and beads were washed twice with 200 µL of 70% ethanol. Ethanol was removed while beads and DNA were still attached to the walls of the wells. After the second wash, the plate was left for 5 minutes to allow the remaining ethanol to evaporate. The 96-well plate was removed from the magnetic plate. The beads and DNA were resuspended in 40 µL of TE. The 96-well plate was then placed back on the magnetic plate. Separation of TE buffer and DNA from beads using magnetism and moved to a new 96-well plate. Amplification by PCR was conducted by adding 4 µL of genomic DNA to 1.5 µL New

England Biolabs Phusion High-Fidelity DNA polymerase, 5  $\mu$ L of New England Biolabs 5X Phusion Buffer, 0.5  $\mu$ L of 10 mM dNTPs, 1.25  $\mu$ L of GBS-Primer A (sequence: 5'-ATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC T-3'), 1.25  $\mu$ L of GBS-Primer B (sequence: 5'-

CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTC CGATCT-3'), and 12.75  $\mu$ L of UltraPure water. The PCR amplification protocol was as follows: 1) 98° C for 30 seconds, 2) 18 cycles of: 98° C for 10 sec, 65° C for 30 sec and 72° C for 30 sec, 3) 72° C for 5 min.

The concentration of each sample was quantified, and volume that contains 100ng of PCR product was calculated for each sample, approximately 2-3 µL per sample. These volumes for each sample were pooled and run on a 2% agarose gel. The band between 300 and 400 base pairs was cut and extracted using the Qiagen Gel Extraction Kit. All concentrations were measured with Invitrogen Qubit dsDNA HS Assay Kit and Qubit 2.0 Fluorometer. Samples were analyzed on an Agilent Technologies 2100 Bioanalyzer to ensure that DNA fragments were of the expected size (300-400 bp).

The resulting library was sequenced on the Illumina HiSeq4000 platform at Genome Québec Facility using 150 bp paired-end read sequencing.

## 2.2.5 Data Analysis for Genomics

Genomic data was processed for 186 individuals using a modified version of the protocol used by Irwin et al. (2016). This results in over 667 billion reads, 200 trillion bases with an average quality of 35. This is roughly 3 billion reads per individual. The GBS data were demultiplexed and trimmed using Trimmomatic (version 0.32; Bolger et al. 2014), using settings 'TRAILING:3 SLIDINGWINDOW:4:10 MINLEN:30'. This removed barcodes and adaptor sequences and low-quality bases. Sequences were mapped to the zebra finch (*Taeniopygia guttata*) reference genome (version 3.2.4, Warren et al. 2010) using BWA-MEM (version 0.7.17, Li and Durbin 2009). Individuals were genotyped using GATK 3.8 (McKenna et al. 2010). Genotypes were called, removing indels, allowing a maximum of two alleles per site, and retaining only variant sites. Sites were removed where more than 60% of individuals were missing genotypes, to analyze only sites with data from a substantial portion of individuals. Sites with MQ <20 were removed, to exclude poorly mapped reads. Finally, sites with heterozygosity above 60% were removed to avoid areas of the genome likely to have arisen through duplication (Irwin et al. 2016).

Analysis was performed on 166 individuals and approximately 1.1 million SNPs. Twenty individuals were removed, because they were missing more than 25% of genotyped SNPs. A PCA using pcaMethods (Stacklies *et al.* 2007) in R v3.4.3 (DevelopmentTeam 2017) was conducted on the genotypes of individuals to examine population structure across regions of Townsend's Warbler range and between coastal and interior populations. I used 'svdImpute' to account for missing data in individuals. In

addition, two individuals clustered tightly together, but away from all other individuals. These samples were collected on the same day in close proximity to one another, so it is likely these are close relatives. The sample with the greater missing data was then excluded from the analysis. Following this, a PCA with all but one of the remaining individuals separated the eight individuals from Valdez, AK, from all other individuals. I have concluded this differentiation is not due to artifacts in the data. Three of these anomalous samples were collected in 1995, and five samples were collected in 2006 in a roughly 10 km radius, so it is unlikely that all samples are close relatives. They did not have extremely high numbers of missing SNPs or uneven sex distribution. The reason for differentiation in this population is beyond the scope of this study. In order to examine how genotype changes from coast to interior, I removed all Alaska samples, as I could not eliminate extreme latitudinal differences between those individuals and more Southern individuals.

To examine how genotype changes based on the distance from the coast, while excluding Latitudinal changes, samples were selected within three degrees of Latitude along a Northern transect (54° N to 57° N) and a Southern transect (48° N to 51°N). There are 34 individuals used in the Northern transect analysis and 41 individuals in the Southern transect analysis. Multilocus genotype was plotted in a PCA using the same methods as above. Then, PC2 score was plotted against distance from the coast, with minimum distance to coast line calculated using the same method as described for the song values. A bivariate analysis was used to test the associate between genotype and geography.

# 2.2.6 Examining Association between Song and Genetics

In order to test for an association between song and population structure, a subset of the samples collected have both song and genetic data (n = 71). For these individuals, I conducted two PCAs: one on song variables, one on genotype, using the same method as described above. I then plotted the genetic PC1 against the song PC1. A bivariate analysis was run to test the association between song and geography.

## 2.3 Results

#### 2.3.1 Type I versus Type II Song

From field observations, most songs matched early qualitative descriptions of songs (Wright *et al.* 1998) and could be auditorily distinguished into Type I and Type II songs. For Type I songs, the notes were clear, whistle-like in quality, and typically composed of five to six ascending introductory notes in part 1, ending with one to three notes in part 2 (Fig 5). Type II songs seemed to be more stereotypical across the range. The song begins with one to six buzzy notes with a variable ending (Fig 6).

Individual repertoires of a bird showed Type I and Type II songs differentiating along PC1 in all cases (Fig. 7). The percent of variation explained by PC1 is almost double the percent of variation in PC2 (Bird A: PC1 28% to PC2 16.7%, Bird B: PC1 39.4% to PC2 17.8%, Bird C: PC1 42.1% to PC2 20%), suggesting that the difference between Type I and Type II is the most important source of variation. However, the variables that contributed most heavily to PC1 differed for each individual. For example, the variables with the highest loadings for bird A were 'maximum frequency of the song' and 'aggregate entropy of the song' (Figure 7A, Table S1). The variables that had the highest loadings for bird B were variables associated with part 2 of the song (Figure 7B, Table S2). The variables that had the highest loadings in for bird C were variables associated with part 1 of the song (Figure 7C, Table S3). Visually, birds appeared to sing one Type I song and one Type II song (Fig 8A and 8C), but one individual sang two

Type II songs, one where the buzzy notes had a wider frequency range, and one where the buzzy notes had a narrower frequency range (Fig 8B).

At the population scale, there was a considerable amount of variation among Type I and Type II in the number of notes a bird sings in a given song. The greatest variation between individuals in both Type I and Type II songs occurred in the number of notes and number of unique notes, with a coefficient of variation greater than 50. Type I songs was highly variable across the range and possessed a greater coefficient of variation than Type II songs for 14 of the 21 variables measured (Table 1). The Type II songs have a narrower range of frequencies and a lower minimum frequency in the song. The mean values of 17 out of the 21 variables measured were significantly different between the Type I and Type II songs. The variables that did not differ between the two groups were 'aggregate entropy', 'length of part 1', 'bandwidth of part 1', and 'bandwidth of part 2'.

