

PHYLOGEOGRAPHY, SONG DIVERGENCE AND REPRODUCTIVE ISOLATION  
IN TWO SPECIES OF SONGBIRD

By

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PHYLOGEOGRAPHY, SONG DIVERGENCE AND REPRODUCTIVE ISOLATION  
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Abstract

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The process of how and why populations diverge and become different species is a topic of widespread interest. A complete understanding of the process of speciation requires that we understand the causes of divergence between populations, as well as the consequences of that divergence. Regardless of the causes of divergence, the consequences must include some degree of reproductive isolation in order for speciation to proceed. For two species of songbird, the American Redstart (*Setophaga ruticilla*) and the Black-throated Blue Warbler (*Dendroica caerulescens*), we find evidence that recent glacial cycles have contributed to allopatric and parapatric divergence, respectively. For the Black-throated Blue Warbler, we find that the causes of the divergence between populations may include sexual selection, as songs in the northern population have faster trills, and sexual selection favors fast trills in this population. The consequences of this divergence may include reproductive isolation, as males in the northern population respond more to local songs compared to non-local songs. However, in the southern population males do not discriminate between northern and southern songs, thus reproductive isolation may be asymmetric and incomplete. These results provide support

for the hypothesis that recent glacial cycles may be contributing to diversification and speciation. In addition, we find a rare example of sexual selection acting to contribute to differences across populations, as well as a rare example of asymmetric discrimination, which may be one of the very first steps in the process of speciation.

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## GENERAL INTRODUCTION

The *Parulid* warblers are an incredibly diverse group of organisms that have experienced a rapid radiation in the past several million years (Lovette & Birmingham 1999), and divergence in this group has likely proceeded, at least to some extent, in the face of gene flow, as the ranges of many of the species in this group, indeed the territories of individuals of different species, largely overlap (Robinson & Holmes 1982).

One period that may have been important for the diversification of this group is Pleistocene, in particular the glacial cycles that predominated this period. The Pleistocene could have facilitated allopatric divergence by separating species into multiple refugia without gene flow between them (Birmingham et al. 1992; Avise & Walker 1998), or through parapatric divergence, as range expansions in the wake of receding glaciers may have facilitated population expansion and ecological and/or sexual differentiation across populations connected by gene flow (e.g. Mila et al. 2000; 2006; 2007a; 2007b).

Regardless of the cause of divergence between populations, in order for speciation to proceed, the consequence must be reproductive isolation. In the case of allopatric divergence, physically isolated populations potentially experienced secondary contact on many occasions (Weir & Schluter 2004), and we would only expect physical isolation to contribute to speciation if divergent populations exhibited reproductive isolation upon secondary contact. In the case of parapatric divergence, reproductive isolation across divergent populations connected by gene flow would have to be strong enough to overcome the homogenizing effects of that gene flow (Smith et al. 1997).

In this dissertation, I was interested in examining these processes in two species of *Parulid* warbler, the American Redstart (*Setophaga ruticilla*) and the Black-throated Blue Warbler (*Dendroica caerulescens*). The American Redstart has a wide distribution across North America (Chapter 1), with the potential for allopatric divergence to have affected its evolutionary history. The Black-throated Blue Warbler has a smaller distribution in the northeastern North America, but has an interesting and ecologically diverse range in that the southern arm of this range extends down the spine of the Appalachian Mountains.

I first ask how populations have been affected by recent glacial cycles, i.e. have current day populations diverged allopatrically, parapatrically or neither? To address this question, I examine the phylogeographic history of the American Redstart, specifically testing the hypothesis that historical populations were separated along the Rocky Mountains/Great Plains, and alternatively that other areas may have been important refugia, such as the now submerged Atlantic Coastal Shelf in northeastern North America (Chapter 1).

The next step in the speciation process is the accumulation of phenotypic differences across populations, and I next ask if we see evidence of phenotypic differentiation, specifically song differences, across populations of the Black-throated Blue Warbler (Chapter 2).

Phenotypic differences can accumulate across populations due to genetic drift, ecological selection or sexual selection, but differences are particularly likely to accumulate rapidly if sexual selection is acting divergently (Coyne & Orr 2004, Price 1998; 2008). Thus I ask if sexual selection is contributing to the evolution of different

songs in different populations of the Black-throated Blue warbler by using male responsiveness and extra-pair paternity to examine sexual selection on song in a northern population (Chapter 3).

Finally, the last step of the speciation process is that phenotypic differences across populations contribute to reproductive isolation. Thus I ask if song differences across populations of the Black-throated Blue warbler are contributing to reproductive isolation by conducting local vs. non-local playback experiments in northern and southern populations of the Black-throated Blue Warbler.

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## CHAPTER ONE

### PHYLOGEOGRAPHY OF A WIDESPREAD NORTH AMERICAN MIGRATORY SONGBIRD (*SETOPHAGA RUTICILLA*)

#### Introduction

Pleistocene glacial cycles and the climate changes that they precipitated have had profound impacts on patterns of genetic diversity within extant species (Avice and Walker 1998; Hofreiter et al. 2004). Changes in climate have likely had diverse effects on different populations, resulting in range shifts as different geographic areas become more or less suitable, and isolation between populations when barriers to dispersal, such as glaciers, arise. The extent to which different species were affected in similar ways by these past climatic events remains unclear. At one end of the spectrum, co-existing extant species may have been affected in similar ways, and therefore share common recent histories. Alternatively, different species may have been affected in different ways by Pleistocene events, such that their recent histories are divergent despite overlapping distributions in the present.

To understand the effects of Pleistocene climate changes, it is necessary to address two inter-related issues. First, it is necessary to determine the number of refugia that may have harbored populations of extant species. One possibility is that populations survived through Pleistocene glacial cycles without being subdivided, subsequently expanding from that location to colonize the continent as glaciers receded. This “single refugium” hypothesis has been supported for some bird species with relatively narrow

current ranges (e.g., Mila et al. 2000; Veit et al. 2005, Davis et al. 2006), but does not seem to hold for most North American species that are currently widespread (i.e., species that currently cover much of North America). For widespread species it is much more common to find a pattern of significant genetic differentiation between populations on either side of a geographic barrier (i.e. mountains, deserts, plains, bodies of water). This pattern indicates the existence of two or more major glacial refugia during Pleistocene glacial cycles (the “multiple refugia” hypothesis; see references below).

Second, in addition to knowing the number of glacial refugia involved, it is necessary to infer the locations of those refugia. Most studies of wide-spread North American species have yielded a pattern of significant genetic differentiation between populations on either side of the Rocky Mountains (or Great Plains) with little genetic differentiation within these phylogroups (Table 1). This pattern suggests the existence of two major glacial refugia during the Pleistocene -- one in the east and one in the west. East-west differentiation is particularly common among species of widespread, migratory birds (Milot et al. 2000; Ruegg and Smith 2002; Kimura et al. 2002; Lovette et al. 2004; Peters et al. 2005), including all warblers with continental distributions studied to date (reviewed in Kelly and Hutto 2005), and also has been found in other taxa (McGowan et al. 1999; Rueness et al. 2003; Ayoub and Riechert 2004; Runck and Cook 2005).

Although the exact locations of these eastern and western refugia are often unclear, some studies have suggested more specific locations. For example, studies of plants (Boys et al. 2005), insects (Berlocher and Dixon 2004), fish (Bernatchez 1997) and mammals (Paetkau and Strobeck 1996, Kyle and Strobeck 2003) have suggested the existence of a glacial refugium on the now-submerged Atlantic coastal shelf near

Newfoundland (Figure 1). Some studies of birds have similarly suggested a glacial refugium in the maritime region of far-eastern Canada (Gill et al. 1993; Holder et al. 1999; Boulet et al. 2005), but the possibility that this was an important refugium for migratory birds is relatively unexplored (but see Zink and Dittmann 1993 and Boulet and Gibbs 2006). Other important glacial refugia have also been suggested, for example the Queen Charlotte Islands (Byun et al. 1997; Burg et al. 2005; 2006) and areas of Alaska (Anderson et al. 2006; Burg et al. 2006) as well as southern regions below the glacial maximum (Mila et al. 2000).

Genetic tools can be used to determine historical biogeography, as described above, but this can be a difficult task as signatures of historical processes and events can be obscured by current day processes. For example, current genetic differentiation can be due historical barriers to gene flow, and/or to current day processes such as isolation by distance (Wilke and Pfenninger 2002; Smith and Farrell 2005). Alternatively, extensive current gene flow would randomize genotypes across the species' range, effectively erasing the geographic signature of earlier events. Recent post-glacial population expansion can also lead to little or no genetic differentiation among locations, even in cases where current gene flow is limited (Timmermans et al. 2005; Spaulding et al. 2006). Indeed, in North American birds the lack of genetic differentiation within phylogroups on either side of the Rockies (above) might be due to post-glacial expansion from glacial refugia, high levels of current day gene flow, or both. To separate these possibilities it is necessary to combine extensive geographic sampling, sensitive molecular markers that can evolve over relatively short time periods, and appropriate analytical methodologies. Thus, a major issue surrounding structuring of genetic variation



is assessing the relative importance of recent historical events, such as glacial cycles, and current day processes, such as gene flow.

The American Redstart (*Setophaga ruticilla*) is a widespread North American songbird occurring on both sides of the Rocky Mountains (Figure 1). If this species has a recent history similar to that of other widespread migratory birds (above), we would expect American Redstarts to show evidence of two Pleistocene glacial refugia, i.e., genetic differentiation between eastern and western North America. However, all of the widely-distributed migratory songbirds listed above consist of sub-species that roughly correspond with east/west divides, whereas American Redstarts lack recognized subspecific designations. Thus, redstarts may have a unique recent evolutionary history that differs from that of other widespread North American birds. For example, populations may have survived in only a single glacial refugium, subsequently colonizing the rest of the continent, or in multiple refugia defined by barriers other than the Rocky Mountains/Great Plains.

We had two primary goals in this study. First, we utilized widespread sampling and two types of molecular markers – mtDNA control region and amplified fragment length polymorphism (AFLP) – to distinguish between the alternative historical scenarios outlined above for the American Redstart. Second, we used a number of analytical approaches to separate the effects of historical events from current day processes in determining the current pattern of genetic variation. To achieve these goals, we used standard measures of genetic differentiation ( $F_{ST}$ , AMOVA) to identify genetic subdivisions across the range of the redstart, and coalescent analyses (Nielsen and Wakeley 2001) to determine if those subdivisions (for mtDNA) are likely due to

historical and/or current day processes. We also tested for the signature limited current gene flow by testing for patterns of isolation by distance.

## **Materials and methods**

### *Sample collection*

We selected collection sites to provide even coverage across the breeding range of the American Redstart (Figure 1, Table 2). All individuals (adults) were captured in mist nets and released after sampling. We captured breeding males with decoys and song playback, while females were captured directly from their nests while they were incubating or provisioning nestlings. U.S. samples were collected in the summer of 2002 whereas Canadian samples were collected in from May – June of 1994 and 1995. Approximately 50  $\mu$ L of blood were collected from the brachial vein of each individual and stored in 200  $\mu$ L lysis buffer for U.S samples (Densmore and White 1991) and Queen’s lysis buffer (Seutin et al. 1991) for Canadian samples. We isolated whole genomic DNA from U.S. blood samples using a standard phenol/chloroform procedure, and from Canadian samples using either phenol/chloroform or DNAZOL genomic DNA isolation reagent (Invitrogen). DNA was quantified using a fluorometer and diluted to a final working concentration of 20  $\mu$ g/ml.

### *Mitochondrial DNA sequencing*

We amplified a 290-bp sequence of the control region I gene using redstart-specific primer sequences F – TTAAGGGTATGTATAGTATG and R –

TTCTTGAAGGCTGTTGGTCG, which were designed from preliminary sequence information generated for redstarts using control region primers DPdl-L5 and DPdl-H4 designed for yellow warblers (*Dendrocia petechia*, Milot et al. 2000). PCR reactions were conducted using 1  $\mu$ L of undiluted whole genomic DNA, 3  $\mu$ L of MgCl<sub>2</sub>, 3  $\mu$ L 10X PCR buffer, 1  $\mu$ L dNTPs, .5  $\mu$ L forward and reverse primers (10  $\mu$ M), 2  $\mu$ L Taq polymerase (Promega) and 10  $\mu$ L ddH<sub>2</sub>O. Reactions consisted of 35 cycles of denaturation at 94°C for one minute, a 55°C annealing temperature held for 90 s, and a 72°C extension temperature, held for 2 min. We are confident that this procedure generated mtDNA sequences as opposed to nuclear homologs because (1) sequences were clear with no double peaks (Sorenson and Quinn 1998), and (2) sequences generated from blood-extracted DNA and purified mtDNA isolated from a single bird were identical (HLG, unpublished data).

We cleaned PCR products using shrimp alkaline phosphatase procedure (Amersham). We then obtained DNA sequences from purified PCR products via cycle sequencing with ABI Big dye version 3.1 (Applied Biosystems) reaction mixture using protocols suggested by the manufacturer, and running products on an ABI 3730 capillary sequencer. Sequence data were visualized using Sequencher software versions 3.0 (Gene Codes). All mtDNA sequences generated have been deposited in Genbank (accession numbers EF999143-EF999326).

#### *Phylogenetic analysis of mitochondrial sequences*

We performed all phylogenetic analyses on the WSU Phylogenetics, Population and Evolutionary Ecology Computer Cluster. We then used PAUP 4.0b10 (Swofford 2003) to

determine phylogenetic relationships among haplotypes using maximum-likelihood. For likelihood analyses, we used DT-MODSEL (Minin et al. 2003) to select the substitution model which best fit the data using an information criteria and executed searches using a heuristic search strategy with a single random addition sequence starting from a random tree. The level of support for each node of the generated tree was evaluated with 100 bootstrap replicates. We used an mtDNA control region sequence from the Chestnut-sided warbler (*Dendroica pennsylvanica*) as an outgroup (Genbank accession #AF206016; Milot et al. 2000).

We used TCS 1.13 (Clement et al. 2000) to generate a haplotype network for all of the sequences. The program was set to estimate the upper limit of the number of mutational steps between haplotypes.

#### *Gene flow and coalescent modeling*

We assessed the genetic structure of redstart populations with ARLEQUIN 2.0 (Excoffier et al. 1992). First, to test whether redstarts exhibit population structure similar to that of other widespread migratory warblers (Kelly and Hutto 2005), we compared two groups: eastern and western. Second, to test for the signature of a separate Atlantic coastal shelf refugium (Figure 1), we grouped all mainland populations together separate from Newfoundland. Patterns of gene flow were evaluated using pairwise and global  $F_{ST}$  values along with Mantel tests for isolation by distance. In order to make comparisons between mtDNA and AFLPs, it is necessary to take into account their differences in effective populations size. We therefore used the equation  $F_{ST}(nuc) = F_{ST}(mt)/4-3(F_{ST}(mt))$  (Brito 2007) to calibrate our mtDNA  $F_{ST}$  values. We used ARLEQUIN to estimate

gene diversity in eastern vs. western sample sites. We also used ARLEQUIN to investigate population expansion by calculating mismatch distributions and evaluating their significance by comparing them to a distribution calculated under a model of sudden population growth (Rogers 1995). MEGA 3.1 (Kumar et al. 2001) was used to infer mean net corrected sequence divergence between populations east vs. west of Rockies, and between Newfoundland vs. continental populations (Nei 1979).

Phylogeographic subdivisions that are associated with a potential barrier to gene flow could have arisen because that barrier has weakly limited gene flow for a long time period, or because it has severely limited gene flow for a short time period. We used a coalescent modeling approach (Nielsen and Wakeley 2001) to determine which of these scenarios was more consistent with our data. Divergence times and migration rates between population groups (both eastern versus western and Newfoundland versus continental) were estimated using coalescent modeling. We used the MDIV software package, which implements the coalescent model described in Nielsen and Wakeley (2001). Aligned sequence data from sample sites were used to estimate the parameters  $\Theta$  ( $= 2N_e\mu$ ),  $M$  ( $= N_e m$  = number of migrants between populations per generation), and  $T$  (the divergence time between populations, where 1 time unit =  $N_e$  generations). These analyses each used a finite sites model and a 3,000,000 generation Markov chain Monte Carlo (MCMC) run with a 500,000 generation burn-in to explore the solution space.  $M$  max was set to 3, and  $T$  max to 10. The coalescent-scaled parameter  $T$  was converted to  $T_{div}$  (time in years since two populations diverged) using the formula  $T_{div} = T\Theta/(2\mu)$  and assuming two years per generation (T.S. Sillett, pers. comm.) and both a low (0.076

mutations/site/million years) and a high (0.3 mutations/site/million years) estimate of mutation rates (Davis et al. 2006).

#### *AFLP analysis of genetic structure*

Our general AFLP procedures followed Vos et al. (1995) with the following modifications. We first digested 1 µL DNA samples (containing 50 - 250 nanograms DNA) by incubating with restriction enzymes *EcoRI* and *MseI* (New England Biolabs - NEB) at 37°C for 3 hours, followed by denaturation at 60°C for 5 minutes. Adapters (Invitrogen) designed to ligate to the *EcoRI* and *MseI* restriction sites were then added to the reaction mix along with T4 Ligation enzyme (NEB) and incubated at 16°C for 2 hours. This mixture was then re-restricted and ligated simultaneously as described by (Vos et al. 1995). This ‘two step’ restriction ligation was done in order to ensure complete and uniform DNA restriction and ligation across reactions, as when we simply employed the simultaneous restriction/ligation described by Vos et al we observed variable results between reactions. Our preselective PCR using the primers *EcoRI* + A and *MseI* + C had an annealing temperature of 59°C and a MgCl<sub>2</sub> concentration of 3.0 mM. We used this annealing temperature, which is slightly above temperatures commonly reported in AFLP studies, to maximize the specificity of the primers. For final selective amplification, 1 µL of the preselective PCR product mix was added to a mixture containing fluorescently labeled *EcoRI* +3 primers (Applied Biosystems) and unlabeled *MseI* +3 or +2 primers. We used a touchdown PCR for the selective amplification (J. Brunelli, pers. com), consisting of 7 initial cycles, starting with an annealing temperature of 65 °C and decreasing one degree per cycle to finish with an annealing temperature of

59 °C, followed by 25 cycles with and annealing temperature of 59 °C. This touchdown methodology was used to maximize the specificity of the primers.

