



## Original Article

# Immigrant song: males and females learn songs after dispersal in a tropical bird

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A fundamental hypothesis about vocal learning is that young animals learn vocalizations in their natal areas and, following postnatal dispersal, they may introduce new types of vocalizations into their breeding areas. We tested this hypothesis in a tropical bird, the Rufous-and-white Wren (*Thryophilus rufalbus*), a species in which both sexes produce learned songs. We collected blood samples and acoustic recordings from 146 adult wrens from 3 populations in northwestern Costa Rica. We genotyped individuals at 10 polymorphic microsatellite loci and identified first-generation migrants using partial Bayesian genotype assignment. We quantified acoustic variation by comparing fine-scale acoustic structure, song sharing, and repertoire novelty between residents and first-generation migrants. We found significant population-level differences in acoustic structure of songs among the 3 populations. Of the 146 individuals genotyped, 9 individuals were identified as first-generation migrants. In contrast to our predictions, however, we found that these first-generation migrants did not exhibit differences in the acoustic structure of their songs from resident individuals in their breeding population, either for males or females. We conclude that song learning in first-generation migrants must be behaviorally influenced by birds in their breeding populations, following postnatal dispersal. We observed population-level acoustic differences among the 3 study sites, which implies sustained divergent selection pressures at each site, possibly reflecting acoustic adaptation to different environments or social pressure to sing local songs. Understanding and quantifying patterns of cultural evolution at multiple scales provides insight into how behavioral barriers, such as acoustic signals, contribute to population differentiation and even speciation.

**Key words:** dispersal, immigration, Rufous-and-white Wren, song learning, *Thryophilus rufalbus*, *Thryothorus rufalbus*.

## INTRODUCTION

The magnitude, direction, and spatial scale of animal dispersal are all important components of gene flow, and these forces contribute to genetic differentiation among populations (Bohonak 1999). In addition to genetic divergence, dispersal influences phenotypic divergence, and in the absence of the gene flow that accompanies dispersal, phenotypic traits can diverge quickly (Lande 1980, 1981; Irwin *et al.* 2001; Clegg and Phillimore 2010). Examining variation in traits and behaviors in the context of dispersal patterns—especially those phenotypes associated with mate attraction and territory defense—can provide key insights into the relationship between phenotypic variation and gene flow, and enhance our comprehension of animal behavior, ecology, and evolution (Wilkins *et al.* 2013).

The signals used by animals in social and sexual communication exhibit considerable geographic variation (reviewed in Bradbury and Vehrencamp 2011). This has been especially well documented for acoustic signals (Prohl *et al.* 2006; Podos and Warren 2007; Campbell *et al.* 2010). Although most animals acquire their vocalizations innately, a few groups of animals have evolved vocal learning, including some birds, bats, primates, elephants, seals, and cetaceans (Janik and Slater 1997; Jarvis 2004; Poole *et al.* 2005; Sanvito *et al.* 2007). Vocal learning, whereby young animals learn to produce vocal signals after hearing sounds of conspecific animals, plays an important role in the cultural evolution of acoustic signals; copying errors (mutations), random cultural drift, and selection can all give rise to new signals or changes in signal characteristics (Lynch 1996). The effect of dispersal on vocal learning, however, is dependent on the timing of vocal learning relative to the timing of dispersal. If animals learn their vocalizations prior to dispersing from their natal populations, then they may introduce new signals into the populations to which they immigrate, and ultimately act

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to homogenize acoustic variation among populations (Ellers and Slabbekoorn 2003). In contrast, if animals learn their vocalizations after dispersal, then this may promote acoustic divergence among populations even when rates of immigration and gene flow are high (Salinas-Melgoza and Wright 2012).

Among birds, vocal learning occurs in 3 orders (Apodiformes, Psittaciformes, and the oscine Passeriformes) and provides a model system for studying the population-level implications of vocal learning (Catchpole and Slater 2008). Birds exhibit considerable variation with respect to the mode of learning (Creanza *et al.* 2016). For example, some birds are able to learn throughout their life span (known as “open-ended learning”) whereas song learning is restricted to a shorter time span in many others, often within the first year (known as “closed-ended learning”; Catchpole and Slater 2008). Although vocal learning is well studied in birds, most studies examining acoustic variation and learning have focused exclusively on the learned vocalizations of males. Female birds also produce and learn songs, especially outside of north-temperate ecosystems (Slater *et al.* 2004), but little is known about song variation, development, and learning in females (Riebel *et al.* 2005), even though female song is an ancestral trait in songbirds (Odom *et al.* 2014). Given that both males and females sing in so many tropical bird species, tropical birds offer an excellent system to study the ecological and behavioral consequences of song learning. Examining song variation in both males and females in the context of dispersal is important, given that sex-biased dispersal is widespread in birds; female birds regularly disperse greater distances than males (Greenwood 1980). As a result, life-history differences between the sexes, including differences in dispersal behavior, may influence acoustic variation and phenotypic evolution of males and females (Ortiz-Ramírez *et al.* 2016).

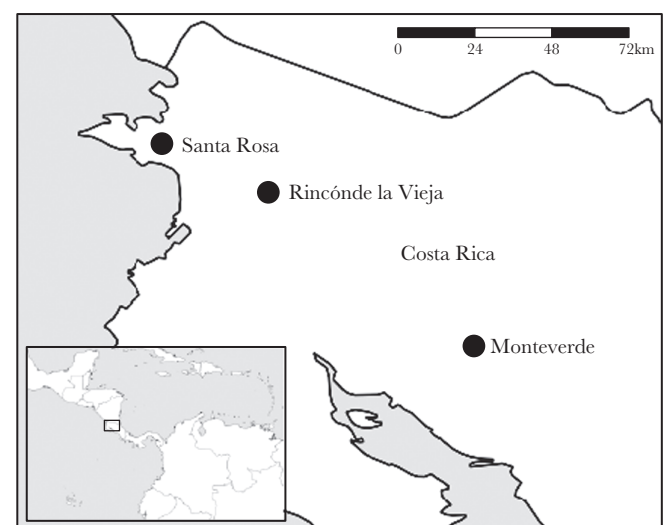
In this study, we examine the role of immigration and acoustic variation in Rufous-and-white Wrens (*Thryophilus rufalbus*), year-round residents of Central America and northern South America. The singing behavior of this species offers a unique system to explore the relationship between dispersal and acoustic variation, given that both sexes sing and that individuals produce a repertoire of different songs (each animal produces up to 15 song types; Mennill and Vehrencamp 2005; Harris *et al.* 2016). Although males and females share some songs, and both sexes use their songs to defend territories, males and females show pronounced sex differences in the fine-detail structure of their songs and rates of song-sharing; females produce songs shorter in duration, with fewer syllables and with higher frequency characteristics, and exhibit lower rates of neighbor-neighbor song-sharing than males (Mennill and Vehrencamp 2005, 2008; Mennill 2006; Graham *et al.* 2017).

We combined molecular genetic and acoustic analyses to determine if immigration influences acoustic variation. Although previous studies have examined this relationship in other bird species, primarily in the Temperate Zone (but see Wright *et al.* 2005), this relationship has never been explicitly examined in female songbirds. Our investigation had 3 components. 1) We identified first-generation migrants in 3 Rufous-and-white Wren populations in northwestern Costa Rica using molecular genetic analyses. 2) We then measured acoustic variation between populations to determine whether songs show population-level distinctiveness, and thereby confirm that we could detect potential acoustic differences between local and nonlocal birds. 3) We then compared the fine structure of the songs of residents to the songs of first-generation migrants. The purpose of this was to determine if first-generation migrants are able to learn and match local songs. If they

are unable to do so, then we expect that first-generation migrants will exhibit acoustic differences from resident birds. As an extension of this third goal, we also used acoustic analyses to compare the repertoire composition and acoustic variation in the fine-structure details of songs between males and females from our 3 study populations. Collectively, these questions allow us to examine the timing of song-learning in Rufous-and-white Wrens and to test whether learning occurs prior to or after dispersal, and whether this pattern differs among populations or sexes.

