

# Divergence in mating signals correlates with genetic distance and behavioural responses to playback

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## Abstract

Animals use acoustic signals to defend resources against rivals and attract breeding partners. As with many biological traits, acoustic signals may reflect ancestry; closely related species often produce more similar signals than do distantly related species. Whether this similarity in acoustic signals is biologically relevant to animals is poorly understood. We conducted a playback experiment to measure the physical and vocal responses of male songbirds to the songs of both conspecific and allopatric-congeneric animals that varied in their acoustic and genetic similarity. Our subjects were territorial males of four species of neotropical *Troglodytes* wrens: Brown-throated Wrens (*Troglodytes brunneicollis*), Cozumel Wrens (*T. beani*), Clarion Wren (*T. tanneri*) and Socorro Wrens (*T. sissonii*). Our results indicate that birds respond to playback of both conspecific and allopatric-congeneric animals; that acoustic differences increase with genetic distance; and that genetic divergence predicts the strength of behavioural responses to playback, after removing the effects of acoustic similarity between subjects' songs and playback stimuli. Collectively, these results demonstrate that the most distantly related species have the most divergent songs; that male wrens perceive divergence in fine structural characteristics of songs; and that perceptual differences between species reflect evolutionary history. This study offers novel insight into the importance of acoustic divergence of learned signals and receiver responses in species recognition.

## Introduction

Animal acoustic signals play an important role in resource defence and mate attraction, and consequently, these traits are under strong forces of ecological and sexual selection (Coyne & Orr, 2004; Wilkins *et al.*, 2013). Differences in the acoustic signals of closely related taxa have been studied in diverse animals, enhancing our understanding of the evolution of animal communication (Ryan, 1986; Wilkins *et al.*, 2013). Many investigations have revealed that variation in acoustic signals parallels phylogenetic relationships (e.g. Ryan & Rand, 1999; Johnson *et al.*, 2000; de Kort &

ten Cate, 2001; Price & Lanyon, 2002; Ord & Martins, 2006; Percy *et al.*, 2006; Farnsworth & Lovette, 2008; Irwin *et al.*, 2008; Sosa-López *et al.*, 2013; but see Price *et al.*, 2007), suggesting that acoustic signals have phylogenetically conserved components. However, the evolutionary processes that drive animal perception of phylogenetically conserved components of acoustic signals are not well studied (Irwin & Price, 1999; Grether, 2011).

If closely related species live in allopatry, they never interact, and therefore, they experience no selection for divergence or convergence in acoustic signals or receiver sensitivity to those signals (e.g. de Kort & ten Cate, 2001; Ryan *et al.*, 2003; Brambilla *et al.*, 2008; Tobias & Seddon, 2009; Uy *et al.*, 2009; Dingle *et al.*, 2010; Kirschel *et al.*, 2011; Hick *et al.*, 2015). Nevertheless, closely related species living in allopatry often exhibit divergent acoustic signals, likely as a result of drift and

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ecological factors (Tilley *et al.*, 1990). Yet divergent signals still sometimes elicit behavioural responses (de Kort & ten Cate, 2001; Ryan *et al.*, 2003; Brambilla *et al.*, 2008; Tobias & Seddon, 2009; Uy *et al.*, 2009; Dingle *et al.*, 2010; Fallow *et al.*, 2011; Kirschel *et al.*, 2011; Getschow *et al.*, 2013), suggesting that receiver responses do not diverge in parallel with signals.

Previous studies propose that receiver responses may be related to phylogenetic relationships. Evidence of an association between receiver responses and genetic distance comes from a few species of birds, frogs and insects suggesting that receiver responses and mating preferences are associated with acoustic signal similarity or phylogenetic relatedness (e.g. Mendelson & Shaw, 2005; Bernal *et al.*, 2007; Boul *et al.*, 2007; Ryan *et al.*, 2007; Tobias & Seddon, 2009). To our knowledge, only one study has linked receiver responses and genetic distance in birds by focusing on two sympatric nonsister species, *Hypocnemis peruviana* and *H. subflava* (Tobias & Seddon, 2009); multiple populations of these two tropical suboscine birds showed a relationship between acoustic similarity, genetic distance and behavioural responses. That study, however, focused on the hypothesis of adaptive signal convergence in two sympatric, competing, nonsister taxa. Whether closely related species living in allopatry perceive the phylogenetically conserved traits in acoustic signals remains poorly understood. Furthermore, this topic has not received any careful attention in oscine songbirds, where animals learn to sing through interactions with adults; song learning is expected to drive more rapid divergence in animal signals and possibly receiver responses (Irwin & Price, 1999; Grant & Grant, 2010; Sznajder *et al.*, 2012).

