Song in a hybrid zone between Townsend's (*Setophaga townsendi*) and black-throated green (*S. virens*) warblers

by

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Abstract

Song is one of the most widely recognized premating barriers to reproduction between avian species. In oscines both genetic and cultural inheritance contribute to an individual’s song phenotype, contributing to difficulty in predicting the role that song may play in reproductive isolation; interspecific song learning could promote interbreeding. Here I seek to better understand this phenomenon by studying song in a narrow hybrid zone between black-throated green (Setophaga virens) and Townsend’s warblers (S. townsendi). I use multivariate analyses to compare songs in the hybrid zone to those found in allopatry, predicting that if song is a strong barrier to interbreeding, then there should be a relationship between song and genotype in the hybrid zone. I employ a genotyping-by-sequencing (GBS) method to identify thousands of markers and develop two hybrid indices. Playback experiments were carried out in the field to test responses to parental songs, and a cline analysis was conducted in order to compare transitions in genotype and song across the hybrid zone. I show that only parental song types are found in the hybrid zone, and furthermore, that there is little if any association between song and genotype in sympatric individuals; this suggests that song is not an important reproductive barrier. Allopatric individuals responded only to local songs, indicating that individuals may exhibit a learned response to songs that they are commonly exposed to. Out of thousands of genomic markers, I identified few that were diagnostic, suggesting that much of the genome may introgress freely between these species. I observed discordance between the song and genotype clines; the song cline was much narrower indicating that song is under stronger selection. Taken together, these findings combine to suggest that song is not a reproductive barrier in this hybrid zone, but is instead a cultural trait that is maintained by frequency-dependent selection. Thus, it may be more beneficial for males to sing a locally common song than to broadcast accurate species-membership information.
Preface

The research question and project design for this thesis were carried out collaboratively between my supervisor, Dr. Darren Irwin, and me. I carried out the majority of the field work for this project; however some recordings and blood samples were obtained in the field by Dave Toews, Darren Irwin and Alan Brelsford. The genotyping by sequencing library preparation and analysis was carried out in collaboration with Dr. Miguel Alcaide. I conducted all analyses and the writing of this thesis. Dr. Irwin assisted with interpretation of the results and provided helpful feedback.

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Chapter 1: Introduction

1.1 Speciation in birds

Reproductive isolation is requisite to speciation (Mayr 1943; Coyne and Orr 2004), and understanding speciation is central to understanding biodiversity. In birds, premating isolation may play a more important role in speciation than postmating isolation (Grant and Grant 1997a; Price and Bouvier 2002; Edwards et al. 2005); it appears that postmating barriers to reproduction, such as reduced hybrid reproductive success (e.g. Bronson et al. 2003) or inferior hybrid migratory routes (e.g. Delmore et al. 2012), may be relatively uncommon. Specifically, closely related avian taxa are thought to be most often reproductively isolated due to geography (Price 2008); however, many other premating barriers to reproduction, including habitat preference (e.g. Gee 2004), mate choice (e.g. Ratcliffe and Grant 1983) and breeding phenology (e.g. Moore et al. 2005) are also known to be important.

1.2 Song as a reproductive barrier

Geography aside, the most widely recognized potential premating barrier to reproduction between avian taxa is song. In many species, males sing in order to both advertise ownership of a territory and attract mates. Song is typically thought to serve as a strong reproductive barrier because males sing songs which serve as species membership signals. Females, in turn, mate assortatively with males singing conspecific songs to avoid any negative consequences of interbreeding (Catchpole and Slater 2008).
Both cultural and genetic inheritance contribute to song phenotype in oscines, adding considerable complexity to the role that song may play in reproductive isolation (Kroodsma 1982; Beecher and Brenowitz 2005; Catchpole and Slater 2008). Genotype can constrain song phenotype by influencing morphology or preference (Podos et al. 2004; Fehér et al. 2009). Culture, on the other hand, may introduce variation in song phenotype, as cultural mutation and transmission can occur with ease (Lynch 1996). For species in which song is not learned but is instead only genetically inherited, song phenotype is simple to understand: individuals sing songs which are reflective of their genotypes (Kroodsma 1984). On the other hand, for species in which cultural transmission also occurs, an individual’s song phenotype is less predictable (Lynch 1996). This complicated interplay between genes and memes, or units of cultural transmission (Dawkins 1976), in cases in which both contribute in determining an individual’s song, can confound the role that song may play as a barrier to interbreeding between avian species. Song learning can, on one hand, give rise to local dialects which substantially decrease interbreeding between conspecific populations (e.g. Baker and Mewaldt 1978), and on the other allow for interspecific learning of songs (e.g. Helb et al. 1985), potentially contributing to interbreeding between species (Grant and Grant 1997b; Kenyon et al. 2011). It is this ability of cultural inheritance to undermine the capacity of song to be an accurate species-membership signal that causes the role of song as a reproductive barrier to be unclear (Baptista 1985).

1.3 Hybrid zones are natural laboratories in the study of speciation

Here I investigate whether song acts as a reproductive barrier in a hybrid zone between two closely related oscine species. Hybrid zones, i.e. areas where distinct species come into contact and interbreed, are considered ‘natural laboratories’ in which we can study speciation (Szymura and Barton 1986; Hewitt 1988; Barton and Hewitt 1989; Rohwer and Wood 1998). Because hybrid zones represent
cases of incomplete reproductive isolation, they can be studied in order to understand which barriers to
reproduction may be central to bringing about divergence (Hewitt 1988). The opportunity to study the
varied genetic combinations often produced through crossing in lab settings (Barton and Hewitt 1985;
Dalziel et al. 2009), in conjunction with any naturally occurring cultural boundaries, makes a hybrid zone
an ideal scenario in which to examine the interplay between genes and memes.

Previous research on song in avian hybrid zones has found that culture often influences song
phenotype. Interspecific learning has been shown to play a role in song phenotype, producing either
heterospecific singers or blended songs, which consist of a combination of components from both
parental songs, in hybrid zones between pied (Ficedula hypoleuca) and collared flycatchers (F. albicollis)
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mourning warblers (G. philadelphia) (Kenyon et al. 2011). Further evidence that learning may affect
song’s role as a reproductive barrier is seen upon the examination of birds’ responses to song; in many
cases individuals living in sympatric regions respond to both conspecific and heterospecific songs
(Robbins et al. 1986; Baker 1991; Qvarnström et al. 2006), whereas in other cases individuals respond
only to conspecific songs (Patten et al. 2004). In spite of resounding evidence that learning affects both
song phenotype and the response to song in many hybrid zones, the fact that in some cases song
continues to act as a reproductive barrier (Baker and Mewaldt 1978; Baker and Boylan 1999a; Patten et
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et al. 2003; Kenyon et al. 2011) is puzzling. The variation seen in such studies provides no clear
conclusions about the relationship between genetic and cultural inheritance of song in determining
whether song is involved in reproductive isolation.
1.4 Study goals

In this study I aim to understand the role of song in reproductive isolation in a hybrid zone between two closely related oscine species. I measured individuals’ responses to different songs and develop hybrid indices using thousands of genetic markers. I examine the transitions in genotype and song across the hybrid zone and the relationship between them. In this way I determine whether song operates primarily as a genetically inherited species membership signal or a culturally transmitted meme between my focal species.
Chapter 2: Does song operate as a reproductive barrier or a culturally transmitted meme in the hybrid zone between black-throated green (*Setophaga virens*) and Townsend's (*S. townsendi*) warblers?

