

# Cultural isolation is greater than genetic isolation across an avian hybrid zone

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## Abstract

Elucidating the relationship between genetic and cultural evolution is important in understanding speciation, as learned pre-mating barriers might be involved in maintaining species differences. Here, we test this relationship by examining a widely recognized pre-mating barrier, bird song, in a hybrid zone between black-throated green (*Setophaga virens*) and Townsend's warblers (*S. townsendi*). We use song analysis, genomic techniques and playback experiments to characterize the cultural and genetic backgrounds of individuals in this region, expecting that if song is an important reproductive barrier between these species, there should be a strong relationship between song and genotype. We show that songs in the hybrid zone correspond to the distinctly different songs found in allopatry but that song and genotype are not tightly coupled in sympatry. Allopatric individuals responded only to local songs, indicating that individuals may have learned to respond to songs they commonly hear. We observed discordance between song and genotype clines; a narrower cline suggests that cultural selection on song is stronger than natural selection on genotype. These findings indicate that song is unlikely to play a role in reproductive isolation between these species, and we suggest that spatial variation in song may nonetheless be maintained by frequency-dependent cultural selection. This decoupling of genes and culture may contribute to hybridization in this region.

## Introduction

Culture and genes often coevolve within populations, as genetic background and learned behaviours each influence the selective regimes that the other is exposed to (Payne, 1996; Irwin, 2012) and genetic background can constrain cultural evolution (Gardner *et al.*, 2005; Fehér *et al.*, 2009). These units are often characterized as undergoing analogous processes in evolution (Dawkins, 1976; Lynch & Baker, 1993; Feldman & Laland, 1996; Lynch, 1996; Wilkins, 1998), yet

they have some notably different properties. In comparison with genes, culture is far less constrained to vertical transmission and tends to be more susceptible to the introduction of new variation (Lynch, 1996; Cardoso & Atwell, 2011). Selection on genetically inherited traits occurs at the level of the individual in terms of how many offspring it leaves, whereas culture can experience selection independently of the fitness of the individual, via transmission through social learning. These factors can allow cultural evolution to produce different patterns compared to genetic evolution (Feldman & Laland, 1996). Such differences can be especially interesting when examining the evolution of pre-mating barriers between species: many traits that can serve as pre-mating reproductive barriers, and thereby affect the transfer of genes to future generations, are themselves culturally transmitted. For example, learned mate choice preferences have been demonstrated in many organisms including guppies (Magurran & Ramnarine, 2004), fruit flies (Dukas, 2005) and birds (Irwin &

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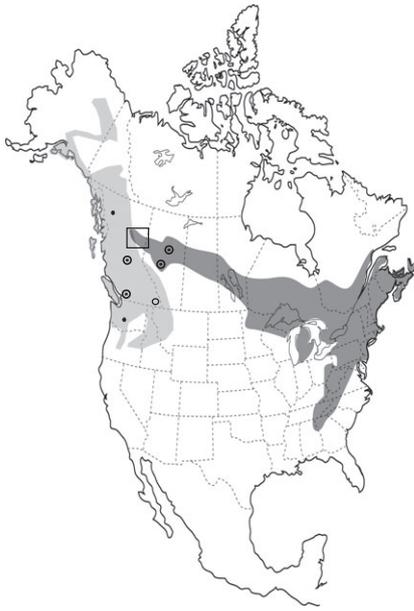
Price, 1999) and can serve as highly effective reproductive barriers (Servedio *et al.*, 2009). Intertaxon learning, however, can undermine the role that such traits play in speciation. Examples of this phenomenon have been observed in birds (e.g. Grant & Grant, 1997b; Kenyon *et al.*, 2011). Within a single species, such as humans, patterns in learned traits often do not correspond well to intraspecific genetic clustering (Cavalli-Sforza, 1997). Here, we seek to better understand the interplay between genetic and cultural evolution and how it influences speciation.

Birds are an ideal taxonomic group in which to investigate this dynamic (Irwin, 2012), as (1) premating isolation is thought to play a very important role in avian speciation (Grant & Grant, 1997a; Price & Bouvier, 2002; Edwards *et al.*, 2005) and (2) bird song is a widely recognized species-membership signal that is partially learned in some groups, especially the oscine passerines (Catchpole & 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 & Brenowitz, 2005; Catchpole & Slater, 2008; Irwin, 2012; Rowell & Servedio, 2012). Genotype can constrain song phenotype by influencing morphology or the preference for which songs are learned (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; Payne, 1996; Irwin, 2012), but can also constrain song phenotype when song is under strong selection to be uniform in a given area (e.g. Baker *et al.*, 1981).

For avian species in which song is not learned but is only genetically inherited (i.e. most nonosine songbirds), individuals sing songs that are reflective of their genotypes (e.g. Kroodsma, 1984). For species in which cultural transmission also occurs (e.g. most oscine passerines – the ‘songbirds’, parrots, some hummingbirds), an individual’s song phenotype may be less predictable, as cultural transmission of song is relatively independent of genetic inheritance (Lynch, 1996). This interplay between genes and culture might complicate the role that song plays as a reproductive barrier between avian species. Song learning can, on the one hand, give rise to local dialects which might substantially decrease interbreeding between conspecific populations (e.g. MacDougall-Shackleton & MacDougall-Shackleton, 2001) and, on the other, allow for interspecific learning or blending of songs in areas of overlap (Helb *et al.*, 1985; Haavie *et al.*, 2004; Olofsson & Servedio, 2008), potentially contributing to gene flow between species (Grant & Grant, 1997b; Kenyon *et al.*, 2011). The sometimes-opposing forces of genetic and cultural evolution cause the role of song as a reproductive barrier to be unclear (Baptista, 1985).

Hybrid zones, or areas where distinct taxa come into contact and interbreed, are considered ‘natural laboratories’ for the study of speciation (Szymura & Barton, 1986; Hewitt, 1988; Barton & Hewitt, 1989; Rohwer & Wood, 1998), and sophisticated analytical approaches involving cline theory (e.g. Barton & Hewitt, 1985) have been developed to understand the selective forces occurring in hybrid zones. However, detailed clinal analyses of hybrid zones have focused primarily on studies of genetic variation and morphological traits that are likely underlain by genetic variation; cultural traits have received comparatively little attention in the context of hybrid zones. Here, we investigate whether song acts as a reproductive barrier in a hybrid zone between two closely related songbird species. Because hybrid zones represent cases of incomplete reproductive isolation, they can be studied to understand which reproductive barriers may be central to bringing about divergence (Hewitt, 1988). The opportunity to study a wide variety of genetic combinations (Barton & Hewitt, 1985; Dalziel *et al.*, 2009), in conjunction with any naturally occurring cultural variants, makes a hybrid zone an ideal scenario in which to examine the interplay between genes and memes. Song has occasionally been demonstrated to become decoupled from other traits in avian hybrid zones (e.g. from genetic structure: Sattler *et al.*, 2007; and from plumage: Greig & Webster, 2013). Furthermore, previous studies have shown variation in the way that song operates in hybrid zones; in some cases, song appears to promote assortative mating (e.g. Patten *et al.*, 2004; Dingle *et al.*, 2010), whereas in others it may contribute to hybridization (e.g. Baker & Boylan, 1999; Secondi *et al.*, 2003; Kenyon *et al.*, 2011), especially in contact zones where heterospecific songs are fairly similar to begin with (Willis *et al.*, 2013). Here, we combine song analysis, genomic analysis and playback experiments in a narrow hybrid zone between Townsend’s (*Setophaga townsendi*) and black-throated green (*S. virens*) warblers to test the hypothesis that song acts as a reproductive barrier between these species; the main prediction of this hypothesis is that songs and genes are closely coupled in the area where these species come into contact.