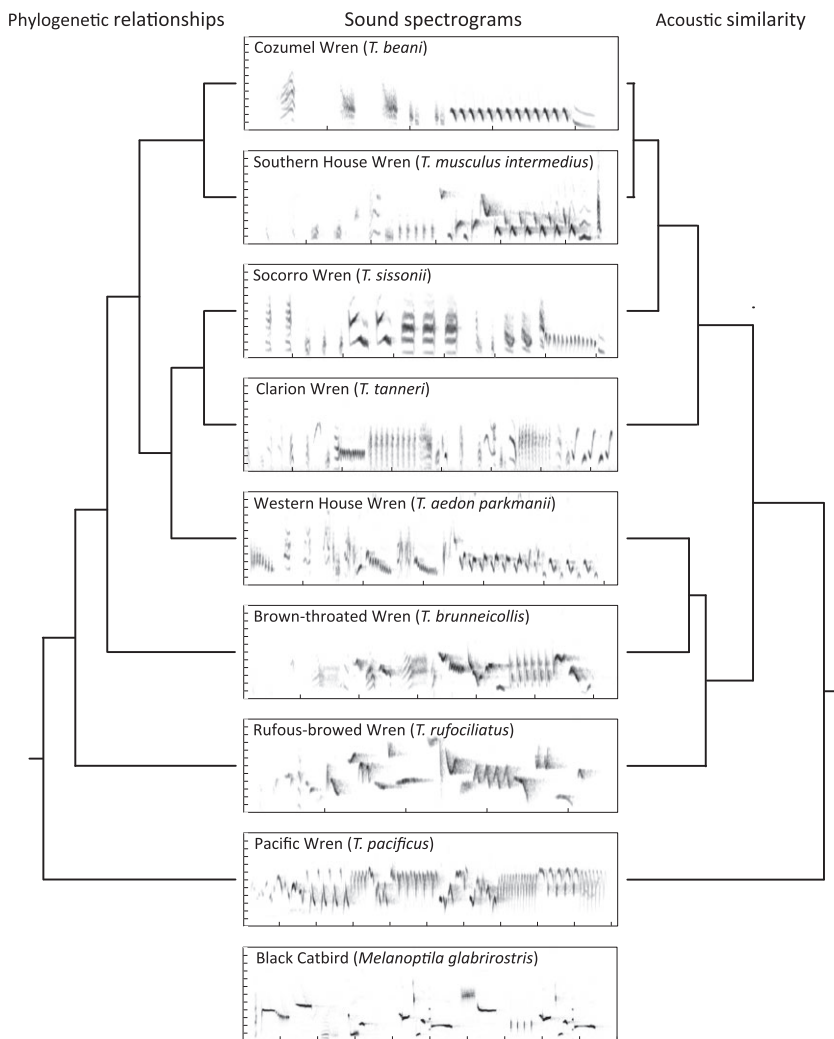
In this study, we investigate receiver responses to conspecific and allopatric-congeneric acoustic signals, and the relationship between receiver responses, acoustic similarity and genetic relatedness. Our study subjects are four species of neotropical wrens in the genus *Troglodytes* – a group of small, insectivorous, highly vocal songbirds – living on isolated oceanic islands and distinct regions of the mainland in Mesoamerica. First, we ask whether acoustic divergence between species of *Troglodytes* wrens is associated with genetic relatedness. If the fine structural characteristics of wren songs reflect evolutionary history, then acoustic divergence should be related to phylogenetic distance (Price & Lanyon, 2002; Percy *et al.*, 2006). Second, we ask whether divergent *Troglodytes* wrens' songs elicit behavioural responses in territorial males. We conducted playback experiments using songs of allopatric-congeneric species as stimuli, involving multiple *Troglodytes* species and encompassing a wide range of acoustic variation and phylogenetic relatedness (Fig. 1). If divergent signals elicit behavioural responses, we predicted that animals would respond to the signals of both conspecific and allopatric-congeneric animals and that they should

exhibit the most intense response to conspecific signals, a moderate intensity of response to allopatric-congeneric signals and little-or-no response to control stimuli (songs from an unrelated bird species). Alternatively, receivers may perfectly discriminate between conspecific and allopatric-congener acoustic signals, especially if selection favours perception of acoustic traits that differ between species (Nelson & Marler, 1989). Third, we ask whether *Troglodytes* wrens' responses to playback are associated with the fine structural characteristics of their songs, and whether responses are better explained by acoustic variation in songs or by variation in the genetic distances between *Troglodytes* Wrens. If signal perception varies with the fine structural characteristics of the signal, then receivers should vary the intensity of their response with acoustic similarity (Derryberry, 2007; Parker *et al.*, 2010; Danner *et al.*, 2011). Furthermore, the variation in responses that is not explained by acoustic signal divergence could represent perceptual differences between species; a relationship between those residual response differences and genetic distances would suggest that perceptual differences between species correspond to evolutionary history (Tobias & Seddon, 2009).

## Materials and methods

### Study species and locations

The distribution of *Troglodytes* wrens in the New World ranges from the far north of North America, in Alaska and northern Canada, to the southern tip of South America, in Chile and Argentina, with a centre of diversity in the tropics (Brewer, 2001). We conducted playback experiments to four *Troglodytes* wren species: Brown-throated Wrens (*Troglodytes brunneicollis*) in the mountains of south-western Mexico in Guerrero and Oaxaca (specifically, we studied the subspecies *T. b. nitidus* which may be a separate species from the other two subspecies of Brown-throated wren; J.A. Chaves, E. Bermingham, P. Escalante, G.M. Spellman & J. Klicka, unpublished data); Socorro Wrens (*T. sissonii*) and Clarion Wrens (*T. tanmeri*) on the deep Pacific oceanic islands Socorro Island and Clarion Island in the Archipelago of Revillagigedo in Mexico; and Cozumel Wrens (*T. beani*) on Cozumel Island off the north-eastern coast of the Yucatan Peninsula in Quintana Roo, Mexico. These four wren species are allopatric with respect to all of their congeners (Brewer, 2001). The taxonomy of the genus *Troglodytes* has been debated for many years (Howell & Webb, 1995; AOU, 1998; Brewer, 2001; Navarro-Sigüenza & Peterson, 2004; Sosa-López & Mennill, 2014). In this study, we followed the taxonomic system used by Brewer (2001) that suggests the existence of 11 species within the genus. Brewer's (2001) taxonomy is in line with a recent genetic study that suggests the existence of 42



**Fig. 1** Phylogenetic relationships among *Troglodytes* wrens and sound spectrograms depicting typical songs of the subject species in this study, and the species used as stimuli. On the left, the phylogenetic tree shows the phylogenetic relationships among *Troglodytes* wrens (J.A. Chaves, E. Bermingham, P. Escalante, G.M. Spellman & J. Klicka, unpublished data); on the right, a dendrogram shows the acoustic similarity among *Troglodytes* wrens based on the mean similarity values calculated in this study. The dendrogram of acoustic distance was calculated based on the Euclidean distances of the first four principal component factors summarizing acoustic measurements, and using within-groups average linkage cluster method. Note that branches in both the phylogenetic tree and the dendrogram only show relationships and similarity between *Troglodytes* wrens, respectively, and not the actual phylogenetic distances or acoustic distances. For all spectrograms, the x-axis tick marks show increments of 0.5 s (note that the time axis varies between spectrograms, to maximize display area), and y-axis tick marks show increments of 1 kHz, from 1 to 13 kHz.

independent evolutionary lineages (J.A. Chaves, E. Bermingham, P. Escalante, G.M. Spellman & J. Klicka, unpublished data). Although the number of species within this genus is still under debate, acoustic data (Sosa-López & Mennill, 2014) and genetic data (J.A. Chaves, E. Bermingham, P. Escalante, G.M. Spellman & J. Klicka, unpublished data) strongly suggest that our four focal wren species are indeed different species (see also Brumfield & Capparella, 1996; Rice *et al.*, 1999; Martínez Gómez *et al.*, 2005; Campagna *et al.*, 2012).

We studied Brown-throated Wrens in San Mateo Rio Hondo, Oaxaca, Mexico (6°07'N, -96°56'W) from 9 June to 15 June 2011; Cozumel Wrens on Cozumel Island, Quintana Roo, Mexico (20°24'N, -87°00'W) from 25 May to 30 May 2011; Socorro Wrens on Socorro Island, Revillagigedo Archipelago, Colima, Mexico (18°46', -110°57'W) from 7 August to 17 August 2011; and Clarion Wrens on Clarion Island, Revillagigedo Archipelago, Colima, Mexico (18°20'N, -114°44'W) from 10 December to 19 December 2011.