## METHODS

In 2012 and 2013, we studied 3 populations of Rufous-and-white Wrens living at 3 sites in northwestern Costa Rica (Figure 1): Sector Santa Rosa of the Guanacaste Conservation Area (10.85°N, 85.60°W; hereafter “Santa Rosa”), Sector Rincón de la Vieja of the Guanacaste Conservation Area (10.78°N, 85.35°W; hereafter “Rincón”), and University of Georgia Campus in the San Luis Valley near Monteverde (10.28°N, 84.79°W; hereafter “Monteverde”). We captured birds at each population using mist nets, and banded each bird with a unique combination of 3 colored leg bands and one numbered aluminum leg band. From each bird we collected a small blood sample (50–100 µL) from the brachial vein, and stored blood samples in 95% ethanol or Queen’s Lysis Buffer (Seutin *et al.* 1991). Individuals were sexed based on the presence of a brood patch (females) and by singing behavior (sexes can be distinguished based on fine structural differences in songs; Mennill and Vehrencamp 2005). Additionally, we included blood samples collected from birds in 2 other populations as part of a separate study: one population was located north of our 3 focal populations in northwestern Nicaragua (13.27°N, 86.31°W; approximately 280 km north of Santa Rosa) and one population was located south of our 3 focal populations in the Central Valley of Costa Rica (9.90°N, 84.25°W; approximately 75 km south of Monteverde). We included these data in our genetic analysis to improve our ability to detect potential first-generation migrants at our 3 focal populations, but these individuals were not included



**Figure 1**

Map of 3 study populations of Rufous-and-white Wrens in Costa Rica where genetic and acoustic samples were collected for comparisons of acoustic variation between resident animals and first-generation migrants. Inset shows map of Central America and northern South America.

in the acoustic analyses in the current study, or in the combined acoustic and genetic analyses, because we did not have sufficient acoustic recordings for these 2 populations.

## Genetic analysis

We genotyped individuals at 10 microsatellite loci, including 4 existing microsatellite primer sets, *ThPl 14*, *ThPl 20*, *ThPl 30* (Brar et al. 2007), *RWWR 2c* (H. Mays personal communication), and 6 newly developed primers (*Tru 08*, *Tru 11*, *Tru 18*, *Tru 20*, *Tru 24*, *Tru 25*). Full details of our PCR technique are provided in Graham et al. (2017).

We genotyped 211 Rufous-and-white Wrens from 5 populations, and tested for departures from Hardy-Winberg Equilibrium (HWE) and linkage disequilibrium using Genepop 4.2 (Raymond and Rousset 1995). The majority of locus  $\times$  population comparisons did not show significant departures from HWE (only 3 of 50 locus  $\times$  population comparisons, i.e., 6%, departed from HWE) or linkage disequilibrium (1 of 225 comparisons, i.e., 0.004%, showed evidence of linkage disequilibrium) following sequential Bonferroni comparisons. Two of the 3 locus  $\times$  population combinations that showed departures from HWE were found at Santa Rosa; to further ensure that departures from HWE were not driving the observed patterns, we performed our analysis with all 10 loci and then repeated the analyses without the 2 loci that showed significant departures from HWE (*ThPl 14* and *ThPl 30*). Removing these loci did not change our results and therefore we present results including all 10 loci.

We used the “detect migrants” function implemented in the program GENECLASS 2.0 (Piry et al. 2004) to identify first-generation migrants. This analysis detects first-generation migrants (i.e., birds born outside of the sampled population), using likelihood ratio statistics and Monte-Carlo resampling methods. We identified first-generation migrants using the likelihood ratio of  $L_{\text{home}}$  and Bayesian resampling method derived by Paetkau et al. (2004) and criteria suggested by Rannala and Mountain (1997). While the  $L_{\text{home}}$  to  $L_{\text{max}}$  likelihood ratio method has greater statistical power than any of the other methods implemented in the program (Paetkau et al. 2004; Piry et al. 2004), we used the  $L_{\text{home}}$  likelihood ratio; this method is recommended by Paetkau et al. (2004), who suggests that  $L_{\text{home}}$  is the most appropriate when all potential source populations of first-generation migrants haven't been sampled. Our resampling method simulated 10,000 individuals, and we identified individuals as first-generation migrants if the probability of originating from a population other than the population where they were banded was greater than 95%.

We complemented our migrant detection analysis with Bayesian exclusion analysis (Rannala and Mountain 1997) in GENECLASS 2.0. The purpose of this analysis was to further characterize dispersal patterns in this species, and to test the accuracy of our first-generation migrant analysis, following Sunnucks (2011). Comparing results from these complementary analyses helped us to avoid potential type I errors, given that the exclusion analysis tests the probability of an individual originating from another population (Sunnucks 2011; García-Navas et al. 2014). For our exclusion analysis, we used the same resampling method that we used for first-generation migrant analysis. We excluded individuals from the population where they were banded if the probability of originating from another population outside of the population a bird was captured and recorded at was less than 0.05.

Finally, we calculated assignment indices for each individual in GeneClass 2.0 following the approach of Favre et al. (1997).

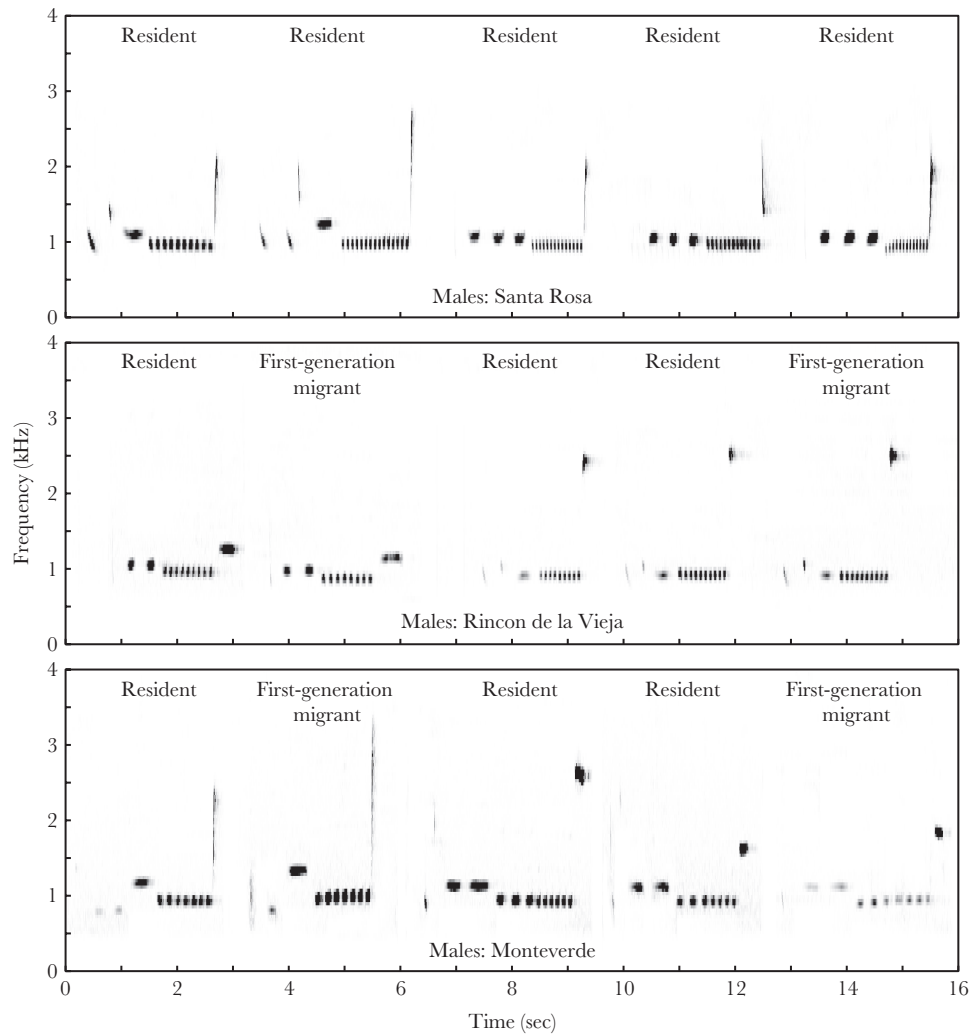
Assignment indices reflect the expected frequency of a single genotype occurring within the population it was sampled. For example, a negative assignment index value indicates an uncommon genotype and the greater the negative number, the more likely that this individual originated from another population. Conversely, a positive value is indicative of a common genotype, so those individuals with high positive values are likely to have been locally recruited. The purpose of calculating these values was to create a continuous variable to characterize immigration that we could use in our linear models analyzing the relationship between immigration and acoustic variation (see Statistical analyses).