We ran products from this selective amplification on an ABI 3730 capillary sequencer with LIZ size standard (Applied Biosystems), and collected the digital gel data using ABI Prism Gene Mapper analysis software (ver. 3.75). Each lane file was analyzed for the presence and absence of AFLP products by eye. To maximize repeatability of fragment scoring, we scored only those fragments between 120 and 400 base pairs, and we considered a locus to be scorable if at least one individual had a peak above 3,000 reflectance units (rfu). At each scorable locus, individuals were scored as having a band at the locus if a distinctive peak was discernable above the background noise. The smallest scorable peaks were usually 200 rfu, although most peaks were at least 1,000 rfu. These scoring criteria were established by conducting two different restriction/ligation reactions on 16 individuals and carrying them all the way through to selective amplification with the selective primer pair Eco AGG/ Mse CCG. Bands meeting the above criteria were 100% repeatable between separate reactions, but repeatability fell off sharply when considering loci smaller than 120 bp, larger than 400 bp, or with maximum reflectance peaks below 3,000 rfu in the brightest individual profiles.

We screened 24 different primer combinations but used only the eight combinations that gave clear, consistent banding patterns (ECO- ACA, AAC, ATG, AGG paired with MSE- CCG, CG). In total, 180 bands met our repeatability standards, and 113 of those were polymorphic (polymorphic bands determined by AFLPOP, Duchesne and Bernatchez 2002). Population genetic analyses of all 180 AFLP bands (pairwise and

global  $F_{ST}$ , AMOVA, and mantel tests for isolation by distance) were conducted using GenAlEx (Peakall and Smouse 2006). We conducted a Bayesian cluster analysis using the program STRUCTURE (Pritchard et al. 2000), treating all 113 polymorphic loci as haplotypic data. Although AFLPs are not haplotypic data, STRUCTURE has been shown to perform well treating AFLP data in this manner (Evanno et al. 2005). For the STRUCTURE runs, we varied the assumed number of populations ( $K$ ) from 1 to 5 (the number of sample sites in the analysis), and each run consisted of a 100,000 iteration burn-in followed by 1,000,000 iterations. For a given value of  $K$ , these run parameters produced nearly identical results in repeated runs.

## **Results**

### *MtDNA and phylogenetic analysis*

We sequenced 290 nucleotides of the control region I of the mitochondrial genome from 184 individuals, and found 106 distinct haplotypes based on 38 variable sites. A maximum likelihood analysis with these 106 haplotypes yielded a tree with little structure (not shown), and no nodes received any bootstrap support above 50. Sequence divergence between most sampled populations was small ( $< 0.10\%$ ), including sequence divergence between populations east and west of the Rockies ( $0.10\%$ ). In contrast, mean net uncorrected sequence divergence between continental sample sites and Newfoundland was  $1.00\%$ .

### *Haplotype analyses*



The constructed parsimony network (Figure 2) showed high levels of homoplasy, with many ( $n = 106$ ) unique haplotypes connected in multiple ways to other haplotypes, and no true "central" haplotypes. The high number of connections between haplotypes made it impossible to group them, precluding the use of nested-clade analysis. Nonetheless, one cluster of haplotypes did separate out from the others: the bulk of the samples from Newfoundland ( $n = 17$ ) clustered together in an unambiguous part of the network. Clustered with these Newfoundland haplotypes were four haplotypes collected from eastern populations (three individuals from New Brunswick and one individual from Maryland) and one haplotype collected from a western population (Montana, the lone "W" in figure 2). This 'Newfoundland' cluster is separated from all other haplotypes by two mutational steps.

#### *Coalescent analyses*

We conducted coalescent analyses to test divisions between putative refugia: east versus west and mainland versus Newfoundland. Estimates of time since divergence between Newfoundland and the mainland, calculated using high and low mutation rates (see methods) and the 95% confidence intervals of the estimates of  $T$ ,  $\Theta$  and  $M$ , as determined by MDIV, yielded a lower estimate of 40.3 Kya and an upper estimate of 2,171.0 Kya (Figure 3a). Migration rates between Newfoundland and the mainland were estimated to be between 0.46 and 2.20 females per generation (Figure 3b). In contrast, divergence estimates between eastern and western populations yielded an irregular probability distribution near zero, indicating that it is likely that these populations have never separated from each other (Nielsen and Wakeley 2001; Davis et al. 2006). The program

also was unable to calculate an upper limit for migration rates between these populations, indicating high levels of contemporary gene flow.

#### *Population genetic differentiation and gene flow*

An AMOVA conducted on mtDNA haplotypes with populations partitioned according to an east-west split showed that 1.19% of the variation was explained between groups ( $\Phi_{st} = 0.012$ ,  $P = 0.17$ ). When the split is defined as occurring between Newfoundland and the mainland, 4.29% of the variation was explained between groups ( $\Phi_{st} = 0.043$ ,  $P = 0.08$ ). The mismatch distribution for mainland sampling sites was unimodal and did not significantly differ from the distribution expected under population expansion (Figure 4a; Rogers and Harpending 1992). The mismatch distribution for the Newfoundland sample site appeared to be different from the distribution expected under population expansion (Figure 4b,  $P = 0.06$ ), potentially suggesting a lack of population growth (Slatkin and Hudson 1991). The mismatch distribution for the western sites also conformed to the population expansion distribution (data not shown).

Pairwise  $F_{ST}$  values calculated from mtDNA haplotype frequencies were much larger (up to an order of magnitude) for comparisons between Newfoundland and all other sites (Table 3). After Bonferroni correction for multiple comparisons, only comparisons involving Newfoundland and sites further to the west remained significant (NF compared to LA, MT, BC, SAS, ONT, all  $P < 0.005$ ). Mantel tests revealed significant isolation by distance (Figure 5a) when considering all sample sites ( $r = 0.47$ ,  $P = 0.004$ ) and all sites excluding Newfoundland ( $r = 0.47$ ,  $P = 0.013$ ). The high correlation coefficients from the Mantel tests indicate that a large proportion (roughly

28% based on  $R^2$  values from a regression analyses of  $F_{ST}$  and distance) of the variation in  $F_{ST}$  is explained by distance, supporting the hypothesis of limited gene flow among populations. For the sake of comparison with the AFLPs, isolation by distance was examined for the U.S. sample sites only and showed a non-significant trend towards isolation by distance (Figure 5b;  $r = 0.50$ ,  $P = 0.12$ ).

#### *AFLP Population Structure*

For the five US sample sites, an AMOVA revealed that the majority of variation (94%) occurred among individuals within populations. However, a moderate but significant global  $Phi_{ST} = 0.06$  ( $P = 0.01$ ) indicated that some structuring exists. Post-hoc groupings of all populations revealed that a grouping of Montana and Michigan vs. Louisiana, Maryland and New Hampshire explained a small (2%) but significant ( $P = 0.01$ ) amount of variation. No pairwise  $Phi_{ST}$  values remained significant after Bonferroni correction for multiple comparisons. MtDNA  $F_{ST}$  values corrected for effective population size (Table 4) show that AFLP differentiation is on average one order of magnitude larger than mtDNA differentiation. We did not find a pattern of isolation by distance among the US populations (Figure 5b;  $r = 0.62$ ,  $P = 0.06$ ), though the number of populations included was small. In the STRUCTURE cluster analysis, the range of priors for  $K$  yielded a flat probability distribution with all potential  $K$  values having similar probabilities.  $K=4$  had the highest value, but each of the 5 sample sites had similar frequencies of each individual  $K$ , and most individuals were not cleanly assigned to one  $K$ . We were therefore unable to make any inference about the number of genetically distinct populations. Thus, although a clear picture of isolation-by-distance did not

emerge, our AFLP analyses show some population structure, with eastern and southern populations being more similar to each other than to mid-western and western populations.

## **Discussion**

### *Phylogeographic history*

Phylogeographic studies of North American migratory birds and other species have shown that most wide-spread species survived Pleistocene glacial cycles in two or more glacial refugia: one in the east and one in the west separated by the great plains and/or Rocky Mountains (e.g., Milot et al. 2000; Ruegg and Smith 2002; Clegg et al. 2003; Lovette et al. 2004; Peters et al. 2005). In sharp contrast, our results for the American redstart show little or no genetic differentiation among populations throughout continental North America. This contrasting pattern agrees somewhat with morphological patterns, as American redstarts are one of only two species of wood warbler with continental distributions (the other being the Black-and-White warbler, *Mniotilta varia*) that lack formal sub-species designations (Sherry and Holmes 1997; Kelly and Hutto 2005). Thus, this species appears to have had just a single continental refugium during the Pleistocene, with expansion from this refugium to populate the rest of the continent. Since the majority of the northern portion of the Redstart's range was glaciated during the Pleistocene (Figure 1), including most or all of the western portion of the range, it is likely that this continental refugium would have been in the southeast. However, although our data indicate that most or all continental redstart populations have spread

out from a single glacial refugium, we cannot make firm conclusions about its exact location.

In contrast to the lack of differentiation among continental populations, our results also show that populations in far eastern Canada are somewhat genetically differentiated from those on the continental mainland (Figures 2, 3a, Table 3), indicating the possibility of a second refugium in the far northeast. The amount of genetic differentiation between continental and Newfoundland populations seen in this study, dating from 40,000 years ago to 2,000,000 years ago and falling within the Pleistocene, is comparable to the differentiation between eastern and western phylogroups found in other studies (Table 1). Moreover, patterns of genetic differentiation and mismatch distributions (Figure 4b) indicate that populations in this region have been relatively stable with relatively modest spread into the remainder of the continent.

While the Atlantic coastal shelf of Canada has been proposed to have been an important refugium for other organisms (Paetkau and Strobeck 1996; Bernatchez 1997, Kyle and Strobeck 2003; Berlocher and Dixon 2004 and Boys et al. 2005), its importance for migratory songbirds remains unclear. This is largely because most studies have failed to incorporate samples from these areas in their studies. To the best of our knowledge, only two studies have sampled populations in Newfoundland: Song Sparrows, *Melospiza melodia* (Zink and Dittman 1993) and Yellow Warblers, *Dendroica petechia* (Boulet and Gibbs 2006). Both studies found indications that individuals could have survived glacial maxima in a northeastern refugium. Thus, studies of additional species, with extensive sampling in eastern Canada as well as other areas, are necessary before firm conclusions

can be drawn about the relative importance of this putative glacial refugium for migratory songbirds.

In sum, our results support the “two refugia” hypothesis but also show that most populations in North America arose from a single continental refugium. Thus, two or more Pleistocene refugia may be the norm for highly mobile species that currently have continental distributions. However, the exact locations of these refugia may differ, even among species with largely overlapping current distributions. The maritime regions of far eastern Canada may have been an important glacial refugium for other North American birds, but more studies are needed before firm conclusions can be drawn.

#### *Current day gene flow and range expansion*

Patterns of genetic divergence found in many species affected by Pleistocene glacial cycles are often accompanied by low levels of differentiation within regions. This pattern can be explained by population contraction during glacial maxima (and thus loss of genetic diversity) followed by rapid range expansion leading to genetic homogeneity across a wide geographic area, or alternatively by high levels of current day gene flow. Historical range expansion may obscure any signal of current gene flow patterns (Mila et al. 2000), and perhaps for this reason only one study of a widespread North American bird that has identified range expansion has additionally found a pattern of isolation by distance (Gibbs et al. 2000). In the current study, mismatch distributions (Figure 4a) indicated range expansion across continental North America, and coalescent analyses indicated high levels of gene flow between populations east and west of the Rockies. Nevertheless, several results suggest that current day gene flow is somewhat limited in

this species. First, our mtDNA analyses detected significant isolation by distance across North America (Figure 5), a pattern supported by significant  $F_{ST}$  values only between Newfoundland and populations further to the west (Table 3), and AMOVA analyses of AFLP data suggest a weak pattern of limited gene flow within the continental US. Taken together, these analyses suggest that continental redstarts expanded from a single glacial refugium (see Ruegg and Smith 2002), but also that gene flow across the current range is somewhat limited.

Additionally, we found much larger levels of genetic differentiation (corrected for effective population size) for nuclear markers than for mitochondrial markers (Table 4). Male-biased dispersal would lead to the opposite pattern, and so our results suggest female-biased dispersal, as has long been thought to be the norm for birds (Greenwood 1980; Clark et al 1997). It is somewhat puzzling that nuclear markers would show greater differentiation than mitochondrial markers, as female dispersal should affect nuclear and mitochondrial gene flow equally. We currently do not have an explanation for this difference.

#### *Comparisons of marker utility*

Studies of widespread species that have revealed low levels of differentiation within refugial populations using mtDNA could be hindered by issues of marker sensitivity -- mtDNA may simply lack useful variation at the regional level (Lovette et al. 2004). More recently AFLP has been purported to generate a large number of highly variable markers with little development time (Mueller and Wolfenbarger 1999), yet very few researchers have used AFLPs to estimate population structure in animals (Bensch and Akesson

2005). In our comparison of the different markers we discovered that, in contrast to mtDNA, AFLPs suffered from one important setback: this method seems highly sensitive to the quality of DNA and hence the sample storage and preparation methods. Laboratory analyses were conducted on samples from the US within months of collection in the field, whereas extracted Canadian DNA samples were frozen for 7-8 years before analysis. The differences in storage time and storage buffer represent the only obvious differences between the two sets of samples, as Canadian DNA originally extracted with a kit was re-extracted with phenol-chloroform and problems persisted. We thus stress that one must be aware of this issue when considering DNA storage and preparation methodologies for AFLP analysis.

### *Conclusions*

Although dispersal distances for migratory songbirds like the American Redstart are potentially very large, our data show that weak but significant population structuring exists, and that this genetic structure is due to both historical and current day processes. Historically, our analyses revealed that continental populations have recently expanded from a smaller mainland refugial population, whereas redstart populations in the maritime regions of Canada are potentially descended from a northeastern refugial population. These results contrast sharply with patterns seen in other widespread North American migratory birds, most of which appear to have survived through the Pleistocene glaciations in eastern and western refugia. Our analyses also revealed a weak pattern of limited contemporary gene flow, seen in both the mtDNA and AFLP analyses, layered on top of the pattern generated by Pleistocene events.



Boulet and Gibbs (2006) used genetic analyses to infer that at least two eastern refugia existed for another widespread migratory North American songbird, the yellow warbler. Furthermore, two of their sample sites, one in Newfoundland and one in New Brunswick, contained common haplotypes along with predominantly maritime haplotypes, raising the possibility that one of the eastern refugia was in the far northeast. Taken together with the genetic differentiation of the Newfoundland sample site presented in this study, these studies support the existence of an Atlantic coastal shelf refugium for at least two species of migratory songbird. Future phylogeographic studies should sample in this region to determine the importance of this refugial area for other extant species.

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**Table 1** Phylogeographic breaks found in species with broad distributions similar to that of the American Redstart

Species	Study	Location of divide	Timing of divergence (years before present/ sequence divergence)
Yellow Warbler	Milot et al (2000)	Great Plains/Rockies	100,000 /2.15%
Swainson's Thrush	Ruegg and Smith (2002)	Rockies	10,000/0.69%
Wilson's Warbler	Kimura et al (2002)	Great Plains/Rockies	33,654-62,500/0.7%-1.3%
Yellow Breasted Chat	Lovette et al (2004)	Great Plains/Rockies	not given/1.8%
Common Yellowthroat	Lovette et al (2004)	Great Plains/Rockies	not given/2.0%
Nashville Warbler	Lovette et al (2004)	Great Plains/Rockies	not given/1.7%-2.3%
Wood Duck	Peters et al (2005)	Great Plains/Rockies	10,000-124,000/not given
American Redstart	present	Newfoundland	40,300-2,171,000/1.0%

**Table 2.** Sampling locations and number of individuals analyzed in AFLP and mtDNA analyses.

Location	Lat.	Long.	AFLP	mtDNA
Revelstoke Natl Park, British Columbia (BC)	50.59	118.12		34
Prince Albert Natl Park, Saskatchewan (SAS)	53.57	106.22		19
Queen's Univ Field Station, Ontario (ONT)	44.35	76.19		16
La Mauricie Natl Park, Quebec (QUE)	45.35	73.41		18
Fundy Natl Park, New Brunswick (NB)	45.37	65.02		13
Gros Morne Natl Park, Newfoundland (NF)	49.36	57.31		20
Bogue Chita NWR, Louisiana (LA)	30.19	89.55	27	11
Jugg Bay, Maryland (MD)	38.45	76.42	28	10
Hubbard Brook, New Hampshire (NH)	43.57	71.43	19	9
Raco, Michigan (MI)	46.24	84.33	33	14
Swan Lake, Montana (MT)	48.02	114.01	33	11

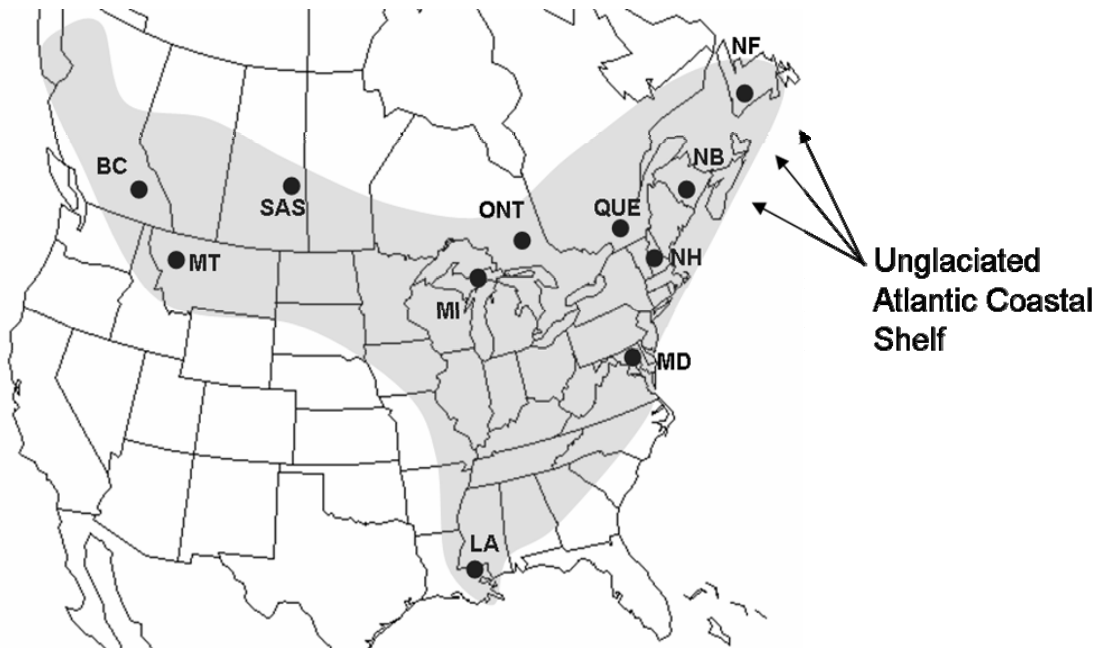


**Table 3.** Results of pairwise  $F_{ST}$  estimates obtained from mtDNA analyses. Bold values are comparisons between Newfoundland and other sites, values significantly different from zero at  $P < .005$  are denoted with an asterisk. Abbreviations as in Table 2.

