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No evidence of song divergence across multiple urban and non-urban populations of Dark-eyed Juncos (Junco hyemalis) in Southern California

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No evidence of song divergence across multiple urban and non-urban populations of Dark-eyed Juncos (*Junco hyemalis*) in Southern California

A thesis submitted in partial satisfaction of the requirements for the degree Masters of Science in Biology

by

Felisha Wong

2020

ABSTRACT OF THE THESIS

No evidence of song divergence across multiple urban and non-urban populations of Dark-eyed Juncos (*Junco hyemalis*) in Southern California

by

Felisha Wong

Masters of Science in Biology

University of California, Los Angeles, 2020

Professor Pamela J. Yeh, Chair

Urbanization can affect species by introducing new selection pressures, such as noise pollution and different environmental transmission properties. These selection pressures can potentially trigger divergence between urban and non-urban populations of conspecifics. Songbirds in particular rely on their vocalizations to defend territories and attract mates. Urban songbirds have been shown in some species and some populations to increase the frequencies and reduce the length and trill rate of their songs. This study compares songs from four urban and three non-urban populations of dark-eyed juncos (*Junco hyemalis*) throughout Southern California. We examined song length, trill rate, minimum frequency, maximum frequency, and bandwidth frequency. All sites showed high variance in these traits. We also analyzed whether there were any differences between songs recorded from one urban junco population in San Diego nearly two decades ago and more recently collected data in 2018-2020. We found no significant differences across sites and between urban and non-urban populations in any of these song features; we also found no significant differences between San Diego junco songs from the 2006/2007 and the 2018-2020 field seasons. These findings partially support and partially are in contrast to previous urban junco song studies. To our knowledge, this is one of only a few studies that found no differences in any song traits examined from multiple urban populations and multiple non-urban populations of the same species.

The thesis of Felisha Wong is approved.

Daniel T. Blumstein

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Pamela J. Yeh, Committee Chair

University of California, Los Angeles

2020

To my advisor, my academic mentors, and the undergraduate assistants who helped me greatly in collecting and analyzing the data. I also wish to dedicate this to the many people who supported my journey and my peers, whom without this would not have been possible.

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study. Pamela J. Yeh conceived of the study. Felisha Wong and Eleanor Diamant developed the study design, collected the data in the field, and performed the statistical analyses. Felisha Wong, Eleanor Diamant, Marlene Walters, and Pamela J. Yeh collectively wrote and edited the manuscript.

INTRODUCTION

Urbanization presents a growing threat for wildlife, altering the landscape of their natural ecosystems and creating new selection pressures (Yeh, 2004; Chace and Walsh, 2006; Isaksson, 2018). For birds that use song for territory defense and mate attraction, these selection pressures could affect song communication due to differences in habitat structure and transmission properties, as well as intense low-frequency ambient noise from cars, planes, and traffic, which can restrict the range over which individuals can be heard successfully (Morton, 1975; Ryan and Brenowitz, 1985; Slabbekoorn and Smith, 2002b, 2002a; Brumm and Slabbekoorn, 2005; Patricelli and Blickley, 2006; Warren *et al.*, 2006; Kirschel *et al.*, 2009; Grabarczyk and Gill, 2019).

Urban landscapes differ from non-urban habitats in at least several ways (Patricelli and Blickley, 2006; Warren *et al.*, 2006; Ouyang *et al.*, 2018). Urban landscapes often consist of hard, flat surfaces (e.g. high buildings and pavement) (Rebele, 1994; Müller *et al.*, 2013) with little vegetation (Slabbekoorn, Yeh and Hunt, 2007; Müller *et al.*, 2013) and potentially more open spaces (Porter, Forschner and Blair, 2001; Slabbekoorn and den Boer-Visser, 2006). Echoes and reverberations reflected by vertical surfaces have been found to affect longer songs with lower frequencies (Wiley and Richards, 1978, 1982; Slabbekoorn, Ellers and Smith, 2002), and faster trill rates fare better in open environments (Brown and Handford, 1996, 2000; Naguib, 2003; Slabbekoorn, Yeh and Hunt, 2007). Furthermore, the typically dense vegetation found in mountainous areas have been found to absorb and scatter higher frequencies more, suggesting that in urban areas where there is low vegetation cover, higher frequencies transmit better (Morton, 1975; Wiley and Richards, 1978; Wiley, 1991; Derryberry, 2009). As a result, it has been hypothesized that urban populations would exhibit longer song lengths, reduced trill rates, and higher frequencies (Morton, 1975; Wiley, 1991; Slabbekoorn, Yeh and Hunt, 2007).

Aside from changes to the landscape composition in urban environments, other anthropogenic changes such as noise pollution have also been shown to change avian song characteristics. Because urban ambient noise is typically high amplitude, low frequency sounds below 2000 Hz (Slabbekoorn and Peet, 2003), some urban songbirds sing with an increased frequency, reduced bandwidth, and longer songs to reduce masking in areas of high ambient noise and traffic noise (Slabbekoorn and Peet, 2003; Han, Jiang and Ding, 2004; Fernández-Juricic *et al.*, 2005; Parris and Schneider, 2009; Luther and Baptista, 2010; Francis, Ortega and Cruz, 2011; Mendes, Colino-Rabanal and Peris, 2011; Seger-Fullam, Rodewald and Soha, 2011; Redondo, Barrantes and Sandoval, 2013; Gough, Mennill and Nol, 2014). However, some studies have found that increasing frequencies might only give a small increase in signal transmission (Nemeth and Brumm, 2010) and may actually be detrimental to an individual's vocal performance (Luther, Phillips and Derryberry, 2016). Other studies have found increases in other song traits such as amplitude, as Lombard effects—the tendency to increase amplitude in response to noise—are known to be common (Brumm, 2004; Derryberry *et al.*, 2017; Zollinger *et al.*, 2017), and song length (Ríos-Chelén *et al.*, 2013), rather than frequency, in response to varying levels of noise.

Frequency shifts as well as song length differences have been found in comparisons between urban and non-urban populations of the same species (Slabbekoorn and den Boer-Visser, 2006; Slabbekoorn, Yeh and Hunt, 2007; Newman, Yeh and Price, 2008; Mockford and Marshall, 2009; Ripmeester *et al.*, 2010; Potvin, Parris and Mulder, 2011; Ivanitskii, Antipov and Marova, 2015; Hill *et al.*, 2018). One study found frequency shifts between urban and rural populations of song sparrows (Wood and Yezerinac, 2006); however, another found no frequency shifts in urban and rural song sparrows (Dowling, Luther and Marra, 2012). This difference found could potentially be attributed to the large geographic distances between these two studies (Wood and Yezerinac, 2006; Dowling, Luther and Marra, 2012).

Here, we study the songs of multiple populations of dark-eyed juncos (*Junco hyemalis*; hereafter, "juncos") in both urban and natural habitat sites across multiple sites in Southern California. While studies conducted on dark-eyed junco populations have illustrated rapid evolutionary changes in physiology, morphology, and behavior such as nesting over the course of just a few decades (Rasner *et al.*, 2004; Yeh, 2004; Newman, Yeh and Price, 2006; Atwell *et al.*, 2012), little is understood about how song traits diverge or converge across multiple urban and non-urban populations of dark-eyed juncos and how they may have changed over time.

Junco song consists of a simple, repetitive trill with approximately two to eight song types (Konishi, 1964; Williams and MacRoberts, 1977; Newman, Yeh and Price, 2008) used for territory defense and mate attraction (Konishi, 1964; Titus, 1998). There is substantial variation in certain elements of their song (Konishi, 1964; Williams and MacRoberts, 1977; Slabbekoorn, Yeh and Hunt, 2007; Newman, Yeh and Price, 2008). For example, Slabbekoorn et al. (2007) and Newman et al. (2008) found that one population of urban juncos sang shorter songs compared to songs from the nearby mountain population. Slabbekoorn et al. (2007) found differences in minimum frequency, while Newman et al. (2008) reported differences in maximum frequency.

In this study, we specifically examine (1) whether the songs from urban and non-urban juncos from locations across Southern California differ in five song characteristics: song length, trill rate, minimum frequency, maximum frequency, and bandwidth frequency; (2) if any differences found can be attributed to differences between urban vs non-urban environments; and (3) whether there have been changes in song in one urban population, the San Diego population, over the course of more than a decade, using songs from 2006/2007 and 2018-2020 breeding seasons.