2.1 Introduction

2.1.1 Song as a reproductive barrier

Due to its role as a signal of species membership among birds it is expected that song should act as a reproductive barrier between closely-related species which come into contact. The fact that, in addition to genetically inherited components, the song an individual sings is also influenced by cultural transmission (Catchpole and Slater 2008, Irwin 2012) is perplexing, as interspecific learning could undermine the role of song in speciation (e.g. Kenyon et al. 2011). How the interaction between memes, or units of cultural transmission (Dawkins 1976), and genes determines how an individual’s song phenotype contributes to the speciation process is not well understood.

In order to better understand which barriers may be important in bringing about divergence we can study hybrid zones, where reproductive isolation is incomplete and species interbreed (Rohwer and Wood 1998). I seek to understand the role of song in reproductive isolation between species in a narrow avian hybrid zone: is song a genetically inherited species-membership signal that acts as a barrier to interbreeding or a culturally transmitted meme which can be shared between species?

2.1.2 The hybrid zone between black-throated green and Townsend's warblers

Townsend’s warblers (*Setophaga townsendi*) interbreed with the closely related black-throated green warbler (*S. virens*), from which they diverged over one million years ago (Weir and Schluter 2004),
in northeastern British Columbia (Fig. 1; Toews et al. 2011) in a region where many other avian species pairs come into contact (Weir and Schluter 2004; Toews and Irwin 2008; Brelsford and Irwin 2009; Irwin et al. 2009). Based on the examination of three molecular markers, this hybrid zone has been shown to be relatively narrow relative to dispersal, indicating that it is being maintained by some form of selection (Toews et al. 2011). If selection were not maintaining the hybrid zone, introgression would bring about a much more gradual transition in genotype across the contact zone (Barton and Hewitt 1985). I investigated whether song might play a role in the selection which maintains this hybrid zone.

2.1.3 Black-throated green and Townsend’s warbler song

Of these two species, song in the black-throated green warbler has been most extensively studied. Male black-throated green warblers have been shown to each sing two song types: Type A, or songs with unaccented endings, and Type B, or songs with accented endings (Morse 1967; Morse and Poole 2005). It has been suggested that the Type A song is predominately used by males in interactions with other males and that the Type B song is used by males in interactions with females (Morse 1967, 1970). An alternative, yet not dissimilar, explanation for the pattern of song use suggests that Type A is sung in response to any form of external stimulus, while Type B acts as a default song, and is sung otherwise (Lein 1972).

Patterns in Townsend’s warbler song are less clear, although some evidence suggests that Townsend’s warbler males also sing two song types (Wright et al. 1998). Furthermore, it has been speculated that these songs show similar functions to those of the black-throated green warbler with one song type used for aggressive interactions and the other for mating interactions (Spector 1992). The two songs of the Townsend’s warbler are referred to as Type I and Type II (Wright et al. 1998). It has
been suggested that the Type II song is more commonly used late in the breeding season and in disputes with other males (Spector 1992; Wright et al. 1998).

Because song type nomenclature varies across species (Spector 1992), here I will refer to song types which are thought to be used primarily in interactions with females as Type I songs and song types which are thought to be used primarily in interactions with other males as Type II songs.

2.1.4 Research questions and predictions

To investigate whether song plays a role in reproductive isolation in the hybrid zone between Townsend’s and black-throated green warblers, I explore several questions. First, I examine song and genotype across the hybrid zone separately. I ask if the allopatric differences in song between these two species are maintained in the hybrid zone between them. I also examine a large number of genetic markers to identify which ones differentiate between allopatric Townsend’s and black-throated green warblers and to describe the genetic background of each individual at a fine scale. Second, I ask if transitions in song across the hybrid zone correspond to transitions in genotype. If song is a strong reproductive barrier, songs and genotype should be highly correlated in the hybrid zone and transitions in song should mirror transitions in genotype. If, on the other hand, song operates as a culturally transmitted meme, I expect that frequency-dependent cultural selection on song will cause males to sing the locally common song (i.e. the song that is sung by other nearby males), regardless of their genetic background, thereby allowing them to defend a territory. This may result in discordance between song and genotype transitions across the hybrid zone and an absence of intermediate songs. Finally, I ask if birds from different sections of the transect across the hybrid zone respond differently to allopatric black-throated green and Townsend’s warbler songs. I expect that if song conveys important information, then allopatric birds may respond differently to conspecific and heterospecific songs.
2.2 Methods

2.2.1 Sampling

Field work for this study was conducted primarily in the Peace region of northeastern British Columbia, Canada along a transect between Mackenzie, BC and Dawson Creek, BC in May and June of 2012. Singing individuals were first recorded and then temporarily captured (< 15 minutes) using a local recording to attract them to a mist net placed within their territory. Each individual was banded using an aluminum leg band with a unique number as well as a unique combination of coloured plastic leg bands for future field identification by sight. A 20 µL blood sample was taken from the brachial vein of each individual captured and stored in lysis buffer. GPS coordinates were taken for locations of each recording and each bird captured. Eighty-three singing individuals were recorded and blood samples were taken from 42 individuals during the 2012 field season.

These samples were supplemented with recordings and blood samples collected between 2006 and 2009 by D. Toews (recordings from 39 individuals, 33 blood samples), D. Irwin (recordings from 33 individuals, 9 blood samples), and A. Brelsford (1 blood sample). In total, this study used recordings from 155 individuals and 85 blood samples (Fig. 1).

2.2.2 Recording analysis

Recordings were analyzed using Raven Pro 1.4. Songs recorded for each individual were qualitatively grouped into song “types” based on groupings previously defined in the literature (Morse 1967; Wright et al. 1998; Morse and Poole 2005) and visual similarity. Fifteen individuals were recorded singing two song types, while one individual was recorded singing three song types. Five songs of each
song type recorded for an individual were selected for measurement using a random number generator. Twenty-one song variables were measured including the number of unique syllable types, total number of syllables, number of repetitions of the first, second, third and fourth syllable types, total number of syllables per song, number of notes in the last syllable from part I and in the first syllable from parts II, III and IV, song duration, duration of parts I, II, III and IV, minimum and maximum song frequencies, minimum and maximum frequencies of the last repeat from part I, and minimum and maximum frequencies of the first repeat from part II (Table 1; Fig. 2). The mean and standard deviation of each variable were calculated for the five songs of each type recorded for every individual.

### 2.2.3 Song scores

I used principal component analyses (PCA) in R v.3.0.1 (DevelopmentTeam 2013) to examine song patterns. Using conservative definitions of allopatry in order to avoid including any intermediate birds, I conducted one PCA on allopatric black-throated green warbler songs, defined as songs found farther east than 95 km east of the crest of the Rocky Mountains (Toews et al. 2011), and another on allopatric Townsend’s warbler songs, defined as songs found to the west of 10 km east of the crest of the Rocky Mountains (Toews et al. 2011). This was done to determine whether songs in each allopatric region fell into the distinct types described in black-throated green warblers (Morse 1967) which were also suggested to exist in Townsend’s warblers (Wright et al. 1998). The allopatric black-throated green warbler PCA used 18 song variables (Table 1) which showed variation across the 33 songs included in the analysis. The allopatric Townsend’s warbler PCA used 18 song variables (Table 1) which showed variation across the 44 songs included in the analysis. I conducted a third PCA on all allopatric songs (i.e. from both species) using all 21 song variables measured and then applied the first two principal
components to songs from the hybrid zone to examine the relationship between allopatric songs and hybrid zone songs.

To determine which variables distinguished well between allopatric songs belonging to the two species, I calculated means and standard deviations in R v.3.0.1 (DevelopmentTeam 2013) for each song variable in allopatric Townsend’s and black-throated green warblers. Because the data were not normally distributed for each variable I tested for significance using a Mann-Whitney U-test.