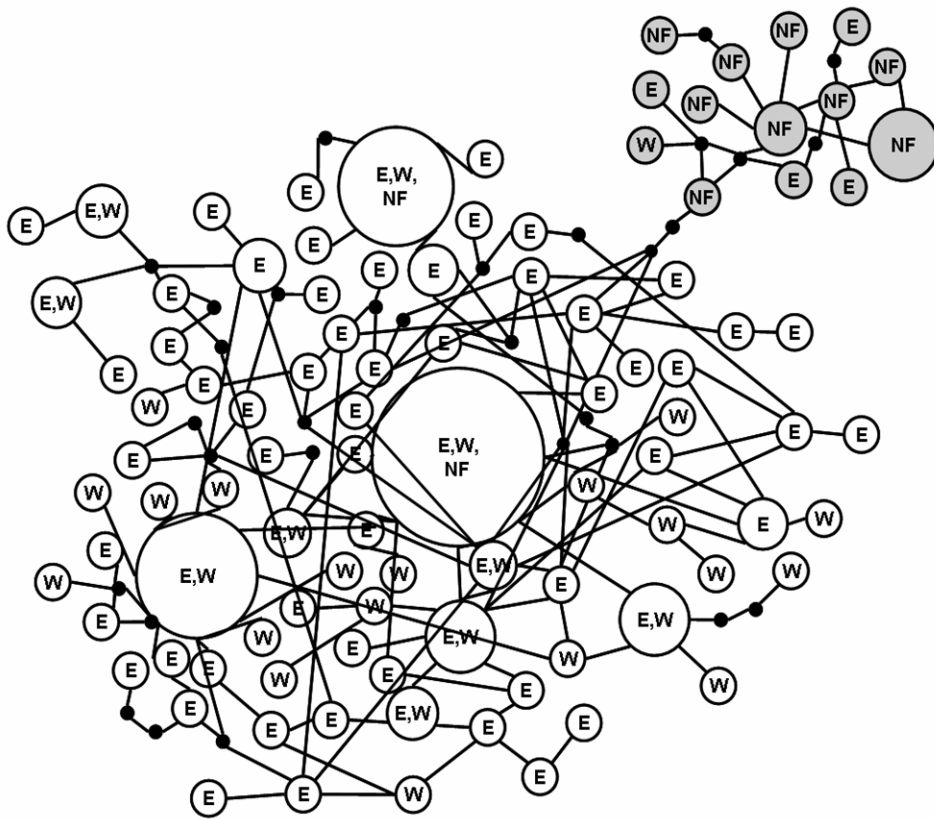
	LA	MD	NH	MI	MT	BC	SAS	ONT	QUE	NB
MD	-0.015									
NH	0.023	0.036								
MI	0.002	0.013	-							
			0.013							
MT	0.021	0.023	0.051	0.035						
BC	0.030	0.040	0.034	0.027	0.002					
SAS	-0.001	0.020	0.027	0.011	0.036	0.043				
ONT	-0.020	0.001	0.024	0.005	0.013	0.020	-0.003			
QUE	0.002	0.014	-	-	0.018	0.011	0.009	-0.008		
			0.008	0.007						
NB	0.008	0.020	-	-	0.042	0.034	0.013	0.001	-	
			0.008	0.020					0.010	
NF	<b>0.075*</b>	<b>0.083</b>	<b>0.060</b>	<b>0.053</b>	<b>0.096*</b>	<b>0.077*</b>	<b>0.075*</b>	<b>0.071*</b>	<b>0.058</b>	<b>0.05</b>

**Table 4** Results of pairwise  $Phi_{ST}$  estimates obtained from AFLP analyses (above diagonal) and  $F_{ST}$  values obtained from mtDNA (calibrated for comparison with nuclear markers – see Brito 2007) below diagonal.

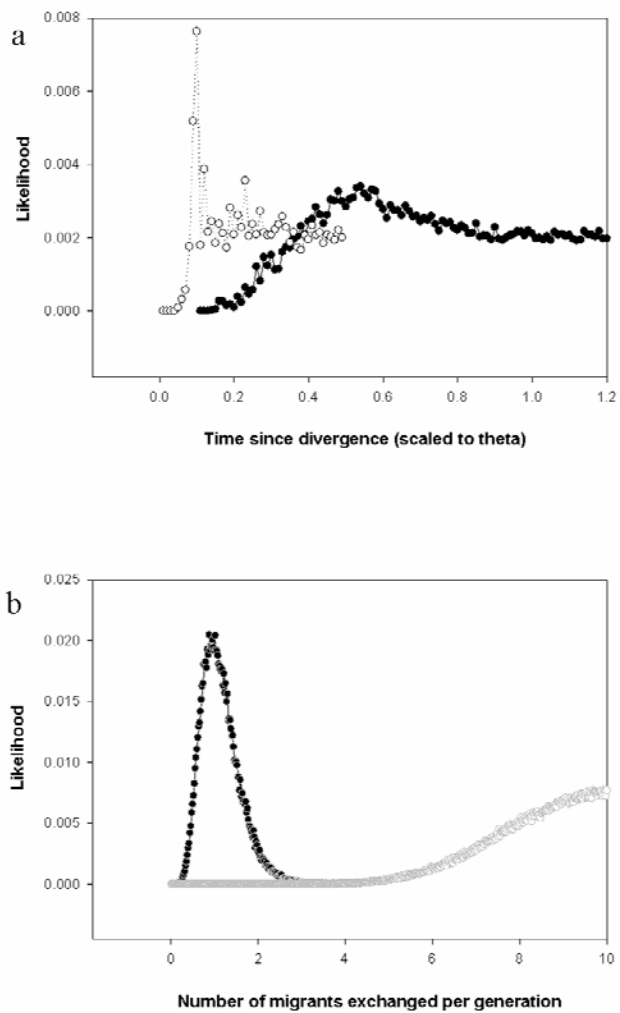
	LA	MD	NH	MI	MT
LA		0.019	0.034	0.034	0.035
MD	-0.004		0.015	0.040	0.035
NH	0.006	0.009		0.039	0.049
MI	0.001	0.003	-0.003		0.031
MT	0.005	0.006	0.013	0.009	



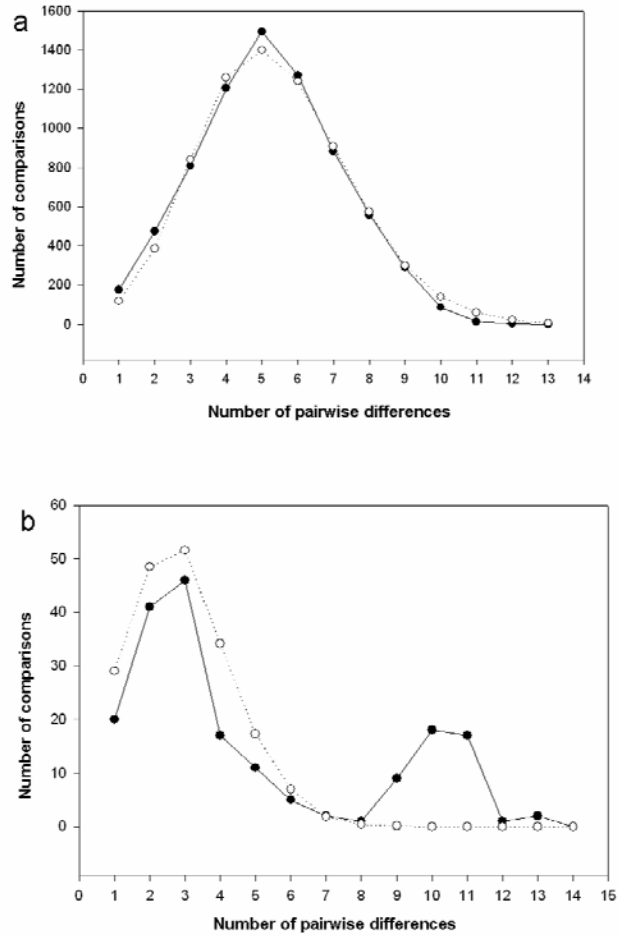
**Figure 1.** The breeding distribution of the American Redstart is shaded in grey. Sampling sites (black dots) are labeled with abbreviations from Table 2. Arrows indicate the approximate location of the proposed Atlantic coastal shelf refugium.



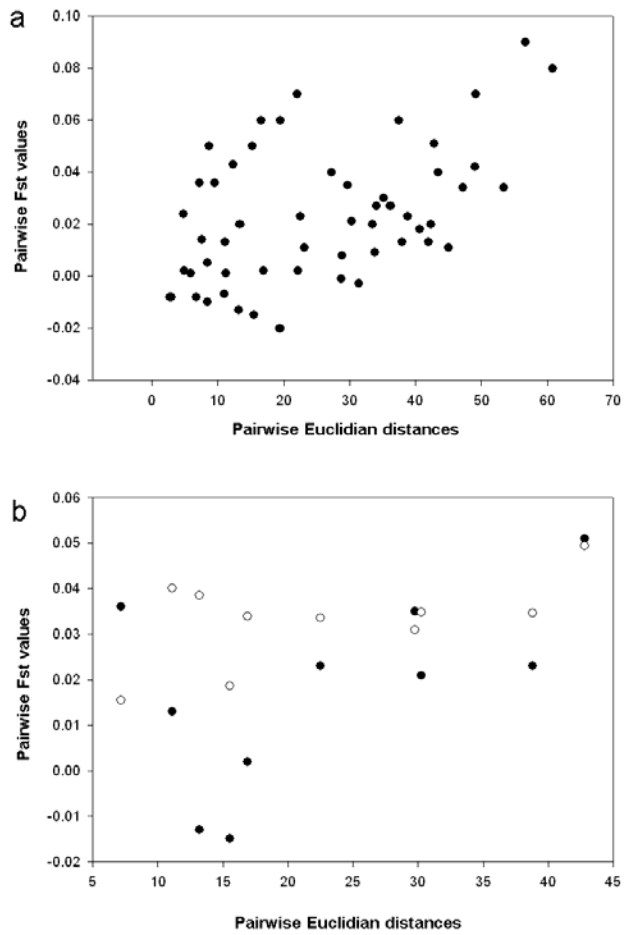
**Figure 2.** Haplotype network produced by TCS. Each circle represents a single haplotype (small circles indicate a single individual, larger circles indicate that the haplotype was sampled from multiple individuals), and circles connected by a line are one mutational step apart. Letters indicate the location(s) where the haplotype was found (W = western North America, E = eastern North America, NF = Newfoundland). Haplotypes in the “Newfoundland Clade” are shaded gray. Filled points represent missing haplotypes.



**Figure 3.** Likelihood graphs produced by the MDIV analysis showing time since divergence between those populations (A) and migration rate between two putative historical populations (B). Black circles are the results of the analysis when run according to a Newfoundland/Mainland split, while the open circles are the results of an east/west split.



**Figure 4.** Distribution of pairwise nucleotide differences between individuals sampled in (A) the mainland and (B) Newfoundland. Black points are the observed distribution, white points are the distribution expected under range expansion.



**Figure 5.** Scatter plot of pairwise distances (x axis) and pairwise  $F_{ST}$  values (mtDNA) for all sample sites (A). Isolation by distance for US sample sites only, black circles are based on mtDNA and white circles are based on AFLPs (B).

## CHAPTER TWO

# SONG DIVERGENCE AND THE POTENTIAL FOR REPRODUCTIVE ISOLATION IN A MIGRATORY SONGBIRD (*DENDROICA CAERULESCENS*)

### Introduction

There is considerable interest in understanding the evolutionary forces that lead to phenotypic divergence between populations of the same species, and also the extent to which such divergence leads to reproductive isolation between those populations, as these are the initial stages in the process of speciation. When populations are fully allopatric, with little or no gene flow between them – for example when populations were isolated in separate glacial refugia during the Pleistocene (Weir & Schluter 2004; Johnson & Cicero 2004), genetic drift and/or selection could have contributed to divergence. However, a number of species appear not to have been separated into multiple glacial refugia during the Pleistocene, and instead have expanded from a single glacial refugium to occupy a more-or-less continuous range today (e.g., Mila et al. 2007; McKay 2009). In such species, putative sister taxa have a parapatric distribution with respect to each other, and on-going gene flow has the potential to counter the diversifying effects of selection and drift. Accordingly, parapatric divergence requires relatively strong selection to overcome the homogenizing effects of gene flow (Slabbekoorn & Smith, 2002), but it is often not



clear what the selective forces might be, nor whether such forces promotes reproductive isolation between adjacent populations.

Divergence could arise if ecological selective pressures vary across the species range; that is if phenotypes perform better in their local habitat than in non-local habitats (Schluter 2001; Rundle & Nosil 2005). This process may be particularly likely for species that have expanded in recent times from a single glacial refugium, as these range shifts may have exposed different populations within a species' distribution to different environments, planting the seeds of ecological divergence (Smith et al. 1997; Hendry et al. 2002; Weir & Schluter 2004).

Alternatively, divergence might result from geographic variation in the strength or direction of sexual selection acting on reproductive traits (Panhuis et al. 2001). Indeed, divergence might be particularly likely, and proceed more quickly, if sexual selection is involved (Ritchie 2007). In other words, the reason why local phenotypes perform better in local habitats is because the distributions of sexual phenotypes, and conspecific responses to those phenotypes (e.g., mate choice), have diverged between populations (Seehausen et al. 1997; Irwin et al. 2001; Boul et al. 2007).

The most commonly cited evidence for sexual selection contributing to divergence between populations is the presence of divergent sexual traits between the populations (Ritchie 2007), but in most cases we do not know what forces have lead to this divergence (Coyne & Orr 2004). Behavioral sexual signals that are learned, such as bird song, might be particularly susceptible to divergence between populations as learning errors can quickly accumulate in different populations (Podos & Warren 2007). However, while learning is thought to speed the process of allopatric speciation (Lachlan

& Servedio 2004), it is generally thought to hinder sympatric or parapatric divergence (Irwin & Price 1999; Ellers & Slabbekoorn 2003; Price 2008). This would occur if individuals are capable of learning new songs after dispersal, obviating any barrier to gene flow due to divergent preferences. While many songbirds are thought to be “close-ended” learners that learn their songs early in life, relatively few experiments have been conducted to determine if learning occurs prior to or after dispersal and little consensus exists as to which mechanism is more prevalent (Ellers & Slabbekoorn 2003). Thus, there appears to be two possible explanations for divergence across a group of populations that differ in song: (1) they may have been allopatrically isolated at one point, with song learning leading to rapid inter-population divergence in song, or (2) they may have never been isolated, with population differences stemming from pre-dispersal song learning accompanied by divergent sexual selection and/or limited dispersal.

While the presence of divergent sexual signals between populations provides a first line of evidence that sexual selection has contributed to divergence, it is not clear whether these signals contribute to reproductive isolation and speciation. For full reproductive isolation to result, it is necessary for individuals across populations to perceive and discriminate against non-local signals. Work from a variety of organisms, from frogs (Boul et al. 2007; Reynolds & Fitzpatrick 2007; Guerra & Ron 2008), to fish (Boughman et al. 2005; Zuarth & Garcia 2006), to crickets (Gray & Cade 2000; Shaw & Lugo 2001; Gray 2005), to birds (Leader et al. 2002; Searcy et al. 2002; Nelson & Soha 2004; Patten et al. 2004; Nicholls 2008; Podos 2007; Uy et al. 2009) has indicated that non-local signals are discriminated against, suggesting that the promotion or maintenance of divergence in sexual signals by sexual selection may be a widespread phenomenon.

However, responses to local versus non-local signals may be asymmetrical, such that discrimination against non-local signals occurs in some populations but not in others of the same species. This situation could result in an asymmetrical and therefore incomplete barrier to gene flow between populations (Kaneshiro 1980; Arnold et al. 1996). Unfortunately, many studies of discrimination against non-local sexual signals have been conducted in single populations and so could not examine the possibility of asymmetrical responses (e.g., Gray & Cade 2000; Searcy et al. 2002; Podos 2007; Guerra & Ron 2008; Uy et al. 2009).

The Black-throated Blue warbler (*Dendroica caerulescens*) is a migratory bird species that shows pronounced phenotypic differences across populations. For example, males from southern breeding populations tend to have darker plumage than do males from more northerly populations (Grus et al. 2009), and isotope studies have revealed a “migratory divide” with birds from southern populations wintering in the eastern Caribbean and those from the north wintering in the western Caribbean (Rubenstein et al. 2002). Such differences in plumage type and migratory behavior between populations are likely to have some genetic basis (e.g., Berthold & Pulido 1994; Mundy 2005), yet phylogeographic analyses have shown that all current day populations of this species likely have expanded since the recent Pleistocene from a single refugial population (Davis et al. 2006; Grus et al. 2009). This finding is remarkable in that it indicates that any heritable differences between Black-throated Blue warbler populations have evolved across parapatric populations very rapidly, and likely in the face of on-going gene flow.

Because song is a sexual signal that has the potential to evolve rapidly between populations (Podos & Warren 2007), we were interested in determining whether song has

diverged between northern and southern populations of this species, and also whether any such divergence contributes to reproductive isolation between them. Divergence in bird song is particularly interesting because of the role that learning might play: we would expect strong divergence in song if males learn their songs on or near the natal territory prior to dispersal, or a lack of divergence in song if males can learn new songs after natal dispersal. Moreover, early song learning should contribute to assortative mating and thus, as a by-product, reproductive isolation (Irwin & Price 1999). Alternatively, even if songs are divergent across populations, non-local songs may not be discriminated against, as the response to such signals is as important as the divergence in the signals themselves. A lack of discrimination between song types would indicate that song differences likely have not contributed strongly to reproductive isolation. A further alternative is that discrimination will be asymmetrical, with some populations discriminating against non-local signals more than others. This result, thought to be particularly likely when one population has recently separated from an ancestral population (Kaneshiro 1980) might imply a uni-directional barrier to gene flow and incomplete reproductive isolation.

## **Methods**

### Song Variation

We recorded songs from color-banded males in the summers of 2006 and 2007 from one population in the north (New Hampshire - Hubbard Brook Experimental Forest,

43.9° N, 71.6° W) and one population in the south (North Carolina - Coweeta Hydrological Laboratory, 35.0° N, 83.4° W). We chose these sites because on-going long term studies at each allowed us to use color-banded males of known age and breeding status. We recorded uncompressed wave files with a Marantz PMD 670 digital recorder and Sennheiser ME66 shotgun microphone with a K6 power supply.

