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Subspecies discrimination on the basis of acoustic signals: a playback experiment in a Neotropical songbird

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When animal mating signals diverge between populations, reproductive isolation and speciation may occur. Variation in animals' responses to these signals may reveal whether differences in perception contribute to behavioural differences between populations. We tested whether signal divergence influences receiver responses to playback in the rufous-capped warbler, *Basileuterus rufifrons*, a Neotropical resident songbird with a contact zone between two divergent subspecies, *B. r. delattrii* and *B. r. rufifrons*, in southern Mexico. Studying nearby populations of birds living in allopatry and sympatry, we presented warblers with playback-simulated territorial male rivals of each subspecies. In sympatry, both *delattrii* and *rufifrons* responded more strongly to songs of their own subspecies than to songs of the other subspecies, whereas in allopatry, *delattrii* responded strongly to songs of both subspecies, suggesting possible reproductive character displacement. Our research demonstrates that sympatric *delattrii* and *rufifrons* discriminate between each other's songs, suggesting that song is a premating isolating barrier between these divergent subspecies. This study adds to the growing literature on receiver response to vocal signal divergence in closely related sympatric and allopatric animal populations.

Signal divergence between animal populations may promote premating reproductive isolation and speciation (Coyne & Orr, 2004; Price & Bouvier, 2002), and occurs through ecological selection, sexual selection, cultural or genetic drift, or a combination of these processes (Wilkins, Seddon, & Safran, 2013). To understand the mechanisms of signal divergence, we cannot rely solely on examinations of patterns of phenotypic or genetic divergence. In some populations, divergence in mating signals such as bright plumage and elaborate song mirrors genetic differentiation (Caro, Caycedo-Rosales, Bowie, Slabbekoorn, & Cadena, 2013; Greig, Baldassarre, & Webster, 2015; Mendelson & Shaw, 2005; Uy, Moyle, & Filardi, 2009). Other closely related taxa may show strong phenotypic differentiation and discrimination despite little genetic difference between them (e.g. Benites, Campagna, & Tubaro, 2015; Mason & Taylor, 2015), or they may discriminate strongly between phenotypically similar signals (e.g. Grace & Shaw, 2012; Grant & Grant, 2002; Tobias & Seddon, 2009). Therefore, it is critical to examine not only the extent of divergence, but also the strength of the behavioural responses to divergent traits by the animals themselves (Hudson & Price, 2014; Seddon & Tobias, 2010). In animals using acoustic and visual modalities to communicate, experimental studies using playback of vocal signals (e.g. Grace & Shaw, 2012; Grant & Grant, 2002; Lemmon, 2009) and presentation of visual models (e.g. Gabor & Ryan, 2001; Hick, Doucet, & Mennill, 2016; Mays & Hopper, 2004) are useful tests of behavioural responses to signal variation.

Although signal divergence between allopatric populations is often considered an important indicator of reproductive isolation (Coyne & Orr, 2004), the extent of signal divergence in closely related allopatric populations does not always reflect the degree of reproductive isolation (Hudson & Price, 2014). Instead, reproductive character displacement (i.e. greater signal divergence, heightened discrimination, or both) between closely related populations that come into secondary contact may maintain premating isolation through reinforcement, especially if there is strong selection against hybrids (Gerhardt, 2013; Hudson & Price, 2014). This pattern has been observed in diverse animal taxa including insects (e.g. Grace & Shaw, 2012; Jang & Gerhardt, 2006), fishes (e.g. Gabor & Ryan, 2001) and anurans (e.g. Lemmon, 2009). In birds, male black-crested titmice, *Baeolophus atricristatus*, and tufted titmice, *Baeolophus bicolor*, showed stronger song discrimination in an

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older hybrid zone because of reduced hybrid fitness (Curry & Patten, 2016). In collared flycatchers, *Ficedula albicollis*, and pied flycatchers, *Ficedula hypoleuca*, plumage and song diverged more in sympatry than in allopatry and corresponded to female preferences, minimizing interspecific mating (Haavie et al., 2004; Sætre et al., 1997). A similar situation can also occur in parapatric populations that potentially hybridize; in two subspecies of grey-breasted wood-wren, *Henicorhina leucophrys*, males in parapatric populations had more acoustically different songs and stronger song discrimination than males in allopatric populations (Dingle, Poelstra, Halfwerk, Brinkhuizen, & Slabbekoorn, 2010). In these cases, reinforcement may promote assortative mating, thereby limiting hybridization, although not all contact zones show this pattern (Wilkins et al., 2018).

Even when they are reproductively isolated, related populations that compete for resources (e.g. food, breeding sites) may respond more strongly to heterotypic signals of competitors (Freeman, 2016; Jankowski, Robinson, & Levey, 2010; Martin & Martin, 2001; Tobias & Seddon, 2009). When one closely related species or subspecies replaces another along an ecological gradient, competitive interactions for mates or territorial resources can promote between-population discrimination upon secondary contact (e.g. Caro et al., 2013; Freeman, Class Freeman, & Hochachka, 2016; Jankowski et al., 2010). Thus, heightened responses to heterotypic signals could be the result of competition for mates or competition for territorial resources under reproductive isolation scenarios (reviewed in Lipshutz, 2018).