Townsend’s warblers and black-throated green warblers are estimated to have started diverging over 1 million years ago (Weir & Schluter, 2004) and now interbreed in a region of north-eastern British Columbia (Fig. 1; Toews *et al.*, 2011) where many other avian species pairs come into contact (Weir & Schluter, 2004; Toews & Irwin, 2008; Brelsford & Irwin, 2009; Irwin *et al.*, 2009). Based on the examination of plumage characteristics and three molecular markers, this hybrid zone has been inferred to be narrow relative to dispersal, indicating that it is maintained by some form of selection (Toews *et al.*, 2011). If selection were not maintaining the hybrid zone, introgression would bring



**Fig. 1** Map of Townsend's (light grey) and black-throated green (dark grey) warbler breeding ranges. These species come into contact and interbreed in the box in the Peace region of north-eastern British Columbia (Toews *et al.*, 2011); this area was sampled extensively. Additional locations that were sampled are also represented here; open circles represent allopatric locations where song recordings were made, whereas filled points represent allopatric locations where blood samples were collected. Allopatric locations where both song recordings and blood samples were collected have an open circle surrounding a filled point.

about a much more gradual transition in genotype across the contact zone (Barton & Hewitt, 1985).

Of these two species, song in the black-throated green warbler has been most extensively studied. In regions far to the east of the hybrid zone, each male black-throated green warbler sings two highly stereotyped song types: one which is used primarily in interactions with other males, and another that tends to be used in interactions with females (Morse, 1967, 1970; Morse & Poole, 2005). These song types exhibit a high degree of similarity across individuals. Patterns in Townsend's warbler song are less clear; although it appears that Townsend's warbler males sing two song types, which have been hypothesized to function similarly to those of black-throated green warblers, the roles they play have not yet been well characterized (Spector, 1992; Wright *et al.*, 1998). Because song type nomenclature varies across species (Spector, 1992), we will refer to song types that are thought to be used primarily in interactions with females as type I songs and song types that are thought to be used primarily in interactions with other males as type II songs.

Here, we test predictions of the hypothesis that song acts as a reproductive barrier between Townsend's and

black-throated green warblers, asking how closely coupled songs and genes are across the hybrid zone between them. To do this, we first examine song and genetic variation separately. We ask whether the allopatric differences in song between these two species are maintained in the hybrid zone between them, or alternatively, whether we observe blended songs in the hybrid zone. We also use high-throughput DNA sequencing to examine a large number of genetic markers to identify those that differentiate between allopatric Townsend's and black-throated green warblers and subsequently describe the genetic background of each individual at a fine scale. To test the hypothesis that song serves as a reproductive barrier between Townsend's and black-throated green warblers, we examine the relationship between song and genotype in the hybrid zone. We expect that if song serves as a strong reproductive barrier between these species, then song and genotype should be highly correlated in the hybrid zone and transitions in song should mirror transitions in genotype. Subsequently, we conduct playback experiments to determine whether interspecific song differences elicit different behavioural responses across species. We compare the ways that males (both allopatric males breeding near to the hybrid zone and males breeding within the hybrid zone) respond to allopatric Townsend's and black-throated green warbler songs. We expect that if song differences are biologically meaningful, then allopatric birds will respond differently to conspecific and heterospecific songs.

## Materials and methods

### Field sampling

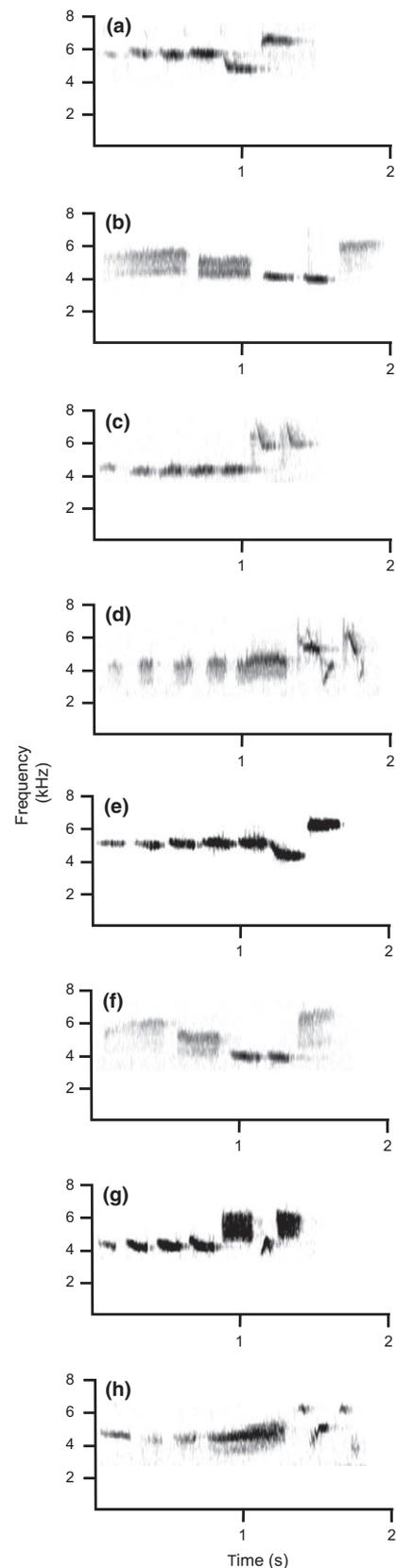
Sampling was conducted primarily in the Peace region of north-eastern British Columbia, Canada, along a transect between Mackenzie, BC, and Dawson Creek, BC, through the centre of the hybrid zone (Toews *et al.*, 2011) in May and June of 2007, 2008, 2009 and 2012. Additional sampling in allopatric regions in British Columbia and Alberta was conducted between 2006 and 2012 (Fig. 1). Males were first recorded singing (in most cases at least ten songs), and then temporarily captured using a local recording to attract them to mist nets placed within their territories. Recordings were made on a Marantz PMD660 solid state recorder with an Audio-Technica 815a microphone. We held each individual for < 15 min and fitted them with a numbered aluminium leg band and a unique combination of coloured plastic leg bands for future field identification by sight. A 5- to 20- $\mu$ L blood sample was taken from the brachial vein of each captured individual and stored in 500  $\mu$ L of 'Queen's lysis buffer' (0.01 m Tris, 0.01 m NaCl, 0.01 m EDTA and 1% *n*-lauroylsarcosine, pH 7.5; Seutin *et al.*, 1991). We took GPS coordinates

for locations of each recording and each bird captured. In total, this study used 85 blood samples and recordings from 169 individuals.