I conducted a discriminant function analysis (DFA) using the MASS package in R v.3.0.1 (Venables and Ripley 2002; DevelopmentTeam 2013) to distinguish between black-throated green and Townsend’s warbler songs using the 19 song variables which vary across the two groups (Table 1). I used 33 allopatric black-throated green warbler songs and 44 allopatric Townsend’s warbler songs as the training data set and then determined the discriminant function (DF) scores of songs from the hybrid zone. I tested the accuracy of group assignment in two ways. First, I applied the discriminant function axis to the songs from the training data set and measured the proportion of songs re-assigned to the correct group based on their score along the DF axis. Second I used a jackknife method, omitting songs in sequence from repeated analyses, and again measured the proportion of assignments which were correct across all analyses.

2.2.4 Genotyping by sequencing library preparation

I used a modified genotyping by sequencing (GBS) approach (Elshire et al. 2011) to estimate the genetic background (i.e. hybrid index) of 85 individuals from in and around the hybrid zone. DNA was extracted from blood samples using a standard phenol-chloroform protocol.
To prepare the library I combined 2.4 ng of barcode adapters (6 µl at 0.4 ng/µl; sequences: 5’-ACACTTTTCCCTACACGACGCTCTTCCGATCTxxxxTGCA-3’ and 5’-xxxxAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG-3’, 2.4 ng of common adapters (6 µl at 0.4 ng/µl; sequences: 5’-AGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG-3’ and 5’-CTCGGCATTCTGCTGAACCGCTCTTCCGATCTTGCA-3’), and approximately 100 ng of genomic DNA (5 µl at 20 ng/µl) for each individual. I added 1 µl Fermentas FastDigest PstI restriction enzyme and 2 µl Fermentas universal FastDigest buffer per individual for a final volume of 20 µL, and then incubated at 37° C for two hours. The PstI enzyme cuts at sites with the nucleotide sequence CTGCAG. Once the restriction digest was complete, I added 1.6 µl New England Biolabs T4 ligase, 5 µl New England Biolabs 10x buffer and 23.4 µl ultrapure water to each reaction and incubated at 22° C for one hour to promote ligation of adapters and DNA fragments. This reaction was then inactivated by incubating for 10 minutes at 65° C. Fifteen µl of each ligation reaction were then cleaned using 23 µl of Beckman-Coulter AMPure XP beads in a magnetic plate. With the DNA bound to the beads at the sides of each well, the remaining liquid was removed from each well using a pipette. Two washes were conducted by adding 200 µl of 70% ethanol to each well, waiting 30 seconds and then removing the ethanol using a pipette while the DNA and beads are still bound to the sides of the well. After the second wash, the plate was left at room temperature for 5 minutes to allow the remaining ethanol to evaporate. The 96-well plate was then removed from the magnetic plate and the DNA and beads were resuspended in 40 µl of TE buffer. After mixing the contents of each well, the 96-well plate was placed back into the magnetic plate and the buffer, now containing the DNA, was removed and placed into a new 96-well plate. PCR amplification was then conducted by combining 2 µl cleaned genomic DNA, 0.5 µl New England Biolabs Phusion DNA polymerase, 10 µl New England Biolabs 5x Phusion buffer, 1 µl 10 mM dNTPs, 0.25 µl GBS-Primer A (sequence: 5’-AATGATACGGGACACCGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-3’), 0.25 µl GBS-Primer B (sequence: 5’-
CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT-3’) and 36 µl of ultrapure water. The thermal profile for the PCR reaction consisted of 1) 98°C for 30 seconds, 2) 20 cycles of 98°C for 20 seconds, 65°C for 30 seconds and 72°C for 30 seconds, and 3) 72°C for 5 minutes, followed by a 4°C holding temperature.

Four µl of each PCR product were pooled and run on a 1.5% agarose gel. The band between 350 and 450 base pairs was cut out of the gel and a gel extraction was performed using a Qiagen Gel Extraction Kit. The final concentration of the library was then measured to ensure that it was greater than 1 ng/µl in a volume of at least 20 µl. All concentrations were measured using an Invitrogen Qubit dsDNA BR Assay Kit and a Qubit 2.0 Fluorometer. An additional quality control step was conducted using an Agilent Technologies 2100 Bioanalyzer to ensure that the genomic DNA fragments were of the expected size (350-450 bp).

The library was then sequenced on the Illumina HiSeq 2000 platform at the University of British Columbia Biodiversity Next Generation Sequencing Facility using paired end read sequencing.

2.2.5 Genotyping

I used the program Geneious v6 to handle, manipulate and analyze the sequence data obtained from the GBS library (Drummond et al. 2011). The two FastQ files associated with the two Illumina sequencing primers were imported into Geneious and then set as paired-end reads. The resulting list of more than 400 million DNA sequences was then sorted by barcode. I conducted a de novo assembly on a single allopatric Townsend’s warbler individual with a large number of reads (approximately 5 million). The contigs, or continuous regions of overlapping sequence fragments, generated by this de novo assembly were concatenated into a single artificial reference sequence in which the consensus sequences for each contig were added in a random order and separated by 25 Ns in order to facilitate
the subsequent reads mapping process. I then annotated all candidate overhang PstI cut sites and putative associate barcode tags in the consensus sequence by searching for the motifs NNNNNNNNTGCAG or CTGCANNNNNNN in order to later avoid calling genetic variations within barcode adapter sequences where insert sizes are small. I then aligned the reads of six allopatric Townsend’s warblers and six allopatric black-throated green warblers (using the same conservative definitions of allopatry) to the artificial reference and annotated regions with coverage higher than 600 reads (approximately 50 reads per individual). I searched for single nucleotide polymorphisms (SNPs) that were variants within the stack of reads from different individuals which mapped to a given position in the concatenated assembly, rather than those that were variants with regards to the artificial reference. To do this I set the minimum variant frequency to 0.15 (to find genetic markers which are highly differentiated between the two species) and the minimum coverage to 72 reads (approximately 6 reads per individual), and ignoring both regions which were annotated as PstI cut sites, which may contain sequenced barcode adapter, and regions which were annotated to have coverage higher than 600 reads, which are likely to be regions repeated in the genome. After putative polymorphic positions were identified and annotated the GBS reads of all 85 birds were mapped against the reference consensus from the previous step in which degenerate bases were placed at loci where minimum variant frequency was at least 15%; including degenerate bases in the reference is critical for the subsequent genotyping process. Once I obtained an assembly for each individual the genotyping of each bird was accomplished by analyzing the array of base calls that aligned to a particular position labeled as a polymorphic site using a degenerate base in the previous step. For this step minimum variant frequency was set to 0.20 (a commonly used threshold to detect heterozygotes) and minimum coverage set to 3 reads.

I obtained two sets of SNP data with a low impact of missing data (at most 10%), each of which was used to construct a separate hybrid index. Two hybrid indices were used to examine different
aspects of genetic differentiation between the two species. Pivot tables were used to transform the original Geneious tables into the input format suitable to run a wide array of genetic analysis in the platform GenAlex ver 6.5 (Peakall and Smouse 2006, 2012). The first set of markers focused on loci which were highly differentiated between allopatric Townsend’s and black-throated green warblers ($F_{st}$ > 0.75). Local BLASTn against the zebra finch genome assembly was run to estimate the origin of each of the fragments analyzed. Where two markers were found to be in close physical linkage, the SNP with the least missing data was retained in the data set. This left 27 markers in this first data set. The second set of markers was used to obtain a more general pattern of genome-wide differentiation and did not include any pairwise $F_{st}$ value filtering. For each data set all markers suspected to involve repetitive loci (e.g. those with significant excess heterozygosity) were removed. To control for sequencing or PCR artifacts, I considered only those variants which were found in at least two individuals. Where two SNPs were called in the same GBS fragment and strong evidence of linkage disequilibrium was observed, only one was included in the data set. This left 4406 SNPs in this second data set.

