



Urban birdsongs: higher minimum song frequency of an urban colonist persists in a common garden experiment

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Environmental changes caused by urbanization and noise pollution can have profound effects on acoustic communication. Many organisms use higher sound frequencies in urban environments with low-frequency noise, but the developmental and evolutionary mechanisms underlying these shifts are generally unknown. We used a common garden experiment to ask whether changes in minimum song frequency observed 30 years after a songbird colonized an urban environment are a consequence of behavioural flexibility. We captured male juvenile dark-eyed juncos, *Junco hyemalis thurberi*, from two populations (urban and mountain) soon after they reached independence (aged 25–40 days), raised them in identical indoor aviaries and studied their songs at an age of 3 years. We found that the large population difference in minimum frequency observed in the field persisted undiminished in the common garden despite the absence of noise. We also found some song sharing between the common garden and natal field populations, indicating that early song memorization before capture could contribute to the persistent song differences in adulthood. These results are the first to show that frequency shifts in urban birdsong are maintained in the absence of noise by genetic evolution and/or early life experiences.

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Anthropogenic noise can alter the biology of diverse animal taxa at organismal, population and even community scales (Cardoso, Hu, & Francis, 2018; Francis, Kleist, Ortega, & Cruz, 2012; Kight & Swaddle, 2011; Kleist, Guralnick, Cruz, Lowry, & Francis, 2018; Schroeder, Nakagawa, Cleasby, & Burke, 2012; Shannon et al., 2016; Slabbekoorn et al., 2010). In particular, the low-frequency background noise often associated with urbanization can interfere with animal communication and has been associated with changes in acoustic signals that improve sound transmission (Brumm & Slabbekoorn, 2005; Slabbekoorn & Ripmeester, 2008). One such change that is widely observed in urban environments is increased minimum frequency of acoustic signals, which may be an adaptation to overcome the masking effects of low-frequency noise (Davidson, Antonova, Dlott, Barber, & Francis, 2017; Derryberry

et al., 2016; Job, Kohler, & Gill, 2016; Redondo, Barrantes, & Sandoval, 2013; Slabbekoorn, 2013). Our understanding of the developmental and evolutionary mechanisms that underlie such changes in acoustic signalling remains limited (Bermúdez-Cuamatzin, Ríos-Chelén, Gil, & Macías García, 2011; Brumm & Slabbekoorn, 2005; Cardoso & Atwell, 2011a; Halfwerk & Slabbekoorn, 2009; Moseley et al., 2018; Verzijden, Ripmeester, Ohms, Snelderwaard, & Slabbekoorn, 2010; Zollinger, Slater, Nemeth, & Brumm, 2017). Species such as oscine songbirds that learn their songs are of particular interest due to the potential for cultural evolution and other forms of behavioural plasticity, which can facilitate rapid change in response to anthropogenic noise (Luther & Baptista, 2010; Luther & Derryberry, 2012; Ríos-Chelén, Salaberria, Barbosa, García, & Gil, 2012; Verzijden et al., 2012).

Several nonmutually exclusive hypotheses have been proposed to explain changes in song frequency in urban environments (Brumm & Slabbekoorn, 2005; Patricelli & Blickley, 2006), including short-term plasticity, ontogenetic effects (hereafter ‘early

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experience') and evolutionary change across generations. The short-term plasticity hypothesis argues that frequency shifts are the result of behavioural flexibility in response to the presence or absence of noise. Some studies in oscine songbirds have found evidence supporting plasticity either through rapid increases in minimum song frequency, which is sometimes associated with increases in amplitude via the Lombard effect (Bermúdez-Cuamatzin, Ríos-Chelén, Gil, & Macías García, 2009; Bermúdez-Cuamatzin et al., 2011; Cardoso & Atwell, 2011b; Goodwin & Podos, 2013; Verzijden et al., 2010), or switching to song types with higher minimum frequencies when noise is present (Halfwerk & Slabbekoorn, 2009). However, evidence from other songbird species has indicated that short-term plasticity in response to noise does not universally explain the frequency shifts present in urban birdsong (Derryberry et al., 2017; Potvin & MacDougall-Shackleton, 2015; Potvin & Mulder, 2013; Ríos-Chelén, Cuatrecasas-Lima, Bautista, & Martínez-Gómez, 2018; Zollinger et al., 2017).

The early experience hypothesis argues that the presence of noise during development affects song structure and production later in life. For example, evidence from black-capped chickadees, *Poecile atricapillus*, and white-crowned sparrows, *Zonotrichia leucophrys*, suggests that experiencing noise early in life may be necessary for the development of noise-induced plasticity in song frequency in adulthood (Gentry, Derryberry, Danner, Danner, & Luther, 2017; LaZerte, Slabbekoorn, & Otter, 2016). In addition to inducing plasticity, noise during development may mask lower-frequency tutor songs and cause selective learning of songs with higher minimum frequencies in urban environments (Lohr, Wright, & Dooling, 2003). Evidence suggests that some species preferentially learn songs that are less degraded by environmental transmission (Morton, Gish, & Vandervoort, 1986; Peters, Derryberry, & Nowicki, 2012; but see Zollinger et al., 2017). Notably, a recent study in white-crowned sparrows found that males developing in an environment with low-frequency noise preferentially learned higher-frequency (less masked) songs (Moseley et al., 2018; but see Zollinger et al., 2017). Collectively, these results indicate that ontogenetic effects of experiencing noise during early life may affect song frequency in adulthood by the preferential learning of certain songs (i.e. cultural selection), or by developing the ability for plastic adjustments to noise.

