

Song structure, not high-frequency song content, determines high-frequency auditory sensitivity in nine species of New World sparrows (Passeriformes: Emberizidae)

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Summary

1. The evolution of vocal signals can be constrained by a host of factors including habitat effects on sound propagation, morphology of sound-producing structures and phylogenetic relationships among species. Here, we asked whether auditory sensitivity over a broad range of frequencies correlates with the spectral content of conspecific vocalizations, or whether it is constrained by the overall structure of vocalizations, habitat effects on sound propagation or relatedness among species.

2. We studied nine New World sparrows (Passeriformes: Emberizidae) including three open-habitat species, three scrub-like habitat species and three forest species. For each habitat, one species had pure-trilled songs, another had tonal songs and another had complex songs with tones, trills and amplitude-modulated buzzes.

3. As predicted by the acoustic adaptation hypothesis, song spectral properties (specifically frequency and entropy) had the highest values in open-habitat species and the lowest values in forest species.

4. Based on our results from song analyses, and the sender–receiver matching hypothesis, we predicted that open-habitat species would be more sensitive to high-frequency sounds compared to forest species. Contrary to this prediction, habitat and high-frequency song content had little effect on audiogram shape. Song type, however, had a strong effect, with species that produce complex songs showing higher sensitivity to high-frequency sounds than all other species.

5. Our results suggest that the use of song frequency by receivers depends on song structure and not necessarily on song spectral content. Therefore, our current understanding of how signal-processing mechanisms should match signal properties appears to be too simple. When thinking about the evolution of signal-processing mechanisms, the multidimensionality of signals, and how the different dimensions can interact, should be considered.

Key-words: acoustic communication, auditory brainstem response, auditory evoked potentials, frequency sensitivity, multidimensional signals, songbirds, vocal communication

Introduction

Acoustic communication mediates biologically important behaviours such as mate attraction, territory defence and group cohesion (Kroodsma & Miller 1996; Gerhardt & Huber 2002; Marler & Slabbekoorn 2004; Bass & Ladich 2008; Janik 2009). Vocal signals are therefore expected to

be shaped in ways that optimize the transmission of information from senders to receivers (Bradbury & Vehrencamp 2011). Moreover, natural selection should favour signal-processing mechanisms in receivers that closely match the physical properties of signals (Endler 1992).

Songbirds have long served as model system to investigate the evolution and function of vocal communication signals (Catchpole & Slater 2008). Consequently, different factors are known to influence the evolution of bird song.

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For instance, body (Wallschager 1980) and bill (Podos 2001) size may limit the frequencies a bird can produce. Overall song structure can also affect song spectral properties because there is a trade-off between repetition rate and frequency bandwidth in trilled elements (Podos 1997). The physical environment is among the most studied factors influencing the evolution of bird song. The acoustic adaptation hypothesis (hereafter AAH) proposes that habitat structure shapes the evolution of acoustic properties (Morton 1975). Reverberations and excess attenuation of high-frequency sounds due to scattering select for tonal vocalizations with low frequencies in forested habitats. In open habitats, slow amplitude modulations imposed by wind favour the evolution of high-frequency vocalizations with rapid amplitude and/or frequency modulations (Morton 1975; Marten & Marler 1977; Wiley & Richards 1978; Richards & Wiley 1980; Wiley 1991). Accordingly, several studies have shown that avian vocalizations tend to have lower frequencies in closed compared to open habitats (reviewed in Boncoraglio & Saino 2007).

The sender–receiver matching hypothesis (hereafter SRMH) suggests that physical properties of vocalizations should be reflected in species-specific auditory processing (Dooling, Lohr & Dent 2000; Woolley *et al.* 2009). For example, studies in songbirds have shown a correlation between the frequency range used in the vocal repertoire and the frequency range of best auditory sensitivity (Koniishi 1970; Dooling, Zoloth & Baylis 1978; Dooling 1982; Henry & Lucas 2008). These studies, however, included only a few species from different families. Comparisons across a few distantly related species could be biased in that differences in song properties and hearing sensitivity may be due to gross morphological and physiological differences, ultimately due to more distant evolutionary relationships. Therefore, to better understand how auditory sensitivity correlates with vocal performance, it is necessary to investigate these patterns within a clade of closely related species taking into account different factors that can shape the evolution of acoustic signals.

The objective of this study was to examine how auditory processing compares with signal properties in a group of closely related species. We analyzed song properties and measured auditory sensitivity in nine species of New World sparrows (Passeriformes: Emberizidae). We measured the spectral entropy of the songs, the minimum, maximum and dominant frequencies, and the frequency range. Based on the AAH, we predicted higher values of these spectral properties in open-habitat species than in forest species. We also used auditory evoked potentials (AEPs) to measure auditory sensitivity in our study species. AEPs, measured with surface electrodes on the scalp, are voltage changes resulting from hair cell (i.e. cochlear) or neural (i.e. auditory nerve, brainstem and possibly mid-brain) activity caused by acoustic input (Hall 2007). Based on the SRMH, we predicted a correlation between the frequency of best hearing sensitivity and the dominant frequency of the song, and a correlation between the range of

hearing sensitivity and the range of frequencies in the song. The combination of our two main hypotheses (AAH and SRMH) generates the prediction that hearing sensitivity would also be correlated with habitat. Alternatively, if song properties and auditory sensitivity are phylogenetically conserved, habitat may have little effect on song properties or auditory sensitivity given the mix of species included in our study. Because the spectral content of songs can be constrained by the production of fast amplitude-modulated elements (Podos 1997), we also explored how song structure influences song and auditory properties.

