Biological Journal of the Linnean Society, 2020, **129**, 74–87. With 6 figures.

Diversification in trophic morphology and a mating signal are coupled in the early stages of sympatric divergence in crossbills

CODY K. PORTER[1,2*] and JULIE W. SMITH[3]

[1]*Department of Zoology and Physiology, University of Wyoming, Laramie, WY 82071, USA*
[2]*Program in Ecology, University of Wyoming, Laramie, WY 82071, USA*
[3]*Department of Biology, Pacific Lutheran University, Tacoma, WA 98447, USA*

Received 25 August 2019; revised 19 September 2019; accepted for publication 19 September 2019

Understanding the mechanisms generating diversity in mating signals is critical to understanding the process of speciation. One mechanism of mating signal diversification occurs when phenotypes that experience divergent ecological selection also affect the production of mating signals, resulting in a coupling between ecological diversification and mating signal diversification. Here, we present evidence that rapid diversification in bill size has resulted in the diversification of some components of song structure in a young adaptive radiation of seed-eating finches (red crossbill, *Loxia curvirostra* complex). Specifically, we find that larger-billed ecotypes sing songs with lower minimum frequencies, lower syllable repetition rates and greater vocal deviation (i.e. lower performance) than smaller-billed ecotypes for pure tonal syllables. In contrast, bill size was not correlated with maximum frequency or frequency bandwidth, and we found no relationship between bill size and any song parameters in buzzy syllables. Furthermore, we found no evidence for a relationship between the degree of bill size divergence and the potential for song discrimination between sympatric ecotypes. Because bill size is correlated with some features of pure tonal syllables (which appear to be most important for courtship in crossbills) in crossbill song, our results suggest that there was an early-evolving link between ecological and mating signal diversification that may have influenced the rapid evolution of reproductive isolation between sympatric ecotypes.

ADDITIONAL KEYWORDS: adaptive radiation – bird song – crossbill – magic trait – reproductive isolation – speciation – vocal tract constraint hypothesis.

INTRODUCTION

A central goal of evolutionary biology is to understand the mechanisms that lead to the formation of new species (Darwin, 1859). A significant characteristic of speciation is diversification in the signals that mediate mate choice (Ptacek, 2000; Coyne & Orr, 2004). Indeed, mating signal diversification is probably a critical component of the speciation process, as it can promote the evolution of reproductive isolation between diverging lineages (Mayr, 1963), thus marking the transition from intraspecific to interspecific diversification (Streelman & Danley, 2003). Therefore, an important component of understanding the drivers of speciation is to understand the evolutionary forces that shape mating signal diversity.

In recent decades, there has been a growing realization that the same mechanisms involved in ecological diversification can also drive mating signal diversification. For example, a direct coupling between ecological and mating signal diversification occurs when the phenotypes that diversify in response to divergent ecological selection influence the production of mating signals, resulting in mating signal diversification as a byproduct of ecological diversification (Wilkins *et al.*, 2013). When mating signals diversify as a pleiotropic effect of ecological adaptation, this may facilitate speciation, even in the face of ongoing gene flow between diverging lineages (Gavrilets, 2004; Servedio *et al.*, 2011).

One phenotype that may often be involved in ecological adaptation and influence the production of mating signals is the bird bill. The size of a bird's bill is a critical determinant of the type of food items that can

---

*Corresponding author. E-mail: cporte16@uwyo.edu be efficiently captured, processed and consumed (Lack, 1968; Newton, 1972). Unsurprisingly, bird bills are a main target of divergent selection during adaptation to alternative food resources (e.g. Schluter & Grant, 1984; Smith, 1990; Benkman, 1993, 2003). Indeed, resource-related differences in bill size characterize many of the most familiar cases of adaptive radiation and speciation in birds (Lack, 1947; Newton, 1972; Grant, 1986; Groth, 1993; Lovette *et al.*, 2002; Schulenberg, 2003; Ryan *et al.*, 2007).

Bird bills are also the terminal component of the vocal tract and play a key role in sound production (Nowicki, 1987; Westneat *et al.*, 1993). Birds have been shown to modulate bill gape while singing to track frequencies produced by the syrinx (Nowicki, 1987; Nowicki & Marler, 1988), with higher frequencies and frequency bandwidth (the difference between minimum and maximum frequency) requiring wider bill opening (Hausberger *et al.*, 1991; Podos *et al.*, 2004). Birds also need to open and close their bills rapidly to produce fast-paced songs, resulting in a tradeoff between the ability to produce fast-paced songs and songs with high frequency bandwidth (Podos, 1997, 2001; Wilson *et al.*, 2014). Because there is a tradeoff in jaw biomechanics between maximal bite force and velocity, birds with larger bills should be less able to rapidly and widely open their bills compared to smaller-billed birds (Podos, 2001). The vocal tract constraint hypothesis thus predicts that larger-billed birds should sing songs with lower frequencies, syllable repetition rates and frequency bandwidths, and be less capable of minimizing the tradeoff between syllable repetition rate and frequency bandwidth (Podos, 1997, 2001; Podos & Nowicki, 2004; Derryberry *et al.*, 2012). Consistent with this hypothesis, several studies have provided evidence that bill size is negatively correlated with minimum and maximum frequency, syllable repetition rate, and frequency bandwidth (Palacios & Tubaro, 2000; Podos, 2001; Seddon, 2005; Ballentine, 2006; Huber & Podos, 2006; Derryberry *et al.*, 2012; Langin *et al.*, 2017; García & Tubaro, 2018; but see Slabbekoorn & Smith, 2000).

Songs are one of the dominant features of courtship in most passerine birds (Darwin, 1871; Hawkins, 1918) and generally appear to be under strong sexual selection (Catchpole, 1987). Given the important role of song in bird mate choice, it is unsurprising that song differences between lineages are a common contributor to prezygotic reproductive isolation (Price, 2008). In many cases of incipient speciation in birds, songs appear to be one of the most important causes of reproductive isolation (Slabbekoorn & Smith, 2002). Thus, the coupling between diversification in bill size and courtship songs may have important consequences for speciation in birds. However, most evidence for a relationship between song and bill size comes from old evolutionary radiations that have primarily diversified in allopatry (Podos, 1997, 2001; Palacios & Tubaro, 2000; Seddon, 2005; Derryberry *et al.*, 2012; García & Tubaro, 2018), complicating interpretations about the role of bill–song correlations in bird speciation. Indeed, studies of recently diverged, sympatric lineages have found more mixed results, with some studies reporting relatively large and consistent effects of bill size on song (e.g. Ballentine, 2006; Huber & Podos, 2006), while others report comparatively weak (Langin *et al.*, 2017; Porzio *et al.*, 2019) or no effects at all (Slabbekoorn & Smith, 2000; Porzio *et al.*, 2018). Furthermore, while most of these recently diverged taxa show ecological differentiation consistent with divergent selection on bill morphology, divergent selection has only been documented in a single case (Hendry *et al.*, 2009). To better understand the role of bill–song correlations in speciation, additional studies of lineages that have recently diverged in sympatry and have experienced divergent selection on bill size are needed.

Here, we test for a correlation between bill size and song features in an adaptive radiation of seed-eating birds, the red crossbill (*Loxia curvirostra*) complex, which has rapidly diversified in just ~10 000 years (Parchman *et al.*, 2016). In North America, there are ten widely sympatric ecotypes (see Supporting Information for geographical ranges of ecotypes included in this study) that differ in bill morphology related to divergent selection on different conifer species (or different varieties within a species; Benkman, 1993, 2003). Crossbill ecotypes also differ in contact calls and courtship song (Benkman *et al.*, 2009; Benkman & Young, 2019; Fig. 1). Field studies have found evidence of strong but incomplete prezygotic reproductive isolation between sympatric crossbills, including high levels of behavioural isolation (Smith & Benkman, 2007), which may be partly due to song divergence between ecotypes. Therefore, crossbills are well suited for examining the role of bill size–song correlations early in the process of divergence without geographical isolation, when the potential effects on reproductive isolation are greatest.