Finally, the evolutionary change hypothesis argues that natural or sexual selection on relevant genetic or cultural variation influences song frequency across generations (Brumm & Slabbekoorn, 2005; Patricelli & Bickley, 2006). In this case, the likely selective pressure is the masking of low-frequency songs by anthropogenic noise, making those songs less effective as signals for territoriality and mate attraction (Moseley et al., 2018). As a result, individuals with cognitive, morphological or sensory phenotypes that make them more likely to learn and/or produce higher-frequency songs will have higher fitness leading to directional selection (Endler, 1992). The colonization of urban environments is associated with a diverse array of phenotypic divergence, such as changes in body size (Meillere et al., 2017; Narango & Rodewald, 2016), bill morphology (Giraudeau et al., 2014) and neural architecture (Møller & Erritzoe, 2015), some of which are likely to be genetic, and all of which may contribute to evolution in song structure.

Here, we used a common garden experiment to evaluate these hypotheses. Common garden experiments are a powerful method for differentiating between the relative effects of genetic and environmental factors in determining phenotypic differences. The only common garden study of divergence in urban acoustic signals thus far used a species of grasshopper (*Chorthippus biguttulus*; Lampe, Reinhold, & Schmoll, 2014). In that study, individuals originating from noisy environments sang at significantly higher

minimum frequencies than individuals from quiet environments, but individuals reared in a noisy environment produced higher-frequency songs as adults regardless of their population of origin. Collectively, these results suggest roles for both evolution and early noise exposure in determining the differences in song frequency of urban populations. It is not known whether a similar interplay of evolutionary and ontogenetic effects applies to birdsong, which can be learned culturally, and should show much greater plasticity in response to noise than the stridulatory songs of insects (Orci, Petroczki, & Barta, 2016).

We studied an urban population of dark-eyed juncos, *Junco hyemalis thurberi*, that was likely established in the early 1980s (Rasner et al., 2004; Yeh, 2004). Although their exact origin is unknown, multiple lines of evidence suggest that these urban colonists rapidly diverged in a variety of behavioural, hormonal and life-history traits from an ancestral population that breeds in the inland mountains and migrates to the coast during the winter (Atwell, Cardoso, Whittaker, Price, & Ketterson, 2014; Cardoso & Atwell, 2011a; Yeh & Price, 2004). The urban and mountain acoustic environments differ strongly, including in anthropogenic noise, which is negligible in the mountains, and urban juncos sing at significantly higher minimum frequencies (ca. 0.5 kHz higher) than juncos in the mountain population (Cardoso & Atwell, 2011a; Cardoso, Atwell, Ketterson, & Price, 2009; Slabbekoorn, Yeh, & Hunt, 2007). If the higher minimum frequency observed in the urban population is caused by behavioural flexibility in song production (short-term plasticity), then the population difference should disappear in the common garden. Alternatively, if the population difference is genetically based or is affected by early experience, including song learning, then the population difference should persist in the common garden. To assess whether early song learning can account for population differences in the common garden, we compared song types of the common garden birds to songs from their natal populations.

METHODS

We studied two populations of dark-eyed juncos (hereafter 'junco') in San Diego County, California, U.S.A. The details of our study populations and the common garden experiment are reported elsewhere (Atwell et al., 2012, 2014; Yeh & Price, 2004). Briefly, the urban population was located at the University of California, San Diego (hereafter 'urban'; elevation 30 m; 32°52'N, 117°10'W) and the wildland population was located at Laguna Mountain Recreational Area (hereafter 'mountain'; elevation 1700 m; 32°52'N, 116°25'W). The landscape of the urban population is dominated by grass lawns, buildings, roads and other impervious surfaces, intermixed with gardens and small stands of trees and shrubs. Anthropogenic influences and noise generated by traffic, construction and pedestrians are widespread, albeit variable within the site (e.g. more intense near busy roads that intersect the site, such as the San Diego Freeway, Gilman Drive or North Torrey Pines Road). In contrast, the mountain population experiences negligible disruption from humans and contains a mixed woodland of coniferous and hardwood trees separated by smaller scrub vegetation. In July 2007, we captured 40 independent, juvenile juncos (day 25–40 posthatch as determined by prior banding in the nest for most subjects; see Atwell et al., 2012) from the urban and mountain populations (20 per sex per population; 80 birds total). The birds were briefly housed in small flocks in outdoor aviaries at the study site before being shipped by air to Indiana University in Bloomington, Indiana, U.S.A. Upon arrival, the birds were housed in mixed-sex flocks in separate but identical indoor aviaries (6.4 × 3.2 × 2.4 m) with photoperiodic conditions that mimicked seasonal shifts in their native ranges (Atwell et al., 2012, 2014). The

two populations were isolated from one another by two walls and a hallway, such that birds could only hear and interact with members of their own population. For the current study, we sampled all remaining males in May 2010 (mountain, $N = 9$; urban, $N = 8$), which was their third spring in the common garden.

