

University of Nevada, Reno

**Elevation Related Variation in Climatic Harshness is Associated with Premating
Mechanisms in Food-caching Mountain Chickadees (*Poecile gambeli*).**

A dissertation submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Ecology, Evolution, and Conservation Biology

by

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May, 2018

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prepared under our supervision by

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DOCTOR OF PHILOSOPHY

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ABSTRACT

Heterogeneous environments are often associated with differential selection pressures and montane habitats present a particularly interesting and useful case for the study of local adaptation, as the environment changes rapidly and predictably along an elevation gradient. Higher elevations are characterized by predictably lower temperatures, more snowfall, and longer periods of winter conditions compared to lower elevations. Due to such environmental heterogeneity, individuals inhabiting higher elevations may experience stronger selection on various ecologically relevant traits compared to their lower elevation counterparts. These local adaptations may be enhanced by reduced movement between elevations and potentially maintained by assortative mating. Throughout my dissertation work, I aim to elucidate potential premating mechanisms involved in reproductive separation of mountain chickadees inhabiting differentially harsh environments along a montane gradient. These birds have previously been shown to differ in several behavioral and neural traits important for survival at their respective elevations, thus making this system ideal for addressing the role of premating separation in a population postulated to be locally adapted. I have addressed this overarching question both indirectly, and directly, throughout the five chapters of my dissertation.

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OVERVIEW

My dissertation research addresses the hypothesis that mountain chickadees experiencing differential selection due to differences in winter climate severity along an elevation gradient, previously shown to differ in several neural and behavioral traits, have evolved premating mechanisms potentially involved in reproductive separation. I have addressed this question both indirectly, and directly, throughout the five chapters of my dissertation.

Chapter 1: Do female mountain chickadees prefer males from their respective elevation versus males from a different elevation?

Chapter 2: Does male song structure differ between males from high elevations compared to males from low elevations?

Chapter 3: Do males from different elevations respond to a playback of a local male intruder more aggressively than a playback of a nonlocal male intruder?

Chapter 4: Does the structure of male song change gradually over a continuous elevation gradient or does it shift abruptly, representing high and low elevation song dialects?

Chapter 5: Do groups of mountain chickadees inhabiting high versus low elevations exhibit population genetic structure?

INTRODUCTION

Heterogeneous environments are often associated with differential selection pressures and present the opportunity to assess the relative strength of selection in populations experiencing local adaptation in the presence of gene flow (Kawecki & Ebert 2004; Sambatti & Rice 2006; Tigano & Friesen 2016). Montane habitats present a particularly interesting and useful case for the study of local adaptation, as the environment changes rapidly and predictably along an elevation gradient, often with no geographic barrier to gene flow (e.g. McCracken et al., 2009; Freas et al., 2012; Wilson et al., 2012; Funk et al. 2015). Higher elevations are characterized by predictably lower temperatures, more snowfall, and longer periods of winter conditions compared to lower elevations (Barbour & Minnich 2000; Levy & Nufio 2014). Due to such environmental heterogeneity, individuals inhabiting higher elevations may experience stronger selection on various ecologically relevant traits, including growth rate, age of maturity, hemoglobin, body size, and spatial memory for recovering food caches, compared to their lower elevation counterparts (Miaud & Merila 2001; McCracken et al. 2009; Freas et al. 2012; Wilson et al. 2012; Pravosudov & Roth 2013; Funk et al. 2015). These local adaptations may be enhanced by reduced movement between elevations (Funk et al. 2015) and potentially maintained by assortative mating (Kondrashov & Shpak 1998). While individuals across taxa use varying phenotypic characteristics to mate assortatively, one trait commonly used by female songbirds in mate choice is male song (Tomback and Baker 1984; Gentner & Hulse 2000).

In temperate songbirds, song is primarily produced by males during the breeding season, and serves a dual function, both in female mate choice (Searcy 1984; Byers 2006)

and conspecific territory defense (Krebs et al. 1978; Mennill et al. 2002). Most notably, the song of oscine passerine birds is learned through the imitation of other local males (Slater 1989; Nelson et al. 2001). While bird song is phenotypically plastic and may be modulated in varying acoustic environments (Slabbekoorn & Ripmeester 2007), many oscine passerines are age-limited learners (Baker 2001) or closed learners, meaning they learn their song early in life and do not learn or produce new songs once their repertoire has been “crystalized” (Nelson 1999). Because song is learned from local conspecifics, it not only varies between species, but also varies among conspecifics experiencing geographic isolation, resulting in geographic dialects within a species (Mundinger 1975; Krebs et al. 1978; Podos & Warren 2007; Caro et al. 2013). These geographic dialects appear to influence female mate choice; with females displaying a preference for dialects from their geographic location or population of origin (Baker et al. 1982; Baker et al. 1987; Slabbekoorn & Smith 2002; Rowell & Servedio 2012).

Several hypotheses have proposed different mechanisms by which such geographic variation may arise:

(1) The acoustic adaptation hypothesis suggests that vocalizations have evolved to maximize transmission in a given environment, and as environments change (i.e. differences in plant density across forests), so will the structure of male song (Wilkins et al. 2012).

(2) The temporal variation hypothesis suggests that song structure may change or drift over time as individuals experience reduced movement and gene flow (Derryberry 2011). This is thought to occur primarily in newly colonized areas where populations are

geographically isolated and males imperfectly imitate the song of male tutors (Gammon & Baker 2004; Gammon et al. 2005).

(3) Finally, the local adaptation hypothesis suggests that individuals within a species that inhabit different environments may experience differential selection pressures on ecologically relevant traits, potentially reducing movement between environments (King et al. 1980; Grant & Grant 2007; Caro et al. 2013). Over time, this reduced gene flow or movement may result in structural changes in male song that can now be used by females to mate assortatively with locally adapted males (MacDougall-Shackleton et al. 2002; Slabbekoorn & Smith 2002). This is interesting, as birds from different habitats may not actually be reproductively incompatible, but assortative mating for a local over nonlocal male may further enhance population-specific adaptations (Baker 1975; Kroodsma et al. 1999; Caro et al. 2013).

Montane habitats are particularly useful for testing hypotheses such as those presented above, as the environment, and subsequent selection pressures, change rapidly and predictably over rather small spatial scales. Mountain chickadees (*Poecile gambeli*) are oscine passerines that inhabit continuous montane gradients in western North America (McCallum et al. 1999). These are resident, food-caching birds that store food in the autumn when food is abundant, to use later, in winter, when food is scarce. Mountain chickadees use spatial memory in part to relocate their food caches (Pravosudov & Roth 2013), and previous research shows that birds inhabiting harsher climates with more and longer snow cover (high elevations) cache 3-4 times more food items, perform better on spatial memory tasks, and have a larger hippocampus with more neurons, than birds inhabiting milder climates (low elevations) (Freas et al. 2012; Freas et al. 2013a; Freas et

al. 2013b; Croston et al. 2016). These differences were found in juvenile birds, prior to their first winter, and persisted in a common garden environment, therefore, the differences in spatial memory ability and hippocampus morphology cannot be attributed to experience alone. Since chickadees are residents, remaining in the mountains throughout the winter, their winter survival is likely affected by the local environmental conditions, and any specific traits enhancing winter survival would likely be favored by selection (e.g., memory for cache recovery).

In addition, high and low elevation birds represent differing behavioral types, such that high elevation males are slower explorers in a novel environment, less proactively aggressive and socially subordinate to low elevation males (Kozlovsky et al. 2014; Branch et al. 2015). Chickadees live in flocks with stable dominance hierarchies outside of the breeding season, and dominant birds have better access to mates and food resources, therefore, dominance status has significant implications for an individual's fitness (Ratcliffe et al. 2007).