MATERIAL AND METHODS

STUDY SPECIES

Red crossbills are cardueline finches that feed almost exclusively on seeds in conifer cones (Newton, 1972; Benkman & Young, 2019). In North America, crossbills have diversified into ten ecotypes including one recently recognized species, the Cassia crossbill (*Loxia sinesciuris*), each with a bill morphology suited to feeding on different conifer species (Benkman, 1993, 2003; Parchman & Benkman, 2002; Irwin, 2010). Strong feeding tradeoffs on different conifer species

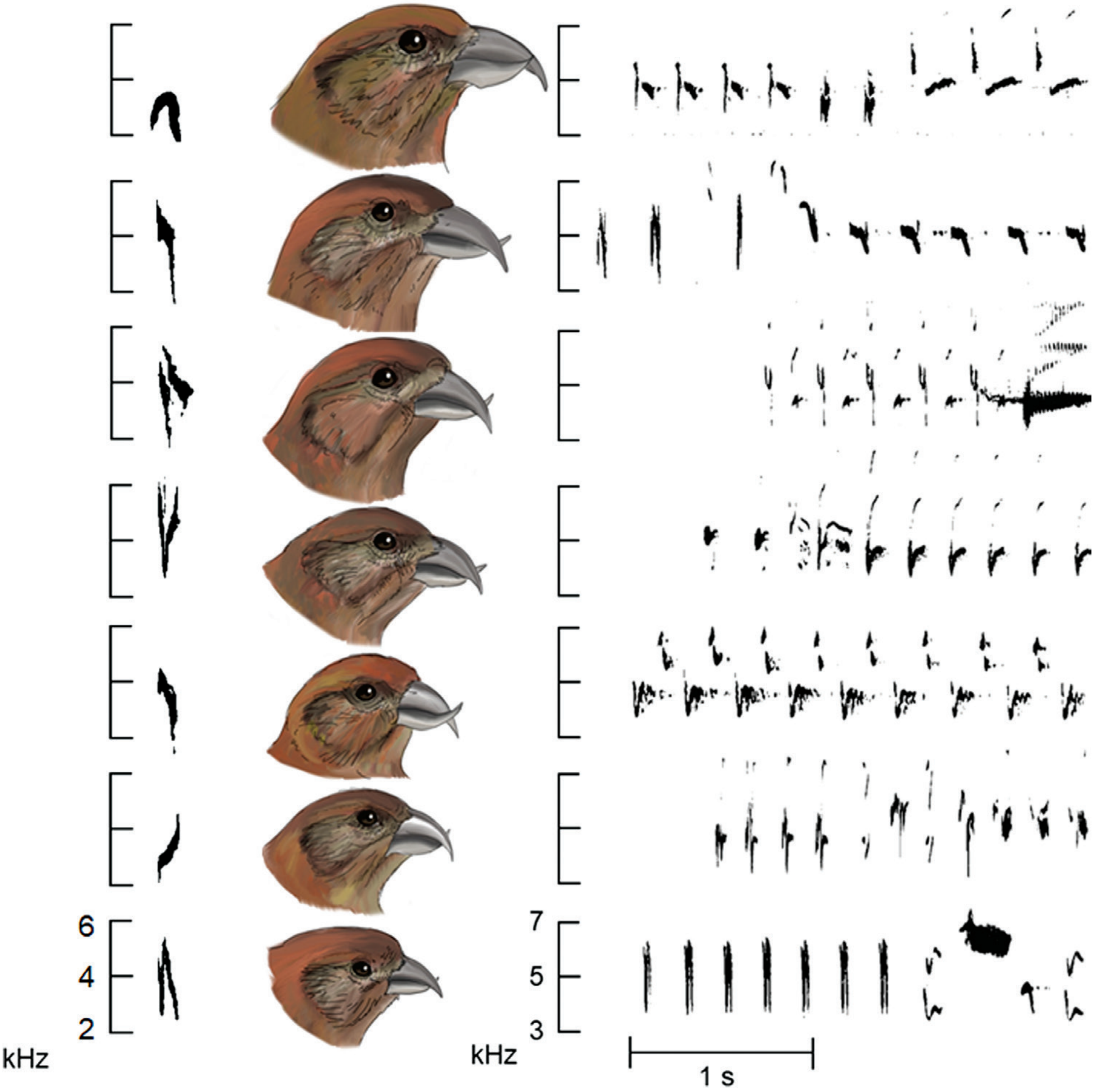

Figure 1. Bill morphology and representative contact call (left) and song (right) spectrograms of the seven red crossbill ecotypes included in this study (from top to bottom: type 9 (or Cassia crossbill, *Loxia sinesciuris*), type 2, type 5, type 4, type 1, type 10, and type 3). Crossbill illustrations by D. J. McNeil, Jr.

result in divergent natural selection on bill structure (particularly bill depth, but also the seed-husking groove of the horny palate; Benkman, 1993, 2003), the primary axis of morphological differentiation among crossbill taxa, with a strong allometric relationship between body mass and bill depth (Benkman, 2003). Multiple lines of evidence suggest that most crossbills have rapidly diversified in the absence of substantial, if any, geographical barriers to gene flow (Benkman, 1993; Smith & Benkman, 2007; Parchman *et al.*, 2016).

As in most passerines, song is a defining feature of courtship in crossbills (Nethersole-Thompson, 1975). Prior to breeding, crossbills occur in mixed-sex feeding flocks, where males spend much of their time singing and displaying to females, with multiple males singing simultaneously and in close proximity to each other (Newton, 1972; Nethersole-Thompson, 1975). Crossbills appear to choose mates from within these flocks, and males do not subsequently defend breeding territories unlike most passerines (Newton, 1972; Nethersole-Thompson, 1975). Observations of singing male crossbills indicate that males sing two general song types in different social contexts. In flocks prior to breeding, males often counter-sing with other males, occasionally resulting in aggressive encounters between males (Nethersole-Thompson, 1975). Of 31 such counter-singing incidents between Cassia crossbill males, 29 involved structurally simple songs that were dominated by loud, broadband (i.e. 'buzzy') syllables repeated multiple times (Benkman & Porter, 2018). In contrast, in ten incidents of singing males actively courting females (e.g. singing while performing display flights near a female; Benkman & Porter, 2018), seven involved more rapid, structurally complex songs dominated by pure tonal syllables with a comparatively narrow frequency bandwidth (Benkman & Porter, 2018). These data are consistent with observations in other crossbill species (Nethersole-Thompson, 1975), suggesting that the use of distinct songs in different social contexts is probably general to all crossbills, as it is for many bird species (e.g. Morse, 1970). Although pure tonal syllables are clearly involved in courtship and probably play a role in behavioural isolation between crossbill lineages, it is unknown whether females eavesdrop on male–male counter-singing and base mate choice decisions on these songs. Therefore, here we analyse both pure tonal syllables and buzzy syllables, but treat them separately in all analyses.

Given the role that song plays in crossbill courtship, the observation that crossbill taxa differ in male courtship songs (Benkman *et al.*, 2009; Fig. 1) suggests that song could be an important component of reproductive isolation. Preliminary field experiments indicate that females are more likely to approach and spend more time near speakers broadcasting homotypic male songs compared to songs from heterotypic males (C.K.P., unpubl. data). Furthermore, habitat isolation and reduced immigrant fecundity are incomplete barriers to gene flow between sympatric ecotypes, resulting in ample opportunity for sympatric ecotypes to intermix during breeding (Smith & Benkman, 2007). However, behavioural isolation between sympatric ecotypes is very high (Smith & Benkman, 2007), resulting in limited interbreeding. Although more data on the importance of song as a component of behavioural isolation in crossbills are needed, it seems likely that song differences play a role in reducing gene flow between ecotypes.