Black-throated blue warbler males typically have repertoires of two songs, one primarily used at dawn and the other primarily used throughout the rest of the day (G. Colbeck pers. obs.). For this study, we used the ‘day’ song, rather than the ‘dawn’ song, of each subject. We used one song with low background noise from each of 40 males per study site for spectrographic analysis. Before analysis, we set the maximum amplitude of each song to 0.5 amplitude units (units of amplitude are arbitrary and specific to different programs) with the sound analysis program AUDACITY 1.3 (Mazzoni 2009). With the same program, we high and low pass filtered all files before exporting them as wave files. We analyzed spectrograms of individual songs using both RAVEN PRO 1.3 (Charif et al. 2008) and Sound Analysis Pro (SAP - Tchernichovski et al. 2001). SAP provides many additional measures not provided by RAVEN, including variance of frequency measures and entropy (the distribution of sound energy across frequency space). Black-throated blue songs consist of one note repeated several times before a terminal buzz (Figure 1), so we collected measurements for notes and buzzes separately from each male’s song (one representative note per song - the last note before the terminal buzz). In the spectrographic view mode of RAVEN, we drew boxes by eye around notes and buzzes and generated measurements. For SAP, we first saved individual notes and buzzes as wave files and converted them to the sampling rate of 44100 Hz with the sound analysis

program Goldwave 5.3 (Goldwave Inc). We then opened the files with SAP in the "explore and score" section. We used the default gain, entropy and dB settings as recommended by the programmer (Tchernichovski et al. 2001). We placed cursors on either side of the note being analyzed, and recorded measurements from the "features across interval" display. We first compared all individual measurements from both programs, excluding variables of pure amplitude, between the two sampling sites with an ANOVA. To account for correlations among song measures, we also conducted principal components analyses to extract orthogonal descriptors of the songs. We conducted these principal component analyses separately for notes and buzzes, and we examined differences in principal component values between the populations with an ANOVA.

### Playback Experiments

We conducted playback experiments on color-banded males ( $N = 20$  in the north, 21 in the south) during the fertile period (building and/or laying) of the test subject's partner. These experiments were conducted in late April in North Carolina and late May in New Hampshire. We scouted each subject the day before the first playback trial to determine the rough area of his territory, and playback arenas were set up in the approximate center of that territory. We constructed six different playback loops for each locality using songs from the 40 males used in population comparison (Figure 1). Each playback loop consisted of three minutes of silence (the pre-playback period) followed by 5 minutes of song. We chose a song rate of 1 song every 7 seconds because this approximates a typical song rate during a single song bout (G. Colbeck pers. obs.). Each

subject received two playbacks, one with a northern song stimulus (chosen randomly from songs A-F in figure 1) and one with a southern song stimulus (chosen randomly from songs G-L in figure 1). The trial order (northern vs. southern song first) was determined randomly, and the two trials were conducted two days apart to avoid habituation.

We conducted all experiments before 10 a.m. to avoid a potential mid-day decrease in territorial motivation. During the experiments, we played playback loops (wave files) from an iPod set at maximum volume. Signal from the iPod was broadcast through an SME-AFS portable field speaker (Saul Mineroff Electronics) placed on the ground, with a standardized output volume of five. We chose these settings as they produced a final broadcast amplitude (when recorded at maximum gain at a 25m distance) that was similar to the amplitude measures produced when recording free living males at the same distance (0.3 amplitude units in AUDACITY).

Playback arenas consisted of measured and flagged (one flag in each of the cardinal directions) four meter and eight meter radius circles around the playback speaker. We waited until the subject was seen or heard near the arena before starting the playback trial. Once the experiment had started, an observer moved to the edge of the arena and vocally dictated the behavior of the subject into a digital recorder, in particular noting flights, the distance of a male after a flight, and "dive bombs" by the male over the speaker. We dictated distances outside the arena as "beyond 8m" and horizontal distances within the 8m arena were dictated in approximate 2m intervals. In addition, we pointed a shotgun microphone at the subject so song rate could be calculated after the experiment. To facilitate discrimination of the subject's song from that of the playback

loop (as well as other males singing in the distance), we vocally dictated “song” each time the subject sang. It was rare that a non-focal male (e.g., a male from a neighboring territory) would approach the playback arena, but in those cases we abandoned the experiment.

The data collected from each trial included length of time from initiation of playback to when the male entered the arena, closest approach to speaker, average distance from speaker, total time in arena, number of perch changes, number of dives over speaker, change in song rate in response to initiation of playback, and change in song rate in response to cessation of playback. We calculated average distance by multiplying each perch distance by the length of time the bird was at that perch, summing across all perch changes, then dividing by the total amount of time. We recorded distances beyond 8m as 25m as an approximate median. We first independently compared all response variables between treatments with a paired t-test. Since different response variables were likely correlated with each other, we also included all variables in a principal components analysis to extract orthogonal measures of male response, and differences in principal component values between treatments were compared with a paired t-test. We also tested for the effect of playback order with an ANOVA.

## **Results**

### Song Variation



Strong song differences were seen, in both the whistle notes and buzzes, between the northern and southern populations (Table 1). Three important classes of differences emerged from the data: (1) those associated with the note length and note repetition, (2) those associated with frequency measures, and (3) those associated with the distribution of song energy across frequency space (purity of tones vs. tones occurring across wide frequency ranges). In the first class, introductions in the north contained more notes and faster trill rates, whereas the length of individual notes was longer in the south. Conversely, terminal buzzes were longer in the north than in the south. In the second class, most frequency variables including mean frequency, maximum frequency, and frequency bandwidth were larger in the south than in the north. This holds for introductory notes as well as terminal buzzes. Thus, songs in the south were on average higher in frequency and occurred over a wider range of frequencies (including lower low frequencies). In the third class, variables such as entropy and frequency variance were larger in the south than in the north, again for both introductory notes and terminal buzzes. Small values for entropy (as well as frequency variance) indicate pure sound that occurs over a narrow range of frequencies. The larger the entropy, the more widely distributed is (across frequency space) the sound's energy (e.g., whistles have low entropy, while buzzes or harmonic overtones have large entropy). The lower entropy of the northern songs can be seen readily in Figure 1 - the northern songs appeared mostly as curved lines while the southern songs tended to be smears of sound.

To further explore quantifiable differences in song between the populations, we conducted a principle components analysis to account for correlations among song measures (Table 2). For introductory notes, a backwards stepwise regression on all PC

variables with eigenvalues larger than one showed that only PC1 explained a significant amount of variation between the sample sites ( $X \pm SD$ : North =  $2.45 \pm 2.05$ ; South =  $2.33 \pm 1.47$ ;  $F_{1,14.85} = 254.7$ ,  $P < 0.0001$ ), indicating that song frequency and entropy were higher in the south than the north. For terminal buzzes, a backwards stepwise regression on all PC variables with eigenvalues larger than one showed that PC1, PC3 and PC4 explained significant amounts of variation between the sample sites ( $X \pm SD$ : North PC1 =  $-2.0 \pm 1.74$ ; North PC3 =  $-0.23 \pm 1.51$ ; North PC4 =  $-0.25 \pm 1.34$ ; South PC1 =  $2.11 \pm 1.86$ ; South PC3 =  $0.24 \pm 1.54$ ; South PC4 =  $0.26 \pm 1.02$ ;  $F_{3,4.15} = 43.7$ ,  $P < 0.0001$ ), indicating that terminal buzzes also have higher frequency measures in the south than in the north.

### Playback Experiments

Males in the northern population responded much more strongly to the local song type than the non-local song type (Figure 3A, Table 3). The response variables 'Dives over speaker' and 'Total time w/in 8m' were significantly greater for the local stimulus (Mean difference  $\pm$  SE: Dives over speaker,  $2.55 \pm 0.61$ ,  $T = 4.19$ ,  $DF = 19$ ,  $P < 0.001$ ; Total time w/in 8m,  $129.80 \pm 34.46$ ,  $T = 3.77$ ,  $DF = 19$ ,  $P = 0.001$ ), and all other response variables trended in the direction of increased responsiveness to the local stimulus but were not significant after Bonferonni correction for multiple comparisons ( $\alpha < 0.006$ , Table 3). Conversely, males in the southern population did not show strong differences in responsiveness to local versus non-local song types, as none of the response variables differed significantly between the treatments (Table 3, Figure 3B).

We also conducted a principal components analysis on response variables for these experiments. This analysis revealed three principal component variables with eigenvalues larger than 1 (Table 4). For PC1, there was no effect of the order in which subjects received playbacks, i.e. subjects did not respond to the first playback more than the second playback ( $X \pm SD$ : North first =  $0.08 \pm 2.49$ ; North second =  $-0.21 \pm 2.46$ ;  $F_{1,0.81} = 0.133$ ,  $P = 0.718$ ; South first =  $-0.03 \pm 2.04$ ; South second =  $0.64 \pm 1.83$ ;  $F_{1,4.9} = 1.31$ ,  $P = 0.26$ ). Nor was responsiveness stronger in one site than the other ( $X \pm SD$ : North =  $.31 \pm 1.94$ ; South =  $-0.07 \pm 2.45$ ;  $T = .771$ ,  $DF = 82$ ,  $P = 0.44$ ).

For experiments conducted in the north, comparisons of response principal components with paired t-tests revealed larger values of PC1 and PC3 in response to local song compared to non-local song, but no significant difference for PC2 (Mean difference  $\pm$  SE: PC1,  $2.31 \pm 0.75$ ,  $T = -3.08$ ,  $DF = 19$ ,  $P = 0.006$ ; PC2,  $0.22 \pm 0.29$ ,  $T = 0.73$ ,  $DF = 19$ ,  $P = 0.47$ ; PC3,  $0.48 \pm 0.17$ ,  $T = 2.81$ ,  $DF = 19$ ,  $P = 0.011$ .  $X \pm SD$ : Local PC1 =  $1.22 \pm 2.61$ ; Non-local PC1 =  $-1.09 \pm 1.64$ ; Local PC2 =  $-0.17 \pm 1.43$ ; Non-local PC2 =  $-0.38 \pm 0.78$ ; Local PC3 =  $0.33 \pm 0.91$ ; Non-local PC3 =  $-0.15 \pm 1.18$ ). Thus these results lead to the interpretation that northern males responded more closely and more quickly to the local stimulus, but their singing behavior did not differ. For experiments conducted in the south, none of the principle components significantly differed between local and non-local song, thus both the physical and singing behavior of southern males did not differ between the stimuli ( $X \pm SD$ : Local PC1 =  $-0.22 \pm 2.16$ ; Non-local PC1 =  $-.39 \pm 1.75$ ;  $T = -0.244$ ,  $DF = 21$ ,  $P = 0.81$ ; Local PC2 =  $0.11 \pm 0.915$ ; Non-local PC2 =  $0.32 \pm 1.08$ ;  $T = -0.701$ ,  $DF = 21$ ,  $P = 0.49$ ; Local PC3 =  $-0.36 \pm 0.76$ ; Non-local PC3 =  $-0.43 \pm 0.71$ ;  $T = 0.39$ ,  $DF = 21$ ,  $P = 0.70$ ).

## **Discussion**

### Divergence in song

When populations are connected by gene flow (parapatrically or sympatrically), the process of speciation becomes challenging, as divergence must proceed in the face of gene flow, which tends to limit genetic differentiation between populations. While variation in ecologies may play a strong role in phenotypic divergence (Boughman et al. 2005), speciation requires that those phenotypic differences contribute to reproductive isolation. Accordingly, some have argued that sexual selection is the most common cause of reproductive isolation in animal species (Ritchie 2007). This hypothesis has been supported theoretically, as non-random mating can rapidly unite favorable alleles from different loci in a single gamete (Kirkpatrick & Ravigne 2002), and also empirically by studies showing that the integrity of diverged populations is more likely to be maintained if divergent sexual selection is involved (Stelkens et al. 2008; van der Sluijs et al. 2008).

Factors contributing to the divergence of traits across populations connected by gene flow could include ecological selection and/or sexual selection. Ecological selection is likely to operate concomitantly with sexual selection, either indirectly by shaping adaptations that subsequently change the phenotypes of sexual signals (Podos 2001; Boughman et al. 2005) or directly by acting on the propagation of signals through a particular environment (Boughman 2001; Slabbekoorn & Smith 2002). Alternatively, sexual selection might act independently of ecology to shape divergent traits across populations (Boul et al. 2007). Here, we present evidence that the songs of the Black-

throated Blue Warbler have diverged across populations, likely in the face of gene flow. The mechanisms behind this divergence remain unclear; however, additional lines of evidence suggest that both ecological and sexual selection may be acting. First, habitats in the south appear to absorb sound much more rapidly than habitats in the north, perhaps facilitating the evolution of more rapid trills in the north (Colbeck unpubl ms). Second, data from playback experiments and studies of extra-pair paternity have revealed strong sexual selection for faster trill rates in the northern population (Chapter 3).

Another factor that could contribute to the divergence of traits between populations is drift. While genetic drift might be an unlikely source of divergence for populations connected by gene flow, cultural drift could be particularly important for learned traits, such as bird song. This cultural drift could stem from different learning 'errors' accruing in different populations, and could be a particularly strong force leading divergence in song (Grant & Grant 1996; Podos & Warren 2007). Divergence in song due to cultural drift should be most likely to occur if two criteria are met: 1. if learning occurs pre-dispersal (Ellers & Slabbekoorn 2003), and 2. if dispersal is limited (reviewed in Podos & Warren 2007). While we do not have any information on song learning in Black-Throated Blue Warblers, song learning has been demonstrated for a closely related species, the Chestnut-sided Warbler (*Dendroica pennsylvanica*, Byers & Kroodsma 1992). In that species, learning appears to occur primarily prior to dispersal. For black-throated blue warblers, feather isotopes examined from first year males show that range-wide dispersal is extremely rare (Betts et al. in prep), and thus this species likely to meet the two criteria under which we would expect cultural drift to contribute to divergence in song.

Interestingly, patterns of geographic variation in song are thought to reflect the processes that generate them. Specifically, gradual clines in variation are thought to reflect cultural drift accompanied by limited dispersal, whereas discontinuous variation in song (the scenario fitting the true definition of 'dialects') is thought to reflect assortative mating (Podos & Warren 2007). Unfortunately, we are not in a position to describe what form of geographic variation in song exists in Black-Throated Blue Warbler, as we have sampled two populations that are inter-connected by unsampled populations.

### Reproductive Isolation

Regardless of the causes of divergence in signals across populations, if these signals are to contribute to parapatric speciation, they must contribute to reproductive isolation. Complete reproductive isolation requires that individuals across populations perceive and discriminate against non-local signals. Over the years there has been much interest and research into the consequences of geographic variation in sexual signals, however, many of those experiments have been conducted at a single site. In those cases (i.e. Gray & Cade 2000; Searcy et al. 2002; Podos 2007; Guerra & Ron 2008; Johannesson et al. 2008; Uy et al. 2009), the answer to the question becomes restricted to one specific site, and we do not know if there is geographic variation in discrimination.

Many of the experiments conducted in multiple populations have found that individuals across populations discriminate against non-local signals (Herring & Verrell 1996; Shine et al. 2002; Patten et al. 2004; Zuarth & Garcia 2006; Boul et al. 2007; Seddon & Tobias 2007). In some situations where local signals are preferred across populations, researchers have looked more closely at the symmetry of that reproductive

isolation, often finding that species A can successfully mate with species B, but B has reduced success when mating with A (i.e. asymmetrical reproductive isolation - Kaneshiro 1980; Arnold et al. 1996; Coyne et al. 2002; Mendelson 2003, Hoskin et al. 2005). The interpretation of this situation, first put forth by Kaneshiro (1980), is that one population may have recently become separated from an ancestral population, with population bottlenecks contributing to a reduction or change in sexual signals. Thus individuals in the derived population still respond relatively strongly to ancestral signals, but individuals in the ancestral population find the derived signals less stimulating. This situation should, in theory, be a common early step in the process of speciation (Kaneshiro 1980; Arnold et. al 1996). Asymmetric reproductive isolation is probably an inappropriate description of what we have found in the Black-throated Blue warbler, as a lack of discrimination in the southern population should translate into a lack of reproductive isolation. Discrimination in one population and a lack of discrimination in another population could perhaps be an early stage in the process of asymmetric reproductive isolation and speciation and to our knowledge has been reported rarely in the literature (Shaw & Lugo 2001; Lemaster & Mason 2003; Matsubayashi & Katakura 2007).

While molecular evidence suggests that extant populations of the Black throated Blue Warbler are genetically similar and have recently expanded from a single refugium (Davis et al. 2006; Grus et al. 2009), ancestral vs. derived songs may still be plausible. Price (2008) argues that new song variants are more likely to establish themselves in peripheral populations. The southern arm of the range of the Black-throated Blue could perhaps be considered peripheral, as populations are restricted to high elevation sites

along the spine of the Appalachian Mountains. Patterns of discrimination also fit this scenario, as individuals from a southern population do not discriminate between local and non-local song, but those from the north respond more strongly to local songs.

In our study we have used male responses to test for discrimination. The assumption is that females will attend to the same signals that a male attends to, and has been made quite frequently in the avian literature. This is largely because of the difficulty involved with surveying female responses in the wild, or bringing them into the lab and conducting estradiol induced preference trials. In support of the hypothesis that females should be more discriminating in their choice of mates than males, one study suggests that females discriminate against non-local signals in a similar manner to males, albeit on a finer geographic scale (Searcy et al. 2002). In light of this study, we might expect females in the north may be even less responsive to southern songs, while females in the south may show a level of discrimination not exhibited by males. While we do not have data on female responsiveness, we have found that trill rate, one of the song variables that differs between populations (Table 1), is important for extra-pair paternity in the northern population (Chapter 3). Thus, to the extent that extra-pair paternity reflects female choice, female choice could be mirroring male responsiveness. In addition, we would expect male responsiveness to be particularly likely to reflect female preferences when a territorial male's pair female is fertile. This would be because during the female's fertile period, it would benefit the male to be attentive to signals preferred by his female, and selectively confront those threats to his paternity. Thus all of our experiments were conducted during the subject's pair female's fertile period.



## Conclusions

Song divergence across populations of the Black-throated Blue warbler has likely occurred in the face of on-going gene flow (Davis et al. 2006; Grus et al. 2009), perhaps as a consequence of sexual selection on song traits (above and Chapter 3). Because males in a northern population responded more strongly to local songs than to non-local songs, but males in a southern population did not, partial or whole reproductive isolation between these populations based on song may be incomplete and unidirectional. The evolutionary consequences of such asymmetric responses, as well as the evolutionary forces acting on the behavioral response to these signals, require further attention.

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Table 1: Comparisons of all introductory note and terminal buzz variables from songs from northern (N = 40) and southern (N = 38) populations. All data are presented as the mean  $\pm$  SD. All significant comparisons ( $P < 0.002$  to account for multiple comparisons) are denoted with an asterisk\*.

