EVOLUTION OF BIRD SONG AFFECTS SIGNAL EFFICACY: AN EXPERIMENTAL TEST USING HISTORICAL AND CURRENT SIGNALS

Elizabeth P. Derryberry

Duke University, Biology Department, P.O. Box 90338, Durham, North Carolina 27708

E-mail: ed8@duke.edu

Mating signals act as behavioral barriers to gene flow in many animal taxa, yet little is known about how signal evolution within populations contributes to the formation of these barriers. Although variation in mating signals among populations is known to affect mating behavior, there is no direct evidence that the evolution of mating signals changes signal effectiveness within a natural population. Making use of historical recordings of bird song, I found that both male and female white-crowned sparrows (*Zonotrichia leucophrys*) respond more strongly to current than to historical songs, indicating that historical songs are less effective as signals in the current contexts of both mate choice and male–male competition. Finding that historical signals are less effective suggests that signal evolution within populations may ultimately contribute to the formation of behavioral barriers to gene flow between populations.

Mating signals play an important role in the process of speciation as behavioral barriers to gene flow among populations (Dobzhansky 1940; Mayr 1963; West-Eberhard 1983). Closely related species often vary dramatically in their mating signals (Darwin 1871; West-Eberhard 1983; Price 1998) and numerous empirical studies suggest that many species discriminate between closely related congeners based on mating signals (reviewed in Andersson 1994). Although there is good evidence that variation in mating signals among populations plays a role in reproductive isolation, there is little evidence of how and when signal evolution contributes to the formation of reproductive barriers (Ryan and Rand 1995; Price 1998).

The prediction that signal evolution may contribute directly to the formation of reproductive barriers is not well tested. The most common approach to testing this prediction uses geographic variation in signal structure as a proxy for signal divergence over time and measures receiver response to geographic variation as an estimate of receiver response to signal evolution (e.g., Irwin et al. 2001). This approach provides evidence that signal divergence among populations may lead to reproductive isolation, yet

provides little information on how signal evolution within populations contributes to this process. Another approach to testing this prediction measures receiver response to statistical reconstructions of ancestral signals (Ryan and Rand 1995). This approach provides evidence that signal evolution within lineages may contribute to the formation of reproductive barriers between species, but is based on assumptions of how ancestral signals vary from current signals (Losos 1999).

The most direct test of the effect of signal evolution within populations on signal efficacy would be to measure the response of receivers to actual mating signals documented at different time points within a single population. This approach has yet to be taken because of the obvious difficulty of preserving historical signals in a manner that allows their use as stimuli in behavioral tests. In the 1960s, the use of audio recording to document birdsong became widespread, and these recordings are now a resource of historical songs. Here, I use historical recordings of birdsong from a population of white-crowned sparrows (*Zonotrichia leucophrys*) at Tioga Pass, California, to test directly whether changes in a mating signal over time affect signal efficacy.

As the song of most songbirds has two functions—to attract females as mates and to repel rival males (Catchpole and Slater 1995)—I tested both female and male response to changes in song over time to ascertain how song evolution has affected signal efficacy in both mate choice and male–male competition. Using a standard laboratory assay of female response to song in the context of mate choice (Searcy 1992), I compared the number of copulation solicitations given by females during playback of historical and current songs. To evaluate the efficacy of historical and current songs in the context of male–male competition, I compared the approach distance of males during playback of each song on their territories (Peters et al. 1980). The closer a male approaches a simulated intruder (i.e., the playback speaker), the more likely he is to attack (Searcy et al. 2006). Male response to intrusion is not a direct measure of function in that this measure does not reflect either a subject's potential to hold a territory or a simulated intruder's potential to set up a territory. However, this type of experiment does provide an accurate measure of song efficacy in an aggressive context (Searcy and Nowicki 2000).