Type I and Type II songs did not form distinct clusters separated along either PC1 or PC2 in a PCA of all songs, but mean scores were significantly different in both PC1 and PC2 (Two Sample t-test PC1: t = -2.51, df = 141.32, p-value: 0.013; PC2: t = 4.57, df = 147.4, p-value < 0.001, Fig 9). Type I songs appeared to form two loose clusters along PC1. One cluster overlapped with Type II songs. PC1 and PC2 axes were largely driven by song length and complexity (number of notes). PC1 explained 24.6% of the variation and 'number of notes in part 1', 'length of part 1', 'bandwidth of part 1', 'number of notes in part 2', and 'number of unique notes in part 2' had the highest loadings on this axis (Table 1). PC2 explained 18.6% of the variation, and 'number of unique notes in song',

'length of song', 'bandwidth of part 2', 'maximum frequency of part 2', and 'aggregate entropy of part 2' had the highest loadings on this axis (Table S4).

A DFA comparing Type I and Type II differentiated these two song types, with slight overlap between groups (Two Sample t-test: t = -17.74, df = 137.98, p-value < 0.001, Fig 10). The linear discriminant was driven primarily by the 'Bandwidth of Part 2' (Table 1). The misclassification rate using the resubstitution error was 5%. The misclassification rate using a jackknife method was 10%.

### 2.3.2 Variation in Type I Songs

Songs were placed into groups based on visual similarity (n = 159), but 173 songs were unique to a single bird and could not be classified into a group. Songs that were members of these recognizable groups showed some geographic clustering (Fig 11). For example, birds in the Vancouver Coast and Mountains area sang primarily category F songs. Birds in the Okanagan region sang primarily category B songs with the B2 song dominating the region. However, many groups were found in more than one location. For example, the B1 song was found all along the coast of British Columbia and in some locations in the interior. In most locations, there is only one or two dominant songs. Within an individual, the songs sung were consistent (within each of Type I and Type II) throughout the recording for all birds.

In a PCA of Type I songs, there seemed to be little distinct clustering into geographic groups, and many groups overlapped (Fig 12). PC1 explained 28% of the

variation, which was driven by the variables with the highest loadings: 'number of notes in part 2', 'number of unique notes in part 2', 'length of part 2', 'bandwidth of part 2', 'aggregate entropy of part 2', and 'minimum frequency of part 2' (Table 1). PC2 explained 16.7% of the variation and was driven by the variables with the highest loadings: 'number of unique notes in song', 'length of song', 'number of unique notes in part 1', 'length of part 1', 'bandwidth of part 1', and 'aggregate entropy of part 1' (Table S5). Though some regions formed clusters along either the PC1 or PC2 axis, variation in song was not driven by regional boundaries. For example, songs found in Northern British Columbia formed two clusters along PC1. The first cluster seemed to consist of the most song found in this region, whereas the second, smaller cluster consisted of less common versions of songs found in this region. This major and minor song pattern appeared to occur in the Gulf Islands region, Kootenay region, and Vancouver Coast and Mountains region, as well. This may have occurred to a lesser extent with other song regions. A PCA of the regional variation of Type I songs showed the same pattern as Type I songs in the PCA comparing all songs in that regard, forming two subtle clusters along PC1. There was significant difference between some regions along PC1 (ANOVA: F = 7.85, p-value < 0.001) and PC2 (ANOVA: F = 7.47, p-value < 0.001). Across 45 pairwise comparisons of mean PC scores between regions, 7 pairs of regions differed significantly along PC1 and 10 pairs of regions differed significantly along PC2 (Table S6 and S7).

#### 2.3.3 Variation in Type II song

A PCA of Type II song showed no geographic pattering (Fig 13). PC1 explained 27.9% of the variation (ANOVA: F = 7.65, p-value = 0.139). The variables that drove this variation were 'total number of notes in song', 'number of unique notes in song', 'length of song', 'bandwidth of song', 'minimum frequency of song', and 'aggregate entropy of song' (Table 1). PC2 explained 20.2% of the variation (ANOVA: F = 2.64, p = 0.009). The variables that drove this variation were 'total number of notes in part 2', 'length of part 2', 'number of unique notes in part 1', and 'total number of notes in part 1' (Table S8). Generally, PC1 seemed to separate songs based on length and complexity of the song, as well as the extent of frequency modulation. Songs were much more tightly clustered along PC2, with only songs that vary in length and complexity in extreme ways being pulled out from the group. The significant difference between groups in PC2 is driven by a single individual in Oregon differing significantly from some of the other regions—Alaska, Northern BC, and the Gulf Islands.

### 2.3.4 Genomic Population Structure

A PCA based on 1.1 million SNPs from a genome-wide marker data set across 165 Townsend's Warblers sampled showed a continuous gradation of population differentiation along PC1 (Fig 14). PC1 represented 1.67% of the variance in SNPs

across all samples. A cluster of a single population—Valdez, AK— was separated from other populations along PC2. PC2 represented 1.5% of the variation in genotypes.

A PCA of genotype of 34 birds collected between 54° and 57°N (here referred to as the Northern transect) showed individual differentiation along PC1 (Fig 15). PC1 represented 5.7% of the variance. PC2 showed individuals clustering by location forming a gradation between populations. PC2 explained 5.6% of the variation.

A PCA of genotype of 41 Townsend's Warblers collected between 48° and 51°N (here referred to as the Southern transect) showed a few outlying individuals separating from a main cluster along PC1 (Fig 16). PC1 represented 5.18% of the variation. Populations differentiated by location along PC2, forming a continuous gradient of genotypes. PC2 represented 4.8% of the variation.

### 2.3.5 Type I Song Variation Based on Genotype

Across all sampling sites, genetic background had a strong linear relationship with geography with a gradient from coastal types to interior types (ANOVA  $r_{adj}^2 = 0.61$ , P = < 0.001, Fig 17). PC2 was used to compare the genetic link to geography, because this axis appears to differentiate populations. This strong linear relationship between genetic background and geography was consistent in the Northern and Southern transect analyses (Northern transect: ANOVA  $r_{adj}^2 = 0.72$ , P = < 0.001, Fig 18; Southern transect: ANOVA  $r_{adj}^2 = 0.72$ , P = < 0.001, Fig 18; Southern transect: ANOVA  $r_{adj}^2 = 0.88$ , P = < 0.001, Fig. 19).

By contrast, Type I song scores showed a weak but significant relationship with geography (ANOVA  $r_{adj}^2 = 0.11$ , P = < 0.001, Fig 17). There was no relationship between Type I song score and geography in the Northern transect (ANOVA  $r_{adj}^2 = 0.03$ , P = 0.14, Fig 18). Along the Southern transect from Vancouver Island to the Rocky Mountains, there was no distinct differences between birds in coastal populations versus interior populations (Fig. 19). Nonetheless, there was a weak but significant relationship between song and geography in Southern transects (ANOVA  $r_{adj}^2 = 0.24$ , P= < 0.001). There appeared to be some geographic clustering among birds in close geographic proximity in some locations. This pattern was not consistent across the transect.

