Territorial responses of male Bermuda White-eyed Vireos (*Vireo griseus* subsp. *bermudianus*) reflect phylogenetic similarity of intruders and acoustic similarity of their songs

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ABSTRACT. For signal divergence to drive speciation, receivers should perceive structural differences in divergent signals; similar-structured signals from closer relatives are expected to elicit stronger responses than dissimilar signals from distant relatives. Two mechanisms can affect receiver responses to passerine song: (1) sympatric song familiarity and (2) an innate auditory template used to assess acoustic similarity. We examined the role of acoustic similarity by comparing behavioral responses of male Bermuda White-eyed Vireos (*Vireo griseus bermudianus*) to playback of the songs of allopatric species from across the family Vireonidae. Phylogenetic distance between the focal and stimulus species predicted response strength. Males uttered fewer vocalizations, had fewer speaker flyovers, and remained farther from the speaker during playback of the songs of more distantly related vireos. We then tested whether structural similarity of playback songs, as defined by three phylogenetically conserved song traits, explained these relationships. As predicted, males uttered fewer vocalizations, had fewer speaker flyovers, and remained farther from the speaker in response to more dissimilar songs. Collectively, our results suggest that male Bermuda Vireos perceive and respond to interspecies variation in the phylogenetically conserved song traits of allopatric species of vireos. This suggests that song divergence, and the ability to distinguish divergent songs, reinforces reproductive isolation and competitor exclusion.

RESUMEN. **Las respuestas territoriales de los machos del vireo *Vireo griseus* subsp. *bermudianus* reflejan la similitud filogenética de los intrusos y la similitud acústica de sus cantos**
Para que la divergencia de señales conduzca a la especiación, los receptores deben percibir las diferencias estructurales de señales divergentes. Se espera que las señales de estructuras similares de parientes más cercanos provoquen respuestas más fuertes que las señales disimilares de parientes distantes. Dos mecanismos pueden afectar las respuestas de los receptores al canto de paserinas: (1) familiaridad de canto simpátrico y (2) una plantilla auditiva innata que se usa para determinar la similitud acústica. Examinamos el papel de la similitud acústica por medio de comparaciones de respuestas de comportamiento en machos del vireo *Vireo griseus bermudianus* a grabaciones de cantos de especies alopátricas de la familia Vireonidae. La distancia filogenética entre las especies focal y estímulo predicen la fuerza de la respuesta. Los machos completaron menos vocalizaciones, tuvieron menos sobrevuelos a la bocina y permanecieron más lejos durante el tiempo en que se tocó la grabación de los vireos más distantemente emparentados. Cuando sometimos a prueba la similitud estructural de los cantos grabados, tal y como lo definen tres características filogenéticamente conservadas de los cantos, éstas explicaron esas relaciones. Como lo predecimos, los machos completaron menos vocalizaciones, tuvieron menos sobrevuelos a la bocina y permanecieron más lejos en respuesta a los cantos más disímiles. En conjunto, nuestros resultados sugieren que los machos de este vireo perciben y responden a variación interespecífica en las características del canto filogenéticamente conservadas de las especies de vireos alopátricos. Esto sugiere que la divergencia del canto, y la capacidad de distinguir cantos divergentes, refuerza el aislamiento reproductivo y la exclusión competitiva.

Key words: allopatric speciation, heterospecific competition, phylogeny, playback experiment, signal evolution, song discrimination, vireos

How birds perceive and respond to conspecific and heterospecific acoustic signals can affect individual fitness. For example, through a sexual selection lens, hybridization may occur if females choose similar-sounding heterospecific males as mates (Ficken and Ficken 1967,

Willis and Symula 2014, Toews et al. 2018). Likewise, interference competition, including males aggressively thwarting mating opportunities of rival males (Grether et al. 2009, Drury and Cowen 2020), is an important mechanism that prevents hybridization. From an acoustic perspective, reproductive barriers between species can be compromised if males fail to repel

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similar-sounding heterospecifics from mating with nearby females. Lastly, through a natural selection lens, distinguishing conspecific and heterospecific signals can facilitate ecological competition for important resources, such as food and territories (Losin et al. 2016, Vogt et al. 2017, Gutema et al. 2018, Beltrão et al. 2021). Aside from conspecifics, the risk of hybridization and resource competition from closely related species should be greater than that from more distantly related species because closely related species have more similar signal structures, niches, behaviors, and physiology. In families where the closest phylogenetic relatives tend to live in sympatry (e.g., Parulidae; Simpson et al. 2021), these risks are immediate; in families where the closest phylogenetic relatives tend to live in allopatry (e.g., diverse, tropical montane birds; Freeman 2015), the risks still exist, but are deferred until secondary contact is made. Therefore, responding more aggressively to closely related species than to distantly related species could further promote and maintain reproductive isolation and resource partitioning.

Bird song facilitates reproductive isolation and speciation by mediating species recognition, mate choice, and interference competition (Borror 1972, Andersson 1994, Slabbekoorn and Smith 2002), allowing receivers to assess the decreasing threat posed by conspecifics, closely related species, and distantly related species, respectively. Two potential mechanisms underly this assessment. First, being exposed to the songs of conspecifics and sympatric heterospecifics during song learning (Beecher and Brenowitz 2005, Phan and Pytte 2006) should cause males and females to respond strongly to conspecific signals and weakly to the songs of heterospecifics that may not pose any threat. This pattern has been found in *Vermivora* warblers (Gill and Murray 1972), *Poecile* chickadees (Hill and Lein 1989), and *Fringilla* Chaffinches (Lynch and Baker 1991). The second mechanism is an innate "auditory template" that stores the structural information of an individual's own song, which can be compared to the songs of another species. Structural similarity between the two signals can determine how the receiver responds (Pinaud and Terleph 2008). In short, songbirds may react strongly to songs that more closely match their own auditory template (Dooling et al.

1992, Brainard and Doupe 2002). Although both learning and acoustic similarity might be important for sympatric species, only acoustic similarity could be relevant for allopatric species where there is no opportunity for juveniles to learn to distinguish their own songs from those of close allopatric relatives. With song familiarity removed, any relationship between the strength of response to a song and phylogenetic distance to the singer would be driven primarily by similarity of acoustic song structure resulting from phylogenetic history (de Kort and ten Cate 2001, Sosa-López and Martínez Gómez 2016). The ability to distinguish close relatives from distant ones based on acoustic similarity could be especially important for families where the most closely related species are allopatric, but where those species then meet in secondary contact.

Playback experiments are a useful tool for investigating the mechanisms underlying avian song recognition. Investigators have used playback experiments to compare receiver responses to the songs of a conspecific and a congeneric, with the general prediction that receivers should respond more strongly to songs of conspecifics (Greenberg et al. 1993, Linhart and Fuchs 2015, Weir and Price 2019, Darolová et al. 2020). Several songbird species have been found to exhibit this predicted pattern, including Black-capped Chickadees (*Poecile atricapillus*; Kershner and Bollinger 1999), White-eared Ground Sparrows (*Melozone leucotis*; Sandoval and Méndez 2013), and Rufous-and-White Wrens (*Thryophilus rufalbus*; Battiston et al. 2015). In other species, however, receivers did not distinguish between conspecific and congeneric songs, e.g., *Hippolais* warblers (Secondi and Faivre 1999), *Dendroica* (now *Setophaga*) warblers (Pearson and Rohwer 2000), and *Vermivora* warblers (Martin and Martin 2001), possibly because these species exhibit conserved responses to heterospecific songs with phylogenetic signal in their song structures (Mejías et al. 2020). These playback experiments have advanced our understanding of avian responses to acoustic signals, but have involved sympatric species where the two song recognition mechanisms (i.e., song learning and acoustic similarity) were both potentially present. Playback studies with allopatric species remove the potential effect of learning,

allowing the independent assessment of acoustic similarity on behavioral responses (Freeman and Montgomery 2017).