The white-crowned sparrow provides an especially good system in which to test the effects of song evolution on signal efficacy. Widespread interest in geographic variation in the song of this species over the past 30 years provides an extensive database of historical recordings. In addition, several studies, including this one, document significant changes in song over time within populations in this species (Harbison et al. 1999; Nelson et al. 2004).

Materials and Methods
SONG SAMPLES
Songs similar in note type and note order may be characterized as the same "song type" (Catchpole and Slater 1995), although the degree of similarity necessary to qualify as the same song type varies among species. White-crowned sparrow males typically learn to sing one song type that is shared among most males in a population (Chilton et al. 1995), and they sing this one song type with very little variation from one rendition to the next (Marler 1970; Baptista 1975). In addition, there is little variation between males singing the same song type, at least within populations of the subspecies tested here, *Z. l. oriantha* (Baptista and King 1980; Harbison et al. 1999). However, among populations, there are consistent differences in the dominant song type, and this pattern of discrete geographic variation in song—known as song "dialects"—has made white-crowned sparrows a model system in which to study the role of mating signals as behavioral barriers to gene flow (Marler and Tamura 1962).

The majority of males in the study population (Tioga Pass) currently sing one of two versions of the local song type, but I restricted song analysis to the one song type version (Fig. 1), which has remained common over the last 30 years (Harbison
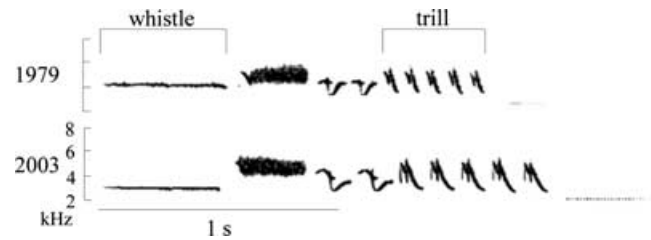

Figure 1. Exemplars of songs recorded at two time points (1979 and 2003). The indicated whistle decreased in dominant frequency whereas the indicated trill decreased in rate and increased in frequency bandwidth (see results for details). The x-axis represents time in seconds, and the y-axis represents frequency in kilohertz.

et al. 1999). This song type is characterized by a series of notes in the following order: an introductory pure tone constant frequency "whistle," a "buzz," two repeated "complex" notes, a variable number of repeated "simple" notes known as the trill, and a low frequency buzz (Fig. 1) (Baptista and King 1980). The second song type version is in most aspects the same, but has a different, unrepeated complex note and is not used in this study because it has only become common in the population relatively recently. All females and males were tested with the version of the local song type illustrated in Figure 1.

Acoustic analysis was based on the songs of 36 male birds recorded at two time points: 21 in 1979 (recorded by L. Baptista, archived at the California Academy of Sciences) and 15 in 2003 (recorded by EPD). In 1979, songs were recorded using a Uher Report-S recorder at a tape speed of 7.5 inch/sec, a Uher microphone, and a parabolic reflector. In 2003, songs were recorded using a Sony TCM-5000EV cassette recorder, a PRO-302 Unidirectional Dynamic microphone, and a parabolic reflector. Males recorded at the two time points were different individuals as the mean life span of white-crowned sparrows is less than two years (Chilton et al. 1995). In addition, within each time point, I used songs that were denoted by the recordist as being sung by different males. I took measurements on a mean of 3.6 songs for each male (range: 1–6). Songs were digitized with 16-bit precision at a 25 kHz sampling rate and high-pass-filtered at 1500 Hz to eliminate noise below the frequency range of white-crowned sparrow song using Syrinx 2.2b (Burt 2001) and an Echo Digital Audio sound card. I took all acoustic measurements using Signal Version 3.1 digital signal processing software (Beeman 1999).

From the 21 historical and 15 current songs, I chose 20 songs for the playback experiments with females and males. The 10 songs used for each time point reflected the overall variation in the song type within the population at each time point. To ensure that test subjects were unfamiliar with the current songs, I used songs recorded from males defending territories at least a kilometer from the test site and at least two years prior to the experiments.

