

Song divergence at the edge of Amazonia: an empirical test of the peripatric speciation model

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The evolutionary divergence of mating signals provides a powerful basis for animal speciation. Divergence in sympatry strengthens reproductive isolation, and divergence in allopatry can reduce or eliminate gene flow between populations on secondary contact. In birds, the first of these processes has empirical support, but the second remains largely hypothetical. This is perhaps because most studies have focused on oscine passerines, whose song learning ability may reduce the influence of vocalizations in reproductive isolation. In suboscine passerines, the role of learning in song development is thought to be minimal, and the resultant signals are relatively fixed. To investigate the role of song in the early stages of peripatric speciation, we therefore studied a suboscine, the chestnut-tailed antbird *Myrmeciza hemimelaena*. We recorded male songs in a natural forest island (isolated for < 3000 years) at the southern fringe of Amazonia, and at two nearby sites in continuous forest. A previous study found the isolated population to be weakly differentiated genetically from the ancestral population suggesting that peripatric speciation was underway. In support of this, although we detected minor but significant differences in song structure between each site, the most divergent songs were those of island birds. On simulating secondary contact using playback, we found that pairs from the forest island responded more strongly to island (i.e. local) songs than to those from both non-island sites, and vice versa. This pattern was not observed in pairs from one non-island site, which responded with equal strength to local songs and songs from the other non-island site. Island females were more likely to approach and sing after hearing local male songs, rather than songs from the non-island populations, and vice versa; non-island females did not appear to discriminate between local songs and those from the other non-island site. These findings are consistent with the idea that vocal divergence arising in small populations at the edge of Amazonia may result in partial reproductive isolation when contact is resumed. They also suggest the possibility that song divergence in peripatry may, after much longer time-frames, act as a barrier to gene flow in suboscines, perhaps because of an inability to learn or recognize divergent songs on secondary contact. © 2007 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2007, 90, 173–188.

ADDITIONAL KEYWORDS: antbirds – geographical variation – peripatry – song – suboscine birds.

INTRODUCTION

Mating signals are powerful isolating mechanisms, and their evolutionary divergence has long been implicated in the speciation process (West-Eberhard, 1983). Classical theory proposes that signals diverge as a by-product of genetic differentiation accumulated over

prolonged periods of allopatry (i.e. through drift; Mayr, 1942, 1963), and that divergent signalling systems stabilize when populations resume contact to allow unambiguous species recognition (i.e. by reinforcement; Dobzhansky, 1951; Butlin, 1995; Servodio & Noor, 2003). According to this hypothesis, mating signals diverge mainly in the sympatric phase (i.e. late in the speciation process), and studies demonstrating pronounced signal divergence between closely-related sympatric species are consistent with this view (Servodio & Noor, 2003). An alternative and more controversial idea is that signals and associated preferences

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diverge at an early stage of speciation (e.g. by sexual selection and/or ecological adaptation), such that pre-mating isolation precedes genome-wide differentiation (West-Eberhard, 1983; Coyne, 1992). Although both theories identify a role for the divergence of mating signals in speciation, the former sees it as a product and the latter as a driving force of the process.

The idea that mating signal divergence drives speciation is supported by studies of African cichlid fish (Galis & Metz, 1998), *Drosophila* spp. fruit flies (Wu *et al.*, 1995), and *Heliconius* spp. butterflies (Jiggins *et al.*, 2001). By contrast, a relatively minor contribution stems from the study of birds (but see Uy & Borgia, 2000), despite the fact that their songs function in mate-choice, species recognition (Catchpole & Slater, 1995), and have been shown in some taxa to prevent hybridization among species that can produce viable offspring (Baker & Boylan, 1999). Although the idea that preferences for local song dialects could lead to reproductive isolation is relatively old (Marler & Tamura, 1962; Baker, 1975), consistent patterns of assortative mating based on intraspecific variation in birdsong structure are few (Slabbekoorn & Smith, 2002a). This may reflect the fact that, with the exception of some work on *Streptopelia* doves (de Kort, den Hartog & ten Cate, 2002a, b; de Kort & ten Cate, 2004), most studies have focused on oscine passerines; for example, white-crowned sparrow *Zonotrichia leucophrys* (Baker, 1975); Darwin's finches *Geospiza* spp. (Grant & Grant, 1996); song sparrow *Melospiza melodia* (Searcy *et al.*, 1997); blue tit *Parus caeruleus* (Doutrelant *et al.*, 2000); greenish warbler *Phylloscopus trochiloides* (Irwin, 2000); and flycatchers *Ficedula* spp. (Haavie *et al.*, 2004). A defining characteristic of oscines is that they learn their songs and song preferences, a trait that might promote speciation in mimetic brood parasites, such as African indigobirds *Vidua* spp. (Sorenson, Sefc & Payne, 2003; Beltman, Haccou & ten Carte, 2004), but which could reduce the efficacy of song as a pre-mating barrier because copying between closely-related forms (Helb *et al.*, 1985) could lead to hybridization (Grant & Grant, 1997).

One way of bypassing this difficulty it is to study inter- and intraspecific geographical variation in the songs of birds that do not learn their vocalizations. In this regard, Neotropical suboscines are good subjects: their songs are simple, stereotypic, and detailed studies in one major suboscine family [the tyrant flycatchers (Tyrannidae)] have found no evidence of learning (Kroodsma, 1984), nor the forebrain cell clusters that control song acquisition in oscines (Kroodsma & Konishi, 1991). Although song-learning may occur in the Cotingidae (Kroodsma, 2004), recent work in another suboscine family, the antbirds (Thamnophilidae), has shown close concordance between vocal and genetic

geographical variation, suggesting that, in this family, song structure is an inherited rather than learned character (Brumfield, 2005; Isler, Isler & Brumfield, 2005; Remsen, 2005). Even if some evidence of learning is eventually demonstrated in the antbirds, the structural simplicity of their songs, low levels of geographical variation, and absence of dialects (Isler *et al.*, 1999, 2001; Isler, Isler & Brumfield, 2005) all indicate that it will be much less than that found in oscines, making suboscines arguably better subjects for examining the role of song in speciation.

Despite this, of the relatively few studies that have examined geographical variation in suboscine songs, none has investigated the circumstances liable to produce divergence in song structure, or whether divergent songs could result in reproductive isolation. Given the diversity of suboscines, which account for at least 20% (1151 species) of all passerine species (Sibley & Monroe, 1990), and the lack of consensus regarding the processes generating high levels of tropical biodiversity (Knapp & Mallet, 2003), such an analysis is overdue.

In Amazonia, where biodiversity reaches its zenith (Myers *et al.*, 2000), and where, at any given site, suboscines account for over half the passerine diversity and around one-fifth of the total avifaunal diversity, the dominant paradigm of vertebrate diversification is allopatric speciation (Mayr & O'Hara, 1986; Terborgh, 1992; Bush, 1994; Haffer, 1997; Hall & Harvey, 2002; Aleixo, 2004). Allopatric speciation can be subdivided into two models: 'vicariant' and 'peripatric' (*sensu* Mayr, 1982). In the former, reproductive isolation evolves in large geographically-isolated populations. In the latter, it evolves either when a few individuals colonize isolated habitats or when small populations become geographically isolated (Coyne & Orr, 2004).

Both models makes two key predictions: (1) climatic and/or tectonic events resulted in fragmentation of populations locally (in peripatric speciation) and/or regionally (in vicariant speciation), and (2) the reproductive traits of isolated populations diverged such that secondary contact did not result in interbreeding. In support of the first prediction, there is evidence that habitat fragmentation occurred in Amazonia, at least peripherally, through a variety of vicariance events mostly predating the Pleistocene (Colinvaux, 2005). These included marine incursions (Lovejoy *et al.*, 1998), climatic cooling (Colinvaux *et al.*, 1996), reduced precipitation (Haffer, 1969; Whitmore & Prance, 1987), and the formation of riverine barriers (Wallace, 1852; Ayres & Clutton-Brock, 1992; Aleixo, 2004). Support for the second prediction derives purely from the study of biogeographical patterns (Mayr & O'Hara, 1986): direct experimental tests have never been carried out. Although the role of song

divergence in premating isolation has been studied in the context of peripatric speciation of birds on oceanic islands (Grant & Grant, 2002a, b), parallel research in small continental forest islands has not been undertaken. Indeed, the only evidence for peripatric speciation on continents is from plants and land snails (Coyne & Orr, 2004); this mode of speciation remains unexplored in birds.