Our playback experiments involved 61 male subjects: 15 Brown-throated Wrens, 16 Cozumel Wrens, 16 Clarion Wrens and 14 Socorro Wrens. To distinguish individuals in the field, we caught most experimental males using mist nets and banded them with a unique combination of coloured leg bands; we distinguished between the unbanded males on the basis of their territory position. We conducted playback experiments during the breeding period of all four species. We confirmed the birds' breeding status on the basis of observing brood patches or cloacal protuberances when banding birds, or observing nest building or nestling feeding behaviour.

### Playback design

We used playback to simulate the acoustic signals of both conspecific and congeneric wrens. Our playback experiment consisted of five or six treatments for each subject, as summarized in Table S1. Treatment A was a

conspecific song, treatments B through E were congeneric songs, and treatment F was a heterospecific control. Congeneric treatment songs were: Brown-throated Wren, Cozumel Wren, Clarion Wren, Socorro Wren, Pacific Wren (*T. pacificus*), Rufous-browed Wren (*T. rufociliatus*), Southern House Wren (*T. musculus intermedius*) and Western House Wren (*T. aedon parkmanii*). The heterospecific control treatment for all subjects was the Black Catbird (*Melanoptila glabrirostris*). Our intention was to present subjects with stimuli from multiple allopatric-congeners that varied in their degree of acoustic divergence and genetic divergence. Therefore, each subject species received a slightly different set of congeneric species (Table S1). For each subject species, we included stimuli from the main two mainland groups of *Troglodytes* wrens (Western House Wren from North America and Southern House Wren from Central and South America), as well as species that are possible sister species. Black Catbirds, in the Mimidae family, are not closely related to the Troglodytidae family (Barker, 2004; Lovette *et al.*, 2012), and thus, we assumed Black Catbirds were equally distantly related to all four *Troglodytes* species. Black Catbirds are also allopatric to three of the four species. Although Cozumel Wrens live in sympatry with Black Catbirds, they do not respond to vocalizations of the latter species (personal observation).

Every subject received playback treatments during a 63-min playback trial. The first 3-min was a preplayback silent period. The experimental period was divided into sections, each with a 2-min stimulus followed by 8-min of silence. During the playback and the first 3-min of silence following the playback, we assessed the response of subjects; we treated the following 5-min as a recovery period before the next stimulus was broadcast. Our preliminary observations in the field indicated to us that 5-min recovery periods were sufficient for wrens to cease interacting with the playback loudspeaker and return to their preplayback activities. All four species received six treatments during the 63-min trials, except for Cozumel Wren, which received five treatments (Table S1), and therefore, playback trials were 53-min for this species.

Stimuli were obtained from personal field recordings by JRSL and from the Macaulay Library of Natural Sounds and the Borror Laboratory of Bioacoustics. Each stimulus consisted of a single exemplar of a given song type (approximately 3 s length) repeated every 7 s for 2 min. Song rates of the stimuli were held constant across all treatments to all four species. Each focal animal received playback of stimuli recorded from a different individual, although some stimuli were used more than once in playback to different species (e.g. one male Socorro Wren may have been used as a conspecific stimulus to a different Socorro Wren, and as a congeneric stimulus to a Clarion Wren). We avoided the use of neighbouring males' songs as stimuli and did not test adjacent males on the same day or subsequent

days. We normalized the amplitude of the playback stimuli to  $-1$  dB, so that amplitude was held constant across all treatments and trials, and filtered them using 1 kHz high-pass filter in ADOBE AUDITION (v2.0; Adobe, San Jose, CA, USA). In choosing the order of the presentation of treatments within trials, we followed a Latin square design (Ott & Longnecker, 2001). In total, we used 166 different birds' songs across nine species for creating stimuli (total number of songs per species: Black Catbird = 18, Brown-throated Wren = 18, Clarion Wren = 20, Cozumel Wren = 16, Pacific Wren = 15, Rufous-browed Wren = 15, Socorro Wren = 28, Southern House Wren = 18 and Western House Wren = 18).

### Playback experiments

We positioned a loudspeaker (model: Foxpro Scorpion X1A, FOXPRO Inc., Lewistown, PA, USA) near the centre of the territory of the experimental subject at a height of 1.8 m. To facilitate distance estimation of the responding male to the loudspeaker, we marked each cardinal direction at 1, 5, 10 and 20 m from the loudspeaker. We standardized the speaker amplitude at  $-90$  dB measured at 1 m from the speaker using a sound level meter (model: Radio Shack 33-2055; C-weighting; fast response), which resulted in songs that were broadcast at a natural amplitude based on our comparison to live birds in the field. We recorded the behaviour of the territorial males as they responded to playback using a digital recorder (model: Marantz PMD660; settings: WAV format, 44.1 kHz sampling frequency; 16 bit accuracy) with two microphones, each connected to a different channel; an observer quietly dictated the subjects' behaviour into a shotgun microphone (model: Sennheiser MHK67) in one channel, and a parabola (model: Telinga) with an omnidirectional microphone (model: Sennheiser K6/ME62) was used to record the songs of the subjects and the loudspeaker in the second channel. A field assistant helped to quantify the behaviour of the subjects as they responded to playback. The field assistant was not familiar with the songs of the species being broadcast in the experiment and was blind to the order of the stimuli. Both observers were positioned at distances of 20–25 m from the speaker.

We quantified the subjects' responses to playback in terms of vocal responses and physical responses. We quantified three aspects of male vocal responses: (1) total number of songs produced in response to playback; (2) number of songs in the longest bout of song produced by the subject (we define a bout of songs as the series of songs delivered by the focal bird with an intersong interval no  $>10$  s); and (3) latency from the start of playback to the subject's first song (in seconds). We quantified four aspects of male physical responses: (4) number of passes over the speaker; (5) closest



approach to the speaker (in metres); (6) latency to the first movement towards the speaker (in seconds); and (7) time spent within 10 m of the speaker.

We reduced the number of response variables by performing a principal component analysis using the response data from all four species ( $n = 61$  subjects). We conducted the principal component analysis using the four physical response variables and the three vocal response variables. We used Varimax rotation and selected principal component factors with eigenvalues  $\geq 1$ , resulting in two factors that together explained 68.9% of the total variation present in the measurements. The first principal component factor, related to physical responses, explained 37.7% of the total variation; the second principal component factor, related to vocal responses, explained 31.2% of the total variation (Table S2).