MATERIALS AND METHODS

Study Sites. Recordings were taken from dark-eyed juncos at seven different Southern California locations: (1) University of California Los Angeles (UCLA; 34° 4' 10" N, 118° 26' 43" W), (2) University of California San Diego (UCSD; 32° 52′ N, 117° 14′ W), (3) University of California Santa Barbara (UCSB; 32° 52' 30.95" N, -117° 14' 10.08" W), (4) Occidental College (34° 07' 40.80" N, -118° 12' 39.60" W), (5) the Angeles National Forest (34° 18' 33.88" N, -117° 57' 31.79" W), (6) the UC Stunt Ranch Santa Monica Mountains Reserve (34° 5′ 27″ N, 118° 39′ 27″ W), (7) and the UC James San Jacinto Mountains Reserve (33° 48’30″ N, 116° 46’40″ W) (Figure 1). The first four locations mentioned are urban environments while the last three locations are considered natural, non-urban mountain environments. The urban locations are all college campuses with high pedestrian traffic that fluctuates throughout the day depending on class schedules and many tall buildings while the non-urban environments have relatively low pedestrian traffic and dense vegetation. All seven of these locations were chosen due to the presence of dark-eyed juncos and their location in Southern California.

Song Recordings. Recordings were taken during the dark-eyed juncos’ breeding seasons during and after the establishment of their territories. UCLA, UCSD, UCSB, and Occidental College juncos were recorded from January to June 2018, 2019, and 2020 from 6:00 - 12:00 h. The UC Stunt Ranch Reserve, the Angeles National Forest, and the UC James Reserve juncos were recorded from mid-April to July 2018, 2019 from 6:00 -12:00 h. All recordings were of spontaneous songs and not in response to playback, which could potentially affect the song's form (Cardoso *et al.*, 2009). Each male was recorded for 1 to 142 song bouts. We define song bouts here as a series of one or more phrases separated by variable intervals of silence (Brenowitz, Margoliash and Nordeen, 1997; Eens, 1997). Individual males were recorded on multiple days throughout the season.

Recordings were made using a Marantz PMD661 solid-state digital recorder, a Sennheiser ME66 omnidirectional microphone, and an Audio-Technica AT815b microphone. All recordings were saved to WAV files using a sampling rate of 44 kHz.

Recordings were made opportunistically after first hearing and locating a singing male. Males were then recorded until they ceased singing for a significant period of time (at least half a minute) or flew too far away (outside of an approximately 10-meter radius of the microphone) to obtain a sufficiently high-quality recording. After starting the recording, the distance between the observer and singer was then halved to the best of our ability, without scaring the individual away, to increase the signal-to-noise ratio; however, exact bird distance to microphone was not recorded. After recording the bird, we marked exact GPS coordinates for each individual recording and used Google Maps (*Google Maps*, 2020) to determine the distance to closest road (m). Date and time were also recorded for each recording. The resulting recording dataset consisted of a total of 130 individual males, with the length of each recording ranging from 1 minute to more than 1 hour. Of the 130 male juncos recorded, 57 were from UCLA, 28 from Occidental College, 17 from UCSD, 7 from UCSB, 3 from UC Stunt Ranch Reserve, 5 from UC James Reserve, and 13 from the Angeles National Forest (Table 1). This gave us a total number of 109 urban juncos recorded and 21 non-urban juncos (Table 1).

Because the sample sizes varied substantially between urban and non-urban populations, we supplemented our sample of non-urban songs with recordings from the Macaulay Library song repository (Cornell Lab of Ornithology), which had a total of 1,142 dark-eyed junco recordings. Of the 1,142 recordings, 42 of them were from locations throughout Southern California. We mapped the coordinate locations of all the Dark-eyed Junco repository recordings from Southern California and then determined whether they were from urban or nonurban sites based on proximity to urbanized areas. In total, we analyzed 19 individual male junco recordings from non-urban mountain sites throughout Southern California through the repository, for a total of 40 non-urban junco individuals compared (Table 1). Distance to microphone was unknown for these data.

To compare song traits from a population of urban juncos from years 2006/2007 to those from years 2018-2020, we used an existing dataset of song measurements from 151 individual male dark-eyed juncos from UCSD and the Laguna mountains taken during the 2006 and 2007 breeding seasons (Cardoso *et al.*, 2007; Cardoso and Atwell, 2011). Of the 151 males recorded, 101 were from UCSD and 50 of them were from the Laguna mountains. For the purpose of our study, we only used the measurements from the 101 UCSD juncos. Distance to microphone as well as latitude and longitude are unknown for these data.

Data Extraction. All songs were transferred into the program Raven Pro 1.5 (Bioacoustics Research Program and Program, 2014). They were input at a sample rate of 44,100 Hz at 16 bits. All songs were normalized and measurements were taken approximately 40 dB from the peak. The following spectrogram parameters were used: window sample size of 512 points (11.6 ms), hop size = 5.8 ms (frame overlap = 50%), frequency grid spacing = 86.1 Hz (DFT size = 512 samples), and 3dB bandwidths of 124 Hz. Following Cardoso and colleagues

(Cardoso *et al.*, 2007), we used spectrogram analysis to obtain: (1) average minimum frequency, (2) average maximum frequency, (3) average song length, and (4) average trill rate. We visually identified the start and end time and minimum and maximum frequency for each song by creating a bounded selection box. After making the selection box, we counted the number of syllables and divided by the song length to obtain the trill rate. To obtain the bandwidth frequency, we subtracted the maximum frequency by the minimum frequency.

Analysis. In all populations where we had a sample size of at least 10 individuals, we found that song features were normally distributed. We ran a linear discriminant analysis (LDA) using the "MASS" package (Venables and Ripley, 2002) in R version 1.1.383 (R Core Team, 2014) for all 7 studied populations by location and by urban/non-urban (with and without the repository data) to determine whether a combination of traits (song length, trill rate, minimum frequency, maximum frequency, and bandwidth frequency) could predict the location or urban/non-urban site grouping. We then used a generalized linear model (GLM) using the R package "stats" (R Core Team, 2014) to model the relationship between LD1 and location, and then urban/non-urban sites with and without the repository data. We also used a GLM to model the relationship between each trait individually and location and urban/non-urban sites with and without repository data. Date, time, distance to closest road, latitude, and longitude were included in these models as covariates when available, and the best model was determined as the model with the lowest AIC score. After determining the best fit model, we used packages "graphics" (R Core Team, 2014) and "emmeans" (Lenth *et al.*, 2020) to perform a post-hoc estimated marginal means (EMMs) analysis to determine the p-values for pairwise comparisons between each location and between urban/non-urban sites. For the urban and non-urban population comparisons, UCLA, Occidental College, UCSD, and UCSB were grouped as urban sites while UC Stunt Ranch Santa Monica Reserve, UC San Jacinto James Reserve, and the Angeles National Forest were considered mountain sites.

After running the aforementioned analyses on all 7 populations studied, we excluded populations that we had less than 10 individual juncos from (UCSB, UC Stunt Ranch Reserve, UC San Jacinto James Reserve) and re-ran all of the analyses to account for the potential effects of small sample sizes.

To compare the 2017-2020 UCSD juncos with the 2006/2007 UCSD juncos, we also ran an LDA to determine whether a combination of traits (trill rate, minimum frequency, maximum frequency, and bandwidth frequency) could predict which field seasons the songs were from. We then ran a GLM using the LD1 score from the linear discriminant analysis as the dependent variable and the field season as the independent variable. Because the dataset from the 2006/2007 UCSD juncos did not contain song length measurements, we did not include song length in our analysis; we also did not include distance to road, latitude, and longitude as covariates in the GLM since that information was not available for the UCSD 2006/2007 field seasons. We then ran a GLM using each trait separately as the dependent variable and field season as the independent variable. We further ran an LDA and then a GLM (LD1 as the dependent variable and location as the independent variable) for all the traits, excluding song length, between all the populations to determine if the UCSD 2006/2007 field season juncos differed from the current populations. For all GLM models, we performed a post-hoc estimated marginal means analysis to determine the p-values.

RESULTS

Across All 7 Sites

After running an LDA on the traits by location for all 7 locations, we found that an increase in LD1 is associated with a decrease in song length and maximum frequency while an increase in LD2 would result in an increase in song length and maximum frequency. A GLM for LD1 scores by location found that LD1 did non-significantly differ between all 7 locations ($p > 0.05$) (Table 2 and Table S1). When plotting each individual juncos' LD2 by their respective LD1s and 95%-confidence interval ellipses for each location, there was high overlap between all of the locations' ellipses, suggesting that all of the populations are broadly similar (Figure 2A). We further found no significant differences when each trait was separately used as the dependent variable with location as the independent variable in their respective GLMs (Table S2; Figure 3).