Differences in receiver perception of signals, whether learned or innate, are expected to influence signal divergence (Endler & Basolo, 1998; Verzijden et al., 2012). Learned discrimination between particular signal features may influence response strength even if the signals show little structural divergence (Gee, 2005; Grace & Shaw, 2012; Grant & Grant, 2002; Seddon & Tobias, 2010), and may give rise to asymmetric discrimination between different populations (e.g. Colbeck, Sillett, & Webster, 2010; Dingle et al., 2010). Populations may also vary in their innate sensory sensitivity to specific signal features (e.g. frequency), further enhancing reproductive isolation in combination with learned discrimination (Dingle et al., 2010; McEntee, 2014). However, learning can reduce reproductive isolation when animals learn signals from neighbouring individuals of a related species or subspecies (e.g. Kenyon, Alcaide, Toews, & Irwin, 2017; McEntee et al., 2016). For instance, in a recently established hybrid zone, male pied flycatchers sang mixed songs containing elements copied from collared flycatcher neighbours, resulting in increased hybridization (Haavie et al., 2004). These examples highlight the importance of comparing the responses of different populations to each other's learned signals.

The rufous-capped warbler, *Basileuterus rufifrons*, is an ideal species in which to study receiver response to signal divergence between populations. This common resident warbler of Mexico, Central America and northwestern South America shows pronounced geographical variation in vocal and visual signal phenotypes. The eight recognized subspecies fall into two groups that vary in plumage and voice: (1) the northern, white-bellied *rufifrons* group of Mexico and northwestern Guatemala, and (2) the southern, yellow-bellied *delattrii* group of southeastern Mexico and Central and South America (Curson, 2010; Fig. 1). The current

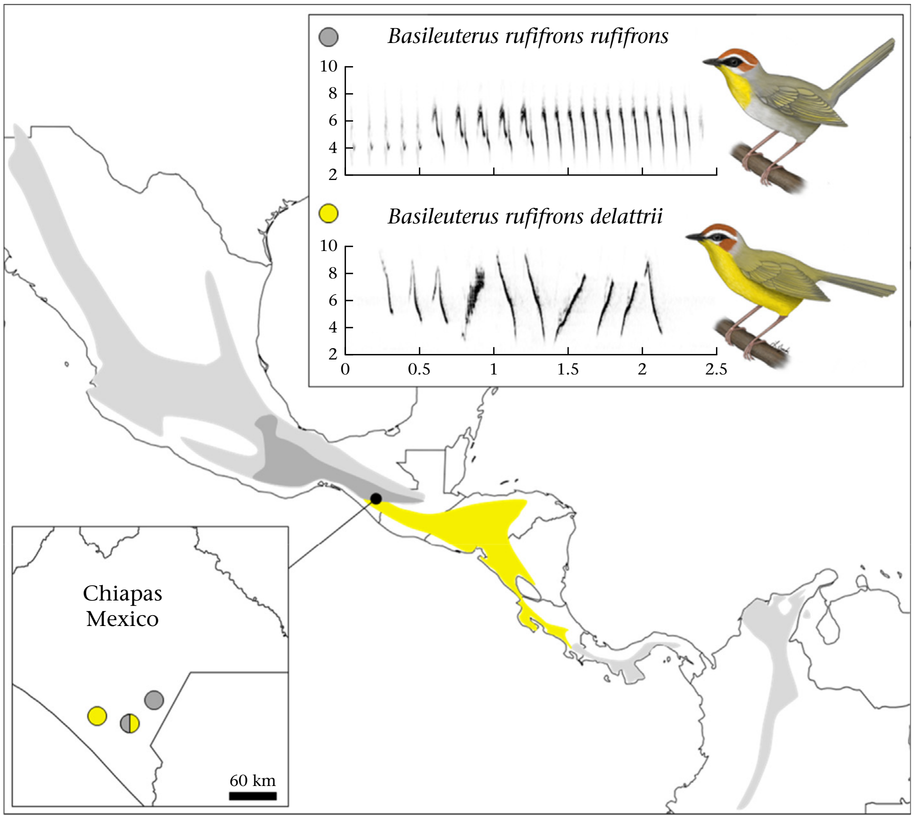

Figure 1. Rufous-capped warblers, *Basileuterus rufifrons*, are distributed from southwestern North America to northwestern South America (light grey shaded region). The ranges of the grey-bellied subspecies *B. r. rufifrons* (dark grey shaded region) and the yellow-bellied subspecies *B. r. delattrii* (yellow shaded region) overlap in Chiapas, Mexico. Inset map shows the location of three study sites: the allopatric population of *B. r. rufifrons* (grey circle), the allopatric population of *B. r. delattrii* (yellow circle) and the sympatric populations (split circle). Sound spectrograms are shown at the top right, highlighting the remarkably different acoustic structure of the two subspecies' songs.

taxonomy recognizes a single species based on the existence of an intermediate-plumaged subspecies, *B. r. salvini*, in the southern Gulf slope lowland region of southern Mexico (north of the study sites indicated in Fig. 1), and possible interbreeding between white-bellied *B. r. rufifrons* and yellow-bellied *B. r. delattrii* in a narrow zone of sympatry in southern Mexico and Guatemala (Curson, 2010; Monroe, 1968; Fig. 1). Other authorities, however, split the two groups into separate species based on their highly divergent plumage and songs (Howell & Webb, 1995; Todd, 1929). The two groups also differ in their habitat preferences; the white-bellied group lives in arid scrub and pine-oak from 1000–3000 m above sea level, whereas the yellow-bellied group inhabits semi-open humid habitats and tropical dry forest from 0–1000 m above sea level (Curson, 2010). Given these phenotypic differences, this system is an interesting one for testing variation in receiver response to vocal signals between populations.