### 2.2.6 Hybrid index scores

In order to compare the genotypes of individuals sequenced, I created hybrid index scores for each of the SNP data sets produced. The GBS method does not sequence every individual at every locus, so despite the fact that there was relatively even coverage for all 85 individuals genotyped, there are data missing from some individuals at some loci. This resulted in data missing from both the high $F_{st}$ marker data set and the genome-wide marker data set. To establish a hybrid index for each of these data sets I conducted a non-linear iterative partial least squares (NIPALS) principal component analysis (PCA), which accounts for missing data, on the genotypes of allopatric individuals using the pcaMethods package (Stacklies et al. 2007) in R v.3.0.1 (DevelopmentTeam 2013).
2.2.7 Song and genotype relationship

To understand the relationship between a bird’s genotype and its song in the hybrid zone, I examined the correlation, first, between high $F_{st}$ marker PC 1 score and song DF score and, second, between genome-wide marker PC 1 score and song DF score. In order to exclude allopatric birds with certainty, here I defined the hybrid zone as the area between 20 and 80 km east of the crest of the Rocky Mountains (Toews et al. 2011). Because the data were non-normal I used the coin package in R v.3.0.1 (Hothorn et al. 2006; DevelopmentTeam 2013) to conduct a Spearman test of independence based on Monte Carlo resampling to test for the significance of these relationships.

Because song differs between allopatric regions, it will tend to be correlated with genotype over broad areas that include both species. In a third analysis I examined how this correlation changes as the area examined shrinks toward the center of the hybrid zone, indicating whether birds can use song to predict the genotype of a singer where the two species meet. Beginning with a 160 km wide region, centered on the approximate center of the hybrid zone (46 km east of the crest of the Rocky Mountains) and extending well into allopatry (Toews et al. 2011), I repeatedly decreased the width by 20 km (i.e. 10 km to the east and 10 km to the west of the center, thereby keeping the center constant) until the study region was only 20 km wide. This allowed me to compare the relationship between genotype and song in regions of decreasing width, eventually focusing on only the very center of the hybrid zone. A Spearman test of independence based on Monte Carlo resampling was used to test the significance of the relationship between high $F_{st}$ marker PC 1 score and song DF score at each interval, but only $R^2$ values were compared across analyses.
2.2.8 Cline analysis

To examine the relative strengths of selection and the changes in both song and genotype across the hybrid zone I used C-Fit 7 (Gay et al. 2008) to conduct a cline analysis, fitting sigmoid clines to trait scores across the hybrid zone. Because I had few allopatric data points far from the hybrid zone I did not fit the tails of the clines. Initially I fit clines for the genome-wide hybrid index score, the high F\textsubscript{st} hybrid index score and the song DF score. I then used only the high F\textsubscript{st} hybrid index and song scores for a more detailed cline analysis, because the cline in the genome-wide hybrid index score has high uncertainty. Based on my previous analyses I treated the high F\textsubscript{st} hybrid index score as a quantitative trait that exhibits a unimodal distribution at each location across the hybrid zone and the song DF score as a quantitative trait that exhibits a bimodal distribution across the hybrid zone. To determine if the transitions in genotype and song across the hybrid zone coincide, I constructed pairs of clines using varying sets of assumptions about the centers and slopes of these transitions and determined which best fit the data. I constructed clines for four different sets of constraints on cline parameters: 1) no constraints, 2) cline slopes constrained to be equal, 3) cline centers constrained to be equal and 4) both cline centers and slopes constrained to be equal. I calculated Akaike Information Criterion (AIC) scores to compare the four cline analyses and used a likelihood ratio test to compare the fit of the model with no constraints (1) to the three other analyses.

2.2.9 Playback experiments

Playback experiments were conducted in the field in June 2012 on three groups of individuals: allopatric Townsend’s warblers, allopatric black-throated green warblers and individuals from the hybrid zone. A paired design was employed for the playback experiments; each individual was exposed to both
a black-throated green warbler song and Townsend’s warbler song on consecutive days. The recordings used were made in allopatry so that the singing individual’s genetic and cultural identity was certain and the recording species order was randomized for each individual. Each recording was used only once within each of the three groups (Kroodsma 1986). Experiments were conducted within an individual’s pre-determined territory and began only once the individual was observed to be present. A speaker playing the song was placed at the estimated center of each individual’s territory and the observers sat 15 m away. Each experiment began with a seven minute observation period followed by a seven minute playback period in which either an allopatric Townsend’s warbler or black-throated green warbler recording was played on a loop. Throughout the 14-minute experiment the individual’s horizontal distance from the speaker was recorded at 15-second intervals. Experiments in which the individual could not be located for the majority of the time period were either aborted or later omitted from analysis. Playback experiments were conducted on 10 allopatric Townsend’s warblers, 15 allopatric black-throated green warblers and 12 individuals from within the hybrid zone. Experiments were conducted only on banded individuals from inside the hybrid zone so that the genetic background of these individuals could be determined.

I used the nlme package (Pinheiro et al. 2013) in R v.3.0.1 (DevelopmentTeam 2013) to create a linear mixed-effects model to analyze response to playbacks for birds from each group. The response variable, ‘closest approach to the speaker’, was examined during the observation and playback periods of experiments featuring either black-throated green or Townsend’s warbler recording treatments. Closest approach to the speaker was transformed to achieve normality by adding one meter, and then log transforming. ‘Individual’ was included as a random effect to control for multiple experiments on the same bird due to the experiment’s paired design. I used pairwise Tukey-Kramer tests in the package multcomp (Hothorn et al. 2008) to test for differences in mean ‘closest approach to the speaker’ across treatments.
2.3 Results

2.3.1 Allopatric songs

In allopatry black-throated green warbler songs are highly stereotyped (Fig. 3 A-D). Only two song types are observed among black-throated green warbler songs recorded east of the hybrid zone. One is composed of three syllable types, the first of which is repeated several times while the second and third syllables are sung only once. The second song type is buzzier in quality and consists of four syllable types. The final syllable type of both song types has the highest frequency of the song. Allopatric Townsend’s warbler songs, on the other hand, show greater variation (Fig. 3 E-H). This variation does not appear to correspond with geography, as individuals from the same area for whom multiple song types were recorded do not necessarily sing the same two songs. These songs tend to have either two or three syllable types and, like black-throated green warbler songs, typically end on the syllable type with the highest frequency.

Song in allopatric black-throated green warblers falls into two distinct PCA clusters (Fig. 4). PC1 explains 59.8% of the variation in allopatric black-throated green warbler song. ‘Number of unique syllable types’, ‘number of repetitions of the fourth syllable type’, ‘number of notes in the first syllable of part IV’ and ‘duration of part IV’ have the highest loadings on this axis (Table 1). ‘Minimum song frequency’, ‘duration of part I’, ‘maximum song frequency’ and ‘song duration’ load highest on PC 2, which explains 15.2% of the variation.

In contrast, a PCA of allopatric Townsend’s warblers shows an absence of distinct song clusters (Fig. 5). PC 1 explains 35.1% of the variation in allopatric Townsend’s warbler song. ‘Number of repetitions of the third syllable type’, ‘duration of part III’, ‘number of notes in the first syllable from part III’ and ‘number of unique syllable types’ have the highest loadings on this axis (Table 1). 'Number of
notes in the first syllable from part II’, ‘number of notes in the last syllable from part I’ and ‘duration of part II’ load highest along PC 2, which explains 22.8% of the variation in song.

A discriminant function analysis distinguished well between allopatric black-throated green warbler song, regardless of type, and Townsend’s warbler song. Duration variables load highest along the discriminant function axis (Table 1). The misclassification rate when re-assigning groups to allopatric individuals based on the DFA axis was 1.0%, whereas the misclassification rate using a jackknife method was 7.8%. The mean values of 19 of the 21 song variables are significantly different between songs of allopatric black-throated green and Townsend’s warblers (Table 1). The only variables that did not differ between the two species were ‘number of unique syllable types’ and ‘minimum song frequency.’