A bivariate analysis comparing song PC score to genotype PC score showed a significant, but weak association between genotype and song (ANOVA  $r^2 = 0.21$ , P = 7.707x10<sup>-5</sup>, Fig. 20).

# 2.4 Discussion

In this study, I examined songs and nuclear DNA patterns from across the Townsend's Warbler range. For song analysis, I used both quantitative and qualitative methods, to describe the song types that exist in this species. I identified two song types—Type I and Type II—in this species. Type I and Type II songs were distinguished by individual repertoires and visually, but at a population scale, they were only weakly differentiated based on the quantitative analysis of song variables. Examinations of song dialect structure showed weak geographic structure in Type I songs (songs used in advertisement to females) and no geographic structure in Type II songs (songs used in male-male competition). Analyses of 1.1 million SNPs revealed there were west-toeast gradients in genomic signatures, but no sharp transition between coast and inland groups. Finally, I examined the association between song and genetic divergence and found that genetic and song patterns were weakly associated.

#### 2.4.1 Type I versus Type II songs

When looking at an individual birds' repertoire, it is apparent that there are at least two song types that a bird commonly sings. This distinction can be easily determined through auditory or visual examinations. At the population scale, this twosong type pattern persists, though these groups are not well defined. In a PCA of all songs from many individuals, Type I and Type II songs were not strongly differentiated

along either axis. A DFA of these two song types distinguishes Type I and Type II songs more clearly. This weak differentiation in quantitative measurements may be driven by the large diversity of Type I songs. First, Type I songs have a high average coefficient of variation (35%), a higher coefficient of variation for most variables in Type I than in Type II songs. When examining individual repertoires, there is greater variation in Type I song than Type II songs. Furthermore, the characteristics that drive these differences between song types are not consistent among the individual birds examined, suggesting that Type I songs vary in a stochastic way.

# 2.4.2 Weak Geographic Variation in Song

Turning from variation between song types to variation between populations within song types, my results indicated weak geographic patterns in Type I song, and no detected geographic structure in Type II song. Type I songs split into two loose clusters. Some regions were significantly different from one another. For example, songs from the Northern British Columbia region were significantly different from songs of every region expect Montana, Kootenay, and Alaska regions, but regions did not diverge from one another consistently.

At the local population level, there was pattern of a major and minor song variety in the qualitative analysis of these songs. In many cases, there was only one stereotypical song, with nine populations with a major and minor song. In only one case—Castelgar, BC—was there a population with more than two stereotypical songs.

Generally, birds commonly sing similar songs to their neighbors, but there are several stereotypical songs that were shared over large distances. For example, the category B1 (dark orange on Fig 11) songs were found along the entire British Columbia coast and in the Okanagan region of BC. It should also be noted that there were many songs that did not appear to resemble other songs closely. Some populations had higher incidences of non-stereotypical songs, such as those near Campbell River, BC, in which all 17 birds recorded did not fit into a song category. Nonetheless, both the regional and location-level quantitative analyses suggested that there was some dialect structure at the microgeographic scale, but this pattern was not evident at the macrogeographic level. This idea is reinforced by comparisons between coastal and interior song types. Birds with similar distances from the coast clustered together on the PC1 axis, but there was a weak relationship between these locations differentiating in line with geographic distance. My data suggest that songs are either being selected differently in different regions, or song divergence is largely a stochastic process in this species (Lynch 1996). Therefore, in this system, songs are likely a reflection of cultural evolutionary forces, rather than primarily genetic differences, as some song categories are readily spread across large distances.

# 2.4.3 Isolation by Distance Pattern in Genetics

Differences in mitochondrial haplotypes have been documented between coastal and interior populations of Townsend's Warbler (Krosby and Rowher 2009). In contrast,

I found that there was a more or less continuous gradient of genotypes in the nuclear DNA, where individuals were most closely related to those in their region. Birds from inland and coastal areas showed gradual differentiation from one another with increasing distance. There was no sharp transition between these populations. Rather, the differentiation I documented follows an isolation-by-distance pattern. An isolation-bydistance (IBD) model suggests there is relatively limited dispersal across large distances in this species (Wright 1946). Examinations of the Northern and Southern transects emphasize this pattern, showing tight clusters of populations based on location identity. One explanation for this pattern is that Townsend's Warblers survived the most recent glaciation in an inland refugium east of the Rocky Mountains and came into contact and hybridized with coastal Hermit Warbler populations during postglacial dispersal (Rowher et al. 2001; Krosby and Rowher 2009). An isolation-by-adaptation model (IBA)—in which populations diverge because of an environmental gradient cannot, however, be ruled out based on my analysis (Nosil et al. 2009; Spurgin et al. 2014). In this case, as the environmental conditions change and the adaptive divergence changes in concordance with the environment (Nosil et al. 2009). The regions of the genome that are driving this population differentiation and how this compares to ecological conditions warrants further investigation to test the idea of IBA. Nonetheless, when comparing genotype to geography, distance from the coast is an adequate predictor of genotype.

#### 2.4.4 Mismatch between Song Divergence and Genetic Divergence

Coastal and Interior populations have been shown to have genetic differences in mitochondrial DNA (Krosby and Rowher 2009), but I found that this pattern is not so clearly observed in song or nuclear DNA. When comparing song score to the distance from the coast, there was some local clustering. There was, however, consistent overlap between coastal song scores and interior song scores. On the Northern transect, there was remarkably similarity between the song scores of the birds, with only a few interior populations showing a differentiation. In the Southern transect, there was weak trend of differences between coastal and interior populations.

The gradual transition from coastal to interior populations, indicates there is low dispersal but no barrier or shift between a coastal type and an interior type. When we directly compared song scores to genotype scores, there was a very weak association between these two factors. Despite evidence that there is limited dispersal of individuals, songs are capable of being shared over very long distances in this species.

In order to understand why genetic patterns are more structured than song patterns Townsend's Warbler song can be examined in the context of the several hypotheses posited for the adaptive significance of song dialects (Catchpole and Slater 2008). The genetic adaptation hypothesis states that young birds learn song in early life, and then mate assortatively as adults based on that song (Marler and Tamura 1962,1964; Nottebohm 1969). If this is the case, then song dialect will inhibit gene flow. Given that Townsend's Warbler songs are readily shared across long distances and not tightly linked to genetic differences, this is likely not the case. The epiphenomenon

hypothesis, first proposed by Andrew (1962), states that song dialect is a byproduct of learning. Drift will act on songs such that songs of distant populations will diverge in different ways. Genetic structure, alternatively, is due to other factors like founder events, genetic drift, geographic barriers, and philopatry (Andrew 1962; Bitterbaum and Baptista 1979; Wiens 1982). As such, there are no strict boundaries to song dialects, but there is greater similarity among nearby individuals than distant individuals. This suggests that the song a male sings is likely learned from other birds in its neighborhood. Furthermore, several studies have shown that song does not pass from male parent to male offspring. Rather, after a young male has dispersed, they learn the local song (Kroodsma 1974; Payne et al. 1987; McGregor et al. 1988). If this is the case, then cultural and genetic lineages are not transmitted together (Payne 1996). In summary, my study found that these song groupings do not coincide with genetic patterns, suggesting that cultural and genetic lineages are not strictly transmitted together in the Townsend's Warbler. Song is heavily influenced by learning and cultural evolution and is likely not preventing gene flow among Townsend's Warbler populations.