The objective of our research was to experimentally test whether song divergence between phenotypically divergent rufous-capped warbler subspecies contributes to behavioural differences in response to these vocal signals. We sought to determine whether vocal divergence is a factor influencing reproductive isolation between two rufous-capped warbler subspecies: *B. r. rufifrons* and *B. r. delattrii*. If the two subspecies are not reproductively isolated (i.e. they interbreed extensively), we predicted that males in both sympatry and allopatry should respond equally strongly to both subspecies. If the two subspecies have a narrow contact zone where they compete for resources and potentially interbreed, we predicted that males in sympatry should respond strongly to both subspecies, whereas males in allopatry should respond strongly to their own subspecies and weakly to the other subspecies. If the two subspecies are reproductively isolated and males also do not compete between subspecies for territories, we predicted that males should respond strongly to their own subspecies and weakly to the other subspecies in both sympatry and allopatry. If reproductive character displacement occurs between the two subspecies (i.e. hybridization can occur, but hybrids have lower fitness), we predicted that males in sympatry should respond strongly to their own subspecies and weakly to the other subspecies, whereas males in allopatry should respond strongly to both subspecies. Finally, if aggression levels or innate perceptual sensitivity differ between subspecies, we predicted an asymmetric response between the subspecies (e.g. Colbeck et al., 2010; Dingle et al., 2010).

METHODS

General Field Methods

We conducted our study in southeastern Chiapas, Mexico, where *B. r. delattrii* (hereafter ‘*delattrii*’) are found in humid forest habitat at low elevations of 200–800 m above sea level, and *B. r. rufifrons* (hereafter ‘*rufifrons*’) live in montane scrub habitat at high elevations of 1200–3000 m above sea level (Howell & Webb, 1995). In this region, *delattrii* and *rufifrons* co-occur in humid, semi-open habitats (e.g. coffee plantations) at middle elevations of 800–1200 m above sea level, but appear to share territories, mate assortatively and have distinct plumage and songs (Demko, 2018). We experimentally tested the importance of vocal signals for within-subspecies discrimination by presenting territorial pairs of *rufifrons* and *delattrii* with song playback of both subspecies (as in Greig et al., 2015; Uy et al., 2009). We conducted our playback study at three localities: (1) allopatric *delattrii* near Mapastepec, Chiapas (15.34°N, 92.52°W; 450 m above sea level), a humid montane deciduous forest (25–31 May 2017); (2) allopatric *rufifrons* near Motozintla, Chiapas (15.38°N, 92.27°W; 1700 m above sea level), an arid montane pine-oak scrub (8–9 June 2017); and (3) sympatric *delattrii* and sympatric *rufifrons* at Finca La Victoria, Chiapas (15.29°N, 92.42°W; 1000 m above sea level), a humid shade coffee plantation (8–21 May 2017; Fig. 1). The timing of playback at all sites coincided with the start of the breeding (rainy) season, which begins earlier in humid localities than in dry localities.

We captured a total of 33 warblers on their territories using mist nets and song playback, and we banded birds with unique colour-band combinations to facilitate individual identification. We banded both the female and the male owners in five territories; in 23 territories we captured the male only; and in 18 territories both birds were unbanded. For unbanded pairs, we verified the identity of the territory holder based on position, and by comparing recordings of the unique song types used by males during each trial; each male has an individually distinct repertoire and pairs are highly territorial prior to and during the breeding season (Demko & Mennill, 2019). Our study included territories of 15 allopatric *delattrii*, 6 allopatric *rufifrons,* 17 sympatric *delattrii* and 8 sympatric *rufifrons* (Table 1).

Playback Design

We presented three treatments to each pair of rufous-capped warblers, with one treatment per day on subsequent days: (1) male *delattrii* song; (2) male *rufifrons* song; and (3) male song of a sympatric noncompetitor species, the banded wren, *Thryophilus pleurostictus*, as a control. Thus, each focal bird received own-subspecies, other-subspecies and heterospecific stimuli. The three stimuli were presented according to a counterbalanced design, and we tested for order effects. We broadcast stimuli from a loudspeaker (FoxPro Scorpion TX200; FoxPro Inc., Lewiston, PA, U.S.A.) placed near the centre of the focal bird's territory. The speaker was placed at a height of 1 m, which is a typical song post height in this species. We estimated the territory centre based on capture locations and 30 min territorial observations of the birds prior to the experiment; males typically sang from the same perch during the dawn chorus, which we presumed to be near the territory centre. We broadcast stimuli at 90 dB(A) SPL measured at 1 m from the speaker using a sound level meter (Casella CEL–240; Casella CEL Inc., Buffalo, NY, U.S.A.). This amplitude approximates the natural volume of rufous-capped warbler broadcast songs and the amplitude used in other warbler song playback studies (e.g. Hof & Hazlett, 2010).

Table 1
Playback locations and origin of rufous-capped warbler stimuli used to produce playback

Playback location	Subspecies	Range	Number of territories	Origin of *rufifrons* stimulus	Origin of *delattrii* stimulus
Finca La Victoria	*delattrii*	Sympatric	17	Motozintla	Mapastepec
Finca La Victoria	*rufifrons*	Sympatric	8	Motozintla	Mapastepec
Mapastepec	*delattrii*	Allopatric	15	Finca La Victoria	Finca La Victoria
Motozintla	*rufifrons*	Allopatric	6	Finca La Victoria	Finca La Victoria

All populations received nonlocal stimuli.