SOUND QUALITY

Because this study makes use of historical recordings, it is necessary to confirm that historical and current recordings do not differ in sound quality. There are three ways in which recordings might differ in sound quality, which would have a critical effect on acoustic measurements or on receiver response: (1) differences between historical and current songs in acoustic characteristics could be due solely to subtle differences in the tape speed of the different recording equipment used, (2) frequency modulation or sound dropout could be introduced by intermittent fluctuations in tape speed due to stick slip playback as a result of tape binder degradation over time, and (3) the background noise in the current and historical recordings could be different due to differences in sound background when recordings were made or to noise accumulated when historical recordings were transferred from reel-to-reel to digital audio tapes during an updating of the sound archives where they were stored.

The methods used to analyze sound quality and the detailed results as well as audio examples of historical and current songs are presented in online Supplementary Material. In summary, (1) the acoustic differences between historical and current songs are not due to differences in tape speed of recording equipment, (2) there is no evidence of frequency modulation or sound dropout in historical recordings, and (3) the relative background noise in current and historical recordings is not significantly different. Both historical and current songs used in the acoustic analysis and in the behavioral experiments were of extremely high quality; therefore, measurements of changes in acoustic features over time reflect actual song evolution and males and females were exposed to differences in these acoustic features of the songs rather than to differences in sound quality.

ACOUSTIC ANALYSES OF CHANGES IN SONG OVER TIME

Acoustic analyses focused on the introductory whistle and the simple note trill (see Fig. 1), because these two song phrases are shared by a large number of populations and thus represent very common features of white-crowned sparrow song (Baptista 1975, 1977; Baptista and King 1980). Both phrases also have been suggested to be particularly salient in other subspecies of white-crowned sparrows, the whistle in the context of song learning (Soha and Marler 2001) and the trill in the discrimination of population differences in the context of male–male competition (Nelson and Soha 2004).

I analyzed three acoustic measurements that captured the variation in the whistle and trill among males: whistle dominant frequency, trill rate, and trill frequency bandwidth. I measured whistle dominant frequency as the frequency at which the most sound energy was transmitted during whistle production from a smoothed power spectrum of each whistle. I calculated trill rate

as the average number of repetitions of the trill syllable produced per second from oscillograms. The frequency bandwidth of each trill was measured by calculating the difference between the maximum and the minimum frequencies measured at -36 dB relative to the peak amplitude frequency in the song from digital spectrograms (256 pt transform, frequency resolution $= 97.7$ Hz). I chose the -36 dB threshold value because it captured variation in frequency bandwidth while excluding background noise. All measurements were taken on each song exemplar and then averaged for each male. I calculated the repeatability statistic, r, of these measurements (Lessells and Boag 1987). Repeatability measurements were high for all three vocal characters (whistle dominant frequency $= 0.99$; frequency bandwidth $= 0.70$; trill rate $= 0.99$). To determine whether these three acoustic features changed significantly over time, I compared measurements taken on 1979 and 2003 songs using two sample t-tests, and P-values were Bonferroni adjusted to reflect the fact that all three acoustic measurements were made on the same pool of songs.

COPULATION SOLICITATION ASSAY

I used a copulation solicitation assay to test whether captive adult females responded differently to historical versus current songs. The copulation solicitation assay is a standard experimental design that quantifies female response to simulated mates by playing songs to receptive females (Searcy 1992). All females tested were collected from an 8-km^2 area around Tioga Pass in the 2006 breeding season during the preincubation period (to avoid taking females with young). Females were housed and tested in individual sound attenuation chambers in the T. Hahn Laboratory at the University of California at Davis. Females were fed water and seed ad libitum. To control for variation in the hormonal state, females were given subcutaneous implants of 17-β-estradiol in silastic tubing four days before testing began. At the end of the experiment, I removed all implants and, after a veterinarian check, released all females near their site of capture.