Table 1. Differences between Type I and Type II songs for 21 variables and the loadings for those variables. Variables with N/A loading values indicates these variables were not used in that analysis. \* indicates p < 0.05.

	Туре І			Туре II			Mann-Whitney U test (df=1)		PC1 Loadings	DEA
Variable Name	Mean	Coefficient of variation	PC1 Loadings	Mean	Coefficient of variation	PC1 Loadings	U	Р	(analysis of all songs)	scalings
Number of unique notes	4.62	33.16	-0.09	5.22	24.97	0.293	9648	<0.001*	-0.091	NA
Total number of notes	7.56	41.46	-0.19	8.65	36.44	0.302	9266.5	0.0012*	-0.194	NA
Length of song (s)	1.45	16.14	-0.07	1.37	22.38	0.249	6301.5	0.054*	-0.073	NA
Bandwidth (Hz)	2265.65	36.55	-0.17	1908.57	33.00	0.248	4983.5	<0.001*	-0.167	NA
Aggregate entropy	4.18	11.77	-0.18	4.12	12.92	0.256	6974	0.44	-0.182	NA
Maximum frequency (Hz)	7224.43	7.84	-0.12	6829.42	10.52	0.222	4545	<0.001*	-0.122	NA
Minimum frequency (Hz)	3757.52	11.98	0.19	3603.78	11.29	-0.252	5859	0.0071*	0.191	NA
Part 1 Number of unique notes	2.63	52.72	0.17	2.00	35.75	0.145	5500	0.0016*	0.165	0.0004
Part 1 Total number of notes	4.67	56.78	0.03	3.81	64.61	0.182	5663	0.0045*	0.031	0.0003
Part 1 Length (s)	0.95	30.98	0.21	0.90	34.46	0.219	6631.5	0.23	0.212	-1.088

Part 1 Bandwidth (Hz)	1134.45	79.24	0.03	1061.48	45.48	0.175	7314.5	0.98	0.028	0.37
Part 1 Aggregate entropy	3.15	23.76	0.15	3.66	12.35	0.179	10374	<0.001*	0.146	0.0003
Part 1 Maximum frequency (Hz)	6042.88	14.38	0.08	5572.96	8.38	0.09	4683	<0.001*	0.083	0.075
Part 1 Minimum frequency (Hz)	4245.26	15.78	-0.04	3636.71	10.85	-0.242	3213	<0.001*	-0.035	0.339
Part 2 Number of unique notes	2.06	51.03	-0.35	3.31	36.43	0.231	11051	<0.001*	-0.346	0.0004
Part 2 Total number of notes	3.00	67.43	-0.35	5.00	51.06	0.205	10723	<0.001*	-0.352	0.0004
Part 2 Length (s)	0.47	44.67	-0.37	0.43	30.52	0.086	5675	0.030*	-0.367	-0.189
Part 2 Bandwidth (Hz)	1890.19	65.61	-0.32	1611.37	50.52	0.228	5586.5	0.019*	-0.317	5.134
Part 2 Aggregate entropy	3.71	26.30	-0.36	3.53	20.84	0.228	5402	0.0076*	-0.36	0.0004
Part 2 Maximum frequency (Hz)	7165.29	10.49	-0.14	6820.91	10.62	0.223	4278	<0.001*	-0.144	-0.13
Part 2 Minimum frequency (Hz)	4294.66	24.38	0.32	3966.83	17.01	-0.182	6097	0.16	0.322	-0.614



Figure 1. Map of the breeding range of Townsend's Warbler and sampling locations in northwestern North America. Green triangle denotes locations where song recordings were collected. Red circles denote locations where blood samples were collected.



Figure 2. Diagram of the variables measured for song analysis in Townsend's Warbler. They are as follows: Duration of the song (s), maximum frequency of song (Hz), minimum frequency of song (Hz), total number of notes in song, number of unique notes in song, bandwidth of song (Hz), aggregate entropy of song (not pictured), duration of Part 1 (s), maximum frequency of Part 1 (Hz), minimum frequency of Part 1 (Hz), total number of notes in Part 1, number of unique notes in Part 1, bandwidth of Part 1 (Hz), aggregate entropy of Part 1 (not pictured), duration of Part 2 (s), maximum frequency of Part 2 (Hz), minimum frequency of Part 2 (Hz), total number of notes in Part 2, number of unique notes in Part 2, bandwidth of Part 2 (Hz), and aggregate entropy of Part 2 (not pictured).



Figure 3. Aggregate Entropy as predicted by Signal-to-Noise Ratio, which measures how much noise is in a recording. The line represents a least squares regression line.



Figure 4. Map of the reference points used to create the composite coastline of northwestern North America in order to calculate minimum distance from coast to sampling points.



Figure 5. A spectrogram example of a stereotypical Type I song in Townsend's Warbler.



Figure 6. A spectrogram example of a stereotypical Type II song in Townsend's Warbler.







Figure 7. PCAs for all songs recorded for an individual Townsend's Warbler, with identity indicated by the letter in the top right corner of each figure. Each graph represents one bird. Each point represents a song. (A) PC1 explains 28% of the variation. PC2 explains 16.7% of the variation. N=19 (B) PC1 explains 39.4% of the variation. PC2 explains 17.8% of the variation. N = 23 (C) PC1 explains 42.1% of the variation. PC2 explains 20% of the variation. N = 20.









Figure 8. Spectrogram examples of individual bird's repertoires for the three Townsend's Warblers analyzed. Each letter refers to one of three individual birds and corresponds to the letter in the PCA. Type I songs are on the left. Type II songs are on the right.



Figure 9. PCA of all songs of Townsend's Warbler. Colors and shapes represent Type I songs (n = 75) versus Type II (n = 75) songs. Each point represents a single bird. PC1 explains 24.6% of the variation (Two-sample t-test: t =-2.51, df = 141.32, p =0. 013). PC2 explains 18.6% of the variation (Two-sample t-test: t =4.56, df = 147.4, p <0.001).



Figure 10. A discriminant function analysis of Type I (n=68) and Type II (n=72) songs of Townsend's Warbler. Linear discriminant score is along the Y-axis. Each point represents a single bird. Points are jittered within each group to better show individual data points. Boxplots are overlaid points. (Two sample t-test: t = -17.741, df = 137.96, p<0.001).



Figure 11. Map of visual characterizations of songs recorded from Townsend's Warbler. Letter categories represent same structure in Part 1 of song. Number subcategory represents exact song match. Each pie chart represents a sampling site. Area of each pie chart represents the number of songs at each location.