Indeed, the phenotypic variation between high and low elevation birds suggests that there may be negative fitness consequences for birds moving between elevations. For example, migrants from low to high elevations may experience decreased fitness due to their inferior spatial memory ability and reduced food caching propensity, while migrants from high to low elevations may experience decreased fitness due to their socially subordinate status. If mountain chickadees inhabiting the Sierra Nevada experience decreased fitness when moving between elevations, it may be expected that females would benefit from selecting males from their respective elevations (MacDougall-Shackleton et al. 2002) and potential differences in song structure may allow females to

discriminate between males from different elevations. Yet, it remains unknown whether females significantly contribute to reduced movement between elevations by mating assortatively with males from their respective elevation.

Throughout my dissertation, I have investigated potential premating mechanisms associated with elevation-related variation in climatic harshness in food-caching mountain chickadees inhabiting the Sierra Nevada. I have approached this question using indirect, behavioral and direct, genomic techniques throughout five chapters:

(1). The first chapter of my dissertation asked, can females from high and low elevations discriminate between high and low elevation males, and if so do they prefer males from their respective elevation? To answer this question, I brought male and female MOCH into the lab to assess high and low elevation female preference given a pairwise choice of a high or low elevation male. Once photostimulated to spring sunlight conditions, high elevation females showed a significant preference for high elevation males over low elevation males; however, low elevation females showed no detectable preference. Females were able to see and hear males in a two-choice testing cage, however, it is unclear what cue(s) females used to discriminate between high and low elevation males.

(2). As mentioned above, song in temperate male songbirds is used by females in mate choice (Searcy 1984), therefore, the second chapter of my dissertation explored variation in song structure, and asked: does male mountain chickadee song structure vary between high and low elevation locations? To address this question, I sampled males from high and low elevations at two different mountain locations. Using bioacoustics

sound analysis software, I found that all four locations differed significantly in song structure, consistent with the hypothesis that local adaptation may limit movement between locations, allowing song structure to change over time and function as a local identifier (Grant & Grant 2007; Caro et al. 2013). My finding that song structure differed among all four locations argues against the idea that song is simply adjusted to the environment (i.e. acoustic adaptation hypothesis), as both high and low locations sampled were similar in habitat structure. Therefore, high and low elevation females may use song structure to mate assortatively with males from their elevation of origin.

(3). Male song also functions in territory defense against conspecific males (Krebs et al. 1978; Searcy 1984). Therefore, the third chapter of my dissertation was driven by the question, can high and low elevation males discriminate between high and low elevation song, and do they respond more strongly to a local elevation intruder compared to a nonlocal elevation intruder? To address this question, I created playback stimuli of high and low elevation male song and presented them to territorial males at high and low elevations. I found that high and low elevation males responded similarly to both types of playback stimuli, suggesting that they do not readily discriminate between local and nonlocal elevation intruders. In chapter one I showed that high elevation females can discriminate between high and low elevation males, therefore the findings of chapter three may have implications for differences in discriminative ability of male and female songbirds, as females may make a costly mistake by failing to identify a local male, resulting in offspring poorly matched to their local environment (Searcy & Brenowitz 1988; Gentner & Hulse 2000; Danner et al. 2011).

(4). In chapter two, I found significant variation in song structure consistent with the local adaptation hypothesis, however, finding variation in the extreme ends of a phenotypic distribution may be a result of isolation-by-distance and short dispersal, and may not necessarily be indicative of local adaptation or adaptive divergence. Therefore, the fourth chapter of my dissertation asked, does variation in song structure along an elevation gradient represent climate-related vocal dialects in mountain chickadees? To address this question, I recorded male song along the continuous elevation gradient at both mountains previously sampled in chapter two. Using criteria laid out in Podos & Warren (2007), I found evidence for steep clines with stepped variation, indicative of ‘high’ and ‘low’ elevation vocal dialects. Finding support for vocal dialects in mountain chickadees at different elevations has important implications for the evolutionary trajectory of these birds (Edwards et al. 2005; Podos & Warren 2007), as these differences in song structure likely represent indicators of local adaptation.

(5). While high and low elevation mountain chickadees appear to be locally adapted to their respective elevations and females may contribute to separation by mating assortatively with males from their respective elevation, it is unclear whether or not they represent genetically differentiated populations or if adaptation may be occurring in the face of gene flow (e.g. Gonzalo-Turpin & Hazard 2009; McCracken et al. 2009). The fourth chapter of my dissertation was driven by the question, is there population genetic structure among mountain chickadees inhabiting high and low elevations across multiple mountain locations? In order to address this question, I collected blood samples from high and low elevation chickadees inhabiting three different mountains in the Sierra Nevada; Red Lake, CA, Mount Rose, NV, and Sagehen, CA. In collaboration with Dr.

Thomas Parchman (UNR), I used a genotyping-by-sequencing approach to obtain ~18,000 single nucleotide polymorphisms in ~200 mountain chickadees for evidence of genetic differentiation among high and low elevation populations. I found no evidence of population genetic structure between high and low elevation birds among all six locations sampled (high and low at three mountain locations). These results suggest that the observed variation in behavioral and neural phenotypes between high and low elevation mountain chickadees may reflect local adaptation in response to elevation-related climatic harshness despite the presence of gene flow (e.g. Niemiller et al 2008; Nosil 2008; Gonzalo-Turpin & Hazard 2009; McCracken et al. 2009).



Elevation-related differences in female mate preference in mountain chickadees: are smart chickadees choosier?



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Heterogeneous environments are often associated with differential selection pressures favouring the evolution of local adaptations, and assortative mating is one of the mechanisms that might enhance such local adaptations. Montane environments present an example in which environment changes rapidly and predictably along an elevation gradient, and such variation may be expected to lead to the evolution of local adaptations. In food-caching mountain chickadees, *Poecile gambeli*, reliance on food stores is likely to increase with elevation, and previous research has shown that individuals living at high elevations cache more food and have superior spatial memory, needed to recover food caches, while also being socially subordinate to low-elevation birds. Here, we asked whether such differences might be associated with assortative mating. Considering that superior spatial memory ability for recovering food caches may be more critical for survival at high elevations because of more severe winter conditions, it should benefit females from high elevations to mate assortatively with males from the same elevation. If spatial memory is costly but not critical at low elevations, females from low elevation should mate assortatively with males from low elevation, especially given their socially dominant status to high-elevation birds. We assessed female preference using a pairwise choice of high- and low-elevation males. We used the amount of time spent in proximity to males from the same versus different elevation to determine female preference. High-elevation females showed significant preference for high-elevation males, however, low-elevation females showed no elevation-related preference. These results suggest that high-elevation females are choosier than low-elevation females, and prefer males from their same elevation. © 2014 The Association for the Study of Animal Behaviour. Published by Elsevier Ltd. All rights reserved.

Heterogeneous environments are often associated with differential selection pressures favouring the evolution of local adaptations, and assortative mating is one of the mechanisms that might enhance such local adaptations (Dieckmann & Doebeli, 1999; Gavrilets, 2003; Nosil, Egan, & Funk, 2008; Via, 2001). Montane environments present an example in which the environment changes rapidly and predictably along an elevation gradient, and such variation may be expected to lead to the evolution of local adaptations (e.g. Freas, LaDage, Roth, & Pravosudov, 2012; McCracken et al., 2009; Wilson, Peters, & McCracken, 2012). High elevations are characterized by predictably lower temperatures, more snowfall and longer period of winter conditions than low elevations (Cook, 2012; Graham, 1983; S. R. Hopkins & Powell, 2001; Shepson & Tinnesand, 2003). Individuals living in harsher

conditions at higher elevations may experience stronger selection on some ecologically relevant traits, including growth rate, age of maturity, haemoglobin, body size and spatial memory for recovering food caches than individuals living in more temperate conditions at lower elevations (Freas et al., 2012; McCracken et al., 2009; Miao & Merila, 2001; Pravosudov & Roth, 2013; Wilson et al., 2012). These local adaptations might be enhanced by reduced movement between elevations and potentially maintained by assortative mating (Kondrashov & Shpak, 1998).