Materials and methods

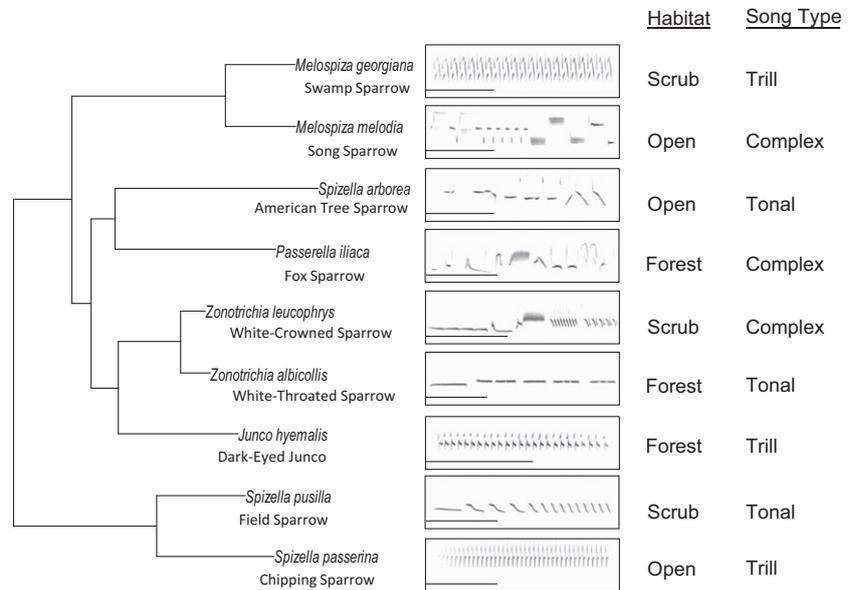
We studied species that most commonly breed in open areas, scrub-like habitats or forests. In each habitat, we chose one species each with a trilled song, tonal song and complex song. Following Podos' (1997) description of song-trilled elements, we defined pure-trilled songs as those composed of only one element (note or syllable) that is repeated throughout the song. We defined tonal songs as those that included more than one element, but where the elements were either pure tones or frequency-modulated tones. Complex songs were defined as songs that included tonal elements, trills and amplitude-modulated buzzes. The open-adapted species were chipping sparrows, *Spizella passerina*, which produce trilled songs (Middleton 1998), American tree sparrows, *Spizella arborea*, which produce tonal songs (Naugler 1993) and song sparrows, *Melospiza melodia*, which produce complex songs (Arcese *et al.* 2002). The three species that inhabit intermediate, scrub-like habitats were swamp sparrows, *Melospiza georgiana*, which produce trilled songs (Mowbray 1997), field sparrows, *Spizella pusilla*, which produce tonal songs (Carey *et al.* 2008) and white-crowned sparrows, *Zonotrichia leucophrys*, which produce complex songs (Chilton *et al.* 1995). The forest-adapted species included dark-eyed juncos, *Junco hyemalis*, which produce trilled songs (Nolan *et al.* 2002), white-throated sparrows, *Zonotrichia albicollis*, which produce tonal songs (Falls & Kopachena 2010) and fox sparrows, *Passerella iliaca*, which produce complex songs (Weckstein, Kroodsma & Faucett 2002). As shown in Figure 1, more closely related species in our study do not occupy similar habitats nor do they have structurally similar songs.

Because some songs of field sparrows and American tree sparrows could be considered a combination of tonal elements and trills, we conducted an additional analysis (see Appendix S1, Supporting Information) in which song structure was classified into two categories: complex songs (including tonal elements trills, and amplitude-modulated buzzes) and tonal/trilled songs (including songs consisting of pure tones, frequency-modulated tones and/or trills).

ACOUSTICAL ANALYSES

For each species, we analyzed 7–12 song exemplars acquired from the Cornell Lab of Ornithology Macaulay Library as digital sound files (number of song exemplars per species: American tree sparrows, $n = 8$; chipping sparrows, $n = 7$; dark-eyed juncos, $n = 12$; field sparrows, $n = 12$; fox sparrows, $n = 11$; song sparrows, $n = 9$; swamp sparrows, $n = 7$; white-crowned sparrows, $n = 12$; white-throated sparrows, $n = 9$). The exemplars selected for analysis were recorded in the field from the Northeast or Midwest United States. If necessary, sound digital files were transformed to mono, resampled at 44.1 kHz and saved with 16-bit resolution in Adobe Audition version 4.0 (Adobe Systems,

Fig. 1. Phylogeny of the emberizid sparrows studied reconstructed from the maximum-likelihood tree generated by Carson & Spicer (2003) using three mitochondrial genes. Gene sequences for tree reconstruction were obtained from GenBank. For each species, a spectrogram (Hanning windows with 512 points and 75% window overlap, 44.1 kHz Sampling rate) representative of the song, the habitat in which it is most commonly encountered, and the type of song is provided. All spectrograms are plotted from 0 to 15 kHz, and the black bar represents one-second.



San Jose, CA, USA) before acoustical analyses. Using custom-written scripts and the *SEEWAVE* package of R (Sueur, Aubin & Simons 2008), we measured the spectral entropy, the frequency range and the minimum-, maximum- and dominant frequencies of each song. For these analyses, we first band-pass filtered each song between 1 and 1.5 kHz to remove unwanted background noise. These filter settings did not bias our analysis as the minimum and maximum frequencies across species were 1.8 and 9.5 kHz, respectively. We then calculated each song's mean frequency spectrum using Hanning windows of 1024 points, and 75% overlap with the *MEANSPEC* function in *SEEWAVE*. From the mean frequency spectrum, we calculated the dominant frequency as the peak with highest amplitude using the *FPEAK* function and the spectral entropy using the *SH* function. Then, we used the *DFREQ* function to calculate the dominant frequency in each window of 1024 samples (i.e. song segments of 2.3 ms) and found the minimum and maximum values throughout the song to obtain the song's minimum and maximum frequencies. Finally, we calculated the frequency range as the difference between the maximum and minimum frequencies.

AUDITORY EVOKED POTENTIALS

Subjects

All protocols for collecting, handling and testing animals were approved by the Purdue Animal Care and Use Committee (PACUC no. 05-058). This study was conducted between February 2012 and November 2013. Birds were collected in the morning with mist nets or trellis traps baited with mixed seed at the Purdue Wildlife Area (40°26'30"N, 87°03'30"W) and at two private residences in Lafayette, IN. Birds were housed individually in 1-m³ stainless steel mesh cages in an indoor aviary at Purdue University and provided with *ad libitum* water, mixed seed and grit. When possible, sex was determined by the presence of a brood patch or cloacal protuberance. Otherwise, we took a blood sample from the brachial vein and used the protocol of Griffiths *et al.* (1998) for genetic sexing. Average \pm SD body mass in grams at the time of capture was 18.86 \pm 1.25 for American tree sparrows ($n = 12$; 7 females, 5 males), 11.87 \pm 0.94 for chipping sparrows ($n = 12$; 6 females, 6 males), 19.74 \pm 1.14 for dark-eyed juncos ($n = 11$; 6 females, 5 males), 12.31 \pm 0.43 for field sparrows ($n = 8$; 2 females, 6 males), 32.59 \pm 3.20 for fox sparrows ($n = 5$; 1 female, 4 males),

21.09 \pm 1.99 for song sparrows ($n = 18$; 1 female, 17 males), 16.65 \pm 1.28 for swamp sparrows ($n = 8$; 3 females, 5 males), 30.63 \pm 1.74 for white-crowned sparrows ($n = 9$; 1 female, 8 males) and 25.50 \pm 1.82 for white-throated sparrows ($n = 16$; 5 females, 11 males). Each bird was fitted with a uniquely numbered aluminium leg band. Auditory tests were typically conducted on the afternoon of the day of capture, and subjects were released at their capture location within 2 days after testing.