Before each trial, I placed a single speaker, microphone, and miniature camera in the sound attenuation chamber (SME-AFS amplified playback speaker, Saul Mineroff Electronics Inc., Elmont, NY; Sennheiser omnidirectional microphone, WAT-660D3.8 surveillance camera). As the camera was visible to the females, I habituated females to its presence using dummy cameras installed four days prior to the trials. During each trial, I counted the number of female solicitations given during a 3-min playback of the song. The amplitude of each stimulus was standardized at 80–83 dB measured at 1 m from the speaker using a sound pressure level meter (RadioShack 33-4050; Fort Worth, TX). Songs were played at a natural rate of six songs per minute.

Each female was tested once with an historical song and once with a current song on the same day with at least 3 h separating the tests. Half the females heard the historical stimulus first while

the other half heard the current stimulus first. This pair of tests was then repeated two days later except with the presentation order of stimuli reversed. The sum of the number of copulation solicitations given to the two presentations of each stimulus was used as the sole response variable. Each pair of historical and current songs was considered a stimulus set, and 10 stimulus sets were used with a different female hearing each set; however, only eight females responded to playbacks ($n = 8$). The two females who did not respond to playback of any songs were excluded from the analysis because it is not possible to distinguish between a lack of motivation and an active no-choice decision in a case of no response. I used a paired t-test to compare the response per set.

TERRITORIAL PLAYBACK EXPERIMENT

I used a territorial playback to test whether free-living adult males responded differently to historical versus current songs. Territorial playback is a standard experimental design that quantifies male response to simulated intruders by playing songs on subjects' territories (McGregor 1992; Catchpole and Slater 1995). All the males tested held territories in a 3-km^2 area around Tioga Pass in the 2005 breeding season, and all playbacks were conducted during the preincubation period to avoid disrupting the nesting period.

Before each trial, a single speaker was placed near the center of a male's territory on the ground, facing up (SME-AFS amplified playback speaker). The amplitude of each stimulus was standardized at 80–85 dB measured at 1 m from the speaker using a sound pressure level meter (RadioShack 33-4050), and the songs were played at a natural rate of six songs per minute. During each trial, I stood at least 20 m from the speaker and recorded the male's distance from the speaker at 10-sec intervals. I recorded this response distance during a 3-min playback period and a 9-min postplayback period. A long postplayback period is used when testing white-crowned sparrows, as their responses tend to vary most between stimuli after playback (Nelson and Soha 2004). Males who approach the speaker more closely are responding more strongly to the playback because a male's distance from a playback speaker is a significant predictor of his likelihood to attack an intruder simulated using a mount (Searcy et al. 2006). To facilitate accurate measures of distance, I placed markers at 4 m and 8 m on either side of the speaker before the trial began. The distance categories used were 0–2 m, 2–4 m, 4–8 m, 8–16 m, and greater than 16 m. In calculating a male's average distance from the speaker during the playback and postplayback period, I used the median distance of each category and 24 m for the "greater than 16 m" category (Peters et al. 1980).

Each male was tested once with a historical song and once with a current song. Half the males heard the historical stimulus first whereas the other half heard the current stimulus first and at least two days separated each male's trials. Neighbors were never tested on the same day. Each pair of songs was considered a stimulus set, and 10 stimulus sets were used with two different males hearing each set. To avoid pseudoreplication (Kroodsma 1990; McGregor et al. 1992), I used a standard method of averaging the response of each pair of males who heard the same stimulus set (Searcy et al. 2002). The sample size is thus the number of stimulus sets ($n = 10$). I used a paired t-test to compare the mean response per set.