Species in the family Vireonidae are ideal for studies of song recognition, with diverse songs learned during development (James 1976a, Mejías et al. 2020). Male responses to song are also conspicuous and easily measured and are thought to also reflect female responsiveness to those stimuli (Naguib and Fichtel 1999, Illes and Hall 2006, Seddon and Tobias 2006). In addition, phylogenetic relationships among the Vireonidae are well-resolved, making broad comparative analyses possible (Slager et al. 2014). Several vireonid song traits, including song duration, minimum peak frequency, maximum peak frequency, and frequency modulation, also exhibit phylogenetic signal, due in part to phylogenetically conserved morphological constraints on song production (Mejías et al. 2020), and so may provide a basis for discriminating the songs of closely related species from those of more distantly related species. Finally, one vireonid, the Bermuda White-eyed Vireo (*Vireo griseus* subsp. *bermudianus*, known locally as the chick-of-the-village), is allopatric to all other vireonids, thus removing potential effects of familiarity and learning on receiver responses to other vireonid songs.

Our objective was to quantify the behavior of territorial, male Bermuda Vireos responding to songs of diverse species from across the family Vireonidae, including some closely related and others more distantly related. After sequencing the DNA of Bermuda Vireos and adding them to the Vireonidae phylogeny, our first objective was to determine whether the strength of receiver responses was correlated with the phylogenetic distance between Bermuda Vireos and the stimulus species. Upon finding that phylogenetic distance predicted behavioral responses, our second objective was to determine whether acoustic similarity between the songs of Bermuda Vireos and those of stimulus species explained this relationship.

METHODS

Only eight species in the family Vireonidae have been recorded in Bermuda (32°310N, 64°750W; Fig. 1), including White-eyed Vireos (*Vireo griseus*), Yellow-throated Vireos (*V. flavifrons*), Blue-headed Vireos (*V. solitarius*), Warbling Vireos (*V. gilvus*), Philadelphia Vireos (*V. philadelphicus*), Yellow-green Vireos (*V. flavoviridis*), Black-whiskered Vireos (*V. altiloquus*), and Red-eyed Vireos (*V. olivaceus*; Amos 1991, Mejías and Mejías 2020). The first seven species are considered rare vagrants, and Red-eyed Vireos are common fall visitors; migratory vireos are even scarcer in the spring when singing intensifies among temperate passerines. In contrast, Bermuda Vireos, a subspecies of the North American White-eyed Vireo found only in Bermuda, are abundant, year-round residents in the archipelago, and the only vireo that breeds in Bermuda (Mejías and Nol 2020, Mejías 2021). Migrant vireos seldom sing on the archipelago (M. A. Mejías, pers. observ., P. Watson, pers. comm.), making Bermuda Vireos largely naive to all songs of heterospecific vireos. As such, we consider Bermuda Vireos allopatric with the North American White-eyed Vireo and all other species in the family Vireonidae.

We conducted fieldwork at 12 sites across Bermuda (Fig. 1) that were primarily comprised of introduced Brazilian pepper (*Schinus terebinthifolius*), allspice (*Pimenta dioica*), and fiddlewood (*Citharexylum spinosum*). From June to September 2017, we captured Bermuda Vireos along walking trails by luring them into mist-nets using playback of consubpecific songs; these songs were not used in subsequent playback experiments. Each netted bird was fitted with an aluminum Porzana identification band on one leg and either one or two color bands on the other leg for individual identification. We collected blood samples by puncturing the brachial vein with a 26.5-gauge needle and pressing filter paper onto the wound. Bleeding was stopped by applying a small dab of Clotisol onto the wound and blowing on it gently until it dried. Birds were released at their point of capture within 10 min, and blood samples were labeled and stored in paper envelopes for subsequent DNA sequencing (see details below). We estimated the territory boundaries of color-banded vireos by opportunistically following them for 1–2 h during favorable weather from June to September 2017, and marking the GPS coordinates of all perches with a handheld GPS unit (Garmin eTrex®
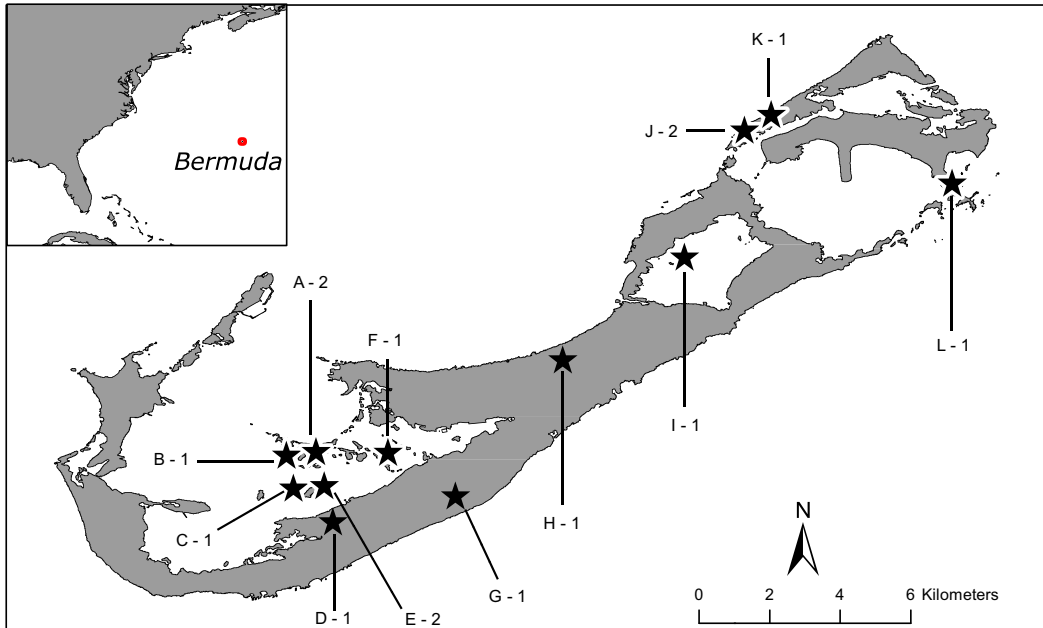
Fig. 1. Sites across Bermuda where 15 territorial, color-banded male Bermuda White-eyed Vireos were recorded reacting to playback stimuli. (A) Port's Island, (B) Gamma Island, (C) Burt's Island, (D) Elm Lodge, (E) Darrell's Island, (F) Hinson's Island, (G) Alfred Blackburn Smith Nature Reserve, (H) Oceanview Golf course, (I) Trunk Island, (J) Ferry Reach, (K) Lover's Lake, and (L) Cooper's Island. Numbers represent the number of male vireos recorded, per site.

10, ~3 m accuracy; Garmin International, Inc., Olathe, KS, USA). We found that at least 1 mo of GPS data collection was sufficient to identify territory boundaries. We mapped territories again before playbacks began in 2018 and, in all cases, found them to be the same as in 2017.

Playback stimuli.   To construct playback stimuli, we passively recorded 10 Bermuda Vireo males in their territories during 2017 using a directional shotgun microphone (Sennheiser K6 handheld) and a digital audio recorder (Marantz PMD-661 MKII; WAV format; 44.1 kHz; 16 bits). We continued to record these males until at least two songs with high signal-to-noise ratio and no overlapping sounds were obtained. Some of these 10 males were the same as the 15 used in playback experiments, but we ensured that songs were never played back to the same male from which they were recorded, or to neighbors. To create heterospecific playback stimuli, we obtained song recordings from two online archives (Macaulay Library:

https://www.macaulaylibrary.org/; Xeno-canto: https://www.xeno-canto.org/) for each of the 51 vireonids in the Vireonidae phylogeny (Mejías et al. 2020). When possible, we obtained 10 recordings of each species. We used the recording quality rating systems of the two song libraries to select recordings with high signal-to-noise ratios. To reduce the probability of including multiple recordings from the same individual, we included recordings of the same species only if they were separated spatially by at least 2 km and temporally by at least 1 yr.

We viewed each recording as a waveform and spectrogram in Raven Pro (Hamming window, FFT size = 512 samples, 87.5% overlap). We annotated all high-quality songs with clear tracings and no overlap with other sounds and clear amplitude pulses on the waveform. We defined a song as an acoustic signal comprising one or more elements, and elements as continuous traces on the spectrogram (Mejías et al. 2020). Elements of a single song were separated by <0.5 s and

successive songs by ≥0.5 s. We chose 0.5 s because it reflected the minimum duration of silent periods between elements of separate songs for all vireos we studied (Mejías et al. 2020). Female song has been documented in at least four North American vireos (Pitelka and Koestner 1942), whereas information about this behavior in tropical vireos appears undocumented. Although the sex of birds recorded was not known, review of both Macaulay Library and Xeno-canto revealed that all our songs approximate the typical songs for each species.