Song Recordings

Juncos produce a loud broadcast song consisting of a simple trill (rapid repetition of the same syllable) and each male sings a repertoire of two to eight distinct song types (Fig. 1; Cardoso & Reichard, 2016; Newman, Yeh, & Price, 2008). To record songs, we isolated each male from the common garden in a ($45.7 \times 45.7 \times 45.7$ cm) cage with a single perch extended across its centre and access to food and water ad libitum. An Audio-Technica shotgun microphone (Model AT835b) was suspended ~30 cm above the centre of the cage's perch and connected to a Marantz digital recorder (Model PMD660). We recorded each male for 3 h over a period of 3 weeks using a 44.1 kHz sampling rate in uncompressed WAV format.

Following Konishi (1964), we visually identified the song types of each male, according to the shape of sound frequency modulation in the trilled syllable (including within-syllable notes, in the case of multinote syllables), before comparing song types between different males to identify instances of song type sharing. Shared song types used syllables of the same shape, but some disparity in duration or frequency bandwidth was deemed acceptable (Fig. 1). To limit bias, assignments of song types and song type sharing were

performed blindly by two independent observers without any knowledge of the population of origin. Mountain males sang an average of three song types (range 1–5), and urban males sang an average of 4.9 song types (range 2–7). We also compared these songs to a catalogue of song types from both field populations collected in 2006 and 2007 (mountain, $N = 115$; urban, $N = 168$; Cardoso & Atwell, 2011a) to assess sharing between the common garden males and their natal populations. These field recordings represent potential tutor songs for the common garden males, which were also captured in 2007.

Song Measurements

We used Raven Pro 1.4 (Center for Conservation Bioacoustics, 2011) to measure minimum, maximum and peak frequency. Measurements were performed by the same observer (M.M.P.) who was blind to population of origin to avoid bias. For each combination of song type and male, we randomly selected a representative exemplar and generated a spectrogram (Hann Window, 512 DFT, 86.1 Hz frequency resolution, 5.8 ms time resolution). We used the cursor to visually draw a 'selection box' around each song type, bounded by the perceived start and end time as well as the minimum and maximum frequency. We recorded the minimum and maximum frequencies of these visual measurements from the spectrogram, and also recorded the peak frequency of the selection (frequency with the highest cumulative amplitude).

Visual measurements from spectrograms have been criticized as a potentially biased technique for determining minimum and

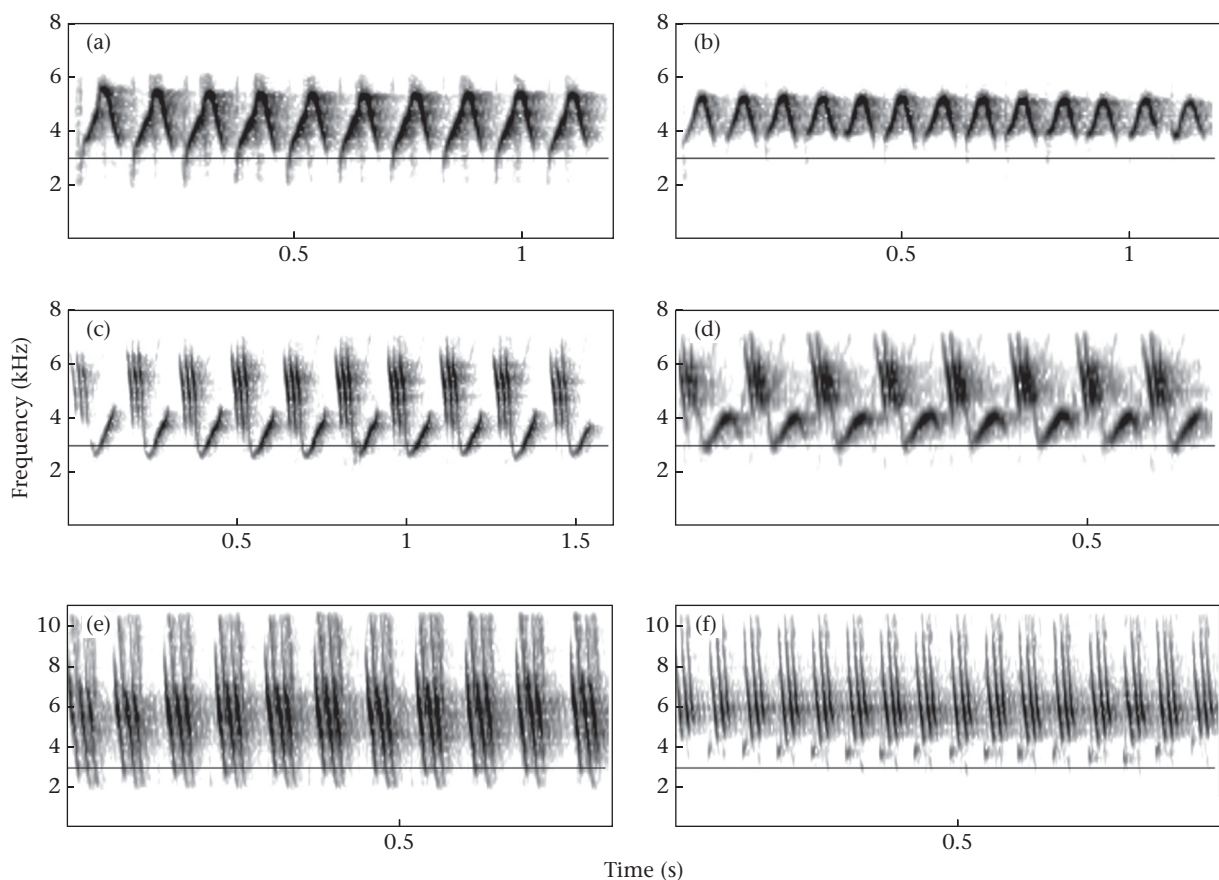


Figure 1. Example spectrograms of shared song types observed between mountain- and urban-captured males in the common garden. Songs (a), (c) and (e) were produced by mountain males, and songs (b), (d) and (f) were produced by urban males. The thin line on each spectrogram marks 3 kHz to highlight frequency shifts between mountain and urban songs.