We recorded or obtained recordings of the songs of six ecotypes (Types 1, 2, 3, 4, 5 and 10 of Groth, 1993; Irwin, 2010) and one recently recognized species (the Cassia crossbill, also referred to as Type 9) of crossbill. An overview of the natural history of all crossbill taxa in this study, including primary geographical range, bill depth, feeding ecology and sympatry among taxa, is available in Table 1. Geographical range maps of each ecotype, as well as recording locality information, are available in the Supporting Information. Importantly, these crossbill taxa exhibit almost the entire range of

Table 1. Natural history overview of the crossbill taxa included in this study

Ecotype	Primary geographical range	Mean bill depth (mm) ± SD (*N*)	Key conifer	Regularly sympatric with	Recording sample size
1	Appalachian Mountains, north-east	8.80 ± 0.26 (39)	Possibly red spruce (*Picea rubens*)	Ecotype 2	31
2	Rocky Mountains	9.67 ± 0.36 (189)	Ponderosa pine (*Pinus ponderosa*)	Ecotypes 4, 5	36
3	Pacific Northwest	8.19 ± 0.24 (28)	Western hemlock (*Tsuga heterophylla*)	Ecotypes 4, 10	30
4	Pacific Northwest	9.00 ± 0.21 (28)	Douglas-fir (*Pseudotsuga menziesii*)	Ecotypes 2, 3	24
5	Rocky Mountains	9.57 ± 0.31 (31)	Lodgepole pine (*Pinus contorta*)	Ecotype 2	14
9	Rocky Mountains (two mountain ranges in southern Idaho)	9.97 ± 0.30 (1408)	Lodgepole pine that has evolved in the absence of squirrels	Ecotypes 2, 5	51
10	Pacific Northwest	8.55 ± 0.37 (54)	Sitka spruce (*Picea sitchensis*)	Ecotypes 3, 4	28

Data on mean bill depth for crossbill taxa were based on studies of crossbill morphology (Benkman, 1993, 2003; Groth, 1993; Irwin, 2010). 'Key conifer' refers to the conifer species that each taxon is morphologically specialized for feeding on based on Benkman (1993, 2003), Irwin (2010) and extensive field observations (Benkman & Young, 2019). The ecotypes that each taxon regularly co-occur with are also listed, but due to regular, long-distance movements by crossbills, most of the taxa in this study can occasionally be found in sympatry. Also listed are the sample sizes of audio recordings available for each taxon in our analyses.

variation in bill size within North American crossbills, with Type 3 being the smallest-billed crossbill (mean bill depth of males = 8.19 mm), Type 9 being the third largest-billed crossbill (9.97 mm) and all other sampled crossbills being intermediate in bill size (Table 1). Therefore, this sample of crossbill taxa is well suited for detecting variation in song related to variation in bill size.

BILL SIZE MEASUREMENTS

Crossbill taxa differ in overall bill size, including common linear bill measurements such as bill width, bill length and bill depth (Groth, 1993). However, bill width measurements are not available for most ecotypes, given that crossbill bill width is difficult to measure consistently (C. W. Benkman, pers. comm.). Bill length can be measured consistently, but the bill length of individuals can vary greatly within a year due to abrasion of the bill tips associated with prying open cones during feeding (Benkman & Porter, 2018). Therefore, we used bill depth as a measure of bill size, given that bill depth can be measured consistently, does not vary due to abrasion and is the main target of divergent selection associated with feeding on alternative conifers (Benkman, 1993, 2003). Because there is sexual dimorphism in crossbill bill size (Groth, 1993; Benkman & Porter, 2018; Benkman & Young, 2019), only measurements of males were used in analyses.

Bill depth data for ecotypes 1, 2, 3, 4 and 5 are from Groth (1993), data for ecotype 9 are from measurements by C. W. Benkman (Benkman & Porter, 2018) and data for ecotype 10 are from Irwin (2010). All authors measured bill depth in a comparable manner (i.e. with calipers at the anterior edge of the nostrils). Although individuals from multiple localities within a taxon were sometimes measured, there appears to be little to no geographical variation in bill size within ecotypes (Benkman & Young, 2019), consistent with morphological specialization of each ecotype being driven by adaptation to a single conifer across its geographical range (Benkman, 1993, 2003) and no evidence for isolation by distance in recent genomic analyses (Parchman *et al.*, 2016). Mean bill depth, standard deviations and sample sizes for all crossbill taxa are presented in Table 1. A plot of male bill depth distributions for ecotypes 2, 3, 4, 5 and 9 is available in the Supporting Information.

SONG DATA

We made digital recordings of ecotypes 2, 3, 4, 5 and 9 during years of fieldwork throughout the western United States between 2003 and 2015. Recordings were made using a Marantz PMD-670 digital recorder (Marantz America, Inc., Aurora, IL, USA) and a Telinga Twin Science MK2 parabolic microphone and dish (Telinga Microphones, Tobo, Sweden). For all other ecotypes, we downloaded digital song recordings from the Macaulay Library at the Cornell Lab of Ornithology. Recording locality information is available in the Supporting Information. Recorded crossbills were identified to ecotype based on ecotype-distinctive contact calls that are consistently associated with differences in bill size, feeding behaviour, geographical range, song and genetic structure (e.g. Benkman, 1993; Groth, 1993; Parchman *et al.*, 2016). All recordings were saved as 16-bit WAVE files with a sampling rate of 44 100 Hz. Spectrograms were visualized and analysed using Raven Pro (v.1.4, Bioacoustics Research Program, 2016) with a Hann window, fast Fourier transform size of 512 points, and window overlap of 50% per window step. To reduce background noise, recordings were bandpass-filtered such that all noise above 20 000 Hz and below 500 Hz was filtered out before analyses. Sample sizes for each crossbill taxon are listed in Table 1.

Syllable repetition rate was calculated by measuring the average number of repetitions of a given syllable per second (Fig. 2A). Next, minimum and maximum frequency were determined using amplitude spectra by measuring the lowest and highest frequencies −24 dB relative to the frequency with the maximum amplitude (Fig. 2B). Similar studies in other bird species have found that the −24 dB threshold effectively excludes background noise while encompassing a majority (99.6% relative to peak amplitude) of the focal acoustic signal (Podos, 1997, 2001; Huber & Podos, 2006). The difference between the minimum and maximum frequencies was used to calculate frequency bandwidth (Fig. 2B). Vocal deviation was calculated using 90% quantile regressions (see below for details on this analysis).

For each individual recorded, we categorized different syllable types as either buzzy or pure tonal based on visual inspection of spectrograms, considering frequency and temporal structure of syllables. Buzzy and pure tonal syllables were treated separately in all subsequent acoustic analyses. When possible, we measured multiple bouts of a given song type for each individual and took the average of these measurements for use in subsequent analyses.

SYLLABLE REPETITION RATE AND FREQUENCY BANDWIDTH

The vocal tract constraint hypothesis predicts a triangular relationship between syllable repetition rate and frequency bandwidth. In other words, most songs will have either high syllable repetition rate, high frequency bandwidth or intermediate values of both,