Results
SONG EVOLUTION

Songs recorded in 1979 and 2003 at Tioga Pass are the same song type in that they are composed of the same note types in the same order (Fig. 1). However, specific acoustic features of this song type have changed (to hear songs, see Supplementary Material available online). The whistle decreased in dominant frequency (historical = 3713 ± 358 (Hz; mean ± SD), current = 3291 ± 204, $t = 4.5$, $n = 21,15$, $P = 0.0001$) whereas the trill decreased in rate (historical = 11.0 ± 1.5 (Hz; mean ± SD), current = 8.0 ± 0.3, $t = 9.3$, $n = 21,15$, $P = 0.0001$) and increased in frequency bandwidth (historical = 2730 ± 348 (Hz; mean ± SD), current = 3024 ± 332, $t = -2.6$, $n = 21,15$, $P = 0.05$). The P-values presented have been Bonferroni adjusted to reflect that all three acoustic measurements were made on the same set of songs.

RESPONSE TO HISTORICAL AND CURRENT SONGS

Females gave more copulation solicitations during the playback of current songs than during the playback of historical songs ($t = 4$, $n = 8$, $P = 0.005$, Fig. 2). If the two females who gave no response are included in the analysis, the results are similar ($t = 3.6$, $n = 10$, $P = 0.008$). Females give more solicitations to songs that they prefer; thus, females prefer current songs over historical songs. Males approached the speaker significantly more closely during playback of current songs than during playback of historical songs
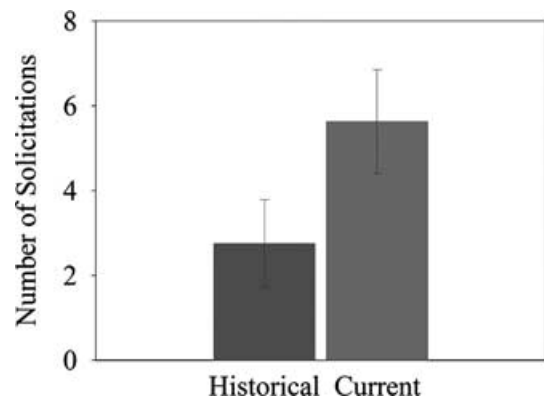

Figure 2. Females gave more copulation solicitations to current than to historical songs (historical: 2.8 ± 1 [copulation solicitations; mean ± SE], current: 5.6 ± 1.2).
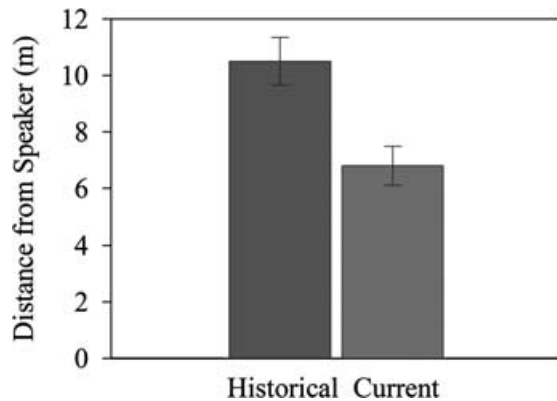
Figure 3. During a simulated territorial intrusion, males approached playback of current song more closely than playback of historical song (historical: 10.5 ± 2.7 [m; mean ± SE], current: 6.8 ± 2.2).

($t = -3.4$, $n = 10$, $P = 0.008$, Fig. 3). Closeness of approach to a speaker within a male's territory is interpreted as a stronger response to a specific stimulus; thus, males respond significantly more strongly to current songs than to historical songs.

Discussion

The data presented here demonstrate that song evolution has affected the efficacy of songs in the population of white-crowned sparrows at Tioga Pass: females perform more solicitations and males approach a simulated intruder more closely during the playback of current songs as compared to songs recorded 24 years earlier (Figs. 2, 3). These results indicate that historical songs are less effective signals in the current population in the context of both female mate choice and male–male competition. Although I am not asserting that birds singing historical sounding songs are currently present in the Tioga population, I am using this analogy of a historical song in a current context as a theoretical construct for understanding how changes over time within populations may contribute to behavioral barriers between populations. To my knowledge, these data represent the first direct demonstration that signal evolution within a population affects signal efficacy. These findings further support the importance of signal evolution within populations in the formation of behavioral barriers to gene flow among populations.