### Acoustic similarity between stimulus and subject species

To investigate whether acoustic similarity had an effect on the response of an individual, we calculated acoustic similarity measurements, comparing the fine structural details of the birds' songs in multidimensional space. The recordings for assessing acoustic distance were collected and analysed following the methods described in our previous research with *Troglodytes* wrens (Sosa-López & Mennill, 2014). We measured acoustic properties of one song from each of the 166 stimuli used in the playback experiments. To increase the sample size, we included songs of 135 additional individuals of the four subject species, including most of the birds in our study populations that we recorded during field expeditions and from libraries of natural sounds (number of additional songs per species: 24 Brown-throated Wrens, 40 Cozumel Wrens, 41 Clarion Wrens, 30 Socorro Wrens). We created high-quality spectrograms of these 301 songs (1024 FFT, 93.75% overlap, Blackman window, 22 Hz frequency resolution, 2.9 ms temporal resolution, 1 kHz high-pass filter) and measured 15 fine structural characteristics of the songs using the automatic parameter measurements tool in AviSoft-SASLab Pro (version 5.2.04; R. Sprecht, Berlin, Germany). We measured: (1) song length (s), (2) minimum frequency (kHz), (3) maximum frequency (kHz), (4) element length (s), (5) number of elements, (6) number of trills, (7) silence (s), (8) mean maximum frequency (kHz), (9) mean bandwidth (kHz), (10) mean peak frequency (kHz), (11) mean entropy, (12) peak frequency shifts per second, (13) standard deviation in maximum frequency (kHz), (14) standard deviation in bandwidth (kHz) and (15) standard deviation in entropy.

We reduced the number of fine structural characteristic measurements by performing a principal component analysis, with Varimax rotation, on the 15 acoustic measurements outlined above. We selected all

principal components with eigenvalues  $\geq 1$  and extracted four component factors that together explained 76.8% of the total variation of the original 15 acoustic variables (Table S3). The first factor explained 21.7% of the variation and was related to minimum frequency, silence, mean maximum frequency, mean peak frequency and peak frequency shifts per second; the second factor explained 20.1% of the variation and was related to song length, element length, number of elements and number of trills; the third factor explained 19.4% of the variation and was related to maximum frequency, standard deviation in maximum frequency, standard deviation in bandwidth and standard deviation in entropy; and the fourth factor explained 15.5% of the variation and was related to mean bandwidth and mean entropy. We plotted each song on these four axes and assessed acoustic similarity as the Euclidean distance between each stimulus (a total of 166 stimuli) and the centroid of the cluster for each subject species (calculated on the basis of the 135 subject species' recordings).

### Genetic distance between stimulus and subject species

To calculate genetic distances between *Troglodytes* species, we used the phylogenetic hypothesis proposed by J.A. Chaves, E. Bermingham, P. Escalante, G.M. Spellman and J. Klicka (unpublished data). They documented the existence of 42 lineages within the genus *Troglodytes*, representing genetically and geographically independent evolutionary units (i.e. phylogroups; Avise *et al.*, 1998). This molecular analysis was based on a thorough geographical sampling that included three mitochondrial and eight nuclear markers, and featured a high level of phylogenetic support ( $> 95\%$  maximum likelihood bootstrap values; J.A. Chaves, E. Bermingham, P. Escalante, G.M. Spellman & J. Klicka, unpublished data). We calculated the genetic distance between different species of *Troglodytes* as the genetic divergences between pairs of lineages (Paradis, 2012) by analysing the topology and metrics of the Bayesian phylogenetic tree. Using TREESNATCHER PLUS software (Laubach *et al.*, 2012), we calibrated our measurements using the scale provided in the figure and then generated a file with information on the tree structure and branch lengths; we used this file to calculate pairwise patristic distances (i.e. distance along the horizontal branches of the shortest path connecting two species; Daltry *et al.*, 1996; Losos *et al.*, 2003) in the R package adephylo (version 1.1-5; Jombart & Dray, 2008; Paradis, 2012; R Core Team, 2013).

### Statistical analyses

To test whether divergence in songs of *Troglodytes* wrens is related to genetic distance, we performed a Mantel

test using matrices representing acoustic and genetic differences between species. The matrices had pairwise divergence values for the eight *Troglodytes* species under analysis: Brown-throated Wren, Clarion Wren, Cozumel Wren, Pacific Wren, Rufous-browed Wren, Socorro Wren, Southern House Wren and Western House Wren. We created the acoustic matrix by calculating the Euclidean distance on the basis of the average value for each species of the four principal component factors summarizing acoustic measurements described above. We created the genetic matrix by calculating the pairwise patristic distance between species as described above. We computed the Mantel test using the software *PASSAGE* (version 2) with 10 000 randomizations (Rosenberg & Anderson, 2011).

To test whether wrens responded differently to conspecific and congeneric signals, we evaluated wrens' responses using linear mixed models. We ran a separate analysis for each of the four subject species. For each analysis, we included the two factors summarizing vocal and physical responses as dependent variables. We included treatment species as well as playback order as fixed effects. Individual identity was included as a subject variable with random effects to account for the fact that stimuli were presented repeatedly to the same subjects. We performed subsequent *post hoc* pairwise tests between conspecific playbacks and congeneric playbacks, using Bonferroni-adjusted correction for multiple comparisons.

To test whether the birds' responses to playback varied with the acoustic similarity between the subject and the playback stimulus, we used linear mixed models to evaluate the response data for all four species. We included the two factors summarizing vocal and physical responses as dependent variables. We included as fixed effects the four subject species and the interaction between subject species and acoustic distance. We included the acoustic distance between the subject species' song and the stimulus song as a covariate. We included individual identity as a subject variable with random effects.

To test whether the birds' responses to playback relate more to the acoustic similarity in their vocalizations, or to the genetic divergence between taxa, we used linear mixed models to evaluate the response data for all four species. Our two dependent variables were the two factors summarizing vocal and physical responses to playback; our three fixed effects were the four subject species, the interaction between subject species and genetic distance between subject species and stimulus species, and the interaction between subject species and acoustic distance; our covariates were the genetic distance between subject species and the stimulus species, and the acoustic distance between the subject species' song and the stimulus song; individual identity was included as a subject variable with random effects.

The control treatment (i.e. Black Catbird) was not included in the latter two analyses because of the low response levels we observed during control treatments (see Results), which could generate a false significant association between behavioural responses, acoustic similarity and genetic distance in the linear mixed model.