Figure 12. A: A reference map of geographic regions used to classify macrogeographic groups, transposed on the Townsend's Warbler range and colored to match the icons used in the PCA. B: PCA of Type I songs. Color and shape

represent region. Each point represents a single Townsend's Warbler (n = 199). PC1 represents 28% of the variation (ANOVA: F = 7.85, p < 0.001). PC2 represents 16.7% of the variation (ANOVA: F = 7.47, p-value < 0.001).



Figure 13. PCA of Type II songs. Color and shape represent region. Each point represents a single Townsend's Warblers (n = 75). PC1 represents 28% of the variation (ANOVA: F = 1.56, p = 0.14). PC2 represents 20.2% of the variation (ANOVA: F = 2.64, p = 0.009).



Figure 14. PCA of genetic data for the whole genome and all SNPs. The colors and shapes represent different regions of Townsend's Warbler range. PC1 represents 1.67% of the variation. PC2 represents 1.5% of the variation.



Figure 15. PCA of mean Townsend's Warbler genotypes along the Northern transect (between 54-57°N). Each point represents an individual Townsend's Warbler. Locations are organized from East to West in the legend. PC1 represents 5.7% of the variation. PC2 represents 5.6% of the variation.



Figure 16. PCA of Townsend's Warbler genotypes along the Southern transect (between 48-51°N). Each point represents an individual Townsend's Warbler. Locations are organized East to West in the legend. PC1 represents 5.18% of the variation. PC2 represents 4.8% of the variation.



Figure 17.Comparison of genetic and Type I song variation from east to west of all Townsend's Warblers sampled. Above: A plot of PC1 for mean genotype from coast to interior of all Townsend's Warblers analyzed. Each point represents a single bird. The line represents a least squares regression line, gray around the line represents the

standard error. The adjusted r<sup>2</sup> value equals 0.6115 (p-value < 0.001). Below: A plot of PC1 for Type I song from coast to interior of all birds analyzed. Each point represents a single bird. The line represents a least squares regression line, gray around the line represents the standard error. The adjusted r<sup>2</sup> value equals 0.11 (p-value < 0.001).



Figure 18. A comparison of genetic and Type I song variation from west to east of Townsend's Warblers along the Northern transect (between 54-57°N). Above: A plot of PC2 for mean genotype from west to east. The line represents a least squares regression line, gray around the line represents the standard error. The adjusted  $r^2$ 

value equals 0.72 (p-value < 0.001). Below: a plot of Type I song PC1 score from west to east. Each point represents a single bird. The line represents a least squares regression line, gray around the line represents the standard error. The adjusted  $r^2$ value equals 0.03 (p-value = 0.14).



Figure 19. A comparison of genetic and Type I song variation from west to east of Townsend's Warblers along the Southern transect (between 48-51°N). Above: A plot of PC2 for mean genotype from west to east. Each point represents a single bird. The line represents a least squares regression line, gray around the line represents the standard

error. The adjusted r2 value equals 0.88 (p-value < 0.001). Below: A plot of Type I song PC1 from west to east. Each point represents a single bird. The line represents a least squares regression line, gray around the line represents the standard error. The adjusted r2 value equals 0.24 (p-value < 0.001).



Figure 20. Type I Song PC1 values versus Gene PC1 values. Each point represents a single bird. The line represents a least squares regression line. The adjusted r2 value equals 0.2119 (p < 0.001).

### **Chapter 3: Conclusion**

### 3.1 Study Goals

In this study, I assessed song types and patterns of song differentiation on a macrogeographic scale in the Townsend's Warbler. I examined song repertoires within individual birds to identify song types. I then looked at population-wide differences in song patterns to determine if there are regional differences in songs. I compared these song patterns to genetic structure, to identify whether geographic variation in song, a trait influenced by both genetics and learning, is concordant with patterns of overall genomic variation.

### 3.2 Diversity in Type I and Type II songs

The Townsend's Warbler appears to follow the two-song-type pattern common in Parulid warblers. This pattern is detectable auditorily, in individual bird repertoires, and in a discriminant function analysis of the entire population. My study highlights the importance of continued use of quantitative and qualitative methods when analyzing song of an individual species. Type I and Type II songs are not easily identified in the Townsend's Warbler using purely quantitative methods. In quantitative analysis at the population scale, there was substantial overlap between these song types, suggesting quantitative analysis is not identifying the characteristics discerned by the ear. Furthermore, there is a tremendous amount of diversity within each song type.

Mine is one of several studies that have shown a great diversity in Type I songs (Moldenhauer 1992, Morrison and Hardy 1983, Janes and Ryker 2006). These systems contradict the idea that Type I song is more stereotypical than Type II song posited by Kroodsma (1981). There are several possibilities why there would be diversity in both Type I and Type II songs. Perhaps, song variability (or lack thereof) could be driven by the cultural forces that are more crucial for a given system. If we assume that Type I songs serve the function of advertisement to females, then there may be pressure to maintain consistency only to the point where a female recognizes and investigates a male. She may use song as a cue to locate a potential mate, then assess him for other characteristics, like feather and color quality or territory quality. In fact, there is some evidence that female mate choice often selects for complex signaling, which would tend to increase variability in song (Byers and Kroodsma 2008). By contrast, a Type II song-or a song used in male-male competition-may be more stereotypical because the song can be used to signal an aggressive action and the accuracy of the song carries information about a male's competitive ability. Furthermore, this song has the potential to be more stereotyped across the range, because it enables competitive interaction after dispersal. This appears to be the case in Greenish Warblers; song is highly variable across their range and seems to have evolved differently as a consequence of the relative strength of selection of male competition and female choice (Scordato 2018). In locations where males are competing for limited resources, the short, stereotyped song used in male countersinging bouts is more frequent. But when

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males have larger territories and abundant resources, they use a longer, more complex song, which seems to be more attractive to females (Irwin 2000, Scordato 2018). Future work could address the strength of selection on song in Townsend's Warblers by conducting playback experiments in order to determine the strength of response to local versus foreign song response in both Type I and Type II song. If males respond to foreign and local Type II song equally, and they respond more strongly to Type II than Type I song, this would suggest that Type II is important in male-male competition and intra-sexual selection may be promoting song stereotypy of Type II song (Kroodsma *et al.* 1984). If females respond more strongly to local Type I song than foreign song, this would suggest that inter-sexual selection is producing the variability in song.

Additionally, several species are known to vary other mating behaviors, such as singing rate or song type switching, to modify the message they are transmitting (Smith 1970, Schroeder and Wiley 1983, Derrickson 1988, Ritchison 1988). This has been described as "performance-encoded" songs (Spector 1992). Perhaps, behaviors associated with song type are driving functional differences of song more strongly than the song components themselves. Finer scale study of the timing, rate, and order in which Townsend's Warbler use their songs would address if other elements of singing behavior are being modified and to what degree.