maximum frequency (Brumm, Zollinger, Niemela, & Sprau, 2017; Cardoso & Atwell, 2012; Rios-Chelen, Lee, & Patricelli, 2016; Zollinger, Podos, Nemeth, Goller, & Brumm, 2012). Instead, researchers have advocated using the power spectrum and a threshold value as a more objective alternative. To assess potential differences in these techniques, we also measured minimum and maximum frequency from the power spectrum of each song using a threshold of minus 30 dB relative to the peak frequency of the song. Minimum and maximum frequency were defined as the points of intersection between the power spectrum curve and the threshold value (Podos, 1997; Rios-Chelen et al., 2016). We were able to use a very large amplitude threshold (minus 30 dB) because of the high signal-to-noise ratio in our aviary recordings. Nevertheless, in a few song types, harmonics caused power spectrum measurements of maximum frequency to far exceed the highest fundamental frequency observed on the spectrogram and the normal range of maximum frequencies measured in various field studies (Cardoso & Reichard, 2016). Similarly, there was one song type where the power spectrum measurement of minimum frequency resulted in a value that was much higher than the lowest observed frequency clearly observed on the spectrogram. We excluded those measurements ($N = 1$ song for minimum frequency, and $N = 10$ songs from 7 different males for maximum frequency) from any analyses involving the power spectrum measurements.

All raw frequency measurements (Hz) were log-transformed before further analysis, because modulation and perception of sound frequency both function on a ratio scale (Cardoso, 2013). Log transformation facilitates the comparison of frequency differences across different frequency ranges; otherwise, differences in maximum or peak frequency would be overestimated compared to differences in minimum frequency.

Across all measurements of minimum frequency, the visual measurements from spectrograms correlated significantly with threshold measurements from power spectra (Pearson correlation: $r_{62} = 0.79$, $P < 0.001$; Appendix, Fig. A1a), but with a slight difference in means between the measurement types ($\bar{x} \pm \text{SD}$: spectrogram: 2598.3 ± 531.9 Hz; threshold: 2767.1 ± 521.6 Hz; \bar{x} difference: 168.7 Hz; $t_{63} = 3.49$, $P < 0.001$; Appendix, Fig. A2). Maximum frequency measurements were also correlated across methods ($r_{53} = 0.96$, $P < 0.001$; Appendix, Fig. A1b), and their means did not differ significantly ($t_{54} = 1.85$, $P = 0.07$; Appendix, Fig. A2). In the main text, we only report analyses using frequency measurements from spectrograms to facilitate a comparison with a data set of field recordings previously analysed in this manner (see above; Cardoso & Atwell, 2011a). In the Appendix we report comparisons between the common garden populations using threshold measurements from power spectra (Tables A1 and A3), which yielded identical results to those reported in the main text.

Statistical Analyses

To compare acoustic traits between populations in the common garden, we conducted linear mixed models (LMM) using the 'lme4' package in R v.3.5.2 (Bates, Mächler, Bolker, & Walker, 2015; R Core Team, 2018). Each model contained a song measurement as the response variable, population of origin as the independent factor and song type as a random factor. We used a likelihood ratio test (LRT) to assess significance by comparing the likelihood of the full model to that of a reduced model that omitted population.

Song types were used as a random factor in the main text, rather than male identity, because junco song traits are a property of the individual song type (high within-type repeatability across males)

rather than a property of the individual males (low repeatability across song types in the repertoire of individual males; Cardoso et al., 2009). In the Appendix we report identical analyses using male identity as a random effect, instead of song type, and our results remain unchanged (Tables A2, A3).

Junco song development is strongly influenced by social learning from peers and by creating novel song types (see below; reviewed in Cardoso & Reichard, 2016). Thus, any differences in minimum song frequency between urban-captured and mountain-captured males may be explained either by early song learning in their natal environments before capture, the development of divergent frequencies later in the common garden through social learning, or some combination of the two. To differentiate between these two explanations, we compared the minimum frequency of song types from urban-captured and mountain-captured males that were shared versus not shared with our field recordings from each natal population. We assume that shared types were learned before capture, and at least some of the unshared song types originated *de novo* in the common garden. Hence, by comparing shared and nonshared song types we can test whether social learning influences acoustic traits in a particular direction (Cardoso & Atwell, 2016). To test this possibility, we used a LMM for each population with minimum frequency as the dependent variable, whether song types were shared or not with field recordings as an independent factor and song type as the random factor, and applied a likelihood ratio test. Finally, we used standard *t* tests to compare frequency measurements from the common garden with those from a previously published field study of both populations (see above; Cardoso & Atwell, 2011a).