All linear mixed models were performed using the restricted maximum likelihood method for estimating fixed effects. To improve linearity in the association between response variables, acoustic distance and genetic distance, we square-root transformed acoustic distances and exponential transformed genetic distances. Residuals were normally distributed. Statistical analyses were performed in *PASW* statistics for Mac (version 18.0; Chicago, IL, USA).

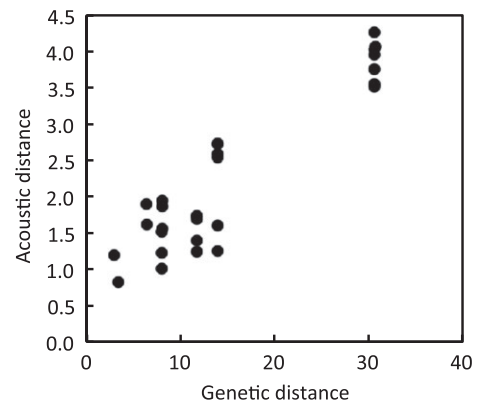
## Results

### Does divergence in *Troglodytes* songs vary with genetic divergence?

Acoustic differentiation paralleled genetic differentiation in eight species of *Troglodytes* wrens (Fig. 1): a Mantel test comparing acoustic divergence and phylogenetic difference scores showed a strong significant relationship ( $r = 0.9$ ,  $P = 0.001$ ; Fig. 2).

### Do wrens respond differently to conspecific and congeneric signals?

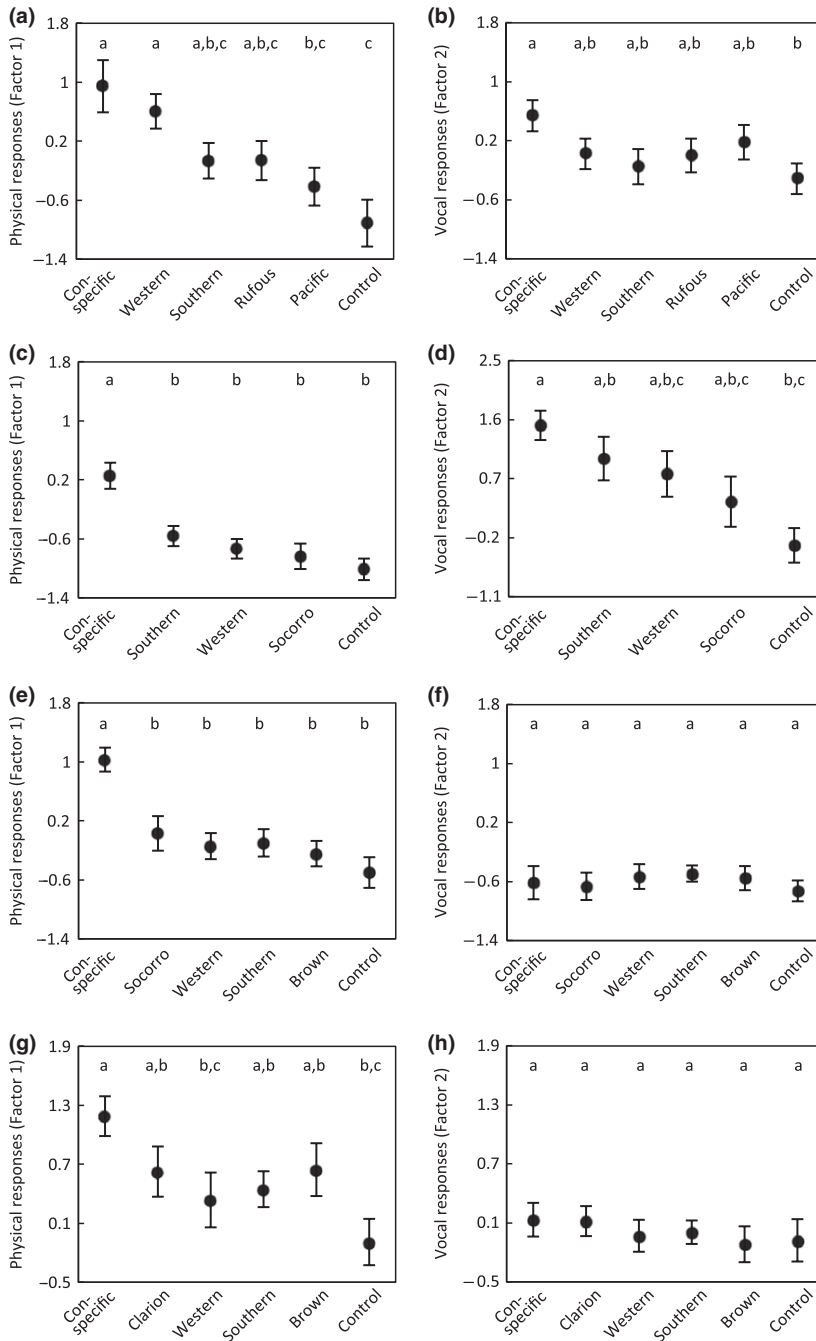
Playback to 61 male *Troglodytes* wrens of four different species at four different sites in Central America (15



**Fig. 2** Pairs of *Troglodytes* wren species with larger genetic differences (shown on the horizontal axis as pairwise Patristic distance calculated as the distance along the horizontal branches of the shortest path connecting two species in a phylogenetic tree) show larger acoustic divergences (shown on the vertical axis as pairwise Euclidean distances based on four principal component scores summarizing variation in song structure). Each pairwise comparison for the seven studied species is shown; the accompanying Mantel test accounts for nonindependence of the data.

Brown-throated Wrens, 16 Cozumel Wrens, 16 Clarion Wrens and 14 Socorro Wrens) elicited strong responses to many playback treatments, with males often approaching the loudspeaker and singing. In general, across the four subject species, physical responses varied with treatments, acoustic distance and genetic distance, with strong responses towards conspecific stimuli, and weaker responses towards congeneric species and more distantly related species.