## Analyses of song structure

We compared the fine-structural details of Rufous-and-white Wren songs to examine the relationship between acoustic variation and immigration. To compare the fine-structural details of the songs of resident birds and first-generation migrants, we measured a subset of each bird's vocal repertoire (see Figure 2, Figure 3). For males we selected and measured 4 song types from each population ( $n = 12$ ). The 4 song types that we chose from each of the 3 populations (12 song types in total) represented the most common song types in each population, and we measured these song types because they were found in the majority of the repertoires of each male we recorded. For females, we followed a similar approach, at first by targeting the 4 most common song types in each population, but given that females sing less often and sing fewer songs overall (Mennill and Vehrencamp 2005), we had to expand our selection criteria and measured up to 16 different song types per population ( $n = 43$  across the 3 populations), to obtain enough songs to measure for each individual. When measuring fine-structural differences in songs, we only included songs with a high signal-to-noise ratio (assessed visually on the basis of spectrograms).

We measured songs from 85 males and 54 females (4 other males, one of which was a first-generation migrant, and 3 other females were excluded from acoustic analyses due to high background noise in recordings). Whenever possible, we tried to include songs from multiple recordings (i.e., from different days, to eliminate any bias from recording on a single day), setting a maximum of 3 exemplars per song type per recording. We measured up to 6 exemplars of each song type for each bird (males: average number of song types measured for each male was 3.26 song types, range 1–4; average number of songs measured=10.08 songs, range 1–21; females: average number of song types measured for each female was 4.16 song types, range 1–9; average number of songs measured=8.74 songs, range 1–32). This left us with 273 male songs to analyze (from an initial pool of 857 songs) and 223 female songs to analyze (from an initial pool of 472 songs).

To quantify fine-structural variation in the songs of male and female Rufous-and-white Wrens we collected 7 temporal and spectral measurements of their songs. For each song, we measured the following 6 features: 1) the duration of the song (s); 2) element rate of the trill (the number of elements per second in the trill portion of the song); 3) length of the terminal syllable (s); 4) bandwidth of the terminal syllable (Hz; calculated by subtracting the minimum frequency of the terminal syllable from the maximum frequency of the terminal syllable); 5) dominant frequency of the trill (Hz), minimum frequency of the song (Hz); and 6) maximum frequency of the song (Hz). We used the automated parameter measurements tool in AviSoft-SASLab Pro (version: 5.2.04; R. Sprecht; Berlin, Germany) to measure these features, thereby minimizing subjectivity in the fine-structural measurements. Songs were resampled to



**Figure 2**

Sound spectrograms of male Rufous-and-white Wren songs from 5 different males from each of our 3 study populations, showing examples of songs recorded from residents and first-generation migrants.

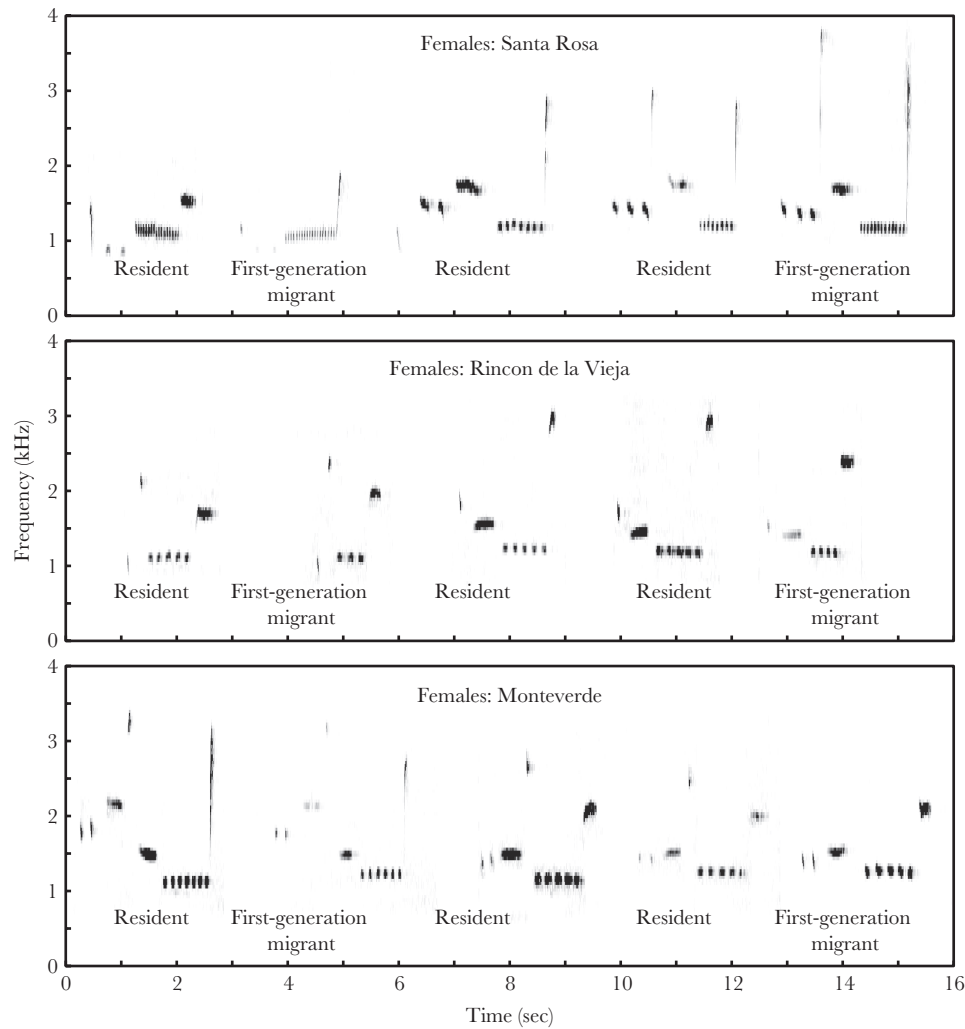
8000 Hz, which allowed maximum spectral resolution in AviSoft. For each song, we created a spectrogram, with an effective resolution of 8 Hz and 4 ms (settings: transform size: 1024 Hz; overlap: 96.86%; window: Hamming). We used a high pass filter of 500 Hz to remove any low-frequency background noise from the sound files. We conducted a Principal Component Analysis (PCA), which included all male and female songs, to reduce our acoustic variables to fewer metrics. We transformed our data using a direct oblimin rotation and retained all principal components with Eigenvalues above 1.0. We were left with 3 principal components, which explained 71.2%, of the variation (Table 1).