When the sites that did not have more than 10 observations (UCSB, UC Stunt Ranch Reserve, and UC San Jacinto James Reserve) were excluded from the analysis, we also found no significant differences between the remaining sites (UCLA, UCSD, Occidental College, and the Angeles National Forest; Table S3). From the LDA, we found that an increase in LD1 is associated with increases in song length and maximum frequency while an increase in LD2 results in an increase in song length primarily. The 95%-confidence interval ellipses for each of these populations show high overlap, suggesting that all of the populations where we had more than 10 observations from are broadly similar (Figure S1A). When each trait was run through the GLM individually as the dependent variable with location as the independent variable, we still found no significant differences between the sites (Table S4).

Comparison between Urban and Non-Urban Populations

When all 7 populations were grouped into urban and non-urban categories, we also found no statistically significant differences using the LD1 scores by urban/non-urban grouping in a

GLM without the repository data (p = 0.0805) and with the repository data (p = 0.3233) (Table S5). An increase in LD1 corresponded with an increase in maximum frequency. Boxplots of each individual juncos' LD1 scores by urban and non-urban sites show high overlap without the repository (Figure 2B) and with the repository data (Figure 2C).

When the sites with less than 10 observations were excluded from the analysis, we still found no significance difference between urban and non-urban sites without the repository data (p = 0.6913) and with the repository data (p = 0.5458) (Table S5). Here, an increase in LD1 was associated with decreases in song length and increases in maximum frequency. The distributions of each individual juncos' LD1 scores by urban and non-urban sites show high overlap without the repository (Figure S1B) and with the repository data (Figure S1C).

The mean song lengths of urban and non-urban sites seem to be roughly similar (Table S6) and when GLMs were run with each trait as the dependent variable and urban/non-urban as the independent variable, we found no significant differences between all sites and when sites with less than 10 observations were excluded (Table S7).

Comparison between UCSD 2006/2007 and UCSD 2018-2020 Populations

In comparing the song characteristics individually in a GLM between the 2006/2007 seasons and the 2018-2020 seasons, we found no statistically significant differences in bandwidth frequency (p=0.8404), trill rate (p=0.1952), minimum frequency (p=0.0603), and maximum frequency (p=0.1133) (Table 3). When plotting the traits by field season, there was overlap in the distributions of each trait between the 2006/2007 and 2018-2020 seasons (Figure 4). However, when song traits were combined with an LDA and then tested LD1 as the response variable in a GLM, we found a statistically significant difference (t-value = 2.740; β = 0.6973;

p=0.0061) (Table 3). The UCSD 2006/2007 juncos have a higher median LD1 score (Figure 5A). For the LDA, an increase in LD1 was associated with an increase in both minimum and maximum frequency.

Comparison between UCSD 2006/2007 and other sites

After combining trill rate, minimum frequency, maximum frequency, and bandwidth frequency using an LDA and then testing the resulting LD1 as the response variable in a GLM by all 7 locations and UCSD 2006/2007, we found statistically significant differences between UCSD 2006/2007 and UCLA ($p<0.0001$), Occidental College ($p<0.0056$), and the Angeles National Forest ($p<0.0057$; Table S8). The UCSD 2006/2007 population had a lower median LD1 score than the aforementioned 3 Los Angeles sites (Figure 5B). An increase in LD1 score was associated with a decrease in both minimum and maximum frequency, suggesting that the UCSD 2006/2007 juncos have a relatively higher minimum and maximum frequency than the Los Angeles populations (Figure 5B). We did not find a statistically significant difference between UCSD 2006/2007 and any of the locations outside of Los Angeles ($p>0.05$; Table S8).

DISCUSSION

Our results indicate that dark-eyed junco songs from urban and non-urban environments in Southern California do not differ in the traits of song length, trill rate, minimum frequency, maximum frequency, and bandwidth frequency across sites and between urban and non-urban groupings. This is a somewhat surprising result given that the urban environments and their nearby non-urban mountain sites appear to differ greatly in terms of their physical composition and levels of noise pollution. We discuss several possible reasons why we would not see any differences in urban and non-urban junco songs: high individual plasticity, possible urban qualities of non-urban sites, a lack of time to evolve, and counteracting transmission demands.

First, there may be individual phenotypic plasticity in adjusting song characteristics and selecting particular song types that allow for better transmission in a particularly noisy environment (Medina and Francis, 2012; Potvin and Parris, 2012). A number of studies have shown that in response to increased and changing ambient noise levels in an urban landscape, urban songbirds can shift their song frequencies with short-term, immediate flexibility (in a time span of minutes), such as in great tits (Halfwerk and Slabbekoorn, 2009; Salaberria and Gil, 2010), house finches (Bermúdez-Cuamatzin *et al.*, 2009, 2011), chiff chaffs (Verzijden *et al.*, 2010), and black-capped chickadees (Goodwin and Podos, 2013); although, work done on vermilion flycatchers found a lack of short-term, immediate flexibility in song minimum frequency (Ríos-Chelén *et al.*, 2018).

Furthermore, evidence suggests that individuals choose song types to match the songs of their neighbors (Nordby, 1999; Nordby *et al.*, 2000). Therefore, repertoire selection may be influenced by the individual's acoustic environment (Marler and Peters, 1982). This ability to modify song provides evidence of short-term vocal plasticity in response to what an individual hears around them (Nordby, Campbell and Beecher, 2001). Urban dark-eyed juncos in San Diego have an repertoire size of approximately 2-6 song types (Newman, Yeh and Price, 2008) and have been shown to select higher performance song types from their repertoire when they are more motivated (Cardoso *et al.*, 2009), suggesting that individual birds have the potential to select the song types they sing depending on the noise in their environment. Thus, it may prove beneficial to maintain variation within their song to be heard in different environments with different noise profiles.

Second, areas considered non-urban for the purpose of comparison may in fact sit along an urban gradient. As urban areas expand and encroach on wildlands, these forest habitats may begin to differ less from urban environments in terms of human interaction, noise levels, and presence of artificial structures due to hiking and tourism (Almeida Cunha, 2010). Tourism in mountain recreation areas such as the Angeles National Forest can modify water and soil dynamics (Stephenson, 1993; Pickering and Buckley, 2003), which can in turn alter animal behavior (Kinnaird and O'Brien, 1996; Berman *et al.*, 2007). Tourism thus can create an urbanization gradient within the forest itself, where there are areas of isolated forest and campground areas frequently visited by humans and automobiles. This kind of gradient can further allow non-urban juncos to habituate to human and car noise and effectively behave similarly to urban juncos. The increased ecotourism and hiking to natural environments could be one possible reason why the trill rate we see at the Angeles National Forest appears to be similar to that of the urban sites (Figure 3).

Third, while it is hypothesized that longer songs of higher frequencies and reduced trill rates allow for better signal transmission in urban environments due to the echoes and reverberations caused by the tall buildings, open spaces, and intense low-frequency ambient noise (Morton, 1975; Wiley, 1991; Slabbekoorn, Yeh and Hunt, 2007), this may be counteracted by differing habitat composition and noise levels within different parts of both urban and non-urban areas. While we did not quantify this, each urban and non-urban area studied visually had differing characteristics in different parts of their habitat. For example, the University of San Diego, California has an urban forest on campus, making that area less urbanized compared to other parts of the campus. These kinds of urban forests, parks, and gardens may attenuate sound and reverberations similarly to non-urban forests (Nemeth and Brumm, 2010). In addition, depending on the time of day, the amount of noise pollution fluctuates in cities depending on people's routines such as work and school. Future studies on the dark-eyed junco populations in these urban sites should consider this urbanization gradient as this may affect song parameters (Mendes, Colino-Rabanal and Peris, 2011).

Fourth, if significant frequency shifts in songs occur, they may be due to population-wide changes that occur over longer evolutionary timescales. Zollinger et al. (2017) found that increased song frequency shifts were not explained by developmental plasticity or chronic noise induced individual plasticity in great tits (Zollinger *et al.*, 2017), suggesting that any observed changes in the song of urban and non-urban birds may be the result of slower evolutionary processes rather than immediate plastic responses. If this is the case, then perhaps there has not been a long enough time period since the urban populations studied here have been established to detect differences in song traits, although birds can learn their song through cultural transmission. For this to happen, however, variation in the trait needs to be expressed. The UCLA population was likely established in the early or mid-2000's—the first reported junco in UCLA on eBird was in April 2007 and juncos have been seen breeding since at least 2008 (P. Yeh, pers. observ.). The Occidental and UCSB populations were first sighted in April and May 2013, respectively (Cornell Lab of Ornithology, Ithaca, 2020).