Trials consisted of a 5 min preplayback, 5 min playback and 2 min postplayback period. We ran all trials during 0700–1230 hours Central Daylight Time (CDT), which is the period of peak daily vocal activity in this species, excluding the early morning dawn chorus when males typically sing spontaneously at a high rate. Observers sat 15–20 m away from the speaker to record the birds' responses and describe their behaviour; in 55 trials, one observer collected observations, and in 70 trials, two observers collected observations, each focusing on a different member of the pair during trials at sites with dense vegetation. We used a Marantz PMD660 digital recorder and an Audiotechnica AT8015 directional microphone to record all playback trials. During the trials, the observer(s) dictated the horizontal and vertical distance of each bird from the playback, and described other activities related to territorial responses (e.g. flights over the speaker).

We considered a response to be a trial where at least one warbler of the focal pair approached within 10 m of the playback speaker during the 5 min playback period (e.g. Gill, Alfson, & Hau, 2007; Jankowski et al., 2010). If both the male and female responded, we recorded all response data for each individual separately, rather than pooling all responses together. We repeated the 5 min playback in a new location within the pair's territory on the following day if there was no response to the own-subspecies stimulus on the first attempt. We used this protocol because we expected all birds to respond territorially to the song of their own subspecies, and thus assumed that the initial playback location was not near the actual warbler territory centre. If there was still no response on the second attempt, we did not include that animal's data in the analysis. We also aborted any trials if a neighbour of the same subspecies approached within 10 m of the playback during the 5 min playback period, or if another animal species approached the playback at the same time as the focal bird, and repeated those trials on a subsequent day. At the sympatric site, we did not repeat the trial if a warbler from the other subspecies also responded, since the territories of the two subspecies frequently overlapped (A. D. Demko, personal observation). When territories overlapped between the two subspecies, we identified the focal subspecies based on capture locations of individuals and dawn singing observations. In all cases where we repeated a trial, we used only the second, successful trial in our analyses. To avoid any effects of familiarity with particular song types, we ensured that neighbouring warbler pairs used in the experiment did not receive the same playback stimuli.

Playback Stimuli

To create playback stimuli, we used our own field recordings collected in Mexico in 2016 and 2017 at the same sites where the playback trials were conducted (Fig. 2). Recordings were collected from birds singing spontaneously, or singing in response to playback consisting of conspecific song from local populations. Since rufous-capped warblers use similar song types in both natural and playback-induced conditions (Demko & Mennill, 2018), we considered songs from both types of recordings to be equivalent. We chose the highest-quality recordings available to produce playback stimuli, typically recorded at close distances of several metres. Our investigation focused on the overall responses of each subspecies to each other rather than their responses to the local population, so birds at all three sites received only nonlocal song playback of both subspecies (an approach modified from Dingle et al., 2010). Furthermore, since we expected warblers at the sympatric site to be more familiar with and potentially more responsive to local song of both subspecies, the use of nonlocal songs at all sites permitted a more conservative approach to data analysis. Birds at each site received the conspecific stimuli indicated

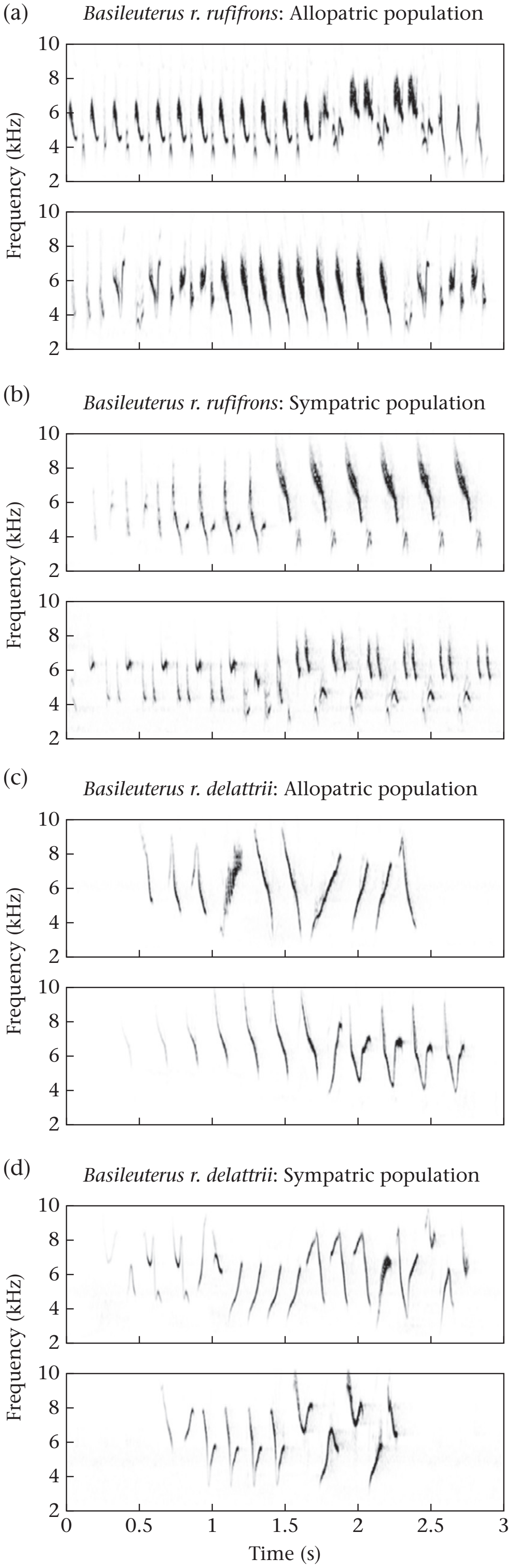

Figure 2. Sound spectrograms of four songs of *Basileuterus r. rufifrons* (a, b) and four songs of *B. r. delattrii* (c, d). Two examples of birds recorded in zones of allopatry (a, c) and sympatry (b, d) are shown for each subspecies.

in Table 1. To produce control stimuli, we recorded banded wren songs opportunistically from five different males at Mapastepec, Motozintla and Parque Nacional Cañon del Sumidero, Chiapas.