Auditory test equipment and procedure

Auditory experiments were conducted inside an anechoic sound chamber (1.2 \times 1.2 \times 1.4 m) lined with 7.2 cm Sonex foam (Acoustics Solutions, Richmond, VA, USA). Subjects were anesthetized with an injection into the breast muscle of midazolam (4.5–5.5 mg kg⁻¹), ketamine (45–55 mg kg⁻¹) and xylazine (45–55 mg kg⁻¹). Subjects were then positioned at the centre of the chamber on a pre-warmed heating pad (Snuggly-Safe pad at 52 °C) covered with several layers of towel. The temperature between the subject's body and the outermost towel layer was monitored with a thermistor and maintained at 39 \pm 2 °C by adding or removing layers of towel (See Henry & Lucas 2008; Gall, Brierley & Lucas 2012).

Stimulus presentation and response acquisition were coordinated with a Tucker Davis Technologies III mounted-rack system (TDT, Alachua, FL, USA) and a Dell PC running BIOSIG32 (TDT, Alachua, FL, USA) software in a room adjacent to the sound chamber. Digital stimuli were converted to analog signals with a TDT RP2 real-time processor, equalized across frequencies with a 31-band equalizer (Ultragraph Pro FBQ 6200, Behringer, Willich, Germany), amplified with a TDT SA1 amplifier and presented through an electromagnetically shielded overhead speaker (JBL Control 25 AV; 0.8–16 kHz frequency response) suspended 50 cm above the subject. Sound levels were calibrated within \pm 2 dB Sound Pressure Level (SPL; re 20 μ Pa) with a Brüel & Kjær 1613 Precision Sound Level Meter and model 4131 2.6-cm condenser microphone at the approximate position of a subject's ear.

Auditory evoked potentials were recorded through subdermal needle electrodes (Nicolet Biomedical, Fitchburg, WI, USA) just below the skin at the crown of the head (non-inverting), the mastoid just posterior to the right ear (inverting) and the nape of the neck (ground). We checked the integrity and placement of the electrodes by measuring interelectrode impedance and proceeded

with testing only when impedance was <7 kohms. The electrodes fed into a TDT RA4LI headstage and responses were amplified (200k) and digitized (24.4 kHz) with a TDT RA4PA Medusa bio-amplifier. Responses were then resampled (48.8 kHz), band-pass filtered between 0.1 and 5 kHz (see Lucas *et al.* 2002, 2007), notch filtered at 60 Hz with a TDT RA16 Medusa Base Station and stored in the computer.

Acoustic stimuli

We used short, pure tones to measure auditory sensitivity in a broad frequency range. Acoustic stimuli were generated in SIG-GENRP (TDT, Alachua, FL, USA) with a sampling rate of 20 kHz and broadcast at a rate of 31.1 stimuli s^{-1} . The stimuli used were 5-ms tone bursts gated with 1-ms \cos^2 rise and fall ramps and frequencies of 0.5, 1, 2, 3, 4, 5, 6 and 7 kHz. Tone bursts were presented with alternating phase values of 90° and 270°. We recorded AEPs at nine levels for each frequency, starting at 72 dB SPL down to 8 dB SPL in 8-dB steps.

AEP analysis

Short tone bursts evoke an auditory brainstem response (ABR); as the tone level decreases, the amplitude of the ABR decreases and the latency increases (Lucas *et al.* 2002; Hall 2007). We obtained two ABR replicates for each frequency and level, each one generated using the average of 400 presentations of the stimulus. We then averaged the two replicates and therefore, our analysis is based on the average response to 800 stimulus presentations. To determine ABR thresholds for each individual, we plotted the average AEPs in order of descending stimulus level for each frequency and used a visual detection method (e.g. Brittan-Powell, Dooling & Gleich 2002; Brittan-Powell & Dooling 2004). As stimulus level varied in 8-dB steps, ABR threshold was operationally defined as the level 4 dB (one-half step) below the lowest stimulus level at which a response could be visually detected. At any given frequency, lower ABR thresholds represent higher auditory sensitivity. Visual detection has proven to yield ABR thresholds that are similar to thresholds obtained behaviourally, that are correlated with estimates based on regression or cross-correlation methods, and that are consistent across trained observers (Gall, Brierley & Lucas 2011; Schrode *et al.* 2014).

STATISTICAL ANALYSES

We ran two types of analyses: repeated measures ANOVA (RMANOVA) and phylogenetic generalized least squares regression analysis (PGLS). RMANOVA provides a powerful approach for data repeated within individuals, but does not account for phylogenetic correlations that can bias the results. PGLS accounts for these phylogenetic effects but with a more limited model.

For the PGLS analyses, we reconstructed the maximum-likelihood phylogenetic tree of the Emberizidae family published by Carson & Spicer (2003). Then, we pruned the tree to include only our study species and mapped the values of mass, habitat (open, scrub or forest), song type (complex, tonal or trilled), mean values of the five song spectral properties and three measurements of auditory sensitivity: mean ABR thresholds, mean frequency of best sensitivity and mean high-frequency limit (see definitions below). To determine the extent to which song and auditory properties are constrained by the relatedness among species, we first measured the phylogenetic signal of each variable independently. We calculated Pagel's lambda and Bloomberg's K with 1000 simulations. We then fitted linear models to explore the effects of habitat and song structure on song spectral properties, and the effects of habitat and song properties on the different measurements of

auditory sensitivity. When necessary, data were log-transformed to achieve normality of residuals. For each model, we optimized Pagel's lambda to find the maximum-likelihood branch-length transformation given the data and the model (Pagel 1999; Revell 2010). Pagel's lambda was optimized between zero and one; when lambda is one, the model tracks the phylogeny following Brownian-motion evolution on the untransformed branches, whereas a value of zero corresponds to no phylogenetic structure (Pagel 1999; Revell 2010). All comparative analyses were conducted in R v. 3.0 (packages *ape*, *geiger*, *phytools* and *nlme*).

Acoustic properties

We conducted a principal components analysis (PCA) using PROC PRINCOMP in SAS (v9.2, SAS Institute, Cary, NC, USA) to examine the extent of covariation between the five acoustic properties measured (spectral entropy, minimum frequency, maximum frequency, dominant frequency and frequency range) and to obtain uncorrelated factors describing spectral properties of the songs. We used univariate ANOVAS (PROC MIXED in SAS) to investigate the effect of habitat and song type on these PCA factors. We used LSMEANS within PROC MIXED to estimate least squares means (LS means) and *post hoc* tests for pairwise comparisons (LSMEANS/DIFF). LS means are useful to describe patterns associated with a specific variable holding other factors constant. Normality of residuals and homogeneity of variances were confirmed using PROC UNIVARIATE (SAS).