2.3.2 Song in the Hybrid Zone

Songs from the hybrid zone tend to be qualitatively similar to those of either allopatric black-throated green (Fig. 6 A-D) or Townsend’s warblers (Fig. 6 E-H) and fit well within the discrete clusters of allopatric songs determined by a PCA describing variation in all allopatric songs (Fig. 7). PC 1 explains 40.4% of the variation in song and the variables which load the highest on this axis include ‘number of notes in the first syllable from part II’, ‘duration of part II’, ‘number of notes in the last syllable from Part I’ and ‘total number of syllables’ (Table 1). ‘Number of unique syllable types’, ‘number of repetitions of the third syllable type’ and ‘duration of part III’ are the variables which load highest on PC 2 which explains 15.2% of the variation in song.

Songs from the hybrid zone are designated with a high degree of certainty as either black-throated green or Townsend’s warbler songs using the DF axis (Fig. 8). Songs from the hybrid zone show either a high posterior probability of being a black-throated green warbler song and a low posterior
probability of being a Townsend’s warbler song, or a low posterior probability of being a black-throated green warbler song and a high posterior probability of being a Townsend’s warbler song. No songs from the hybrid zone are equally likely to be a black-throated green warbler song and a Townsend’s warbler song. Hence all songs in the hybrid zone can be clearly identified as similar to either black-throated green warbler or Townsend’s warbler songs; no blended songs are found in the hybrid zone.

2.3.3 Genetic patterns contrast dramatically with the discrete clusters seen in song

Out of 4406 SNPs identified in the genomic analysis, only three independent fixed SNP loci were identified between Townsend’s and black-throated green warblers based on the de novo assembly. A PCA of the 27 SNPs from the high Fst marker data set shows that many individuals of intermediate genotype are found in the hybrid zone (Fig. 9A). PC 1 explains 89.6% of the variation in genotype between allopatric Townsend’s and black-throated green warblers and all 27 markers load approximately equally on the axis. PC 2 explains only 2.3% of the variation in genotype.

A PCA of the 4406 SNPs from the genome-wide marker data set similarly shows that many intermediate individuals are found along the transect (Fig. 9B). PC 1 explains 8.6% of the variation in genotype between allopatric Townsend’s and black-throated green warblers whereas PC 2 explains 1.4% of this variation.
2.3.4 The relationship between genotype and song in the hybrid zone

No significant relationship was found in the hybrid zone (the region from 20 to 80 km east of the crest of the Rocky Mountains) between the high F\textsubscript{st} marker hybrid index score and song DF score (Spearman test of independence $R^2 = 0.07$, $P=0.48$; Fig. 10A) or between the genome-wide marker hybrid index score and song DF score (Spearman test of independence $R^2 = 0.06$, $P=0.92$; Fig. 10B).

Furthermore, when examining the relationship between the high F\textsubscript{st} marker hybrid index score and song DF score at decreasing intervals toward the center of the hybrid zone, we see song cannot be predicted based on high F\textsubscript{st} marker hybrid index score near the center of the hybrid zone (Fig. 11). Only when individuals as far as 70 km from the center of the hybrid zone were included in the analysis (i.e. a zone at least 140 km wide was examined) was a statistically significant relationship between hybrid index score and song uncovered (Spearman test of independence $R^2 = 0.29$, $P=0.003$).

2.3.5 Song and genotype clines

Cline analysis indicates that transitions in genotype and song across the hybrid zone do not closely coincide. Estimates of slope and center for the high F\textsubscript{st} marker hybrid index score and the song DF score clines vary across four different sets of constraints on parameters (Table 2). A Likelihood ratio test indicates that the model in which each cline has its own center and slope is the best fit to the data (log-likelihood=-345.02, AIC= 720.04). According to this model the center of the broader high F\textsubscript{st} marker hybrid index score cline (center=34.47 km East of the crest of the Rocky Mountains, width=slope/4=93 km (Gay et al. 2008); Fig. 12B) is broader and further west of the narrower song DF hybrid index score cline (center=43.87 km East of the crest of the Rocky Mountains, width=22 km; Fig. 12C). The estimated slope of the genome-wide hybrid index score cline (slope=0.04) is similar to that of the high F\textsubscript{st} marker
hybrid index score cline (Fig. 12A). These analyses indicate that the shift in the two discrete song types occurs over a much smaller distance than would be predicted by the genomic cline.

2.3.6 Playback experiments

Playback experiments demonstrated that birds in allopatry show a significant response to playback only when exposed to conspecific songs (Fig. 13). Allopatric black-throated green warblers approached the speaker 80% closer during a conspecific song playback than during the corresponding observation period ($z=3.71$, $P=0.001$), but showed no significant response to heterospecific song ($z=0.81$, $P=0.85$; Fig. 13C). Similarly, allopatric Townsend’s warblers approached the speaker 84% closer during a conspecific song playback than during the corresponding observation period ($z=3.49$, $P=0.003$), but showed no significant response to heterospecific song ($z=-0.32$, $P=0.99$; Fig. 13A).

In the hybrid zone no significant differences were seen in an individual’s ‘closest approach to the speaker’ between the observation period and the playback period of experiments using either song type (black-throated green warbler playback experiments: $z=1.61$, $P=0.37$; Townsend’s warbler playback experiments: $z=1.97$, $P=0.20$; Fig. 13B). Despite this lack of significance, mean ‘closest approach to the speaker’ during playback periods of both black-throated green and Townsend’s warbler songs in the hybrid zone are similar to approach distances observed in response to conspecific songs in allopatry.

In summary, the playback experiments showed that in allopatry birds respond strongly only to conspecific songs, while in the hybrid zone there are no significant differences seen in response.
2.4 Discussion

The existence of a contact zone between black-throated green and Townsend’s warblers (Toews et al. 2011) facilitates the study of song as a potential reproductive barrier between species in which individual males sing multiple song types. The role that song can play as a reproductive barrier has been addressed extensively in the literature (Baker and Mewaldt 1978; Grant and Grant 1996; Irwin 2000; Slabbekoorn and Smith 2002; Patten et al. 2004; Qvarnström et al. 2006; Price 2008; Toews and Irwin 2008), yet cultural transmission of song has not always been well integrated into these discussions (Slabbekoorn and Smith 2002). The goal of this study was to use a combination of detailed genetic clines, song recording analysis and playback experiments to determine whether song operates primarily as a species-membership signal or as a culturally transmitted meme in a region in which hybridization occurs at relatively high frequencies, but species differences are maintained by some form of selection against hybrids (Toews et al. 2011).

I found evidence for extensive hybridization between Townsend’s and black-throated green warblers, corroborating earlier work on these taxa which was conducted using fewer markers (Toews et al. 2011). Using SNP data obtained through a modified genotyping-by-sequencing method (Elshire et al. 2011), I found many individuals with intermediate genotypes in the region of contact. A surprisingly small proportion of SNPs discovered (27 out of 4406) were highly differentiated between these species, indicating that interspecific gene flow may be higher than previously suspected. The juxtaposition of substantial gene flow between species with the existence of a narrow hybrid zone between them indicates that selection on some small section of the genome may be maintaining species differences between Townsend’s and black-throated green warblers. A small number of genomic islands of divergence have recently been shown to maintain differences between parapatric Heliconius butterflies in the face of extensive introgression (Martin et al. 2013). I suggest that a similar genomic pattern may
exist in the taxa studied here; a more fine scale genomic analysis in Townsend’s and black-throated green warblers is warranted based on my observations.