Consistent with previous descriptions of vireo singing behavior (Borror 1972, Kroodsma 1983), our recordings revealed three distinct singing styles among our study species, including (1) immediate variety, where each new song produced is a different song type, (2) eventual variety, where a single song type is repeated several times before switching to another, and (3) no variety, where a single song type is repeated. Because our goal was to design playback stimuli that matched the natural singing style of each species, we used a random number generator (https://www.random.org/) to randomly select two songs (each a different song type) per recording per species for species that sing with immediate or eventual variety, and one song per recording per species for species that sing with no variety. Some species were excluded if (1) we obtained less than two recordings (to reduce pseudoreplication, $N = 2$ species), (2) for a species that sings multiple song types, we had fewer than two recordings containing two or more song types ($N = 10$ species), and (3) song duration was considered an outlier within the family ($N = 1$ species; mean song duration = 13.5 s; Table S1; Mejías et al. 2020). For all retained species, we exported the selected songs as standalone clips with 0.3 s of silence before and after songs, filtered them with a 600-Hz high-pass filter, and normalized them to a peak amplitude of −1 dB. In total, we exported 221 songs from 137 individuals across 38 species (mean number of individuals per species = 3.5 ± 1.9 [SD], range = 1–10; Tables S1 and S2).

We used Audacity software (2.1.3; Audacity Team, 2012; https://audacityteam.org/) to create a separate 2-min playback sequence (WAV format, 16-bit amplitude encoding, 44.1 kHz

sampling rate) for each of the 137 stimulus males. We standardized the intersong interval of all playback sequences by inserting 5 s of silence between individual song clips. For species that sing with immediate variety, each playback sequence included two song types from a given individual, presented alternately (i.e., A-B-A-B-A-B-). For species that sing with eventual variety, each sequence included five repetitions of one song type followed by five repetitions of the second song type from a given individual, repeated during the 2-min sequence (i.e., A-A-A-A-A-B-B-B-B-B-A-A-A-A-A-A-). For species that sing with no variety, each sequence included a single song from a given individual, repeated during the 2-min sequence (i.e., A-A-A-A-A-).

We assigned playback sequences to subjects such that each would be presented songs from species evenly distributed across the Vireonidae phylogeny. To do this, we divided the phylogenetic tree (Mejías et al. 2020) into eight clades and two grades (Fig. 2). These 10 groups comprised all Vireonidae genera, except the monotypic *Erpornis* because we obtained fewer than two recordings for this species. For each of our 15 subjects, we randomly selected one stimulus species from each of the eight clades and two grades and then assigned one randomly selected playback sequence, without replacement until all sequences were used, from that stimulus species. Due to the limited number of recordings obtained for some species, some recordings were reused on multiple test subjects. We also assigned one consubpecific playback sequence to each subject. Subjects therefore received two playback sequences from the clade containing Bermuda Vireos (dark green clade in Fig. 2).

We also created lure sequences with scolding calls of Bermuda Vireos that are often used during agonistic consubspecific interactions (M. A. Mejías, pers. observ.). We elicited scolding calls from vireos by "pishing." Scolding bouts were recorded in 2018, prior to playback trials. Using Audacity, we trimmed each recording to a 30-s bout of continuous calls, added 3 s of silence before and after the bout, applied a 1000 Hz high-pass filter, and normalized it to a peak amplitude of 0 dB.

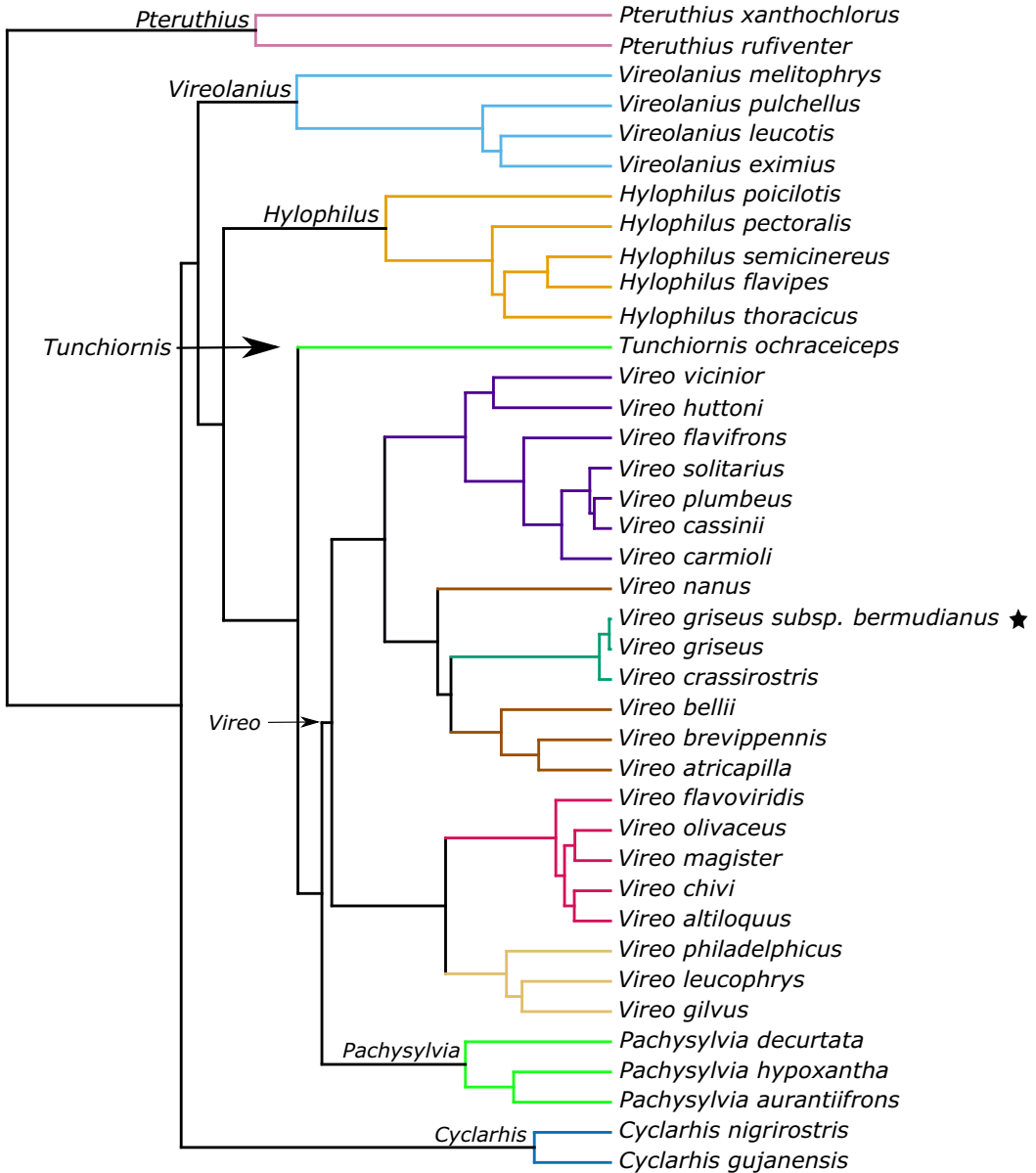All playback sequences were transferred to our playback device (Apple iPod nano, 7th

Fig. 2. Maximum clade credibility (MCC) tree for 38 Vireonidae species and Bermuda White-eyed Vireo subspecies (marked with a star) used as playback stimuli. We performed a Bayesian analysis in BEAST v2.5.2 using ND2 sequence data from Slager et al. (2014) and our 10 Bermuda Vireo ND2 sequences. We partitioned our phylogenetic tree into eight clades and two grades, each represented by a different color, to facilitate our assignment of diverse playback stimuli to test subjects. From top to bottom: (1) dark pink: *Pteruthius* clade, (2) light blue: *Vireolanius* clade, (3) gold: *Hylophilus* clade, (4) lime green: *Tunchiornis* and *Pachysylvia* grade, (5) navy blue: *Vireo* clade 1, (6) dark brown: *Vireo* grade, (7) dark green: *Vireo* clade 2, (8) dark red: *Vireo* clade 3, (9) light brown: *Vireo* clade 4, and (10) dark blue: *Cyclarhis* clade.

generation) for playback in the field. We did not measure the amplitude of the scolding lures or song stimuli during playbacks because

we were concerned that the measurement process would disrupt the trial. Rather, because our speaker was calibrated to broadcast a

calibration tone (4000 Hz sine wave, normalized to −10 dB) at 80 dB SPL, it automatically broadcast scolding lures (normalized to 0 dB) at 90 dB SPL and songs (normalized to −1 dB) at 89 dB SPL; we confirmed these amplitudes in a series of pilot trials.