Ethical Note

This research was conducted in compliance with best practices for animal research, including the approval of the Indiana University Animal Care and Use Committee (Study No. 06-242, 09-037) and with permits from the U.S. Federal Bird Banding Lab (Permit No. 20261), U.S. Fish and Wildlife Service (Scientific Collecting Permit No. MB093279), the California Department of Fish and Game (Scientific Collecting Permit No. 802039-02, SC-007852), the U.S. Forest Service (Special Use Permit No. DRD018701) and the Indiana Department of Natural Resources (Scientific Purposes License No. 07-001, 10-0005). The birds used in this study were extracted as soon as possible after capture in mist nets or walk-in traps to minimize any discomfort associated with these widely used capture techniques. Transport between the capture site and study site was done as rapidly as possible via air freight. Birds were placed in small boxes immediately before departure and released into large flight aviaries (see above) immediately after delivery to minimize discomfort associated with transport. While in transport and captivity, birds had constant access to food and water, including during the song recording process. Unless they were isolated for song recording or for another experiment unrelated to this study, birds were housed in small flocks so that normal social interactions and bonds could be established and maintained.

RESULTS

Song Types and Sharing

Mountain-captured males sang a total of 17 song types in the common garden, of which 7 (41%) were shared between two or more individuals. Urban-captured males sang 28 song types in the common garden, of which 8 (29%) were shared between two or

more individuals. This population difference in song sharing was not significant (contingency table chi-square: $\chi^2_1 = 0.43$, $P = 0.51$). Three song types were shared between mountain- and urban-captured males in the common garden (Fig. 1). We also identified two song types from the mountain-captured males (out of 17; 12%) that had been previously recorded from mountain males in the field (out of 115 song types recorded in the field) and six song types from the urban-captured males (out of 28; 21%) that had been previously recorded from urban males in the field (out of 168 song types recorded in the field), which might be due to the urban population being more extensively recorded in the field.

Frequency Comparisons within the Common Garden

In the common garden, urban-captured males produced songs with significantly higher minimum frequencies than mountain-captured males (LRT: $\chi^2_1 = 11.7$, $P < 0.001$; Fig. 2 and examples in Fig. 1). In contrast, maximum ($\chi^2_1 = 0.14$, $P = 0.71$) and peak frequency ($\chi^2_1 = 0.10$, $P = 0.75$) did not differ significantly between urban- and mountain-captured males. For each frequency measure, we noted a small number of extreme outliers that exceeded three times the interquartile range (Fig. 2). Although there was no biological justification for their exclusion, removing these outliers from the analyses produced qualitatively identical results (Appendix, Tables A4, A5).

Frequency Comparisons Between the Captive and Natal Field Populations

For urban-captured males, the minimum frequency of song types shared with recordings from the urban field site ($\bar{x} = 3.434$ logHz (2761.2 Hz), $N = 6$ song types) did not differ from that of song types not found in field recordings ($\bar{x} = 3.437$ logHz (2761.6 Hz), $N = 22$ song types; $\chi^2_1 = 1.21$, $P = 0.27$). In fact, the means for the minimum frequency of song types were almost identical between those shared and not shared with the urban field recordings. A similar analysis for mountain song types yielded the same result, as shared song types had a similar mean ($\bar{x} = 3.510$ logHz (2344.4 Hz), $N = 2$ song types) and did not differ from nonshared types in the field recordings ($\bar{x} = 3.527$ logHz (2344.0 Hz), $N = 15$ song types; $\chi^2_1 = 0.33$, $P = 0.57$). However, the analysis of mountain song types

may be less reliable as only two song types were shared between our mountain-captured males and the field recordings.

Compared to field recordings from the mountains, mountain-captured males in the common garden sang with significantly lower minimum frequencies ($t_{130} = 8.06$, $P < 0.001$; Fig. 3a), higher peak frequencies ($t_{130} = 2.16$, $P = 0.03$; Fig. 3b) and higher maximum frequencies ($t_{130} = 3.42$, $P < 0.001$; Fig. 3c). Urban-captured males also sang with significantly lower minimum frequencies ($t_{194} = 8.12$, $P < 0.001$; Fig. 3a), higher peak frequencies ($t_{194} = 3.02$, $P < 0.01$; Fig. 3b) and higher maximum frequencies ($t_{194} = 2.21$, $P = 0.03$; Fig. 3c) than urban field recordings.

DISCUSSION

Consistent with differences observed in the field (Cardoso & Atwell, 2011a), urban male juncos captured early in life sang at significantly higher minimum frequencies than mountain-captured juncos when both were held in a quiet, common garden environment for 3 years. This result supports the prediction of both the early experience and the evolutionary change hypotheses and indicates that the higher minimum frequency of the urban junco population is established early in life, through genetic and/or cultural mechanisms, rather than occurring as a result of short-term behavioural plasticity in response to noise. No significant differences were found in maximum or peak frequency between the two populations in the common garden, indicating that acoustic adaptation in the urban population has acted predominantly on minimum frequency rather than on the frequency of the entire song. We identified shared songs between the captive and natal field populations, indicating that early song learning from tutors in the field before capture could contribute to the differences observed in the common garden.

The songs of oscine birds develop through a combination of cultural transmission and genetically based influences on the morphology and physiology of the vocal production apparatus, on the auditory system and on learning preferences (Fehér, Wang, Saar, Mitra, & Tchernichovski, 2009; Podos, Huber, & Taft, 2004). While genetic evolution relies on selection or drift acting on standing genetic variation or new mutations, cultural evolution allows for selectively learning pre-existing memes (i.e. cultural selection) or generating novel memes (i.e. cultural mutation) in a nonrandom, adaptive way (Cardoso & Atwell, 2011a). For example, white-crowned sparrows in noisy environments were shown to preferentially learn higher-frequency songs and also to elevate the frequency of the learned songs over those of their tutor (Moseley et al., 2018). A similar combination of cultural selection and cultural mutation has also been inferred for urban juncos based on population comparisons of the song type meme pools present in the field populations (Cardoso & Atwell, 2011a).