### Analyses of song repertoires

We recorded birds during the breeding season, in April through July of each year of the study, when vocal output is high for this species (Topp and Mennill 2008). Overall, we recorded songs from 146 birds (89 males and 57 females) from our 3 study populations. We recorded each individual on at least 2 separate occasions (average:  $5.63 \pm 0.33$  recording sessions; range: 2–12). The majority of our recordings were collected during focal recordings, where we followed each bird around its territory (each morning, from 0445h to 1100h) and confirmed the bird's identity during the recording.

Songs were collected during focal recordings using a solid-state digital recorder (Marantz PMD-660; 44.1 kHz sampling rate; 16-bit accuracy; WAVE format) and a shotgun microphone (Sennheiser MKH70). We supplemented these recordings with recordings from automated digital recorders (model SM2, Wildlife Acoustics Inc., Concord, MA; details in Mennill *et al.* 2012). We placed these recorders within the center of the territories of each focal pair, usually within 10 m of the pair's nest. We confirmed that the songs collected by these automated recorders were those of the intended pair by resighting the focal individuals in their territory after each automated recording session, and by matching the songs collected during focal recordings (as in Harris *et al.* 2016).

We used several methods to quantify repertoire variation and composition in Rufous-and-white Wrens. First we calculated repertoire size for each individual, as in previous studies of this species (Mennill and Vehrencamp 2005; Harris *et al.* 2016). To classify song types, we inspected the fine-structural characteristics of songs following the approach outlined in Harris *et al.* (2016). Previous work by Barker (2008) has shown that discriminant analysis can differentiate song types based on fine-structural measurements (i.e., duration of song, maximum frequency, minimum frequency, and intersyllable interval), and we incorporated these methods to help



**Figure 3**

Sound spectrograms of female Rufous-and-white Wren songs from 5 different females from each of our 3 study populations, showing examples of songs recorded from residents and first-generation migrants.

assign song types correctly. Across our 3 populations, we identified 51 male and 44 female song types at Santa Rosa, 36 male and 24 female song types at Rincón, and 33 male and 20 female song types at Monteverde. For each male and female, we built a song library of all the song types in their repertoire, using simple enumeration (*sensu* Harris *et al.* 2016). Next, we calculated the percentage of song types that an individual shared with all other individuals of the same sex within the population where they were recorded. We defined song sharing as the proportion of song types shared between 2 individuals. To measure song sharing, we calculated an adjusted Jaccard's coefficient of sharing ( $S_j$ ) using the following formula (Tracy and Baker 1999) in R 3.2.3 (R Core Team 2017):

$$S_j = c / ((a + b + c) - d)$$

where  $a$  = the number of song types in individual A's repertoire but not individual B's;  $b$  = the number of song types in individual B's repertoire but not individual A's;  $c$  = the number of song types shared between 2 individuals; and  $d$  = the difference in repertoire size between individual A and B. We chose this coefficient, because this method accounts for differences in repertoire size ( $d$ ) providing a more accurate estimate of sharing between 2 individuals. In our

analysis of repertoire sharing we present the average percentage of songs that an individual shares with all members of the same sex in their population.

We considered a song to be shared between 2 individuals if those songs met the following criteria: 1) songs shared the same sequence of elements in the introductory part of the song (although we ignored differences of up to 2 introductory syllables because birds sometimes produce introductory notes very quietly, making them difficult to detect); 2) introductory syllables were produced at the same frequency (within 100 Hz); 3) trills were composed of the same type of syllables (*i.e.* syllables were the same length and shape); 4) trills were produced at the same frequency (within 100 Hz); 5) trills were delivered at the same rate (within 2 elements per second); and 6) terminal syllables were the same shape (*e.g.*, long tonal syllables that covered a short bandwidth versus short syllables with a broad bandwidth).

To quantify how unique an individual's repertoire was, relative to the rest of the population where it was recorded, we calculated a measure we call "repertoire novelty." Following Fayet *et al.* (2014), each song type was given a weighted value based on how common it was in the population (*i.e.*, the number of individuals with this

**Table 1**  
Principal component loadings for male and female Rufous-and-white Wrens (*Thryophilus rufalbus*)

	Males and females		
	PC1	PC2	PC3
<b>Song length (s)</b>	0.062	-0.219	<b>0.841</b>
<b>Trill Rate (elements/s)</b>	-0.198	0.228	<b>0.473</b>
<b>Length of terminal syllable (s)</b>	0.057	<b>-0.818</b>	0.316
<b>Bandwidth of terminal syllable (kHz)</b>	0.124	<b>0.724</b>	0.356
<b>Dominant Frequency of the trill (kHz)</b>	<b>0.926</b>	-0.105	-0.013
<b>Maximum Frequency (kHz)</b>	<b>0.649</b>	<b>0.529</b>	0.233
<b>Minimum Frequency (kHz)</b>	<b>0.884</b>	0.030	-0.121
<b>Eigen Value</b>	2.41	1.50	1.11
<b>% Variance</b>	34.46%	21.44%	15.79%

Variables with strong loadings to the 3 principal components are shown in bold. Principal Component 1 summarized variables associated with song frequency; Principal component 2 was summarized variables associated with terminal syllable length and bandwidth; Principal Component 3 summarized variables associated with song duration and trill rate.

song type in their repertoire); common song types received a low value (e.g., a song present in 80% or more of the individuals in the population received a value of 1), whereas rare song types received a higher value (e.g., a song sung by a single individual received a value of 6; see [Supplementary Methods](#) for details). We then added the accumulated values for every song in each bird's repertoire and divided this sum by the repertoire size of the individual; this gave us an estimate of how novel the bird's repertoire was. For example, an individual with a repertoire that included 10 widespread songs would have a novelty score of 1, while an individual who sang 10 songs that were not shared with any other bird in the population would receive a novelty score of 6. Additionally, we counted the number of unique songs that an individual sang and calculated the proportion of unique songs that an individual possessed in their repertoire.

## Statistical analyses

To determine whether we could detect differences in the acoustic structure in the songs of first-generation migrants and residents, we first compared fine-structure details of songs between populations. Previous work has demonstrated that male and female songs show sex-based acoustic differences (Mennill and Vehrencamp 2005); in general, female songs are shorter, contain fewer syllables and have higher frequency characteristics than male songs. Further, although some song-types are shared between the sexes, many song types are sex-specific (Mennill and Vehrencamp 2005). Therefore, in addition to analyzing both sexes together, we also examined each sex individually. For our full model (males and females combined), we conducted linear mixed models on the 3 principal components. For each of the 3 models, the principal component was set as the response variable, and we set population and sex as our independent variable. Additionally, we examined the 2-way interaction between population and sex and we included individual as a random factor, given that we measured multiple song types from each individual.

Next, we performed redundancy analysis models (Legendre and Legendre 1998) to examine the relationship between immigration

and the fine-structure details of male and female songs. The purpose of these analyses was to compare fine-structure details among individuals to determine if the songs of first-generation migrants differed from the songs of resident birds. Redundancy analysis is an extension of multiple linear regression techniques, and because it uses ordination techniques, it tests the effects of multiple variables on the variance of a set of response variables. We employed this approach given that there was overlap in several of the variables we included in our analysis, and this method allowed us to determine which variables are significant drivers of acoustic variation in our study. We conducted 3 different redundancy models, a full model that combined both males and females and then a separate model for each sex. We performed full redundancy models on the 3 first principal components for each of our models. For each model we examined the effect of population, immigration (we examined both migrant status and assignment index, see below), individual identity, and song type on acoustic variation. In addition, we examined the effect of sex in our full model.