Playback experiments. From May to August 2018, we simulated intrusions of consubspecific and heterospecific vireos in the territories of 15 color-banded male Bermuda Vireos. Intrusions were simulated by broadcasting the songs of the intruding consubspecific or heterospecific for 2 min through a loudspeaker (Monster SuperStar High Definition Bluetooth Speaker; Monster Power, San Francisco, CA, USA) in the subject's territory. Each subject received 11 treatments in random order, with each treatment presented on a different day to reduce the likelihood of habituation (Sosa-López et al. 2016, Fernández-Gómez et al. 2021). Although 11 trials is more than the number used in some previous playback studies, the within-subjects design provides a powerful test of treatment effects because intermale variability in responsiveness can be accounted for statistically (Akçay and Campbell 2014). Nevertheless, we acknowledge that subjects may have habituated to our playbacks after repeated trials. We therefore randomized treatment order to prevent any potential confound between treatment and treatment order and accounted for the potential habituation effects by including trial order in our statistical analyses. One treatment included songs of a Bermuda Vireo; the other 10 included songs of heterospecifics from each of the 10 clades composing the Vireonidae (Fig. 2; see details in "Playback stimuli," above). We tested subjects in batches comprising 3–5 individuals from the same general location, and ensured subjects were separated by at least 100 m to reduce the probability that subjects would hear and respond to playback trials conducted in another territory. Within each batch, we randomly selected a maximum of five subjects to test on a given day, repeating this process each day until all males in the batch had received their 11 treatments. All trials were conducted between 08:00 and 13:00 h when there was no precipitation and little to no wind.

Before beginning a trial, we placed the loudspeaker facing upwards on top of a tripod (76 cm above ground) in a natural clearing at the approximate center of the subject's territory. We selected locations where males would be visible for at least 7 m in all directions from the speaker, and with multiple perches at varying distances from the speaker. We chose 7 m because dense vegetation made birds difficult to observe at greater distances. To facilitate distance estimates, we hung colored trail tape ribbons, 30-cm long, from branches at 1, 3, and 7 m from the speaker in each of the four cardinal directions before trials began; the tape remained in place until all 11 treatments were completed. To calibrate speaker volume, we connected it to our digital playback device, broadcast a calibration tone (4000 Hz sine wave, normalized at −10 dB), and adjusted the volume until the tone measured 80 dB sound pressure level (SPL) measured 1 m from the speaker with a Proster sound level meter (±1.5 dB(A), fast time weighting (125 ms)). We then stood 7 m from the speaker and immediately began the trial.

Trials began by broadcasting an audio lure to attract the subject to the 7-m radius around the speaker, thereby standardizing each male's distance from the speaker before the treatment phase of trials began. The lure was one of 15 recordings (selected at random) of a 30-s bout of continuous Bermuda Vireo scolding calls, flanked with 3-s of silence before and after the bout. The lure was repeated for 3-min, and the 3-min sequence then played in alternation with 5 min of silence for up to 1 h. If we observed or heard the subject during the lure playback or a 5-min silence period, we immediately played the lure one final time. Bermuda Vireos move through territories with short flutter hops, which favored our approach of waiting for subjects to approach playback sites after the audio lure ceased. If a subject came within 7 m of the speaker at any time during the 1-h lure phase, we immediately switched to the treatment phase of the trial. Focal males were sometimes accompanied by mates, but we only monitored the behavior of focal males. If focal males did not approach to within 7 m of the speaker before 1 h elapsed, trials were aborted and repeated on a another day using a different lure.

When focal males moved within 7 m of the speaker, we began the treatment phase of the

trial by broadcasting a pre-selected 2-min song treatment. We recorded the male's vocal behaviour during the 2-min playback and for 1 min after playback. We continued recording even if males left the 7-m radius during either the playback or post-playback periods; recording ceased the moment the test subject left the 7-m radius any time after the 1-min post-playback period. During playback trials, we also quietly dictated the focal male's behavior, including distance from the speaker with each change of perch and the number of flights or flutter hops over the speaker, into the same microphone used to record focal males.

We subsequently reviewed trial recordings as spectrograms (settings as above) in Raven Pro sound analysis software (1.5; Charif and Waack 2010). For each trial, and while the focal male was within 7 m of the speaker, we counted the number of vocalizations, including songs and scolding calls (Fig. 3), and number of speaker flyovers. We also noted the distance of closest approach (i.e., perch) to the speaker (1-m resolution) and time spent within 7 m of the speaker. We interpret strong responses as those with many vocalizations and flyovers, and those with close approaches and more time spent within 7 m of the speaker.

Song characteristics. Spectrograms of our playback stimuli revealed that vireonid songs are structurally diverse, thus limiting the number of structural traits common to all species. Following Mejías et al. (2020), we used Raven Pro to measure four song traits applicable to all vireonid songs and that exhibit phylogenetic signal, including song duration, minimum and maximum peak frequency, and frequency modulation. Song duration was defined as the time from the start of the first song element to the end of the last element. To measure frequency traits, we used the "split border" function to split songs into 2-ms time bins and then automatically determined the peak frequency (i.e., frequency with the greatest energy) of each bin. Minimum and maximum peak frequency (Hz) were the 5th and 95th percentiles, respectively, of the peak frequency values from across all 2-ms time bins across the song (excluding silent periods between elements). Frequency modulation (Hz/s) was the cumulative absolute change in peak frequency across all consecutive 2-ms time bins

(excluding silent periods between elements), divided by the cumulative duration of all song elements. For species with two song variants in their playback sequences, we calculated the average between them for each playback sequence. We estimated the four song traits for each species by averaging values from all available playback sequences for that species. To ensure that average song traits were not affected by the number of individuals contributing recordings, we ran three simple linear regressions where each averaged song trait, per species, was regressed against the number of individuals sampled, per species; all were non-significant (i.e., $P > 0.05$).