#### 3.3 Song as a Reproductive Barrier

Type I songs showed a degree of geographic clustering, but there were not sharp dialect boundaries. There was evidence that some dialect structure may exist at the microgeographic level, but not at the range-wide scale. Because of the range-wide sampling goals of this study, finer-scale patterns were not clearly evident. The possible microgeographic patterns to song warrant further investigation in this system. Hermit Warblers, the sister species to the Townsend's Warbler, have been demonstrated to have microgeographic structure, with dialect ranges between 688-3600 km<sup>2</sup> (Janes and Ryker 2009). By contrast, the present study was comparing songs across macrogeographic space, over 1,00,000km<sup>2</sup>. Consequently, it is likely that the cultural differences within Townsend's Warbler populations are occurring across a smaller scale.

This contrasts with the genetic pattern that I found, which displayed an isolationby-distance pattern. A lack of strong differentiation among populations in both song and genetics suggests there is very little reproductive isolation among populations. This does not rule out the possibility that song is contributing to reproductive isolation between the Townsend's Warbler and more distantly-related species.

There is an increasing body of knowledge about the connection between song and genetics. There are a few cases where dialect or regiolect adequately predict genotype (Baker 1981, Danner *et al.* 2011, MacDougall-Shakleton and MacDougall-Shakleton 2001, Lipshutz 2017, Wilkins *et al.* 2018). In most cases this has been found in non-migratory populations of sparrows with only one song type, such as White-

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crowned Sparrow and Rufous-collared Sparrow. But there are many cases that suggest that song and genetics are often uncoupled or only weakly correlated with each other (Soha et al. 2004, Ortiz-Ramirez et al. 2016, Kenyon et al. 2017). For instance, in the comparison of songs at the hybrid zone between Townsend's Warblers and Blackthroated Green Warblers, hybrids readily sing either Townsend's or Black-throated Green warbler song regardless of genotype (Kenyon et al. 2018). Further, in a subspecies of migratory White-crowned Sparrows, genetic distances between populations did not correspond to dialect differences (Soha et al. 2004) This is potentially due to the learning component of oscine song and overproduction associated with migratory behavior (Marler and Peters 1982). During the 'plastic song' phase of learning, birds will imitate notes and pieces of songs they have heard in various orders before transitioning into a more stereotyped song (Hultsch and Todt 2004). Overproduction is when a bird practices plastic song excessively and with greater variability (Marler and Peters 1982). Migratory populations of White-crowned Sparrows have been shown to sing twice as many songs and sing the non-stereotyped 'plastic song' longer than sedentary populations during the sensitive phase of learning (Nelson et al. 1996).

In cases where song and genetics are diverging in concert, these situations usually involve primarily non-migratory, low dispersing populations. These populations could be forming regiolects simply because song forms are diverging in isolation (Martens 1996, Toews 2017). For example, Savannah Sparrows (*Passerculus sandwichensis*) have been shown to sing songs of distant populations when exposed to these songs during the critical period of learning (Mennill *et al.* 2018). This suggests that

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the only barrier to song sharing is lack of exposure across long distances.

Subsequently, when dialect boundaries represent dispersal boundaries, genetic and song differences should align (Toews 2017). Song divergence, then, could be a signal that reproductive isolation has already occurred. Alternatively, it could act as reinforcement of pre-existing reproductive isolation. In the Townsend's Warbler, song seems to be shared readily across different populations and there are no sharp transitions between populations in genetic structure. This indicates that there is no evidence of barriers to reproduction across the range of this species.

This study helps expands our understanding of the role song plays in Townsend's Warblers and the natural variation that exists in this species. We can apply this knowledge to its hybrid zone interactions, to better understand processes of speciation in Parulid warblers. Furthermore, this work contributes to the body of knowledge about the role that song learning plays in cultural evolution of dialects and the ways songs can diversify within a species. This study serves as another piece toward understand the complex interaction between cultural and genetic forces in the behavior of bird species.

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# Appendices

Appendix A Supplemental Figures and Tables

# A.1 Supplemental Figures



Supplemental Figure 1. Map of visual characterizations of songs of the Townsend's Warbler, including songs that are not categorized. Letter categories represent similar songs. Number subcategory represents exact song match. Each pie chart represents a sampling site. Area of each pie chart represents the number of songs at each location.

# A.2 Supplemental Tables

	PC1	PC2	PC3	PC4
Number of unique notes	-0.162	-0.219	-0.174	-0.064
Total number of notes	-0.091	-0.042	-0.181	-0.455
Length of song (s)	-0.083	0.371	-0.068	-0.358
Bandwidth (Hz)	-0.189	0.364	-0.204	0.113
Aggregate entropy	-0.330	0.046	-0.298	0.171
Maximum frequency (Hz)	-0.404	0.045	0.016	-0.056
Minimum frequency (Hz)	0.270	-0.263	-0.174	-0.107
Part 1 Number of unique notes	0.326	0.020	-0.089	0.051
Part 1 Total number of notes	0.150	0.246	-0.105	-0.512
Part 1 Length (s)	0.321	0.157	-0.223	-0.263
Part 1 Bandwidth (Hz)	-0.089	0.373	-0.299	0.131
Part 1 Aggregate entropy	0.105	-0.033	-0.445	0.360
Part 1 Maximum frequency (Hz)	0.264	-0.120	-0.353	0.151
Part 1 Minimum frequency (Hz)	0.308	-0.220	-0.159	-0.090
Part 2 Number of unique notes	-0.181	-0.256	-0.145	-0.090
Part 2 Total number of notes	-0.181	-0.256	-0.145	-0.090
Part 2 Length (s)	-0.161	-0.129	-0.214	-0.192

# Supplemental Table 1. Bird A PCA loadings

Part 2 Bandwidth (Hz)	-0.062	0.124	-0.392	0.057
Part 2 Aggregate entropy	-0.154	-0.259	-0.095	-0.126
Part 2 Maximum frequency (Hz)	-0.186	-0.250	-0.119	-0.124
Part 2 Minimum frequency (Hz)	-0.094	-0.160	0.032	-0.069

	PC1	PC2	PC3	PC4
Number of unique notes	-0.090	-0.343	-0.296	0.033
Total number of notes	0.210	0.017	-0.063	0.571
Length of song (s)	-0.172	0.392	0.113	-0.006
Bandwidth (Hz)	0.186	0.317	0.059	0.367
Aggregate entropy	-0.059	-0.186	0.438	0.249
Maximum frequency (Hz)	-0.169	-0.050	0.422	0.052
Minimum frequency (Hz)	-0.192	-0.019	0.274	0.057
Part 1 Number of unique notes	-0.134	-0.393	-0.234	0.060
Part 1 Total number of notes	0.288	0.061	-0.005	-0.122
Part 1 Length (s)	-0.091	0.446	0.124	-0.144
Part 1 Bandwidth (Hz)	0.258	0.264	0.070	-0.109
Part 1 Aggregate entropy	0.150	-0.222	0.396	0.031
Part 1 Maximum frequency (Hz)	0.138	-0.260	0.394	-0.026
Part 1 Minimum frequency (Hz)	0.213	-0.136	0.218	-0.338
Part 2 Number of unique notes	0.316	0.043	-0.025	-0.010
Part 2 Total number of notes	0.239	0.007	-0.045	0.490
Part 2 Length (s)	-0.310	0.147	-0.022	0.083