In addition to treating individuals as either a resident or first-generation migrant in our first set of models, we also calculated assignment indices (Paetkau *et al.* 1995) for each individual to examine the effect of immigration in our second set of models. This analysis allowed us to treat immigration as a continuous variable, and thereby determine if birds with nonlocal genotypes (i.e., more negative assignment indices) exhibit different repertoire characteristics than those individuals with local genotypes (i.e., more positive genotypes). Assignment index and migrant status were moderately correlated with each other (correlation coefficient =  $-0.33$ ,  $P < 0.001$ ), so we ran separate models with each genetic variable to determine if either genetic variable was a significant predictor of song variation. In each set of models, we included individual and song type as independent variables because we measured multiple song types from each individual and because we included multiple song types in our analyses. Given that song types can be quite variable in this species (Mennill and Vehrencamp 2005; Harris *et al.* 2016), the song type measured may have a greater influence on variation, in contrast to species where only a single variant of a song is sung. For this reason, each song type was given a unique code (1 through 12 for males and 13 through 55 for females), so that we could include the effect of song type in our analyses of the fine-structure detail of songs.

Finally, we compared the effect of immigration on repertoire characteristics (i.e., repertoire size, within-population song sharing, repertoire novelty, and the proportion of unique songs in an individual's repertoire) of male and female Rufous-and-white Wrens using multiple linear regression models. We created 4 models, one for repertoire size, and one for each of our repertoire composition variables. Repertoire measurements were treated as the response variable in each model and we included population, sex, and assignment index as our independent variable, and examined all 2-way interactions. We tested all variables for normality by viewing Q-plots of the residuals and all values are presented as mean  $\pm$  standard error. All statistical analyses were carried out in R (R core development team 2017).

## RESULTS

### Genetic identification of first-generation migrants

Our genetic analyses revealed immigration between 5 populations of Rufous-and-white Wrens in Central America: we detected 16 first-generation migrants overall (based on genetic data from 211

birds from 5 populations in our dataset; Table 2), and 9 first-generation migrants from one of our 3 focal populations (Santa Rosa, Rincón, and Monteverde). Fourteen of the 16 individuals (88%) identified as first-generation migrants were also identified as mismatches in our complementary GENECLASS population exclusion analysis, demonstrating that the same individuals were identified as first-generation migrants using 2 different approaches.

Overall, 6.3% (8 of 127) of the males we genotyped were identified as first-generation migrants, and 9.5% (8 of 84) of the females we genotyped were identified as first-generation migrants; we found no significant difference in the proportion of males and females identified as first-generation migrants (binomial test,  $P = 1.0$ ).

### Acoustic comparisons between populations

Our analysis of the fine-structure details of songs revealed both population-specific and sex-specific differences (Figure 4a–f). Songs varied among populations with respect to song frequency characteristics (PC1; ANOVA:  $F_{2,496} = 23.78$ ,  $P < 0.001$ ), terminal syllable characteristics (PC2;  $F_{2,496} = 17.67$ ,  $P < 0.001$ ), and song duration and trill rate (PC3;  $F_{2,496} = 15.65$ ,  $P < 0.001$ ), while song frequency characteristics (PC1;  $F_{1,496} = 464.79$ ,  $P < 0.001$ ) and song duration and trill rate (PC3;  $F_{1,496} = 13.29$ ,  $P < 0.001$ ) varied between sexes. The interaction between population and sex was significant for our analysis of the first principal component ( $F_{2,496} = 23.10$ ,  $P < 0.001$ ; Figure 4g), revealing that female songs from Rincón and Monteverde had significantly different frequency characteristics from all other songs, whereas we found no significant interaction between population and sex in our analysis of the first and second principal components (PC2:  $F_{2,490} = 1.97$ ,  $P = 0.14$ ; PC3:  $0.32$ ,  $P = 0.73$ ; Figure 4h,i).

### Song structure of first-generation migrants versus residents

Overall, we found limited support for the idea that immigration influences fine-structural features of songs for male and female Rufous-and-white Wrens (Table 3). Assignment index (a continuous variable representing the degree of “localness” of a bird) was a significant predictor in our full model (but not in our 2 other models examining males and females independently), but only a very small portion of variation in the songs of males and females was attributed to assignment index (1%,  $P = 0.03$ ; Table 4). Furthermore, migrant status (resident vs. first-generation migrant) was a nonsignificant predictor in all 3 of our models, and again only a small portion of variation was attributed to this variable (0.1–1%,  $P > 0.08$ ).

### Population and sex-specific differences in song structure

Although migrant status did not explain acoustic variation, we did observe significant effects in our models. We found an effect of sex in our full model, and an effect of population in all 3 models examining acoustic variation; sex explained 16% of the variation, and population explained between 5% and 11% across our 3 models. Not surprisingly, song type was a significant predictor of acoustic variation, accounting for between 71% and 76% of the variation across models ( $P < 0.001$ ). Finally, individual accounted for 21–39% of the variation across the 3 models, but individual was a significant predictor for our full model and our analysis of females only ( $P < 0.02$ ).

### Repertoires of first-generation migrants versus residents

In our analyses of repertoire composition and repertoire size, 3 of our 4 models were significant, but assignment index was not a significant predictor of repertoire size, song-sharing repertoire novelty, or the number of unique songs in an individual’s repertoire for male and female Rufous-and-white Wrens (Table 4, Figure 5).

Although we saw no relationship between immigration and repertoire variation, we did observe sex-specific and population-specific differences. Males had significantly larger repertoires (males:  $11.56 \pm 0.27$  song types; females  $6.53 \pm 0.33$  song types;  $t = 5.99$ ,  $P < 0.001$ ), and exhibited greater within-population song-sharing (males:  $0.47 \pm 0.01$  song types; females  $0.41 \pm 0.01$ ;  $t = -4.85$ ,  $P < 0.001$ ) than females, although we observed no significant differences between sexes with respect to repertoire novelty ( $t = 1.25$ ,  $P = 0.21$ ) or the proportion of unique songs in their repertoire ( $t = -1.77$ ,  $P = 0.08$ ; Table 4).