Phylogenetic distance between Bermuda Vireos and playback species. To calculate the phylogenetic distance between Bermuda Vireos and each stimulus species, we added the Bermuda Vireo to the existing Vireonidae phylogeny (Slager et al. 2014, Mejías et al. 2020). To do this, we extracted total genomic DNA from 10 Bermuda Vireo blood samples with a QIAGEN DNeasy Blood and Tissue Kit following the blood protocol. The QIAGEN TopTaq master mix kit (Qiagen Inc, Valencia, CA, USA) was then used to amplify the mitochondrial ND2 locus. Amplifications were performed on a 25-µl solution containing 12.5-µl TopTaq master mix 2×, 0.4 µM of each ND2 primer (L5215 and H6313, Brumfield et al. 2007), and ~50–116 ng of the template DNA. Thermal cycling conditions were as follows: denaturation at 94°C for 30 s, followed by 40 cycles of 94°C for 30 s, annealing temperature range of 56° to 60°C for 45 s, and 72°C for 1 min. This was followed by a 10-min extension at 72°C. We sent samples to the Centre for Applied Genomics at the Hospital for Sick Children (Canada, http://www.tcag.ca/) for Sanger sequencing. The resulting chromatograms were observed, assembled, and edited in Geneious 7.1.8 (https://www.geneious.com, Kearse et al. 2012). We aligned the 10 Bermuda Vireo ND2 sequences with the full Vireonidae ND2 alignment from Slager et al. (2014) using MAFFT 7.271 (Katoh and Standley 2013), followed by manual refinement. We conducted a Bayesian inference in BEAST v2.5.2 (Bouckaert et al. 2014), tested for tree convergence in Tracer v1.7 (Rambaut et al. 2018), and generated a maximum clade
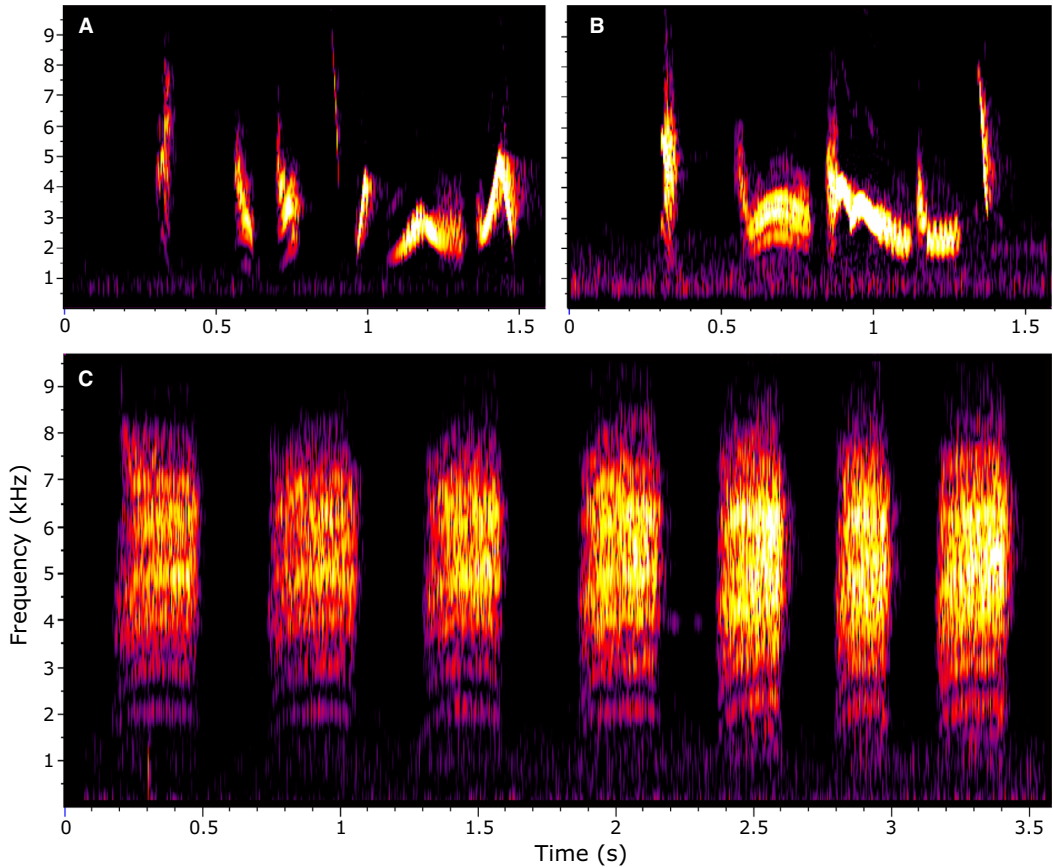
Fig. 3. Spectrograms depicting two kinds of vocalizations produced by Bermuda White-eyed Vireos during territorial encounters: discrete songs (Bradley 1980; A and B) and scolding calls (C). Discrete song is produced only by males, whereas scolding calls are produced by both sexes. The two discrete songs were recorded from the same male. Note the differences in element structure, representing two discrete song types. Bermuda Vireos sing discrete songs with eventual variety, repeating the same song type several times before switching to another. Each male produces approximately 6–10 discrete song types. Spectrograms were created using a Hamming window, 512-point fast Fourier transform, and 87.5% overlap.

credibility (MCC) tree in Tree Annotator v.2.5.2 (Bouckaert et al. 2014), all following Mejías et al. (2020). All 10 Bermuda Vireos sampled formed a monophyletic group sister to the North American White-eyed Vireo (*V. griseus*). Bayesian branch support values (posterior probabilities) ranged from 0.47 to 1 and were concordant with those on the same nodes in Fig. 1 of Slager et al. (2014). We pruned the Bayesian MCC tree in R using the *drop.tip* function in the *ape* package (3.5.3; Paradis and Claude 2004) to include only the 38 (59% of Vireonidae) vireonid species with playback sequences (Fig. 2). We

used the pruned Bayesian MCC tree to calculate the phylogenetic distances (i.e., patristic distance, substitutions per site; the sum of the lengths of the branches that link two species or subspecies in a tree) between the Bermuda Vireo and the other stimulus species using the *distTips* function in the *adephylo* package (3.5.3; Revell, 2012) in R.

Statistical analysis. All statistical analyses were conducted in R (3.5.2; R Development Core Team, 2008, R Foundation of Statistical Computing, Vienna, Austria). Although principal component analysis can be used to reduce the number of correlated

response variables, it is inappropriate for repeated measures data (Budaev 2010), particularly when the number of subjects is <30 (Jiang and Eskrdge 2000). Therefore, we assessed collinearity among our independent and dependent variables using Spearman's correlation tests and considered variables for exclusion if highly correlated (Spearman's rho ≥0.4). For our dependent variables, time within 7 m of the speaker was strongly correlated with the total number of vocalizations (N = 165, rho = 62). We chose to exclude time within 7 m of the speaker because it was based on estimated distances and thus prone to error. Our remaining response variables (i.e., number of vocalizations and speaker flyovers, and closest approach to speaker) showed low correlation (N = 165, all *rho* < 0.4) and were used as measures of response strength.

For our predictor variables, maximum peak frequency was strongly correlated with frequency modulation (Spearman's correlation: N = 165, rho = 0.85) so we excluded it from subsequent analyses. The remaining song traits (i.e., song duration, minimum peak frequency, and frequency modulation) were not correlated (N = 165, all rho ≤ 0.4). We therefore used these traits that all exhibit phylogenetic signal (Mejías et al. 2020) to estimate the overall acoustic distance between the structure of an average Bermuda Vireo song and the structure of each playback stimulus. To do this, we rescaled each song trait to between 0 and 1 and then treated these scaled traits as the x, y, and z axes of a three-dimensional acoustic space. We then calculated the Euclidean distance between each stimulus song and the mean Bermuda Vireo song in three-dimensional acoustic space (Simpson et al. 2021).

We used linear mixed models and generalized linear mixed models in the lme4 package (Bates et al. 2015) to test our prediction that males respond more strongly to the songs of more closely related species. We regressed each dependent variable (total vocalizations, number of speaker flyovers, and closest approach) against phylogenetic distance, which was included as a fixed effect in three separate models. If relationships were significant (see Results), we tested our second prediction that that these relationships could be explained by the acoustic distance between the Bermuda Vireo's song and stimulus songs. Specifically, we regressed each dependent variable against acoustic distance, which was included as a fixed effect in three separate models. For all six models, subject identity was included as a random factor to account for possible dependencies among repeated trials from the same subject. We also included trial number (1–11) as a covariate with fixed effects to account for any effect of trial order and habituation on responses.

To ensure that our method of attracting focal males using consubpecific calls did not prime subjects to respond weakly to subsequent heterospecific song (i.e., mismatched stimuli) and strongly to subsequent consubpecific song (i.e., matched stimuli), we re-ran our six statistical models, but excluded the trials corresponding to the consubspecific treatment. The models yielded similar results, and so we present results for the more comprehensive models that include the consubspecific treatment. We present the results for when the consubspecific treatment is excluded in Tables S3 and S4. Finally, we also ran a separate linear mixed effects model to determine whether acoustic distance (dependent) was related to singing style (fixed effect), again including subject identity as a random factor. The overall effect of singing style was assessed with the Anova function in the car package (Fox and Weisberg 2019), and pairwise comparisons among singing styles were conducted using Tukey's contrasts in the multcomp package (Hothorn and Bretz 2008). Due to our small sample size (N = 15 males), we chose not to include singing style in the models assessing vireo responses to playback because an additional categorical variable would reduce the statistical power needed to detect treatment effects related to our hypotheses.