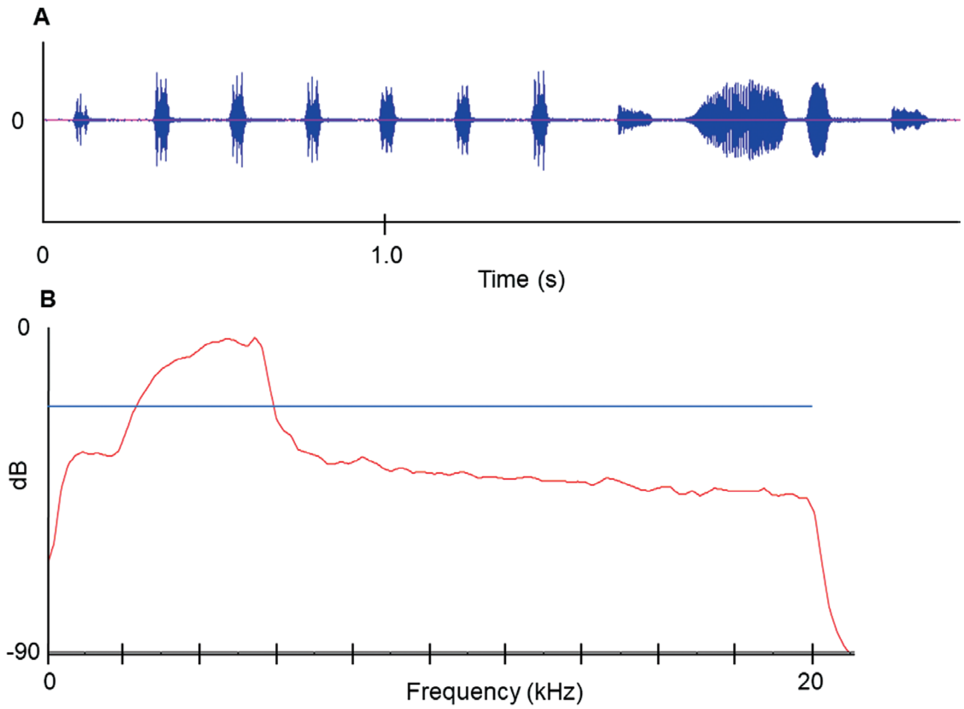

Figure 2. A, oscillogram of the representative type 3 song in Figure 1. Syllable repetition rate was measured as the number of syllables produced per second (5.01 Hz here). B, amplitude spectrum of the second syllable from the oscillogram. The solid line at −24 dB from the maximum amplitude frequency represents the threshold used to estimate minimum and maximum frequencies (2.33 and 5.95 kHz, respectively). Frequency bandwidth was calculated as the difference between minimum and maximum frequencies.

but no songs will have both high syllable repetition rate and frequency bandwidth, which represent the performance limits of song (Wilson *et al.*, 2014). To estimate the relationship between syllable repetition rate and frequency bandwidth at the maximum limits of song performance, we calculated a 90% quantile regression based on the recommendation of Wilson *et al.* (2014) using the quantreg package in R (Koenker, 2018). A trade-off between syllable repetition rate and frequency bandwidth is indicated by a negative quantile regression slope and is predicted by the vocal constraint hypothesis (Wilson *et al.*, 2014). The slope of the quantile regression was estimated using the bootstrap function in the quantreg package in R v.3.3.3.

Because the 90% quantile regressions used here are assumed to approximate the maximum performance limits of song (Wilson *et al.*, 2014), the degree to which a song deviates from this upper bound can be considered a measure of relative song performance. Relative song performance can thus be measured by calculating the orthogonal distance between each song and the 90% quantile regression line. Podos (2001) refers to this measure of song performance as the 'vocal deviation' of a song, with higher values of vocal deviation representing low-performance songs and lower values of vocal deviation representing high-performance songs. Importantly, mate choice experiments in at least one species (swamp sparrow, *Melospiza georgiana*) indicate that females prefer songs of low vocal deviation over songs of high vocal deviation (Ballentine *et al.*, 2004), suggesting that vocal deviation is a biologically meaningful measure of song performance. We calculated the vocal deviation of each song using R v.3.3.3.

MORPHOLOGICAL CONSTRAINTS ON SONG

The vocal tract constraint hypothesis predicts that crossbill taxa with larger bills should sing songs with lower minimum and maximum frequencies, syllable repetition rates, and frequency bandwidths, and higher vocal deviation. To test this hypothesis, we performed Jonckheere–Terpstra tests between the mean bill depth of each crossbill taxon (Table 1) and each song variable in R using the 'clinfun' package. The Jonckheere–Terpstra test is a non-parametric, rank-based trend test that tests for an ordered difference in means among groups (where x is an ordinal variable and y is a continuous variable). Importantly, this test requires that we specify a priori the direction of the ordered differences among groups. For each song variable, we defined the alternative hypothesis according to the predicted relationship with bill depth based on the vocal tract constraint hypothesis. For each test we ran 1000 permutations. We note that similar studies on older evolutionary radiations conducted comparisons of song variables while correcting for the phylogenetic structure of taxa. However, a study based on 238 615 single nucleotide polymorphisms found little evidence for phylogenetic structure in North American red crossbills (Parchman *et al.*, 2016), probably due to very recent (i.e. post-Pleistocene) divergence and low levels of ongoing gene flow (e.g. Smith & Benkman, 2007), suggesting that phylogenetic structure is unlikely to confound the comparisons we present here.

CLASSIFYING CROSSBILL SONGS TO TAXA

Bill depth might influence reproductive isolation through its effects on song structure if the songs of sympatric crossbill taxa can be reliably distinguished based on variables related to bill size. To evaluate this possibility, we ran a discriminate function analysis that included all song variables that were found to be related to bill size as predicted by the vocal tract constraint hypothesis. From each discriminant function analysis, we evaluated the extent to which six pairs of highly sympatric crossbill taxa (based on geographical ranges in Benkman & Young, 2019) were distinguishable from each other based on the percentage of songs that were misclassified to taxa. To further evaluate the influence of bill size differences on song structure divergence (i.e. the potential for song discrimination), we ran a Mantel test with 9999 replicates to test for a negative relationship between mean bill depth difference and percentage of misclassified songs for each pair of crossbill taxa using the 'ade4' package in R (Dray & Dufour, 2007). Finally, we ran step-forward discriminant function analyses using all song variables to test whether variables constrained by bill size were more important in discriminating crossbill taxa compared to song variables unrelated to bill size. Analyses were run using JMP Pro, v.13.1.0 (SAS Institute Inc., 2016) and were only performed for pure tonal syllables, given that we found no evidence for bill depth–song correlations in buzzy syllables (see Results).

RESULTS

SYLLABLE REPETITION RATE AND FREQUENCY BANDWIDTH

Consistent with the hypothesis that there is a motor constraint on song production in crossbills, we found the predicted triangular relationship between syllable repetition rate and frequency bandwidth for both buzzy and pure tonal syllables (Fig. 3). In other words, fast paced songs tended to have low frequency bandwidth, whereas slow songs had either high or low frequency bandwidth. Furthermore, the slope of the 90% quantile regression for these data was significantly negative for pure tonal syllables ($frequency\ bandwidth = -0.57 syllable\ repetition\ rate + 10.06$, $t_{150} = -3.22$, $P = 0.0016$) and buzzy syllables ($frequency\ bandwidth = -0.66 syllable\ repetition\ rate + 8.17$, $t_{68} = -6.26$, $P < 0.0001$).

MORPHOLOGICAL CONSTRAINTS ON SONG

For pure tonal syllables, we found that minimum frequency [Jonckheere–Terpstra test value (JT) = 3835, $P = 0.001$; Fig. 4], syllable repetition rate ($JT = 3688.5$, $P = 0.001$; Fig. 5) and vocal deviation ($JT = 5442$, $P = 0.013$; Fig. 6) showed the relationship with bill depth predicted by the vocal tract constraint hypothesis. We did not find evidence for the negative relationships between bill depth and maximum frequency or frequency bandwidth ($P > 0.9$ in both cases) predicted by the vocal tract constraint hypothesis. We found no relationship between bill depth and any acoustic variables for buzzy syllables ($P > 0.1$ for all other comparisons).

CAN SONG DIFFERENCES DISTINGUISH CROSSBILL TAXA?

Overall, discriminant function analyses based on minimum frequency, syllable repetition rate and vocal deviation of pure tonal syllables misclassified a total of 72% of songs to taxa. In pairwise comparisons of six highly sympatric ecotypes, an average of 13.9% of songs were misclassified (range: 4.3–26.1%; Table 2). In contrast to our prediction, we did not find a negative relationship between bill size difference and percentage of songs misclassified between pairs of crossbill taxa ($P = 0.37$), suggesting that the potential for song discrimination does not increase as bill size diverges.

Step-forward discriminant function analyses based on pure tonal syllables revealed that minimum frequency, syllable repetition rate and vocal deviation were the least important variables for discriminating among the songs of crossbill taxa (Table 3). These results indicate that variables constrained by bill size do not tend to be more important for distinguishing among the songs of crossbill taxa than those unconstrained by bill size.