Here, we asked whether female mountain chickadees, *Poecile gambeli*, are contributing to potential separation between high- and low-elevation birds by mating assortatively with males from their respective elevation. Mountain chickadees are food-caching, resident montane birds that inhabit a range of elevations and use spatial memory, at least in part, to relocate their scattered caches (Pravosudov & Smulders, 2010). At our study site, high-elevation chickadees start breeding, on average, 2 weeks later than low-elevation birds, probably due to climatic differences in elevation (Kozlovsky, Branch, & Pravosudov, in press). Harsher winters associated with higher elevations have been hypothesized to

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generate higher dependence on food caches necessary to survive winter and hence successful cache recovery might have stronger fitness consequences at higher elevations than at lower elevations (Freas et al., 2012). Previous work has confirmed that compared to low-elevation chickadees, birds from high elevation have higher food-caching propensity and superior spatial memory associated with large morphological differences in the hippocampus, a brain region involved in spatial memory function (Freas et al., 2012). That these elevation-related differences were found in first-year juvenile birds residing in a uniform common garden laboratory environment prior to their first winter suggests that variation in memory and hippocampus morphology may be heritable (Freas, Bingman, LaDage, & Pravosudov, 2013; Freas et al., 2012; Pravosudov & Roth, 2013). In addition, high-elevation birds perform better on a novel problem-solving task than low-elevation birds (Kozlovsky, Branch, & Pravosudov, 2014) and overall high- and low-elevation chickadees seem to represent group-level behavioural types with high-elevation males additionally being slower explorers, less aggressive and socially subordinate to low-elevation males (Branch, Kozlovsky, & Pravosudov, 2014; Kozlovsky, Branch, Freas, & Pravosudov, 2014). Differences seen in social dominance status are particularly interesting given that chickadees form permanent flocks of unrelated individuals outside of the breeding season and individuals within flocks maintain a linear dominance hierarchy, with subordinate individuals usually experiencing lower fitness than dominant individuals (e.g. Ratcliffe, Mennill, & Schubert, 2007).

Taken together, these findings suggest that high-elevation birds may be somewhat restricted to high elevation because of their socially subordinate status, while low-elevation birds may be restricted to low elevation because of their inferior spatial memory, notwithstanding the short distance separating these birds. If birds have the highest fitness at their respective elevations, and their locally adapted traits are heritable (W. D. Hopkins, Russell, & Schaeffer, 2014), it would benefit females to mate with males from their respective elevation to ensure the highest fitness of their offspring.

We assessed female mountain chickadee preference for males from their respective elevation using a standard pairwise choice paradigm (Amundsen, Forsgen, & Hansen, 1997; Collins, Hubbard, & Houtman, 1994; Leitao, Monteiro, & Mota, 2014; Nolan & Hill, 2004; Woolley & Doupe, 2008). If high- and low-elevation birds are locally adapted to their environments, and females are able to discriminate between males from different environments, then given a pairwise choice of a high- or low-elevation male, we predicted that high-elevation females would spend more time adjacent to high-elevation males and that low-elevation females would spend more time adjacent to low-elevation males.

METHODS

Study Subjects and Capture

Twenty-four mountain chickadees were trapped using mist nets at established feeders on 30 November – 1 December 2013 from high-elevation (2400 m) and low-elevation (1800 m) sites at Sagehen Experimental Forest, Truckee, California, U.S.A. (sensu Freas et al., 2012; 'high' and 'low' elevation used here are the same elevations as 'high' and 'mid' in Freas et al., 2012; we use 'high' and 'low' here because they represent the largest differences in spatial memory and hippocampus morphology). Birds were captured from multiple flocks at multiple feeders to form pairwise choices using unfamiliar individuals. Approximately 100 µl of blood was taken from the brachial vein at the time of capture for genetic analysis of sex. All birds were transferred to the laboratory facilities at the

University of Nevada, Reno, U.S.A., and were held until 20 April 2014, when they were released back at Sagehen. Six males and six females each from both high and low-elevation sites were used in behavioural tests; sex was determined using genetic analyses following Fox, LaDage, Roth, and Pravosudov (2009). Birds were individually colour banded and housed singly in wire-mesh cages (42 × 60 and 60 cm high), visually but not acoustically isolated from each other. Cages were covered with a translucent white cloth to allow birds to habituate to individual cages with minimal outside disturbance for 3 weeks before testing (Pravosudov, Mendoza, & Clayton, 2003). Male and female birds were housed in separate rooms to reduce familiarization with vocalizations. Birds were initially maintained on a 10:14 h light:dark cycle to mimic winter conditions. On 8 February 2014, birds were photostimulated by shifting photoperiod to a 14:10 h light:dark cycle, mimicking spring breeding conditions. All birds showed signs of being affected by photostimulation (e.g. cloacal protuberances, increased singing).

Testing Apparatus

During preference testing, a female was housed in a double cage with two joining compartments (84 × 60 and 60 cm high) and two males (one from high elevation and one from low elevation) placed in smaller cages (each 42 × 60 and 60 cm high) on either side of the female's cage. A cardboard divider with a small square opening (7.62 × 7.62 cm) in the centre separated the two adjoining compartments of the female testing cage (sensu Woolley & Doupe, 2008; see Fig. 1). Females were given a 2 h acclimation period, after which males were placed in both side cages and left for additional 1.5 h. All preference tests were videorecorded for a total of 2 h (last 30 min of female by herself and 1.5 h with males). The first 30 min with the males present was considered the females' assessment period. The final hour of recording was coded for female preference, blind to the position of males. The total amount of time spent on either side of the double cage was used to indicate preference (Amundsen et al., 1997; Collins et al., 1994; Leitao et al., 2014; Nolan & Hill, 2004; Woolley & Doupe, 2008); time spent on the centre cardboard divider was not included as preference time.

Testing

Mate preference testing took place in two rounds, one prior to photostimulation (mimicking autumn/winter when chickadees form pairs within flocks; McCallum, Grundel, & Dahlsten, 1999) from 26 December 2013 to 7 February 2014 and the second, 12 days after photostimulation (to mimic actual breeding conditions) from 20 February 2014 to 30 March 2014. All 12 females were habituated to one of two identical testing rooms for 7 h prior to testing. Each of the 12 females was presented with the same six unique pairs of males across six trials prior to photostimulation. Then, after photostimulation, the male pairs were switched to form six new unique pairs, which were again presented to each of the 12 females. Therefore, during each of the six trials prior to and following photostimulation, each female experienced a unique pair of males. All high- and low-male pairings were size-matched using wing chord length. Two females were tested per day in separate, identical testing rooms, and females were tested sequentially such that 5 days passed between each testing. Female placement into the testing cage was counterbalanced for the left or right side of the cage across both testing rooms. Male placement in testing cages relative to the female (e.g. left or right) was also counterbalanced between trials to ensure that any preference a female might show was due to preference for a male rather than for a cage side. To assess female preference, we calculated the mean amount of time