For our primary statistical models, number of vocalizations and number of flyovers were modeled using generalized linear mixed models with a negative binomial distribution and Poisson distribution, respectively, and a log link. Closest approach was modeled using a linear mixed effects model. Because we tested three different measures of response strength, we controlled experimentwise type I error by applying a Bonferroni correction at the level

of the hypothesis (i.e., alpha = 0.05/3). Results pertaining to the hypotheses were therefore considered statistically significant where $P < 0.0167$.

We used the DHARMA package (Hartig 2020) to validate our statistical models. Its diagnostic tests, combined with our visual inspection of scaled residual plots, did not reveal any issues with the distribution of residuals, over- or underdispersion, frequency of outliers, or zero-inflation. We also simulated the responses of all six models and found strong agreement between the simulated data and our original data. Finally, using the *car* package, we calculated variance inflation factors (VIFs) to test for possible collinearity between the two predictor variables in each of the six models predicting vireo responses. VIFs greater than five indicate possible problems associated with collinearity (Zuur and Ieno 2010); our greatest VIF was 1.15 (Tables 1 and 2). Values are provided as means ± 1 SD.

RESULTS

The 38 species of vireonids used as playback stimuli produced songs with diverse structures (Table S2, Figs. 4 and 5). Average song traits, across the family Vireonidae, were 0.91 ± 0.75 s for duration, 2535 ± 471 Hz for minimum peak frequency, and 18129 ± 8798 Hz/s for frequency modulation. Compared to other vireos, songs of Bermuda Vireos were of intermediate duration (mean = 1.01 ± 0.22 s, $N = 10$) and had high frequency modulation (25,327 ± 5485 Hz/s, $N = 10$; Table S2, Figs. 4 and 5A and C).

We conducted 165 trials with 15 male Bermuda Vireos. The number of days needed to complete the 11 playback trials varied among males (mean = 22 ± 7.22 d, range = 11–39 d). Typical responses of males to playback included flutter hopping in the foliage while vocalizing within 7 m of the speaker. Overall, focal males produced 2797 vocalizations during 120 of the 165 trials, including 1663 songs during 110 trials and 1134 scolding calls during 29 trials. Focal males flew over the speaker 42 times during 19 trials and approached to an average minimum distance of 4.0 m from the speaker.

The strength of Bermuda Vireo responses increased with decreasing phylogenetic distance to the stimulus species (Fig. 6, Table 1). When responding to songs of more closely related species, Bermuda Vireos produced more vocalizations, flew over the speaker more often, and approached the speaker more closely (Table 1). Excluding consubspecific stimuli trials, the relationship between the number of vocalizations and

Table 1. Relationships between the strength of response of 15 male Bermuda Vireos to playbacks and the phylogenetic distance between Bermuda Vireos and the playback stimulus species.

Model	Factor	Coefficient (±SE)	Test stat	P	VIF
Total vocalizations (GLMM)	Intercept	3.87 ± 0.42	9.3	**<0.0001**	
	Phylogenetic distance	−0.03 ± 0.01	−2.5	**0.012**	1.15
	Trial number	−0.14 ± 0.04	−3.1	**0.0019**	1.15
Flyovers (GLMM)	Intercept	0.05 ± 0.72	0.1	0.94	
	Phylogenetic distance	−0.16 ± 0.02	−6.5	**<0.0001**	1.04
	Trial number	−0.23 ± 0.07	−3.5	**0.0004**	1.04
Closest approach (LMM)	Intercept	1.58 ± 0.48	3.3	**0.0014**	
	Phylogenetic distance	0.09 ± 0.02	4.8	**<0.0001**	1.02
	Trial number	0.20 ± 0.05	4.1	**<0.0001**	1.02

Model results for trial order are also presented. Statistically significant *P* values, relative to an adjusted alpha of 0.0167, are in bold. All variance inflation factors (VIFs) were <5, indicating low collinearity among model predictor variables.

$N = 165$ trials distributed evenly among 15 subjects. Random effects (variance ± SD) for total vocalizations: 0.19 ± 0.44; flyovers: 2.93 ± 1.71; closest approach: 0.44 ± 0.66, residual = 4.07 ± 2.02. Test statistic for generalized linear mixed model (GLMM) was *z*; test statistic for linear mixed model (LMM) was *t*.

Table 2. Relationships between the strength of response of 15 male Bermuda Vireos to playbacks and the acoustic distance between Bermuda Vireos and the playback species.

Model	Factor	Coefficient (±SE)	Test statistic	*P*	VIF
Total vocalizations (GLMM)	Intercept	4.12 ± 0.43	9.6	**<0.0001**	
	Acoustic distance	−2.45 ± 0.78	−3.2	**0.0016**	1.07
	Trial number	−0.13 ± 0.04	−3.1	**0.0019**	1.07
Flyovers (GLMM)	Intercept	0.56 ± 0.76	0.7	0.47	
	Acoustic distance	−7.95 ± 1.40	−5.7	**<0.0001**	1.04
	Trial number	−0.22 ± 0.06	−3.7	**0.0002**	1.04
Closest approach (LMM)	Intercept	1.99 ± 0.55	3.6	**0.0004**	
	Acoustic distance	2.71 ± 1.02	2.7	**0.0083**	1.02
	Trial number	0.19 ± 0.05	3.7	**0.0003**	1.02

Model results for trial order are also presented. Statistically significant *P* values, relative to an adjusted alpha of 0.0167, are in bold. All variance inflation factors (VIFs) were <5, indicating low collinearity among model predictor variables.
N = 165 trials distributed evenly among 15 subjects. Random effects (variance ± SD) for total vocalizations: 0.21 ± 0.46; flyovers: 3.10 ± 1.76; closest approach: 0.45 ± 0.67, residual = 4.46 ± 2.11. Test statistic for generalized linear mixed model (GLMM) was *z*; test statistic for linear mixed model (LMM) was *t*.

phylogenetic distance became non-significant, although all other effects remained the same with respect to statistical significance (Table S3). The strength of responses also increased with decreasing acoustic distance between the songs of Bermuda Vireos and the playback stimulus (Fig. 6, Table 2). In response to more similar songs, focal males uttered more vocalizations, flew over the speaker more often, and approached the speaker more closely (Table 2). Excluding trials involving con-subspecific stimuli, the relationship between closest approach and acoustic distance became non-significant, although all other effects remained the same with respect to statistical significance (Table S4). In all analyses, the strength of responses was negatively related to trial order, with significantly more vocalizations and flyovers, and closer approaches to the speaker, during earlier trials (Tables 1 and 2, Tables S3 and S4). Lastly, acoustic distance between the songs of Bermuda Vireos and those of stimulus species differed with singing style (linear mixed effects model, ANOVA: $X^2_2 = 50.7$, $P < 0.0001$). Species singing with immediate variety had significantly greater acoustic distance than those singing with eventual variety (pairwise comparison: $Z = 2.1$, $P = 0.0382$), and species singing with no variety had greater acoustic distance than species singing with either eventual

($Z = 7.0$, $P < 0.0001$) or immediate ($Z = 4.8$, $P < 0.0001$) variety.

DISCUSSION

We show that male Bermuda White-eyed Vireos distinguish among the songs of vireos based on phylogenetic distance. This relationship was expected because some vireonid song traits exhibit phylogenetic signal (Mejías et al. 2020). Using three phylogenetically conserved song traits, we derived a single measure of acoustic distance and found that it explained the responses of male Bermuda Vireos. Collectively, our results suggest that acoustic similarity, because of phylogenetic relatedness, plays a significant role in how songbirds perceive and respond to song.