In order to compare songs from the hybrid zone to those sung in allopatry I first characterized song in allopatric black-throated green and Townsend’s warblers using a separate principal component analysis for each species. While the allopatric black-throated green warblers sampled here sing only the two song types which have been previously characterized across the black-throated green warbler breeding range (Morse 1967; Spector 1992; Morse and Poole 2005), song in allopatric Townsend’s warblers is much less stereotyped. Instead of the expected two clusters in the PCA of allopatric Townsend’s warbler songs, much greater variation is observed. This variation in song cannot be solely attributed to geographic clustering across the Townsend’s warbler breeding range. This pattern suggests that song may be more complex in Townsend’s warblers, with the possibility of more than two song types being sung across individuals. It remains unclear as to whether song usage patterns in Townsend’s warblers mirror those in black-throated green warblers and other congeners (e.g. Morse 1967; Price and Crawford 2013) where one song type is sung in territorial interactions and the other in mating interactions, but such a stereotyped pattern is unlikely due to the variation described here in Townsend’s warbler songs. Canada warblers (Cardellina canadensis) have been recently shown to share phrases between the two song types that they sing (Demko et al. 2013). Similar mixing of song types could produce the lack of clear distinction between song types I observed in Townsend’s warbler songs.

Using a discriminant function analysis I demonstrated that black-throated green and Townsend’s warbler songs can be easily distinguished from one another regardless of within-species song type. It is interesting to note that the variables that distinguish best between Townsend’s and black-throated green warblers are duration variables; a change in duration variables has been previously suggested to
have strong effects on the ability of Indigo buntings (*Passerina cyanea*) to recognize the song of their own species (Emlen 1972).

Both the PCA conducted based on all allopatric songs and the posterior probabilities from the DFA which I conducted to distinguish allopatric Townsend’s warbler songs from allopatric black-throated green warbler songs indicate that no clearly intermediate songs are being sung in the hybrid zone. Instead I found only parental species song types. I would expect to find intermediate songs in the hybrid zone if either 1) genetic inheritance dominates song transmission and song is not controlled by a single dominant locus or 2) cultural transmission contributes strongly to song phenotype, but songs are not inherited as single units (i.e. components of song are transmitted independently). On the other hand, if songs have a strong cultural component and are transferred as distinct units (i.e. memes), then we would see no song blending and little association with genetic background.

Despite the fact that only parental song types are found in the hybrid zone, an individual’s genotype is not a good predictor of its song. In fact, at the center of the hybrid zone the correlation between genotype and song is close to zero, indicating that song is a signal that carries little or no accurate species membership information where the highest potential for interbreeding exists (Barton and Hewitt 1985). This indicates that song is not an important reproductive barrier between black-throated green and Townsend’s warblers. Similar patterns have been attributed to indiscriminate song learning in hybrid zones between other taxa (Baker and Boylan 1999a; Secondi et al. 2003; Kenyon et al. 2011).

The width of clines in various traits is often used to infer the strength of selection on those traits; all else being equal, a narrower cline indicates stronger selection (Barton and Hewitt 1985). I found that the song cline across the hybrid zone is greater than four times more narrow than that of a genetic index for markers which are highly differentiated between Townsend’s and black-throated green
warblers, indicating stronger selection on song than on genotype. Based on this evidence, along with the discovery that only parental songs are sung in the hybrid zone, I suggest that song may be under frequency-dependent selection whereby locally common songs are favored over novel songs (Lynch 1996; Price 2008). Furthermore, results from the playback experiment show that both allopatric Townsend’s and black-throated green warbler males respond more strongly to the local song than to heterospecific song. Similar results have been found in playback experiments of ‘coo’ vocalizations in a hybrid zone between Streptopelia doves, where males producing non-local ‘coo’s are thought to be unable to hold territories in a given area (den Hartog et al. 2008). Here I suggest that it may be advantageous for males to sing locally common songs in order to hold a territory, as the local song appears to be perceived as a stronger threat (Baker et al. 1981; Searcy et al. 1997; Wilson et al. 2000).

Ironically, frequency-dependent cultural selection on song may, in fact, promote interbreeding between Townsend’s and black-throated green warblers. In much of the hybrid zone females are exposed to the song of only one parental species and are thereby presented with no species-membership information through song by which to choose a mate, and even in the narrow region of overlap between songs there is no relationship between genotype and song. As a result, females in the hybrid zone are not presented with accurate song information with which to choose a conspecific mate.

In conclusion, I have shown that no intermediate songs are sung in the northeastern British Columbia hybrid zone between Townsend’s and black-throated green warblers, despite the extensive genetic hybridization that occurs there. Furthermore, no relationship exists between genotype and song in sympatry, indicating that song is unlikely to be an important reproductive barrier between these species. Instead, it appears that song in the hybrid zone is under frequency-dependent cultural selection whereby males are more likely to maintain a territory when singing a locally common song. The fact that this selection on song is stronger than selection on genotype may, in fact, promote interbreeding
between sympatric Townsend’s and black-throated green warblers. In addition, I found evidence suggesting that introgression may occur across much of the genome while small regions may be responsible for maintaining genetic differences between these taxa. This warrants further investigation at a finer scale. It appears that in this hybrid zone song operates as a culturally transmitted meme rather than as a genetically-inherited species membership signal.
Table 1: Differences between allopatric Townsend’s (TOWA) and black-throated green (BTNW) warbler songs for 21 song variables and those variables’ loadings in multivariate analyses. Variables with N/A loading values were not included in the given analysis.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Allopatric TOWA</th>
<th>Allopatric BTNW</th>
<th>Mann-Whitney U-Test (df=1)</th>
<th>PC 1 loading (analysis of all allopatric songs)</th>
<th>DFA loading</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Number of unique syllable types</td>
<td>2.2 0.4 0.33</td>
<td>3.5 0.5 -0.30</td>
<td>638.5 0.36</td>
<td>-0.34</td>
<td>-0.71</td>
</tr>
<tr>
<td>2. Total number of syllables</td>
<td>5.5 1.1 -0.16</td>
<td>5.4 0.8 0.18</td>
<td>1369.5 &lt;0.0001</td>
<td>0.10</td>
<td>-0.69</td>
</tr>
<tr>
<td>3. Number of repetitions of the first syllable type</td>
<td>3.6 1.0 -0.25</td>
<td>2.3 1.6 0.27</td>
<td>400.5 0.0008</td>
<td>0.27</td>
<td>-0.55</td>
</tr>
<tr>
<td>4. Number of repetitions of the second syllable type</td>
<td>1.6 0.5 -0.30</td>
<td>1.0 0.0 N/A</td>
<td>313.5 &lt;0.0001</td>
<td>0.25</td>
<td>0.83</td>
</tr>
<tr>
<td>5. Number of repetitions of the third syllable type</td>
<td>0.3 0.6 0.35</td>
<td>1.5 0.5 -0.30</td>
<td>1315 &lt;0.0001</td>
<td>-0.33</td>
<td>0.83</td>
</tr>
<tr>
<td>6. Number of repetitions of the fourth syllable type</td>
<td>0.0 0.0 N/A</td>
<td>0.5 0.5 -0.30</td>
<td>1122 &lt;0.0001</td>
<td>-0.29</td>
<td>N/A</td>
</tr>
<tr>
<td>7. Number of notes in the last syllable from part I</td>
<td>1.2 0.3 0.04</td>
<td>1.0 0.03 N/A</td>
<td>576 0.006</td>
<td>0.06</td>
<td>2.59</td>
</tr>
<tr>
<td>8. Number of notes in the first syllable from part II</td>
<td>1.5 0.8 -0.02</td>
<td>1.0 0.0 0.03</td>
<td>544.5 0.004</td>
<td>0.12</td>
<td>-1.72</td>
</tr>
<tr>
<td>9. Number of notes in the first syllable from part III</td>
<td>0.5 0.9 0.35</td>
<td>1.0 0.0 N/A</td>
<td>1171.5 &lt;0.0001</td>
<td>-0.21</td>
<td>0.12</td>
</tr>
<tr>
<td>10. Number of notes in the first syllable from part IV</td>
<td>0.0 0.0 N/A</td>
<td>0.5 0.5 -0.30</td>
<td>1122 &lt;0.0001</td>
<td>-0.29</td>
<td>N/A</td>
</tr>
<tr>
<td>11. Duration of song (s)</td>
<td>1.3 0.2 0.02</td>
<td>1.5 0.7 -0.09</td>
<td>1064 0.0004</td>
<td>-0.12</td>
<td>12.15</td>
</tr>
<tr>
<td>12. Duration of part I (s)</td>
<td>0.7 0.2 -0.28</td>
<td>0.7 0.7 0.04</td>
<td>528 0.04</td>
<td>0.05</td>
<td>-12.28</td>
</tr>
<tr>
<td>13. Duration of part II (s)</td>
<td>0.4 0.1 -0.04</td>
<td>0.3 0.1 -0.27</td>
<td>319 &lt;0.0001</td>
<td>0.09</td>
<td>-5.62</td>
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<tr>
<td>14. Duration of part III (s)</td>
<td>0.1 0.1 0.35</td>
<td>0.3 0.2 -0.28</td>
<td>1286 &lt;0.0001</td>
<td>-0.32</td>
<td>-11.75</td>
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<td>15. Duration of part IV (s)</td>
<td>0.0 0.0 N/A</td>
<td>0.1 0.1 -0.29</td>
<td>1122 &lt;0.0001</td>
<td>-0.29</td>
<td>-18.33</td>
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<tr>
<td>16. Minimum frequency (Hz)</td>
<td>4111.6 395.7 -0.19</td>
<td>4024.1 247.2 0.001</td>
<td>536 0.05</td>
<td>0.09</td>
<td>0.002</td>
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</tr>
<tr>
<td><strong>17. Maximum frequency (Hz)</strong></td>
<td>7164.3</td>
<td>564.2</td>
<td>-0.19</td>
<td>6913.9</td>
<td>399.1</td>
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<tr>
<td><strong>18. Minimum frequency of last syllable from part I (Hz)</strong></td>
<td>4353.0</td>
<td>317.4</td>
<td>-0.11</td>
<td>5086.8</td>
<td>346.5</td>
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<td><strong>19. Maximum frequency of last syllable from part I (Hz)</strong></td>
<td>5401.1</td>
<td>513.1</td>
<td>-0.006</td>
<td>6100.3</td>
<td>485.9</td>
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<tr>
<td><strong>20. Minimum frequency of the first syllable from part II</strong></td>
<td>4952.1</td>
<td>1000.3</td>
<td>-0.26</td>
<td>4456.0</td>
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<td><strong>21. Maximum frequency of the first syllable from part II</strong></td>
<td>6826.9</td>
<td>797.0</td>
<td>-0.33</td>
<td>5536.6</td>
<td>423.3</td>
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Table 2: Cline analysis for high $F_{st}$ hybrid index score and song DF score for four different sets of parameter constraints 1) no constraints on slope or center, 2) slopes constrained to be equal, 3) centers constrained to be equal and 4) both centers and slopes constrained to be equal. A likelihood ratio test was conducted to compare the parameter set with no constraints on slope or center (1) to the other sets.