Brown-throated Wrens showed significant variation across treatments in both their physical responses ( $F_{5, 10.2} = 11.4, P = 0.001$ ; Fig. 3a) and vocal responses ( $F_{5, 14.7} = 3.3, P = 0.03$ ; Fig. 3b). *Post hoc* tests revealed significant differences in physical and vocal responses between treatments. Brown-throated Wrens showed the strongest responses to conspecific playback and playback of Western House Wrens, the weakest responses to control stimuli (Black Catbirds), and



**Fig. 3** Physical (left) and vocal (right) responses of males to playback of allopatric-congeneric songs by Brown-throated Wrens (a, b), Cozumel Wrens (c, d), Clarion Wrens (e, f) and Socorro Wrens (g, h). Playback stimuli were conspecific, Southern House Wrens (Southern), Western House Wrens (Western), Socorro Wrens (Socorro), Clarion Wrens (Clarion), Brown-throated Wrens (Brown) and Black Catbirds as a control stimulus. Data are shown as means  $\pm$  SE. Lower-case letters indicate the results of the *post hoc* test using Bonferroni correction for multiple comparisons; levels connected by the same letter are not significantly different.

intermediate values for the remaining three congeneric stimuli (Fig. 3a). Similarly, they showed the most vocal responses to conspecific playback, the least vocal responses to the control species (Black Catbirds) and intermediate intensities of response to the four congeners (Fig. 3b).

Cozumel Wrens showed significant variation across treatments in both physical responses ( $F_{4, 19.5} = 10.7$ ,  $P < 0.001$ ; Fig. 3c) and vocal responses ( $F_{4, 23.9} = 9.2$ ,  $P < 0.001$ ; Fig. 3d). *Post hoc* tests revealed significant differences in physical and vocal responses between treatments. Cozumel Wrens' physical responses were significantly stronger in response to conspecific playback than all other treatments (Fig. 3c). Their vocal responses were strongest to conspecific playback and weakest to the control species (Black Catbirds), with intermediate intensities of response to the three congeners (Fig. 3d).

Clarion Wrens showed significant variation across treatments in physical responses ( $F_{5, 15.8} = 23.1$ ,  $P < 0.001$ ; Fig. 3e), but not in vocal responses ( $F_{5, 20.5} = 0.6$ ,  $P = 0.6$ ; Fig. 3f). *Post hoc* tests revealed significantly stronger physical responses to conspecific treatments than all of the other treatments (Fig. 3e).

Socorro Wrens also showed significant variation across treatments in physical responses ( $F_{5, 16.2} = 7.2$ ,  $P = 0.001$ ; Fig. 3g), but not in vocal responses ( $F_{5, 12.4} = 0.5$ ,  $P = 0.7$ ; Fig. 3h). Socorro Wrens showed the strongest responses to conspecific playback and weakest responses to the control species (Black Catbirds), with intermediate intensities of response to the four congeners (Fig. 3g).

The subjects' physical and vocal responses showed no significant variation in the order of playback treatments for Cozumel Wrens, Clarion Wrens, Socorro Wrens and Brown-throated Wrens (physical responses:  $F$  range = 0.9–1.4,  $P > 0.2$ ; vocal responses:  $F$  range = 0.1–0.9,  $P > 0.4$ ), except for the Brown-throated Wren physical responses, which showed a significant effect of the order of the treatments ( $F_{5, 24.5} = 3.6$ ,  $P = 0.01$ ); for this species, *post hoc* tests revealed that the birds exhibited weaker physical responses to the treatments presented in fourth position compared to the seventh position; all other treatments did not vary with order.

### Do playback responses vary with acoustic similarity?

We tested whether there was a significant relationship between wrens' responses to playback and the acoustic similarity between the subject species' songs and the playback stimuli. *Troglodytes* wrens' physical responses showed a significant association with acoustic distance ( $F_{1, 212.9} = 20.1$ ,  $P < 0.001$ ), where birds showed stronger responses towards the most acoustically similar stimuli, and the lowest response levels to the most acoustically dissimilar stimuli (Fig. 4a–d). Vocal

responses, in contrast, showed no significant association with acoustic distance ( $F_{1, 194.8} = 2.6$ ,  $P = 0.1$ ). This analysis, including acoustic divergence, revealed no significant differences in the physical responses between the four subject species ( $F_{3, 190.9} = 1.6$ ,  $P = 0.1$ ), and the interaction between species and acoustic distance was not significant ( $F_{3, 149.2} = 0.2$ ,  $P = 0.8$ ). Vocal responses, however, varied between the four subject species ( $F_{3, 156.3} = 4.1$ ,  $P = 0.007$ ), with Cozumel Wrens showing the highest responses to treatments, Brown-throated Wrens and Socorro Wrens showing intermediate responses, and Clarion Wrens showing the lowest responses. The interaction between species and acoustic distance was not significant ( $F_{3, 135.7} = 1.1$ ,  $P = 0.3$ ).

### The relative importance of acoustic similarity versus genetic divergence

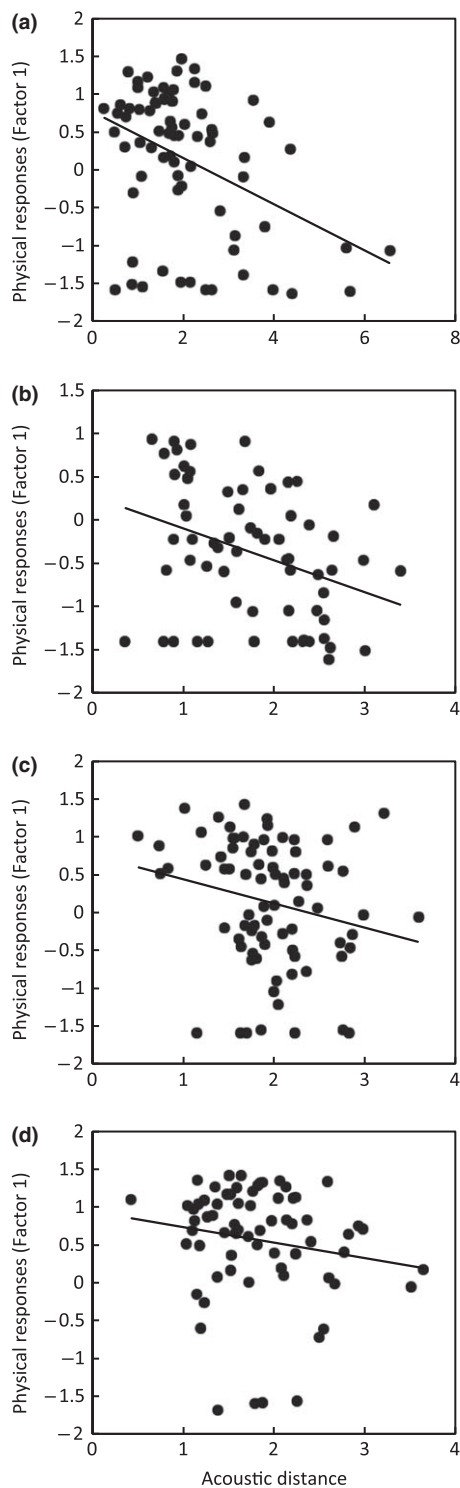
We tested whether the birds' responses to playback relate more to the acoustic similarity or to the genetic divergence among *Troglodytes* Wrens. After controlling for the effect of acoustic distance, birds' physical and vocal responses showed a significant association with genetic distance ( $F_{1, 121.5} = 62.1$ ,  $P < 0.001$ ;  $F_{1, 108.0} = 13.3$ ,  $P < 0.001$ , respectively), where birds showed stronger responses towards more closely related congeners, and the lowest response levels to the most distantly related congeners (Fig. 5a–h). In contrast, after controlling for the effect of genetic divergence, birds' physical and vocal responses did not show a significant association with acoustic distance ( $F_{1, 210.2} = 3.8$ ,  $P = 0.05$ ;  $F_{1, 210.9} = 0.008$ ,  $P = 0.9$ , respectively).