Each playback stimulus consisted of a single song type recorded from one individual male, repeated at the study species' natural daytime song rate of six songs/min (Demko & Mennill, 2018). We prepared stimuli by filtering each song with a 1000 Hz high-pass filter, editing out background noise around the song using the lasso selection tool, and normalizing the amplitude of the final playback files to −1 dB using Adobe Audition 3.0 software (Adobe, San Jose, CA, U.S.A.). We produced the following number of different stimuli for each subspecies and location: sympatric *rufifrons* ($N = 6$); sympatric *delattrii* ($N = 9$); allopatric *rufifrons* ($N = 9$); allopatric *delattrii* ($N = 11$); and banded wren ($N = 5$). No particular conspecific stimulus was used in more than four playback trials per site (range 1–4).

Ethical Note

Our research was approved through permits from the University of Windsor (Animal Care Committee; Permit No. AUPP–13–15) and the Government of Mexico (SEMARNAT; Permit No. SGPA/DGVS/00853/17) and adhered to ASAB/ABS guidelines for ethical treatment of animals. Prior to playback experiments, focal birds were captured using mist nets and playback, banded with one aluminium numbered band and two to three coloured plastic leg bands and released unharmed into the wild. We minimized stress to birds when capturing and banding them by holding them for as short a period as possible and releasing them as quickly as possible after capture (within 10 min). Following playback experiments, all birds resumed normal behaviours (e.g. foraging, feeding young) within 5–10 min. No adverse effects on the birds' health or behaviour from banding or use of song playback were noted during follow-up observations.

Analysis

We annotated recordings of playback trials using Syrinx PC sound analysis software (J. Burt, Seattle, WA, U.S.A.), focusing on the observers' description of the birds' physical responses to the playback, and all songs and calls produced by each warbler during the trials. We recorded the following response measures for each focal male and female: (1) closest distance of approach to speaker; (2) latency to approach within 10 m of speaker; (3) time spent within 10 m of speaker; (4) number of songs given; and (5) song duration in seconds (calculated by averaging the song duration of all songs recorded during the playback trial). To test for a significant difference in the number of responses (i.e. whether the focal birds approached within 10 m of the playback or not) between own- and other-subspecies playback, we ran Fisher's exact tests for each site. To test the overall response strength by warblers to playback, we used principal components analysis (PCA) to create uncorrelated response variables from the original five measures (McGregor, 1992). We first log-transformed the latency to approach and time within 10 m variables to meet the assumption of linearity between variables for PCA (Quinn & Keough, 2002). The first principal component, PC1 (eigenvalue: 2.92), represented 58.3% of the total variation in response strength and was the only principal component with an eigenvalue greater than 1.0. Factor loadings for PC1 corresponded to both physical and vocal responses of warblers to playback; a positive PC1 score corresponded to a stronger response, including closer approach distance (factor loading: −0.46), shorter latency to approach (factor loading: −0.31), more time spent near the speaker (factor loading: 0.50), more songs sung (factor loading: 0.49) and longer songs produced (factor loading: 0.45). We then ran linear mixed models with PC1 as the response variable, playback treatment (*delattrii*, *rufifrons*, or control), site (allopatric *delattrii*, sympatric *delattrii*, allopatric *rufifrons*, or sympatric *rufifrons*) and treatment∗site interaction as fixed effects and bird identity and playback stimulus file as random effects (Greig et al., 2015). We included an order main effect in the initial models, but as this was nonsignificant ($\chi^2_1 = 0.2$, $P = 0.67$), we excluded it from the final models. We conducted likelihood ratio tests to estimate fixed effects P values, and we conducted separate post hoc analyses for each site using the 'glht' function in the 'multcomp' package in R (Hothorn et al., 2017). Data met LMM assumptions aside from the presence of three outliers; we elected to retain the outliers in our final analysis because they represented actual responses of pairs to playback, and a separate analysis with the outliers removed yielded the same significant effects and post hoc test results. We conducted all statistical analyses using R v.3.4.3 (R Core Team, 2017).

RESULTS

Male Response Rate

Male rufous-capped warblers at all three sites showed high response rates to playback; 88–100% of individuals responded (i.e. approached within 10 m of the speaker) during own-subspecies trials and 20–87% responded during other-subspecies trials. Typical responses by males to playback included moving rapidly between multiple perches, singing frequently within 10 m of the speaker and performing flyovers (i.e. flying 1–2 m high over top of the speaker). Out of the 71 trials where males approached within 10 m, males performed at least one flyover (range 1–7) in 21 trials (29.6%). No males were observed to physically attack the speaker or perform low-amplitude song, and only one male performed wing-waving displays, which are behaviours associated with highly aroused responses in other playback studies of songbirds (e.g. Anderson, DuBois, Piech, Searcy, & Nowicki, 2013; Hof & Podos, 2013). Sympatric *delattrii* responded significantly more often to own-subspecies playback than to other-subspecies playback (Fisher's exact test: $P < 0.001$). Sympatric *rufifrons*, allopatric *delattrii* and allopatric *rufifrons* did not differ significantly in the number of responses to own- and other-subspecies playback (Fisher's exact test: all $P > 0.10$; Fig. 3). Response rates to the control playback were low overall.