### Song recording analysis

We analysed song recordings using Raven Pro 1.4 (Bioacoustics Research Program). We qualitatively grouped songs from each individual into song ‘types’ based on groupings previously defined in the literature (Morse, 1967; Wright *et al.*, 1998; Morse & Poole, 2005) and visual similarity of song spectrograms; in the previous literature, individuals from both species have been described as having two song types. Of 169 individuals recorded, we captured two song types from fifteen individuals during the recording period. In addition, one allopatric Townsend’s warbler individual was recorded singing three song types, which, to our knowledge, has not been documented previously. We selected five songs of each song type recorded for an individual for measurement using a random number generator. We measured twenty-one song variables (Table S1; Fig. S1), which together capture most acoustic differences among the recorded song types (Fig. 2). We conducted all measurements manually on spectrograms in Raven Pro 1.4 using Hann spectrogram windows with 512 samples, 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. For each individual, we calculated the mean and standard deviation of each variable for each song type recorded.

We conducted principal component analyses (PCAs) in R v.3.0.1 (R Core Team 2013) to examine song patterns. Using conservative definitions of allopatry to avoid including any hybrid birds, we conducted one PCA on allopatric black-throated green warbler songs, defined as the mean song measurements for each individual 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 the mean song measurements for each individual 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 two distinct types previously described in black-throated green warblers (Morse, 1967), and which were also suggested to exist in Townsend’s warblers (Wright *et al.*, 1998). For individuals that were recorded singing multiple song types, data from only one randomly chosen song type per



**Fig. 2** Song spectrogram examples: allopatric black-throated green warbler songs from the area Dawson Creek, BC: (a) type I song and (b) type II song; allopatric Townsend’s warbler songs from the area Mackenzie, BC (c, d), show higher variation and do not cluster well into discrete song types; and songs from the hybrid zone (e–h) tend to be similar to parental song types.

individual were included in the analysis. The allopatric black-throated green warbler PCA used 18 song variables (Table S1) that showed variation across the mean song measurements for 29 individuals included in the analysis. The allopatric Townsend's warbler PCA used 18 song variables (Table S1) that showed variation across the mean song measurements for 42 individuals included in the analysis. We 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. PCA axis loadings were calculated in R.

To determine which variables distinguished well between allopatric songs belonging to the two species, regardless of song type, we calculated means and standard deviations for each song variable in allopatric Townsend's and black-throated green warblers. Because the data were not normally distributed for each variable, we used Mann-Whitney U-tests to test for significance. We also calculated Hedges's *g* (Hedges, 1981) to show the amount of divergence in each variable between allopatric populations.

We conducted a discriminant function analysis (DFA) using the MASS package in R v.3.0.1 (Venables & Ripley, 2002; R Core Team 2013) to distinguish between black-throated green and Townsend's warbler songs using the 19 song variables that vary within each of the allopatric groups; two syntax variables showed no variation among allopatric Townsend's warblers (i.e. were always zero values) due to a shorter song structure in this group and were therefore omitted from the analysis (see Results; Fig. 2c,d; Table S1). We again used mean song measurements of 29 allopatric black-throated green warblers and 42 allopatric Townsend's warblers as the training data set and then determined the discriminant function scores ('song score') of songs from the hybrid zone. To avoid pseudoreplication, only one randomly chosen song type per individual was included in the analysis ( $n = 169$ ). We tested the accuracy of group assignment in two ways. First, we applied the discriminant function axis to the songs from the training data set and measured the proportion of songs reassigned to the correct group based on their score along the DF axis. Second, we used a jackknife method, repeatedly running new discriminant function analyses and omitting the song of a different individual from each analysis until all individuals had been omitted once. Accuracy was again assessed by measuring the proportion of assignments that were correct across all analyses.

## Genotyping

We used a modified genotyping-by-sequencing (GBS) approach (Elshire *et al.*, 2011) similar to the approach

described by Alcaide *et al.* (2014) to estimate the genetic background (i.e. hybrid index score) of 85 individuals from in and around the hybrid zone. We extracted DNA from blood samples using a standard phenol-chloroform protocol.

We used the program Geneious v6 (Biomatters) to analyse the sequence data obtained from the GBS library. The two FastQ files associated with the two Illumina sequencing primers were imported into Geneious and then set as paired-end reads. We sorted the resulting list of more than 400 million DNA sequences by barcode. We used a single allopatric Townsend's warbler individual with a large number of reads (approximately 5 million) to assemble a reference library of GBS reads, for the purpose of aligning reads from other individuals to this reference. We concatenated more than 300 000 contigs, or continuous regions of overlapping sequence fragments, from this individual into a single artificial reference sequence (approximately 60 Mb) in which the consensus sequences for each contig were added in a random order and separated by 25 Ns to facilitate the subsequent reads mapping process. We then annotated all candidate overhang PstI cut sites and putative associate barcode tags in the consensus sequence by searching for the motifs NNNNNNNNTGCAG or CTGCANNNNNNNN to later avoid calling genetic variations within barcode adapter sequences where insert sizes are small. We then aligned the reads of six allopatric Townsend's warbler individuals and six allopatric black-throated green warbler individuals (using the above conservative definitions of allopatry) to the artificial reference and annotated regions with coverage higher than 600 reads (approximately 50 reads per individual) to avoid calling SNPs in such regions, which likely are the result of multiple areas in the genome having similar sequences. We searched for single nucleotide polymorphisms (SNPs) that were variants within the stack of reads from different individuals that mapped to a given position in the concatenated assembly, rather than those that were variants with regard to the artificial reference. To do this, we set the minimum variant frequency (the minimum frequency of the rare allele for each SNP remaining after filtering) to 0.15 (to increase our chances of finding genetic markers that are highly differentiated between the two species) and the minimum coverage to 72 reads (approximately six reads per individual; average coverage of our GBS regions per individual was 18X). We did not search for SNPs in regions that were annotated as either PstI cut sites or as having coverage higher than 600 reads. After we identified putative polymorphic positions and annotated them using degenerate bases, we mapped the GBS reads of all 85 birds against the reference consensus from the previous step. Once we obtained an assembly for each individual, we genotyped each bird by analysing the array of base calls that aligned to each position labelled with a degenerate base (i.e. polymorphic). For this step, the

minimum variant frequency was set to 0.20 (a commonly used threshold to detect heterozygotes) and minimum coverage set to three reads.