This analysis, including both acoustic similarity and genetic divergence, revealed significant differences in the physical responses between the four species of subjects ( $F_{3, 169.5} = 2.8$ ,  $P = 0.03$ ), with Cozumel Wrens showing the highest responses to treatments, Brown-throated Wrens and Clarion Wrens showing intermediate responses, and Socorro Wrens showing the lowest responses. Vocal responses, in contrast, show no variation between the four subject species ( $F_{3, 162.1} = 0.4$ ,  $P = 0.7$ ). Physical responses showed no significant interaction in both cases, between subject species and genetic divergence, and between subject species and acoustic distance ( $F_{3, 112.5} = 1.7$ ,  $P = 0.1$ ;  $F_{3, 148.6} = 0.4$ ,  $P = 0.7$ , respectively). Vocal responses showed a significant interaction between subject species and genetic divergence ( $F_{3, 91.2} = 4.7$ ,  $P = 0.004$ ) and a no significant interaction between subject species and acoustic distance ( $F_{3, 148.6} = 0.4$ ,  $P = 0.7$ ).

### Discussion

We investigated the relationship between acoustic divergence and phylogenetic distance in four allopatric species of *Troglodytes* wrens, and whether divergence in





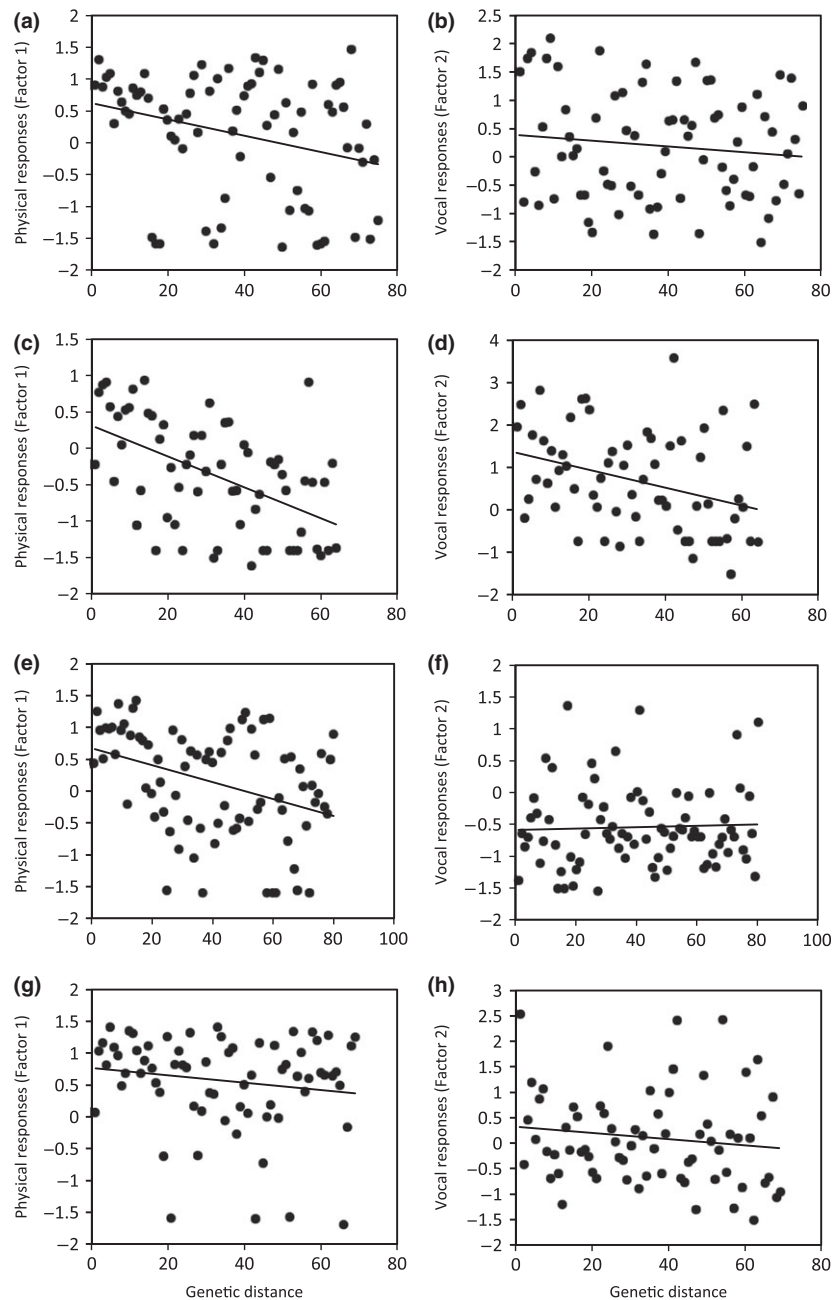
**Fig. 4** Physical responses to playback of allopatric-congeneric songs by Brown-throated Wrens (a), Cozumel Wrens (b), Clarion Wrens (c) and Socorro Wrens (d) are predicted by the acoustic divergence of the stimuli to the subject species. Physical response is a principal component score summarizing variation in multiple measures of the birds' playback responses.

acoustic and genetic features are associated with receiver responses to song playback. Acoustic analysis revealed that acoustic similarity between species of *Troglodytes* wrens decreases with genetic distance. Playback responses revealed that, in general, males of four species of *Troglodytes* wren respond to conspecific and allopatric-congeneric animals, that the response is related to acoustic similarity between the subject and the species simulated by playback, and that there is a relationship between genetic divergence and wrens' responses after controlling for the effects of acoustic distance.