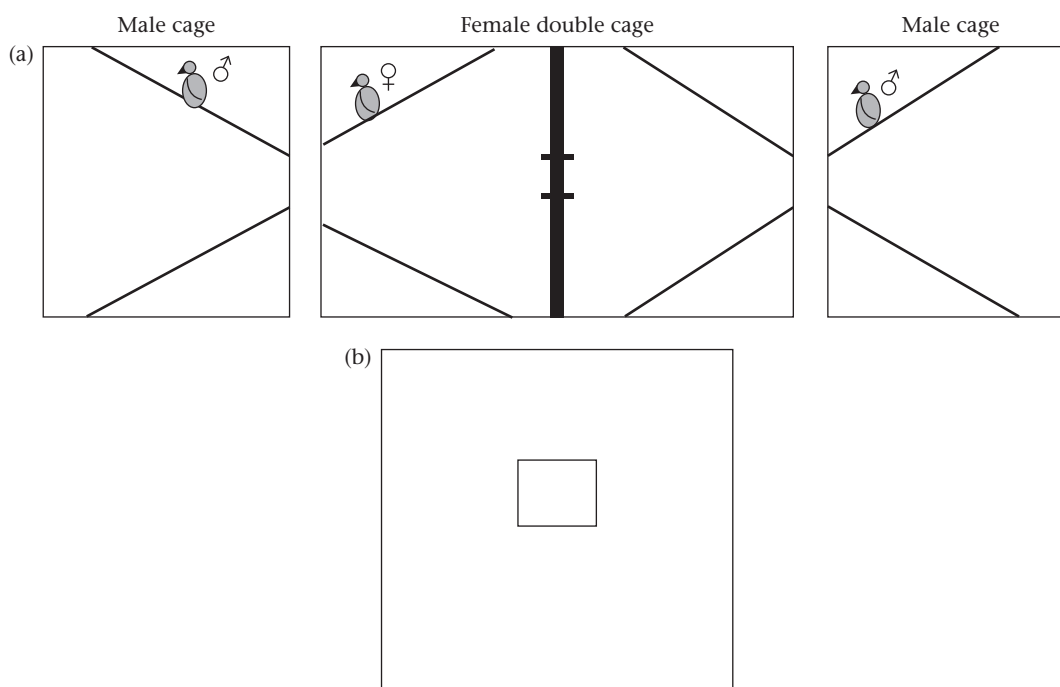


Figure 1. (a) Schematic of preference-testing cage set-up. Thin lines represent perches; the thick line represents the cardboard separator; the two lines on the thick line demarcate the 7.62×7.62 cm square opening in the centre of the cardboard separator. (b) Side view of cardboard separator showing placement of the square opening.

that each female spent next to the male from the same versus different elevation regardless of cage side.

Statistical Analysis

We analysed the data using a repeated measures general linear model (GLM) (STATISTICA 12) where the mean total time spent adjacent to males from the same versus different elevation across six trials was the repeated measure and elevation was the between-subject variable. Preference times were analysed separately for pre-photostimulation and post-photostimulation. Planned comparisons were used to assess pairwise statistical differences for high- and low-elevation females' preference for males from the same or different elevation.

We also calculated individual preference scores for each female by dividing the total amount of time each female spent with the same male over the total amount of time in the testing cage (maximum of 3600 s). Preference scores ranged from 0 to 1, spending no time on the side of the cage with the same male and spending all time on the side of the cage with the same male, respectively. We used paired *t* tests to compare preference scores to chance preference of 0.5 for high- and low-elevation females, separately for pre- and post-photostimulation. We also used a general linear model to compare high- and low-elevation female preference scores, separately for pre- and post-photostimulation.

Ethical Note

No birds were injured by mist netting or while collecting blood samples in the present study. We observed no infections or other detectable effects of mist netting or bleeding. Birds were handled for about 2–5 min after capture before being placed in transport cages covered with white translucent cloth to minimize disturbance. Birds always ate and drank water in these transport cages during the transport. All birds were released at the end of the

experiment at our study site. Some of these birds were later resighted successfully breeding.

RESULTS

Pre-photostimulation

Prior to photostimulation, there were no significant differences between the mean amount of time that females from both elevations spent adjacent to males from the same versus different elevation (GLM: $F_{1,10} = 0.19$, $P = 0.67$). There was a significant difference in the mean amount of time that high- and low-elevation birds spent in both sides of the testing cage that counted towards preference time ($F_{1,10} = 10.62$, $P = 0.009$). The interaction between elevation and time spent adjacent to males from the same versus different elevation, however, was not significant ($F_{1,10} = 0.74$, $P = 0.410$; Fig. 2a). Planned comparisons showed no significant differences in the amount of time that high- and low-elevation females spent on the side of the cage with the male from the same versus different elevation (all $P > 0.05$).

There were no significant differences in preference scores of females from high and low elevations ($F_{1,10} = 0.82$, $P = 0.39$). The individual preference scores for high- and low-elevation females also did not differ significantly from chance prior to photostimulation (paired *t* test: high elevation: $t_5 = 1.06$, $P = 0.34$; low elevation: $t_5 = 0.28$, $P = 0.79$, respectively; Fig. 3a).

Post-photostimulation

Following photostimulation to mimic breeding conditions, there were no significant differences in the mean amount of time that females from both elevations spent adjacent to males from the same versus different elevation (GLM: $F_{1,10} = 0.99$, $P = 0.34$). There was a significant difference in the mean amount of time that high- and low-elevation birds spent in the testing cage that counted

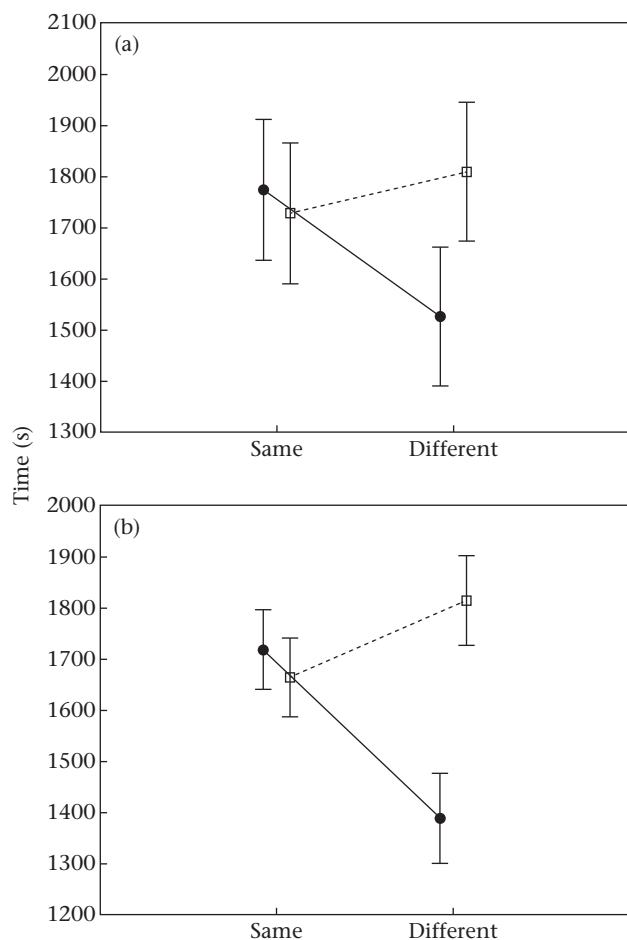


Figure 2. Total mean time (a) before and (b) after photostimulation that female mountain chickadees from high-elevation (solid circles) and low-elevation (open squares) sites spent on the side of the cage adjacent to the male from the same versus different elevation. Error bars represent SE.

towards preference time ($F_{1,10} = 6.24$, $P = 0.032$), and the interaction between elevation and time spent adjacent to males from the same versus different elevation was also statistically significant ($F_{1,10} = 7.07$, $P = 0.024$; Fig. 2b). Planned comparisons revealed that high-elevation females spent significantly more time adjacent to high-elevation males ($P = 0.027$), while females from low elevation showed no significant preferences ($P > 0.05$).