The UCSD population has been established for approximately 40 years and a recent study on juvenile dark-eyed juncos from UCSD raised in captivity found that the juvenile juncos maintained the significantly higher minimum frequency seen previously in the UCSD population (Reichard *et al.*, 2019), suggesting that these frequency shift differences in song can be heritable. Thus, it is possible that while evolutionary changes in some song traits could occur relatively rapidly, some junco populations may not have been established long enough to have shown significant song frequency shifts. Dark-eyed juncos have established populations in other urban areas such as Los Angeles and Santa Barbara within the last 10-20 years, making their explosion into these areas a relatively recent development.

Our findings are only partially in line with previous research studying similar characteristics in dark-eyed junco songs in Southern California. Our data supports Slabbekoorn et al. (2007)'s finding of no statistically significant difference in song length and trill rate, but we did not find an increased minimum frequency. On the other hand, our results do support Newman et al.'s (2008) finding of no statistically significant difference between trill rate and minimum frequency, but not their finding of an increased maximum frequency and reduced song length ($p<0.1$ alone, $p<0.05$ combined with Slabbekoorn et al. (2007)). We suspect our very low sample sizes in some of these populations could affect our results.

Approximately two decades have passed since the juncos studied in the Slabbekoorn et al. (2007) and Newman et al. (2008) papers were recorded (1998 and 2001; 2002 and 2003, respectively); therefore, there may have been significant changes to the population since then that could affect differences in their song. At that time, UCSD was an island population that experienced founder effects (Rasner *et al.*, 2004); however, since the mid-2000s, their population has expanded beyond their prior borders (Figure 6; Cornell Lab of Ornithology, Ithaca, 2020). UCSD may no longer be an isolated population. This increased gene flow could counteract founder effects. Indeed, urban populations throughout Southern California are rapidly expanding (Figure 6). While we know the first reported sightings on eBird of dark-eyed juncos in all the locations studied (Cornell Lab of Ornithology, Ithaca, 2020), we do not know the actual first instances of juncos in these urban areas, and we do not know the sources of these populations nor the extent of gene flow between our urban and natural populations. This could be a possible explanation of why we found non-statistically significant differences in dark-eyed junco song between UCSD 2006/2007 and the sites outside of Los Angeles. Juncos do, however, learn their songs from what they hear around them and can improvise novel song types during development (Marler and Tamura, 1962; Titus, 1998). Juncos can also undergo rapid divergence in song and behavior in short timescales (Rasner *et al.*, 2004). In spite of this, we still do not see much divergence in song characteristics between the different sites studied. It is, however, important to note that we found significant differences between UCSD 2006/2007 and the Los Angeles populations, suggesting that there could be geographic variation and potential heterogeneity over time.

Our results further show that there are no significant differences when each trait (bandwidth frequency, trill rate, minimum frequency, and maximum frequency) is individually analyzed and compared between the UCSD juncos from the 2006/2007 breeding seasons and the 2018-2020 breeding seasons although the 2018-2020 minimum frequencies seem to trend lower (p=0.0603) (Table 3). There is also extensive overlap in each trait between the breeding seasons (Figure 4). However, when song traits are combined with a linear discriminant analysis, there is a significant difference between the 2 field seasons (p=0.0061; Table 3). The UCSD 2006/2007 juncos have a relatively higher median LD1 score compared to the UCSD 2018-2020 juncos (Figure 5A). Since LD1's increase is associated with an increase in minimum and maximum frequency, this likely suggests that UCSD 2006/2007 juncos have a relatively higher minimum and maximum frequency compared to UCSD 2018-2020. There was a large difference between the sample sizes compared from the UCSD 2006/2007 (101 males) and UCSD 2017-2020 (17 males) and it is possible that further data collection could yield different or additional insights.

Finally, while much of song difference research focuses on the cultural evolution aspect of song itself, it is possible that these song differences come as a by-product of the evolution of other adaptations to urban environments. For example, song characteristic differences may not always be directly a result of selection due to noise pollution (Zollinger *et al.*, 2017); they can also be the result of morphology changes (Ryan and Brenowitz, 1985; Catchpole and Slater, 1995). Body size and bill shape directly affect the pitch a bird produces (Ryan and Brenowitz, 1985; Catchpole and Slater, 1995). Several studies point out that the smaller the body size, the higher the frequency of song and vice versa, and that larger bills produce lower-pitched, narrower bandwidth songs with slower trill rates (Ryan and Brenowitz, 1985; Podos, 2001; Derryberry, 2009; Giraudeau *et al.*, 2014; Liu *et al.*, 2017). Urbanization can cause changes in morphology—selecting for changes in beak and body sizes—as fluctuation in food abundance and type often characterize the urban landscape (Giraudeau *et al.*, 2014; Narango and Rodewald, 2016; Meillère *et al.*, 2017). There is, however, evidence that differences in body size were not correlated with song frequencies in dark-eyed juncos (Cardoso *et al.*, 2008) and this lack of correlations was also found in 529 and 842 urban and non-urban populations of songbird species, respectively (Hu and Cardoso, 2009; Moiron *et al.*, 2015).

Longer, faster, and higher frequency songs could also be associated with bird population densities (Nemeth and Brumm, 2009), which tends to be higher in urban environments (Hamao, Watanabe and Mori, 2011; Narango and Rodewald, 2016). Weather has also been shown to affect song traits, and weather patterns can be influenced by pollution levels in the city (Schäfer *et al.*, 2017). Thus, song in urban habitats can be complex and nuanced, with many potential factors affecting song traits.

To our understanding, this is one of very few studies that has found no significant differences in song characteristics between multiple urban and multiple non-urban populations of the same species and to find no changes across all traits collectively over time in an urbanized population of birds. Future studies could expand on this work, focusing on other species and their similarities and differences across multiple urban and non-urban habitats and on predicting the response of a bird species to urban noise. This would allow us to start answering questions regarding how generalizable species song responses are to urbanization.

FIGURES

Figure 1. Map of all seven locations where male juncos were recorded: UC Los Angeles, UC Santa Barbara, UC San Diego, UC James Reserve, UC Stunt Ranch, and Angeles National Forest. A blue diamond indicates the location is a non-urban site while a red star indicates an urban site.