We therefore investigated the effects of geographical isolation on the songs of the chestnut-tailed antbird (*Myrmeciza hemimelaena*), a suboscine inhabiting the Amazonian understory. We studied this species in a natural forest island at the southern fringe of Amazonia, and at two sites in adjacent continuous forest (from which the island is thought to have been isolated for 1000–3000 years). All three populations belong to the same subspecies (Isler *et al.*, 2002). However, previous work using mtDNA showed the isolated population to be weakly differentiated from the ancestral population (Bates, 2000, 2002). This suggested reduced gene flow to and from the forest island and hinted that peripatric speciation might be underway. In support of this, our analysis of male songs detected subtle yet significant levels of differentiation in structure. As a test of premating isolation, we used playback to simulate secondary contact between populations, following the model of previous avian speciation studies (Irwin, Bensch & Price, 2001; Grant & Grant, 2002b). In so doing, we replicated an event that probably occurred repeatedly in Amazonia, as demonstrated by the large number of sympatric suboscine species that are morphologically cryptic yet vocally distinct (Isler *et al.*, 1998; Zimmer, Whittaker & Oren, 2001; Seddon, 2005).

By working in and around a forest island at the interface between southern Amazonian humid forest and the *cerrado* grasslands of the Brazilian Shield, we investigated whether a small geographically-isolated and weakly genetically-differentiated population has diverged, or has begun the process of divergence, in a trait likely to be involved in reproductive isolation. In so doing, we examine the role of song in the early stages of suboscine speciation, and carry out the first experimental test in birds of a key prediction of the peripatric model of faunal diversification in Amazonia.

MATERIAL AND METHODS

STUDY SPECIES

The antbird family (Thamnophilidae, Order Passeriformes), one of the major groupings of Neotropical suboscines, contains at least 209 species (Zimmer & Isler, 2003). Our study centres on one of these, the chestnut-tailed antbird (i.e. *M. hemimelaena*). Recently thought to comprise a single species with two

races, the *M. hemimelaena* complex is now known to contain at least two cryptic species that occur sympatrically in north Peru, and four subspecies, all virtually identical in morphology and plumage but with divergent songs (Isler *et al.*, 2002). The focus of the present study is *Myrmeciza hemimelaena pallens*, a subspecies found from central Brazil to eastern Bolivia.

The chestnut-tailed antbird is a widespread, medium-sized (length 11–12 cm, weighing 14.5–16.5 g), sexually dimorphic, and socially monogamous (Zimmer & Isler, 2003) resident of Amazonia. Pairs maintain year-round territories (Terborgh *et al.*, 1990) and their offspring have low levels of dispersal (Bates, 2000). In common with many antbirds, males and females produce loud sex-specific vocalizations on a daily basis, consisting of multiple notes delivered in a stereotyped pattern. Because this differs from the traditional, rather narrow definition of song (i.e. complex male vocalizations used in mate-advertisement; Catchpole & Slater, 1995), the term ‘loudsong’ has been coined (Willis, 1967; Zimmer & Isler, 2003). Although we employ this term for the sake of consistency with previous work, antbird loudsongs are produced in both territorial and sexual contexts and they are probably functionally analogous to ‘songs’. The highly energetic response of resident male antbirds to playback of conspecific signals indicates that loudsongs function in territory defence, whereas the fact that they are sexually dimorphic and elicit highly sex-specific responses (Bard *et al.*, 2002; Seddon & Tobias, 2006) strongly suggests that they are subject to sexual selection. Even though antbirds have long-term, year-round pair-bonds, and hence may rarely advertise for mates, it is likely that the vocal signal used for mate attraction is the same as (or a slight variation upon) the usual loudsong, as found in dusky antbirds *Cercomacra tyrannina* (Morton, 1996).

Although we did not colour-ring individuals, the points at which experiments were carried out and recordings made were marked with coded flagging; no further experiments or recordings were made from the same pair. Chestnut-tailed antbirds are highly territorial, sedentary and vocal, and we were able to delimit territories by tracking the movement of singing birds and marking the location of counter-singing individuals. Where territory boundaries were less clear, we ignored any male heard singing within 100 m of our flagged locations. Given the small size of known territories, this safeguarded against pseudoreplication.

STUDY SITES

Fieldwork was carried out in October to December 2002 on and near the Serranía de Huanchaca, an iso-

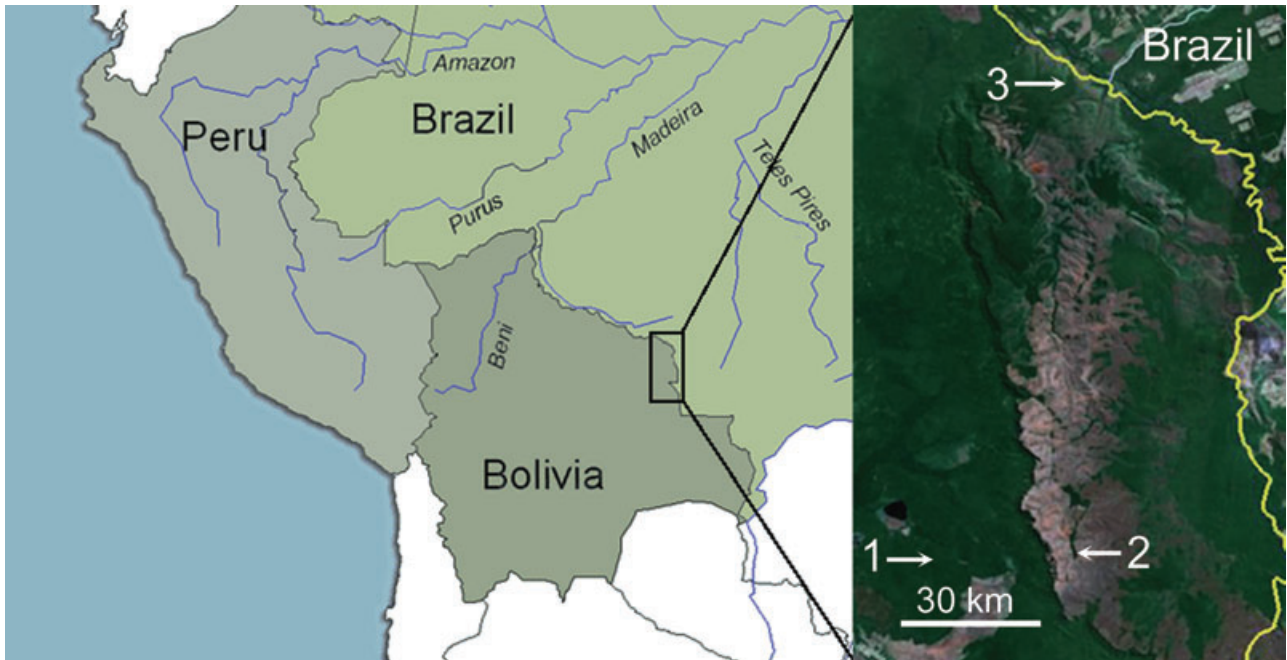


Figure 1. Location of study sites in Amazonia. The inset is a satellite image of the Serranía de Huanchaca in the Department of Santa Cruz, Bolivia. Site 2 (Huanchaca II: 14°34'S, 60°40'W) is a 350-ha forest island located on top of the plateau. Sites 1 (Los Fierros: 14°30'S, 61°00'W) and 3 (Lago Caiman: 13°36'S, 60°55'W) lie in continuous forest just below the plateau to the south-west and north, respectively.

lated 275 km² sandstone escarpment rising *c.* 300 m from the surrounding forest in Noel Kempff Mercado National Park, Bolivia (Fig. 1). We worked in a 350-ha forest-island on top of the plateau (Huanchaca II) and at two nearby sites within 200 km of each other (Los Fierros and Lago Caiman) in continuous forest at the south-western and northern bases of the plateau, respectively. The isolated population is thought to contain approximately 50 pairs of chestnut-tailed antbirds.