Male Response Strength

The two subspecies of rufous-capped warbler showed different patterns of response to playback in sympatry and allopatry. A significant treatment main effect ($\chi^2_2 = 16.6$, $P < 0.001$) and treatment∗site interaction (likelihood ratio test: $\chi^2_6 = 39.0$, $P < 0.001$) indicated that response strength to each treatment type varied between sites (Table 2). In sympatry, *delattrii* and *rufifrons* both discriminated between songs of the two subspecies (Fig. 4). Sympatric *delattrii* males responded significantly more strongly to *delattrii* playback than to either *rufifrons* (estimate ± SE = 2.18 ± 0.47, $t = 4.6$, $P < 0.001$) or control (estimate ± SE = −2.34 ± 0.50, $t = -4.7$, $P < 0.001$) playback, whereas their responses to *rufifrons* and control playback did not differ (estimate ± SE = −0.16 ± 0.50, $t = -0.3$, $P = 0.94$). Sympatric *rufifrons* males responded significantly more strongly to *rufifrons* playback than to either *delattrii* (estimate ± SE = −2.34 ± 0.66, $t = -3.6$, $P = 0.002$) or control (estimate ± SE = −2.58 ± 0.67, $t = -3.8$, $P < 0.001$) playback, whereas their responses to *delattrii* and control playback did not differ (estimate ± SE = −0.24 ± 0.69, $t = -0.3$, $P = 0.94$; Table 2).

In allopatry, however, *delattrii* males did not discriminate between the two subspecies; instead they responded significantly

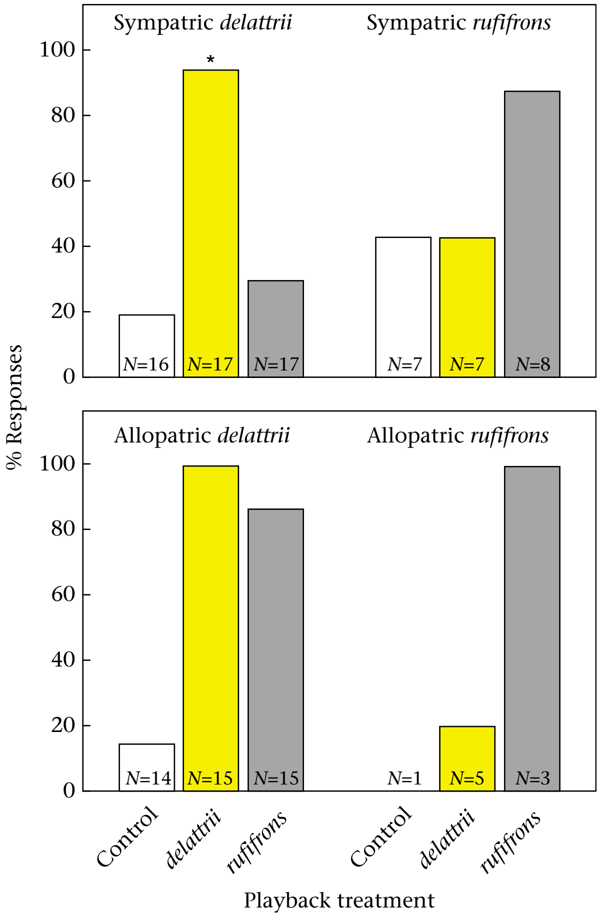

Figure 3. Percentage of responses by sympatric and allopatric populations of rufous-capped warbler males to a heterospecific control (banded wren) and own- and other-subspecies playback. $*P < 0.05$.

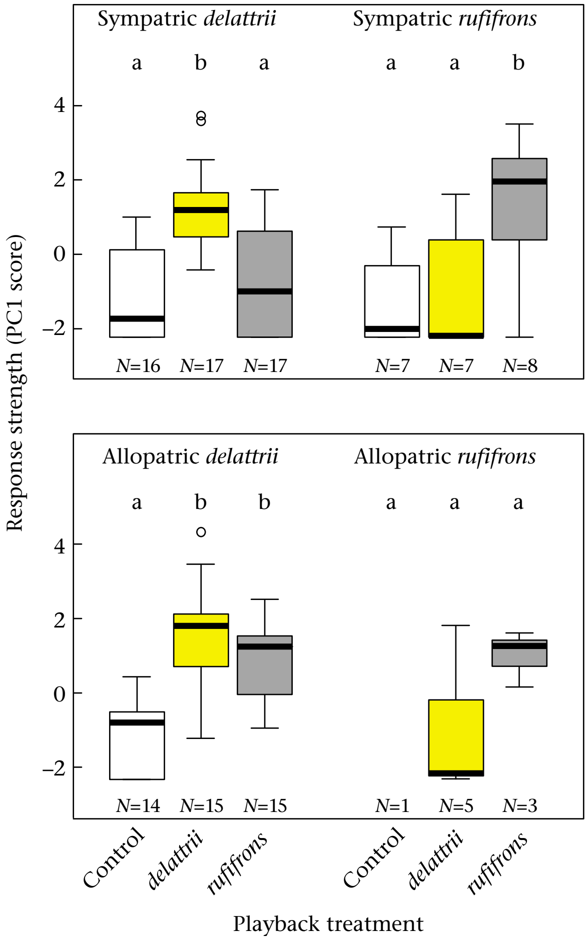

Figure 4. Response strength of sympatric and allopatric populations of rufous-capped warbler males to a heterospecific control (banded wren) and own- and other-subspecies playback. Box plots show 25% and 75% quartiles (boxes), medians (lines in the boxes), outermost values within the range of 1.5 times the respective quartiles (whiskers) and outliers (circles). Letters above box plots indicate significant post hoc test results.