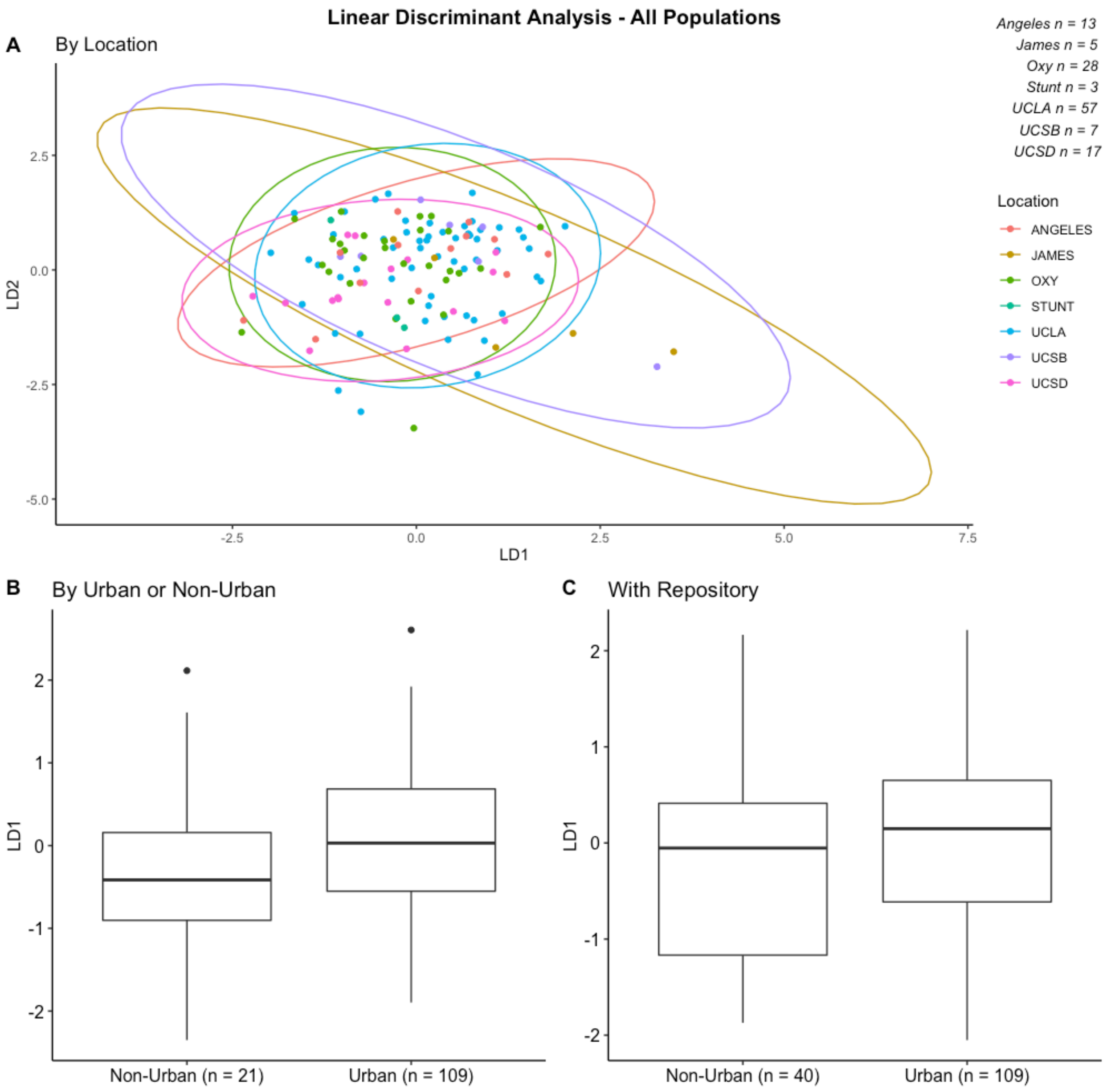

Figure 2. Linear Discriminant Analysis graphs for all 7 studied populations A) Scatterplot graph of LD1 by LD2 scores for each individual bird color coded by location. Ellipses at the 95% level for each location illustrate overlap across all populations. B) Boxplots of LD1 scores for each bird classified as non-urban or urban without the repository and C) with the repository data. Each boxplot is a representation of the LD1 scores for each individual bird classified by urban and non-urban.

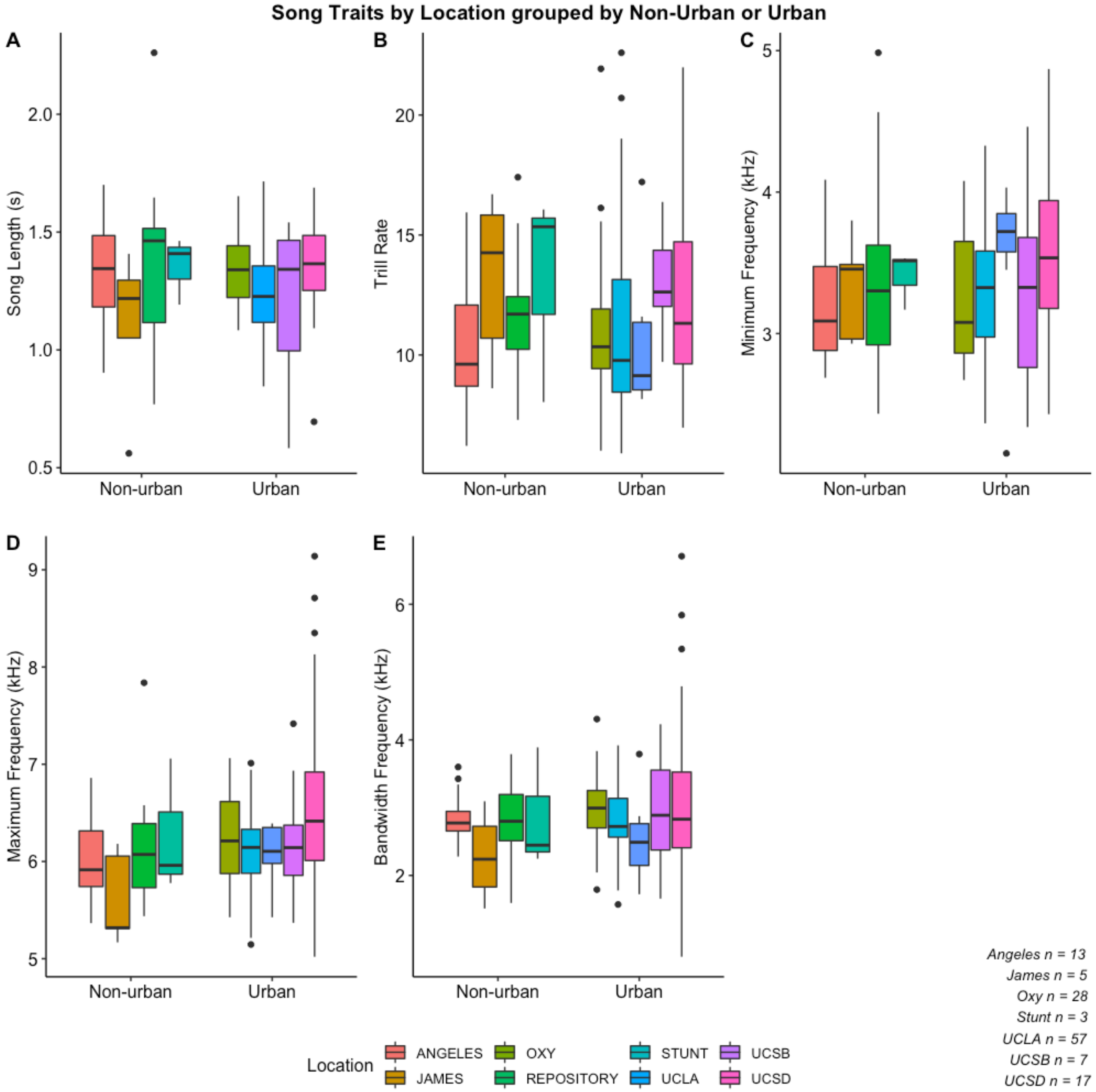

Figure 3. Boxplots of differences across sites in each of the different characteristics studied: (A) Song length in seconds, (B) trill rate, (C) minimum frequency (kHz), (D) maximum frequency (kHz), and (E) bandwidth frequency (kHz). In all characteristics, there is overlap in the ranges. There are no statistically significant differences in these five characteristics across sites and between non-urban and urban populations (multivariate ANOVA; p-value>0.05). Each boxplot is a representation of the distribution of its respective song characteristic for all populations. The

vertical lines illustrate the range of the data from the minimum value to the maximum value while the bottom of the box represents the lowest value of quartile 1, the horizontal line represents the median, and the top of the box represents the highest value of quartile 3.