Huanchaca II is a fragment of intact, moist gallery forest isolated from the nearest forest by 15 km of *cerrado*, a complex savanna ecosystem occurring across the southern edge of Amazonia (Goodland, 1971). Differing greatly in physiognomy, and sharing few plant and animal species (for birds, see da Silva, 1995), the *cerrado* surrounding the forest island is likely to act as a strong deterrent to dispersal for sedentary forest understory species (Bates, 2002). Although there are several forest islands on top of the plateau, fieldwork was restricted to Huanchaca II because it is the only easily accessible island that is completely isolated from forest; others are interconnected by gallery forest through which antbirds could potentially disperse. For full descriptions of the three study sites, see Killeen & Schulenburg (1998).

ACOUSTIC ANALYSIS

A Sennheiser ME66-K3U microphone and a Sony TC-D5 Pro II were used to record loudsongs onto 60-min TDK metal tapes. Using Avisoft SASLabPro, version 4.15 (Raimund Spect), loudsongs were digitized and audiospectrograms generated. Using only high-quality recordings (i.e. those with low background noise made within 5–15 m of vocalizing birds), loudsongs were described quantitatively by taking a variety of temporal and frequency measurements (Fig. 2). To achieve maximum resolution (1.3 ms), temporal features were taken from audiospectrograms of recordings sampled at 24 kHz using standard broadband (320 Hz) filter settings on Avisoft (FFT = 512, Frame = 55%, Window = FlatTop, Overlap = 93.75%; Fig. 2A). To maximize frequency resolution (7 Hz), audiospectrograms were also produced from recordings digitized at 8 kHz using standard narrow-band (59 kHz) filter settings (FFT = 1024, Frame = 50%, Window = FlatTop, Overlap = 96.87%; Fig. 2B).

Our sample comprised 211 high-quality loudsongs from 45 males. For each male, at least three loudsongs were chosen for analysis (mean = 4.4 ± 1.4 loudsongs per male). Given that variation between individuals exceeded that within individuals for all acoustic mea-

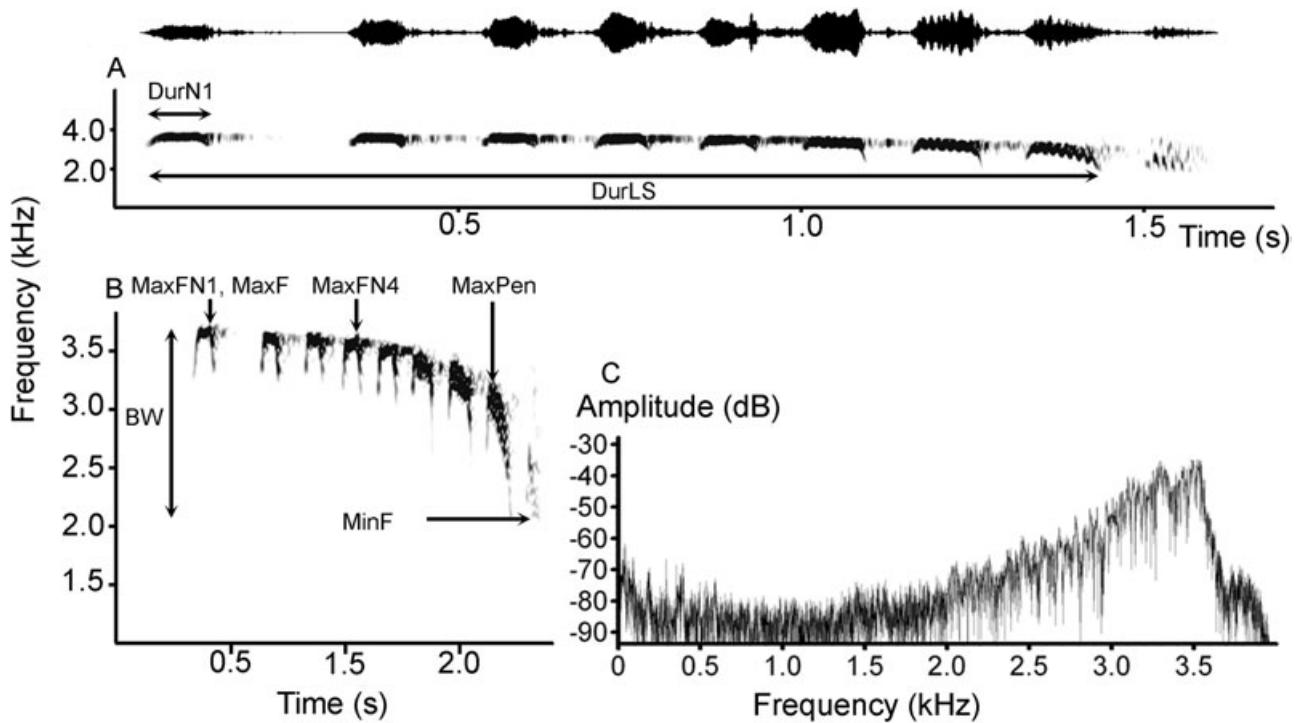


Figure 2. Annotated audiospectrograms of a single male loudsong recorded at Lago Caiman, produced using (A) broadband and (B) narrowband settings on Avisoft from recordings digitized at 24 kHz and 8 kHz, respectively. Loudsong structure was quantified using on-screen cursors to measure the following time (s) and frequency (Hz) traits, moving in increments of 1.3 ms and 7 Hz, respectively: (i) duration of loudsong (excluding final raspy note; DurLS); (ii) number of notes per loudsong; (iii) pace (number of notes per s); (iv) duration of first note (DurN1); (v) maximum frequency of first note (MaxFN1); (vi) maximum frequency of fourth note (MaxFN4); (vii) maximum frequency of penultimate note (MaxPen); (viii) maximum frequency of loudsong (MaxF); (ix) minimum frequency of loudsong (MinF); (x) bandwidth of loudsong (BW); (xi) first to fourth note frequency shift (maximum frequency of fourth note divided by max frequency of first note); (xii) fourth to penultimate note frequency shift (max frequency of final note divided by maximum frequency of fourth note). Because of reverberation between and above notes, other variables could not be measured reliably. C, average power spectrum, showing the energy distribution with respect to frequency.

tures ($F_{7,47} > 6.71$, $P < 0.0001$) for the eight individuals from which six to ten loudsongs were sampled, three loudsongs was sufficient to encompass intraindividual variation.

PLAYBACK EXPERIMENTS

We used playback to assess whether pairs distinguished between local but non-neighbouring (homotypic) loudsongs, and those recorded in nearby populations (heterotypic loudsongs). To prepare stimuli for these experiments, we first viewed broadband audiospectrograms of stimulus songs with Avisoft to ensure that there was no background noise in the frequency range of chestnut-tailed antbird songs. We then normalized their amplitude, and digitally filtered them to remove low and high frequency background noise (FIR bandpass filter set between 1–8 kHz). Stimulus sound ('wav') files, 1-min long, were then created using Avisoft. Each consisted of the same song

repeated every 15 s, matching the natural song rate of four songs per minute. Stimulus files were then burned onto 52 × CD-R compact discs. Where possible, stimulus loudsongs were made by males singing alone under natural conditions.

Playbacks were conducted in November and December 2002 at 06.00–11.00 h on a total of 57 pairs: 17 pairs at Huanchaca II, 15 at Los Fierros, and 24 Lago Caiman. Each pair received two playback treatments separated by an interval of 2–3 days to minimize habituation and any seasonal effects: (1) local homotypic and (2) heterotypic loudsongs from nearby populations. Ordering effects were counteracted by playing the local cut first to half of the pairs, and the heterotypic cut first to the remainder. However, to minimize observer bias, the source of cuts was encrypted on the CDs and we were unaware of the order of presentation when carrying out experiments.

We used unique sets of male loudsongs as stimuli for each pair to avoid pseudoreplication (Kroodsma,

1989). All playbacks were given from the same location and at a similar time of day (to within 1 h). To avoid problems of habituation and non-independence of trials, pairs on adjacent territories were not tested on the same day. Although no evidence of neighbour-stranger recognition was found in the spotted antbird *Hylophylax naevioides* (Bard *et al.*, 2002), it does occur in another species of suboscine, the alder flycatcher *Empidonax alnorum* (Lovell & Lein, 2004). In recognition of neighbours confounding our results, we ensured that all pairs received songs recorded from birds at least two territory diameters away (> 200 m).