The set of SNPs we obtained had a low impact of missing data (at most 10%) and was used to construct a hybrid index to examine genetic differentiation between Townsend's and black-throated green warblers. We used GenALEX 6.5 (Peakall & Smouse, 2006, 2012) to run a wide array of genetic analyses on this data set. We ran local BLASTn against the zebra finch genome assembly to estimate the genomic location of each of the fragments analysed. Where two markers were either 1) found to be in close physical linkage based on local BLASTn or 2) were called in the same GBS fragment and showed strong evidence of linkage disequilibrium, only the SNP with the least missing data was retained in the data set. We removed all markers suspected to involve repetitive loci (e.g. those with significant excess heterozygosity) and variants that were found in only one individual (to control for sequencing or PCR artefacts). This resulted in a large set of 4406 markers that did not include any pairwise  $F_{st}$  value filtering (Fig. S3B). Of these markers, we identified 27 loci that were highly differentiated between allopatric Townsend's and black-throated green warblers ( $F_{st} > 0.75$ ).

We conducted a principal component analysis to develop a hybrid index for this highly differentiated data set (i.e. the 27 SNPs with  $F_{st} > 0.75$ ; Fig. S3). The GBS method does not sequence every individual at each locus, so despite the fact that there was relatively even coverage for all 85 individuals genotyped, there are data missing from some individuals at certain loci in our data set. For this reason, we conducted a nonlinear iterative partial least squares (NIPALS) principal component analysis (PCA), which accounts for missing data, on the genotypes of allopatric individuals ( $n = 25$ ) using the *pcaMethods* package (Stacklies *et al.*, 2007) in R v.3.0.1 (R Core Team 2013). The resulting first principal component, PC1, differentiated well between allopatric forms and explained 89.6% of the variation in genotype, and was therefore used as the hybrid index; an individual's PC1 score based on its genotype at all of these 27 highly differentiated loci was used as its hybrid index score. Hybrid index scores calculated by the same methods but using the entire genomewide SNP data set consisting of 4406 markers were highly correlated with this hybrid index ( $r^2 = 0.8$ ,  $P < 0.0001$ ).

### Song and genotype relationship

To test the hypothesis that song and genotype are tightly coupled in the hybrid zone, such that song can act as an indicator of genotype and thereby function as a reproductive barrier between Townsend's and black-throated green warblers, we examined the relationship between hybrid index score and song score for birds in

the hybrid zone ( $n = 33$ ). To exclude allopatric birds with certainty, we conservatively 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, we used the *coin* package in R v.3.0.1 (Hothorn *et al.*, 2006; R Core Team 2013) to conduct a Spearman test of independence based on Monte Carlo resampling to test for the significance of this relationship.

Song and genetic background differ between allopatric regions, so the two will tend to be correlated when sampling across a broad region that includes both species. Hence, in a second analysis we examined how the relationship between hybrid index score and song score changes as the area examined shrinks towards the centre 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, centred on the approximate centre of the hybrid zone (46 km east of the crest of the Rocky Mountains) and extending well into allopatry (Toews *et al.*, 2011), we repeatedly decreased the width by 20 km (i.e. 10 km to the east and 10 km to the west of the centre, thereby keeping the centre constant) until the study region was only 20 km wide. This allowed us to compare the relationship between genotype and song in regions of decreasing width, eventually focusing only on the very centre of the hybrid zone. We used a Spearman test of independence based on Monte Carlo resampling to test the significance of the relationship between hybrid index score and song score at each interval, but only  $r_s^2$  values were compared across analyses.

### Cline analysis

To examine whether the changes in song and genotype across the hybrid zone were concordant (i.e. had the same width) and coincident (i.e. were centred in the same location), we used C-Fit 7 (Gay *et al.*, 2008) to conduct cline analyses, fitting sigmoid clines to trait scores across the hybrid zone. Based on both previous analyses (Toews *et al.*, 2011) and information introduced in the present genomic analysis (see Results; Fig. 7), we treated hybrid index score as a quantitative trait that exhibits a unimodal distribution at each location across the hybrid zone and song score as a quantitative trait that exhibits a bimodal distribution (see Results; Figs 5 and 7) across the hybrid zone. To determine whether the transitions in genotype and song across the hybrid zone coincide, we constructed pairs of clines using varying sets of assumptions about the centres and slopes of these transitions and determined which best fit the data while appropriately minimizing the number of parameters. We constructed clines for four different sets of constraints on cline parameters: (1) no constraints, (2) cline slopes for genotype and song constrained to be equal, (3) cline centres

constrained to be equal and (4) both cline centres and slopes constrained to be equal. We 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.

### Playback experiments

To examine patterns of song recognition, we conducted song playback experiments in the field in June 2012 on three groups of individuals: allopatric Townsend's warblers ( $n = 9$ ), allopatric black-throated green warblers ( $n = 14$ ) and individuals within the hybrid zone ( $n = 12$ ). We exposed most individuals to both a black-throated green warbler recording and Townsend's warbler recording on consecutive days, randomizing treatment order for each individual and using recordings made in allopatry so that the genetic and cultural identities of singing individuals were certain. Several individuals were exposed to only a single treatment (allopatric Townsend's warblers  $n = 4$ , hybrid zone individuals  $n = 1$ , allopatric black-throated green warblers  $n = 3$ ) and were also included in analyses. Each recording was used only once within each of the three groups to avoid pseudoreplication (Kroodsma, 1986), and its natural song rate was maintained (Townsend's warbler =  $4.9 \pm 1.6$  songs  $\text{min}^{-1}$ ; black-throated green warbler =  $4.9 \pm 1.0$  songs  $\text{min}^{-1}$ ). We conducted experiments within an individual's predetermined territory and began each experiment only once the focal individual was present. A speaker playing the song was placed at the estimated centre of each individual's territory and the observers sat 15 m away. Each experiment began with a 7-min observation period followed by a 7-min playback period in which either an allopatric Townsend's warbler or allopatric black-throated green warbler recording (mean recording duration = 2 min, 14 s) was played on a loop. Throughout the 14-min experiment, we recorded the individual's horizontal distance from the speaker within 15-s time intervals.

We used the nlme package (Pinheiro *et al.*, 2013) in R v.3.0.1 (R Core Team 2013) to create linear mixed-effects models to analyse response to playbacks for birds from each group. We examined the response of 'closest approach to the speaker' to a categorical predictor variable with four possible values describing the experimental period (observation/playback) and the recording treatment (black-throated green warbler/Townsend's warbler) in which each observation was made. Closest approach to the speaker in metres was transformed to achieve normality: for black-throated green warblers and hybrid zone birds by adding one metre and then log-transforming, and for Townsend's warblers by adding one metre and then square-root-transforming (no statistical comparisons were made between target groups, enabling us to use different transformations on

each group). 'Individual' was included as a random effect to control for multiple experiments on the same bird due to the paired design of the experiment. Based on these models, we used pairwise Tukey–Kramer tests in the package multcomp (Hothorn *et al.*, 2008) in R v.3.0.1 (R Core Team 2013) to test for differences in mean 'closest approach to the speaker' across treatments.