<table>
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<th></th>
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<th>Center constrained</th>
<th>Center and slope constrained</th>
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<td>High $F_{st}$ score</td>
<td>Song DF score</td>
<td>High $F_{st}$ score</td>
<td>Song DF score</td>
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<tr>
<td>Center (km)</td>
<td>34.47</td>
<td>43.87</td>
<td>5.81</td>
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<td>Slope</td>
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<td>-0.18</td>
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<td>-356.94</td>
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<td>755.43</td>
<td>726.01</td>
<td>739.88</td>
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<tr>
<td>Likelihood ratio test P-value</td>
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<td>&lt;0.005</td>
<td>&lt;0.001</td>
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Figure 1: Breeding ranges of Townsend’s warblers (blue) and black-throated green warblers (green) (Wright et al. 1998; Morse and Poole 2005; Ridgely et al. and BirdLife International 2012). The region
encompassing the hybrid zone, where the majority of recordings (n=133) and blood samples (n=72) were obtained, is denoted with a rectangle. Additional allopatric sites where blood samples were obtained (White Pass, WA (n=3); Todagin, BC (n=1); Squamish, BC (n=2); Prince George, BC (n=1); Whitecourt, AB (n=1); and Lesser Slave Lake, AB (n=5)) are denoted by filled black dots. Additional allopatric sites where recordings were made (Squamish, BC (n=2); Manning Park, BC (n=2); Fernie, BC (n=6); Prince George, BC (n=4); Whitecourt, AB (n=4); and Lesser Slave Lake, AB (n=4)) are denoted by hollow black circles.
Figure 2: An illustration of the twenty-one song variables measured on a simplified illustration of a song spectrogram: (A) 1: ‘number of unique syllable types’, 3: ‘number of repetitions of the first syllable type’, 4: ‘number of repetitions of the second syllable type’, 5: ‘number of repetitions of the third syllable type’ and 6: ‘number of repetitions of the fourth syllable type’; (B) 2: ‘total number of syllables’, 7: ‘number of notes in the last syllable from part I’, 8: ‘number of notes in the first syllable from part II’, 9: ‘number of notes in the first syllable from part III’, and 10: ‘number of notes in the first syllable from part IV’; (C) 11: ‘song duration’ (s), 12: ‘duration of part I’ (s), 13: ‘duration of part II’ (s), 14: ‘duration of part III’ (s), 15: ‘duration of part IV’ (s), 16: ‘minimum song frequency’ (kHz), and 17: ‘maximum song frequency’ (kHz); (D) 18: ‘minimum frequency of the last repeat from part I’ (kHz), 19: ‘maximum frequency of the last repeat from part I’ (kHz), 20: ‘minimum frequency of the first repeat from part II’ (kHz), and 21: ‘maximum frequency of the first repeat from part II’ (kHz).
Figure 3: Examples of allopatric song spectrograms: allopatric black-throated green warbler songs from the Dawson Creek, BC area: (A) Type I song and (B) Type II song and from Lesser Slave Lake, AB: (C) Type I song (D) Type II song; (E-H) allopatric Townsend’s warbler songs from the Mackenzie, BC area show higher variation and do not cluster well into discrete song types.
Figure 4: Principal component analysis of all allopatric black-throated green warbler songs distinguishes clearly between song types. PC 1 explains 59.8% of the variation in allopatric black-throated green warbler song while PC 2 explains 15.2%.
Figure 5: Principal component analysis of allopatric Townsend's warbler songs shows no clearly distinct song types. PC 1 explains 35.1% of the variation in allopatric Townsend's warbler song, while PC 2 explains 22.8%.
Figure 6: Examples of song spectrograms from the hybrid zone. Spectrograms A-D are black-throated green warbler-like songs, while spectrograms E-H are Townsend’s warbler-like songs.
Figure 7: Plotting the first two axes from a principal component analysis of allopatric songs applied to both allopatric (light green: allopatric black-throated green warbler (BTNW) type I song, dark green: allopatric black-throated green warbler type II song, blue: Townsend’s warbler (TOWA) song) and hybrid zone songs (grey) shows that songs from the hybrid zone fall into similar clusters to allopatric songs. PC 1 explains 40.4% of the variation in song between allopatric black-throated green and Townsend’s warblers, while PC 2 explains 15.2%.
Figure 8: Discriminant function analysis Posterior Probabilities for hybrid zone songs shows that most songs are classified clearly as being either a black-throated green warbler (BTNW) or Townsend’s warbler (TOWA) song, with none having roughly equal assignment probability.
Figure 9: Genotype principal component analysis results show many individuals of intermediate genotype in the hybrid zone (A) PCA on 27 SNPs with Fst>0.75 between allopatric Townsend’s (TOWA) and black-throated green (BTNW) warblers (B) PCA on 4406 genome-wide SNPs. Both PCAs were conducted on only allopatric individuals and the resulting first two axes were applied to individuals from the hybrid zone.
Figure 10: In the hybrid zone an individual’s hybrid index score has no significant relationship to its song when (A) hybrid index is defined using the high $F_{st}$ marker hybrid index score ($R^2=0.06735$, $P=0.4781$) or (B) hybrid index is defined using the genome-wide hybrid index score ($R^2=0.06423$, $P=0.9244$).
Figure 11: The $R^2$ value representing the relationship between high $F_{st}$ marker PC 1 score and song DF score for study areas of varying widths centered on 46 km east of the crest of the Rocky Mountains, the approximate center of the hybrid zone.
Figure 12: Genotype and song clines across the black-throated green and Townsend’s warbler hybrid zone. (A) A genome-wide hybrid index score cline is estimated to be centered at 21.07 km east of the Rocky Mountains and have a slope of -0.0406. Clines for (B) high F$_{st}$ hybrid index score and (C) song DF...
score for the best fitting parameter set where cline centers and slopes are not constrained to be equal are displayed. The high F_{st} hybrid index score is centered at 34.47 km east of the Rocky Mountains with a slope of -0.043 and the song DF score is centered at 43.87 km east of the Rocky Mountains with a slope of -0.
Figure 13: Closest approach to the speaker (mean ± SE) during playback experiments exposing (A) allopatric Townsend’s warblers, (B) individuals from the hybrid zone and (C) allopatric black-throated green warblers to allopatric black-throated green (BTNW) warbler or Townsend’s warbler (TOWA) songs.
Chapter 3: Conclusions