Each trial lasted 10 min (1 min of playback and 9 min of silence). During the first minute, songs were played during through a Sony SRS-58 loudspeaker connected to a portable Sony Walkman CD player. The loudspeaker was placed at least 30 m from a territory boundary, approximately 0.2 m up and facing the subjects, which were always located 15–20 m away. Peak sound pressure level was adjusted to approximate that of natural loudsongs (i.e. 60 dB SPL at 10 m; mean amplitude \pm SE = 59.2 \pm 6.7 dB, $N = 9$ males) measured with an Aadastra hand-held analogue sound level meter (set at 'C' weighting and fast response). Experiments began only after both the subject and its neighbour had been silent for 5 min. The timing of trials started at the beginning of the first loudsong of the playback.

QUANTIFYING RESPONSE TO PLAYBACK

During the 9 min after playback had ceased, one observer used a tape-recorder to record as much of the vocal response as possible, and a second observer used a Sanyo Dictaphone to record the location and behaviour of subjects. We quantified male response by recording several continuous measures: (1) the time from the start of playback to the first visible flight towards the speaker (latency to approach, s); (2) the closest distance (m) to the speaker; (3) the time (s) spent < 10 m from the speaker; (4) the time from the start of playback to the first loudsong (latency, s); and (5) the duration (s) of the first bout of singing following playback; bouts were defined as a series of loudsongs between which all intervals were less than 20 s (twice the average intersong interval). In addition, we recorded (6) whether or not the white interscapular feathering was exposed and (7) whether or not the male flew around the speaker in short rapid flights; the latter two measures indicated especially high levels of territorial aggression (Robinson & Terborgh, 1995). The female always responded less aggressively and less obtrusively than the male, and thus we were only able to record: (1) whether or not she flew to < 10 m of the speaker and (2) whether or not she sang within 10 min from the start of playback.

STATISTICAL ANALYSIS

Males were given a mean value for each acoustic variable measured from their loudsongs. Number of notes per loudsong and pace were square-root transformed; all other loudsong parameters were log-transformed. Residual diagnostics revealed that four variables failed to meet parametric assumptions of error normality and constant variance: maximum frequency of first note, maximum frequency of fourth note, minimum frequency of loudsong, and fourth to penultimate note frequency shift. These were therefore excluded from parametric analyses. Because many of the acoustic variables were intercorrelated, we carried out principal component analysis to reduce the separate measures to three uncorrelated synthetic variables (PC1, PC2 and PC3). To test for intersite loudsong differentiation, we used Kruskal–Wallis tests in which separate measures and PC scores were the dependent variables, and location was the independent variable. Experiment wide error rates were adjusted with sequential Bonferroni tests (Rice, 1989), and Kruskal–Wallis tests were followed by post-hoc multiple comparison tests to examine pairwise differences. To identify which acoustic features best discriminated between loudsongs island and non-island loudsongs, and may therefore have formed the basis of the discrimination revealed by playbacks, we carried out discriminant function analyses (DFA), using F -tests (Wilks' λ) to examine whether the overall discriminant model was significant. Canonical structure matrices reveal the Pearson correlation between a given independent variable and the discriminant scores associated with a given discriminant function (Statsoft, 2003). These were used to show the order of importance of the discriminating variables by total correlation as well as the relative importance of each variable on each discriminant function. We tested the generality of the classification using cross-validation to estimate error rates. This method generates a discriminant function by withholding one observation at a time and then classifying that observation (Bard *et al.*, 2002; Westcott & Kroon, 2002), thus controlling for the bias involved in constructing discriminant functions with the same observations that they are then used to classify (Statsoft, 2003).

We first describe the response of males to different playback treatments by calculating mean values for the continuous variables (1) to (5). When there was no approach and/or no vocal response, latency to approach and latency to first loudsong was scored as 601 s, and bout duration was given a score of zero. If a bout of singing exceeded 10 min, it was scored as 601 s. We compared these variables for each male using Wilcoxon matched-pairs signed-rank tests. To control the error rate at 0.05 in making pairwise com-

parisons, we used the False Discovery Rate method (Benjamini & Hochberg, 1995). Because some of the response variables could not be transformed to achieve the error normality and constant variance required by principal components analysis, the strength of the overall response of pairs of antbirds to playback could not be quantified using this method. Following other key playback and speciation studies (Robinson & Terborgh, 1995; Irwin, 2000), we therefore quantified the overall response by devising a rank scoring system wherein response to playback was graded on a scale of zero (no response) to 30 (maximum aggressive response) (see Appendix).

All statistical tests were carried out using SPSS (version 11.01); data are means \pm SD unless otherwise stated; and all P -values are two-tailed and corrected for ties where appropriate.

RESULTS

GEOGRAPHIC DIFFERENTIATION IN LOUDSONG STRUCTURE

There was evidence of minor but significant geographical variation in the structure of male loudsongs, as described by separate acoustic measures and three principal components (Table 1), with the loudsongs of the Huanchaca population being the most distinct (Fig. 3). Although these structural differences are difficult to detect by ear or eye (Fig. 4), we found that five acoustic measures differed significantly between the sites: number of notes ($P = 0.003$), loudsong duration ($P = 0.002$), duration of first note ($P = 0.011$), maximum frequency of the penultimate note ($P = 0.003$), minimum frequency of the loudsong ($P = 0.003$), and loudsong bandwidth ($P = 0.032$). Of these variables, two (number of notes and duration of the first note) distinguished Huanchaca II loudsongs from those recorded at Los Fierros and Lago Caiman.

That these two variables may have formed the basis of the discrimination revealed by playback (see below) was also highlighted by DFA. Specifically, this analysis revealed that island and non-island loudsongs were best discriminated by the synthetic variable, PC2 which had a correlation with the discriminant function (DF) of 0.509. PC2 was positively correlated with the duration of the first note and maximum frequency of the penultimate note (Table 1). PC1, which was strongly correlated with the number of notes, duration of first note and first frequency shift, also made an important contribution to the discrimination (correlation with DF = 0.381). PC3, which reflected the pace of notes, was weakly correlated (-0.054) and was therefore deemed unimportant in distinguishing island from non-island loudsongs. The descriptive DF thus generated had an Eigenvalue of 0.64 and was strongly significant (Wilks' $\lambda = 0.610$, $\chi^2_4 = 20.3$,

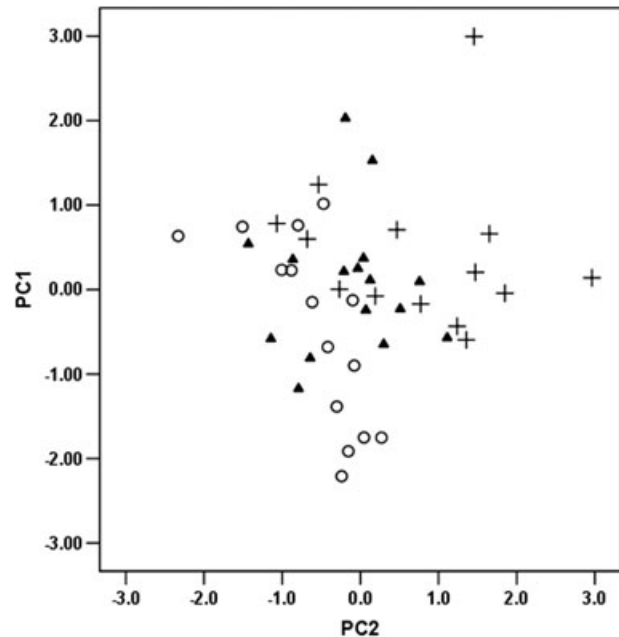


Figure 3. Scatterplot of the first two principal components from an analysis of variation in chestnut-tailed antbird song structure between Huanchaca II (crosses), Los Fierros (triangles), and Lago Caiman (open circles). PC1 explains 24.7% of the variation and PC2 explains 22.5%. For factor loadings of song variables on each principal component, see Table 1.