DISCUSSION

Our results suggest that some components of song structure in crossbills are constrained by bill size, which has diversified for feeding on alternative food

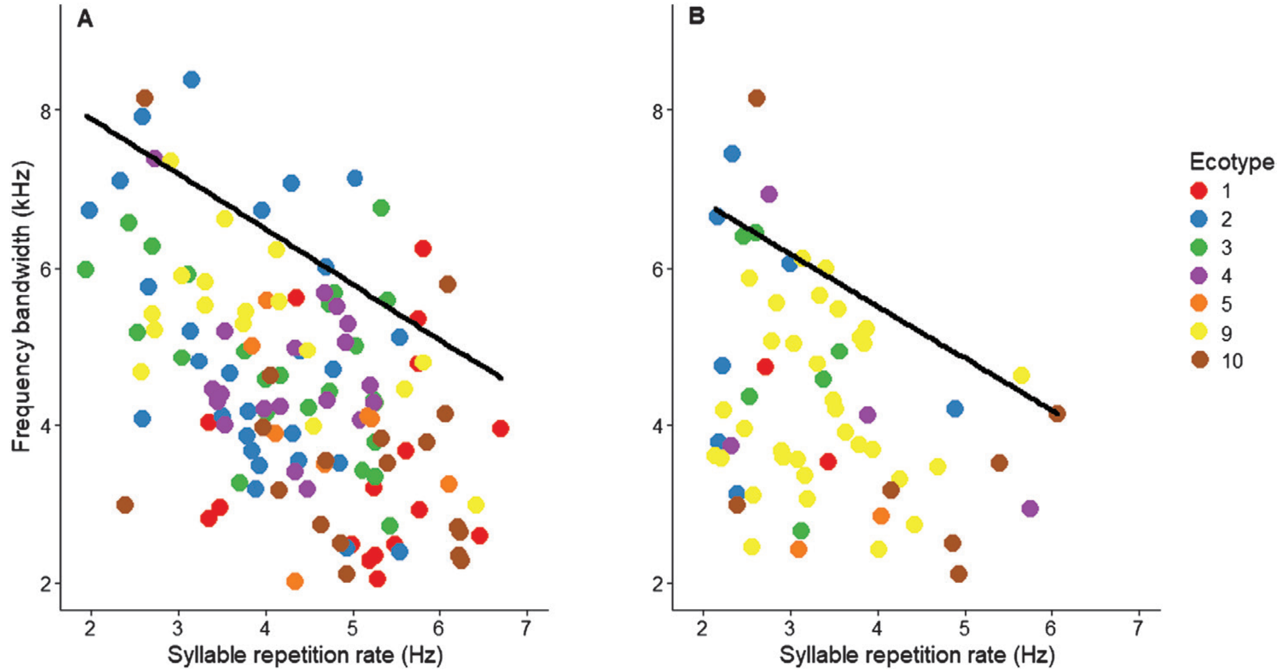

Figure 3. The relationship between syllable repetition rate and frequency bandwidth for (A) pure tonal syllables ($N = 153$ individuals) and (B) buzzy syllables ($N = 71$ individuals) of crossbill songs. Colours denote the different ecotypes for which recordings were obtained (note that ecotype 9 is synonymous with the Cassia crossbill). The black line is the 90% quantile regression. Note that the vocal deviation of songs was estimated as the orthogonal distance between each song and the 90% quantile regression.

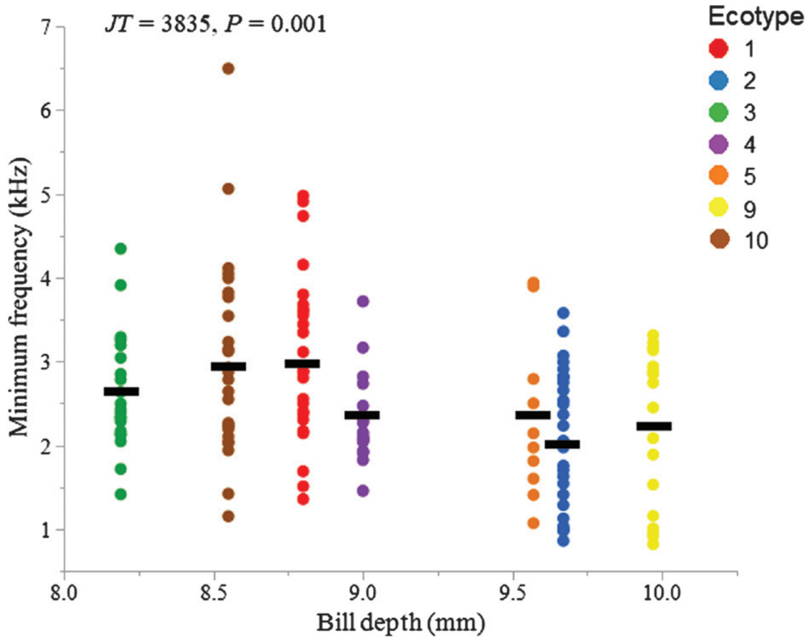

Figure 4. The minimum frequency of pure tonal syllables in crossbill song decreases as the mean bill depth of ecotypes increases, consistent with the vocal tract constraint hypothesis. Black lines represent the means for each ecotype. Colours denote the different ecotypes for which recordings were obtained (note that ecotype 9 is synonymous with the Cassia crossbill). The results of a Jonckheere–Terpstra test are reported on the graph.

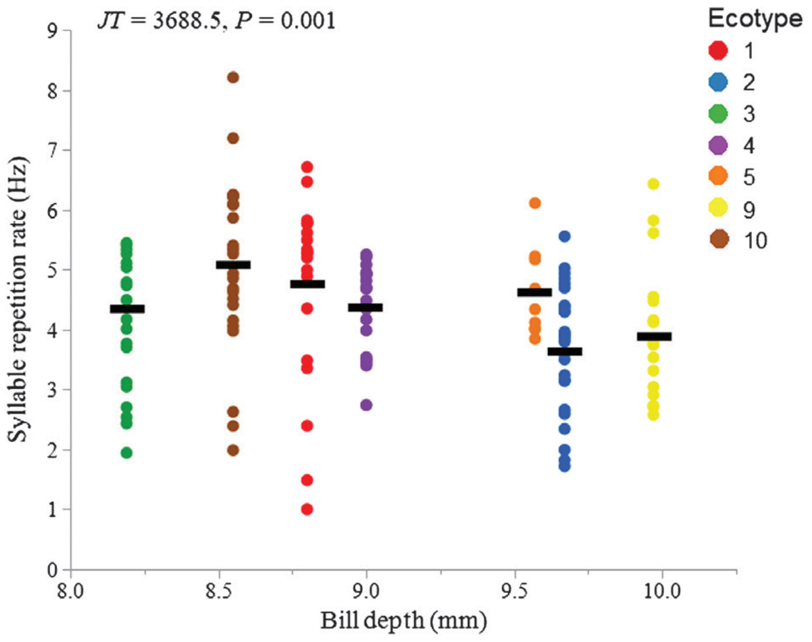

Figure 5. The syllable repetition rate of pure tonal syllables in crossbill song decreases as the mean bill depth of ecotypes increases, consistent with the vocal tract constraint hypothesis. Black lines represent the means for each ecotype. Colours denote the different ecotypes for which recordings were obtained (note that ecotype 9 is synonymous with the Cassia crossbill). The results of a Jonckheere–Terpstra test are reported on the graph.