Acoustic Variable	Introductory notes				Terminal Buzzes			
	Locality		Between-site comparison		Locality		Between-site comparison	
	North	South	F value	P value	North	South	F value	P value
Mean frequency (kHz)	4.69 $\pm$ 0.25	5.27 $\pm$ 0.27	93.87	< .001*	5.62 $\pm$ 0.25	6.10 $\pm$ 0.26	69.32	< .001*
High frequency (kHz)	5.81 $\pm$ 0.36	6.59 $\pm$ 0.23	127.66	< .001*	6.69 $\pm$ 0.25	7.31 $\pm$ 0.28	110.20	< .001*
Frequency bandwidth (kHz)	2.1 $\pm$ 0.50	3.15 $\pm$ 0.42	98.95	< .001*	2.73 $\pm$ 0.33	3.26 $\pm$ 0.28	58.20	< .001*
Mean entropy	-5.09 $\pm$ 0.33	-4.36 $\pm$ 0.22	128.34	< .001*	-4.67 $\pm$ 0.24	-4.35 $\pm$ 0.24	33.75	< .001*
Frequency variance (kHz <sup>2</sup> )	194.73 $\pm$ 162.05	666.24 $\pm$ 411.23	45.06	< .001*	263.38 $\pm$ 236.97	404.76 $\pm$ 343.37	4.52	0.037
Frequency at max power (kHz)	4.6 $\pm$ 0.41	5.17 $\pm$ 0.74	17.96	< .001*	5.43 $\pm$ 0.64	6.17 $\pm$ 0.50	32.14	< .001*
Note length (s)	0.13 $\pm$ 0.04	0.17 $\pm$ 0.03	17.65	< .001*	0.44 $\pm$ 0.10	0.35 $\pm$ 0.08	18.90	< .001*
Mean pitch goodness	89.78 $\pm$ 34.78	106.41 $\pm$ 13.93	7.36	0.008	96.95 $\pm$ 21.2	103.28 $\pm$ 16.28	2.17	0.145
Mean frequency modulation (Hz)	32.40 $\pm$ 8.85	35.85 $\pm$ 3.47	4.92	0.030	32.58 $\pm$ 4.90	33.19 $\pm$ 3.08	0.42	0.519
Frequency modulation variance (Hz <sup>2</sup> )	456.42 $\pm$ 95.76	454.35 $\pm$ 59.72	0.01	0.910	392.8 $\pm$ 56.52	361.61 $\pm$ 31.23	8.97	0.004
Pitch goodness variance	9.75 $\pm$ 15.24	74.81 $\pm$ 2.46	0.80	0.375	7.35 $\pm$ 3.80	7.00 $\pm$ 2.20	0.23	0.630
Entropy variance	0.24 $\pm$ 0.11	0.21 $\pm$ 0.06	2.86	0.095	0.023 $\pm$ 0.07	0.20 $\pm$ 0.05	4.97	0.029
Song length (s)	1.10 $\pm$ 0.20	0.97 $\pm$ 0.19	9.05	0.004	N/A	N/A	N/A	N/A
Low frequency (kHz)	3.71 $\pm$ 0.37	3.44 $\pm$ 0.41	9.03	0.004	3.96 $\pm$ 0.34	4.05 $\pm$ 0.30	1.53	0.221
Trill rate (notes/s)	6.28 $\pm$ 2.22	5.31 $\pm$ 0.80	6.31	0.014	N/A	N/A	N/A	N/A
Number of notes	6.95 $\pm$ 2.79	5.13 $\pm$ 1.27	13.11	0.001*	N/A	N/A	N/A	N/A

Table 2: Correlations between initial variables and principal components (eigenvalues  $\geq 1$ ) for songs from a northern and a southern population. Introductory notes and terminal buzzes were analyzed separately. The variable that loads most heavily onto each principle component is in bold. The percentage of song variation explained by each principle component is given in parentheses.

Acoustic variable	Introductory notes					Terminal buzzes				
	PC1 (37.01%)	PC2 (19.83%)	PC3 (8.63%)	PC4 (6.14%)	PC5 (5.3%)	PC1 (35.61%)	PC2 (18.06%)	PC3 (11.16%)	PC4 (7.03%)	PC5 (5.12%)
Mean frequency (kHz)	<b>0.955</b>	-0.124	-0.102	0.032	-0.050	<b>0.966</b>	-0.079	-0.034	-0.020	-0.087
High frequency (kHz)	0.904	-0.028	0.007	0.102	-0.155	0.903	0.083	0.228	0.064	-0.023
Frequency bandwidth (kHz)	0.862	0.288	0.060	-0.129	0.048	0.548	-0.058	0.390	0.701	-0.009
Mean entropy	0.821	0.224	0.308	-0.025	0.087	0.623	0.546	0.363	-0.170	0.039
Frequency variance (kHz <sup>2</sup> )	0.622	-0.001	0.590	-0.191	-0.180	0.300	0.714	0.174	-0.094	0.044
Frequency at max power (kHz)	0.608	0.170	<b>-0.604</b>	-0.015	-0.141	0.798	0.036	-0.314	0.110	0.187
Note length (s)	0.475	-0.684	0.222	0.183	-0.016	-0.455	0.087	0.092	0.098	<b>-0.225</b>
Mean pitch goodness	0.402	0.728	0.266	0.304	0.153	0.123	<b>0.946</b>	-0.113	0.012	0.073
Mean frequency modulation (Hz)	0.342	<b>0.840</b>	0.119	-0.068	0.075	0.017	0.871	-0.235	0.070	0.131
Frequency modulation variance (Hz <sup>2</sup> )	-0.010	0.638	0.134	-0.131	-0.357	-0.311	0.401	<b>-0.589</b>	0.145	-0.124
Pitch goodness variance	-0.069	0.574	0.194	<b>0.580</b>	0.070	-0.118	0.852	-0.073	0.041	-0.062
Entropy variance	-0.122	0.422	0.162	0.503	0.116	-0.420	0.182	-0.306	0.309	0.165
Song length (s)	-0.209	0.324	-0.077	0.178	<b>-0.774</b>	N/A	N/A	N/A	N/A	N/A

Table 3: Comparisons of responses to local and non-local stimuli for all response variables in the north (N = 20) and south (N = 22). All data are presented as the mean  $\pm$  SD. All significant comparisons ( $P < 0.006$  to account for multiple comparisons) are denoted with an asterisk\*.

Response variable	North				South			
	Stimulus		Between-stimulus comparison		Stimulus		Between-stimulus comparison	
	Local	Non-local	T value	P value	Local	Non-local	T value	P value
Number of perch changes	8.65 $\pm$ 8.35	2.05 $\pm$ 3.33	3.04	0.007	4.27 $\pm$ 5.1	2.64 $\pm$ 3.58	0.55	0.592
Total time w/in 8m	158.5 $\pm$ 149.29	28.7 $\pm$ 42.65	3.77	0.001*	86.91 $\pm$ 109.47	61.14 $\pm$ 96.03	0.73	0.476
Dives over speaker	2.7 $\pm$ 2.8	0.15 $\pm$ 0.37	4.19	<0.001*	0.86 $\pm$ 1.36	0.64 $\pm$ 1.0	0.19	0.851
Songrate difference (postplayback)	0.72 $\pm$ 1.8	-0.69 $\pm$ 1.58	2.52	0.021	0.59 $\pm$ 1.96	0.46 $\pm$ 2.3	0.60	0.558
Songrate difference (playback)	0.8 $\pm$ 2.14	0.6 $\pm$ 1.88	0.28	0.780	0.77 $\pm$ 1.36	0.25 $\pm$ 1.68	0.28	0.784
Average distance	10.51 $\pm$ 9.76	17.52 $\pm$ 9.53	-2.19	0.042	15.22 $\pm$ 10.1	13.96 $\pm$ 9.58	-0.83	0.420
Time of first approach	82.45 $\pm$ 287.09	255.75 $\pm$ 287.07	-1.72	0.102	192.77 $\pm$ 300.17	176.73 $\pm$ 265.52	-0.69	0.501
Closest approach	8.2 $\pm$ 11.37	16.9 $\pm$ 10.42	-2.38	0.028	13.77 $\pm$ 11.69	13.05 $\pm$ 10.58	-1.04	0.312



Table 4: Correlations between initial variables and principal components (eigenvalues  $\geq 1$ ) for responses to all playback experiments. The variable that loads most heavily onto each principle component is in bold. The percentage of response variation explained by each principle component is given in parentheses.

Response variable	PC 1 (53.87%)	PC 2 (14.84%)	PC 3 (11.28%)
Number of perch changes	0.886	-0.120	0.283
Total time w/in 8m	0.881	-0.069	0.238
Dives over speaker	0.769	-0.070	0.169
Songrate difference (postplayback)	0.233	<b>0.744</b>	0.157
Songrate difference (playback)	0.198	-0.743	0.154
Average distance	-0.922	-0.098	0.147
Time of first approach	-0.927	-0.028	0.132
Closest approach	<b>-0.944</b>	-0.067	<b>-0.298</b>

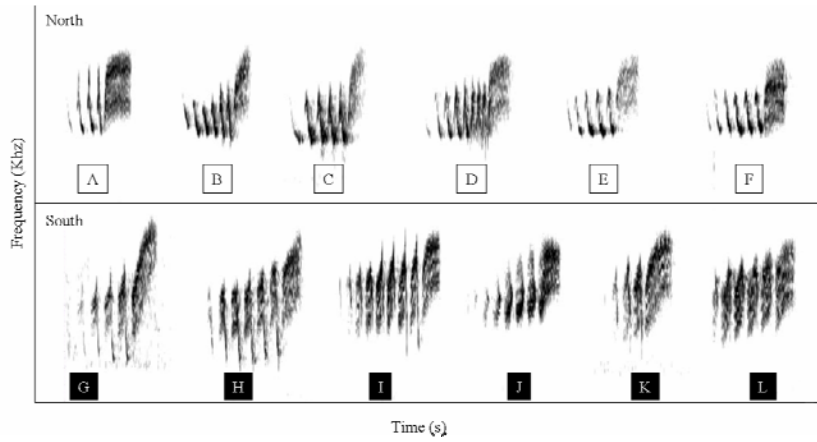


Figure 1. Example spectrograms of songs recorded from six males at the northern study site (A-F) and six males recorded at the southern study site (G-L). These were also the songs used as stimuli in the playback experiments.

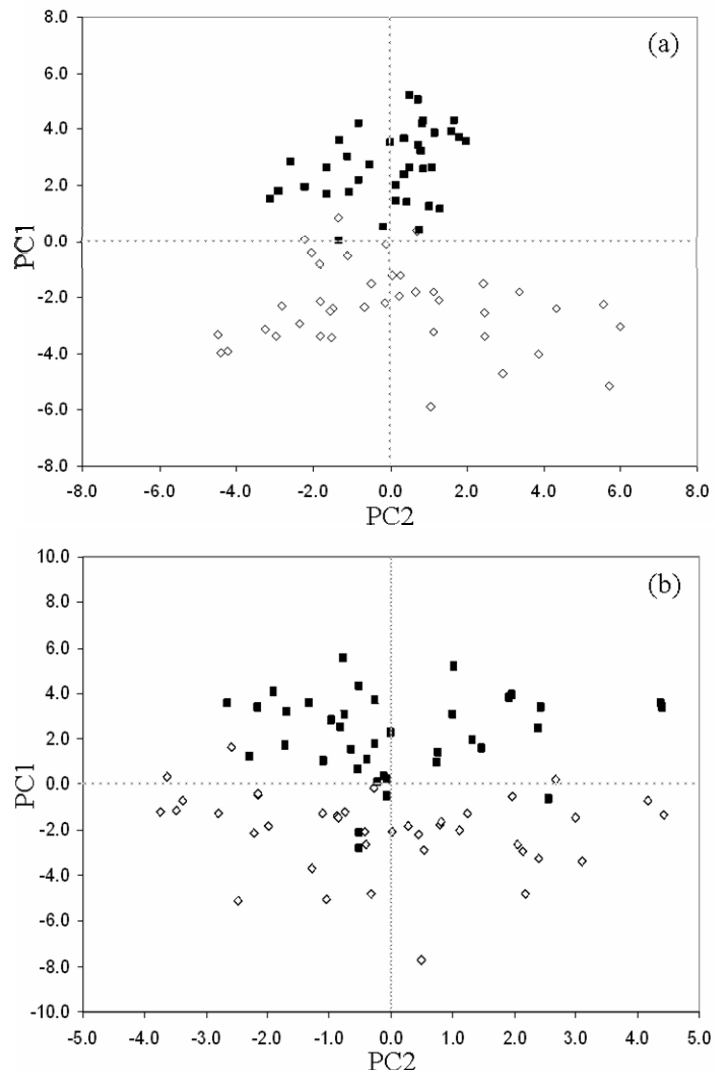


Figure 2. Differences between the north ( $\diamond$ ) and the south ( $\blacksquare$ ) in PC1 (e.g. frequency bandwidth) and PC2 (e.g. trill rate) for (a) introductory notes, and in PC1 (e.g. maximum frequency) and PC2 (e.g. entropy) for (b) terminal buzzes.

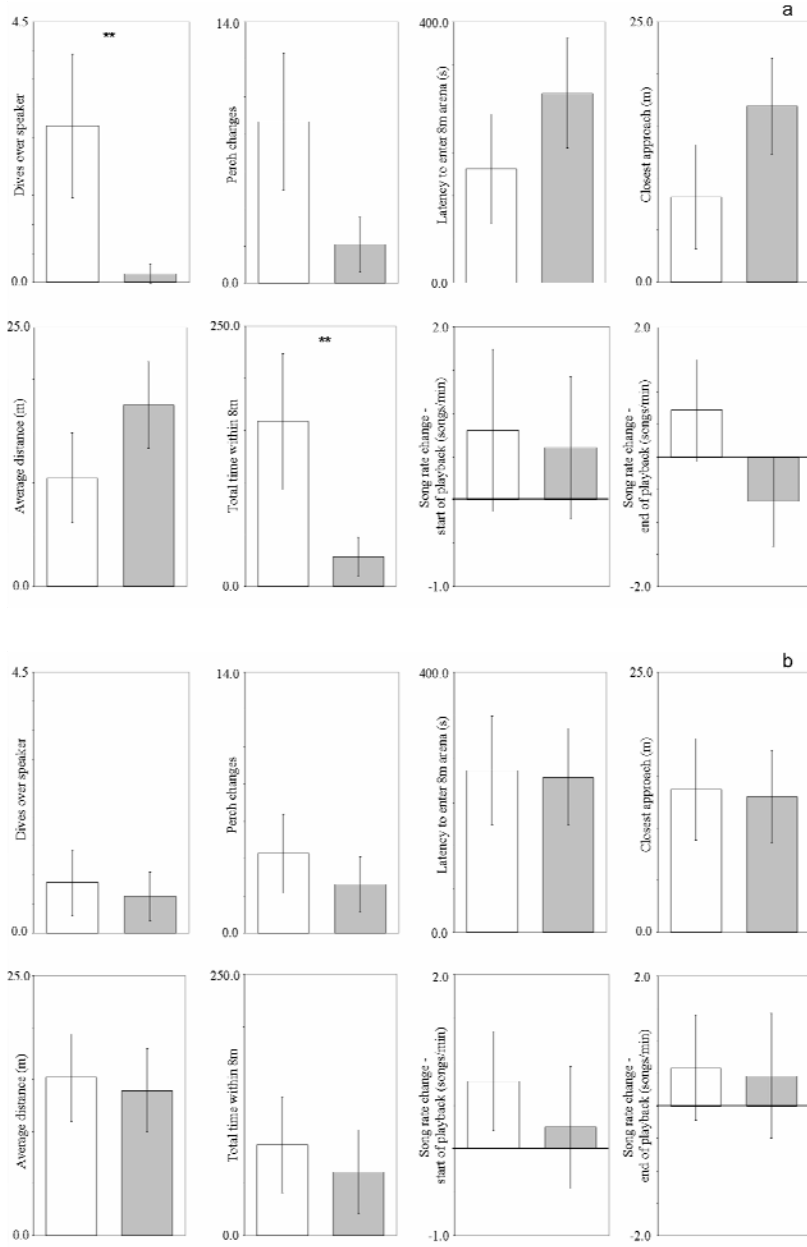


Figure 3. Playback experiment results in the north (a) and south (b): differences in subject responsiveness to local (white bars) and non-local (gray bars) stimuli are given for each response variable. Error bars represent the 95% CI around the mean. Significance after accounting for multiple comparisons ( $P < 0.006$ ) is denoted with a double asterisk \*\*. Full statistics are provided in Table 3.

## CHAPTER THREE

# SEXUAL SELECTION ACTS ON TRILL RATE, AN AGE-INDEPENDENT ASPECT OF SONG QUALITY, IN A NORTHERN POPULATION OF A MIGRATORY SONGBIRD (*DENDROICA CAERULESCENS*)

### Introduction

Conspicuous secondary sexual traits, such as elaborate coloration and vocal signals, generally are thought to be the products of sexual selection. Yet, explaining why particular traits (and not others) attract mates or repel rivals has proved very difficult and is one of the main goals of sexual selection theory (Andersson 1994). Under Zahavi's (1975) Handicap model, sexual signals are only meaningful to potential mates and competitors if their production is physiologically and/or socially costly, yet inferring how production and maintenance costs differ amongst traits has proven challenging.

Costly sexual signals may often be associated with age; if access to or ability to acquire resources increases with age (e.g. Miller & Brooks 2005), then older individuals should be better able to deal with the production/maintenance costs of elaborate signals. Accordingly, mate choice for more elaborate traits should result in mate choice for older individuals, and may benefit the choosers by ensuring that good genes underlying longevity are passed to their offspring (Kokko & Lindstrom, 1996). Examples of this process seem to be widespread (Komdeur et al. 2005; Siefferman et al. 2005; Nakagawa et al. 2007; Hegyi et al. 2008; Schmoll et al. 2009), and in many vertebrates the signals

that are the targets of sexual selection become more elaborate as individuals age (Brooks & Kemp 2001; Jennions et al. 2001).

Conversely, because they are not susceptible to changing environmental influences, traits that do not change with age might be particularly likely to reflect heritable condition (Hunt et al. 2004; Miller & Brooks 2005; Garamszegi et al. 2006a). A good example of a trait that appears to change little over an individual's lifetime can be found in songbirds, parrots and hummingbirds. In these bird species, 'crystallized' songs develop at a young age and change little throughout an individual's adult life (Hultsch & Todt 2004). In particular, the developmental stress hypothesis (Nowicki et al. 1998) posits that an individual's condition during the sensitive phase (the period of time in which songs are learned and become crystallized) is directly related to the quality of song that the individual is able to learn. Thus, this crystallized song becomes a badge of early developmental condition, a period of time in which condition is likely to reflect heritable differences among individuals (Nowicki et al. 2002; Ritchie et al. 2008).

For many songbirds, the rapidity with which notes of a song are repeated (trill rate) and the range of frequencies that those notes encompass (frequency bandwidth) trade off with each other (Podos 1997; 2001). This occurs because the larger the frequency bandwidth, the wider a bird has to open and close its bill, consequently the slower it can repeat the note (the slower the trill rate), resulting in an upper limit to the relationship between frequency bandwidth and trill-rate (see below). Podos (1997) hypothesized that only males in good condition can learn and/or sing songs near the upper limit of this relationship. Accordingly, females should prefer males near the limit,

and sexual selection should 'push' songs toward this biomechanical maximum via increasing trill rate, increasing frequency bandwidth, or both.