$P < 0.0001$). The overall degree of discrimination was high at 80.0%, with cross-validation assigning 71.4% of island loudsongs and 83.9% of non-island loudsongs to their correct site. This is significantly higher than expected by chance $\chi^2_1 = 11.4$, $P = 0.001$).

SIMULATION OF SECONDARY CONTACT

Males responded by singing and/or approaching the speaker in 95.6% (109/114) of playbacks, and females in 33.3% (38/114; Table 2). Although males were equally likely to approach and/or sing in response to local and heterotypic loudsongs ($\chi^2_2 = 0.08$, not significant), females were more likely to respond to the former $\chi^2_2 = 5.16$, $P < 0.05$; Table 2). Analysing this categorical response data by site, we found that, although males did not appear to discriminate between local and heterotypic loudsongs at any site, females at Huanchaca II and Los Fierros were more likely to approach and/or sing in response to local loudsongs, although the difference was only significant for Huanchaca II (Table 2). At Lago Caiman, females were more likely to approach or sing in response to local loudsongs than to those recorded at Huanchaca II but, crucially, they did not appear to discriminate between local loudsongs and those from Los Fierros.

Table 1. Mean \pm SD values of 12 temporal and frequency measurements taken from male loudsongs recorded at the three study sites, given in seconds (s) and kilohertz (kHz), respectively

| Acoustic measure | Sites | | | Statistics | | Post-hoc tests [†] | | | Factor loadings [‡] | | |
|------------------------------------|----------------------------------|----------------------------------|----------------------------------|------------|-----------------------|-----------------------------|-----------------|----------------|------------------------------|--------|--------|
| | Huanchaca II (HII) | Los Fierros (LF) | Lago Caiman (LC) | χ^2 | <i>P</i> | HII \times LF | HII \times LC | LF \times LC | PC1 | PC2 | PC3 |
| Number of notes | 9.5 \pm 1.8 (8.8–11.7) | 8.9 \pm 0.6 (8.0–10.3) | 8.6 \pm 5.8 (7.5–9.5) | 11.8 | 0.003 [¶] | * | ** | NS | 0.815 | 0.470 | -0.021 |
| Loudsong duration | 1.48 \pm 0.14 (1.30–1.83) | 1.41 \pm 0.11 (1.22–1.58) | 1.31 \pm 0.07 (1.19–1.45) | 12.7 | 0.002 [¶] | * | ** | * | 0.366 | -0.193 | 0.607 |
| Overall pace | 5.7 \pm 0.4 (5.1–6.6) | 5.6 \pm 0.4 (4.9–6.3) | 5.8 \pm 0.48 (4.9–6.6) | 0.94 | 0.624 | NS | NS | NS | -0.239 | -0.403 | 0.667 |
| Duration of first note | 0.09 \pm 0.01 (0.07–0.11) | 0.10 \pm 0.01 (0.07–0.12) | 0.10 \pm 0.01 (0.09–0.12) | 8.98 | 0.011 [¶] | * | * | NS | 0.543 | 0.637 | -0.748 |
| Maximum frequency first note | 4.06 \pm 0.23 (3.75–4.41) | 4.01 \pm 0.17 (3.75–4.42) | 3.99 \pm 0.18 (3.75–4.42) | 1.14 | 0.565 | NS | NS | NS | - | - | - |
| Maximum frequency fourth note | 4.02 \pm 0.17 (3.77–4.31) | 4.01 \pm 0.16 (3.81–4.47) | 3.91 \pm 0.14 (3.64–4.16) | 3.22 | 0.200 | NS | NS | NS | - | - | - |
| Maximum frequency penultimate note | 3.31 \pm 0.19 (3.06–3.70) | 3.18 \pm 0.15 (2.96–3.55) | 3.12 \pm 0.13 (2.86–3.37) | 11.5 | 0.003 [¶] | ** | * | ** | -0.008 | 0.545 | 0.085 |
| Maximum frequency of loudsong | 4.14 \pm 0.22 (3.84–4.45) | 4.04 \pm 0.17 (3.78–4.53) | 4.03 \pm 0.20 (3.73–4.42) | 1.86 | 0.395 | NS | NS | NS | - | - | - |
| Minimum frequency of loudsong | 1.92 \pm 0.25 (1.47–2.27) | 1.88 \pm 0.21 (1.59–2.23) | 1.63 \pm 0.19 (1.16–1.84) | 11.6 | 0.003 [¶] | NS | ** | ** | - | - | - |
| Bandwidth of loudsong | 2.21 \pm 0.32 (1.64–2.75) | 2.16 \pm 0.25 (1.68–2.69) | 2.41 \pm 0.24 (2.05–3.02) | 6.86 | 0.032 | * | NS | ** | -0.519 | 0.300 | -0.331 |
| First frequency change | 0.99 \pm 0.03 (0.93–1.05) | 1.00 \pm 0.02 (0.97–1.03) | 0.98 \pm 0.03 (0.94–1.04) | 3.58 | 0.167 | NS | NS | NS | 0.545 | -0.110 | 0.119 |
| Second frequency change | 0.91 \pm 0.03 (0.85–0.96) | 0.91 \pm 0.05 (0.85–1.07) | 0.90 \pm 0.19 (0.86–0.94) | 0.02 | 0.986 | NS | NS | NS | - | - | - |
| PC1§ | 0.43 \pm 0.90 (-0.60–3.00) | 0.08 \pm 0.08 (-1.17–2.03) | -0.48 \pm 1.10 (-2.20–1.02) | 3.43 | 0.042 | * | NS | NS | - | - | - |
| PC2 | -0.78 \pm 1.14 (-2.21–3.00) | -0.14 \pm 0.67 (-1.43–1.11) | -0.57 \pm 0.67 (-2.33–0.27) | 9.46 | < 0.0001 [¶] | ** | ** | NS | - | - | - |
| PC3 | -0.06 \pm 0.91 (-1.73–1.27) | -0.49 \pm 0.99 (-1.49–2.51) | -0.47 \pm 0.90 (-1.89–1.60) | 4.11 | 0.023 | NS | NS | NS | - | - | - |
| <i>N</i> (individuals) | 14 | 16 | 15 | | | | | | | | |

[†]Post-hoc multiple comparison tests examining differences in male loudsong structure between pairs of sites; * $P < 0.05$, ** $P < 0.01$.

[‡]Factor loadings on the first three principal components for the acoustic measures taken from male loudsongs; -, variables that could not be included in the analyses because they violated parametric assumptions.

[§]Principal components accounted for 24.7%, 22.5%, and 19.6% of the variance in song structure, respectively; their Eigenvalues were 1.98, 1.80, and 1.07.

[¶]Statistical tests derive from Kruskal–Wallis tests and significant differences after sequential Bonferroni corrections are denoted by [¶].