To account for non-independence among species, we first conducted a phylogenetic PCA (Revell 2009) on the mean values of the five acoustic properties measured. Then, we fitted PGLS models to investigate the effect of habitat and song structure on the phylogenetic PCA factors associated to song spectral properties.

Auditory evoked potentials

We analyzed ABR thresholds using linear models in SAS v.9.2. We used RMANOVA (PROC MIXED) specifying the between-within method to calculate degrees of freedom and a first-order autoregressive (ar (1)) covariance structure. Within PROC MIXED, we used the command LSMEANS to estimate least squares means and the DIFF option for *post hoc* pairwise comparisons. In each analysis, all interaction terms were initially included and non-significant interaction terms were deleted in order of decreasing *F*-statistic. We used PROC UNIVARIATE to confirm that the assumptions of normality of residuals and homogeneity of variances were met.

We were particularly interested in investigating whether habitat or song properties have an effect on auditory sensitivity. However, other factors like time of year or sex can also affect auditory sensitivity. Therefore, we included sex and month in the RMANOVA as covariates in our analyses of factors affecting ABR thresholds. For this analysis, we labelled each month starting with March as month 1 and finishing with February as month 12. We did this so that the order of the months better reflects a transition starting from the beginning of the breeding season to the non-breeding season for our study species and location.

To determine the extent to which song properties correlate with auditory sensitivity, we examined the effect of song spectral properties (PCA factors from acoustical analysis described above) on ABR thresholds. Next, to determine whether song type (trilled, complex and tonal) or habitat (open, scrub or forest) effects on sound propagation affect auditory sensitivity, we conducted an analysis including stimulus tone frequency, habitat, and song type as independent variables and ABR threshold as the dependent variable in the RMANOVA.

For the PGLS analyses that account for effects associated with phylogenetic relatedness, we used three measurements of auditory sensitivity that relate to the audiograms and the RMANOVA

analyses: mean ABR threshold, frequency of best sensitivity and high-frequency limit. The mean ABR threshold was calculated as the average threshold for all frequencies tested (0.5–7 kHz). We defined the frequency of best sensitivity as the frequency with lowest ABR threshold. We then calculated the high-frequency limit as the frequency at which ABR threshold was 10 dB higher than the threshold at the frequency of best sensitivity. This 10-dB threshold was estimated using linear interpolation between ABR thresholds measured at the frequencies used in our experiment (0.5–7 kHz). We then fitted linear models to explore the effects of habitat, song structure and song spectral properties (phylogenetic PCA factors from acoustical analysis) on these three measurements of auditory sensitivity.

Results

PHYLOGENETIC SIGNAL

We found little phylogenetic signal in song and auditory properties, as evidenced by low values of Pagel’s lambda (all Pagel’s $\lambda < 0.1$, $P > 0.9$) and low values of Bloomberg’s k (all $k < 0.72$, $P > 0.2$). Similarly, there was little phylogenetic signal in the PGLS models reported below.

ACOUSTICAL ANALYSES

The five spectral properties measured (spectral entropy, minimum frequency, maximum frequency, dominant frequency and frequency range) could be summarized by two principal components with eigenvalues >1.0 , which together accounted for 85.6% of the total variance (Table 1). The first PCA factor explained 55.1% of the variance and loaded most heavily on spectral entropy, frequency range and maximum frequency. The second PCA factor described an additional 30.5% of the variance and loaded most heavily on minimum and dominant frequencies.

The RMANOVA revealed significant effects of habitat ($F_{2,78} = 61.04$, $P < 0.0001$), song type ($F_{2,78} = 28.84$,

Table 1. Loadings of song spectral properties on each of the two principal components analysis (PCA) and phylogenetic (phyl) PCA factors with eigenvalues larger than 1

Song property	PCA factor 1	PCA factor 2	phyl PCA factor 1	phyl PCA factor 2
Minimum frequency	-0.14	0.72	-0.11	0.98
Dominant frequency	0.25	0.63	0.56	0.81
Maximum frequency	0.57	0.08	0.96	-0.01
Frequency range	0.56	-0.25	0.92	-0.37
Spectral entropy	0.54	0.07	0.94	0.01
Eigenvalue	2.75	1.53	3.00	1.75
Variation explained (%)	55.1	30.5	60.0	34.94

Values of the variables loading on each factor are in bold.

$P < 0.0001$) and their interaction ($F_{4, 78} = 9.76$, $P < 0.0001$) on PCA factor 1 (spectral entropy, frequency range and maximum frequency). As predicted by the AAH, open-habitat species have higher values of song spectral properties associated with PCA factor 1 compared to forest species ($t_{78} = 10.91$, $P < 0.001$; Fig. 2a) and scrub species ($t_{78} = 7.58$, $P < 0.001$; Fig. 2a). In addition, tonal songs had overall lower values of song spectral properties associated with PCA factor 1 than trilled and complex songs (both $t_{78} > 6.0$, $P < 0.001$; Fig. 2a). However, scores of PCA factor 1 were significantly lower in tonal songs in forest and scrub species (all $t_{78} > 5.3$, $P < 0.001$), but no significant pattern was found in open-habitat species (all $t_{78} < 2.9$, $P > 0.11$).

Song spectral properties associated with PCA factor 2 (minimum and dominant frequencies) differed across habitats ($F_{2,78} = 4.49$, $P = 0.014$) and were higher in open-habitat species than forest and scrub species (both $t_{78} > 2.18$, $P < 0.032$; Fig. 2b); this result is also consistent with predictions from the AAH. In addition, there was a significant difference between song types ($F_{2,78} = 51.22$, $P < 0.0001$) with trilled songs having the highest values of PCA factor 2, followed by tonal songs and, with the lowest values, complex songs (all $t_{78} > 5.04$, $P < 0.0001$; Fig. 2b).