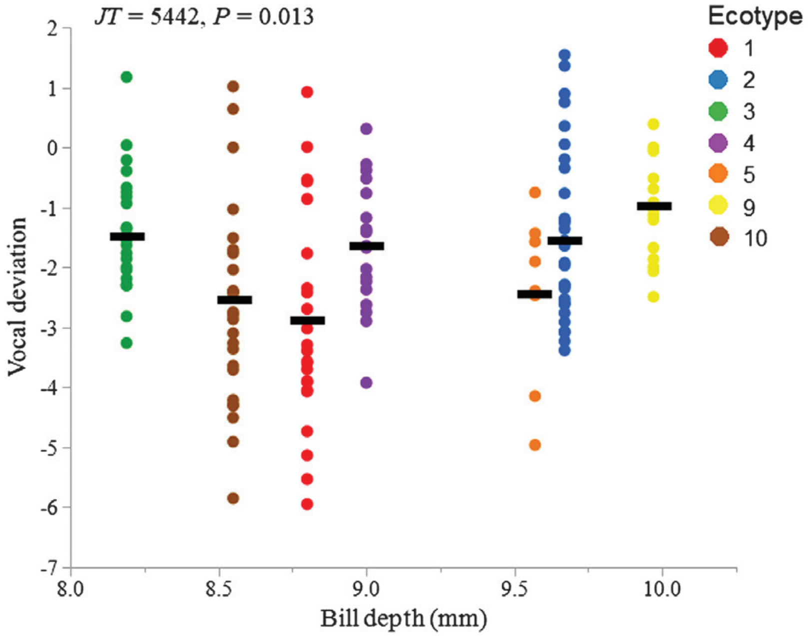

Figure 6. The vocal deviation of pure tonal syllables in crossbill song increases as the mean bill depth of ecotypes increases, consistent with the vocal tract constraint hypothesis. Black lines represent the means for each ecotype. Colours denote the different ecotypes for which recordings were obtained (note that ecotype 9 is synonymous with the Cassia crossbill). The results of a Jonckheere–Terpstra test are reported on the graph.

resources (Benkman, 1993, 2003). As in other studies of animal vocalizations (Wilson *et al.*, 2014), we found a tradeoff between syllable repetition rate and frequency bandwidth in crossbill song (Fig. 3). Crossbills with larger bills were less likely than smaller-billed birds to sing songs with combinations of high syllable

Table 2. Estimated potential for song discrimination of highly sympatric pairs of crossbill taxa in North America

Region	Ecotype pair	Mean bill depth difference (mm)	Percentage misclassified
Pacific Northwest	3 & 4	0.81	18.2
	3 & 10	0.36	6
	4 & 10	0.2	4.3
Central Rocky Mountains	2 & 5	0.45	18.4
South Hills	5 & 9	0.40	10.3
	2 & 9	0.30	26.1

The percentage of misclassified songs is based on discriminant function analyses for song variables related to bill depth as predicted by the vocal tract constraint hypothesis in pure tonal syllables (minimum frequency, syllable repetition rate and vocal deviation).

Table 3. Results from a step-forward discriminant function analysis, showing the relative importance of five song variables in pure tonal syllables for discriminating among crossbill taxa

Song variable	Model rank	*F* ratio	*P*-value
Frequency bandwidth	1	11.81	<0.0001
Maximum frequency	2	6.18	<0.0001
Vocal deviation	3	6.25	<0.0001
Minimum frequency	4	4.96	0.0002
Syllable repetition rate	5	4.06	0.0009

Model rank refers to the order in which variables were incorporated into the model. Variables in bold font are those that are constrained by bill size as predicted by the vocal tract constraint hypothesis.

repetition rates and wide frequency bandwidths in pure tonal syllables (Fig. 6), as has been found for Darwin's finches (Podos, 2001; Huber & Podos, 2006) and swamp sparrows (Ballentine, 2006). Of the four additional song variables we measured, minimum frequency and syllable repetition rate of pure tonal syllables varied with bill depth as predicted by the vocal tract constraint hypothesis (Figs 4, 5). Overall, our findings indicate that ecological diversification and mating signal diversification have been coupled to some degree during the adaptive radiation of crossbills.

Nonetheless, our results also suggest that some features of crossbill song are not constrained by bill size. In particular, we found no evidence for bill size constraints on the production of buzzy syllables. Additionally, pairwise comparisons from discriminant function analyses suggest that the potential for song discrimination does not increase as bill size divergence increases, even for song variables constrained by bill size. While discriminant function analyses do not indicate that song variables constrained by bill size are more important for discriminating among the songs of sympatric crossbill taxa, the importance of bill size–song correlations to reproductive isolation depends ultimately on which song features mediate behavioural isolation between taxa (Hudson & Price, 2014). Our results thus serve as an important guide for future mate choice experiments by allowing tests of whether song variables constrained or unconstrained by bill size are more important to reproductive isolation among crossbill taxa.

While our results are consistent with multiple predictions of the vocal tract constraint hypothesis, the evolution of bird song can be influenced by factors other than variation in bill size (Slabbekoorn & Smith, 2002). Indeed, numerous examples of sensory drive, wherein mating signals are adapted for efficient transmission of information through the habitat of organisms (Endler & McLellan, 1988; Endler, 1992), come from bird song (Morton, 1975; Wiley, 1991; Slabbekoorn & Smith, 2002; Derryberry, 2009; Tobias *et al.*, 2010). For example, birds occupying open environments tend to have highly modulated and trilled songs with high frequencies, whereas birds from densely vegetated environments have predominately pure tonal songs with a narrower frequency range (Morton, 1975) and lower frequencies overall (Wiley, 1991). Given that the crossbill taxa studied here occupy different habitats which vary structurally, divergence in songs due to adaptation to different environments might seem possible. However, crossbills do not defend territories (Newton, 1972; Nethersole-Thompson, 1975) and thus do not broadcast songs over long distances unlike many territorial songbirds. Indeed, crossbill singing is most intense just prior to breeding while crossbills are in large, mixed-sex flocks. In these flocks, male crossbills will often direct song towards an individual female while only a few metres away (Nethersole-Thompson, 1975). Furthermore, the majority of singing by all crossbill taxa occurs at or near the tops of trees (Nethersole-Thompson, 1975) such that male–female courtship interactions of all ecotypes occur in open environments above the tree canopy, which should not favour song divergence related to signal transmission through alternative forest environments.

Song divergence in crossbills could also be influenced by traits other than bill size. Apart from bill size, crossbills differ in body size (Groth, 1993), which has been shown to influence the frequency characteristics of bird song, namely minimum and maximum frequency and frequency bandwidth (Wiley, 1991; Wild *et al.*, 1998). Body size is strongly correlated with bill depth among crossbills (Benkman & Young, 2019), making it difficult to disentangle the effects of these two traits. While we did find evidence for divergence in minimum frequency among crossbills that is consistent with both bill and body size predictions, we also found divergence in song variables for which there are not biomechanical predictions of the relationship between body size and song (syllable repetition rate and vocal deviation; Derryberry *et al.*, 2012). Indeed, none of the eight studies that have quantified the relationship between body size and syllable repetition rate and/or vocal deviation in birds have documented significant associations between these variables (Podos, 1997, 2001; Slabbekoorn & Smith, 2000; Seddon, 2005; Langin *et al.*, 2017; García & Tubaro, 2018; Porzio *et al.*, 2018, 2019), while some studies have found significant associations between bill size and these song variables (Podos, 2001; Seddon, 2005; Ballentine, 2006; Huber & Podos, 2006; Derryberry *et al.*, 2012; Langin *et al.*, 2017; García & Tubaro, 2018). Therefore, we are confident that the patterns we document for syllable repetition rate and vocal deviation (Figs 5, 6) are due to bill size variation among ecotypes. Finally, we note that body size has probably experienced disruptive selection as crossbills diversified to exploit a wide variety of seed and cone sizes. Small-billed crossbills that specialize on small seeds probably experience net selection favouring smaller body size, as large-bodied birds have high energetic demands (Calder, 1974) that may not be satisfied on a diet of small seeds (e.g. Benkman & Pulliam, 1988). Conversely, large-billed crossbills that specialize on large seeds probably experience net selection favouring larger body size. Increased body mass relative to bill size shifts the centre of gravity between and slightly below the wings, thereby improving aerodynamic efficiency and acceleration for predator evasion (Benkman, 1991). Additionally, some of the force involved in prying open conifer cones appears to be made with the movement and positioning of the entire body, suggesting that there may be a more direct link between body size and resource use in crossbills. Therefore, even if body size directly influences song diversification in crossbills, this would still represent a coupling of ecological and mating signal diversification.