Three limitations to our study are worth addressing. First, we found habituation among subjects, with responses to stimuli decreasing during later trials (Dong and Clayton 2009). Although birds are known to habituate after multiple exposures to the same stimuli (Verner and Milligan 1971, Harris and Haskell 2013), finding such strong habituation when the stimulus species changed between trials was unexpected. Focal males may have habituated to our playback apparatus or to lure calls, resulting in weaker responses in later trials. Second, male status
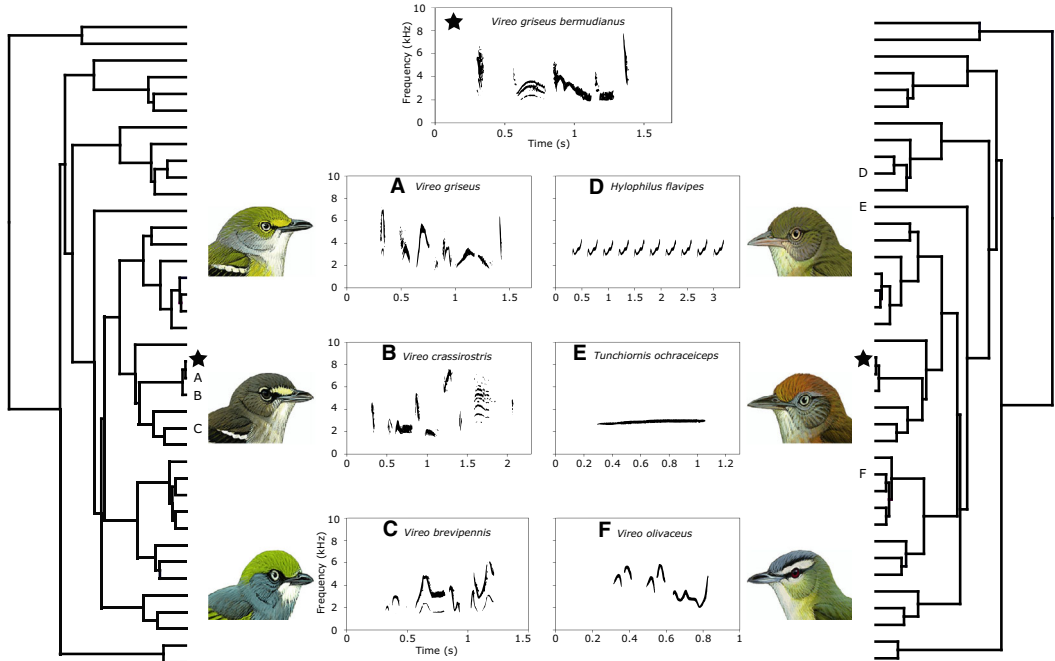
Fig. 4. Spectrogram of a Bermuda White-eyed Vireo song coupled with spectrograms and illustrations of six other vireonid species and their respective phylogenetic positions. Time on spectrogram x-axes is variable to accommodate differences in song lengths. Species illustrated include three heterospecific vireonids (A, B, and C) whose phylogenetic position and song structure elicited strong vocal and physical responses in Bermuda Vireos ("star"), and examples of vireonids (D, E, and F) that did not. Vireonids on the left are more closely related to the Bermuda Vireo and have similar acoustic structure to this subspecies, whereas vireonids on the right show greater phylogenetic distances and dissimilarity in song structure to the Bermuda Vireo. Illustrations reproduced with permission of Lynx Edicions; Brewer, D., Orenstein, R., & Bonan. A. (2019). Vireos (Vireonidae). In J. del Hoyo, A. Elliott, J. Sargatal, D. A. Christie, & E. de Juana (Eds.). Handbook of the Birds of the World. Vol. 15. Weavers to New World Warblers. Lynx Edicions, Barcelona, Spain.

(i.e., paired or unpaired and whether they have nests with eggs or young) can affect singing rates (Liu and Kroodsma 2007), and a subsequent study revealed that male Bermuda Vireos that were building nests, incubating eggs, or feeding young sang significantly less than males without nesting duties (Mejías and Wilson, unpubl. data). We did not determine the pairing or breeding status of males in our study so cannot assess their potential effects on responses by focal males. Lastly, we found that the singing style of vireos, a variable that was not part of our hypotheses, was strongly correlated with acoustic distance. Species that sang with eventual variety, like Bermuda Vireos, had songs most similar to those of Bermuda Vireos. We were unable, therefore, to disentangle the correlated effects of acoustic distance and singing style on the responses of male Bermuda Vireos.

Our results support the hypothesis that Bermuda Vireos are sensitive to phylogenetically conserved acoustic traits, as also shown in *Troglodytes* wrens (Sosa-López and Martínez Gómez 2016) and *Streptopelia* doves (de Kort and ten Cate 2001). Similarly, James (1976b) found that Warbling Vireos responded strongly to conspecific songs and weakly to those of two vireos with different song structures, i.e., Red-eyed and Yellow-throated vireos. The heightened responses to songs of consubspecifics and closely related heterospecifics may facilitate speciation through reproductive isolation. Hybridization is commonly reported in some songbird
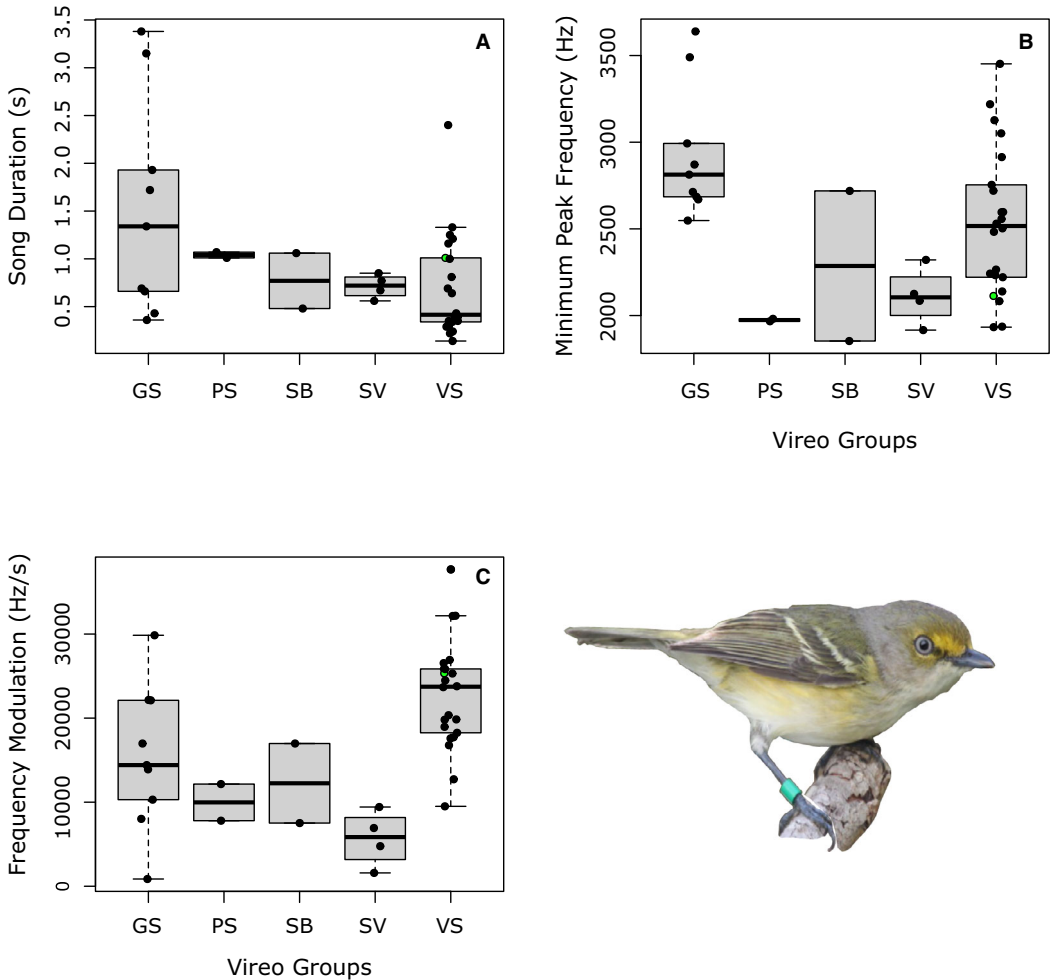
Fig. 5. Boxplots showing the average song duration (A), minimum peak frequency (B), and frequency modulation (C) of vireonid species' songs across the five following groups: Greenlets (GS), Peppershrikes (PS), Shrike-babblers (SB), Shrike-vireos (SV), and Vireos (VS). The Bermuda Vireo is depicted within the Vireo group with a green circle. In general, compared to the other species, Bermuda Vireo songs had an intermediate duration, lower minimum peak frequency, and higher frequency modulation. Photograph of the Bermuda White-eyed Vireo, colour-ringed by the author on Nonsuch Island, was taken by Jonathan Pierce.

genera, like new-world warblers (Graves 1996, Irwin et al. 2009, Toews et al. 2018), but not among vireos. The few reported cases usually involve vagrancy (e.g., Blundell and Kus 2011, McKee et al. 2016). Similarly, Battey and Klicka (2017) reported introgression among vireonids in the Red-eyed Vireo complex, but these levels were low and they proposed that this hybridization reflected historic rather than present-day gene flow. Thus, divergence in vireo songs may be an effective

prezygotic reproductive isolation barrier that reduces hybridization.