When controlling for the phylogenetic relationships among species, the phylogenetic PCA also summarized the

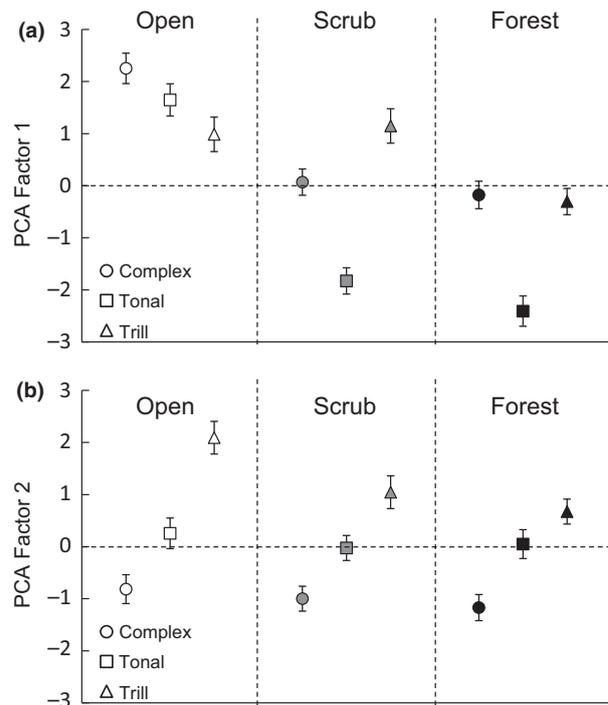


Fig. 2. LS Mean (\pm SE) values of (a) principal components analysis (PCA) factor 1 and (b) PCA factor 2 for complex songs (circles), tonal songs (squares) and trilled songs (triangles), for songs of species that occupy open habitats (white), scrub-like habitats (grey) and forests (black). PCA factor 1 loads on frequency range, maximum frequency and spectral entropy. PCA factor 2 loads most heavily on minimum and dominant frequencies.

five song properties in two phylogenetic PCA factors with eigenvalues >1.0 , which together accounted for 95% of the total variance (Table 1). Consistent with the non-phylogenetic PCA, the phylogenetic PCA factor 1 loaded most heavily on spectral entropy, frequency range and maximum frequency. Similarly, the second phylogenetic PCA factor loaded most heavily on minimum and dominant frequencies (table 1). The PGLS models revealed that song spectral properties associated with phylogenetic PCA factor 1 differed across habitats ($F_{2,6} = 4.18$, $P = 0.037$; model parameters: $\lambda < 0.01$, hypothesis test for λ not different than zero: $P > 0.9$) and were higher in open-habitat species than forest species ($t_6 = 2.81$, $P = 0.03$). This result is consistent with predictions from the AAH. There were no differences across habitats in song spectral properties associated with phylogenetic PCA factor 2 ($F_{2,6} = 0.07$, $P = 0.93$; model: $\lambda < 0.01$, $P > 0.9$). While we found no effect of song type on values of phylogenetic PCA factor 1 ($F_{2,6} = 0.76$, $P = 0.51$; model: $\lambda < 0.01$, $P > 0.9$), phylogenetic PCA factor 2 values differed significantly between song types ($F_{2,6} = 18.92$, $P = 0.003$; model: $\lambda = 0.20$, $P = 0.84$), with trilled songs presenting the highest values, followed by tonal songs and complex songs (all $t_6 > 4.0$, $P < 0.006$).

Together, the $rMANOVA$ and PGLS results suggest that the frequency range, maximum frequency and entropy levels (PCA factors 1) follow the AAH. Moreover, some song spectral properties differ between song types with trills having higher minimum and dominant frequencies (PCA factors 2) than tonal or complex songs.

AUDITORY EVOKED POTENTIALS

Sex, month, species and frequency effects on ABR thresholds

Using $rMANOVAS$, we found a significant month \times species interaction effect ($F_{8,625} = 2.26$, $P = 0.022$) on ABR thresholds. The solution for fixed effects reveals a positive relationship between month and ABR threshold for song sparrows ($t_{625} = 2.97$, $P = 0.0031$) and dark-eyed juncos ($t_{625} = 2.61$, $P = 0.0091$). Because there is no month \times species \times frequency interaction, this result suggests that the shape of the audiogram does not change over time, but rather that these species become less sensitive across all frequencies after the spring. Because of the cyclic nature of the breeding behaviour of these species and the concomitant cyclic changes in hormonal profile, we also explored nonlinear month effects on ABR thresholds using a month² term and found no effect on ABR thresholds nor significant interactions with species, sex or frequency (all $P > 0.16$). We found significant differences across frequencies ($F_{7,602} = 137.53$, $P < 0.0001$), species ($F_{8, 85} = 2.17$, $P = 0.038$) and a significant species \times frequency interaction ($F_{56,602} = 2.80$, $P < 0.0001$). Averaged across all species, the audiogram has a 'U' shape with lowest ABR thresholds between 3 and 4 kHz and highest ABR

thresholds at 0.5 kHz (Fig. 3). Species and frequency differences are further analyzed in the context of habitat and song properties.

Effect of song spectral properties on auditory sensitivity

Based on the SRMH, we predicted higher high-frequency auditory sensitivity in species that produce songs with higher frequencies. However, our $rMANOVA$ revealed no effect of PCA factor 1 (spectral entropy, frequency range and maximum frequency) on ABR thresholds ($F_{1,92} = 0.04$, $P = 0.84$) nor an interaction between frequency and PCA factor 1 ($F_{7,676} = 1.23$, $P = 0.28$). This result is contrary to our predictions. We found a significant, and positive, effect of PCA factor 2 (minimum and dominant frequencies) on ABR thresholds ($F_{1,92} = 5.09$, $P = 0.026$). This effect was due to a frequency \times PCA factor 2 interaction ($F_{7,676} = 6.26$, $P < 0.0001$). $rMANOVAS$ also indicate significant positive relationships between PCA factor 2 and ABR thresholds for frequencies above 3 kHz; that is, as PCA factor 2 increases, ABR thresholds are higher at frequencies above 3 kHz. This result is also contrary to our predictions.