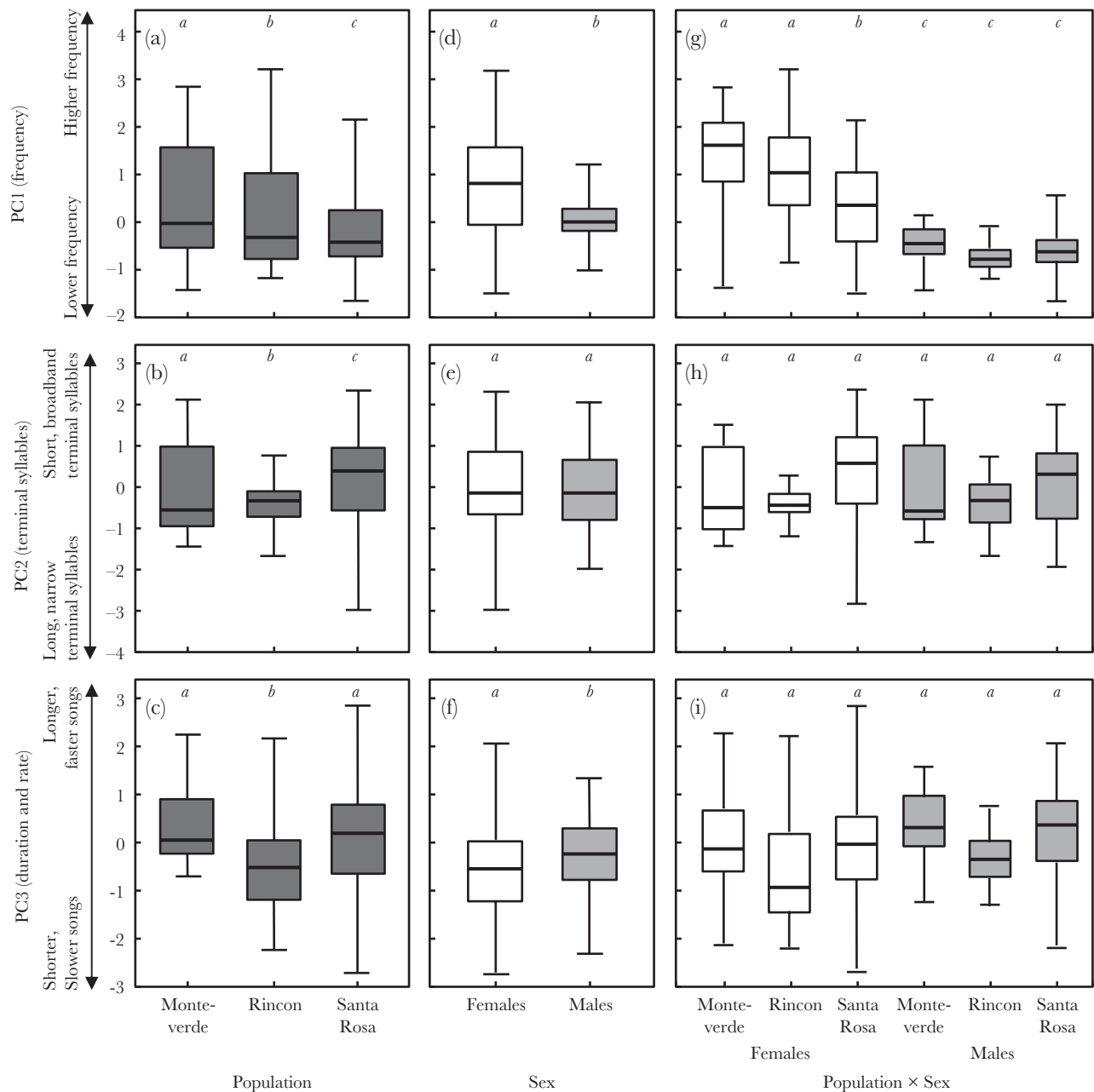
Across populations, we did not observe any differences in repertoire size within each sex, nor did we observe any differences in the proportion of unique songs in an individual’s repertoire. We did however observe population differences in within population song-sharing and repertoire novelty respectively following post-hoc comparisons (Figure 6). Males exhibited significantly greater within population song sharing at Rincón ( $0.53 \pm 0.02$ ) and Santa Rosa ( $0.46 \pm 0.01$ ) than at Monteverde ( $0.42 \pm 0.02$ ), while the reverse was true for females; females exhibited greater within population song-sharing at Monteverde ( $0.58 \pm 0.03$ ) than at Rincón ( $0.39 \pm 0.03$ ) or Santa Rosa ( $0.25 \pm 0.01$ ). Further, males from Monteverde ( $2.74 \pm 0.10$ ) had significantly higher repertoire novelty scores than males from Rincón ( $2.40 \pm 0.10$ ) and Santa Rosa ( $2.56 \pm 0.05$ ), while females at Santa Rosa had higher

**Table 2**

**Number of Rufous-and-white Wrens determined to be first-generation migrants through genetic analysis, and the number of individuals assigned to another population based on exclusion analysis at each population**

Population	First-generation migrant analysis		Population exclusion analysis				
	Residents	First-generation migrants	Nicaragua	Santa Rosa	Rincón	Monteverde	Central Valley
<b>Nicaragua</b>	44	3	45	1	0	0	1
<b>Santa Rosa</b>	95	2	0	96	0	1	0
<b>Rincón</b>	26	4	0	2	26	2	0
<b>Monteverde</b>	23	4	0	1	2	23	1
<b>Central Valley</b>	7	3	0	0	1	2	7

Two assignment methods showed high concordance, and we found no significant difference between sexes with respect to the number of males and females identified as first-generation migrants or assigned to an alternative population.



**Figure 4**

Acoustic differences in the songs of Rufous-and-white Wrens, summarized through principal components analysis, showing variation between 3 populations and 2 sexes ( $n = 496$ ): (a–c) population-specific differences in song structure; (d–f) sex-specific differences in song structure; and (g–i) population  $\times$  sex interactions for song structure. Error bars represent the lower and upper quartiles, while the bold vertical line represents the median. Different italicized letters above the bars represent values that are significantly different from each other following post-hoc comparisons.

repertoire novelty scores ( $3.96 \pm 0.06$ ) than females from both Rincón ( $3.03 \pm 0.13$ ) and Monteverde ( $2.53 \pm 0.13$ ).

## DISCUSSION

Dispersal plays an important role in the evolution of animal acoustic signals (Lynch 1996) and given the important role that acoustic signals play in resource competition and mate choice (Bradbury and Vehrencamp 2011), the interplay between dispersal and signal divergence may shape patterns of evolution. We studied 3 features of the songs of Rufous-and-white Wrens in 3 populations in Costa

Rica: fine-scale acoustic structure, repertoire size, and repertoire composition of male and female songs. We compared these acoustic features between birds classified as first-generation migrants or residents on the basis of genetic analyses. Although previous work has suggested that immigration and dispersal increases song diversity and influences acoustic structure (Stewart and MacDougall-Shackleton 2008; Fayet *et al.* 2014), we did not observe this in our study. Despite population-specific differences, we observed no significant relationship between immigration and fine-scale acoustic structure, within-population song sharing, or repertoire novelty, and these patterns were consistent for both males and females. Overall,



our results imply that male and female Rufous-and-white Wrens continue to learn songs after juvenile dispersal to their breeding populations, as opposed to learning songs in natal populations and then dispersing. Although we did not observe any differences in the songs and repertoires of residents versus first-generation migrants, we did observe differences in singing behavior among populations: the 3 study populations showed acoustic differences that were present in the songs of both male and female birds. Below we discuss our findings and the factors that may influence the limited role that immigration appears to play in acoustic traits in our study system.

### Dispersal and acoustic variation

Genetic assignment methods revealed gene flow among 5 populations of Rufous-and-white Wrens in Central America. Eight percent of the individuals we genotyped were first-generation migrants, which is comparable to migrant rates observed in other resident bird species (Moore *et al.* 2005; Pruett and Winker 2005; García-Navas *et al.* 2014). By comparison, these rates are much lower than those observed in 2 nonpasserine species studied in the same region of Central America (McDonald 2003; Wright *et al.* 2005); these

**Table 3**

**Results of Redundancy Analysis testing the effect of assignment index, immigrant status (i.e., first-generation migrant vs. resident) population, individual identity, and song type on acoustic variation in male and female Rufous-and-white Wrens**

Full Model	df	<i>F</i>	<i>P</i>	Inertia	Variation <sup>a</sup>
Sex	<b>1,494</b>	<b>97.06</b>	<b>0.001</b>	<b>0.47</b>	<b>16</b>
Population	<b>2,490</b>	<b>15.82</b>	<b>0.001</b>	<b>0.17</b>	<b>6%</b>
Assignment Index	<b>1,494</b>	<b>3.01</b>	<b>0.03</b>	<b>0.02</b>	<b>1%</b>
Migrant status	1,494	2.26	0.09	0.01	0.5%
Individual	<b>136,359</b>	<b>1.65</b>	<b>0.001</b>	<b>1.09</b>	<b>38%</b>
Song type	<b>58,347</b>	<b>24.27</b>	<b>0.001</b>	<b>2.17</b>	<b>76%</b>
<b>Females</b>					
Population	<b>2,218</b>	<b>13.23</b>	<b>0.001</b>	<b>0.34</b>	<b>11%</b>
Assignment Index	1,219	0.63	0.57	0.01	0.30%
Migrant status	1,219	2.35	0.08	0.03	1.00%
Individual	<b>53,167</b>	<b>1.29</b>	<b>0.02</b>	<b>0.91</b>	<b>29%</b>
Song type	<b>45,175</b>	<b>9.47</b>	<b>0.001</b>	<b>2.21</b>	<b>71%</b>
<b>Males</b>					
Population	<b>2,272</b>	<b>7.76</b>	<b>0.001</b>	<b>0.10</b>	<b>5%</b>
Assignment Index	1,273	1.36	0.23	0.01	0.50%
Migrant status	1,273	0.32	0.75	0.002	0.10%
Individual	82,192	0.68	0.99	0.4	22%
Song type	<b>12,262</b>	<b>58.24</b>	<b>0.001</b>	<b>1.3</b>	<b>73%</b>

Values significant at  $P < 0.05$  are shown in bold.