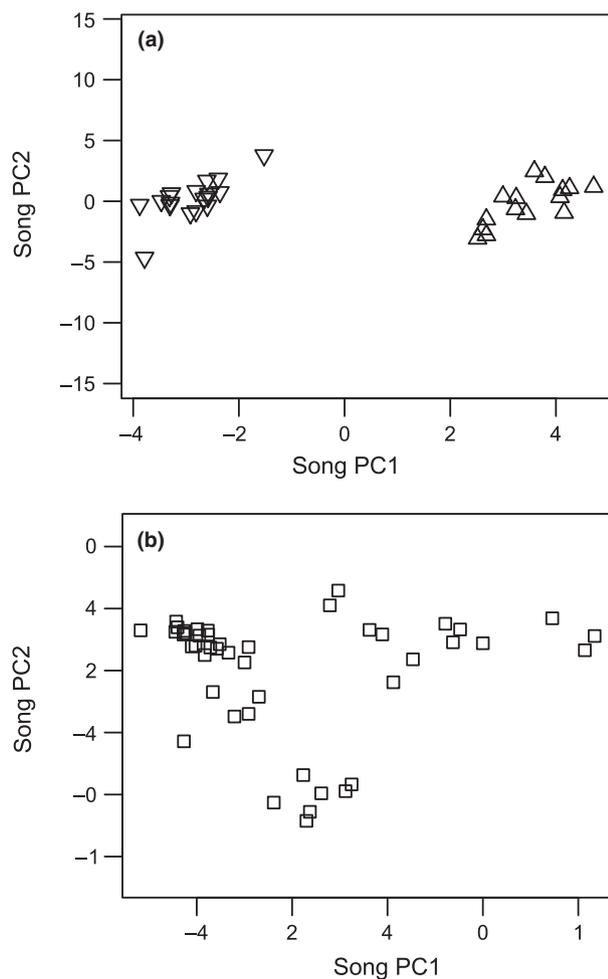
## Results

### Allopatric songs

Qualitatively, allopatric black-throated green warbler songs are highly stereotyped; we observed only two song types (Fig. 2a,b) among the black-throated green warbler songs recorded east of the hybrid zone. One song type is composed of three syllable types, the first of which is repeated several times, whereas the second and third syllables are sung only once. The second song type is buzzy in quality and consists of four syllable types, of which only the third is repeated (i.e. sung twice). The final syllable type of both song types has the highest frequency of the song. Allopatric Townsend's warbler songs (examples in Fig. 2c,d), on the other hand, show greater variation among individuals; when birds from the same area were recorded singing multiple song types, they were different song types as those being sung by other individuals nearby. These songs tend to have either two or three types of syllables within each song and, like black-throated green warbler songs, typically end on the syllable type with the highest frequency.

Songs in allopatric black-throated green warblers ( $n = 33$ ) fall into two distinct PCA clusters (Fig. 3a). PC1 explains a majority (59.8%) of the variation in allopatric black-throated green warbler song, and a variety of syntax and duration variables have the highest loadings on this axis (Table S1). Several frequency and duration variables load highest on PC2, which explains only 15.2% of the variation. In contrast, the PCA of allopatric Townsend's warblers ( $n = 42$ ) shows an absence of two distinct song clusters (Fig. 3b). PC1 explains 34.8% of the variation in allopatric Townsend's warbler song. Syntax and duration variables load highest on both PC1 and PC2 (22.9% of variation). Allopatric Townsend's warbler songs do not appear to cluster geographically on the PCA plot.

A discriminant function analysis distinguished well between allopatric black-throated green warbler song, regardless of type, and allopatric Townsend's warbler song (Fig. 4). Duration variables load highest along the discriminant function axis (Table S1). The misclassification rate when reassigning groups to allopatric individuals based on the DFA axis was 1.4%, and the misclassification rate using a jackknife method was 4.2%. The mean values of 17 of the 21 song variables

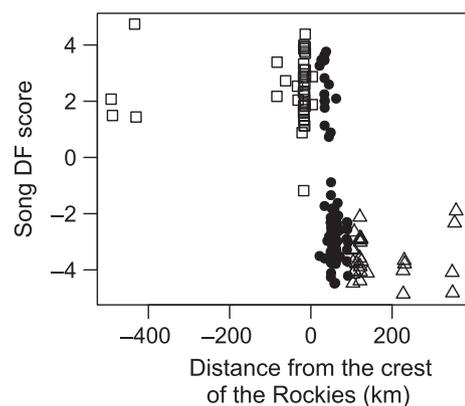


**Fig. 3** (a) Principal component analysis of allopatric black-throated green warbler songs ( $n = 29$ ) distinguishes clearly between type I (upward pointing triangle) and type II (downward pointing triangle) songs. PC1 explains 59.8% of the variation in allopatric black-throated green warbler song, whereas PC2 explains 15.2%. (b) Principal component analysis of allopatric Townsend's warbler songs ( $n = 42$ ; squares) shows no clearly distinct song types. PC1 explains 34.8% of the variation in allopatric Townsend's warbler song, whereas PC2 explains 22.9%.

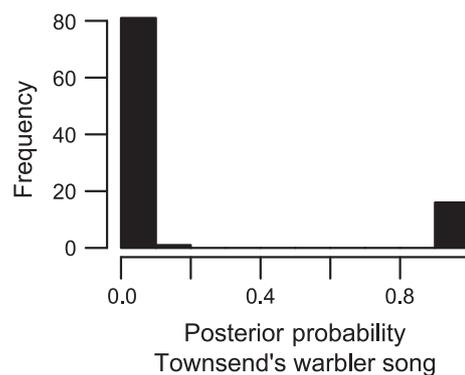
are significantly different (at the  $P < 0.05$  level) between songs of allopatric black-throated green and Townsend's warblers (Table S1).

### Song in the hybrid zone

Songs from the hybrid zone (Fig. 2e–h) tend to be qualitatively similar to those sung by either allopatric black-throated green (Fig. 2e,f) or Townsend's warblers (Fig. 2g,h), and most songs fit well within the discrete clusters of allopatric songs determined by both the PCA (Fig. S2) and the discriminant function analysis (Fig. 4). Songs from the hybrid zone are designated with a high degree of certainty as either black-throated



**Fig. 4** The discriminant function axis discriminates well between allopatric Townsend's ( $n = 42$ ; open squares) and black-throated green ( $n = 29$ ; open triangles) warbler songs. Most songs from the hybrid zone ( $n = 98$ ; filled circles) fall into the clusters of parental songs.