Table 2

Linear mixed model and post hoc comparison results of physical and vocal approach (PC1) of male rufous-capped warblers in response to playback

	χ^2	*df*	*P*
Full LMM			
Treatment	**16.6**	**2**	**<0.001**
Site	7.4	3	0.06
Treatment × site	**39.0**	**6**	**<0.001**
Post hoc comparisons	**Estimate ± SE**	***t***	***P***
Sympatric *delattrii*			
control − *delattrii*	**−2.34±0.50**	**−4.7**	**<0.001**
control − *rufifrons*	−0.16±0.50	−0.3	0.94
delattrii − *rufifrons*	**2.18±0.47**	**4.6**	**<0.001**
Sympatric *rufifrons*			
control − *delattrii*	−0.24±0.69	−0.3	0.94
control − *rufifrons*	**−2.58±0.67**	**−3.8**	**<0.001**
delattrii − *rufifrons*	**−2.34±0.66**	**−3.6**	**0.002**
Allopatric *delattrii*			
control − *delattrii*	**−2.64±0.52**	**−5.1**	**<0.001**
control − *rufifrons*	**−2.02±0.54**	**−3.8**	**<0.001**
delattrii − *rufifrons*	0.62±0.51	1.2	0.45
Allopatric *rufifrons*			
control − *delattrii*	−1.19±1.35	−0.9	0.65
control − *rufifrons*	−3.07±1.44	−2.1	0.09
delattrii − *rufifrons*	−1.88±0.94	−2.0	0.12

Significant effects are highlighted in bold.

more strongly to both *delattrii* (estimate ± SE = −2.64 ± 0.52, $t = -5.1$, $P < 0.001$) and *rufifrons* (estimate ± SE = −2.02 ± 0.54, $t = -3.8$, $P < 0.001$) playback than to the control playback, whereas their responses to *delattrii* and *rufifrons* playback was similar (estimate ± SE = 0.62 ± 0.51, $t = 1.2$, $P = 0.45$; Fig. 4). In allopatric *rufifrons*, males tended to discriminate between subspecies; they responded more strongly to *rufifrons* playback than to *delattrii* (estimate ± SE = −1.88 ± 0.94, $t = -2.0$, $P = 0.12$) or control (estimate ± SE = −3.07 ± 1.44, $t = -2.1$, $P = 0.09$; Table 2) playback, although neither comparison was significant. The observed pattern only suggests discrimination between subspecies, since a small sample size at this site precluded rigorous statistical analysis. Although some males did respond to control trials (0–43% by site; Fig. 3), response strength (PC1) was greater to conspecific trials than to control trials at all sites (Fig. 4).

At the sympatric site, an opposite-subspecies neighbour sometimes responded to playback when the focal bird did not respond (e.g. during a *rufifrons* playback treatment on a *delattrii* territory, a neighbouring *rufifrons* sometimes responded). Some *rufifrons* individuals responded to off-territory playback of both *rufifrons* (6 of 17 trials) and *delattrii* (2 of 17 trials) playback by moving outside of their usual territory to respond to playback. During both of these *delattrii* trials and during one of the six *rufifrons* trials, the *delattrii* territory holder also responded to the playback. Only one *delattrii* individual responded to *delattrii* playback (1 of 8 trials) on a *rufifrons* territory. Two of the *rufifrons* intruders were confirmed as neighbours of the focal *delattrii* male based on their colour band combinations. The remaining unbanded off-territory responders were presumed to be males based on the song types they sang on the playback recording; both *delattrii* and *rufifrons* males sing acoustically distinct songs from females (Demko, 2018).

Female Playback Responses

Although our focus was on males, female rufous-capped warblers also occasionally responded to playback along with their male partners. Results suggested that female *rufifrons* showed stronger responses to playback than did female *delattrii*. Female *rufifrons* responded in 33% (1 of 3) of *rufifrons* trials and 20% (1 of 5) of *delattrii* trials at the allopatric site, and 25% (2 of 8) of *rufifrons* trials at the sympatric site. All four responding *rufifrons* females sang in response to the playback, which has been identified previously as an intense female response in this species (Demko & Mennill, 2018). Female *delattrii* responded in 13.3% (2 of 15) of *delattrii* trials and 6.7% (1 of 15) of *rufifrons* trials at the allopatric site, and in 5.9% (1 of 17) of *delattrii* trials and 6.2% (1 of 16) of control trials at the sympatric site. In contrast, only one of five responding *delattrii* females sang during playback.

DISCUSSION

Our results demonstrate that rufous-capped warblers of two subspecies discriminate between each other's songs when they are living in sympatry; male *B. r. delattrii* and *B. r. rufifrons* responded more strongly to their own-subspecies versus other-subspecies song playback. The strong responses to own-subspecies playback and the weak responses to other-subspecies playback in sympatry indicate that the two subspecies are not interspecifically territorial, and may be reproductively isolated in spite of their co-occurrence. The lack of discrimination between the two subspecies' songs by allopatric *delattrii* suggests that reproductive character displacement may explain enhanced discrimination in sympatry. Furthermore, the two subspecies appeared to differ in intensity of response or discrimination ability, since males showed asymmetric responses to playback of the two subspecies; our results suggest that *delattrii* showed larger differences in discrimination between sympatric and allopatric populations than did *rufifrons*.