The coupling of bill size and song diversification in crossbills may have been key to their rapid radiation despite extensive sympatry among ecotypes. Early-acting components of reproductive isolation that restrict gene flow between sympatric ecotypes (habitat isolation, reduced immigrant fecundity and assortative flocking; Smith & Benkman, 2007; Smith *et al.*, 2012) periodically break down when ecotypes converge in their use of abundant, easily accessible seeds produced by some conifers (e.g. Engelmann spruce, *Picea engelmannii*) in mast years (C.K.P., unpubl. data). However, behavioural isolation remains moderately strong between ecotypes and appears to be the only barrier to gene flow in scenarios of abundant, easily accessible seeds (C.K.P., unpubl.data). Mate choice based on song differences between ecotypes may be responsible for maintaining behavioural isolation under these conditions when the scope for gene flow is greatest, although more research in this regard is needed.

ACKNOWLEDGEMENTS

We thank M. Young at the Macaulay Library for giving us access to several crossbill recordings and for developing the range maps of ecotypes. We also thank C. K. Akcali, C. W. Benkman, P. Edelaar and three anonymous reviewers for providing valuable feedback on the manuscript. The Robert Berry Chair Endowment awarded to C. W. Benkman provided financial support to C.K.P. during production of the manuscript. The M. J. Murdock Charitable Trust awarded J.W.S. funding for this project. The authors declare no conflicts of interest.

REFERENCES

Ballentine B. 2006. Morphological adaptation influences the evolution of a mating signal. *Evolution* **60:** 1936–1944.

Ballentine B, **Hyman J**, **Nowicki S. 2004.** Vocal performance influences female response to male bird song: an experimental test. *Behavioral Ecology* **15:** 163–168.

Benkman CW. 1991. Predation, seed size partitioning, and the evolution of body size in seed-eating finches. *Evolutionary Ecology* **5:** 118–127.

Benkman CW. 1993. Adaptation to single resources and the evolution of crossbill (*Loxia*) diversity. *Ecological Monographs* **63:** 305–325.

Benkman CW. 2003. Divergent selection drives the adaptive radiation of crossbills. *Evolution* **57:** 1176–1181.

Benkman CW. 2007. Red crossbill types in Colorado: their ecology, evolution and distribution. *Colorado Birds* **41:** 153–163.

Benkman CW, **Parchman TL**, **Favis A**, **Siepielski AM. 2003.** Reciprocal selection causes a coevolutionary arms race between crossbills and lodgepole pine. *The American Naturalist* **162:** 182–194.

Benkman CW, **Porter CK. 2018.** Cassia Crossbill (*Loxia sinesciuris*), version 1.0. In: Rodewald PG, ed. *The Birds of North America*. Ithaca: Cornell Lab of Ornithology.

Benkman CW, **Pulliam HR. 1988.** The comparative feeding rates of North American sparrows and finches. *Ecology* **69:** 1195–1199.

Benkman CW, **Smith JW**, **Keenan PC**, **Parchman TL**, **Santisteban L. 2009.** A new species of red crossbill (Fringillidae: *Loxia*) from Idaho. *The Condor* **111:** 169–176.

Benkman CW, **Young M. 2019.** Red crossbill (*Loxia curvirostra*), version 1.0. In: Rodewald PG, ed. *The Birds of North America*. Ithaca: Cornell Lab of Ornithology.

Bioacoustics Research Program. **2016.** *Raven Lite: Interactive Sound Analysis Software (Version 2.0)* [Computer software]. Ithaca: The Cornell Lab of Ornithology. Available at: http://www.birds.cornell.edu/raven.

Calder WA III. **1974.** Consequences of body size for avian energetics. In: Paynter RA Jr, ed. *Avian energetics*. Cambridge: Nuttall Ornithology Club, 86–151.

Catchpole CK. 1987. Bird song, sexual selection and female choice. *Trends in Ecology and Evolution* **2:** 94–97.

Coyne JA, **Orr HA. 2004.** *Speciation*. Sunderland: Sinauer.

Darwin CR. 1859. *On the origin of species by means of natural selection, or the preservation of favoured races in the struggle for life*. London: John Murray.

Darwin CR. 1871. *The descent of man, and selection in relation to sex*. London: John Murray.

Derryberry EP. 2009. Ecology shapes birdsong evolution: variation in habitat and morphology explains variation in white-crowned sparrow song. *The American Naturalist* **174:** 24–33.

Derryberry EP, **Claramunt S**, **Seddon N**, **Tobias J**, **Baker A**, **Brumfield RT. 2012.** Correlated evolution of beak morphology and song in the Neotropical woodcreeper radiation. *Evolution* **66:** 2784–2797.

Dray S, **Dufour A. 2007.** The ade4 package: implementing the duality diagram for ecologists. *Journal of Statistical Software* **22:** 1–20.

Endler JA. 1992. Signals, signal conditions, and the direction of evolution. *The American Naturalist* **139:** S125–S153.

Endler JA, **McLellan T. 1988.** The processes of evolution: toward a newer synthesis. *Annual Review of Ecology, Evolution, and Systematics* **19:** 395–421.

García NC, **Tubaro PL. 2018.** Dissecting the roles of body size and beak morphology in song evolution in the “blue” cardinals (Passeriformes: Cardinalidae). *The Auk* **135:** 262–275.

Gavrilets S. 2004. *Fitness landscapes and the origin of species*. Princeton: Princeton University Press.

Grant PR. 1986. *Ecology and evolution of Darwin’s finches*. Princeton: Princeton University Press.

Groth JG. 1993. Evolutionary differentiation in morphology, vocalizations, and allozymes among nomadic sibling species in the North American Red Crossbill (*Loxia curvirostra*) complex. *University of California Publications in Zoology* **127:** 1–143.

Hausberger M, **Black JM**, **Richard J. 1991.** Bill opening and sound spectrum in barnacle goose loud calls: individuals with “wide mouths” have higher pitched voices. *Animal Behavior* **42:** 319–322.

Hawkins CJ. 1918. Sexual selection and bird song. *The Auk* **35:** 421–437.

Hendry AP, **Huber SK**, **De León L**, **Herrel A**, **Podos J. 2009.** Disruptive selection in a bimodal population of Darwin’s finches. *Proceedings of the Royal Society B: Biological Sciences* **276:** 753–759.

Huber SK, **Podos J. 2006.** Beak morphology and song features covary in a population of Darwin’s finches. *Biological Journal of the Linnean Society* **88:** 489–498.

Hudson EJ, **Price TD. 2014.** Pervasive reinforcement and the role of sexual selection in biological speciation. *Journal of Heredity* **105:** 821–833.

Irwin K. 2010. A new and cryptic call type of the Red Crossbill. *Western Birds* **41:** 10–25.

SAS Institute Inc. **1989–2016.** *JMP®, Version 13.1.0*. Cary: SAS Institute Inc.

Koenker R. 2018. *Quantreg: quantile regression. R package version 5.38*. Availablae at: http://CRAN.R-project.org/packagepquantreg. Accessed 20 December 2018.

Lack D. 1947. *Darwin’s finches*. Cambridge: Cambridge University Press.

Lack D. 1968. *Ecological adaptations for breeding in birds*. London: Methuen.

Langin KM, **Sillett TS**, **Morrison SA**, **Ghalambor CK. 2017.** Bill morphology and neutral genetic structure both predict variation in acoustic signals within a bird population. *Behavioral Ecology* **28:** 866–873.

Lovette IJ, **Bermingham E**, **Ricklefs RE. 2002.** Clade-specific morphological diversification and adaptive radiation in Hawaiian songbirds. *Proceedings of the Royal Society B: Biological Series* **269:** 37–42.

Mayr E. 1963. *Animal species and evolution*. Cambridge: Harvard University Press.

Morse DH. 1970. Territorial and courtship songs of birds. *Nature* **226:** 659–661.

Morton ES. 1975. Ecological sources of selection on avian sounds. *The American Naturalist* **109:** 17–34.

Nethersole-Thompson D. 1975. *Pine crossbills: a Scottish contribution*. Calton: T. & A. D. Poyser.