High-elevation females had significantly higher preference scores than low-elevation females ($F_{1,10} = 7.96$, $P = 0.018$). At the same time, the preference scores for high-elevation females were also significantly higher than expected by chance (paired t test: $t_5 = 3.90$, $P = 0.011$), while low-elevation female preference scores were not significantly different from chance ($t_5 = 0.94$, $P = 0.39$; Fig. 3b).

DISCUSSION

Overall, we found that high-elevation females showed a significant preference for males from the same elevation while no significant preference was detected in low-elevation females, albeit the preference among high-elevation females was dependent on photoperiod. Prior to photostimulation, when high- and low-elevation females were still experiencing winter daylength, they spent similar amounts of time adjacent to both high- and low-elevation males, suggesting no particular preference. After being

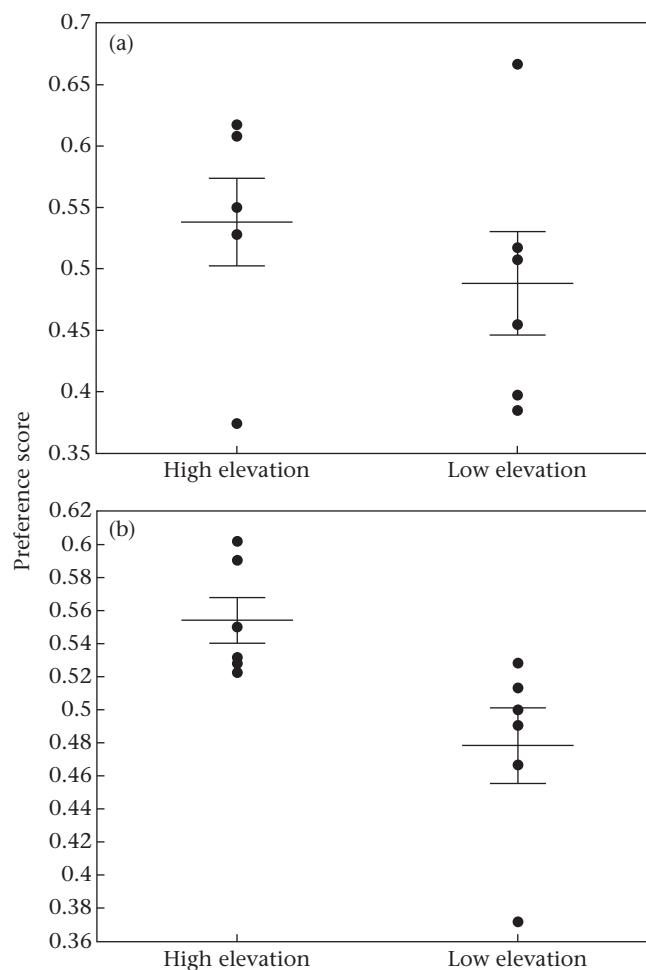


Figure 3. Individual (closed circles) and group-level (long horizontal lines: means; whiskers: SE) preference scores, calculated by dividing the total amount of time spent with the same male over the total amount of preference time, for female mountain chickadees from high- and low-elevation sites (a) before and (b) after photostimulation.

photostimulated to mimic breeding conditions, however, females from high elevation spent significantly more time adjacent to males from high elevation than they spent adjacent to males from low elevation. Low-elevation females, on the other hand, continued to spend similar amounts of time adjacent to males from both elevations. The fact that these patterns of preference were shown using multiple unique male pairs formed from unfamiliar birds (captured in different social flocks) suggests that such preferences are unlikely to be based on familiarity.

If the amount of time that a female spends on the same side of the cage with a male is indicative of her preference for that male in a breeding context (Amundsen et al., 1997; Collins et al., 1994; Leitao et al., 2014; Nolan & Hill, 2004; Woolley & Doupe, 2008), our results suggest that high-elevation female mountain chickadees are able to discriminate between high- and low-elevation males, and subsequently prefer males from their respective elevation. Our finding that high-elevation females prefer high elevation males only after photostimulation, and despite their socially subordinate status compared to low-elevation males (Kozlovsky, Branch, Freas, et al., 2014), socially subordinate birds experience reduced fitness compared with dominant individuals (e.g. Ratcliffe et al., 2007), suggests that their mate preference might be driven by the males' local adaptation to the females' local environment (e.g. superior spatial memory among other potential adaptations). If

spatial memory is in part heritable (W. D. Hopkins et al., 2014), high-elevation females might be expected to be choosier than low-elevation females because of the strong reliance on spatial memory to successfully retrieve food caches necessary to survive winters at high elevations. Conversely, because low-elevation birds might have less selective pressure on their spatial learning abilities, they might show less elevation-based mate preference, again assuming the discrimination made by high-elevation females is somehow linked to this particular local adaptation.

Interestingly, our original hypothesis, that low-elevation females would prefer low-elevation males over high-elevation males because of their socially dominant status (Kozlovsky, Branch, Freas, et al., 2014), was not supported: low-elevation females spent similar amounts of time near high- and low-elevation males. It remains unclear whether no significant preference among low-elevation females was due to their inability to discriminate between males from different elevations or due to a lack of preference. Our results only suggest that, at the population level, there was no discrimination or significant preference among low-elevation females using a high- and low-elevation male dichotomy. This lack of preference suggests that low-elevation females may not prefer males based on any particular known elevation-related characteristic including spatial memory/hippocampus morphology, social dominance, novel exploration, aggression or problem solving (Branch et al., 2014; Freas, Bingman, et al., 2013; Freas et al., 2012; Freas, Roth, LaDage, & Pravosudov, 2013; Kozlovsky, Branch, Freas, et al., 2014; Kozlovsky, Branch, & Pravosudov, 2014).

That elevation-related differences in social dominance had no detectable effect on female preference might have several potential explanations. Even though high-elevation males are socially subordinate to low-elevation males (Kozlovsky, Branch, Freas, et al., 2014), they cache more food and have superior spatial memory needed to recover food caches and superior problem-solving ability (Freas et al., 2012; Kozlovsky, Branch, & Pravosudov, 2014). The question is whether higher dominance status can compensate for cognitive deficiencies. If that were the case, dominant individuals may be expected to cache less and to show inferior spatial memory even within the same social groups, as they might simply capitalize on taking food from subordinate individuals. Yet, the opposite has been reported, with dominant individuals caching more food and having superior spatial memory compared to subordinate individuals (Pravosudov et al., 2003; Pravosudov & Omanska, 2005). In other words, females at high elevation should value traits related to more successful food caching and cache retrieval over dominance status. However, it is also possible that within elevations, female may additionally prefer socially dominant males. In addition, it may be that females are unable to assess relative dominance status in the absence of any male–male interactions.

Several concerns with the present study should be noted. (1) Because males from high and low elevations were housed in the same room, there could be a potential issue of call convergence (Mammen & Nowicki, 1981), if females use calls to identify males from their respective elevation. Although we did not measure call convergence, the fact that high-elevation females were able to discriminate between the high- and low-elevation males suggests that either call convergence did not occur, or females might use multiple cues in discrimination. In addition, although there is evidence of within-flock call convergence in chickadees from the same population (black-capped chickadees, *Poecile atricapillus*; Mammen & Nowicki, 1981), it is not clear whether chickadees from different populations can even be expected to converge.