3.1 General conclusions

The goal of this study was to determine whether song operates as a reproductive barrier in the hybrid zone between black-throated green and Townsend’s warblers and to understand how song phenotype is affected by the interplay between genetic and cultural inheritance. This hybrid zone is known to be relatively narrow indicating that selection is acting to maintain it (Toews et al. 2011) and it was hypothesized that song could play a role in maintaining species differences.

3.1.1 Song behaves as a culturally transmitted trait, not a reproductive barrier

I showed that there is no relationship between a bird’s song and genotype, suggesting that song is not a strong barrier to interbreeding. Furthermore, I show that the transition in song across the hybrid zone is much narrower than that of genotype. I suggest that the narrow song transition in conjunction with the absence of blended songs in the hybrid zone indicates that song is maintained by frequency-dependent cultural selection; males likely learn the locally common song regardless of their genetic background. The idea that males may be better able to defend a territory when singing a locally common song is supported by playback experiments. The behavior of song as a cultural, rather than genetic, trait may promote interbreeding, as singing males in the hybrid zone do not broadcast accurate species-membership information for females to use to choose a conspecific mate.
3.1.2 Narrow clines in culturally inherited traits do not indicate important reproductive barriers

I found discordance between genotype and song clines across the hybrid zone. Most striking was the fact that the song cline was much narrower than the genotype cline, indicating stronger selection on song. Typically traits which have steeper clines are under stronger selection across the hybrid zone and as a result are more important reproductive barriers (Barton and Hewitt 1985; Szymura and Barton 1986). Here I show that this is not always the case. The lack of agreement between genotype and song clines in conjunction with the more fine-scaled analysis showing no relationship between genotype and song in the hybrid zone clearly indicates that despite experiencing strong selection, song is not a barrier to interbreeding between Townsend’s and black-throated green warblers and instead likely contributes to hybridization. The very narrow transition in song observed across the hybrid zone does not indicate that song is a strong reproductive barrier between these species due to the fact that selection on memes, rather than on genes, appears to be playing the dominant role in shaping song phenotype.

3.1.3 Selection may act on a small section of the genome

Along with the confirmation of widespread interbreeding occurring in the hybrid zone between Townsend’s and black-throated green warblers, few diagnostic markers were found in a broad genomic analysis. This is indicative of either the occurrence of ongoing substantial introgression between black-throated green and Townsend’s warblers or the fact that these species diverged very recently. The latter explanation is highly unlikely, as mitochondrial DNA divergence estimates that these species are over one million years old (Weir and Schluter 2004). As it therefore follows that this pattern is likely due to substantial gene flow between these species, it is interesting that the narrow hybrid index cline
indicates selection on genotype in the hybrid zone. An explanation for these apparently opposing patterns is that much of the genome may be shared between black-throated green and Townsend’s warblers, but a small section may be under strong selection and involved in maintaining the differences between these species. Small regions which may control other potentially strong reproductive barriers such as migratory behavior or plumage could be involved. Further investigation of the genome at a finer scale will be key to better understanding this pattern.

3.2 Future directions

3.2.1 Future directions in the black-throated green and Townsend’s warbler hybrid zone

In order to better understand song in the hybrid zone between Townsend’s and black-throated green warblers I suggest that an effort should be made to gain more knowledge of the song types found in the Townsend’s warbler breeding range. Here I have shown that allopatric Townsend’s warbler song types do not follow the highly stereotyped patterns in use which are seen across the black-throated green warbler breeding range (Morse 1967; Morse and Poole 2005). Increased sampling of recordings, observational studies and playback experiments at a finer scale across the Townsend’s warbler breeding range may uncover geographic clusters in song or gather contextual information associated with song types which I was not able to elucidate through limited sampling. If these undoubtedly more complex patterns in song type usage do exist in the Townsend’s warbler repertoire it would be interesting to compare homologous song types across the hybrid zone. Perhaps song types used in different contexts undergo slightly different transitions across the hybrid zone.

In addition to learning more about song in allopatric Townsend’s warblers, I suggest that a genomic analysis be conducted at a finer scale on these species. For this study I was relatively
conservative in selecting markers with which to construct hybrid indices. Whole genome sequencing would provide much higher resolution across the genome. I believe that the next step in this study is to search for divergence peaks in order to identify which regions may be responsible for maintaining species differences in the face of introgression across the majority of the genome, evidence for which was found in this study.

Finally, this study focused on black-throated green and Townsend’s warbler males by examining a male-specific behavioural trait. Furthermore, only male territorial responses to song were measured. While I have little information about female behaviour in this hybrid zone, it is noteworthy that female preferences can promote the choice of heterospecific males as mates (Brodsky and Weatherhead 1984; Pearson and Rohwer 2000). Female choice has been shown to greatly influence hybrid zone dynamics in other systems in this way. I suggest that the cultural nature of song may promote interbreeding between black-throated green and Townsend’s warblers because females may rarely be exposed to songs of both species. Quantifying female responses to black-throated green and Townsend’s warbler songs, while logistically difficult, would clarify whether females prefer locally common songs or show an affinity for the song of one species over the other.

3.2.2 Future directions for the study of song as a reproductive barrier

Here I have shown that it is important to take learned inheritance of song into account when examining song as a reproductive barrier between oscine species. Through the integration of song analysis, cutting-edge molecular techniques, cline theory and playback experiments I found that culture dominates genotype in determining song phenotype in the hybrid zone between black-throated green and Townsend’s warblers. Many studies examining the role of song as a reproductive barrier focus exclusively on a single aspect of this complex phenotype. This disregard of the broader context in which
song operates at species boundaries may contribute to the a lack of consensus which has been observed across different systems (Slabbekoorn and Smith 2002). I suggest that in future studies the use of methods similarly integrated to my own will help us to understand whether any generalizations about the role of song as a reproductive barrier between species can be made.
References