A reduction—not just a complete loss—in signal efficacy is evidence of a behavioral barrier to gene flow, as any breakdown in communication between potential mates is likely to result in a reduced probability of mating. In studies measuring receiver response to geographic variation in mating signals, a significantly lower response to signals from nonlocal, conspecific populations is considered evidence of reproductive isolation among populations (e.g., Verrell and Arnold 1989; Irwin et al. 2001; Svensson et al. 2006). Because these populations are often not in direct

contact, these barriers to gene flow are likely not the result of selection on individuals to discriminate against the signals of different populations (Searcy et al. 2002; Ryan et al. 2003); rather the formation of these barriers is more likely the result of selection on signals and on receiver recognition in other contexts (Ryan 1990; Podos 2001), such as selection on females to recognize quality mates within populations (Andersson 1994; Searcy et al. 2002). Evolution of a signal within a population may therefore affect the effectiveness of that signal as a mating signal in another population or species. Although this hypothesis is suggested by studies demonstrating that individuals respond less to signals of allopatric heterospecifics (Ryan et al. 2003) or to signals from distant, conspecific populations (Searcy et al. 2002), there is no direct evidence that actual changes in signals over time within populations affect signal efficacy. In the study presented here, males and females respond significantly more strongly to current songs than to historical songs. The finding that changes in song within a population affects signal efficacy suggests that these changes within populations ultimately contribute to the formation of behavioral barriers among populations, although further study is need to address this hypothesis.

It is intuitively clear how a reduction in signal efficacy in the context of female mate choice can act as a behavioral barrier to gene flow, but a reduction in signal efficacy in the context of male–male competition also may contribute to reproductive isolation. Reduction in communication between males and females during mate choice can lead to a reduced probability of mating (Andersson 1994). By contrast, reduction in communication between males during competition contributes indirectly to reproductive isolation. A less effective signal in a male–male interaction may not directly affect a male's ability to attract a mate; however, if this loss in efficacy leads to a reduction in a male's ability to gain or to hold resources such as a territory, then a male's ability to attract a mate may be affected indirectly (Andersson 1994). Although the experimental design used here to test male response is not a direct measure of a song's function as a territorial "keep-out" signal, it is an accurate measure of song efficacy in an aggressive context (Searcy and Nowicki 2000). Thus, the results of this experiment suggest that males singing historical songs would likely have reduced competitive ability, which might affect their ability to attract a mate. It is important to emphasize here that one cannot interpret a lower response to historical songs as an indication that males singing historical songs would theoretically have an easier time establishing a territory in the current population because the experimental design is not a direct test of function. More direct evidence could be obtained by male removal/speaker replacement tests (Krebs et al. 1978), although this method may be problematic in addressing specific questions about song function (Nowicki et al. 1998). Results presented here would predict that the broadcast of historical songs would be less effective compared to current

songs at repelling intruders from a current territory after the territory owner is removed.

The effects of familiarity may be important in understanding the difference in response to historical and current songs, as experience with particular songs and song types likely affects response (Milligan and Verner 1971; O'Loghlen and Beecher 1997, 1999). Familiar songs may be used as a standard to which individuals give their strongest response and response to unfamiliar songs may then be proportional to the degree to which these songs deviate from the standard (Milligan and Verner 1971; Shepard 1987). As males and females are likely most familiar with the current version of the local song type, current songs may act as the standard with response to historical songs being proportional to how much historical songs differ from current songs. The effects of familiarity could be controlled for by testing the response of individuals who have been exposed equally to historical and current songs in the laboratory. However, it is important to note that individuals have also been found to respond more strongly to novel (hence, unfamiliar) signals (Burley and Symanski 1998; Verzijden et al. 2007). Thus, the unfamiliarity of historical songs did not necessarily predict that males and females would respond less to historical songs. Although it is worthwhile addressing the mechanism by which historical songs are less effective, the results presented here, nonetheless, demonstrate that historical songs are less effective than current songs in the biologically relevant contexts of both mate choice and male–male competition.