(2) We do not know whether time spent in proximity to a male directly translates into mate choice or breeding in our birds. Our assumption of this was based on results of other studies that have

used this methodology and that have shown that it is indicative of mate preference (Amundsen et al., 1997; Collins et al., 1994; Leitao et al., 2014; Nolan & Hill, 2004; Woolley & Doupe, 2008). However, the fact that high-elevation females only showed a preference for high-elevation males once they were photostimulated to spring breeding conditions supports the notion that this discrimination or preference may be important for breeding. In addition, in resident pairs such as mountain chickadees, pairs form prior to winter when birds join stable social groups after natal dispersal (e.g. Ekman, 1989). Essentially, we changed the birds' social settings and so preferences expressed in the new social settings are likely to reflect new pair formation.

(3) Prior to photostimulation, high-elevation females also tended to prefer males from the same elevation, but the preference for high-elevation females was only significant after photostimulation. That we saw a preference after, but not before, photostimulation using the exact same sample sizes suggests that it was not an issue of power, but rather that the preference actually became stronger or more biologically relevant once all birds were in spring breeding conditions (all birds showed cloacal protuberances and singing rates increased).

Note, however, that we did not test spatial memory in this particular set of birds, and thus, we are not suggesting that females' preference is based on memory ability per se, but rather that there may be a proxy by which females can discriminate males from high versus low elevations. Previous studies have established significant elevation-related differences in spatial memory and the hippocampus (Freas, Bingman, et al., 2013; Freas et al., 2012; Freas, Roth, et al., 2013), and other behavioural traits (see above; Branch et al., 2014; Kozlovsky, Branch, Freas, et al., 2014; Kozlovsky, Branch, & Pravosudov, 2014). Our results suggest that high-elevation females might constrain movement between high and low elevations by preferentially selecting high-elevation males, despite a small distance (ca. 10 km). Such preference indirectly suggests that adaptations to high elevations in these resident birds might be especially crucial for survival and hence for female fitness. Our first step was to address whether females aid in maintaining local adaptations at high and low elevations by assessing female discriminability or preference for males from their respective elevation. Future research will now aim to assess potential proxies used by females to discriminate between high- and low-elevation males. We are specifically interested in potential structural variation in male song, because of its importance in mate choice (Searcy, 1984), and differences in achromatic contrast in males (see Doucet, Mennill, Montgomerie, Boag, & Ratcliffe, 2004; Mennill, Doucet, Montgomerie, & Ratcliffe, 2003) from different elevations that may serve as potential mechanisms for group-level identification and female mate choice in mountain chickadees.

Species experiencing disparate environmental conditions leading to local adaptations are of particular importance in evolutionary biology because they can shed light on the process of adaptive divergence (Streicher et al., 2014). The mountain chickadee system of the Sierra Nevada is a particularly useful system for addressing this question because these are resident birds, locally adapted to their respective elevation, and this local adaptation occurs across a small spatial scale. It will be paramount to gain insight into the heritability of spatial memory used in food cache recovery as well as the level of gene flow between birds at high and low elevations. Given the high mobility of avian species and the lack of a geographical barrier between the groups of individuals, it seems unlikely that there is no gene flow between these populations. However, the results found here suggest that females from high elevations may be contributing to separation between populations by mating assortatively with males from their respective elevation. Although we currently have no data on genetic population

structure, nonrandom mating may be important for maintaining local adaptations regardless of the presence of gene flow (Kondrashov & Shpak, 1998; McCracken et al., 2009; Wilson et al., 2012). In our study system, these new findings provide further support for elevation-related adaptations associated with differences in winter climate.

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Mountain chickadees from different elevations sing different songs: acoustic adaptation, temporal drift or signal of local adaptation?

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1. Summary

Song in songbirds is widely thought to function in mate choice and male–male competition. Song is also phenotypically plastic and typically learned from local adults; therefore, it varies across geographical space and can serve as a cue for an individual's location of origin, with females commonly preferring males from their respective location. Geographical variation in song dialect may reflect acoustic adaptation to different environments and/or serve as a signal of local adaptation. In montane environments, environmental differences can occur over an elevation gradient, favouring local adaptations across small spatial scales. We tested whether food caching mountain chickadees, known to exhibit elevation-related differences in food caching intensity, spatial memory and the hippocampus, also sing different dialects despite continuous distribution and close proximity. Male songs were collected from high and low elevations at two different mountains (separated by 35 km) to test whether song differs between elevations and/or between adjacent populations at each mountain. Song structure varied significantly between high and low elevation adjacent populations from the same mountain and between populations from different mountains at the same elevations, despite a continuous distribution across each mountain slope. These results suggest that elevation-related differences in song structure in chickadees might serve as a signal for local adaptation.

2. Introduction

In many songbird species, male song serves as an important cue in female mate choice [1,2] and may be used to defend territories from invasion by conspecific males [3]. Song is typically learned from other local males [4], is phenotypically plastic [5] and can be shaped by female preference [6]. Variation in song not only aids in species recognition, but is particularly useful for female mate choice because it allows females to discriminate between males from local versus non-local populations. Females have been shown to exhibit a preference for local males, which may be indicated by local dialects [7,8]. This preference may benefit females because males occupying the same local habitat may possess genetic material beneficial for survival at that specific location (local adaptation, e.g. [9]).

While song dialects or geographical variation in song within a species allow for identification of locally suited individuals, there are several mechanisms by which such variation could arise. The acoustic adaptation hypothesis suggests that song dialects or geographical variation in song may arise as a result of changes in the environment affecting signal transmission [10]. In this case, shifts in song could either evolve and become fixed or simply be adjusted accordingly by individuals, with functional significance such that signal transmission is optimized in a new environment (e.g. differences in plant density in different forests). The temporal variation hypothesis suggests that song may simply drift or change over time within a population with no particular adaptive significance and, therefore, different populations with limited gene flow might evolve different song dialects simply by drifting apart [11]. Finally, the local adaptation hypothesis suggests that individuals locally adapted to their environment may experience limited movement, and over time female preference for local males may produce divergence in song, creating different song dialects within a species [12,13]. These changes may serve as an additional cue for females to mate assortatively with males locally adapted to their location of origin (*sensu* individuals colonizing new areas and mistakes in males' song imitation resulting in new dialects [14]). The local adaptation hypothesis predicts that vocal dialects should evolve via sexual selection in heterogeneous environments regardless of spatial scale [15,16] when such heterogeneity is associated with local adaptations limiting movement [12,17]. However, evidence linking specific local adaptations—beyond acoustic adaptation—with song dialects is lacking [9].

Montane environments present an especially striking example of rapid environmental change over an elevation gradient, which is usually associated with highly predictable climatic changes. As such, individuals living along montane elevation gradients are predicted to vary phenotypically [18–21]. Likewise, songbirds would be predicted to have different song dialects associated with such elevation-specific adaptations, despite small spatial scale or lack of geographical barriers [13,15,17].