Additional research supports the hypothesis that the relationship between trill rate and bandwidth could be crystallized during the sensitive period. In particular, developmental stress seems to selectively affect the size of a brain region important for song learning and song production (the Higher Vocal Center, HVC), and the complexity of the songs that a bird is able to learn appear directly related to the size of the HVC during the sensitive period (Spencer et al. 2003; Buchanan et al. 2004; Macdonald et al. 2006; Soma et al. 2009). Complimentary research shows that 'high performance' songs are more difficult for juveniles to faithfully imitate and learn relative to 'low performance' songs (Podos 1996; Podos et al. 1999), suggesting that the performance level of an individual's song could be an honest badge of his condition during development. In addition, lab and field experiments are beginning to reveal that trill rate performance is a salient signal for potential mates and competitors (Ballentine et al. 2004; Illes et al. 2006; Cramer & Price 2007; Dalziell & Cockburn 2008; Schmidt et al. 2008; de Kort et al. 2009; Dubois et. al 2009). Thus, in the context of the trade off between frequency bandwidth and trill rate and the developmental stress hypothesis, we are provided with a rare opportunity to predict ahead of time which aspects of a trait should sexually selected.

The Black-throated Blue Warbler (*Dendroica caerulescens*) is a migratory songbird that breeds in eastern hardwood forests of North America, from southern populations along the spine of the Appalachian Mountains to northern populations in eastern Canada. Song differs significantly between a northern and southern population, with the individuals in the north differing from each other primarily along a trill rate axis,

and individuals in the south differing from each other primarily along a frequency bandwidth axis (Chapter 2; see also below). This led us to hypothesize that, within the constraints of a trade-off between frequency bandwidth and trill rate, selection might be acting on different components of song in the different populations.

In the present study, we tested the hypothesis that sexual selection is acting on song performance, specifically trill rate but not frequency bandwidth, in a northern breeding population. In addition, we examined whether song performance reflects current or early life condition: if song performance is an early developmental badge, as proposed by the developmental stress hypothesis, then measures of song performance should not increase with age, whereas if song performance is a badge of current condition, then these variables should increase with male age (e.g. Ballentine 2009).

Regardless if song performance is an early developmental badge or a current condition badge, to the extent that extra-pair paternity relates to variance in reproductive success, males singing high performance songs should be more successful at siring extra-pair young than males singing low performance songs. We use four years of data on song and paternity to test the predictions that: (1) males singing faster trill rates (but not larger frequency bandwidths) should be less likely to lose paternity in their own nests; (2) extra-pair sires should have higher faster trill rates (but not larger frequency bandwidths) than the males they cuckold; and (3) males with faster trill rates (but not larger frequency bandwidths) should sire more total genetic offspring per year. Finally, we used a playback experiment to test the prediction that males should perceive and respond differently to simulated intruders singing high vs. low performance songs.



## **Methods**

### *Study species*

Black-throated Blue Warblers are small (ca. 10 g) insectivorous passerines that overwinter in the Caribbean and breed in relatively undisturbed northern hardwood forests in the eastern United States and Canada (Holmes 2005). In New Hampshire, Black-throated Blue Warblers begin arriving on their breeding grounds in early May and initiate first broods by early June. Males defend breeding territories of 1-4 ha. Females build open-cup nests in low shrubs, lay 2-5 eggs per clutch (usually 4), and incubate the eggs alone (12-13 days). Both parents feed nestlings and attend fledglings (Holmes 2005). Natal dispersal is high, but 40-60% of adults return to the same general breeding area in subsequent years (Silllett & Holmes 2002). Black-throated Blue Warblers are socially monogamous, but extra-pair paternity is common: from 1995 through 1998, 34% of all broods sampled (N = 117) contained extra-pair young (Webster et al. 2001). Extra-pair sires have significantly higher return rates than do males who were cuckolded (67% versus 42%; Webster & Silllett, unpubl. data), suggesting that higher quality males are more successful at obtaining extra-pair paternity.

Black-throated Blue Warblers typically have two songs in their repertoire, one sung primarily at dawn and in male-male interactions, and a second sung during the day and prior to pairing (G. Colbeck, pers. obs.). In a northern population, the structure of the dawn song varies little between males across the population, but the day song shows discrete variation between males, with males forming song neighborhoods based on the similarity of their day song (Colbeck et al, in prep.).

### *Field protocol*

We monitored territoriality, breeding behavior and reproductive success of Black-throated Blue Warblers from the time that birds arrive on the breeding grounds (early May) until all young have fledged (mid-August) during the summers of 2004-2007. We lured adult males into mist nets with song playback, while females were flushed into mist nets from their nests during the incubation stage. We color banded, aged, and collected a small (30  $\mu$ l) blood sample from all adults. Most newly arriving birds on the plot (>90%, T.S. Sillett, pers. comm.) are in their first breeding season, but older individuals (ASY) are easily distinguished by the large size of their white wing spot and a lack of greenish alula feathers. We located all nests by following building females and monitored the nests through fledging. We banded, measured and collected blood from all nestlings on day 6.

We attempted to record songs from each color banded male on the study plot in each year of the study. We spent at least 15 minutes recording the 'day' songs of each male on a given day, and recorded each male on a minimum of two days in a given year. We recorded all songs as wave files with a Marantz PMD670 digital recorder and Sennheiser MKH 70 shotgun microphone. Before analysis, all songs were high and low pass filtered, and adjusted to a normalized .5 amplitude units with the program AUDACITY 1.3 (Mazzoni, 2009). Black-throated Blue Warbler songs consist of one note repeated several times before a terminal buzz (Figure 2), thus in the spectrogram view mode of RAVEN PRO 1.3 (Charif et al. 2008) we drew boxes by eye around the series of introductory notes to extract measurements. For a given individual, the structure of songs sung during the 15 minute daily recording sessions appeared identical, so one song was selected for measurement. To get a male's song measures for a particular

breeding season, we averaged the measurements from recording sessions on separate days (as they were also very similar).

#### *Performance constraints in Parulidae*

In order to determine if we would expect Black-throated Blue Warblers to experience the same trade-offs between frequency bandwidth as described for the family *Emberizidae* by Podos (1997), we used the measurement methods above on recordings from Stokes Field Guide to Birdsongs, eastern region (Stokes & Stokes 1997) and western region (Stokes & Stokes 1999) to plot trill rate vs. frequency bandwidth for 46 species of Parulid warbler. In the spectrograms, we searched for easily identifiable series of repeated notes (trills), and for those species with multiple distinct recordings containing trills, we used up to three songs per species, giving us a total sample size of 56. To estimate the upper bound regression, we divided individual measures into trill rate categories, one category every 5 notes/s. From each category, we selected the individual with the largest frequency bandwidth and conducted a linear regression on those individuals only (N = 6, e.g. Ballentine et al. 2004).

#### *Statistical analysis of age and song*

We first performed a cross sectional analysis, using a linear regression to test for the effect of age on acoustic variables for 123 males across 4 years (only the song measurements from one year, chosen at random, were included for males occurring in multiple years). We next performed a longitudinal analysis on 23 males whose songs were recorded in multiple years. For these males, we tested the prediction that acoustic variables would change with age by comparing measurements in one year to those in a subsequent year with a paired, 2-tailed t-test.

### *Paternity methodology*

We used 6 microsatellite loci to evaluate paternity (Table 2). One locus was isolated for the Golden-winged Warbler (*Vermivora chrysoptera*), VECR8 (Stenzler et al. 2004). A second locus was isolated for the Swainson's Thrush (*Catharus ustulatus*), CU $\mu$ 4 (Gibbs et al. 1999). Two more loci were isolated for the Yellow Warbler (*Dendroica petechia*), DP $\mu$ 1 and DP $\mu$ 16 (Dawson et al. 1997). The remaining two loci were extracted for *Dendroica caerulescens*, DCA28 and DCA32 (Webster et al. 2001).

We amplified genomic DNA in 10  $\mu$ L PCR reactions with 100  $\mu$ M dNTPs (Applied Biosystems), 0.5  $\mu$ M forward and reverse primers, a final concentration of 1X PCR Buffer (Promega), 3.0 mM MgCl<sub>2</sub> (Promega) and .75 units taq polymerase (Promega). Each reaction consisted of 25 cycles of 94°C for 60 s, the primer specific annealing temperature (Table 2) for 60 s, and 72°C for 45 s. Each forward primer was labeled with an Applied Biosystems fluorescent dye - FAM (blue), VIC (green), NED (yellow) or PET (red), and .2  $\mu$ L of PCR product from loci representing each of the 4 colors was combined with .3  $\mu$ L LIZ size standard (Applied Biosystems) and 10.5  $\mu$ L de-ionized formamide (Roche). We ran these mixtures in batches of 96 on an Applied Biosystems 3730 automated sequencer. To control for 1 base pair 'plate shifts' which can occur between different runs on the sequencer, we included between 1 and 5 individuals as repeats with every plate of 96.

We used Genemapper 3.7 (Applied Biosystems) to visualize DNA fragments. We scored all alleles by eye to avoid scoring errors associated with the automated scoring procedure used by Genemapper. We obtained estimates of scoring errors from the repeat individuals present on each plate (N = 100).

We used Cervus 3.0 (Kalinowski et al. 2007) to assign paternity to all nestlings. We first verified that the social mother was the true mother by checking mismatches between offspring and mother. We excluded nests where the social mother mismatched her offspring by 3 or more loci, or two loci of which at least one mismatch could not be explained by the presence of null alleles. For the assignment of paternity, we first conducted an 'Allele Frequency Analysis' using the default parameters in Cervus. We next conducted a 'Simulation of Paternity Analysis' entering 0.9 for the proportion of sires sampled (a conservative approximation representing unsampled males at the periphery of the study site), 0.99 for the proportion of loci typed and 0.05 for the proportion of loci mistyped (an estimate based on mismatches between offspring and their putative mother). We chose to calculate confidence with the LOD methodology and used 80% for the relaxed confidence and 95% for the strict confidence. We then conducted the paternity assignment, allowing Cervus to identify the sire of each offspring with strict confidence (95%, LOD score taking mother's genotype into account).

We then followed the protocol of Webster et al. (2004) to double check all of Cervus' paternity assignments. Specifically, we called paternity in favor of the social sire if he differed from the putative extra-pair offspring by one or zero mismatches. In cases where it was clear the most likely genetic sire was not the within pair male, we checked the list of males with the highest LOD scores and determined that we could not assign paternity if all of the potential sires mismatched at three or more loci, or two of which at least one could not be explained by null alleles. If the CERVUS identified sire mismatched at two loci, we assigned paternity to a male with a lower LOD score if he mismatched at only one locus.

### *Statistical analysis of song and paternity*

To determine if features of a male's song affected the probability he would lose paternity in his own nest, we conducted a logistic regression on song variables, age, male id and year against cuckolded vs. uncuckolded status. We conducted this analysis on first broods only as well as on first and second broods combined. For offspring in which we had data for the within pair sire and the extra pair sire, we conducted paired analyses on frequency bandwidth, trill rate and introduction length (N = 79) as well as age (N = 146). In cases where a male was cuckolded on multiple occasions by another male, we included their comparison only once. Because success at extra-pair paternity may not necessarily translate into increased fitness, we conducted a backwards stepwise regression on song variables, age, male id and year against the total number of genetic offspring sired in a given year. We conducted this analysis for first broods only, as well as for first and second broods combined.

### *Playback protocol*

We conducted playback experiments on color-banded males, but waited at least one week after a male was initially banded before using him as an experimental subject. We conducted experiments during the fertile period of the subject's female (building and/or laying) in order to maximize the probability that a male's responsiveness would mirror that of his female. We scouted each subject the day before experimentation so that we could have a good grasp of his territory, and playback arenas were set up in the approximate center of a male's territory. We were interested in testing for the effect of trill rate, independently of other factors, thus we constructed playback loops with songs that were artificially sped up and slowed down (Figure 2). We constructed playback

loops from five original songs recorded from males in 2004 and 2005 that were no longer present in the 2007 population. With the program AUDACITY 1.3 (Mazzoni 2009), we first took the original song, high and low pass filtered it and normalized its amplitude to .5 units. Next, under the 'Effect' menu, we selected 'change tempo' and made two copies of the song, one which we increased in speed by 30% and one which we decreased in speed by 30%. 30% was chosen as this maximized the amount of change without the resulting song being beyond the range of normal variation. Changing the tempo allowed us to change the speed of the songs without affecting the pitch. We used this procedure with caution on many songs before deciding on those that would be used in experiments. For some songs, changing the tempo led to the splitting of individual notes, and these songs were excluded. The resulting stimuli ranged in trill rate from 3.37 notes/s to 6.75 notes/s (Table 3), while natural variation in trill rate varies from 3 note/s to 14 notes/s, thus our stimuli span the lower end of natural variation where the bulk of individuals occur (Figure 1).

Each playback loop consisted of three minutes of silence (the pre-playback period) followed by 5 minutes of song. We chose a song rate of 1 song every 7 seconds in order to mimic a normal male's song rate during a song bout (G. Colbeck, pers. obs.). Each of 19 subjects received both a sped up and slowed down version of one of the songs in Figure 2, in random order. Experiments were conducted 2 days apart to avoid potential habituation.

We conducted all experiments before 10 a.m. to avoid a potential mid day decrease in territorial motivation. During the experiments, we played playback loops (wave files) from an iPod set at maximum volume. Signal from the iPod was broadcast

through an SME-AFS portable field speaker (Saul Mineroff Electronics) placed on the ground, with a standardized output volume of five. We chose these settings as they produced a final broadcast amplitude (when recorded at maximum gain at a 25m distance) that was similar to the amplitude measures produced when recording free living males at the same distance (0.3 amplitude units in AUDACITY 1.3). Playback arenas consisted of measured and flagged (one flag in each of the cardinal directions) four meter and eight meter circles around the playback speaker. We waited to start each experiment until the subject was seen or heard near the arena. Once the experiment had started, an observer moved to the edge of the arena and dictated the behavior of the subject, in particular noting flights, the distance of a male after a flight, and attempts by the male to "dive bomb" the speaker. We dictated distances outside the arena as "beyond 8m" and horizontal distances within the 8m arena were dictated in approximate 2m intervals. In addition, we pointed a shotgun microphone at the subject so his song rate could be calculated after the experiment. To facilitate discrimination of the subject's song from that of the playback loop (as well as other males singing in the distance), we vocally dictated "song" each time the subject sang. It was rare that a male neighboring the subject would approach the playback arena, but in those cases we abandoned the experiment. The data collected from the experiments included length of time after initiation of playback before male entered the arena, closest approach to speaker, average distance from speaker, total time within 8m, number of perch changes, number of dives over speaker, change in song rate in response to initiation of playback, and change in song rate in response to cessation of playback. We calculated average distance by multiplying each perch distance by the length of time the bird was at that perch, adding



amongst all perch changes, then dividing by the total amount of time. We recorded distances beyond 8m as 25m as an approximate median.

We independently compared all response variables between treatments with a paired t-test and Bonferroni correction for multiple comparisons. Independent comparisons and Bonferroni correction are useful for the detection of strong effects, but may be inappropriate for the detection of weak effects (Garamszegi et al. 2006b). We therefore used Hedges'  $g$  to calculate the effect size of each response variable, as well as the 95% CI around the mean effect size for all variables following Garamszegi et al. (2006b).

$$g = (x_1 - x_2)/s^*, s^* = \sqrt{((n_1 - 1)\text{stdeva}_1^2 + (n_2 - 1)\text{stdeva}_2^2) / (n_1 + n_2 - 2)}$$

Hedges'  $g$  is more appropriate for analyses with small sample sizes than Cohen's  $d$  because it is the difference between two means divided by the pooled variance, as opposed to the un-pooled variance (Garamszegi et al. 2006b).

Since different variables are likely correlated with each other, we also included all variables in a principal components analysis, and differences in principal component values between treatments were compared with a paired t-test. We also tested for the effect of playback order to see if responsiveness differed between first and second experiments.

## **Results**

### *Frequency bandwidth vs. trill rate*

The slope of the Parulidae upper bound regression (Figure 1a, Regression coefficient  $\pm$  SE:  $-210.07 \pm 54.53$ ,  $P = 0.018$ ) is defined by  $y = -210.07x + 7751.10$  and

is superficially very similar to the upper bound regression described for Emberezidae by Podos (1997), thus Black-throated Blue Warblers are likely to be susceptible to similar constraints on frequency bandwidth and trill rate.

### *Acoustic variables*

In a principle components analysis conducted using all measurements extracted with RAVEN from a sample of 40 songs, the acoustic variables trill rate, frequency bandwidth and introduction length loaded heavily onto the first three principle components, respectively (Table 1). Thus, we used those three variables only for all analyses.

### *Song and age*

In the cross sectional analysis, age did not explain any of the variation in song variables, nor was there an effect of year (regression coefficient  $\pm$  SE: introduction length,  $0.090 \pm 0.590$ ,  $P = 0.880$ ; trill rate,  $0.034 \pm 0.053$ ,  $P = 0.522$ ; frequency bandwidth,  $<0.001 \pm <0.001$ ,  $P = 0.264$ ; Year,  $0.084 \pm 0.099$ ,  $P = 0.400$ ;  $F_{4,0.92} = 0.606$ ,  $P = 0.659$ ), thus, within a given year, older males did not have faster trill rates, longer introductions or larger frequency bandwidths. In the longitudinal analysis, none of the song variables were significantly different between years (Figure 3; Mean difference  $\pm$  SE: trill rate,  $-0.11 \pm 0.37$ ,  $T = -0.30$ ,  $DF = 22$ ,  $P = 0.77$ ; frequency bandwidth,  $-31.11 \pm 91.29$ ,  $T = -0.34$ ,  $DF = 22$ ,  $P = 0.74$ ; introduction length,  $-0.02 \pm 0.04$ ,  $T = -0.42$ ,  $DF = 22$ ,  $P = 0.68$ ). Thus, for a given male across years, trill rate does not become faster, introduction length does not become longer and frequency bandwidth does not become

larger. In particular, most males do not change their trill rate appreciably across seasons, although a minority does (Figure 3).