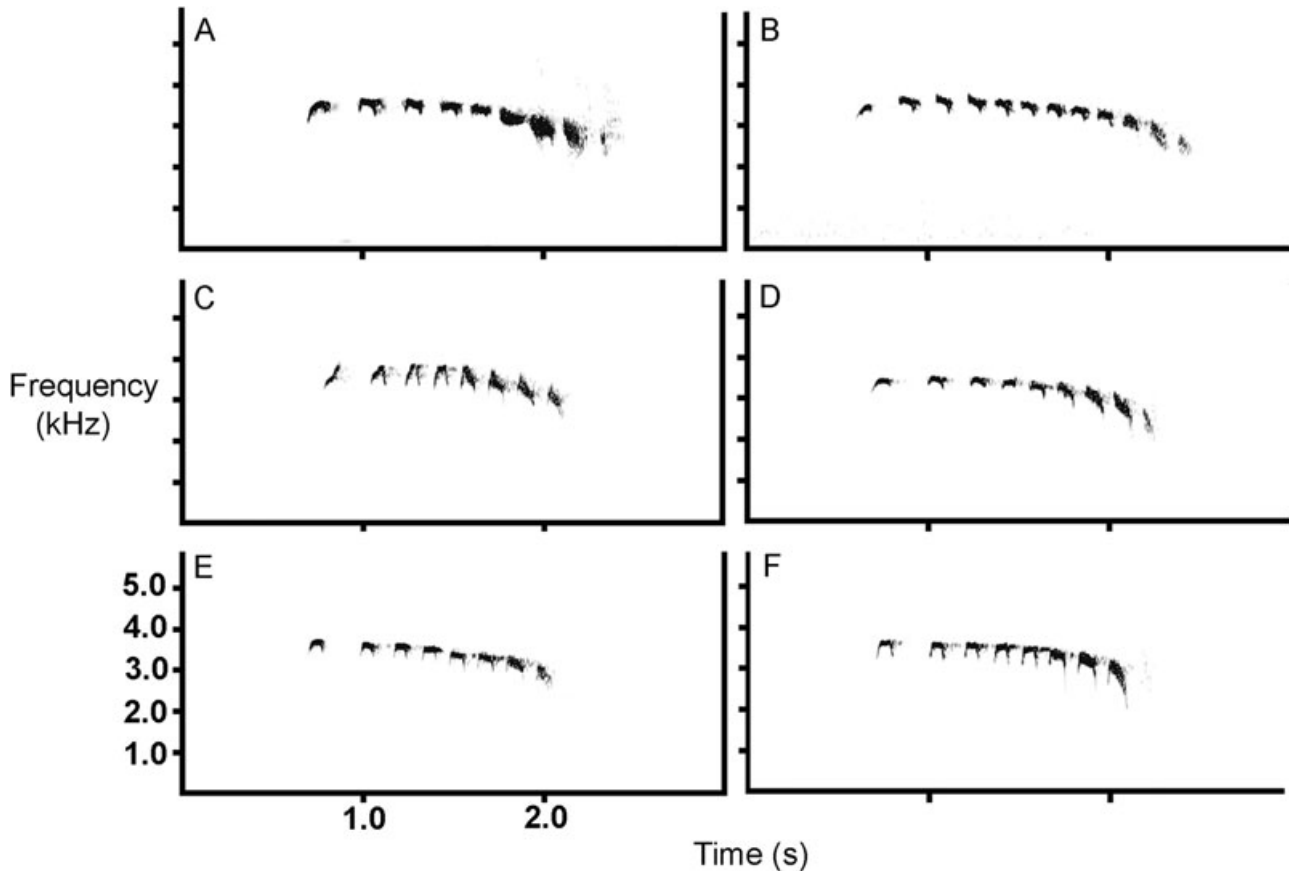


Figure 4. Audiospectrograms of loudsongs recorded from two different males at each of the three study sites: (A, B) Huanchaca II; (C, D) Los Fierros; and (E, F) Lago Caiman.

Analysing the continuous response data, we found that overall, pairs responded more strongly to local loudsongs than to heterotypic loudsongs from nearby sites (mean response score = 18.8 ± 6.0 vs. 14.2 ± 8.24 ; $t = 3.95$, d.f. = 55, $P < 0.0001$). However, looking at the data by site, a more interesting pattern emerged. Males at Huanchaca II approached local loudsongs significantly more quickly and closely, and remained in the vicinity for longer (Table 2). After correcting for multiple comparisons, only latency to approach varied significantly between treatments ($P = 0.020$) but, when other response variables were incorporated (including female response), we found that pairs responded much more strongly to local loudsongs than to loudsongs from the nearby Los Fierros population ($P = 0.004$, Fig. 5A). Conversely, when local loudsongs were played to birds from Los Fierros, males approached the speaker significantly more closely ($P = 0.016$), and the overall response score of pairs was significantly higher ($P = 0.007$, Fig. 5B) compared with their response to loudsongs from Huanchaca II. At Lago Caiman, males also approached the speaker more closely, stayed in the vicinity for longer and

tended to sing for a greater duration in response to local vs. island loudsongs. Only the closest approach was significant after correcting for multiple comparisons ($P = 0.013$), but the overall response score of pairs was significantly higher for local loudsongs than those recorded at Huanchaca II ($P = 0.030$, Fig. 5C). Interestingly, birds from Lago Caiman responded equally strongly to local loudsongs as to those from Los Fierros ($P = 0.586$, Fig. 5D).

In summary, at all sites males and females appeared to discriminate between island and non-island loudsongs, with island loudsongs eliciting strong responses within the island population but relatively weak responses within non-island populations. Conversely, responsiveness between non-island sites was relatively constant.

DISCUSSION

In evaluating the role of song during an early stage of peripatric speciation in a subsocial bird, we found: (1) geographical variation in song structure and (2) reduced responsiveness in a small geographically-

Table 2. Results of playback experiments showing approach or singing frequency by males and females, and absolute values for continuous response variables from male responses and overall response scores (see Appendix)

| Location/response variable | Playback treatment with mean \pm SD response scores | | Statistic* | P |
|---|---|--------------------|------------|---------|
| | <i>Huanchaca II</i> | <i>Los Fierros</i> | | |
| <i>Huanchaca II</i> | | | | |
| No. of times ♂♂ approached or sang | 17 | 15 | 0.03 | NS |
| No. of times ♀♀ approached or sang | 7 | 1 | 4.50 | < 0.05 |
| Latency to approach | 155 \pm 218 | 357 \pm 282 | 4.43 | 0.035¶ |
| Latency to song | 348 \pm 118 | 421 \pm 189 | -2.06 | 0.039¶ |
| Closest distance | 9 \pm 9 | 16 \pm 10 | -0.99 | 0.320 |
| Time < 10 m | 99 \pm 82 | 40 \pm 67 | -1.96 | 0.050¶ |
| Duration of song | 229 \pm 167 | 158 \pm 173 | -2.33 | 0.020¶ |
| Overall response score ($N = 17$ pairs)† | 17.5 \pm 5.4 | 8.6 \pm 7.2 | -2.90 | 0.004¶ |
| <i>Los Fierros</i> | | | | |
| No. of times ♂♂ approached or sang | 14 | 13 | 0.04 | NS |
| No. of times ♀♀ approached or sang | 9 | 3 | 3.00 | < 0.1 |
| Latency to approach | 141 \pm 160 | 162 \pm 196 | -0.47 | 0.638 |
| Latency to song | 269 \pm 147 | 368 \pm 212 | -1.73 | 0.084 |
| Closest distance | 7 \pm 5 | 10 \pm 8 | -2.41 | 0.016¶ |
| Time < 10 m | 215 \pm 179 | 156 \pm 139 | -0.98 | 0.325 |
| Duration of song | 337 \pm 120 | 265 \pm 170 | -1.72 | 0.085 |
| Overall response score ($N = 15$ pairs) | 20.1 \pm 5.5 | 16.2 \pm 6.4 | -2.68 | 0.007¶ |
| <i>Lago Caiman</i> | | | | |
| No. of times ♂♂ approached or sang | 15 | 15 | 0 | NS |
| No. of times ♀♀ approached or sang | 9 | 2 | 6.40 | < 0.01¶ |
| Latency to approach | 201 \pm 179 | 255 \pm 233 | -1.02 | 0.310 |
| Latency to song | 359 \pm 189 | 435 \pm 170 | -1.22 | 0.221 |
| Closest distance | 9 \pm 4 | 13 \pm 6 | -2.49 | 0.013¶ |
| Time < 10 m | 207 \pm 168 | 119 \pm 167 | -2.11 | 0.035¶ |
| Duration of song | 348 \pm 118 | 267 \pm 195 | -1.79 | 0.074 |
| Overall response score ($N = 15$ pairs) | 18.9 \pm 6.4 | 14.2 \pm 7.5 | -2.17 | 0.030¶ |
| <i>Lago Caiman</i> | | | | |
| No. of times ♂♂ approached or sang | 9 | 9 | 0 | NS |
| No. of times ♀♀ approached or sang | 2 | 5 | 0.64 | NS |
| Latency to approach | 138 \pm 183 | 101 \pm 190 | 32 | 0.301 |
| Latency to song | 312 \pm 196 | 377 \pm 175 | 19 | 0.469 |
| Closest distance | 12 \pm 10 | 7 \pm 5 | 29 | 0.141 |
| Time < 10 m | 204 \pm 200 | 271 \pm 141 | 15 | 0.438 |
| Duration of song | 358 \pm 85 | 299 \pm 164 | 7 | 0.625 |
| Overall response score ($N = 9$ pairs) | 19.0 \pm 7.6 | 21.2 \pm 7.9 | 28 | 0.586 |

*Statistics derives from chi-square tests for data on frequency of approach or singing, and Wilcoxon signed-rank tests for continuous measures. T -values with exact P -values are given where $N < 15$; otherwise Z -values with asymptotic P -values are given.