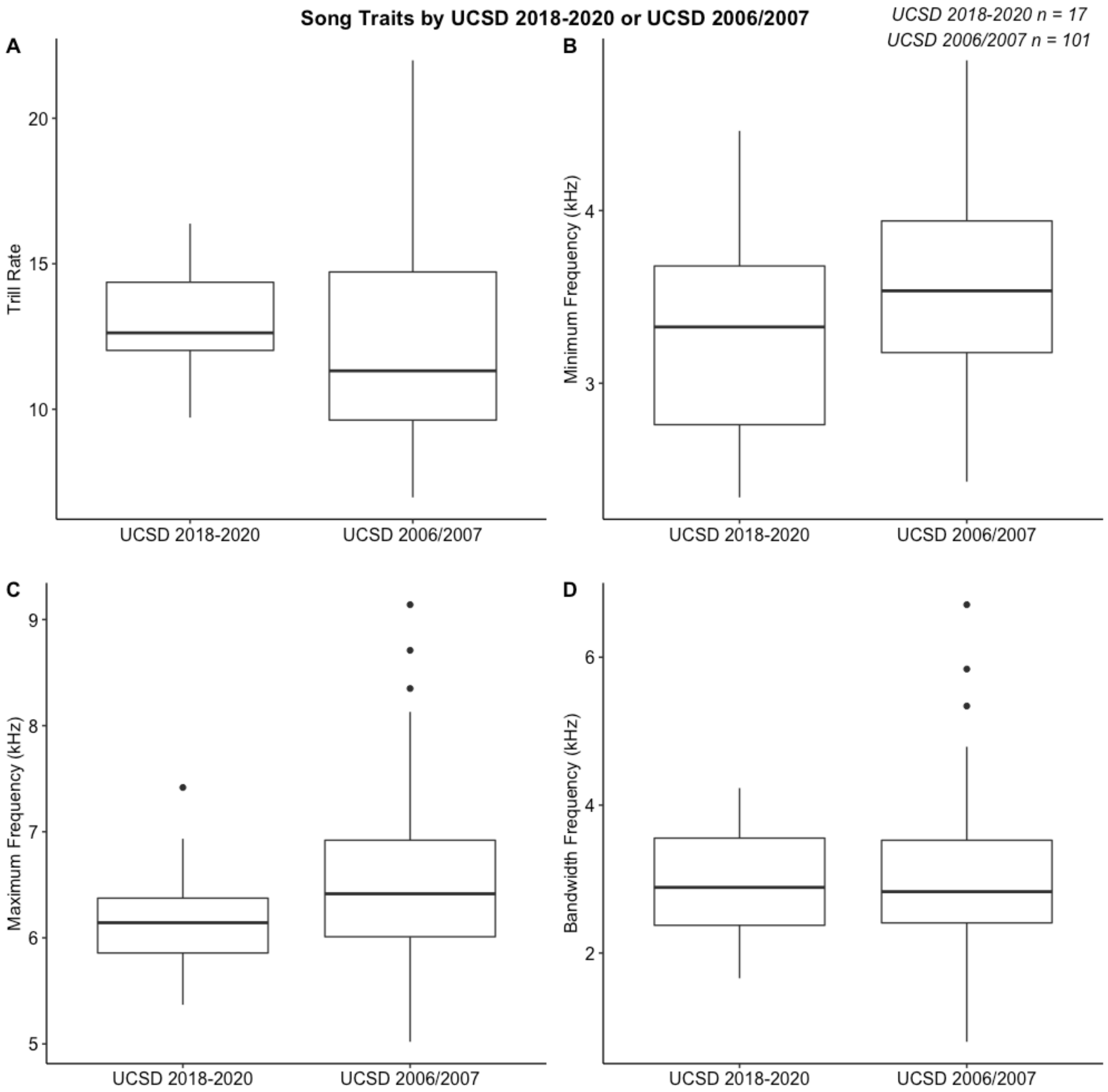

Figure 4. Boxplot of differences in the UCSD population during the 2006/2007 and the 2018-2020 breeding seasons in (A) trill rate, (B) minimum frequency (kHz), (C) maximum frequency (kHz), and (D) bandwidth frequency (kHz). For all traits, there are no statistically significant differences between the songs taken from UCSD juncos during the 2006/2007 breeding seasons and those taken during the 2018-2020 breeding seasons (Table 3).

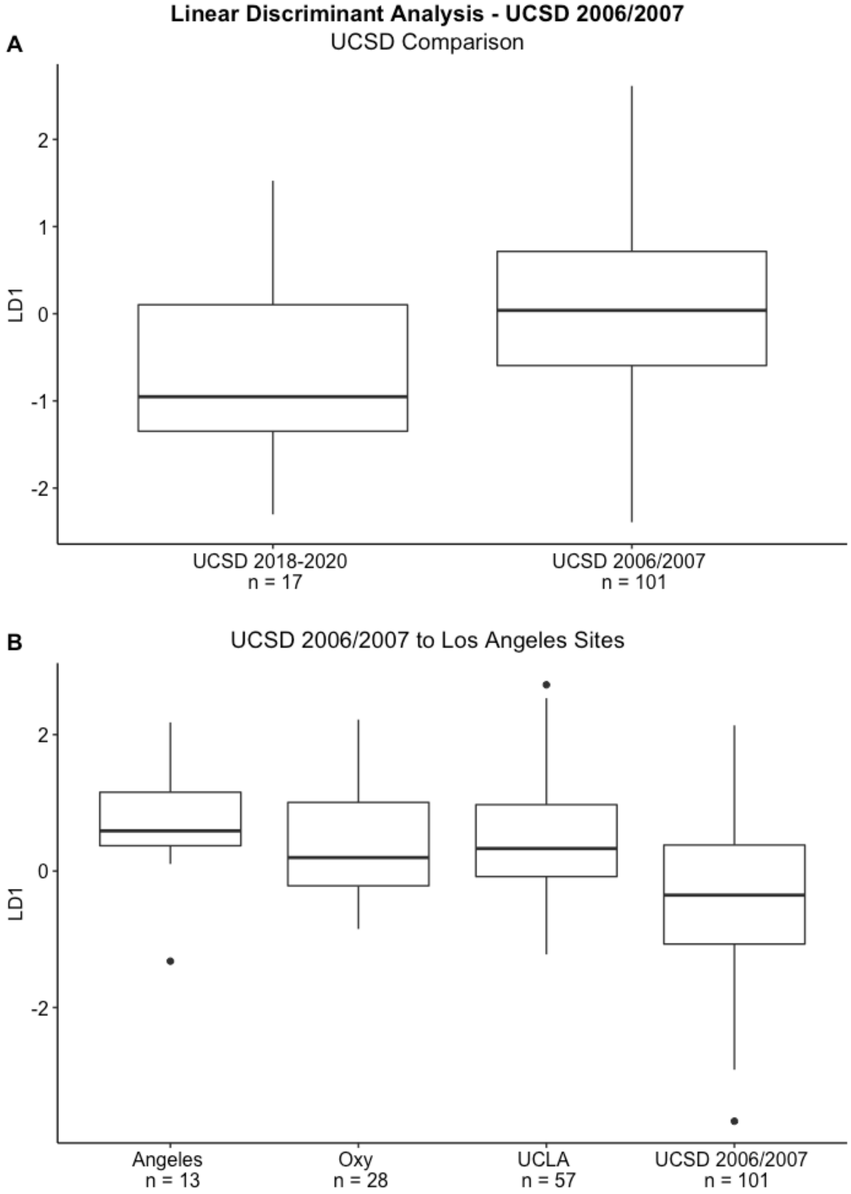

Figure 5. A) Boxplot distributions of each individual junco's LD1 scores for the UCSD 2018-2020 and UCSD 2006/2007 field seasons. The median LD1 score is marginally higher for the UCSD population in 2006/2007, implying that the UCSD 2006/2007 juncos have a relatively higher minimum and maximum frequency. An increase in the LD1 score is associated with an increase in both minimum and maximum frequency (p-value = 0.0061). B) Boxplot distributions

of each individual junco's LD1 scores for UCSD 2006/2007 and the 3 Los Angeles populations (the Angeles National Forest, p-value = 0.0057; Occidental College, p-value = 0.0056; and UCLA, p-value < 0.0001). The median LD1 score for UCSD 2006/2007 is marginally lower, implying that the UCSD 2006/2007 juncos have a relatively higher minimum and maximum frequency. An increase in the LD1 score is associated with a decrease in both minimum and maximum frequency.

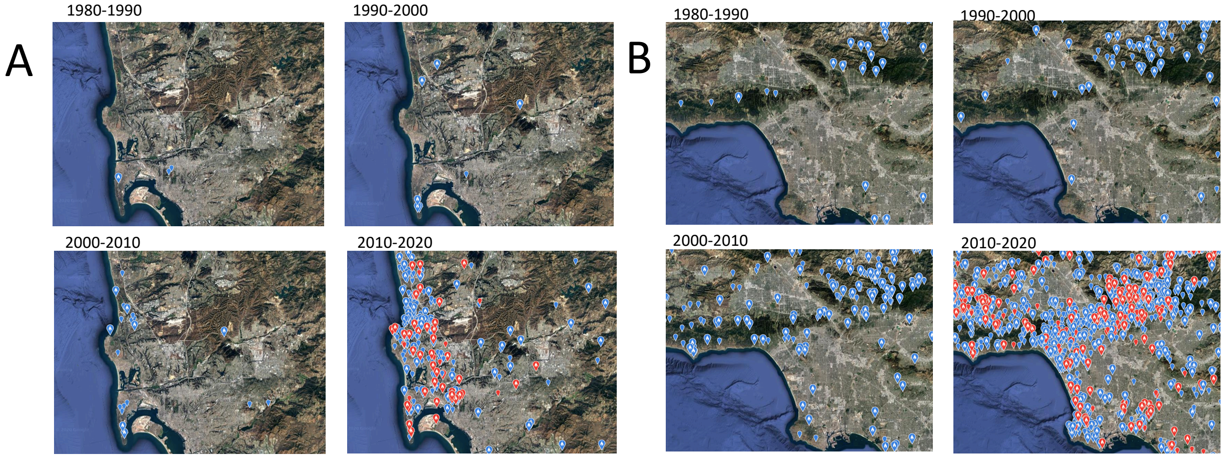

Figure 6. The expansion of dark-eyed juncos from 1980-2020 at 10-year intervals in the (A) San Diego area and (B) Los Angeles area with community science data collected from eBird (Cornell Lab of Ornithology, Ithaca, 2020). Pins are placed in locations where dark-eyed juncos have been observed. Large pins indicate "hotspots" with multiple individual juncos sighted and small pins indicate single sightings. Blue pins indicate spots with former sightings and orange pins are recent hotspots and sightings (as of June 2020).