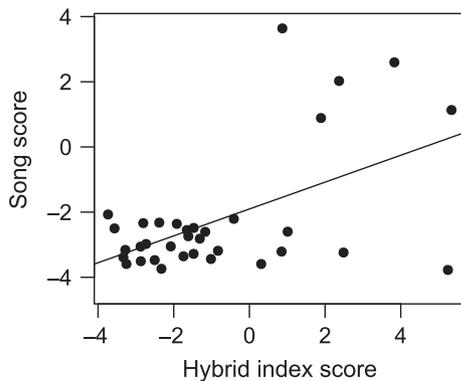


**Fig. 5** Histogram of posterior probabilities of songs from the hybrid zone being classified as a Townsend's warbler song. Songs are classified clearly as being either strongly Townsend's warbler (1) or strongly black-throated green warbler (0), with none having roughly equal assignment probability.

green or Townsend's warbler songs using the discriminant function axis (Fig. 5). Hence, nearly all songs in the hybrid zone can be clearly identified as similar to either black-throated green warbler or Townsend's warbler songs. We did not observe any intermediate or mixed songs.

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

Of 4406 high-quality SNPs identified in the genomic analysis, only three SNP loci were identified as fixed differences between our samples of allopatric Townsend's and black-throated green warblers. However, 27 SNPs differed strongly in frequency (at the  $F_{st} > 0.75$  threshold) between these two allopatric groups. A PCA



**Fig. 6** In the hybrid zone, an individual's hybrid index score (PC1 based on 27 highly differentiated markers between Townsend's and black-throated green warblers) has a weak but nonsignificant relationship to its song score (Spearman test of independence  $r_s^2 = 0.2589$ ,  $P = 0.1937$ ,  $n = 33$ ). Negative values indicate song or hybrid index scores that are more black-throated green warbler-like, whereas positive values indicate song or hybrid index scores that are more Townsend's warbler-like.

of these 27 SNPs shows that many individuals of intermediate genotype are found in the hybrid zone (Fig. S3). PC1 explains 89.6% of the variation in genotype between allopatric Townsend's and black-throated green warblers; all 27 markers load approximately equally on this axis, which was subsequently used as the hybrid index. PC2 explains only 2.3% of the variation in genotype.

### The relationship between genotype and song in the hybrid zone

In the hybrid zone (conservatively, the region from 20 to 80 km east of the crest of the Rocky Mountains; Toews *et al.*, 2011), the relationship between hybrid index score and song score was weak but nonsignificant ( $r_s^2 = 0.26$ ,  $P = 0.19$ ; Fig. 6). When examining the relationship between hybrid index score and song score at

increasing intervals moving away from the centre of the hybrid zone (Fig. S4), we uncovered a statistically significant relationship between hybrid index score and song score only when individuals as far as 60 km from the centre of the hybrid zone were included in the analysis (i.e. when a zone at least 120 km wide was examined;  $r_s^2 = 0.45$ ,  $P = 0.009$ ). Near the centre of the hybrid zone, song cannot be predicted based on hybrid index score.

### Song and genotype clines

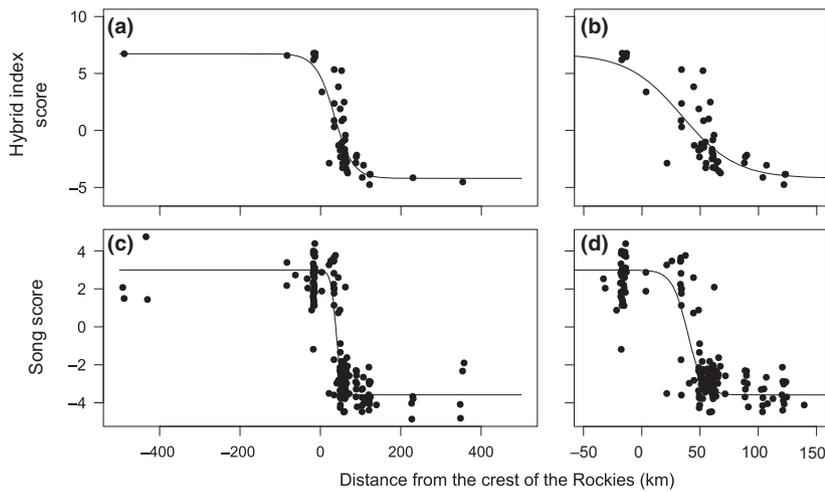
Cline analysis (Gay *et al.*, 2008) indicates that transitions in genotype and song across the hybrid zone differ in width, with the song cline being narrower. Estimates of slope and centre for the hybrid index score and the song score clines vary across four different sets of constraints on parameters (Table 1). A likelihood ratio test indicates that the two models in which the slopes of the hybrid index cline and song score cline are different best fit our data (Table 1). These results indicate that the song cline is narrower than the genomic cline, but their centres are roughly coincident. According to the model with no constraints, the hybrid index score cline (centre = 34.37 km east of the crest of the Rocky Mountains, width = 92 km; Fig. 7a,b) is broader than the song score cline (centre = 39.35 km east of the crest of the Rocky Mountains, width = 27 km; Fig. 7c, d). Similar differences in width (94 km vs. 29 km; Table 1) are also estimated when cline centres are constrained to be equal (Table 1). The shift in the two discrete song types occurs over a much smaller distance than would be predicted by the genomic cline.

### Playback experiments

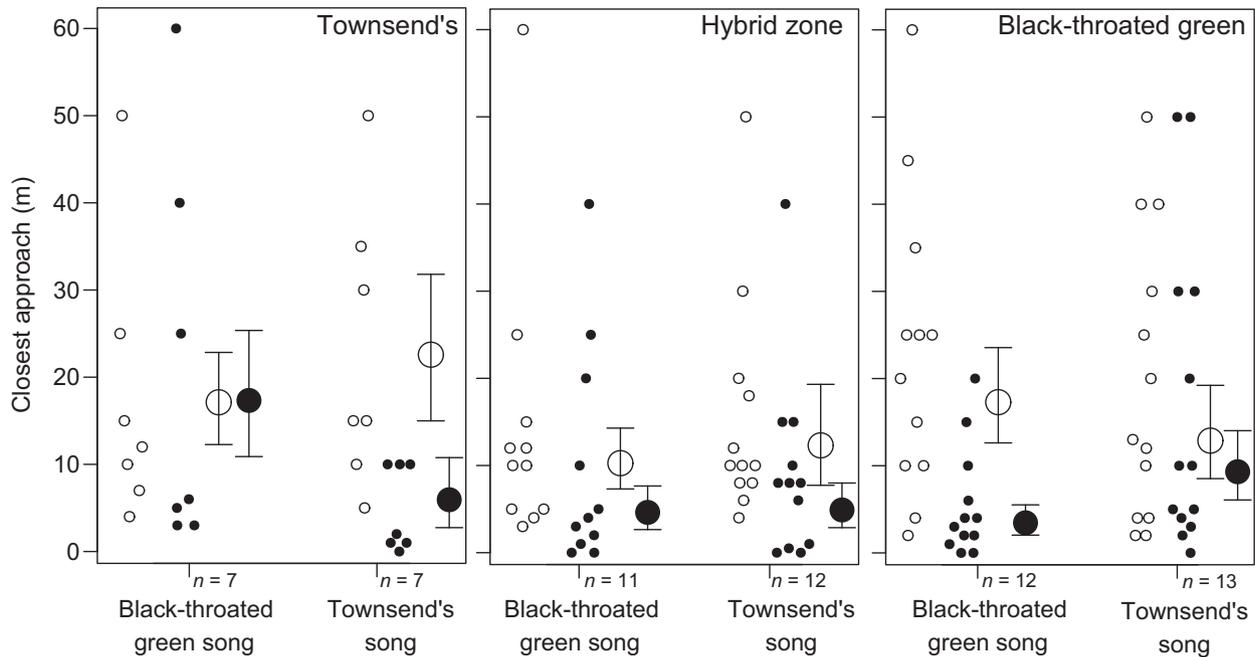
Playback experiments demonstrated that birds in allopatry show a significant response to playback only when exposed to conspecific songs (Fig. 8). Results from linear mixed-effects models indicate that allopatric black-throated green warblers approached the speaker