As in other songbirds, juncos partially rely on conspecific tutors early in life to develop species-typical songs (Marler, Kreith, & Tamura, 1962), but the exact duration of this sensitive period for song learning is not known. We observed some song type sharing between the common garden birds and field recordings from their natal populations, implying that song learning occurred in the field before capture at around 25–40 days of age. This time frame is consistent with the timing of the sensitive period in other closely related species (Marler & Peters, 1987, 1988). However, the majority of song types in both common garden populations were not shared with any known field tutors despite our extensive catalogue of field recordings (>100 song types per population), particularly in the spatially confined, urban population (Cardoso & Atwell, 2011a). In addition to cultural transmission from adult tutors, juncos are known to experience frequent cultural mutations in the form of modifications to learned song types (i.e. improvisation, Beecher &

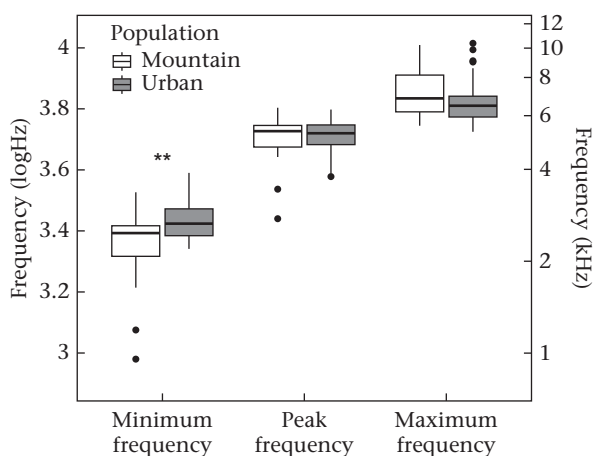


Figure 2. Comparison of the minimum, peak and maximum frequencies of song types produced by mountain- and urban-captured males raised in a common garden environment. Each box represents the interquartile range and median, whiskers represent range of data within 1.5 times the interquartile range, and dots represent data points exceeding that range. ** $P < 0.001$.

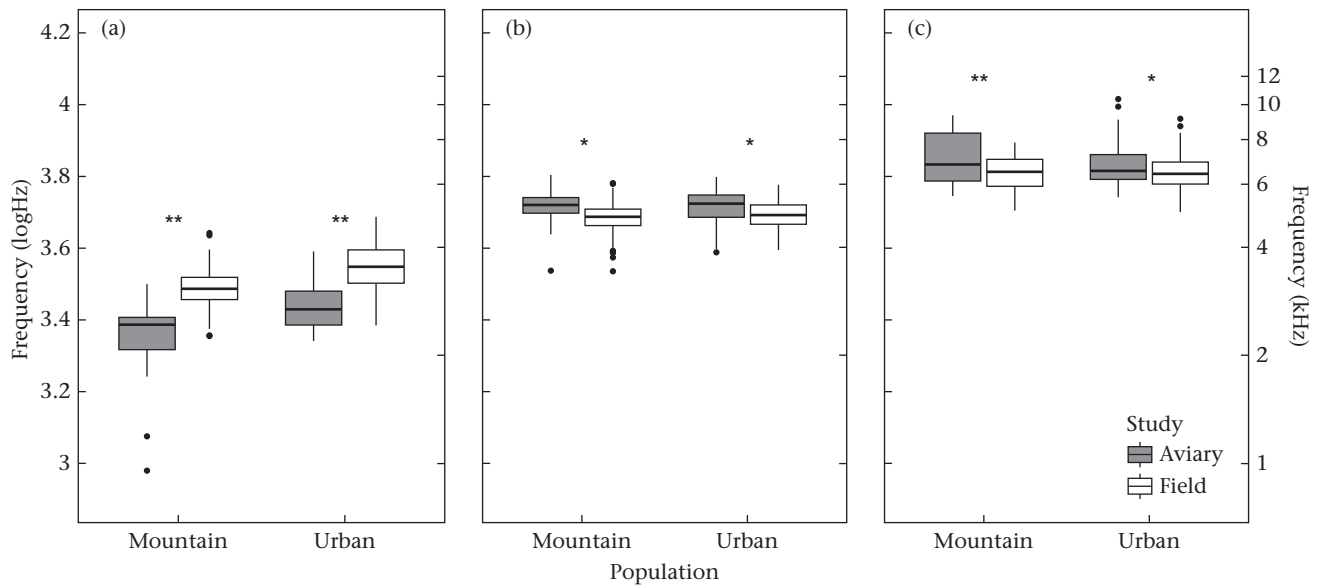


Figure 3. Comparison of the (a) minimum, (b) peak and (c) maximum frequency of songs produced by mountain and urban males in the field (Cardoso & Atwell, 2011a) and in a common garden aviary environment. Box plots as in Fig. 2. * $P < 0.05$; ** $P < 0.001$.