<sup>a</sup>Variation is the percent variation explained by each variable.

**Table 4**

**Main effects and factor interactions for multiple linear regression models analyzing repertoire size and repertoire composition of male and female Rufous-and-white Wrens**

	df	Repertoire size		Song sharing		Repertoire novelty		Proportion of unique songs	
		<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Model	9,136	<b>18.4</b>	<b>&lt;0.001</b>	<b>27.86</b>	<b>&lt;0.001</b>	<b>39.3</b>	<b>&lt;0.001</b>	1.75	0.08
Sex	1,136	<b>151.18</b>	<b>&lt;0.001</b>	<b>103.92</b>	<b>&lt;0.001</b>	<b>224.59</b>	<b>&lt;0.001</b>	2.74	0.10
Population	2,136	1.66	0.19	<b>23.66</b>	<b>&lt;0.001</b>	<b>21.99</b>	<b>&lt;0.001</b>	<b>3.19</b>	<b>0.04</b>
Assignment Index	1,136	0.30	0.58	0.51	0.48	1.61	0.21	0.10	0.75
Sex × Population	2,136	<b>3.86</b>	<b>0.02</b>	<b>47.86</b>	<b>&lt;0.001</b>	<b>40.9</b>	<b>&lt;0.001</b>	1.01	0.37
Sex × Assignment Index	1,136	0.34	0.56	0.04	0.84	0.25	0.62	0.08	0.78
Population × Assignment Index	2,136	1.35	0.26	1.60	0.21	0.71	0.49	2.21	0.11

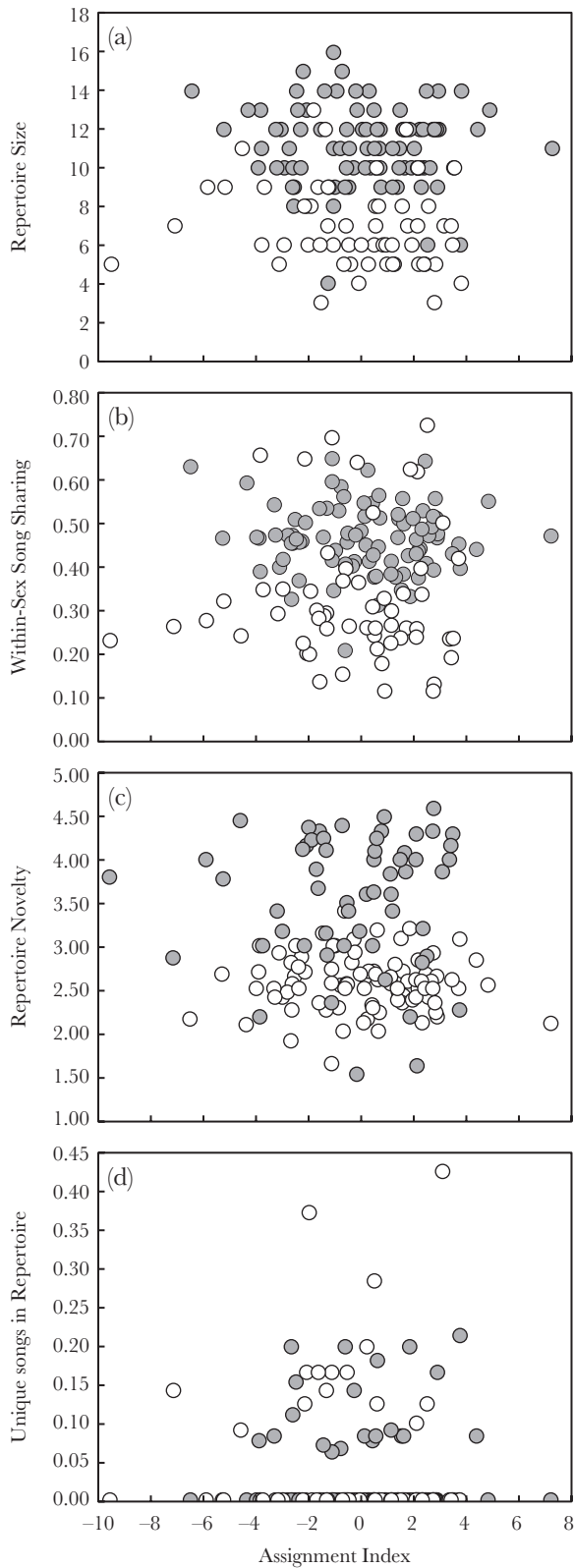
Values significant at  $P < 0.05$  are shown in bold.

studies showed that parrots and manakins move among populations, resulting in little genetic differentiation among populations. Differences in genetic patterns between wrens, parrots, and manakins, suggest that differences in dispersal capabilities among the 3 species may explain differences in genetic divergence (Claramunt *et al.* 2012). In particular, parrots and manakins are known to have high dispersal capabilities (McDonald 2003; Wright *et al.* 2005), whereas insectivorous understory birds, like wrens, are thought to have lower dispersal capabilities (Stouffer and Bierregaard 1995; Şekerciöglu *et al.* 2002; Moore *et al.* 2008).

Similar to our findings, many studies of vocal-learning animals have demonstrated that genetic and acoustic variation are not necessarily correlated (Wright and Wilkinson 2001; Ruegg *et al.* 2006; Ortiz-Ramírez *et al.* 2016; but see MacDougall-Shackleton and MacDougall-Shackleton 2001). Therefore, the results in our study may arise from the lack of a correlation between acoustic and genetic variation. While acoustic and genetic traits often show similar patterns of variation (Ruegg *et al.* 2006; Ortiz-Ramírez *et al.* 2016), this may arise because other factors, such as drift and selection in the presence of isolation, act on both cultural and biological evolution simultaneously. This pattern may be even more prominent in tropical animals (which exhibit strong philopatry, Stutchbury and Morton 2008), given that dispersal and ecological specialization are considered to be strong drivers of speciation in the tropics (Claramunt *et al.* 2012; Salisbury *et al.* 2012; Smith *et al.* 2014).