**Table 1** Cline analysis for hybrid index score and song DF score for four different sets of parameter constraints: (1) no constraints on slope or centre, (2) slopes constrained to be equal, (3) centres constrained to be equal and (4) both centres and slopes constrained to be equal. A likelihood ratio test was conducted to compare the parameter set with no constraints on slope or centre (1) to the other sets. Because the  $P$ -value of the 'centre constrained' model is  $> 0.05$ , it is interpreted as equally parsimonious to the 'no constraints' model.

	No constraints		Slope constrained		Centre constrained		Centre and slope constrained	
	Hybrid index score	Song DF score	Hybrid index score	Song DF score	Hybrid index score	Song DF score	Hybrid index score	Song DF score
Centre (km)	34.37	39.35	36.38	32.28	37.71	37.71	34.67	34.67
Width (km)	92	27	58	58	94	29	54	54
Log-Likelihood		-301.51		-308.57		-301.61		-308.91
AIC score		633.03		645.14		631.22		643.83
Likelihood ratio test $P$ -value				< 0.001		> 0.05		< 0.001



**Fig. 7** Genotype and song clines across the black-throated green and Townsend's warbler hybrid zone. Clines for one of the best fitting parameter sets ('no constraints') are displayed. Clines for hybrid index score and song score are illustrated showing all of the data (a and c, respectively) and scaled to show just the hybrid zone and nearby areas (b and d, respectively). The transition in hybrid index score is estimated to be centred at 34.37 km east of the Rocky Mountains with a width of 92 km, and the transition in song score is estimated to be centred at 39.35 km east of the Rocky Mountains with a width of 27 km.



**Fig. 8** Closest approach to the speaker (mean  $\pm$  SE), representative of an aggressive response, during playback experiments exposing allopatric Townsend's warblers, individuals from the hybrid zone and allopatric black-throated green warblers to allopatric black-throated green warbler or Townsend's warbler songs. Filled circles represent each individual's closest approach during the playback period, whereas open circles represent each individual's closest approach during the observation period. Larger filled and open circles for each song category represent mean approaches to playback and observation periods, respectively. Bars around the means are standard error.

80% closer during a conspecific song playback than during the corresponding observation period ( $Z_{3,33} = -3.71$ ,  $P = 0.001$ ), but showed no significant response to heterospecific song ( $Z_{3,33} = -0.81$ ,  $P = 0.85$ ). Similarly, allopatric Townsend's warblers approached the speaker 74% closer during a conspecific song playback than during the corresponding observation period ( $Z_{3,16} = -2.71$ ,  $P = 0.03$ ), but showed no

significant response to heterospecific song ( $Z_{3,16} = 0.03$ ,  $P = 1.0$ ).

In the hybrid zone, we observed no significant differences in individuals' closest approach to the speaker between observation periods and the playback periods of experiments using allopatric songs of either species (black-throated green warbler song playback:  $Z_{3,31} = -1.61$ ,  $P = 0.37$ ; Townsend's warbler song

playback:  $Z_{3,31} = -1.97$ ,  $P = 0.20$ ; Fig. 8). It is interesting to note, however, that despite this lack of significance, there is a weak trend in the direction of response to both groups' song in the hybrid zone, and mean 'closest approach to the speaker' during playback periods of both black-throated green and Townsend's warbler songs in the hybrid zone is 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, whereas in the hybrid zone there are no significant differences seen in response, although there is a trend towards responses to both species song.

## Discussion

Using SNP data obtained through a modified genotyping-by-sequencing method (Elshire *et al.*, 2011; Alcaide *et al.*, 2014), we found evidence for extensive hybridization in the region of contact between Townsend's and black-throated green warblers, corroborating earlier work on these taxa that was conducted using three molecular markers and morphological traits (Toews *et al.*, 2011). A surprisingly small proportion of SNPs (27 of 4406) were highly differentiated ( $F_{st} > 0.75$ ) between these species, despite evidence that mitochondrial DNA sequence divergence time is estimated at over 1 million years ago (Weir & Schluter, 2004). The juxtaposition of the possibility of substantial gene flow between species with the existence of a narrow hybrid zone between them suggests that selection on some small sections of the genome may be maintaining species differences between Townsend's and black-throated green warblers. Small numbers of genomic islands of divergence have been shown to maintain differences between pairs of other closely related taxa in the face of extensive introgression (Martin *et al.*, 2013; Poelstra *et al.*, 2014; Toews *et al.*, 2016). A similar genomic pattern may exist in the taxa studied here; a genomic analysis conducted at a finer scale involving mapping along chromosomes in Townsend's and black-throated green warblers is warranted.

To compare songs from the hybrid zone to those sung in allopatry, we first characterized song in allopatric black-throated green and Townsend's warblers using separate principal component analyses for the two species. Whereas the allopatric black-throated green warblers sampled here sing only the two song types that have been previously characterized across their breeding range (Morse, 1967; Spector, 1992; Morse & Poole, 2005), song in allopatric Townsend's warblers is much more variable and does not fall into such distinct clusters. This variation in song cannot be solely attributed to geographic structuring across the Townsend's warbler breeding range, as songs that were geographically close did not cluster together on the PC axes. This pattern suggests that song variation may be more complex

in Townsend's warblers, with either more than two song types being sung by each individual or, perhaps more likely, different song types being used for analogous purposes in different individuals. Whether song use patterns in Townsend's warblers mirror those in black-throated green warblers and other congeners (e.g. Morse, 1967; Price & Crawford, 2013), where one song type is used more in territorial interactions and the other in mating interactions, is unclear; however, such a simple, stereotyped pattern is unlikely due to the variation described here in Townsend's warbler songs. Intraspecific mixing of song types, as has been described in Canada warblers (*Cardellina canadensis*; Demko *et al.*, 2013), could perhaps produce the lack of clear distinction between song types that we observed in Townsend's warbler songs.