One explanation for the subspecies discrimination we observed in sympatry is that selection against hybrids in the contact zone may promote stronger discrimination in sympatry than in allopatry through reproductive character displacement (Gerhardt, 2013; Uy, Irwin, & Webster, 2018). This is the case for collared and pied flycatchers, where hybrid females are sterile and hybrid males have lower pairing success, favouring character displacement of both song and plumage in sympatry to avoid interspecific mating (Sætre & Sæther, 2010). This scenario seems plausible for rufous-capped warblers as well, since sympatric *delattrii* and *rufifrons* showed strong discrimination against other-subspecies playback whereas allopatric *delattrii* did not. Although songs do not differ acoustically within subspecies between sympatric and allopatric sites (Demko, 2018), enhanced discrimination in sympatry is still an important component of reproductive character displacement (e.g. Dingle et al., 2010; Kirschel, Blumstein, & Smith, 2009). Furthermore, we found no phenotypic hybrids at our sympatric site based on plumage coloration, and all warblers that we observed at this site mated assortatively (over 40 *delattrii* and 25 *rufifrons* pairs). This apparent absence of hybrids in the contact zone suggests selection against hybrids, or that the two subspecies are completely reproductively isolated.

Alternatively, the weak other-subspecies responses in sympatry and the apparent absence of hybrids in the contact zone could also suggest complete reproductive isolation, where populations that have diverged over a long period come into secondary contact and no longer recognize one another as potential mates or competitors (Uy et al., 2018). A similar pattern was found in two related species of African tinkerbirds (*Pogoniulus bilineatus* and *Pogoniulus subsulphureus*), which are not interspecifically territorial and respond less to heterospecifics in sympatry than in allopatry (Kirschel et al., 2009). This seems plausible for rufous-capped warblers, since both subspecies share overlapping territories in sympatry and sing in adjacent trees without apparent antagonism (A. D. Demko, personal observation). However, allopatric *delattrii* did not discriminate between the two subspecies; such discrimination would be expected if reproductive isolation had been complete. We therefore suggest that character displacement is a more likely explanation.

In contrast to sympatric *delattrii*, the majority of allopatric *delattrii* pairs responded strongly to playback of both subspecies. Interestingly, data on rufous-capped warblers from a recent study involving 72 pairs of closely related songbirds found the same pattern; 60% of pairs (total $N = 15$) responded to songs of both local *delattrii* and allopatric *B. r. caudatus*, a northern Mexican subspecies with similar songs and plumage as *rufifrons* (Freeman & Montgomery, 2017). In contrast, allopatric *rufifrons* in our study appeared to show weaker responses to other-subspecies songs, although our sample size was relatively small. One explanation for the differing responses of *delattrii* and *rufifrons* is that *delattrii* have a broader acoustic perceptual sensitivity than *rufifrons*, as suggested for other closely related songbird species when birds exhibit different perceptual sensitivities to particular acoustic features (Dingle et al., 2010; McEntee, 2014) or respond preferentially to songs that are acoustically similar to their own (Sosa-López, Martínez Gómez, & Mennill, 2016). An analysis comparing acoustic features of *delattrii* and *rufifrons* songs has shown that *delattrii* songs have a broader syllable bandwidth than *rufifrons* songs and that the frequency ranges of the two subspecies overlap (Demko, 2018), suggesting that *delattrii* may be sensitive to a broader frequency range than *rufifrons*. The small sample size for allopatric *rufifrons* in our study, however, precluded any detailed statistical comparisons between the two subspecies in allopatry. Further experiments with a larger sample size of allopatric *rufifrons* would be useful, although this proved impossible in the current study owing to access-to-property issues.

Allopatric *delattrii* may also have responded strongly to *rufifrons* because they are behaviourally dominant and therefore more aggressive towards intruders with which they compete for resources. Overall, *delattrii* are larger than *rufifrons* and are more numerous at the sympatric site; both characteristics are associated with the dominant species in other similar animal relationships (Freshwater, Ghalambor, & Martin, 2014; Hick et al., 2016; Martin & Martin, 2001). However, *rufifrons* responses were in fact stronger in some respects than those of *delattrii*. For example, sympatric male *rufifrons* had stronger own-subspecies responses to playback than did sympatric *delattrii* (Fig. 4).

Female song, once thought to be a rare trait, has received increasing attention (Odom & Benedict, 2018). Although males are the primary singers in rufous-capped warblers, our observations suggest that females might also discriminate between the two subspecies. In both subspecies, female choice for specific song characteristics or heightened female discrimination between signals could therefore result in lower other-subspecies responses and assortative mating. Indeed, females of other bird species respond more strongly to local songs than do males, suggesting a preference and heightened discrimination for those signals (Danner et al., 2011; Seddon & Tobias, 2010; Wheatcroft & Qvarnström, 2017). Playback experiments comparing female responses to songs of local and nonlocal own- and other-subspecies populations would be useful to investigate the importance of vocal geographical variation in female mate choice (Danner et al., 2011; Jang & Gerhardt, 2006).

Our research suggests that song is an important trait promoting premating reproductive isolation between the *delattrii* and *rufifrons* subspecies of rufous-capped warbler, since birds preferentially responded to their own subspecies in sympatry. This discrimination between own- and other-subspecies songs in sympatry suggests that reproductive character displacement may contribute to divergence between the two subspecies. In contrast, the strong response of allopatric *delattrii* to songs of both subspecies may indicate a wider range of perceptual sensitivity or higher aggression levels in *delattrii* compared to *rufifrons*, although further experimental studies incorporating additional allopatric populations and visual signals would be useful to explore these ideas (e.g. Gabor & Ryan, 2001; Hick et al., 2016; McEntee, 2014). Overall, our work adds to the growing number of studies examining receiver response differences to learned signal divergence between bird populations and reveals the importance of vocal signal divergence as a premating isolating barrier in secondary contact zones.

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