### *Patterns of Paternity*

Our microsatellite loci were highly variable (between 14 and 29 alleles, Table 2), providing us with a high probability (0.999) of excluding a randomly selected male as the sire. We had a total of 665 mother/offspring/father trios in which all individuals were genotyped at 5 or 6 loci. Of those, 26 offspring (4%) from 14 broods and 12 mothers were un-assignable due to mismatches with the mother (3 loci or more, or 2 loci of which at least one could not be explained by null alleles). In these instances, all individuals were re-genotyped and re-scored to ensure mismatches were not lab artifacts. Of the remaining 639 offspring, 15 (2.3%) mismatched the mother at two loci (of which at least one mismatch could be explained by null alleles), 186 (29%) mismatched the mother at 1 locus, and 438 (68.5%) had zero mismatches. CERVUS assigned paternity with 95% confidence to all of these 639 offspring. In 291 cases in which CERVUS identified an extra-pair sire, we overturned 43 cases (14.8%) in favor of the within-pair sire because he mismatched at one or zero loci. Of the remaining 248 putative extra-pair offspring, we excluded 9 offspring (3.6%) from 8 broods because the most likely sire mismatched at two loci, of which at least one could not be explained by null alleles. In 16 cases (6.5%), we assigned paternity to a male with a lower LOD score because he mismatched at one locus, whereas the CERVUS identified sire mismatched at two.

Based on these paternity assignments, we found that 45% to 66% of first broods contained at least one extra pair offspring, and 27% to 45% of first brood offspring were

assigned an extra pair sire (Table 4). For second broods, 50% to 100% of broods contained extra pair offspring, and 27% to 67% of offspring were assigned to an extra pair sire (Table 4).

### *Song and paternity*

None of the song variables, age, year, or male id were significantly associated with the probability that a male would be cuckolded in their first broods (Regression coefficient  $\pm$  SE: trill rate,  $-0.072 \pm 0.090$ ,  $P = 0.421$ ; Frequency bandwidth,  $0.000 \pm 0.000$   $P = 0.893$ ; introduction length,  $0.298 \pm 0.947$ ,  $P = 0.753$ ; age,  $0.489 \pm 0.376$ ,  $P = 0.193$ ; year,  $-0.082 \pm 0.142$ ,  $P = 0.564$ ; male id,  $0.002 \pm 0.008$ ,  $P = 0.812$ ), or for first and second broods combined (Regression coefficient  $\pm$  SE: trill rate,  $0.101 \pm 0.078$ ,  $P = 0.199$ ; frequency bandwidth,  $0.000 \pm 0.000$   $P = 0.667$ ; introduction length,  $-0.214 \pm 0.827$ ,  $P = 0.796$ ; age,  $0.593 \pm 0.335$ ,  $P = 0.076$ ; year,  $-0.042 \pm 0.125$ ,  $P = 0.736$ ; male id,  $0.001 \pm 0.007$ ,  $P = 0.838$ ). Thus males with faster trill rates, longer introduction lengths, wider frequency bandwidths or older age were not less likely to be cuckolded.

In paired comparisons between extra-pair sires and within-pair sires, extra-pair sires had significantly faster trill rates (Figure 4a, Mean difference  $\pm$  SE: trill rate,  $-0.700 \pm 0.260$ ,  $T = -2.689$ ,  $DF = 78$ ,  $P = 0.009$ ) and were significantly older than the males they cuckolded ( $-0.493 \pm 0.124$ ,  $T = -3.983$ ,  $DF = 145$ ,  $P < 0.001$ ), but did not differ in other song parameters tested (frequency bandwidth,  $-2.939 \pm 68.027$ ,  $T = -0.043$ ,  $DF = 78$ ,  $P = 0.966$ ; introduction length,  $0.016 \pm 0.031$ ,  $T = 0.521$ ,  $DF = 78$ ,  $P = 0.604$ ). Although we did not find an association between age and trill rate, age might nonetheless confound our results for trill rate, and so we conducted a second paired analysis restricted to cases

where males two years or older cuckolded other males two years or older. In this analysis, extra-pair sires still had faster trill rates than the males that they cuckolded (Figure 4b, Mean difference  $\pm$  SE: trill rate,  $-1.143 \pm 0.449$ ,  $T = -2.584$ ,  $DF = 19$ ,  $P = 0.020$ ).

Male trill rate also affected total male reproductive success. In a backwards stepwise regression, the total number of genetic offspring sired in the first broods of each year ( $F_{6,7.428} = 2.080$ ,  $P = 0.062$ ) was significantly related to male trill rate (Figure 5a, Regression coefficient  $\pm$  SE:  $0.237 \pm 0.085$ ,  $P = 0.006$ ), but not other song parameters (frequency bandwidth,  $0.000 \pm 0.000$ ,  $P = 0.920$ ; introduction length,  $0.180 \pm 0.968$ ,  $P = 0.853$ ), male id ( $-0.011 \pm 0.013$ ,  $P = 0.403$ ), age ( $0.175 \pm 0.202$ ,  $P = 0.388$ ) or year ( $0.051 \pm 0.212$ ,  $P = 0.811$ ). For first broods and second broods combined, total number of offspring sired per year ( $F_{6,19.957} = 4.021$ ,  $P = 0.001$ ) was significantly related to trill rate (Figure 5b, Regression coefficient  $\pm$  SE:  $0.312 \pm 0.100$ ,  $P = 0.002$ ), male age ( $0.521 \pm 0.238$ ,  $P = 0.031$ ) and year ( $-0.527 \pm 0.250$ ,  $P = 0.038$ ), but not to frequency bandwidth ( $0.000 \pm 0.000$ ,  $P = 0.609$ ), introduction length ( $-0.045 \pm 1.142$ ,  $P = 0.969$ ) or male id ( $0.004 \pm 0.016$ ,  $P = 0.775$ ). For first and second broods combined, we then included only age and trill rate in an ANOVA, with year as the covariate. In this analysis, only trill rate was a good predictor of the total number of genetic offspring that a male sired in a particular year (trill rate,  $F_{12,9.096} = 2.073$ ,  $P = 0.026$ ; age,  $F_{12,1.485} = 1.145$ ,  $P = 0.334$ ).

### *Playback experiments*

In comparisons of responses to fast vs. slow stimuli with a paired t-test, none of the response variables significantly differed between the treatments (Table 5). A

principal components analysis yielded two principal component variables with eigenvalues greater than 1 (Table 6). For these principal components, a large value of PC1 indicates that the subject responded quickly and closely, whereas a large value of PC2 indicates that the subject had an increased song rate in response to the beginning of playback, and a decreased song rate in response to the cessation of playback. Neither PC1 nor PC2 differed significantly between stimuli (Mean difference  $\pm$  SE: PC1,  $0.627 \pm 0.796$ ,  $T = -0.788$ ,  $DF = 18$ ,  $P = 0.441$ ; PC2,  $0.059 \pm 0.325$ ,  $T = 0.182$ ,  $DF = 18$ ,  $P = 0.858$ .  $X \pm SD$ : Fast PC1 =  $0.598 \pm 2.061$ ; Slow PC1 =  $-0.029 \pm 2.394$ ; Fast PC2 =  $0.073 \pm 1.221$ ; Slow PC2 =  $0.014 \pm 1.416$ ). There was no effect of the order in which subjects received playbacks on PC1, i.e. subjects did not respond to the first playback significantly more or less than they did to the second playback ( $X \pm SD$ : First =  $-0.122 \pm 2.074$ ; Second =  $-0.447 \pm 2.415$ ;  $T = 0.445$ ,  $DF = 36.0$ ,  $P = 0.659$ ).

In our playback experiments males appeared to respond more strongly to fast trill rate songs than to the slow trill counterpart, albeit subtly. Specifically, all of the effects were positive, i.e. the mean response to the fast stimulus was stronger than mean response to the slow stimulus (Figure 6; effect size - Hedges'  $g$ : dives over speaker = 0.060; number of perch changes = 0.213; time of first approach = 0.255; closest approach = 0.407; average approach = 0.319; total time w/in 8m = 0.316; change in song rate in response to the beginning of playback = 0.101; change in song rate in response to the cessation of playback = 0.168). We calculated an overall mean effect size from these individual responses and found that the 95% CI around the average effect size did not overlap zero (Figure 6; Mean  $\pm$  95% CI:  $0.230 \pm 0.082$ ).

## Discussion

A central goal of sexual selection theory is to explain how and why female choice or male-male competition leads to the elaboration of male secondary sexual traits. How and why particular traits, or aspects of traits, and not others become the targets of sexual selection remains a topic of great interest. Under the condition-dependent theory of sexual selection, we would expect traits that are costly, either physiologically or socially, to be the targets of sexual selection, but identifying and understanding how different traits might involve different costs has proven challenging.

The relationship between trill rate and frequency bandwidth has recently gained traction as a valuable theoretical model for predicting how and why sexual selection should act on bird song. Specifically, Podos (1997) described a biomechanical performance maximum resulting from the trade off between trill rate and frequency bandwidth which allows researchers to predict *a priori* that sexual selection should be acting to increase frequency bandwidth, trill rate or both.

In previous research on geographic variation in the song of the Black-throated Blue warbler, we found that song differed significantly between a northern and southern population, and variation in the northern population occurs primarily along a trill rate axis, while variation in the southern population occurs primarily along a frequency bandwidth axis (Chapter two). This allowed us to hypothesize that sexual selection might be acting on different components of song in the different populations, and predict that, in the northern population, selection should be acting on trill rate and not frequency bandwidth. We found support for this prediction in that extra pair sires had faster trill rates than the males they cuckolded (but not larger frequency bandwidths), and trill rate

was positively and significantly associated with the number of genetic offspring that a male sired in a given year, but frequency bandwidth was not.

Our work adds to a growing body of research that suggests that trill rate and frequency bandwidth are important sexual signals, and is among the first to address the importance of these signals in extra-pair paternity. Interestingly, we did not find that males who did not lose paternity were older or had faster trill rates. This finding is surprising, as extra-pair sires were older, and independently had faster trill rates than the males they cuckolded. The lack of association with paternity loss could arise if males who sire extra pair young are also likely to lose paternity in their own nests; however this is not supported by our finding that males with faster trill rates sire more total offspring than males with slower trill rates. When we looked at first broods only, trill rate is the only variable that correlates with the number of genetic offspring, but when we look at double broods as well age and year become significant. Year becomes important because second broods are much more likely to occur in some years than others (Table 3), and age might become a factor because older males are more likely to have second broods (Nagy & Holmes 2005).

According to the condition dependent theory of sexual selection, a signal has meaning to potential mates and competitors because it is costly. One way that the costs of a signal can be assessed is through an association with age: if only older individuals (with better access to resources) can develop elaborate signals, then the production of the signal is likely costly (Brooks & Kemp 2001). Accordingly, many investigations of sexual signals have found that signals tend to become more elaborate as individuals age (Andersson 1994). One important reason for this could be that selection for elaborate



traits benefits the chooser in the form of genes conferring longevity (Kokko & Lindstrom, 1996).

Alternatively, we might predict that traits that become more elaborate with age should be less likely to be the targets of sexual selection. This would arise if traits that became more elaborate with age are also more susceptible to environmental variation, and thus less likely to reveal heritable condition (Miller & Brooks 2005, Garamszegi et al. 2006a). In other words, traits that signal heritable condition should be less likely to be influenced by environmental conditions, and should therefore be impervious to the increased experience and/or resources of older individuals.

Examples of sexual signals that are impervious to age are rare (Andersson 1994), but include aspects of bird song that are crystallized during early development, a point in time in which heritable condition is likely to be on display (Nowicki et al. 1998). Podos (1997) hypothesized that songs approaching the biomechanical maximum of the tradeoff between trill rate and frequency bandwidth should be challenging to sing and/or learn. While some evidence indicates that such high performance songs may be difficult to learn (Podos 1996; Podos et al. 1999), more recent evidence suggests that males can increase performance as they age (Ballentine 2009) and during territorial conflicts (Dubois et al. 2009), suggesting that the singing of high performance songs may also be costly.

Because trill rate does not increase with age in our population of the Black-throated Blue Warbler, its value as a sexual signal could come from a link between trill rate and developmental condition, following the developmental stress hypothesis (Nowicki et al. 1998). A key line of evidence that this condition dependent process is

involved in the selection of faster trill rates would be evidence of genetic benefits for females producing offspring with fast trill males. If males singing fast trills are signaling their badges of developmental condition, they and their offspring should have relatively high survivorship. Preliminary work indicates that extra-pair sires have higher survivorship than males who are cuckolded (Webster & Sillett unpubl. data), and we find that extra-pair sires have faster trill rates than the males they cuckold, suggesting there may be an association between trill rate and adult survivorship. In addition, we have followed adult survival in four years, and offspring survival in two years and hope to examine thoroughly examine the relationship between trill rate and survival in the near future.

The condition dependent hypothesis relies on fast trill rate songs being challenging to learn and/or sing. There are several alternative hypotheses for why trill rate might be favored in this northern population that do not explicitly require that fast trill rates are costly, including ecological selection, Fisherian sexual selection, and selection for song matching. In the case of ecological selection (Nicholls and Goldizen, 2006; Nicholls 2008), faster trills might transmit through the habitat in this northern population better than slower trills, and thus they might be more effective signals. This seems unlikely; however, as cross-species analyses generally reveal slower trills in forests, and faster trills in open habitats (Wiley 1991).

Under Fisherian sexual selection, females might prefer faster trills because of some pre-existing bias, and pass the trait to their sons, and the preference to their daughters. While it seems plausible that females could pass on the preference for faster trill rates to their daughters, song is not necessarily learned from the social father, and as

long as there are plenty of adults singing slow trills, sons have the opportunity to learn "un-sexy" songs. Thus the Fisherian process may be unlikely for learned bird song, especially when the song is not necessarily learned from the social father.

With regards to song matching, we have found that males in this northern population form song neighborhoods and on average are more likely to match the trill rates of their neighbors than the trill rate of a randomly selected male (Colbeck et al. in prep). Such song neighborhoods might arise because territorial interactions are facilitated by song matching (Beecher & Brenowitz 2005). Thus, if there are some slow trill males in the population, selection might favor additional males to have slower trill rates because they can better communicate with their neighbors. This process may be important in this system, especially if the benefits of matching neighbors outweigh the costs (in terms of paternity loss) of singing a slow trill rate. We found that trill rate does not significantly increase with age, but for a small number of individuals, trill rate did change appreciably between year one and year two, and for two individuals between year two and year three. We would predict that individuals should increase their trill rate with age, but of the handful of individuals who change appreciably, the majority decrease their trill rate from one year to the next. This may have to do with the costs and benefits associated with matching neighbors, but will require further investigation.

If a putative sexual signal is important in sexual selection, then a straightforward prediction is that males with more elaborate signals should be more successful at siring extra-pair young than males with less elaborate traits, as in monogamous birds extra-pair paternity can be an important component of male reproductive success (Freeman-Gallant et al. 2005; Webster et al. 2007). Extensive research in birds has revealed that sexually

selected plumage signals are important in extra-pair paternity. Yet most studies, excluding that of Sheldon & Ellegren (1999) which shows no association, reveal a positive association between age and expression of the sexually selected trait (Sundberg & Dixon 1996; Dunn & Cockburn 1999; Thusius et al. 2001; Budden & Dickinson 2009) or do not report if there is an association between age and the signal (Safran et al. 2005; Kleven et al. 2006; Balenger et al. 2009). Similarly, for extra pair paternity and bird song, Forstmeier et al. (2002) report no association between age and the trait, but most studies report a positive association (Hasselquist et al. 1996; Kempenaers et al. 1997; Gil et al. 2007; Dalziell & Cockburn 2008; Suter et al. 2009) or do not report if there is an association (Byers et al. 2007; Dolan et al. 2007; Chiver et al. 2008). Thus our finding that trill rate does not increase with age, but is important for success in extra pair paternity provides rare evidence of a trait that is not associated with age but is nonetheless an important sexual signal.

An alternative method for the evaluation of the importance of a sexual signal in sexual selection is to test the responsiveness of territorial males to more elaborate signals vs. less elaborate signals. However, the predictions in these experiments are not as straightforward as with extra-pair paternity. There appear to be two hypotheses to explain how territorial males should respond to intruders of varying quality. *The fighting ability hypothesis* suggests that conspicuous displays are favored if they make good fighters recognizable (Andersson 1994). Under this scenario males are able to win contests by successfully advertising their high quality, and withdraw from contests where another individual advertises superiority. Thus, territorial males should be more responsive to low quality intruders compared to high quality intruders, because they

should be more likely to dominate the low quality intruders. This hypothesis has received support from several studies (Pryke et al. 2001; Cramer & Price 2007; Hardouin et al. 2007; de Kort et al. 2009). Alternatively, *the priority hypothesis* suggests that males will defend their territories vigorously in relation to the perceived quality of the intruder. Under this hypothesis, males should respond more strongly to higher quality intruders because they represent more of a perceived threat to the territorial owner. This hypothesis has also received support from several studies (Hansen & Rohwer 1986; Slabbekoorn & ten Cate 1997; Garamszegi et al. 2006c; Illes et al. 2007; Chaine & Lyon 2008). Furthermore, Collins (2004) argues that these two hypotheses are not distinct, and that territorial responsiveness should follow a unimodal curve, with males responding the most to intruders of intermediate quality, and the least to intruders of low quality and high quality (because they don't represent a threat, and because they represent too strong a threat, respectively). In an analysis of effect sizes, we find that male Black-throated Blue Warblers respond more strongly to increased trill rates compared to decreased trill rates. Thus, we find support for the *priority hypothesis*, as males are responding more strongly, albeit subtly, to the higher performance intruder.

We are not in a position to test the unimodal response curve hypothesis, as we have not included the highest quality intruders in our experiment - our stimuli span the lower end of natural variation (Table 3). Nonetheless, our results can still be interpreted in light of the unimodal response curve hypothesis, as we have essentially provided males a medium quality intruder and a low quality intruder, and in accordance with Collins (2004) we find that males respond more strongly to the medium quality intruder.

Whether we expect males to be more or less responsive to intruders of varying quality, many of the above cited studies in support of the two hypotheses involve sexual signals that become more elaborate with age, and those involving song performance (Illes et al. 2006; Cramer & Price, 2007; de Kort et al. 2009) make no mention of how song performance is related to age in their study populations. Thus, once again, our finding that males discriminate amongst sexual signals that do not increase with age provides rare evidence of an age-independent signal that is salient to receivers.

We are interpreting our results in the light of males responding to the difference in trill rate between the stimuli. However, when we sped and slowed the tempo of the songs, we also affected the length of individual notes, and the length of the song. As songs naturally increase in trill rate, note length becomes shorter (Table 2), thus these two variables are strongly correlated. However, introduction length varies independently of trill rate (Table 2), but all of our sped up songs have shorter introductions, and all of our slowed down songs have longer introductions. Intuitively, we might expect a longer introduction to solicit a stronger response, because the signal is being transmitted for a longer period of time. However, we found that males responded more strongly to the sped up trill rates with shorter introductions. Thus, we feel the effect of trill rate likely overwhelmed any effect of introduction length.