# Supplemental Table 2. Bird B PCA loadings

Part 2 Bandwidth (Hz)	-0.336	-0.019	0.007	0.207
Part 2 Aggregate entropy	-0.315	0.057	0.038	0.027
Part 2 Maximum frequency (Hz)	-0.296	0.023	0.087	0.110
	PC1	PC2	PC3	PC4
-------------------------------	--------	--------	--------	--------
Number of unique notes	0.284	-0.042	0.034	-0.282
Total number of notes	0.113	-0.039	0.325	-0.419
Length of song (s)	-0.218	-0.310	0.136	-0.038
Bandwidth (Hz)	-0.233	-0.294	0.095	0.132
Aggregate entropy	-0.190	-0.179	-0.144	-0.333
Maximum frequency (Hz)	0.095	-0.045	0.357	-0.054
Minimum frequency (Hz)	0.060	0.053	-0.472	-0.297
Part 1 Number of unique notes	-0.201	0.140	0.151	-0.524
Part 1 Total number of notes	-0.309	0.038	0.148	-0.190
Part 1 Length (s)	-0.317	-0.085	0.051	-0.041
Part 1 Bandwidth (Hz)	-0.306	-0.150	-0.079	0.068
Part 1 Aggregate entropy	-0.204	-0.146	-0.264	0.032
Part 1 Maximum frequency (Hz)	-0.217	0.249	0.246	-0.146
Part 1 Minimum frequency (Hz)	0.183	0.204	0.260	-0.069
Part 2 Number of unique notes	0.315	-0.093	-0.036	-0.007
Part 2 Total number of notes	0.322	-0.049	-0.006	0.007
Part 2 Length (s)	0.217	-0.303	0.099	0.045

# Supplemental Table 3. Bird C PCA loadings

Part 2 Bandwidth (Hz)	-0.050	-0.466	0.010	0.001
Part 2 Aggregate entropy	0.152	-0.372	-0.018	-0.294
Part 2 Maximum frequency (Hz)	0.156	-0.374	0.101	-0.042
Part 2 Minimum frequency (Hz)	0.061	0.054	-0.471	-0.300

	PC1	PC2	PC3	PC4	
Number of unique notes	-0.091	0.338	-0.107	-0.186	
Total number of notes	-0.194	0.253	-0.268	-0.155	
Length of song (s)	-0.073	0.306	-0.215	-0.160	
Bandwidth (Hz)	-0.167	0.234	0.195	0.400	
Aggregate entropy	-0.182	0.132	0.083	0.277	
Maximum frequency (Hz)	-0.122	0.161	0.363	-0.247	
Minimum frequency (Hz)	0.191	-0.036	0.337	-0.106	
Part 1 Number of unique notes	0.165	0.333	-0.112	-0.161	
Part 1 Total number of notes	0.031	0.285	-0.297	-0.151	
Part 1 Length (s)	0.212	0.330	-0.109	-0.153	
Part 1 Bandwidth (Hz)	0.028	0.334	0.217	0.353	
Part 1 Aggregate entropy	0.146	0.321	0.110	0.309	
Part 1 Maximum frequency (Hz)	0.083	0.228	0.344	-0.234	
Part 1 Minimum frequency (Hz)	-0.035	-0.096	0.315	-0.420	
Part 2 Number of unique notes	-0.346	0.061	-0.005	-0.049	
Part 2 Total number of notes	-0.352	0.021	-0.022	-0.038	
Part 2 Length (s)	-0.367	-0.110	-0.081	0.029	
Part 2 Bandwidth (Hz)	-0.317	0.152	0.193	0.130	

Supplemental Table 4. PCA loadings of all songs.

Part 2 Aggregate entropy	-0.360	-0.063	0.044	-0.093
Part 2 Maximum frequency (Hz)	-0.144	0.088	0.346	-0.224
Part 2 Minimum frequency (Hz)	0.322	0.020	0.153	0.055

Supplemental Table 5. PCA loadings of Type I songs.

	PC1	PC2	PC3	PC4
Number of unique notes	-0.091	0.338	-0.107	-0.186
Total number of notes	-0.194	0.253	-0.268	-0.155
Length of song (s)	-0.073	0.306	-0.215	-0.160
Bandwidth (Hz)	-0.167	0.234	0.195	0.400
Aggregate entropy	-0.182	0.132	0.083	0.277
Maximum frequency (Hz)	-0.122	0.161	0.363	-0.247
Minimum frequency (Hz)	0.191	-0.036	0.337	-0.106
Part 1 Number of unique notes	0.165	0.333	-0.112	-0.161
Part 1 Total number of notes	0.031	0.285	-0.297	-0.151
Part 1 Length (s)	0.212	0.330	-0.109	-0.153
Part 1 Bandwidth (Hz)	0.028	0.334	0.217	0.353
Part 1 Aggregate entropy	0.146	0.321	0.110	0.309
Part 1 Maximum frequency (Hz)	0.083	0.228	0.344	-0.234
Part 1 Minimum frequency (Hz)	-0.035	-0.096	0.315	-0.420
Part 2 Number of unique notes	-0.346	0.061	-0.005	-0.049
Part 2 Total number of notes	-0.352	0.021	-0.022	-0.038
Part 2 Length (s)	-0.367	-0.110	-0.081	0.029

Part 2 Bandwidth (Hz)	-0.317	0.152	0.193	0.130
Part 2 Aggregate entropy	-0.360	-0.063	0.044	-0.093
Part 2 Maximum frequency (Hz)	-0.144	0.088	0.346	-0.224
Part 2 Minimum frequency (Hz)	0.322	0.020	0.153	0.055

Supplemental Table 6. Tukey Kramer results for differences in mean PC1 scores for Type I songs by region. Values above the diagonal represent the q value. Values below the diagonal represent the p-value. Significant values indicated with \*.

	WA	ID	МТ	Gulf Islands	Vancouver Coast/Mtns	Okanagan	Kootenay	Cariboo- Chilcotin	Northern BC	AK
WA		-0.59	0.31	-1.32	1.44	3.56	2.05	0.18	-0.46	-0.1
ID	1		0.7	-0.03	1.4	2.53	0.23	0.72	0.41	0.56
МТ	1	1		-1	0.44	1.58	0.77	-0.22	-0.57	-0.37
Gulf Islands	0.95	1	0.99		4.07	7.14	4.88	1.9	1.46	1.51
Vancouver Coast/Mtns	0.91	0.93	1	0.003*		2.86	0.83	-1.51	-2.89	-1.87
Okanagan	0.02	0.26	0.86	<0.001*	0.13		-1.99	-4.06	-6.04	-4.41
Kootenay	0.57	0.78	1	<0.001*	1	0.61		-2.23	-3.74	-2.59
Cariboo- Chilcotin	1	1	1	0.67	0.89	0.003*	0.44		-0.84	-0.33
Northern BC	1	1	1	0.91	0.12	<0.001*	0.009*	1		0.44
AK	1	1	1	0.89	0.69	<0.001*	0.23	1	1	

Supplemental Table 7. Tukey Kramer results for differences in mean PC2 scores for Type I songs by region. Values above the diagonal represent the q value. Values below the diagonal represent the p-value. Significant values indicated with \*.