TABLES

Table 1. How many dark-eyed juncos were recorded from each of the 7 locations visited and added together for the sum total of how many urban and non-urban male dark-eyed juncos were recorded with the addition of the 19 natural songs from male juncos in Southern California from the Macaulay Library Repository of Cornell Lab of Ornithology.

Location	Number of Males Recorded		Number of Males Recorded
UC Los Angeles	57	Urban	109
Occidental College	28		
UC San Diego 2017-2020	17		
UC Santa Barbara	7		
UC Stunt Ranch reserve	3	Natural	21 + **19** = 40
UC James Reserve	5		
Angeles National Forest	13		

The following dark-eyed junco recordings from the Macaulay Library at the Cornell Lab of Ornithology were used: 104914591, 141793701, 158949451, 165278021, 165792881, 166068651, 166509621, 166509641, 171008801, 47853201, 47853301, 104914551, 165792981, 54608211, 62694621, 65154251, 98625141,102524971, and 103606961.

Table 2. Song measurements from different populations across California.

	UCLA	UCSD	UCSB	Oxy	Stunt Reserve	James Reserve	Angeles NF
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
Song Length (s)	1.25 ± 0.03	1.34 ± 0.06	1.25 ± 0.03	1.33 ± 0.03	1.35 ± 0.08	1.11 ± 0.15	1.33 ± 0.07
Trill Rate	10.9 ± 0.51	12.98 ± 0.49	10.62 ± 1.21	10.97 ± 0.63	13.15 ± 2.56	13.22 ± 1.54	10.6 ± 0.79
Minimum Frequency (kHz)	3.29 ± 0.06	3.28 ± 0.16	3.54 ± 0.24	3.23 ± 0.08	3.40 ± 0.12	3.33 ± 0.17	3.19 ± 0.12
Maximum Frequency (kHz)	6.11 ± 0.06	6.23 ± 0.12	6.08 ± 0.13	6.21 ± 0.09	6.27 ± 0.4	5.61 ± 0.21	6.02 ± 0.11
Bandwidth Frequency (kHz)	2.83 ± 0.07	2.95 ± 0.18	2.55 ± 0.25	2.98 ± 0.11	2.86 ± 0.52	2.28 ± 0.29	2.83 ± 0.11

SE = Standard Error, UCLA = University of California, Los Angeles, Oxy = Occidental College, UCSD = University of California, San Diego, UCSB = University of California, Santa Barbara, NF = National Forest

Table 3. Song measurements for both UCSD from the 2006/2007 data set and our 2018-2020 data. All p-values obtained for each trait separately through a generalized linear model show a lack of significance (p-value >0.05) in song characteristics between the UCSD 2006/2007 and UCSD 2018-2020 field seasons. However, the p-value obtained from the post hoc estimated marginal means analysis for the LD1 generalized linear model showed that a combination of traits resulted in significance (p-value <0.05)

	UCSD 2006/2007	UCSD 2018-2020	Between the Years	LDA -> GLM
	Mean ± SE		P-value	
Bandwidth Frequency (kHz)	2.99 ± 0.07	2.95 ± 0.18	0.8404	**0.0061**
Trill Rate	12.14 ± 0.27	12.98 ± 0.49	0.1952	
Minimum Frequency (kHz)	3.53 ± 0.04	3.28 ± 0.16	0.0603	
Maximum Frequency (kHz)	6.52 ± 0.06	6.23 ± 0.12	0.1133	

SUPPLEMENTARY MATERIALS

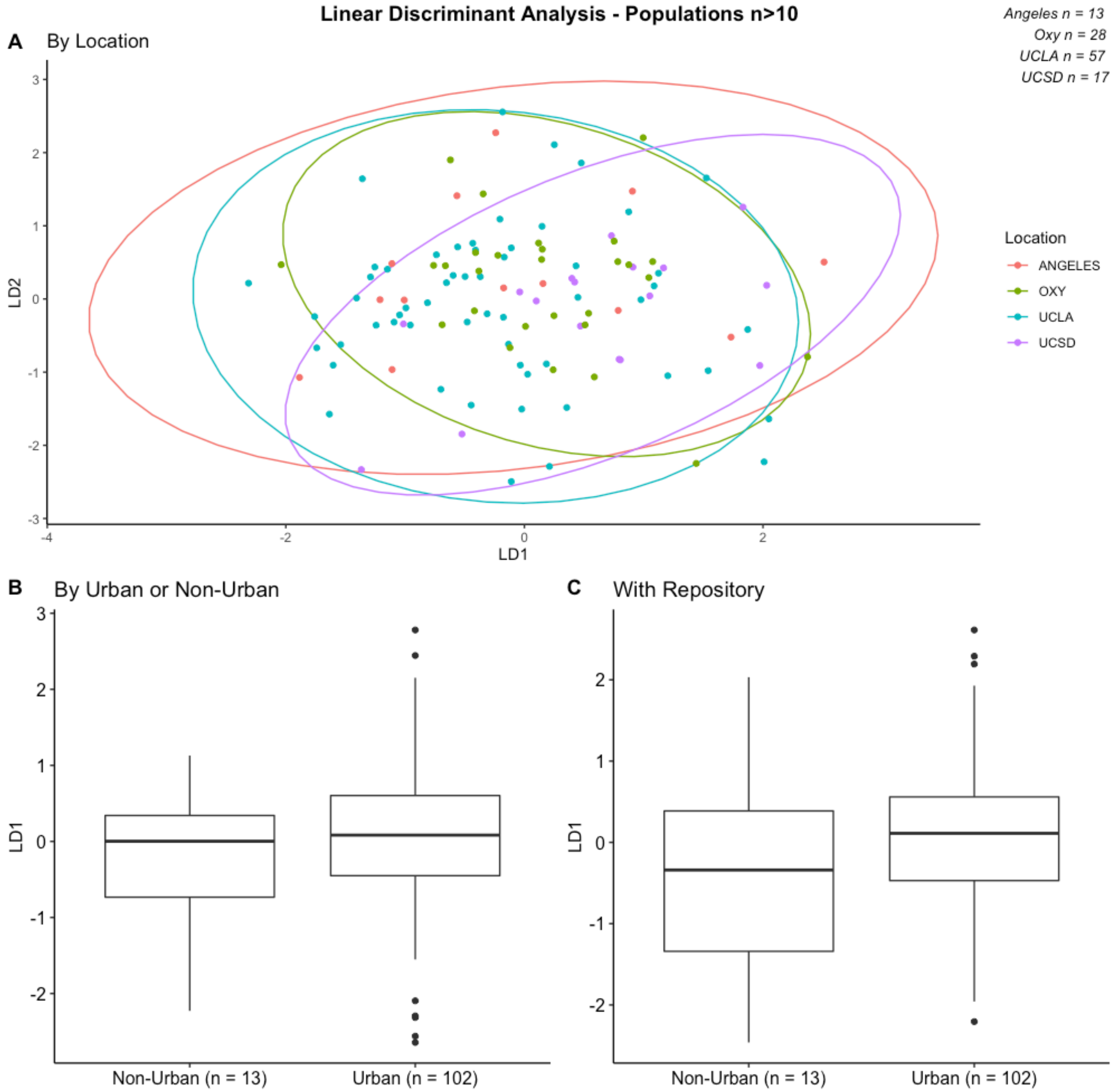

Figure S1. Linear Discriminant Analysis graphs for populations that had a sample size greater than 10 individuals. A) Scatterplot graph of LD1 by LD2 scores for each individual bird color coded by location. Ellipses for each location illustrate overlap across shown populations. B) Boxplots of LD1 scores for each bird classified as natural or urban without the repository and C) with the repository data.

Table S1. P-values from the post hoc estimated marginal means analysis for the generalized linear model of linear discriminant scores by site for all 7 locations. All p-values between each site are non-significant (p-value > 0.05).

Location	P-values						
	UCLA	UCSD	UCSB	Oxy	Stunt	James	Angeles
UCLA	-	0.1515	0.3101	0.3895	0.5479	0.3735	0.4208
UCSD	0.1515	-	0.1515	1	0.185	0.6304	0.9756
UCSB	0.3101	0.1515	-	0.3247	0.2715	0.3453	0.3427
Oxy	0.3895	1	0.3247	-	0.4437	0.3715	0.4699
Stunt	0.5479	0.185	0.2715	0.4437	-	0.3909	0.4486
James	0.3735	0.6304	0.3453	0.3715	0.3909	-	0.3525
Angeles	0.4208	0.9756	0.3427	0.4699	0.4486	0.3525	-

Table S2. P-values from the post hoc estimated marginal means analysis for the generalized linear model for all 7 locations studied with each trait individually as the dependent variable and location as the independent variable. All p-values between each site are non-significant (p-value > 0.05).