## Conclusions

To conclude, the theoretical framework laid out by Podos (1997) and our previous work on geographic variation in songs of black-throated blue warblers allowed us to

make *a priori* predictions about which aspects of song should be important in sexual selection, and which should not. In accordance with our predictions, we found that extra-pair sires had faster trill rates than the males they cuckolded, and trill rate was the only variable significantly associated with the total number of genetic offspring sired per year. In addition, we found that males responded more strongly, albeit subtly, to playbacks that had their trill rates artificially increased compared to those that had their trill rates artificially decreased. In addition, we found that trill rate did not increase with age. Thus, our results provide a rare example of sexual selection acting on a signal that does not honestly indicate age, and may signal heritable condition in the context of the developmental stress hypothesis (Nowicki et al. 1998).

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Table 1: Correlations between initial variables and principal components (eigenvalues  $\geq 1$ ) from a principal components analysis conducted on 40 songs. The variable that loads most heavily onto each principle component is in bold. The percentage of song variation explained by each principle component is given in parentheses.

Acoustic variable	PC 1 (41.7%)	PC 2 (23.5%)	PC 3 (15.5%)
Trill rate (notes/s)	<b>0.936</b>	-0.191	-0.050
Number of notes	0.934	-0.096	0.287
Frequency bandwidth (kHz)	0.366	<b>0.906</b>	-0.209
Introduction length (s)	0.247	0.191	<b>0.851</b>
Maximum frequency (kHz)	-0.264	0.895	0.144
Frequency at maximum amplitude (kHz)	-0.293	0.200	0.413
Minimum frequency (kHz)	-0.749	-0.358	0.422
Note length (s)	-0.823	0.102	-0.145

Table 2: Annealing temperatures and variability of microsatellite loci based on analysis of 2007 samples (N = 235 to 238). CU $\mu$ 4 is the only locus to deviate from HWE ( $X^2 = 45.28$ , DF = 6,  $P < 0.001$  with Bonferroni correction).

Locus	$T_a$ ( $^{\circ}$ C)	No. Alleles	Heterozygosity		Probability of paternal exclusion	Null allele frequency	Genotyping error rate
			Observed	Expected			
<i>CU<math>\mu</math>4</i>	50	24	0.686*	0.788	0.616	0.069	0.009
<i>DCA28</i>	52	29	0.748	0.949	0.896	0.118	0.050
<i>DCA32</i>	52	21	0.889	0.892	0.782	0.001	0.006
<i>DP<math>\mu</math>1</i>	50	26	0.896	0.945	0.886	0.026	0.031
<i>DP<math>\mu</math>16</i>	54	14	0.778	0.783	0.584	0.003	0.000
<i>VECR8</i>	54	20	0.918	0.910	0.818	0.005	0.047
Combined					0.999		

Table 3: Trill rates before and after manipulation of the five songs used in the playback experiments.

	+30%	Original	-30%
A	6.51	4.84	3.66
B	6.76	5.17	3.37
C	5.95	4.29	3.41
D	6.43	4.51	3.44
E	6.09	4.33	3.54



Table 4: Table 4. Extra pair parentage characteristics across years. EPY indicates offspring sired by an extra pair male.

year	First broods				Second broods			
	No. broods analyzed	Broods w/ EPY	Offspring analyzed	EPY	No. broods analyzed	Broods w/ EPY	Offspring analyzed	EPY
2004 (main)	25	13 (52%)	73	20 (27%)	11	8 (73%)	36	21 (58%)
2004 (ridge)	22	10 (45%)	71	20 (28%)	11	7 (64%)	36	12 (33%)
2005	37	23 (62%)	110	34 (31%)	4	2 (50%)	11	3 (27%)
2006	39	26 (66%)	127	52 (41%)	12	9 (75%)	39	14 (36%)
2007	37	20 (54%)	106	48 (45%)	5	5 (100%)	18	12 (67%)

Table 5: Comparisons of responses to fast (+30%) and slow (-30%) stimuli for all response variables (N = 19). All data are presented as the mean  $\pm$  SD.

Response variable	Stimulus		Between-stimulus comparison	
	+30%	-30%	<i>T</i> value	<i>P</i> value
number of perch changes	6.21 $\pm$ 6.31	4.90 $\pm$ 7.26	0.55	0.592
total time w/in 8m	130.37 $\pm$ 121.80	92.58 $\pm$ 141.00	0.73	0.476
dives over speaker	1.05 $\pm$ 1.75	0.95 $\pm$ 2.12	0.19	0.851
songrate difference (postplayback)	0.74 $\pm$ 2.60	0.37 $\pm$ 2.34	0.60	0.558
songrate difference (playback)	0.19 $\pm$ 1.62	0.02 $\pm$ 2.03	0.28	0.784
average distance	10.60 $\pm$ 9.01	13.40 $\pm$ 10.33	-0.83	0.420
time of first approach	94.00 $\pm$ 252.53	157.16 $\pm$ 2917.45	-0.69	0.501
closest approach	8.37 $\pm$ 10.62	12.42 $\pm$ 11.33	-1.04	0.312

Table 6: Correlations between initial variables and principal components (eigenvalues  $\geq 1$ ) for responses to playback experiments. The variable that loads most heavily onto each principle component is in bold. The percentage of response variation explained by each principle component is given in parentheses.

Response variable	PC 1 (59.49%)	PC 2 (17.29%)
Number of perch changes	0.895	-0.001
Total time w/in 8m	0.903	0.067
Dives over speaker	0.721	0.04
Songrate difference (postplayback)	0.344	-0.779
Songrate difference (playback)	0.058	<b>0.870</b>
Average distance	-0.905	<0.001
Time of first approach	-0.898	-0.111
Closest approach	<b>-0.937</b>	-0.032

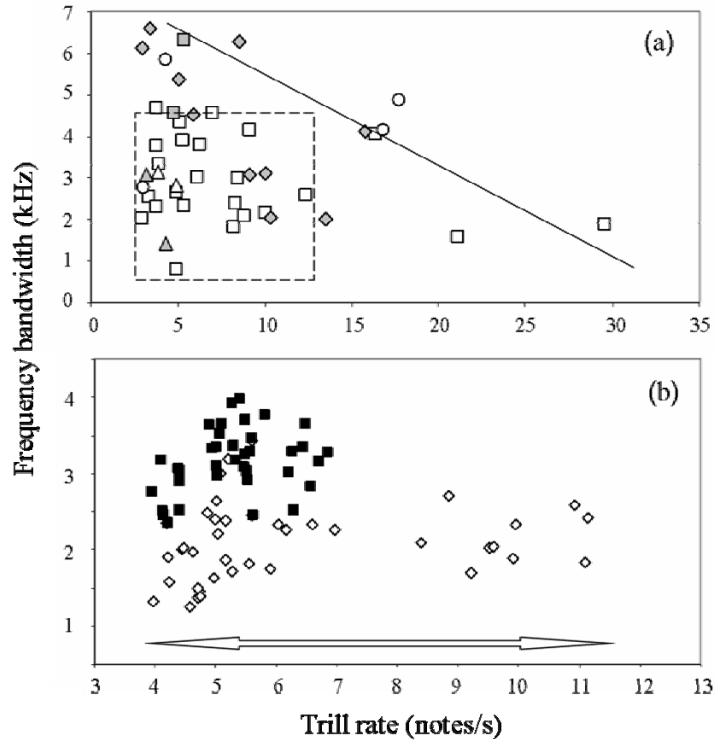


Figure 1. Relationship between frequency bandwidth and trill rate for (a) 46 species of *Parulid* warbler (N = 1 to 3 per species) and (b) a northern  $\diamond$  (N = 40) and southern  $\blacksquare$  (N = 38) population of the Black-throated Blue Warbler. The solid diagonal line in figure 1a represents the upper bound regression. The data in figure 1b occurs within the dashed box in figure 1a. The double ended white arrow in figure 1b emphasizes that Black-throated Blue Warblers in a northern population differ primarily along the trill rate axis. In figure 1a, different genera are represented by different symbols (*Dendroica* = white square, *Vermivora* = grey diamonds, *Seiurus* = white circles, *Setophaga* = grey squares, *Parula* = white triangles, *Oporornis* = grey triangles).

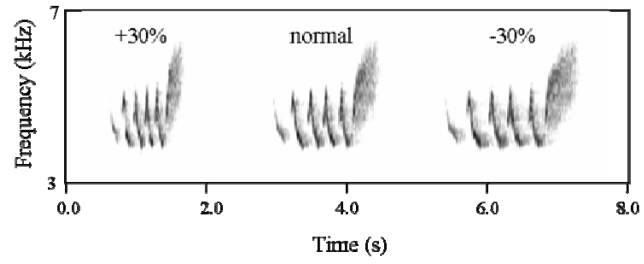


Figure 2. Example spectrograms of songs used in the playback experiments. The song on the left had its tempo increased by 30% while the song on the right had its tempo decreased by 30%.



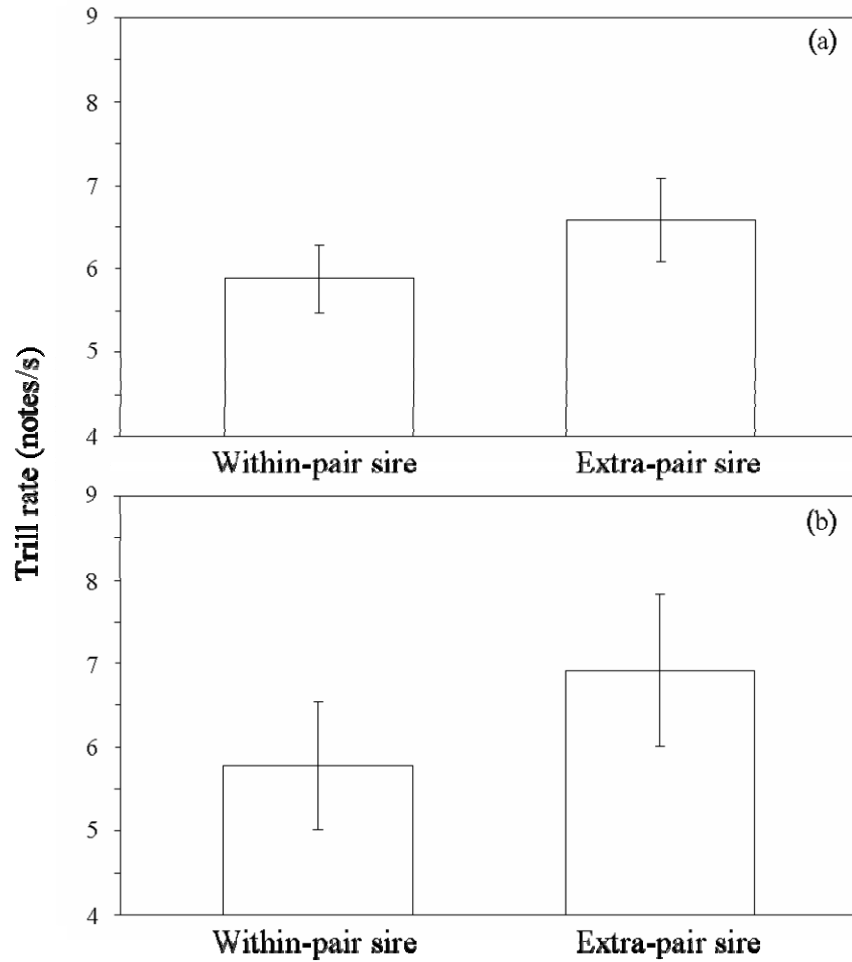


Figure 4. Comparison of trill rate between within pair and extra pair sires for (a) 79 comparisons involving all age classes, and (b) 20 cases in which both males were two years or older. Error bars represent 95% CI around the mean. For both comparisons, extra pair sires have faster trill rates than the males they cuckold (Paired, two-tailed T-test, mean difference  $\pm$  SE: (a)  $-0.700 \pm 0.260$ ,  $T = -2.689$ ,  $DF = 78$ ,  $P = 0.009$ ; (b)  $-1.143 \pm 0.449$ ,  $T = -2.584$ ,  $DF = 19$ ,  $P = 0.020$ ).

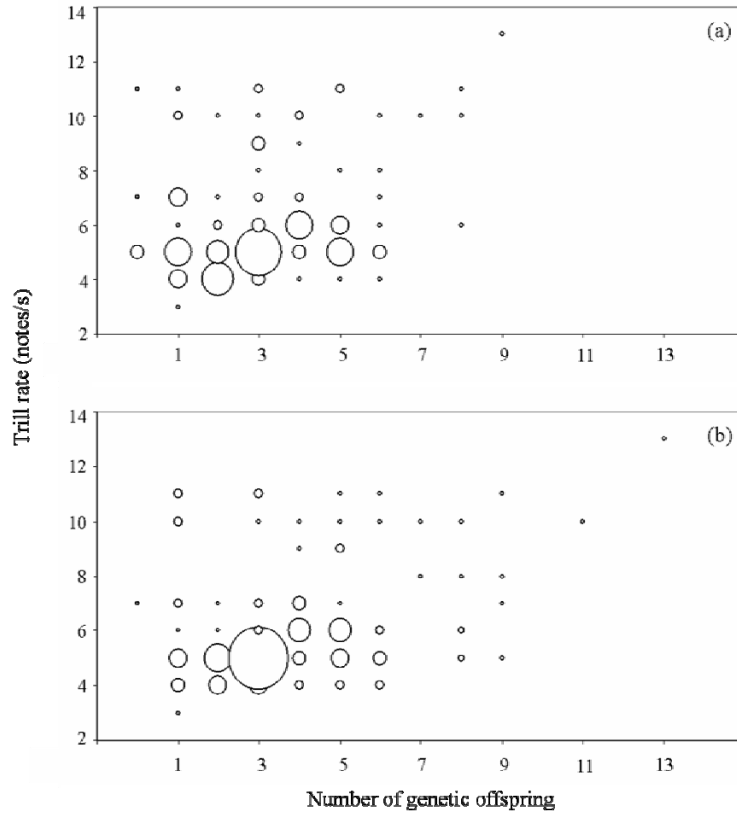


Figure 5. Trill rate vs. total number of genetic offspring for 80 males across four years for (a) first broods only and (b) first and second broods combined. To simplify visualization, trill rates were rounded to the nearest whole number. The size of each circle is proportionate to the number of individuals, with the smallest circle representing one individual and the largest circle representing 13 individuals. Trill rate is the best predictor of total number of genetic offspring (Regression coefficient  $\pm$  SE: (a)  $0.237 \pm 0.085$ ,  $P = 0.006$ ; (b)  $0.312 \pm 0.100$ ,  $P = 0.002$ ).



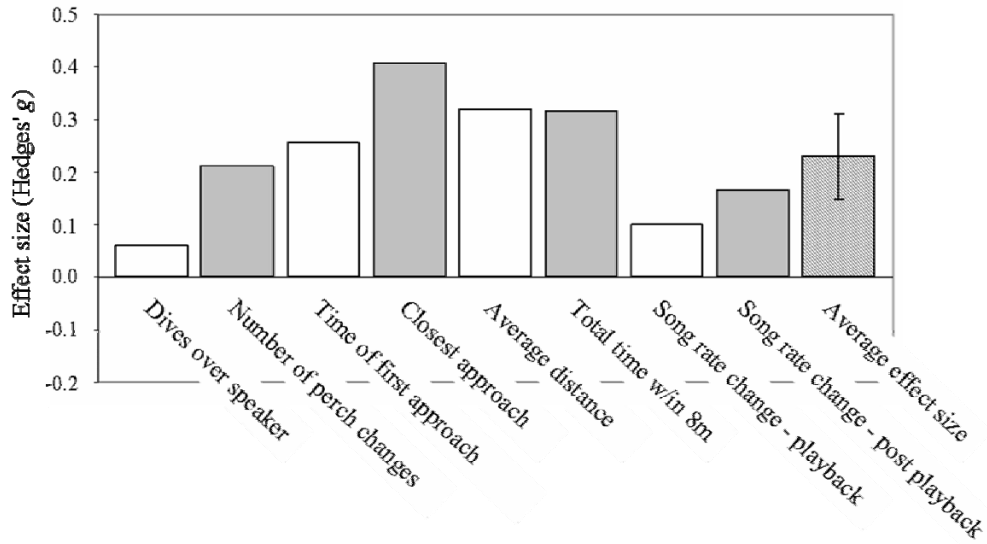


Figure 6. Effect sizes and mean effect size for each of the response variables from the playback experiments. A positive effect size indicates that the responses, on average, were stronger for the fast stimulus than for the slow stimulus. The 95% CI around the mean effect size does not overlap zero (Mean  $\pm$  95% CI: 0.230  $\pm$  0.082).

## CONCLUSIONS

During the recent Pleistocene, populations may have been physically isolated into multiple refugia, or restricted to single refugia, and in either case populations may have experienced rapid population expansion following the retreat of the glaciers. For the American Redstart, we find evidence of allopatric divergence between the now submerged Atlantic Coastal Shelf and the mainland, and for the Black-throated Blue warbler, its current day populations were restricted to a single refugium (Davis et al. 2006; Grus et al. 2009). In both cases, populations have expanded rapidly from small population sizes, and for the Black-throated Blue warbler this expansion has accompanied parapatric divergence in migratory strategies (Rubenstein 2002) and plumage characteristics (Grus et al. 2009).

If speciation is to result from divergence during the Pleistocene, phenotypic differences must accumulate between populations. For the Black-throated Blue Warbler, we find that song differences have accompanied, and perhaps facilitated, the evolution of the migratory and plumage differences described above. Specifically, songs in the north have faster trill rates and smaller frequency bandwidths than the songs in the south, and the faster trill rates in the north may, in part, be facilitated by more open habitats.

Sexual selection may be contributing to the rapid parapatric divergence in this species, with songs in different populations evolving along different trajectories. For many songbirds, frequency bandwidth and trill rate trade off with each other, such that sexual selection acts to increase frequency bandwidth, trill rate or both (Podos 1997). We find that songs in the south vary primarily along a frequency bandwidth axis, while those

in the north vary primarily along a trill rate axis, thus we were able to predict *a priori* that sexual selection should be acting on trill rate and not frequency bandwidth in the northern population. We found that extra pair sires sing faster trill rates (but not larger frequency bandwidths) than the males they cuckold, trill rate was the best (and only significant) predictor of the number of genetic offspring sired by a male per year, and males respond more strongly to trill rates that have been artificially sped up compared to those that have been artificially slowed. Thus sexual selection indeed appears to be acting on trill rate, and not frequency bandwidth, in this northern population.

In addition, song differences between populations may be contributing to reproductive isolation, as in the northern population, non-local signals are discriminated against. In the south, however, local and non-local signals are responded to equally, thus reproductive isolation may be asymmetric and therefore incomplete. This asymmetric discrimination may be an early stage of reproductive isolation and speciation between parapatric populations.

These results provide support to the hypothesis that recent glacial cycles have contributed to current day patterns of diversity. We find support for the ideas of allopatric as well as parapatric divergence, and we find that sexual selection may be contributing to the rapid phenotypic differences across populations, as well as reproductive isolation between divergent populations.

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