†Overall scores include noncontinuous response variables from male and female pair-members (see Appendix). Time variables (s) and distance variables (m); significant probabilities are denoted by ¶.

isolated population when secondary contact was simulated using playback. These findings have several interesting implications for the study of birdsong and its potential role in speciation.

The traditional view of song in suboscine birds as an invariant trait is contradicted by our finding that loudsongs varied across all sites. Whether clinal or discontinuous, this regional variation suggests that

complete geographical isolation is not a prerequisite for vocal divergence in suboscine birds (see also Isler *et al.*, 2005). Instead, variation may be underpinned by limited gene flow through relatively continuous habitat, resulting from specialized habitat requirements and a sedentary lifestyle: chestnut-tailed antbirds, in common with most passerines of the rainforest understorey, rarely move far from their

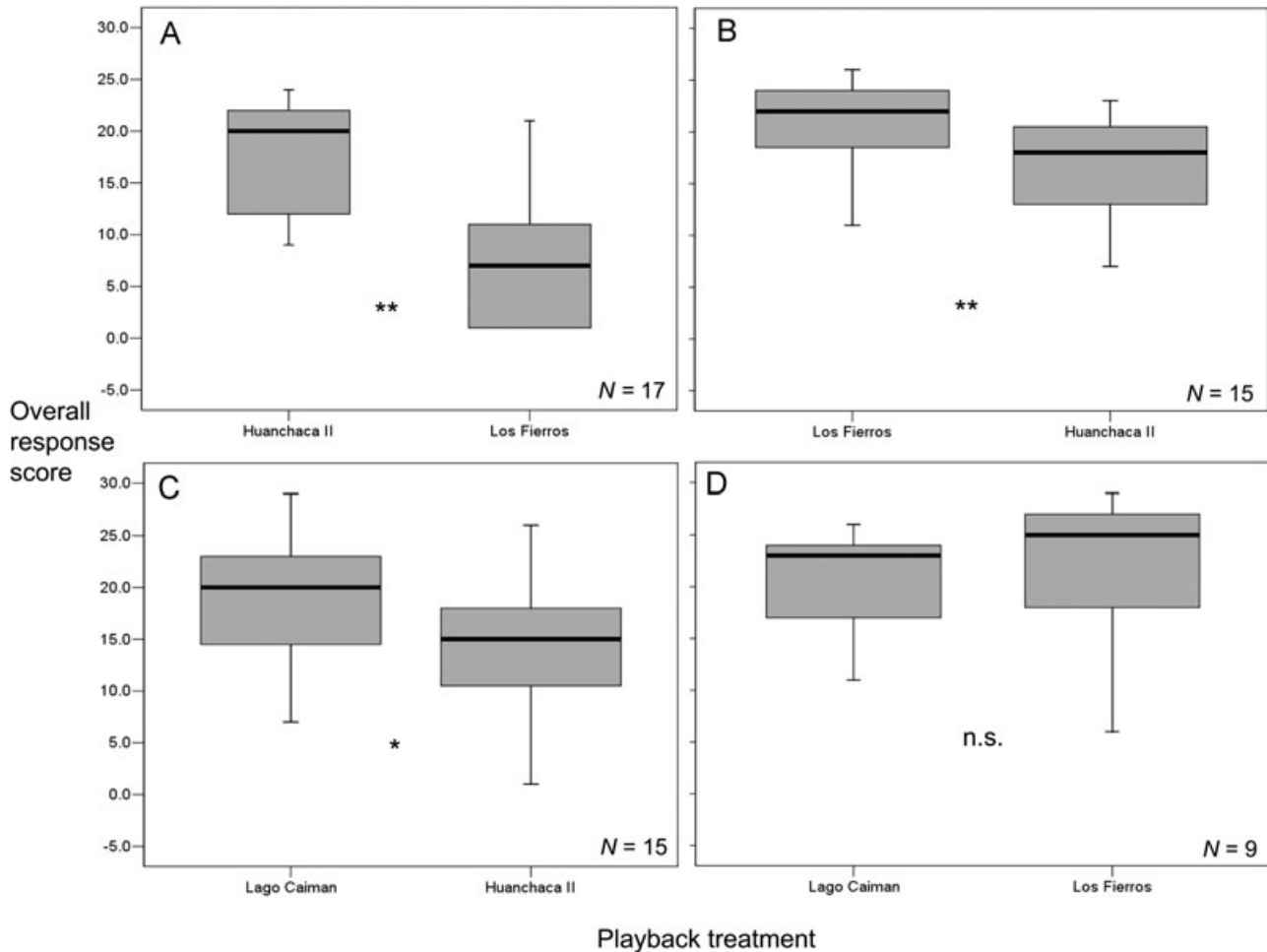


Figure 5. Boxplots showing overall strength of the response of pairs (mean \pm SD) to the different playback treatments at (A) Huanchaca II; (B) Los Fierros; and (C, D) Lago Caiman. Boxes indicate the 25th and 75th percentiles, the line in the box marks the median and the whiskers denote 10th and 90th percentiles. Asterisks denote the significance of Wilcoxon signed-rank tests between pairs of treatments (* $P < 0.05$, ** $P < 0.01$).

natal area and probably never cross broad stretches of unsuitable habitat such as grassland or water (Bates, 2002; Zimmer & Isler, 2003). Despite variation in loud-song structure, our playback experiments revealed that, only in the case of the isolated population, was a significant dip in responsiveness detected. This hints that only total isolation results in the type of vocal change involved in species recognition.

We found that temporal measures were important in distinguishing island and non-island loudsongs. Although our quantification of loudsong structure was not comprehensive, and we are unlikely to have measured all the traits used by the birds themselves, we suggest that temporal traits may be important cues used in species recognition, and possibly also in establishing premating isolation. Recent comparative studies are in general agreement, identifying number of notes, note duration, and loudsong duration amongst

the acoustic features maintaining species barriers in the antbird family (Isler *et al.*, 1998; Seddon, 2005; Isler, Isler & Whitney, in press).

According to Isler *et al.* (1998), at least three non-overlapping vocal differences are required to assign species-status to closely related antbirds, or at least to those occurring sympatrically. As might be expected, the chestnut-tailed antbirds at Huanchaca II fall far short of these requirements because we found overlap in all loudsong characteristics. However, considerable overlap occurs in the properties of loudsongs produced by two species of antbird: the Peruvian and the yellow-breasted warbling-antbird *Hypocnemis peruviana* and *Hypocnemis subflava* (Isler *et al.*, in press). The fact that these distinct species occur sympatrically without obvious evidence of hybridization, indicates that, in antbirds at least, species barriers can be maintained in the presence of very minor differences in song struc-

ture (N. Seddon and J. Tobias, unpubl. data). These recent findings in warbling-antbirds support the idea that speciation is underway, although far from completion, in our isolated population of chestnut-tailed antbirds.

If vocal signals play a role in peripatric speciation, what drives their divergence in isolation? Mating signals, similar to other heritable features, alter gradually over time because of random microevolutionary processes, including genetic drift. Drift is most likely to cause phenotypic change when small populations, founded by few individuals, are isolated for millions of years (Wright, 1931). However, there is little evidence that drift alone can cause significant phenotypic differentiation or reproductive isolation over short time scales (Rice & Hostert, 1993; Clegg *et al.*, 2002). Although the population in the forest fragment at Huanchaca II is small (approximately 100 individuals), the islands are thought to have formed when forest retreated and *cerrado* expanded across the plateau (O'Conner *et al.*, 1987). Thus, resident chestnut-tailed antbirds represent a remnant population rather than one derived from a small number of founders. Moreover, although the exact age of the island is unknown, it is probably relatively young: carbon isotope analysis suggests that forest has persisted on the plateau for several thousand years at least (Panfil, 1992), and pollen data indicate that extensive forest in the region is 700–2300 years old (Mayle, Burbridge & Killeen, 2000). This suggests that the fragment has been isolated from the continuous forest for at least 1000 years (Bates, 2002), and probably less than 3000 years. Thus, it is unlikely that genetic drift is the main agent of vocal change and direct natural selection may be more important.