Song Length	P-values						
	UCLA	UCSD	UCSB	Oxy	Stunt	James	Angeles
UCLA	-	0.8469	0.9227	0.9471	0.9619	0.9414	0.9551
UCSD	0.8469	-	0.7971	1	0.6648	0.988	1
UCSB	0.9227	0.7971	-	0.928	0.9134	0.9338	0.9342
Oxy	0.9471	1	0.928	-	0.9522	0.9404	0.9651
Stunt	0.9619	0.6648	0.9134	0.9522	-	0.9437	0.9437
James	0.9414	0.988	0.9338	0.9404	0.9437	-	0.9336
Angeles	0.9551	1	0.9342	0.9651	0.9437	0.9336	-
Trill Rate	P-values						
	UCLA	UCSD	UCSB	Oxy	Stunt	James	Angeles
UCLA	-	0.1634	1	0.9999	1	0.8081	0.9244
UCSD	0.1634	-	0.6303	0.2062	0.9292	1	1
UCSB	1	0.6303	-	1	1	0.9166	0.9552
Oxy	0.9999	0.2062	1	-	1	0.7943	0.8801
Stunt	1	0.9292	1	1	-	0.9772	0.9846
James	0.8081	1	0.9166	0.7943	0.9772	-	1
Angeles	0.9244	1	0.9552	0.8801	0.9846	1	-
Minimum Frequency	P-values						
	UCLA	UCSD	UCSB	Oxy	Stunt	James	Angeles
UCLA	-	0.8207	0.6885	0.9797	1	0.8022	0.9987
UCSD	0.8207	-	0.7881	0.8262	0.821	0.8431	0.8359
UCSB	0.6885	0.7881	-	0.6431	0.7801	0.7168	0.6728
Oxy	0.9797	0.8262	0.6431	-	1	0.8233	1
Stunt	1	0.821	0.7801	1	-	0.8525	1

James	0.8022	0.8431	0.7168	0.8233	0.8525	-	0.8758
Angeles	0.9987	0.8359	0.6728	1	1	0.8758	-
Maximum Frequency	P-values						
	UCLA	UCSD	UCSB	Oxy	Stunt	James	Angeles
UCLA	-	0.9999	0.7209	0.7204	0.8734	0.7519	0.7576
UCSD	0.9999	-	0.7145	0.9811	0.9626	0.8036	0.926
UCSB	0.7209	0.7145	-	0.7193	0.6855	0.7386	0.7315
Oxy	0.7204	0.9811	0.7193	-	0.7837	0.7614	0.8062
Stunt	0.8734	0.9626	0.6855	0.7837	-	0.767	0.7889
James	0.7519	0.8036	0.7386	0.7614	0.767	-	0.7528
Angeles	0.7576	0.926	0.7315	0.8062	0.7889	0.7528	-
Bandwidth Frequency	P-values						
	UCLA	UCSD	UCSB	Oxy	Stunt	James	Angeles
UCLA	-	0.6883	0.7036	0.5789	0.9947	0.8055	0.7484
UCSD	0.6883	-	0.6955	0.7205	0.7643	0.9787	0.69
UCSB	0.7036	0.6955	-	0.6825	0.6256	0.7602	0.7069
Oxy	0.5789	0.7205	0.6825	-	0.8729	0.8393	0.9327
Stunt	0.9947	0.7643	0.6256	0.8729	-	0.8405	0.8699
James	0.8055	0.9787	0.7602	0.8393	0.8405	-	0.8393
Angeles	0.7484	0.69	0.7069	0.9327	0.8699	0.8393	-

Table S3. P-values from the post hoc estimated marginal means analysis for the generalized linear model of linear discriminant scores by site for populations that had a sample size greater than 10. All p-values between each site are non-significant (p-value > 0.05).

	P-values			
	UCLA	UCSD	Oxy	Angeles
UCLA	-	0.2227	0.242	0.246
UCSD	0.2227	-	1	0.8996
Oxy	0.242	1	-	0.2564
Angeles	0.246	0.8996	0.2564	-

Table S4. P-values from the post hoc estimated marginal means analysis of each trait individually in the generalized linear model by location for sites with greater than 10 populations. All p-values were non-significant (p-value > 0.05).

Song Length	P-values			
	UCLA	UCSD	Oxy	Angeles
UCLA	-	0.3051	0.2505	0.5035
UCSD	0.3051	-	0.9982	0.999
Oxy	0.2505	0.9982	-	1
Angeles	0.5035	0.999	1	-
Trill Rate	P-values			
	UCLA	UCSD	Oxy	Angeles
UCLA	-	0.0621	0.9819	0.6974
UCSD	0.0621	-	0.0809	0.9966
Oxy	0.9819	0.0809	-	0.6063
Angeles	0.6974	0.9966	0.6063	-
Minimum Frequency	P-values			
	UCLA	UCSD	Oxy	Angeles
UCLA	-	0.5008	0.5093	0.9485
UCSD	0.5008	-	0.5093	0.5189
Oxy	0.5093	0.5093	-	0.9991
Angeles	0.9485	0.5189	0.9991	-
Maximum Frequency	P-values			
	UCLA	UCSD	Oxy	Angeles
UCLA	-	0.9889	0.4093	0.4455
UCSD	0.9889	-	0.83	0.6771
Oxy	0.4093	0.83	-	0.4983
Angeles	0.4455	0.6771	0.4983	-

Bandwidth Frequency	P-values			
	UCLA	UCSD	Oxy	Angeles
UCLA	-	0.3802	0.289	0.4379
UCSD	0.3802	-	0.4107	0.3814
Oxy	0.289	0.4107	-	0.7008
Angeles	0.4379	0.3814	0.7008	-

Table S5. P-values from the post hoc estimated marginal means analysis for the generalized linear model of linear discriminant scores by urban/non-urban for all 7 sites and sites with n>10 with and without the repository. P-values between sites classified as urban or non-urban were non-significant (p-value > 0.05) with and without the repository included.

	All Sites		Sites With n>10	
	Without Repository	With Repository	Without Repository	With Repository
Urban – Non-urban	0.0805	0.3233	0.6913	0.5458

Table S6. Song measurements with each population studied categorized as urban and non-urban.

	Urban	Non-urban
	Mean ± SE	Mean ± SE
Song Length (s)	1.28 ± 0.02	1.32 ± 0.05
Trill Rate	11.23 ± 0.33	11.54 ± 0.46
Minimum Frequency (kHz)	3.29 ± 0.05	3.30 ± 0.08
Maximum Frequency (kHz)	6.16 ± 0.04	6.04 ± 0.08
Bandwidth Frequency (kHz)	2.87 ± 0.06	2.75 ± 0.09

Table S7. P-values from the post hoc estimated marginal means analysis for each trait individually in the generalized linear model by urban and non-urban for all sites and for sites with n>10. All p-values are non-significant (p-value >0.05).

Urban/Non-urban	All Sites		Sites With n>10	
	Without Repository	With Repository	Without Repository	With Repository
Song Length	0.9764	0.1997	0.4385	0.0764
Trill Rate	0.1619	0.616	0.2155	0.8311
Minimum Frequency	0.9599	0.8963	0.8458	0.5334
Maximum Frequency	0.1436	0.1996	0.4983	0.4684
Bandwidth Frequency	0.3456	0.2516	0.8192	0.4628

Table S8. P-values from the post hoc estimated marginal means analysis for the generalized linear model of linear discriminant scores by location between UCSD 2006/2007 and the other sites. P-values between UCSD 2006/2007 and the Los Angeles populations (UCLA, Oxy, and Angeles National Forest) were significant (p-value < 0.05).

Location	UCLA	UCSD 2018-2020	UCSB	Oxy	Stunt	James	Angeles	UCSD 2006/2007
UCLA	-	0.9668	0.9998	1	0.9936	0.9887	0.9949	**<0.0001**
UCSD 2018-2020	0.9668	-	1	0.9968	1	0.8723	0.8467	0.4144
UCSB	0.9998	1	-	1	0.9999	0.9749	0.9877	0.7042
Oxy	1	0.9968	1	-	0.9981	0.9768	0.9849	**0.0056**
Stunt	0.9936	1	0.9999	0.9981	-	0.9331	0.9575	0.9987
James	0.9887	0.8723	0.9749	0.9768	0.9331	-	1	0.1193
Angeles	0.9949	0.8467	0.9877	0.9849	0.9575	1	-	**0.0057**
UCSD 2006/2007	**<0.0001**	0.4144	0.7042	**0.0056**	0.9987	0.1193	**0.0057**	-

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