Here, we examined song variation associated with differences in elevation in food caching mountain chickadees (*Poecile gambeli*) from different elevations. Chickadees are resident songbirds inhabiting the mountains of western North America that form permanent flocks of unrelated individuals outside of the breeding season [22]. Chickadees are scatter-hoarders, caching large quantities of food in numerous locations during the autumn, to be consumed over winter when food is scarce [22]. Chickadees from different elevations vary across a number of behavioural and neural traits thought to reflect local adaptations to elevation-specific environments [23–26]. For example, birds occupying habitat at higher elevations experience harsher winter conditions (lower temperature, more snow, extended periods of snow cover [27]) and be likely to have a higher reliance on previously cached food to survive winter [23]. As such, chickadees exhibit large elevation-related differences in memory needed to retrieve caches associated with large differences in the hippocampus, a brain region known to be involved in spatial memory [23–25]. These differences were discovered in juvenile birds prior to their first winter and therefore prior to the period of largest climate-related mortality. In addition, recent data comparing high versus low elevation chickadees suggests group level behavioural types, such that high elevation males are slower explorers, less aggressive and socially subordinate to low elevation males [26,28]. What is most striking is that these differences exist on very a small spatial scale in a highly mobile species—chickadees from high and low elevations are separated by just a few kilometres with only 600 m difference in elevation.

Taken together, these data suggest that birds hatched at high elevations would be likely to fare poorly at low elevations, owing to their socially subordinate status, whereas low elevation birds would be likely to fare poorly at high elevations with harsher winter conditions, owing to their inferior memory limiting successful cache retrieval. As a result, it may be expected that females would benefit from selecting males from their respective elevations. Indeed, given a pairwise choice of a high or low elevation male, high elevation females preferred high elevation males, whereas low elevation females showed no preference [29]. It is unclear how high elevation females are able to discriminate between high and low elevation

males; however, given the pervasiveness of geographical variation in the songs of songbirds as well as the role of song in mate choice [1,7], variation in song may be one cue females use to discriminate between males from high and low elevations.

Here we assessed the song structure of male mountain chickadees using two exemplars of high and low elevation habitats on two different mountains in the Sierra Nevada with similar mixed-conifer forest structure. The structure of male song collected from multiple individuals at four locations was compared using basic acoustic parameters: duration, frequency and amplitude. Sampling song from males at high and low elevations from two different mountains allowed us to (i) assess the presence of any differences in song structure at different elevations and (ii) consider the potential mechanism generating variation or song dialects on a small spatial scale. If there are any differences in song structure among these four locations, the acoustic adaptation hypothesis predicts that song should differ between but not within elevations, because the composition of the mixed-conifer forests in Sierra Nevada are fairly standard and do not vary significantly between sites at similar elevations [30] and should therefore shape the song in similar ways in order to optimize signal transmission. The temporal variation hypothesis predicts that songs are selectively neutral and may drift and change over time. If there is no movement between birds from different elevations and/or mountains, then song structure could be different at all four locations. Finally, the local adaptation hypothesis predicts that limited movement between high and low elevation birds due to local adaptations, as well as limited movement between mountains due to short dispersal distances, would result in differences in song structure among all four locations, resulting in local songs or dialects used by females to choose local males [31].

3. Material and methods

3.1. Subjects and song collection

Male mountain chickadee song was recorded from high and low elevation sites at two spatially distinct mountain slopes separated by approximately 35 km, Sagehen, CA (Mountain 1) and Mt. Rose, NV (Mountain 2), between 06.00 and 13.00 h PST from 13 April to 9 June 2013. The high elevation sites were approximately 2400 m and the low elevation sites were approximately 1800 m at both locations (following [23]). At Sagehen (Mountain 1), we recorded at exactly the same elevations and locations used previously [23–26,29]. Males in these areas are not individually colour banded; therefore to avoid duplicating subjects, we recorded males with a minimum of 500 m separation between sites [32]. A minimum of 20 songs from at least 12 males per site were recorded using a Marantz PMD661 Compact Flash Card digital recorder and Sennheiser ME-66 unidirectional microphone with a sampling rate of 44 000 Hz and 16 bit resolution. Males were located auditorily and approached with microphone and recorder in hand. Each male was recorded on 1 day.

3.2. Acoustic analysis

Song recordings were first viewed in 10 s interval spectrograms from large .wav files using SIGNAL 5 digital signal analysis system (Engineering Design, Berkeley, CA, USA); each song was then edited and saved in an individual .wav file. For each male (Mountain 1 low, $n = 15$; Mountain 1 high, $n = 17$; Mountain 2 low $n = 12$; Mountain 2 high $n = 12$), we analysed a minimum of six quality songs limited by amount of noise (range: 6–23 songs per male). To obtain a comprehensive description of the song, we extracted 19 acoustic measures based on previous work on mountain and black-capped chickadees (table 1 and figure 1) [33–35]. Mountain chickadee song typically consists of either three or four notes, with variation in the presence/absence of an introductory note [33]. We extracted measures from the entire song as well as the individual notes within each song; therefore, the number of measures per song was partially dependent on the presence/absence of the introductory note.

Temporal and spectral measures were taken from sound spectrograms: eight temporal measures including (i) total song duration (SD), (ii) note duration (ND; 3 or 4 depending on the song) and (iii) internote interval (II; 2 or 3 depending on song); and nine spectral measures including (i) start frequency for each note (SF; 3 or 4 depending on song), (ii) frequency ratio (FR; mountain chickadee songs have a frequency shift between Notes 2 and 3, where the frequency of the note drops (calculated by dividing the end frequency of Note 2 by the SF of Note 3)) and (iii) the glissando of each note (NG; 3 or 4 depending on the song, calculated by dividing the SF of the note by the end frequency of the note). We also measured the number of notes (NN) in each song. The one amplitude measure, (i) relative amplitude (RA), was taken from a power spectrum and calculated by dividing the maximum amplitude of Note 3

Table 1. Means and standard errors for each song characteristic measured from high and low elevation males at both Mountain 1 and Mountain 2 locations. Bold values represent significant difference between elevations within a location using Tukey post hoc comparisons at $p < 0.05$. Italics indicate very closely approaching significant values, 0.056. Acoustic measures in bold and italics represent the 11 variables used in DFA.

acoustic measure	Mountain 1			Mountain 2		
	low	high	<i>p</i> -value	low	high	<i>p</i> -value
NN	3.85 ± 0.08	3.85 ± 0.08	0.999	3.81 ± 0.09	3.36 ± 0.11	0.011
N1 SF	4356.82 ± 20.22	4388.43 ± 23.50	0.828	4338.56 ± 20.79	4357.82 ± 56.17	0.976
N2 SF	4291.04 ± 17.78	4306.97 ± 23.62	0.999	4297.15 ± 25.97	4288.22 ± 32.64	1
N3 SF	3503.35 ± 18.78	3659.19 ± 20.82	<0.001	3608.64 ± 21.10	3611.20 ± 21.79	1
N4 SF	3590.68 ± 16.41	3730.89 ± 19.26	<0.001	3682.67 ± 21.18	3660.37 ± 20.03	0.999
FR	705.23 ± 20.64	591.33 ± 18.10	0.005	641.78 ± 21.02	609.48 ± 38.73	0.823
N1 G	0.99 ± 0.003	0.99 ± 0.002	0.993	0.98 ± 0.003	0.99 ± 0.009	0.039
N2 G	1.02 ± 0.003	1.01 ± 0.002	0.896	1.01 ± 0.003	1.02 ± 0.005	0.976
N3 G	0.99 ± 0.003	0.1 ± 0.003	0.275	0.99 ± 0.003	0.99 ± 0.003	0.999
N4 G	0.1 ± 0.003	1.01 ± 0.002	0.414	0.1 ± 0.002	0.99 ± 0.002	0.992
SD	1201.42 ± 16.99	1129.55 ± 19.11	0.079	1140.70 ± 31.84	1094.03 ± 21.39	0.514
N1 D	44.72 ± 2.07	40.62 ± 1.43	0.421	39.29 ± 2.20	36.96 ± 3.28	0.905
N2 D	316.98 ± 4.22	279.88 ± 5.91	<0.001	303.89 ± 7.74	290.68 ± 5.33	0.839
N3 D	188.73 ± 2.78	160.10 ± 2.21	<0.001	193.59 ± 4.29	182.36 ± 1.93	0.942
N4 D	294.23 ± 5.73	279.82 ± 4.17	0.539	278.61 ± 6.58	301.18 ± 5.89	0.106
N1–N2 II	127.78 ± 2.78	140.67 ± 3.19	0.056	127.26 ± 3.06	119.41 ± 8.38	0.614
N2–N3 II	138.12 ± 3.06	140.46 ± 3.88	0.999	138.68 ± 4.37	145.77 ± 6.00	0.931
N3–N4 II	114.84 ± 3.13	109.35 ± 2.67	0.962	99.78 ± 3.15	117.98 ± 4.98	0.056
RA	0.93 ± 0.015	0.92 ± 0.016	0.984	0.96 ± 0.016	0.86 ± 0.015	<0.001