	WA	ID	МТ	Gulf Islands	Vancouver Coast/Mtns	Okanagan	Kootenay	Cariboo- Chilcotin	Northern BC	AK
WA		1.14	-1.73	-0.95	-1.16	-1.96	-3.57	-1.24	-4.63	-2.42
ID	0.98		-2.24	-1.71	-1.82	-2.25	-3.12	-1.87	-3.57	-2.53
МТ	0.78	0.43		1.38	1.22	0.78	-0.08	1.11	-0.47	0.46
Gulf Islands	0.99	0.79	0.93		-0.4	-1.6	-4	-0.56	-6.15	-2.22
Vancouver Coast/Mtns	0.98	0.72	0.97	1		-1.09	-3.25	-0.19	-4.89	-1.72
Okanagan	0.63	0.43	1	0.85	0.98		-2.11	0.79	-3.52	-0.71
Kootenay	0.02*	0.06*	1	0.004*	0.04*	0.52		2.73	-1.08	1.24
Cariboo- Chilcotin	0.97	0.69	0.98	1	1	1	0.17		-4.04	-1.39
Northern BC	<0.001*	0.02*	1	<0.001*	<0.001*	0.012*	0.99	0.003*		2.35
AK	0.32	0.26	1	0.45	0.78	1	0.97	0.93	0.36	

# Supplemental Table 8. Type II PCA loadings

	PC1	PC2	PC3	PC4
Number of unique notes	0.293	-0.124	-0.139	-0.201
Total number of notes	0.302	-0.039	-0.225	-0.154
Length of song (s)	0.249	0.111	-0.271	-0.102
Bandwidth (Hz)	0.248	0.041	0.359	0.112
Aggregate entropy	0.256	0.060	0.290	0.203
Maximum frequency (Hz)	0.222	0.008	0.210	-0.402
Minimum frequency (Hz)	-0.252	0.028	0.202	-0.380
Part 1 Number of unique notes	0.145	0.313	-0.173	-0.051
Part 1 Total number of notes	0.182	0.320	-0.218	-0.089
Part 1 Length (s)	0.219	0.261	-0.249	-0.082
Part 1 Bandwidth (Hz)	0.175	0.249	0.246	0.143
Part 1 Aggregate entropy	0.179	0.282	0.059	0.216
Part 1 Maximum frequency (Hz)	0.090	0.295	0.179	-0.203
Part 1 Minimum frequency (Hz)	-0.242	-0.010	0.244	-0.367
Part 2 Number of unique notes	0.231	-0.306	-0.053	-0.180
Part 2 Total number of notes	0.205	-0.374	-0.069	-0.104

Part 2 Length (s)	0.086	-0.354	-0.055	-0.040
Part 2 Bandwidth (Hz)	0.228	-0.103	0.335	0.141
Part 2 Aggregate entropy	0.228	-0.161	0.281	0.172
Part 2 Maximum frequency (Hz)	0.223	0.016	0.199	-0.410
Part 2 Minimum frequency (Hz)	-0.182	0.267	0.152	-0.208

### Appendix B Credits for Song Recordings and Blood Samples

## B.1 Macauley Library Recordings

The following recordings from the Macauley Library at the Cornell Lab of Ornithology

were used:

10027
10028
10029
10030
22965
47702
47704
47707
50312
50314
50325
51226
51236
51237
51239
62228
111134
118756
130940
193564
195071
195075
195218
204812
207265
207279
207281
516709
516711

#### **B.2 Xeno-Canto Recordings**

The following recordings were obtained from <u>www.xeno-canto.org</u> and were recorder by

the recordist following the xeno-canto ID:

36563 Tayler Brooks76413 Andrew Spencer107827 Jelmer Poelstra114348 Ian Cruickshank

- 134887 Tom Forwood Jr.
- 137649 Randy Dzenkiw
- 139862 GABRIEL LEITE
- 150338 Paul Marvin
- 150351 Paul Marvin
- 150356 Paul Marvin
- 150357 Paul Marvin
- 150359 Paul Marvin
- 153709 Paul Marvin
- 154215 Ian Cruickshank
- 156181 Ian Cruickshank
- 156182 Ian Cruickshank
- 156184 Ian Cruickshank
- 156194 Ian Cruickshank
- 156213 Ian Cruickshank
- 156215 Ian Cruickshank
- 156219 Ian Cruickshank
- 156220 Ian Cruickshank
- 156550 Ian Cruickshank
- 156837 Ian Cruickshank
- Gwaii Haanas National Park Reserve and Haida Heritage Site songbird 157557 research group
- Gwaii Haanas National Park Reserve and Haida Heritage Site songbird 157559 research group
- Gwaii Haanas National Park Reserve and Haida Heritage Site songbird 157603 research group
  - Gwaii Haanas National Park Reserve and Haida Heritage Site songbird
- 157610 research group
- 157613 Ian Cruickshank
- 160177 Ian Cruickshank
- 160207 Ian Cruickshank
- 160215 Ian Cruickshank

160216 Ian Cruickshank 160219 Ian Cruickshank 160220 Ian Cruickshank 160221 Ian Cruickshank 160225 Ian Cruickshank 160229 Ian Cruickshank 160232 Ian Cruickshank 160233 Ian Cruickshank 160830 Ian Cruickshank 160831 Ian Cruickshank 160838 Ian Cruickshank 160851 Ian Cruickshank 179100 Ian Cruickshank 182395 Ian Cruickshank 182398 Ian Cruickshank 182404 Ian Cruickshank 182421 Ian Cruickshank 182424 Ian Cruickshank 182425 Ian Cruickshank 187462 Richard E. Webster 187464 Richard E. Webster 187466 Richard E. Webster 187467 Richard E. Webster 193985 Richard E. Webster 255648 Ian Cruickshank 255649 Ian Cruickshank 255816 Ian Cruickshank 255829 Ian Cruickshank 255830 Ian Cruickshank 255832 Ian Cruickshank 256447 Ian Cruickshank 269030 Frank Lambert 269031 Frank Lambert 269234 Frank Lambert 296039 Paul Marvin 297528 Ross Gallardy 302557 James Bradley 322898 Peter Boesman 322899 Peter Boesman 322900 Peter Boesman 322903 Peter Boesman 322907 Peter Boesman 322939 Peter Boesman

323056 Peter Boesman
323058 Peter Boesman
323646 Richard E. Webster
323648 Richard E. Webster
333511 Ted Floyd
335339 Richard E. Webster

### B.3 Burke Museum of Natural History Samples

The following blood samples were obtained from the Burke Museum of Natural History:

50368
50374
50377
50378
50389
50390
50398
50403
50405
50416
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50425
50746
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50755
50756
50760
53585
53586
53587
53588
53590
53592
53594
53828
53829
53842
53849
72399
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