The hypothesis that changes in mating signals over time can affect the efficacy of these traits has received indirect support from measures of receiver response to geographic variation in signals. Measuring receiver response to geographic variation provides an estimate of the current degree of behavioral isolation among populations, but only an indirect estimate of the effects of signal evolution within populations on reproductive isolation. The mechanisms generating and maintaining divergence in signals among populations may be different from those generating changes in signals over time within populations. Because these different mechanisms can lead to differences in the patterns of variation in signals across time and across space, measuring receiver response to geographic variation does not necessarily predict receiver response to temporal variation within populations.

For example, in white-crowned sparrows, patterns of variation in song across populations are different from patterns of variation in song over time within populations. Geographic variation in the white-crowned sparrow song consists of differences in note type and note order among populations (Baptista 1977; Baptista and King 1980), and playbacks have demonstrated that both male and female white-crowned sparrows generally respond more strongly to the song type of their own population than to the song types of other populations (Baker et al. 1981, 1982; Baker

1983; Thompson and Baker 1993; Maney et al. 2003; Nelson and Soha 2004; but see Milligan and Verner 1971). These playback studies suggest that changes in note type or a reordering of note types may affect signal efficacy in this species. However, the pattern of within-population song evolution in this species consists of more subtle changes in acoustic features of songs and not typically in a reordering or emergence of new note types (Harbison et al. 1999). Thus, playback studies measuring response to geographic variation would not necessarily have predicted how individuals would respond to temporal variation in this species. Although studies in other species have demonstrated a functional response to subtle acoustic variation in song (e.g., review in Becker 1982), this variation often existed along dimensions that reflected individual or geographic variation but not necessarily evolutionary changes. Altogether, the findings reported here suggest that the mechanisms contributing to changes in song within populations affect signal efficacy, supporting the need to test response to changes in signals over time and not just to geographic variation in signals.

Testing the response of receivers to reconstructed ancestral mating signals is another approach to understanding how signal evolution contributes to the formation of behavioral barriers to gene flow over time (Ryan and Rand 1995, 1999). Phylogenetic methods used to reconstruct ancestral character states are, however, prone to error (reviewed in Cunningham et al. 1998). In particular, the reconstruction of continuous ancestral characters, such as mating signals, depends on the evolutionary model used (Cunningham et al. 1998), and the use of different models can yield substantially different versions of ancestral mating signals (Losos 1999). By contrast, documented historical signals, such as those used in the present study, represent a known state in evolutionary history. Thus, measuring receiver response to historical signals provides an understanding of how behavioral barriers may form over time without any assumptions made about how ancestral signals may differ from current ones.

Determining the mechanisms driving signal evolution is also critical to understanding how behavioral barriers to gene flow form over time. The results presented here demonstrate that changes in song over time lead to meaningful differences in songs, but they do not provide insight into the mechanisms driving song evolution. Birdsong evolution may be influenced by the mechanics of sound production (Nowicki et al. 1992; Suthers and Goller 1997; Podos and Nowicki 2004), environmental factors affecting signal transmission (Morton 1975; Wiley and Richards 1978; Slabbekoorn and Smith 2002), sexual selection (Searcy and Andersson 1986; Nowicki et al. 2001), and learning (Marler and Peters 1989; Hughes et al. 1998). Studies have demonstrated how these individual forces may influence song evolution, but song diversification is likely driven by multiple forces acting on song. Further research should investigate the mechanisms driving song

evolution in this system to understand more broadly the role of signal evolution in speciation.

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Supplementary Material

The following supplementary material is available for this article:

MATERIALS AND METHODS

RESULTS

This material is available as part of the online article from:
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