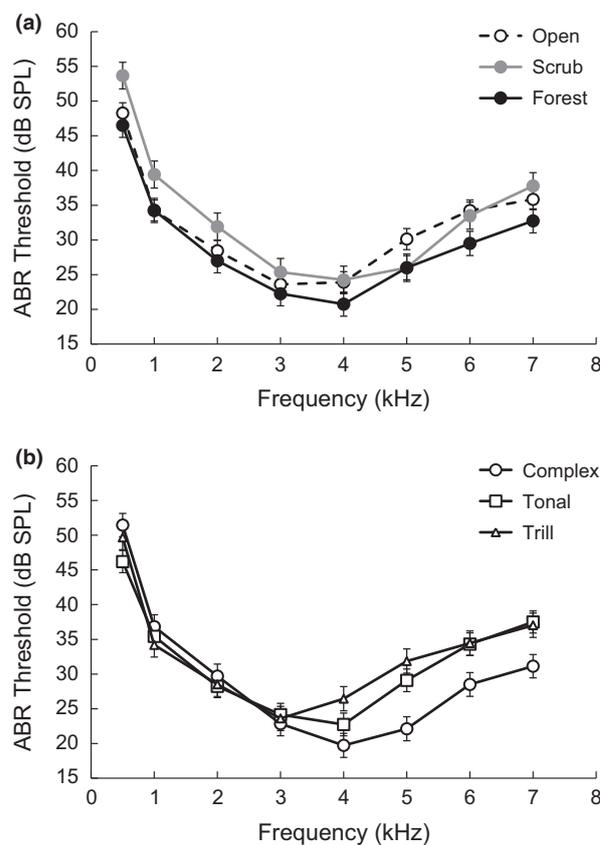


Fig. 3. (a) Audiograms plotting LS Mean (\pm SE) auditory brainstem response (ABR) threshold as a function of frequency and for species that occupy open habitats (white), scrub-like habitats (grey) and forests (black). (b) LS Mean (\pm SE) ABR thresholds as a function of frequency for species that produce pure-trilled songs (triangles), tonal songs (squares) or complex songs (circles).

When controlling for the phylogenetic relationships among species, we found no effect of song spectral properties associated with phylogenetic PCA factor 1 on any of the measurements of hearing sensitivity (mean ABR threshold: $F_{1,7} = 0.029$, $P = 0.87$; model: $\lambda < 0.01$, $P > 0.9$; frequency of best sensitivity: $F_{1,7} = 2.24$, $P = 0.18$; model: $\lambda < 0.01$, $P > 0.9$; high-frequency limit: $F_{1,7} = 0.43$, $P = 0.53$; model: $\lambda < 0.01$, $P > 0.9$). Similarly, we found no effect of phylogenetic PCA factor 2 on mean ABR threshold ($F_{1,7} = 3.14$, $P = 0.12$; model: $\lambda < 0.01$, $P > 0.9$). In contrast, we found a significant (and negative) effect of phylogenetic PCA factor 2 on hearing sensitivity measures associated with audiogram shape (frequency of best sensitivity: $F_{1,7} = 5.24$, $P = 0.05$; model: $\lambda = 0.21$, $P = 0.79$; high-frequency limit: $F_{1,7} = 5.8$, $P = 0.046$; model: $\lambda < 0.01$, $P > 0.9$). The negative effect suggests that as PCA factor 2 (minimum and dominant frequencies) increases, the frequency of best sensitivity and the high-frequency limit decrease. Thus, the patterns detected when controlling for phylogenetic relationships are contrary to those expected from the SRMH and consistent with our RMANOVA analyses.

Effects of habitat on ABR thresholds

The combination of the AAH and the SRMH predicts higher auditory sensitivity in open-habitat species than forest species. The analysis of ABR thresholds revealed a significant main effect of frequency ($F_{7,630} = 126.67$, $P < 0.0001$) but not habitat ($F_{2,90} = 2.97$, $P = 0.056$). There was, however, a significant frequency \times habitat interaction ($F_{14,630} = 2.30$, $P = 0.0044$): scrub species tended to have higher ABR thresholds at low frequencies than species from the other two habitats, and forest species had lower ABR thresholds at higher frequencies than open-habitat species (Fig. 3a). This latter result is contrary to our predictions. Similarly, the PGLS models revealed no main effects of habitat on the different measurements of auditory sensitivity (mean ABR threshold: $F_{2,6} = 1.62$, $P = 0.27$; model: $\lambda < 0.01$, $P > 0.9$; frequency of best sensitivity: $F_{2,6} = 0.25$, $P = 0.79$; model: $\lambda < 0.01$, $P > 0.9$; high-frequency limit: $F_{2,6} = 0.40$, $P = 0.69$; model: $\lambda < 0.01$, $P > 0.9$).

Effects of song type on ABR thresholds

Neither the song spectral properties nor the habitat effects matched our predictions. In our model, however, we included a factor not considered in the original tests of the AAH: song type. Our results from RMANOVA analyses show that song type is indeed an important correlate of the variation in ABR thresholds. While the main effect of song type was not significant ($F_{2,90} = 2.05$, $P = 0.13$), we found a significant frequency \times song-type interaction ($F_{14,630} = 4.27$, $P < 0.0001$) due to lower ABR thresholds at higher frequencies (>4 kHz) in species with complex songs (Fig. 3b; $t_{630} > 3.6$, $P < 0.05$).

When controlling for phylogeny, there was no effect of song type on mean ABR threshold ($F_{2,6} = 0.45$, $P = 0.66$; model: $\lambda < 0.01$, $P > 0.9$) or high-frequency limit ($F_{2,6} = 0.40$, $P = 0.69$; model: $\lambda < 0.01$, $P > 0.9$). There was, however, a significant effect of song type on frequency of best sensitivity ($F_{2,6} = 6.14$, $P = 0.014$; model: $\lambda < 0.01$, $P > 0.9$) with significantly higher frequencies in complex songs than trilled songs ($t_6 = 2.78$, $P = 0.032$). Thus, both RMANOVA and PGLS indicate that auditory sensitivity at high frequencies is greater in species with complex song types compared to those with tonal or trilled songs.

RELATIONS WITH BODY MASS

Body mass was negatively related to phylogenetic PCA factor 2 ($F_{1,7} = 6.92$, $P = 0.034$; model $\lambda = 0.34$, $P = 0.62$) but not to phylogenetic PCA factor 1 ($F_{1,7} = 0.09$, $P = 0.78$; model: $\lambda = 0.88$, $P = 0.07$). There were no differences in body mass across habitats ($F_{2,6} = 0.04$, $P = 0.95$; model: $\lambda = 0.9$, $P = 0.05$) or song types ($F_{2,6} = 3.40$, $P = 0.10$; model: $\lambda = 0.74$, $P = 0.29$). Body size had no effect on mean ABR threshold ($F_{1,7} = 0.94$, $P = 0.36$; model: $\lambda = 0.72$, $P = 0.32$), frequency of best sensitivity ($F_{1,7} = 0.69$, $P = 0.43$; model: $\lambda = 0.91$, $P = 0.09$) or high-frequency limit ($F_{1,7} = 0.7394$, $P = 0.42$; model: $\lambda = 0.95$, $P = 0.1$).

ANALYSES WITH TWO SONG-TYPE CATEGORIES

We analyzed song spectral properties and AEPs using two song types (complex vs. tonal/trilled). The main conclusions of this analysis are the same as the ones reported above for our analyses with the original classification of trilled, tonal and complex songs. These are that (i) song spectral properties associated with PCA factor 1 are higher in open-habitat species than scrub and forest species and (ii) species with complex songs are more sensitive to high frequencies (i.e. have lower ABR thresholds) than all other species (see Appendix S1).