Several studies have suggested that behavioural discrimination between conspecific and heterospecific signals is a widespread behaviour across diverse animal taxa (de Kort & ten Cate, 2001; Ryan *et al.*, 2003; Deryberry, 2007; Amézquita *et al.*, 2011; Danner *et al.*, 2011). Our results provide strong evidence that this pattern holds true for Cozumel Wrens and Clarion Wrens, which both showed statistically higher intensities of response to conspecific playback than congeneric playback, and to a lesser degree for Brown-throated Wrens and Socorro Wrens, which showed their highest intensities of response to conspecific playback, but these differences were not statistically higher than some congeneric treatments. This result, in combination with our previous analysis of acoustic differences throughout the *Troglodytes* wrens (Sosa-López & Mennill, 2014), suggests that *Troglodytes* songs contain features that vary between species, and that birds use these features – or the lack of them – to recognize potential competitors.

In line with several studies that report behavioural responses to playback of allopatric-heterospecific signals in animals (Nevo & Capranica, 1985; Coyne & Orr, 1989; Verrel & Arnold, 1989; Ryan & Rand, 1993; de Kort & ten Cate, 2001; Ryan *et al.*, 2003), our results demonstrate that *Troglodytes* wrens also respond aggressively to the acoustic signals of allopatric-congeneric animals, suggesting that the songs of allopatric-congeners contain sufficient information to trigger territorial responses (Emlen, 1972; Höbel & Gerhardt, 2003; Ryan *et al.*, 2003). When the amount of species-specific information varies with the degree of phylogenetic relatedness, signals may elicit responses that are proportional to the genetic distance between species (Ryan *et al.*, 2003). For example, in a study of doves, de Kort & ten Cate (2001) suggested that the relationship between behavioural responses and phylogenetic relatedness could be the result of either similarity in signals or phylogenetic effects on perceptual mechanisms. In our study, the association between behavioural responses and acoustic distance suggests that *Troglodytes* wrens respond based on the similarity between stimulus species' song and the receiver's species' song.

In some cases, animals might respond to the signals of closely related congeners because of previous experience, including prior aggressive interactions while competing for similar resources (Grant & Grant, 2010;



**Fig. 5** Physical (left) and vocal (right) responses to playback of allopatric-congeneric songs by Brown-throated Wrens (a, b), Cozumel Wrens (c, d), Clarion Wrens (e, f) and Socorro Wrens (g, h) are predicted by the genetic distance between the species stimuli to the subject species. Physical and vocal responses are principal component scores summarizing variation in multiple measures of the birds' playback responses. Graphs show raw data, whereas the statistical analyses were conducted on residuals.

Amézquita *et al.*, 2011). We ruled out this effect by studying allopatric species. Given the isolated geographic distribution of our four study species, we can be confident that our subjects had not previously encountered the heterospecific stimuli we presented to them in this experiment.

Our analysis shows a direct relationship between genetic divergence and the variation in birds' responses, after controlling for acoustic similarity, suggesting that perceptual differences between wren species reflect evolutionary history. Further evidence supporting this

idea comes from the very few studies that have explored phylogenetic relationships and receiver responses; existing data come from experimental analyses of nonlearning animal species including birds (de Kort & ten Cate, 2001; Tobias & Seddon, 2009) and frogs (Ryan *et al.*, 2003, 2007; Bernal *et al.*, 2007). Interestingly, song-learning animals – such as the wrens we studied here – are expected to experience rapid changes in signals and receiver responses across time (Irwin & Price, 1999; Grant & Grant, 2010; Sznajder *et al.*, 2012). Our results, however, show that diver-

gence in learned signals may not occur as rapidly as is sometimes thought (discussed in Price & Lanyon, 2002). Our results are in line with other studies that show a parallel divergence between songs and genetics in other oscine bird species (Price & Lanyon, 2002; Farnsworth & Lovette, 2008; Irwin *et al.*, 2008; Cadena & Cuervo, 2010; Sosa-López *et al.*, 2013), suggesting a bias for learning the song of the parental population and perhaps a partially genetically controlled sound production system (Remsen, 2005).

A possible perceptual mechanism that could explain *Troglodytes* wrens' responses to playback is known as generalization, where subjects associate specific traits in common between similar signals and the similarity between signals should predict the strength of the response (Shepard, 1987). Our findings that response intensity is affected by the similarity between the subject species' songs and the playback stimuli, and that *Troglodytes* wrens respond to unfamiliar allopatric-congeneric signals, support the idea that recognition might be influenced by generalization. Others have made similar suggestions in frogs and other songbirds (Ryan *et al.*, 2001, 2003; Derryberry, 2011). Birds may generalize using an 'auditory template' (a neuronal representation defining the characteristic of a song in a given species; Catchpole & Slater, 2008), comparing the features of playback stimuli to their auditory template. In this case, the strength of the response should be associated with the similarity between the auditory template and the external stimuli. Describing the auditory template in birds is a challenging task, although studies in frogs using signals modified in the laboratory demonstrate that it is possible (Amézquita *et al.*, 2011).

One interesting finding of our playback study is that *Troglodytes* wrens showed stronger physical responses to more similar songs, but their vocal responses did not vary. This result supports the idea that physical vs. vocal engagement with rivals may have different costs (Searcy & Beecher, 2009). Further research in this and other taxa should provide insight into the costs associated with physical vs. vocal behaviours and how these costs mediate interactions.

In conclusion, we analysed whether behavioural responses to allopatric-congeneric songs are predicted by acoustic distance and phylogenetic relatedness in *Troglodytes* wrens. We showed that birds respond most strongly to playback of conspecific songs, but still respond to allopatric-congeneric songs, and that the intensity of response varies with both the acoustic similarity between the playback subject's song and the playback-simulated species song, as well as the phylogenetic distance between the species. We provided replication by testing the same hypothesis in four related species distributed throughout different parts of the Neotropics. Our investigation is one of very few to focus on phylogenetic differences in animals' responses to signals, rather than phylogenetic differences in signals them-

selves; wrens perceive fine structural variation in acoustic signals across related species, suggesting an effect of evolutionary history on perceptual differences between species.

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## Supporting information

Additional Supporting Information may be found in the online version of this article:

**Table S1.** The playback treatments given to four different species of *Troglodytes* wrens.

**Table S2.** Details of the principal components analysis performed on physical and acoustic responses of four *Troglodytes* species.

**Table S3.** Details of the principal components analysis performed to reduce 15 fine structural characteristics of 301 stimuli songs used to investigate whether acoustic similarity influenced the responses of the playback subjects.

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