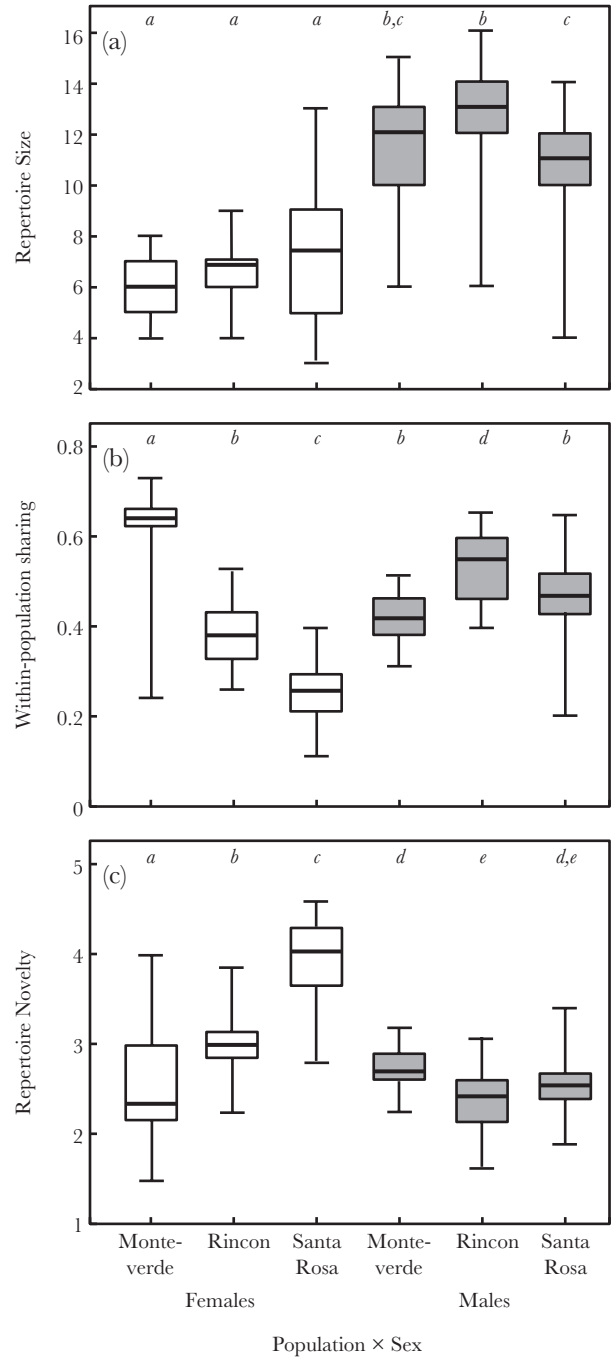
### Song learning

Our hypothesis that immigration increases song-diversity in populations was based on the assumption that young birds learn some or all of their songs prior to dispersal, and then introduce new songs following dispersal. Our study suggests that such a phenomenon does not occur in Rufous-and-white Wrens, contrary to the pattern observed in other systems where immigrant birds provide an influx of novel acoustic information (e.g., Payne 1996; Wright *et al.* 2005; Gammons and Baker 2006; Stewart and MacDougall-Shackleton 2008). This observation provides insight into the role of dispersal on cultural patterns, and also the timing of dispersal and song-learning in this species. Rufous-and-white Wrens are closed-ended learners; in more than a decade of study we have no evidence of a bird incorporating a new song type after their first year (Harris *et al.* 2016). All together, these observations suggest that dispersal events must occur during birds' natal year, when these animals are still learning their songs, and that young birds learn songs after dispersal, from birds in their breeding population.



**Figure 5**

Repertoire size and repertoire composition showed no significant relationship with assignment index for male (gray circles) and female (white circles) Rufous-and-white Wrens. Individuals with nonlocal genotypes (i.e., more negative AIC values) did not show any acoustic differences when compared to individuals with local-genotypes (i.e., more positive AIC values).



**Figure 6**

Repertoire size and repertoire composition measurements of male and female Rufous-and-white Wrens, showing the interaction between population  $\times$  sex. Error bars represent the lower and upper quartiles, while the bold vertical line represents the median. Different italicized letters above the bars represent values that are significantly different from each other following post-hoc comparisons.

Our results also imply that young Rufous-and-white Wrens follow a selection-based model of song-learning, where young birds match their songs and repertoires with those of neighboring birds postdispersal (Nelson 2000; Nelson and Poesel 2014). Predispersal learning is also likely to occur, although selective attrition may result in young birds dropping those songs learned while on their

natal population from their song repertoires (Peters and Nowicki 2017). Combined together, postdispersal learning and selective attrition likely help to maintain acoustic divergence between populations. Although the song-learning patterns of males have been well studied in both laboratory and wild settings, this is the first study to examine these hypotheses in female songbirds. Our results suggest that male and female birds exhibit similar processes with respect to song-learning, although other life-history traits may explain acoustic differences between males and females (Price 2015; Graham et al. 2017).

Our 3 study populations shared relatively few song types, but we did observe a number of population-specific song types that were common to the repertoires of males and females in each population. Furthermore, song type accounted for greater than 70% of the variation in both male and female songs and was a greater predictor of acoustic variation than among-individual variation. The prevalence of local song types may occur due to male and female song type preferences that develop during the song-learning period (Grant and Grant 1996). Additionally, females and males may select mates based on their ability to produce local song types (Nowicki et al. 1998; Nowicki et al. 2002; Reinhold 2004). Many birds have been shown to respond more strongly to local songs or local dialects (Searcy et al. 2002; Dingle et al. 2008, 2010; Danner et al. 2011; Derryberry et al. 2011; Garamszegi et al. 2012; Caro et al. 2013), including Rufous-and-white Wrens (Hick et al. 2015). Several of these studies have suggested that the decreased response to nonlocal songs may indicate that song acts as a reproductive barrier (Irwin et al. 2001). Alternatively, the reduced response may be due to these songs containing less information than local songs, given that different song types are known to be used in different contexts by some species (Trillo and Vehrencamp 2005; Cardoso et al. 2009; Demko et al. 2013), including Banded Wrens (*Thryophilus pleurostictus*), a closely-related congener of Rufous-and-white Wrens.

Although we did not observe any differences between the songs and song repertoires of residents and first-generation migrants, we did observe considerable variation among our 3 study populations. Between-population behavioral differences may be influenced by genetic factors, developmental factors (Nowicki et al. 1998; Reinhold 2004), social factors (Williams and Slater 1990), or a combination of the three. For example, males in the Rincón population had larger repertoires, and female song-sharing was higher in the Monteverde population. Among our 3 study sites, we observed differences in the territory sizes of individuals: Rufous-and-white Wrens at Monteverde and Rincón occupy much smaller territories than males at Santa Rosa (60 m<sup>2</sup> vs. 100 m<sup>2</sup>, respectively). The former populations have higher densities and therefore males in these populations may learn more songs because they have more neighbors nearby, creating more opportunity for hearing tutor songs. Differences in female song-sharing between Monteverde and Santa Rosa could also reflect the higher population density at Monteverde. Demographic factors may play a role in song sharing, but further studies are necessary to see how factors such as the proximity of neighbors and population density influence song sharing, repertoire size, and aspects of vocal behavior (Williams and Slater 1990).

## CONCLUSION

We studied the influence of first-generation migrants on the cultural diversity of male and female Rufous-and-white Wrens. Contrary to 2 other studies of temperate birds (Stewart and MacDougall-Shackleton 2008; Fayet et al. 2014), we did not find

that first-generation migrants introduce unique songs into their breeding territories or that they differ in song structure from that of residents. Our results suggest that annual dispersal among populations is relatively low in this species, and this likely reflects the strong philopatric nature of this species. Importantly, our study suggests that first-generation migrants learn songs in their breeding population after dispersal, ultimately producing songs that are similar to resident males and females. Additionally, our results suggest that dispersal events in this species must be restricted to the first year of life when animals are still learning their songs. Furthermore, the prevalence of local song types may reflect selection for specific songs within each population. Future studies are necessary to better understand the forces that drive multiple song types to persist in each population, and to determine why these animals possess song repertoires and how they use their repertoire. Additional studies will also help to provide greater insight into female song, duetting, and the evolution and function of these complex acoustic signals.

## SUPPLEMENTARY MATERIAL

Supplementary data are available at *Behavioral Ecology* online.

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Data accessibility: Analyses reported in this publication can be reproduced following the data provided by Graham et al. (2018).

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