Discussion

Our results from the phylogenetic comparative analyses parallel those from the non-phylogenetic RMANOVA analyses and can be summarized as follows: First, the effect of habitat on song spectral features is consistent with the AAH. Open-adapted species have higher scores of PCA factor 1 (entropy, frequency range and maximum frequency). There was only a weak effect of habitat on scores of PCA factor 2 (dominant and minimum frequency). Secondly, song type (tonal, trill and complex) is not associated with any particular habitat and is strongly correlated with PCA factor 2, with lower values in complex songs and higher values in trilled songs. Thirdly, contrary to the SRMH, high-frequency auditory sensitivity does not correlate with high-frequency song content in our sample of nine species. In fact, as song dominant and minimum

frequencies (PCA factor 2) increase, auditory sensitivity to high frequencies decreases. Finally, high-frequency sensitivity correlates with song type, with species that produce complex songs showing higher high-frequency sensitivity than all other species. The strong correlation between song type and PCA factor 2 can therefore explain the negative relationship between PCA factor 2 and high-frequency hearing sensitivity.

We acknowledge that interpretation of our phylogenetic comparative analyses should be taken with care for two related reasons. The sample size in these analyses ($n = 9$ species) is small and offers only weak power to detect significant patterns in PGLS models (Freckleton, Harvey & Pagel 2002). Related to the small sample size, there was little phylogenetic signal in the variables and the models. Therefore, in our study, non-phylogenetic analyses are more robust, not only because of the lack of phylogenetic signal and low power of the phylogenetic analyses, but also because they account for the repeated-measures nature of our experimental design. Nevertheless, when there was a detectable effect in the phylogenetic comparative models, the pattern supported that of the non-phylogenetic analyses. We now discuss these patterns, how they relate to previous work and the implications for the evolution of communication systems.

HABITAT AND SONG PROPERTIES

Consistent with the AAH (Morton 1975), songs of open-habitat species have higher frequencies and higher entropy than those of forest-adapted species. While some song properties were correlated with body mass, habitat differences are likely not a by-product of morphological differences given that body mass did not differ across habitats. Spectral entropy, maximum frequency and frequency range (PCA factor 1) are highly correlated with habitat and show a weak phylogenetic signal. While also affected by habitat and showing weak phylogenetic signal, minimum and dominant frequencies (PCA factor 2) are less sensitive to habitat properties. Cardoso & Price (2010) recently showed that song frequency differed across habitats and was independent of phylogeny and body mass in passerines from Europe and North America. Similarly, our results parallel those of Wiley's (1991) study on 120 species of North American songbirds showing that maximum frequency, but not minimum or dominant frequencies, was strongly associated with habitat, both with and without a correction for phylogenetic relationships. In his study, the presence of buzzes in the song was also strongly associated with habitat. This result contrasts our study system, in which songs with buzzes were present in all habitats. More in line with our study system, McCracken & Sheldon (1997) showed that frequency range is strongly associated with habitat and has little phylogenetic signal, whereas the effect of habitat on syllable structure is weak and potentially more phylogenetically informative. Similarly, Rheinhardt, Grafe & Abouheif (2004) showed that the presence of

buzzes has little phylogenetic signal and a weak association with habitat. Together, these results suggest that different song types may not be restricted to specific habitats and that habitat constraints on sound propagation have a stronger effect on song spectral properties than overall song structure.

AUDITORY SENSITIVITY AND THE SRMH

Two predictions that derive from the SRMH are that (i) sensitivity to high-frequency sounds should correlate with the presence of high-frequency vocalizations and (ii) the frequency of best sensitivity of the auditory system should match the dominant frequency of songs. Accordingly, studies on hearing sensitivity across taxa have revealed a close match between the range of frequencies emphasized in conspecific signals and the frequency regions of highest sensitivity in the auditory periphery (e.g. Konishi 1970; Capranica & Moffat 1983; Szymanski *et al.* 1999; Sisneros & Bass 2003). Our results, however, are in stark contrast with these predictions. High values of spectral entropy, maximum frequencies and frequency range of the songs (PCA factor 1) did not correlate with higher auditory sensitivity to high-frequency sounds. Similarly, forest species, whose songs have on average lower frequencies than those of open-habitat species, tend to be more sensitive to high-frequency sounds. Additionally, species with songs that have higher dominant and minimum frequencies (PCA factor 2) tend to have lower frequencies of best sensitivity and to have less sensitive auditory processing at higher frequencies. However, our results show that overall song structure is a stronger predictor of auditory sensitivity at higher frequencies than habitat or song spectral properties. Therefore, processing of high frequencies by receivers is strongly affected by song type, but receiver sensitivity to this frequency range is not restricted to specific habitats.

Our results contrast those of previous studies showing that species with high frequencies in their vocal repertoire tend to be more sensitive at higher frequencies (Konishi 1970; Dooling 1982; Henry & Lucas 2008). One reason for this discrepancy could be the level at which comparative analyses were conducted. Konishi (1970) studied hearing sensitivity and song spectral properties in 10 species of birds belonging to five different families: Emberizidae, Turdidae, Passeridae, Sturnidae and Fringillidae. Dooling (1982) described frequency spectra and audiograms of four species belonging to the Icteridae, Emberizidae, Psittaculidae and Fringillidae families. Similarly, Henry & Lucas (2008) generated audiograms for three species of birds, each one from a different family: Passeridae, Paridae and Sittidae. In these studies, differences among species could be due to more distant evolutionary relationships and not to differences associated with song properties. For instance, all the species in our study (family Emberizidae) are more sensitive at higher frequencies than house sparrows (family Passeridae), white-breasted nuthatches (family Sittidae), and tufted titmice (family Paridae) (this study;

Henry & Lucas 2008). However, not all emberizids have vocalizations with higher frequencies (e.g. the dominant frequency and frequency range of white-throated sparrows' vocalizations are narrower than both house sparrows and tufted titmice). By focusing on closely related species, the present study provides a better understanding of the effects of habitat and song properties on auditory sensitivity, revealing patterns not easily detected in studies with a more diverse set of species.

The combination of the AAH and the SRMH predicts higher auditory sensitivity to high-frequency sounds in open-habitat species than forest species. Our results show that habitat has only weak effects on auditory sensitivity and in the opposite direction: the forest species we tested tend to have higher auditory sensitivity to high-frequency sounds than open-adapted species. One possible explanation for this pattern is an adaptation of receivers in forested habitats to environmental effects on sound propagation. Scattering due to foliage and trunks result in strong attenuation of high-frequency sounds in forests (Wiley & Richards 1978; Wiley 1991). Therefore, in forested habitats, higher sensitivity to high-frequency sounds may be a way to compensate for such excess attenuation of high-frequency sounds. High-frequency sensitivity in forest habitats may have important fitness consequences as the alarm calls of different forest species, including species in our study, tend to be having high frequencies (Nolan *et al.* 2002; Weckstein, Kroodsma & Faucett 2002; Falls & Kopachena 2010).