Brenowitz, 2005) or de novo creation of new song types (i.e. invention, Beecher & Brenowitz, 2005; reviewed in Cardoso & Reichard, 2016). These frequent cultural mutations likely contribute to the low incidence of song sharing between the captive and field populations.

We also observed frequent song sharing among captive males (41% of song types for the mountain-captured birds, and 29% for the urban-captured) at a much higher rate than typically found in the field, where most neighbouring males do not share song types (Cardoso & Reichard, 2016; Konishi, 1964; Newman et al., 2008). This disparity suggests that song development in the common garden was strongly influenced by peers rather than by adult tutors in the field before capture. The importance of peer interactions is consistent with a previous experiment that showed that when young juncos are reared together without adult tutors, they are stimulated to create novel sounds (cultural innovation), copy them from each other and modify them (cultural improvisation) into a species-typical song (Marler et al., 1962). This type of cultural mutation may be biased towards higher frequencies if it occurs in a noisy urban environment (Cardoso & Atwell, 2011a; Moseley et al., 2018), but our common garden environment was quiet and lacked even naturally occurring noise such as wind or rain. Furthermore, all vocal interactions in the common garden occurred over short distances, which should limit the effects of song degradation between the sender and receiver (Wiley & Richards, 1982). When these transmission constraints are reduced, juncos can effectively communicate using the extremes of their vocal range, such as a wider frequency bandwidth that is normally restricted to a distinct class of low-amplitude songs produced during close-proximity interactions (i.e. 'short-range song'; Reichard, Rice, Vanderbilt, & Ketterson, 2011). Consistent with this prediction, we found that both populations of captive juncos sang at significantly lower minimum frequencies and higher maximum frequencies as well as higher peak frequencies than field recordings from their natal populations.

Importantly, all of the changes in song from the wild to the common garden (wider frequency bandwidth, learning from peers) did not erode the population difference in minimum frequency. The difference in minimum song frequency between mountain- and

urban-captured birds in the common garden was large (417 Hz), and close to the difference reported between the wild populations (540 Hz; Cardoso & Atwell, 2011a). Both the evolutionary change and the early experience hypotheses may explain this persistent difference. These hypotheses are not mutually exclusive and may even reinforce each other. For example, besides the difference in minimum song frequency, the urban junco population studied here also diverged in morphological, reproductive and endocrinological traits (Atwell et al., 2012, 2014; Rasner et al., 2004; Yeh, 2004; Yeh & Price, 2004). Some of these traits appear to have changed by a combination of phenotypic plasticity, which provides an immediate and approximate adaptation to the urban environment, as well as selection causing genetic assimilation and the adjustment of the plastic response (Atwell et al., 2014; Price, Yeh, & Harr, 2008). Song traits, including minimum frequency, are also likely to undergo such synergy of plasticity and selection, especially in sedentary populations that experience a stable acoustic environment after colonizing a new habitat like these urban juncos. Initially, behavioural flexibility can change songs to provide an immediate reduction of masking by noise (Bermúdez-Cuatatzin et al., 2011; Goodwin & Podos, 2013; Halfwerk & Slabbekoorn, 2009), and this plasticity simultaneously creates cultural models for which genetically based learning preferences may be selected upon. Interestingly, song types of both urban-captured and mountain-captured males that were shared with field recordings, and thus likely to have been memorized from tutors in their natal environments, had an identical average minimum frequency to the unshared song types, many of which may have been acquired later in the common garden. This result suggests that cultural learning early in life is not the most important explanation for the persistent population difference in song frequency. Instead, around 30 years after colonization of the urban environment (Rasner et al., 2004; Yeh, 2004), it seems probable that the population difference in song frequency already has a substantial genetic component.

A genetic difference in traits that influence minimum song frequency could also explain why the population difference persisted even in the face of an overall decrease in minimum frequency by both the urban and mountain populations when held in captivity. The divergent traits that may be responsible for the persistent

population difference could be cognitive, such as learning or singing preferences (e.g. the genetic song template: Fehér et al., 2009; Mets & Braind, 2018; Mundinger, 1995; Mundinger & Lahti, 2014), or related to auditory perception and processing (Prather, Nowicki, Anderson, Peters, & Mooney, 2009), or even anatomical or physiological traits that affect song production (e.g. body size: Narango & Rodewald, 2016; Ryan & Brenowitz, 1985; bill morphology: Giraudeau et al., 2014). Morphologically mediated population differences in sound frequency are perhaps less likely because these two populations are similar in body weight, tarsus length and bill morphology (Rasner et al., 2004), and any minor differences in body size are insufficient to fully explain the large interpopulation difference in minimum song frequency (Wallschläger, 1980). Whether the genetic song template, auditory perception, or other aspects of neuroanatomy have diverged between the urban and mountain juncos is unknown and represents an intriguing direction for future research.

Broadly, our results suggest that urban environments, and particularly urban noise, can drive the rapid evolution of higher minimum frequencies in oscines through a combination of cultural and genetic changes. The urban junco population studied here exhibits one of the largest documented differences in minimum song frequency relative to a rural population, and this difference was likely generated in less than 30 years, indicating that if evolutionary changes are the primary driver, they can happen relatively rapidly (Cardoso & Atwell, 2011a; Slabbekoorn, Yeh, & Hunt, 2007). Although behavioural flexibility may provide an immediate escape from masking by environmental noise (Bermúdez-Cuamatzin et al., 2011; Goodwin & Podos, 2013; Halfwerk & Slabbekoorn, 2009), a combination of cultural and genetic evolution can drive more permanent shifts in minimum frequency in chronically noisy environments (Zollinger et al., 2017). The juncos in this study experienced less than 40 days of life with adult song tutors and the noise present in their natal environments, which also suggests that possible developmental mechanisms were triggered in very early life (e.g. memorization of song types, experiencing noise) and had lasting effects.