For example, song divergence may be related to environmental selection pressures, especially those imposed by the sound transmission properties of different habitats (Slabbekoorn & Smith, 2002b). The habitat at Huanchaca II consists of gallery forest, lower in stature and more densely vegetated than the mature forest present at Los Fierros and Lago Caiman (Killeen & Schulenburg, 1998). Given the coupling of song structure to the physical properties of habitat across a broad spectrum of bird species, including antbirds (Seddon, 2005), shifts in the structure of chestnut-tailed antbird loudsongs may have arisen to optimize transmission through this habitat. The degradation of sound caused by attenuation and reverberation in dense foliage is less pronounced for songs with low frequency (Morton, 1975) and short, well-spaced notes (Ryan & Brenowitz, 1985; Badyaev & Leaf, 1997), which are acoustic properties that might therefore be expected in Huanchaca II loudsongs. However, although these loudsongs had shorter introductory notes, their minimum frequencies did not differ signifi-

cantly from those recorded at Los Fierros, and there was no difference between sites in the pace of note delivery. Although a detailed analysis of habitat differences between the sites is needed to test this idea, the apparent lack of habitat-associated acoustic divergence may relate to the fact that chestnut-tailed antbirds occupied similar microhabitats (tree-falls and relatively dense undergrowth) at all sites.

Another possibility is that loudsong divergence is being shaped by sexual selection, a potentially powerful cause of rapid divergence in mating signals (Panhuis *et al.*, 2001), especially in peripatric populations (Kaneshiro, 1989; Odeen & Florin, 2002). When two isolated populations diverge in a mating signal and the preferences associated with it, and if those populations no longer prefer one another as mates when contact is resumed, sexual selection is implicated in the speciation process (Schulter & Price, 1993). Although we did not directly test female preferences, two lines of evidence are consistent with this view. First, Huanchaca II females were more likely to approach island loudsongs than non-island loudsongs, and vice versa, whereas Lago Caiman females did not appear to discriminate between local loudsongs and those recorded at Los Fierros. Second, it is likely that the intensity of the male's response is correlated with the strength of the female's preference. This assumption is common to almost all studies attempting to show assortative mating based on song (Slabbekoorn & Smith, 2002a) and is justified on the basis that the high costs (in terms of energy and predation risk) of noisy and aggressive behaviour ensures that males are only likely to adopt this strategy in the presence of sexual competitors (Price, 1998). Moreover, not only are female birds usually more discriminating than males (Searcy & Brenowitz, 1988), but also they have been shown in some species respond to extremely subtle variation in the structure of male songs (Dabelsteen & Pedersen, 1993; Vallet *et al.*, 1998). Thus, if male antbirds can discriminate between local and heterotypic loudsongs, females are likely to share this ability.

The sympatric occurrence of morphologically cryptic species with divergent songs not only in the *M. hemimelaena* complex (Isler *et al.*, 2002), but also in a number of other antbird species (Isler *et al.*, 1998; Seddon, 2005), as well as in other avian families (Rensen, 2005), supports the idea that premating isolation may develop more rapidly between allopatric populations than postmating isolation (Tregenza, 2002). It also implicates a role for song divergence at an early stage of the speciation process. Indeed, although the historical biogeography of Amazonian birds is unclear (Haffer, 1997), the existence of such species confirms that secondary contact can occur when vocal differences are great enough to produce almost complete

reproductive isolation at a time when differences in morphology and genetics are not great enough to result in isolation on their own.

The situation outlined above differs from that found in the Darwin's finches *Geospiza* spp. of the Galapagos archipelago. In this oscine assemblage, song learning probably promotes signal divergence in allopatry through cultural evolution and the rapid accumulation of copying errors (Grant & Grant, 1996), but the divergence of learnt songs does not always lead to pre-mating isolation (Grant & Grant, 2002a, b). Indeed, song learning may ultimately reduce the influence of signal divergence when secondary contact is established, chiefly because immigrant birds will then acquire the songs of residents (Grant & Grant, 2002b).

If a lack, or at the most, low levels of song learning raises the likelihood that prolonged geographical isolation leads to pre-mating isolation, it may partly explain the dramatic diversification of suboscines in Amazonia. This makes sense in view of the fact that Amazonian rainforest was repeatedly fragmented, at least peripherally, through a variety of geological and ecological mechanisms during the Cenozoic: there is general agreement that these events created suitable conditions for allopatric speciation (for an overview, see Hall & Harvey, 2002).

Conversely, there is long-standing disagreement regarding the precise timing, scale and cause of vicariance events (Bush, 1994; Knapp & Mallet, 2003), uncertainty that has led to the resurrection of the idea of 'isolation-by-distance' (Darwin, 1859) as an alternative, although more probably as an additional (Coyne & Orr, 2004) explanation of Amazonia's prolific diversity (Knapp & Mallet, 2003). In the present study, loudsongs varied geographically, but only in an isolated population did vocal change cause a dip in responsiveness. The findings support the peripatric model, but are in contrast to the predictions of isolation-by-distance. However, testing these models more rigorously will require working in a larger number of isolated and non-isolated populations, as well as carrying out genetic studies using rapidly evolving genes (microsatellites or amplified fragment length polymorphisms).

At first glance, the concept that evidence of pre-mating isolation might arise in a relatively young forest fragment appears to be surprising given current thinking about allopatric speciation. The completed process is traditionally assumed to span several million years (Mayr, 1963; Haffer, 1969), whereas we show that detectable vocal change, and suggest that the beginnings of reproductive isolation, can occur in an isolated population of antbirds within a more modest time-frame (probably between one and three thousand years) and at a relatively local scale. This discovery seems more plausible, however, set against

the finding that a sexually selected plumage trait became 22% smaller in an isolated population of white-eyed juncos, *Junco hyemalis*, within 20 years of its establishment in a novel habitat (Yeh, 2004).

Divergence between distinctive taxa and major lineages of antbirds certainly dates back millions of years (Bates *et al.*, 1999), but our results suggest that cryptic taxa may be able to speciate relatively rapidly in small peripheral isolates. Furthermore, they support the idea that conditions suitable for peripatric speciation in Amazonia are likely to develop in regions susceptible to disturbance and climatic change, such as the fluctuating boundary between forest and savanna (Bush, 1994; Nores, 2000). The potential importance of ecotones for generating biodiversity in tropical Africa has already been noted (Smith *et al.*, 1997), and the same may apply to Amazonia.

In summary, by showing how minor song divergence in an isolated forest fragment may lead to incipient reproductive isolation, the present study provides the first indication that allopatric, specifically peripatric, divergence in song may constitute an early step in the speciation process for suboscine birds and, although further studies are necessary, highlights one plausible route by which Amazonia's high avian diversity may have evolved.

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APPENDIX

Rank scoring system for playback experiments. Responses to playback were graded on a 30-point scale, each of nine response variables being awarded 0–5

| Sex | Response variable | Score | | | | | |
|--------|---------------------------------------|-------------|-------|---------|---------|--------|-------|
| | | 0 | 1 | 2 | 3 | 4 | 5 |
| Male | Latency to approach (s) | No approach | > 600 | 301–600 | 121–300 | 61–120 | ≤ 60 |
| | Closest approach (m) | No approach | 20–30 | 16–20 | 11–15 | 6–10 | ≤ 5 |
| | Time < 10 m away (s) | No approach | < 60 | – | 61–300 | – | > 301 |
| | Latency to first loudsong (s) | No song | > 600 | 361–600 | 241–360 | 61–240 | ≤ 60 |
| | Duration of first bout of singing (s) | No song | < 60 | – | 61–300 | – | > 301 |
| | White interscapular region exposed? | No | Yes | – | – | – | – |
| | Rapid flights around speaker? | No | Yes | – | – | – | – |
| Female | Approach to < 10 m? | No | Yes | – | – | – | – |
| | Loudsong given? | No | – | Yes | – | – | – |