Few studies have explored how the SRMH applies to acoustic properties outside the spectral domain. A recent study showed that peripheral auditory processing of fast sound onsets correlates with the rise time of vocalizations in five songbird species, including three New World sparrows (Gall, Brierley & Lucas 2012). Similarly, previous studies showed a correlation between the harmonic structure of vocalizations and the auditory processing of harmonics (Lohr & Dooling 1998; Dooling *et al.* 2002; Lohr, Dooling & Bartone 2006). Our results show that yet another dimension, overall song structure, can also correlate with auditory processing. Interestingly, the correlation is multidimensional in the sense that song structure is not strongly correlated with the high-frequency properties of a song, but it is strongly correlated with high-frequency auditory sensitivity. Why song structure correlates with auditory sensitivity is an open question, but one possibility concerns the ability to decode information from different song elements.

From an information-theory perspective (Hailman 2008), the internal organization of complex songs, which include trills, buzzes and tonal elements, contains more information than that of pure-trilled songs, in which only one element is repeated throughout the song. Hence, there is potentially more information available to be decoded in the different elements of complex songs. The ability to decode all of the note types of the song may therefore be important for receivers of species that use complex song types. Accordingly, we show that species that produce

complex songs are sensitive to a wide range of frequencies, including those present in the songs. On the other hand, our acoustical analyses show that trilled songs can have high frequencies, but the audiograms reveal that receivers are not very sensitive to high-frequency components of these songs. This suggests that auditory processing of high-frequency portions of the song may not be as important as processing of low-frequency portions in these species. Alternatively, processing of high-frequency portions of the song may be more important for receivers at relatively short distances from the sender, where signal amplitude is high enough to facilitate signal processing.

Our results also show a mismatch between the dominant frequencies of songs and the frequencies of best sensitivity. This mismatch has been previously reported for other species of birds (Konishi 1970; Dooling, Zoloth & Baylis 1978; Gall, Brierley & Lucas 2011) and contrasts the expected pattern from the SRMH. The reason for this mismatch is unclear. Konishi (1970) hypothesized that the shift of songs to higher frequencies improved the signal to noise ratio. Another possibility is that the mismatch is a mechanism underlying preferences for certain signal properties. For instance, physical constraints, such as size of the resonator, may impose low-frequency limits for sound production (Wallschager 1980; Ryan & Brenowitz 1985). Minimum song frequencies (as defined in our study) are negatively correlated with body size and may be an honest indicator of competitive ability in purple-crowned fairywrens (*Malurus coronatus coronatus*; Hall, Kingma & Peters 2013) and Montezuma oropendolas (*Psarocolius Montezuma*; Price, Earnshaw & Webster 2006). Similarly, in the subdesert mesite (*Monias benschi*), song frequency correlates with male body size, territory size and genetic quality (Seddon *et al.* 2004). Higher sensitivity at lower frequencies may then be a mechanism that imposes selection favouring low-frequency songs, in turn selecting for higher quality males. Therefore, a mismatch between song frequency and auditory sensitivity is one way in which sensory physiology may impose selection pressures on signal properties and explain patterns of signal preferences.

One example in which the mismatch between song frequency and auditory sensitivity could affect song preferences comes from species producing trilled songs. Our results show that species with trilled songs have the highest values of dominant and minimum frequencies and the lowest frequencies of best sensitivity. Interestingly, the frequencies of best sensitivity in these species closely match the minimum frequencies of their songs. In New World sparrows, there is a well-known trade-off between trill repetition rate and frequency bandwidth in trilled song elements (Podos 1997). At slow trill rates, songs may have narrow or wide frequency bandwidths; at high trill rates, however, songs are constrained to narrow frequency bandwidths. Therefore, when trill bandwidth is plotted as a function of trill rate, the result is a triangular distribution of songs where the diagonal represents an estimate of performance limit (Podos 1997). Ballentine, Hyman &

Nowiki (2004) showed that male swamp sparrow songs vary in their proximity to the performance limit and that females prefer songs closer to this limit. This means that at a given trill rate, female swamp sparrows should prefer songs with a broader frequency bandwidth. Our data suggest that salience may vary across the different frequencies of a sound of a given bandwidth, with species that specialize on trilled songs, in particular, relatively insensitive to the higher frequencies and highly sensitive to the lower frequencies in the trill elements. It would be interesting to know whether, at a particular trill rate, narrow-bandwidth songs are restricted to low or high frequencies. It is possible that songs that are closer to the performance limit have a broader frequency range because they extend to lower frequencies than those farther from the performance limit. Interestingly, our acoustical analysis reveals greater variability in minimum frequencies than maximum frequencies in the three species that produce trilled songs (coefficients of variation of minimum and maximum frequencies were 0.08 and 0.04 for swamp sparrows, 0.27 and 0.11 for dark-eyed juncos and 0.20 and 0.11 for chipping sparrows). Higher auditory sensitivity to lower frequencies, closer to the minimum frequencies of the songs, could therefore be a mechanism that imposes selection favouring songs with a broader range of frequencies that, depending on the trill rate, could be closer to the performance limit.

Conclusions

To conclude, our results suggest that our understanding of signal design and our expectations of how signal processing mechanisms should match signal properties are too simple. For example, the fact that the overall song structure has a stronger effect on auditory sensitivity than song spectral content raises the question whether the amount of information encoded in signals, and not the spectral properties of the signals, correlates with auditory processing. In turn, this suggests that the multidimensionality of signals (e.g. Nelson & Marler 1990), and how the different dimensions can interact, should be considered when we think about the evolution of signal-processing mechanisms.

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Data accessibility

Data on song spectral properties and hearing sensitivity have been deposited in the Dryad Digital Repository <http://dx.doi.org/10.5061/dryad.2n96q> (Véléz et al. 2014).

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Supporting Information

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

Appendix S1. Analyses with two song-type categories.

Fig. S1. (a) Audiograms plotting LS Mean (\pm SE) ABR threshold as a function of frequency and for species that occupy open habitats (white), scrub-like habitats (gray) and forests (black). (b) LS Mean (\pm SE) ABR thresholds as a function of frequency for species that produce pure tonal/trilled songs (triangles), or complex songs (circles).