Newton I. 1972. *Finches*. London: Collins.

Nowicki S. 1987. Vocal tract resonances in oscine bird sound production: evidence from birdsongs in a helium atmosphere. *Nature* **325:** 53–55.

Nowicki S, **Marler P. 1988.** How do birds sing? *Music Perception: An Interdisciplinary Journal* **5:** 391–426.

Palacios M, **Tubaro PL. 2000.** Does beak size affect acoustic frequencies in woodcreepers? *The Condor* **102:** 553–560.

Parchman TL, **Benkman CW. 2002.** Diversifying coevolution between crossbills and black spruce on Newfoundland. *Evolution* **56:** 1663–1672.

Parchman TL, **Buerkle CA**, **Soria-Carrasco V**, **Benkman CW. 2016.** Genome divergence and diversification within a geographic mosaic of coevolution. *Molecular Ecology* **25:** 5705–5718.

Podos J. 1997. A performance constraint on the evolution of trilled vocalizations in a songbird family (Passeriformes: Emberizidae). *Evolution* **51:** 537–551.

Podos J. 2001. Correlated evolution of morphology and vocal signal structure in Darwin’s finches. *Nature* **409:** 185–188.

Podos J, **Nowicki S. 2004.** Beaks, adaptation, and vocal evolution in Darwin’s finches. *BioScience* **54:** 501–510.

Podos J, **Southall JA**, **Rossi-Santos MR. 2004.** Vocal mechanics in Darwin's finches: correlation of beak gape and song frequency. *Journal of Experimental Biology* **207:** 607–619.

Porzio NS, **Repenning M**, **Fontana CS. 2018.** Evolution of beak size and song constraints in Neotropical seedeaters (Thraupidae: *Sporophila*). *Acta Ornithologica* **53:** 173–180.

Porzio NS, **Repenning M**, **Fontana CS. 2019.** Do beak volume and bite force influence the song structure of sympatric species of seedeaters (Thraupidae: *Sporophila*)? *Emu* **119:** 71–78.

Price TD. 2008. *Speciation in birds*. Greenwood Village: Roberts and Company Publishers.

Ptacek MB. 2000. The role of mating preferences in shaping interspecific divergence in mating signals in vertebrates. *Behavioural Processes* **51:** 111–134.

Ryan PG, **Bloomer P**, **Moloney CL**, **Grant TJ**, **Delport W. 2007.** Ecological speciation in South Atlantic island finches. *Science* **315:** 1420–1423.

Schluter D, **Grant PR. 1984.** Determinants of morphological patterns in communities of Darwin's finches. *The American Naturalist* **123:** 175–196.

Schulenberg TS. 2003. Vangidae, vangas. In: Goodman SM, Benstead JP, eds. *The natural history of Madagascar*. Chicago: University of Chicago Press, 1138–1143.

Seddon N. 2005. Ecological adaptation and species recognition drives vocal evolution in neotropical suboscine birds. *Evolution* **59:** 200–215.

Servedio MR, **van Doorn GS**, **Kopp M**, **Frame A**, **Nosil P. 2011.** Magic traits in speciation: 'magic' but not rare? *Trends in Ecology and Evolution* **26:** 389–397.

Slabbekoorn H, **Smith TB. 2000.** Does bill size polymorphism affect courtship song characteristics in the African finch *Pyrenestes ostrinus*? *Biological Journal of the Linnean Society* **71:** 737–753.

Slabbekoorn H, **Smith TB. 2002.** Bird song, ecology and speciation. *Philosophical Transactions of the Royal Society B: Biological Series* **23:** 1–11.

Smith TB. 1990. Natural selection on bill characters in the two bill morphs of the African finch *Pyrenestes ostrinus*. *Evolution* **44:** 832–842.

Smith JW, **Benkman CW. 2007.** A coevolutionary arms race causes ecological speciation in crossbills. *The American Naturalist* **169:** 455–465.

Smith JW, **Sjoberg SM**, **Mueller MC**, **Benkman CW. 2012.** Assortative flocking in crossbills and implications for ecological speciation. *Proceedings of the Royal Society B: Biological Series* **279:** 4223–4229.

Streelman JT, **Danley PD. 2003.** The stages of vertebrate evolutionary radiation. *Trends in Ecology and Evolution* **18:** 126–131.

Tobias JA, **Aben J**, **Brumfield RT**, **Derryberry EP**, **Halfwerk W**, **Slabbekoorn H**, **Seddon N. 2010.** Song divergence by sensory drive in Amazonian birds. *Evolution* **64:** 2820–2839.

Westneat MW, **Long JH Jr**, **Hoese W**, **Nowicki S. 1993.** Kinematics of birdsong: functional correlation of cranial movements and acoustic features in sparrows. *Journal of Experimental Biology* **182:** 147–171.

Wild JM, **Goller F**, **Suthers RA. 1998.** Inspiratory muscle activity during bird song. *Journal of Neurobiology* **36:** 441–453.

Wiley RH. 1991. Associations of song properties with habitats for territorial oscine birds of eastern North America. *The American Naturalist* **138:** 973–993.

Wilkins MR, **Seddon N**, **Safran RJ. 2013.** Evolutionary divergence in acoustic signals: causes and consequences. *Trends in Ecology and Evolution* **28:** 156–166.

Wilson DR, **Bitton PP**, **Podos J**, **Mennill DJ. 2014.** Uneven sampling and the analysis of vocal performance constraints. *The American Naturalist* **183:** 214–228.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website.

Figure S1. Bill depths of males from five of the seven ecotypes included in this study. Bill depth data were taken from Groth (1993) and Benkman & Porter (2018).
Figure S2. Geographical range of ecotype 1 from Benkman & Young (2019). The Core Zone of Occurrence (solid dark purple) represents the area where key conifers are found and where birds can regularly be found utilizing their key conifer and breeding in large numbers during most typical years. The Secondary Zone of Occurrence (solid light purple) represents the area where lesser numbers of birds can be found and breeding in many years with some regularity. The Primary Zone of Irruption (solid dark blue) represents the area where birds commonly move to when key conifer cone crops fail in the core zone of occurrence. The Secondary Zone of Irruption (solid light blue) represents the area where birds move to only during widespread cone failures on many conifers they typical use in core zones. Dashed blue represents the area of irruptive migration. Recordings for this ecotype were obtained from the following locations: Cayuga, NY; Chenango, NY; Madison, NY; Onondaga, NY; Schohaire, NY.
Figure S3. Geographical range of ecotype 2 from Benkman & Young (2019). Details as in Figure S2, plus: solid light yellow represents the tertiary zone of irruption where movement is uncommon and breeding israre and local. Recordings for this ecotype were obtained from the following locations: San Bernardino, CA; Sierra, CA; La Plata, CO; Teller, CO; Deschutes, OR; Douglas, OR; Chelan, WA; Albany, WY; Big Horn, WY.
Figure S4. Geographical range of ecotype 3 from Benkman & Young (2019). Details as in Figure S2. Solid black dots represent vagrant records. Recordings for this ecotype were obtained from the following location: Lane, OR.

Figure S5. Geographical range of ecotype 4 from Benkman & Young (2019). Details as in Figure S2. Solid black dots represent vagrant records. Recordings for this ecotype were obtained from the following location: Chelan, WA.
Figure S6. Geographical range of ecotype 5 from Benkman & Young (2019). Details as in Figure S2. Solid black dots represent vagrant records. Recordings for this ecotype were obtained from the following locations: Teller, CO; Cassia, ID; Albany, WY.
Figure S7. Geographical range of ecotype 10 from Benkman & Young (2019). Details as in Figure S2. Recordings for this ecotype were obtained from the following locations: Hancock, ME; Washington, ME; Chippewa, MI; Tompkins, NY.
Figure S8. Geographical range of Cassia crossbills (ecotype 9) from Benkman & Porter (2018). Unlike other ecotypes in the red crossbill complex, Cassia crossbills are sedentary. Recordings for this ecotype were obtained from the following location: Cassia, ID.