Heterospecific vireonid competition is well-documented in continental habitats. For example, Red-eyed Vireos and Philadelphia Vireos (Rice 1978, Robinson 1981) breed in sympatry in parts of Canada, where their similarities in foraging behavior (Robinson 1981) and song structures (Barlow and Power 1970, Mejías et al. 2020) are thought to drive their ecological competition (Robinson 1981).
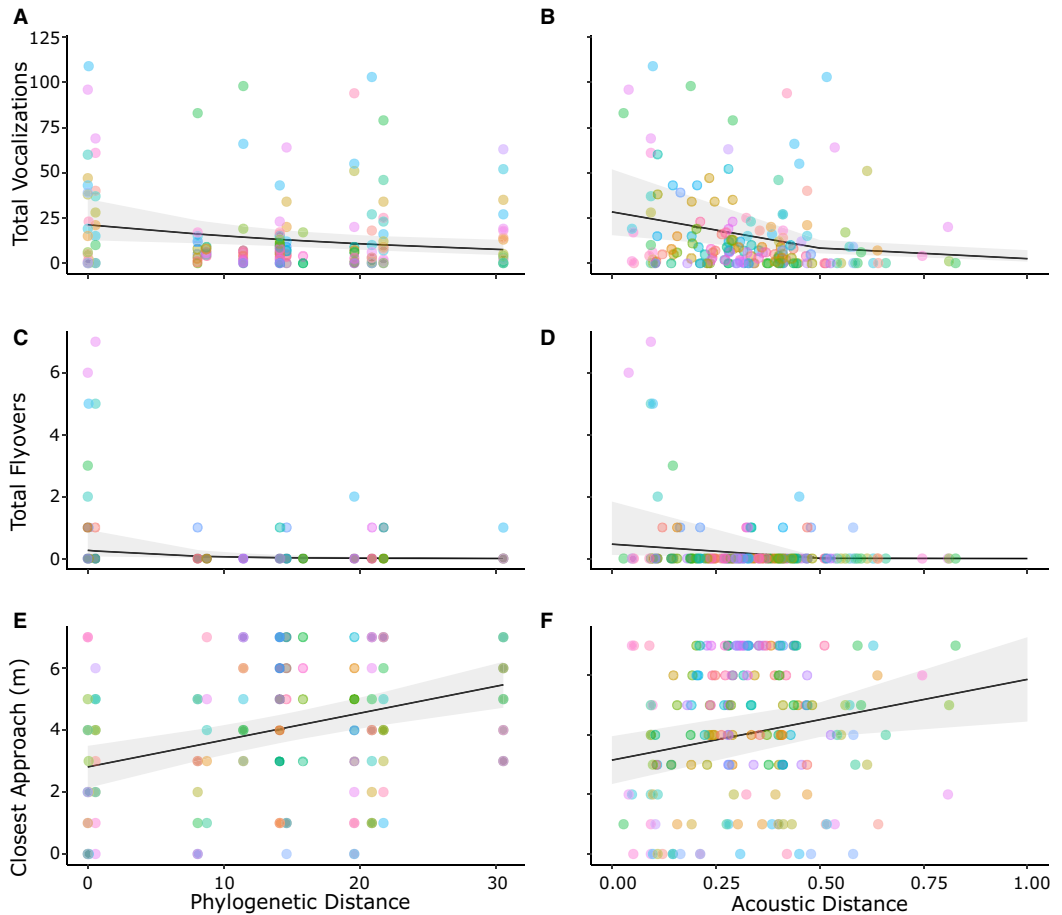
Fig. 6. Scatter plots depicting relationships of phylogenetic distance and acoustic distance with total vocalizations (A, B), total flyovers (C, D), and closest approach (E, F), respectively. Regression lines and their 95% confidence intervals (clouded, gray outlines) are based on estimated marginal means derived from the statistical models. Different colored dots correspond to different color-banded male Bermuda Vireos used as test subjects. A point corresponding to 260 vocalizations is not shown to better illustrate the relationships between number of vocalizations and patristic distance/acoustic distance (A, B). In general, both vocal displays and number of speaker flyovers significantly decreased with increasing patristic and acoustic distance. A positive relationship was found between closest speaker approach and increasing patristic and acoustic distance.

Bermuda Vireos, however, are exempt from heterospecific vireonid competition because they are the only viroenids that breed on the island. Despite their allopatry, our observations of male Bermuda Vireos showing stronger responses to congenerics with similar song structures and singing styles, such as White-eyed Vireos, Thick-billed Vireos, and Slaty Vireos, are notable because all three species live in semi-open, shrubby habitat (Mejías et al. 2020). This further supports the idea that these three species might compete with

Bermuda Vireos for ecological resources if they occurred on the island, making the responses of male Bermuda Vireos beneficial with respect to competitive exclusion.

At least two perceptual mechanisms may be important in avian song recognition, and disentangling the two depends in part on the extent of range overlap among species. In the first mechanism, receiver responses to songs are learned through previous experience with conspecifics and heterospecifics (Gill and Murray 1972, Matyjasiak 2004). However, we ruled out this

mechanism in our study by using the songs of allopatric species as stimuli. The second mechanism, more in line with our results, is an inherent ability to adjust responses based on acoustic similarity (Fallow and Gardner 2011). Specifically, songbirds use an "auditory template," a neuronal representation of song that guides song development (Soha 2017). Using this template, songbirds compare elements of heterospecific songs to those of their own song and adjust their responses accordingly. Receivers are expected to show stronger vocal and behavioral responses to songs that generally match their own, even songs of unfamiliar species. The responses of male Bermuda Vireos in our study suggest that they use an innate "template" to identify potential rivals based on acoustic similarity.

Our results, along with those of Mejías et al. (2020), provide compelling evidence that significant divergence in song structure and song perception is present among species in the family Vireonidae and that allopatric passerines have an innate ability to respond to heterospecific songs that most resemble their own. This parallel divergence between song structure and song perception likely contributed to speciation in this family. Thus, our results advance our understanding of how the phylogenetic history and song structure of signalers affects allopatric receivers, and how this may ultimately drive speciation.

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SUPPORTING INFORMATION

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

Table S1. Song measurements from 137 individuals from 38 species of male vireonids. Songs were collected from Macaulay (ML) and Xeno-canto (XC) libraries; songs from *V. griseus bermudianus* were recorded in the field by MM for this study. We viewed each recording as a waveform and spectrogram (Hamming window, FFT size = 512 samples, 87.5% overlap) using Raven Pro v1.5 sound analysis software (Charif et al., 2010).

Table S2. Song structure of 38 vireonid species and Bermuda Vireo subspecies used as stimuli in 165 playback trials to 15 male Bermuda Vireos. Also shown are the phylogenetic distances between each species and Bermuda Vireos.

Table S3. Relationships between the strength of response of 15 male Bermuda Vireos to playbacks and the phylogenetic distance between Bermuda Vireos and the playback stimulus species. Model results for trial order are also presented. Trials involving consubspecific stimuli have been excluded (N = 15). Statistically significant P-values, relative to an adjusted alpha of 0.0167, are in bold. All variance inflation factors (VIFs) were < 5, indicating low collinearity among model predictor variables.

Table S4. Relationships between the strength of response of 15 male Bermuda Vireos to playbacks and the acoustic distance between Bermuda Vireos and the playback species. Model results for trial order are also presented. Trials involving consubspecific stimuli have been excluded (N = 15). Statistically significant P-values, relative to an adjusted alpha of 0.0167, are in bold. All variance inflation factors (VIFs) were < 5, indicating low collinearity among model predictor variables.