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Appendix

Table A1

Results of linear mixed model analyses comparing frequency characteristics between common garden populations measured from power spectra, rather than from spectrograms, using song type as a random effect

Song measurement	χ^2_1	<i>P</i>	<i>N</i>
Minimum frequency	16.55	<0.001	64
Maximum frequency	0.007	0.935	55

Significant outcomes are shown in bold.

Table A2

Results of linear mixed model analyses comparing frequency characteristics between common garden populations measured from spectrograms using individual as a random effect rather than song type

Song measurement	χ^2_1	<i>P</i>	<i>N</i>
Minimum frequency	7.62	0.006	65
Maximum frequency	2.03	0.154	65
Peak frequency	0.183	0.669	65

Significant outcomes are shown in bold.

Table A3

Results of linear mixed model analyses comparing frequency characteristics between common garden populations measured from power spectra, rather than from spectrograms, using individual as a random effect rather than song type

Song measurement	χ^2_1	<i>P</i>	<i>N</i>
Minimum frequency	4.075	0.044	64
Maximum frequency	0.518	0.472	55

Significant outcomes are shown in bold.

Table A4

Results of linear mixed model analyses comparing frequency characteristics between common garden populations measured from spectrograms using song type as a random effect with extreme outliers removed from the data set

Song measurement	χ^2_1	<i>P</i>	Number of outliers removed
Minimum frequency	8.28	0.004	2
Maximum frequency	0.447	0.504	1
Peak frequency	0.142	0.706	1

Outliers were defined as any points that exceeded three times the interquartile range. Significant outcomes are shown in bold.

Table A5

Results of linear mixed model analyses comparing frequency characteristics between common garden populations measured from power spectra using song type as a random effect with extreme outliers removed from the data set

Song measurement	χ^2_1	<i>P</i>	Number of outliers removed
Minimum frequency	12.89	<0.001	2
Maximum frequency	0.007	0.935	0

Outliers were defined as any points that exceeded three times the interquartile range. Significant outcomes are shown in bold.

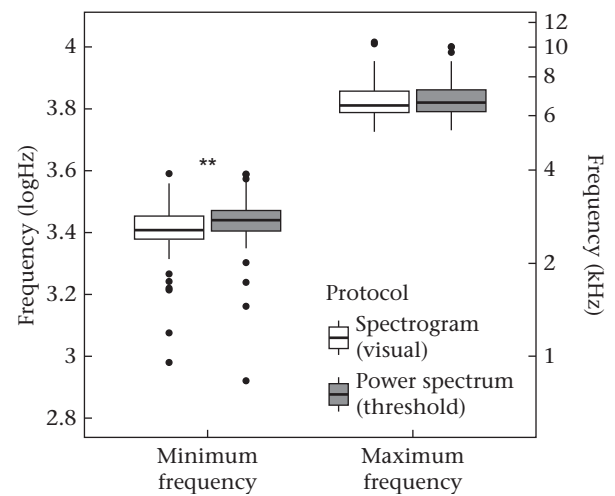


Figure A2. Comparison of the spectrogram and power spectrum techniques for measuring minimum and maximum frequency of songs recorded in the common garden environment. Each box represents the interquartile range and median, whiskers represent range of data within 1.5 times the interquartile range, and dots represent data points exceeding that range. ***P* < 0.001.

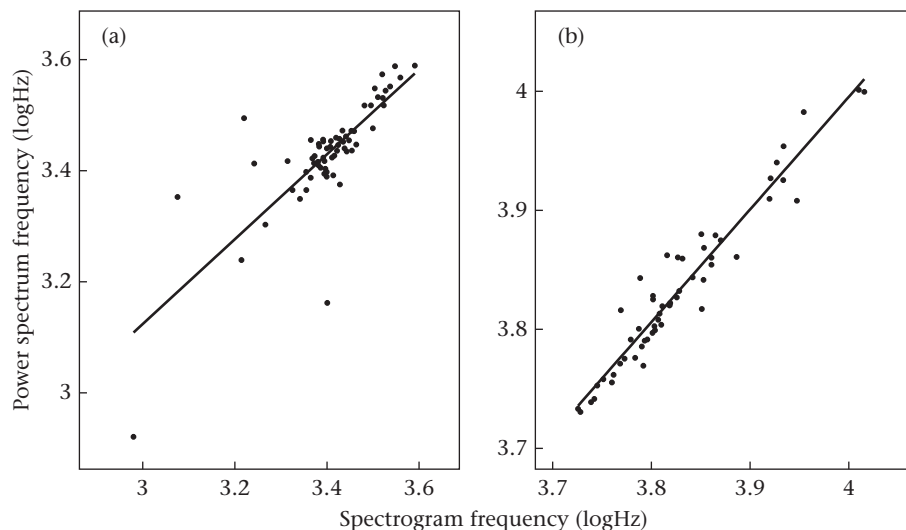


Figure A1. Correlation of (a) minimum and (b) maximum frequencies measured visually from the spectrogram and using a minus 30 dB threshold from the power spectrum.