Despite the variation among songs within each species, a discriminant function analysis demonstrated that black-throated green warbler songs and Townsend's warbler songs can be distinguished from one another with high confidence, regardless of song type (and therefore function). Duration variables, which have been previously suggested to have strong effects on the abilities of other species to recognize conspecifics (e.g. Indigo buntings (*Passerina cyanea*); Emlen, 1972), best distinguish Townsend's and black-throated green warbler songs from one another.

Both the PCA conducted based on all allopatric songs and the posterior probabilities from the DFA conducted to distinguish allopatric Townsend's warbler songs from allopatric black-throated green warbler songs indicate that few, if any, intermediate songs are found in the hybrid zone. Instead, hybrid zone songs cluster closely with allopatric song types. We 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 (i.e. genetically intermediate individuals sing intermediate songs) or (2) cultural transmission contributes strongly to song phenotype, but songs are not inherited as single units (i.e. components of song are learned from multiple tutors and subsequently blended). On the other hand, if songs have a strong cultural component and are transmitted as distinct units, then we expect to see no song blending and little association with genetic background. This is the observed pattern. It is interesting to note that this pattern is consistent with the concept of memetics in which cultural inheritance occurs through the transmission of discrete units of culture, known as memes, just as genetic inheritance operates through the transmission of genes (Dawkins, 1976). Here, whole song types appear to operate as memes, with each type learned in entirety and no mixing between types occurring.

Despite the fact that most songs in the hybrid zone are similar to either allopatric black-throated green or Townsend's warbler songs, an individual's genotype is not a good predictor of its song in this region. In fact,

at the centre of the hybrid zone there is no significant correlation between genotype and song (although there is some evidence for a very weak relationship), indicating that song is a signal that carries little species-membership information where the highest potential for interbreeding exists (Barton & Hewitt, 1985). This indicates that song is largely decoupled from genetic background in the centre of the zone, suggesting that the distinct types of songs are learned rather than genetically based, and that song is unlikely to play an important role in reproductive isolation between these two species groups.

The width of clines in various traits is often used to infer the strength of selection on those traits; all else being equal, and presuming an old and stable hybrid zone (Toews *et al.*, 2011), a narrower cline indicates stronger selection (Barton & Hewitt, 1985). We found that the song cline across the hybrid zone is more than three times narrower than that of the genomic transition between Townsend's and black-throated green warblers; this pattern is consistent with stronger selection on song than on genotype. Based on this evidence, along with the findings that (1) the songs sung in the hybrid zone are those found in allopatry and (2) there is little, if any, correlation between song and genomic background in the hybrid zone, we suggest that song may be under frequency-dependent cultural selection whereby locally common songs are favoured over rare songs (Lynch, 1996; Price, 2008). This may explain why entire songs, rather than components of songs, appear to be transmitted as cultural units. 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 in other species have been attributed to the fact that local vocalizations may be perceived as a stronger threat (Baker *et al.*, 1981; Searcy *et al.*, 1997; Wilson *et al.*, 2000; Den Hartog *et al.*, 2008). We suggest that this system is another case in which it may be advantageous for males to sing locally common songs in order to hold a territory.

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 hear the song of only one species and are thereby presented with no species-membership information through song by which to choose a mate. Even in the narrow region where both songs are found, there is little or 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, we have shown that few, if any, intermediate songs are sung in the north-eastern British Columbia hybrid zone between Townsend's and black-throated green warblers, despite the extensive hybridization that occurs there. There is, at best, a

weak relationship between genotype and song in sympatry, indicating that song is unlikely to be an important reproductive barrier between these species. Instead, the difference in cline widths between song and genomic signatures suggests that there is stronger selection on culturally transmitted song characteristics than on overall genomic patterns. Our interpretation is that song is likely under frequency-dependent cultural selection, whereby males may be more likely to maintain a territory when singing a locally common song (e.g. Baker *et al.*, 1981; Wilson *et al.*, 2000). The hybrid zone between Townsend's and black-throated green warblers can therefore be thought of as an area where genomic and cultural patterns of variation have become somewhat decoupled, and where cultural isolation is in fact stronger than genetic isolation. We suggest that the concept of speciation can be applied to both gene pools and culturally inherited traits; in this case, it appears that culture has reached a further stage of isolation and incompatibility between these species than gene pools have. Ironically, the strong cultural component in song appears to have caused a breakdown of the association between genes and culture in the centre of the zone, such that song is inhibited from playing a strong role in genetic isolation. This pattern contrasts with other situations in which song has been implicated as a strong reproductive barrier (e.g. Baker & Mewaldt, 1978; Irwin, 2000; Patten *et al.*, 2004; Qvarnström *et al.*, 2006). Future work in this area should focus on understanding what conditions cause learned traits to either promote or prevent reproductive isolation between genomically divergent groups.

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## Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article: **Table S1** Means and standard deviations (SD) for allopatric Townsend's ( $n = 42$ ) and black-throated green warbler ( $n = 29$ ) songs for 21 song variables, those

variables' Hedges'  $g$  scores and loadings in principal component analyses (PCA) for each allopatric group, as well as a PCA and discriminant function analysis (DFA) for all allopatric birds ( $n = 71$ ; 29 black-throated green warblers and 42 Townsend's warblers).

**Figure S1** An illustration of the twenty-one song variables measured on a simplified illustration of a typical black-throated green warbler type II 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 S2** Songs from the hybrid zone (gray) fall into

similar clusters to allopatric songs (light green: allopatric black-throated green warbler type I song, dark green: allopatric black-throated green warbler type II song, blue: Townsend's warbler song) on the first two axes of a principal component analysis based only on allopatric songs (i.e. those in colour here).

**Figure S3** Principal component analyses on two sets of markers show many individuals of intermediate genotype in the hybrid zone ( $n = 60$ ; gray) between Townsend's ( $n = 12$ ; blue) and black-throated green ( $n = 13$ ; green) warblers. The first axis of variation (PC1) of a principal component analysis conducted on 27 SNPs with  $F_{st} > 0.75$  between allopatric populations (a) was used as a hybrid index score to describe the genetic background of individuals in this study and was highly correlated with a second principal component analysis conducted on all 4406 SNPs identified (b;  $r^2 = 0.8$ ,  $P < 0.0001$ ).

**Figure S4** The  $r_s^2$  value representing the relationship between hybrid index score and song DF score for study areas of varying widths centered on 46 km east of the crest of the Rocky Mountains, the center of the hybrid zone as approximated by Toews *et al.*, 2011.

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