by the maximum amplitude of Note 2. Temporal and spectral measures were made using a spectrogram window size of 512 points, a time resolution of 11.6 ms and a frequency resolution of 86.1 Hz.

3.3. Statistical analysis

For each measure described, we calculated means and standard deviations across all songs produced by the same male. This way each bird is only represented once in all statistical analyses and pseudoreplication is avoided. We analysed SF, NG, ND and II of Notes 2, 3 and 4 using four repeated measures general linear models (GLMs; STATISTICA v. 12), where the acoustic measure from male song was the repeated measure (e.g. SF of Notes 2, 3 and 4) and elevation (high, low), location (Mountain 1, Mountain 2), and an interaction between elevation and location were the between-subject variables. Univariate GLMs were used to assess differences between male song at each of the four locations on NN, SD, FR, RA, as well as SF, NG, ND and II of birds with at least one song containing Note 1 (the introductory note). If a bird did not have the introductory note (Note 1), the analyses for that bird started with Note 2. Tukey posthoc tests were used for all pairwise comparisons, with significance established at $p < 0.05$. In addition, we calculated location-specific individual variation using the coefficient of variation (CV) for each acoustic measure for every bird and used GLMs to assess how consistently male songs were produced (used to assess potential differences in male quality [36–38]).

To further address differences in songs from these four locations, we ran two ‘all-variables-together’ discriminant function analyses (DFAs) (STATISTICA v. 12) to assess song classification by location. In an ‘all-variables-together’ DFA, all predictor variables are used at the same time to derive the discriminant functions model [39,40]. We ran two models using the same 11 predictor variables, one based on mean

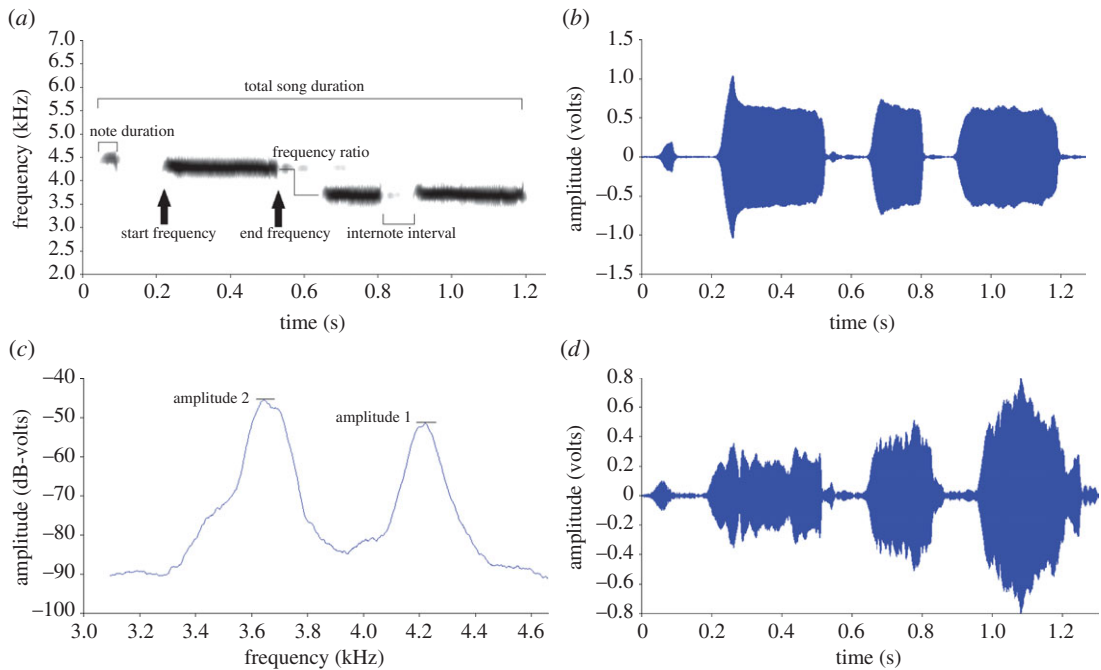


Figure 1. Schematic of sound spectrogram and power spectrum showing acoustic measurements used for analysis. Other measurements not physically shown in diagram were calculated using the measurements depicted. (a) Sound spectrogram (transform length of 512 points, time resolution of 11.6 ms, and frequency resolution of 86.1 Hz); measurements include temporal: total SD, ND, II and spectral: SF, end frequency (used with SF to calculate NG), change in frequency from end of Note 2 to start of Note 3 used to calculate FR, also the NN can be counted from the spectrogram, there are four notes in this example. (b) Waveform of 1a. (c) Power spectrum (FFT window = 65 536 points; 88 Hz smoothing) showing amplitude 1 and 2 used to calculate RA. (d) Waveform of 1c.

values and one based on CV values for each predictor. The number of variables in a DFA are limited by the least number of objects in a group minus one [39]; our lowest sample size was 12, therefore we used 11 predictor variables. Seven variables (NN, Note 3 SF, FR, Note 2 D, Note 3 D, Note 3–4 II, RA, see bolded variables in table 1) were chosen based on significant differences from GLMs and four (Note 4 D, Note 2 G, Note 3 G, Note 4 G, see italicized variables in table 1) were added to increase likelihood of discrimination. Location-specific individual variation was assessed by using the CV of each acoustic measure for each song within a birds' repertoire, therefore the CVs represent how consistently an individual produces its song [41].

We used the 'hold-out-sample' method to cross-validate the location classifications, which simply involves running a DFA without a set of observations from each group and then using those results to classify the observations that were held out [39,40]. For our analysis, we held out three randomly chosen males from each location (12 total). The same cases were held out for both DFAs.

4. Results

4.1. General linear models

4.1.1. Temporal

There was no significant effect of overall location ($F_{1,52} = 1.93$, $p = 0.171$) for ND of Notes 2, 3 and 4, however there was a significant effect of elevation ($F_{1,52} = 13.85$, $p < 0.001$), and significant interaction between location and elevation ($F_{1,52} = 12.62$, $p < 0.001$; figure 2a). There was a significant difference in ND for Notes 2 and 3 between high and low elevation male song from Mountain 1, but no differences within Mountain 2 (table 1). Additionally, there were significant differences in Note 2 duration between Mountain 1 low and Mountain 2 high ($p = 0.012$) and between Mountain 1 high and Mountain 2 low ($p = 0.026$); and in Note 3 duration between Mountain 1 high and Mountain 2 low ($p < 0.001$), and Mountain 1 high compared with Mountain 2 high approached significance ($p = 0.059$).

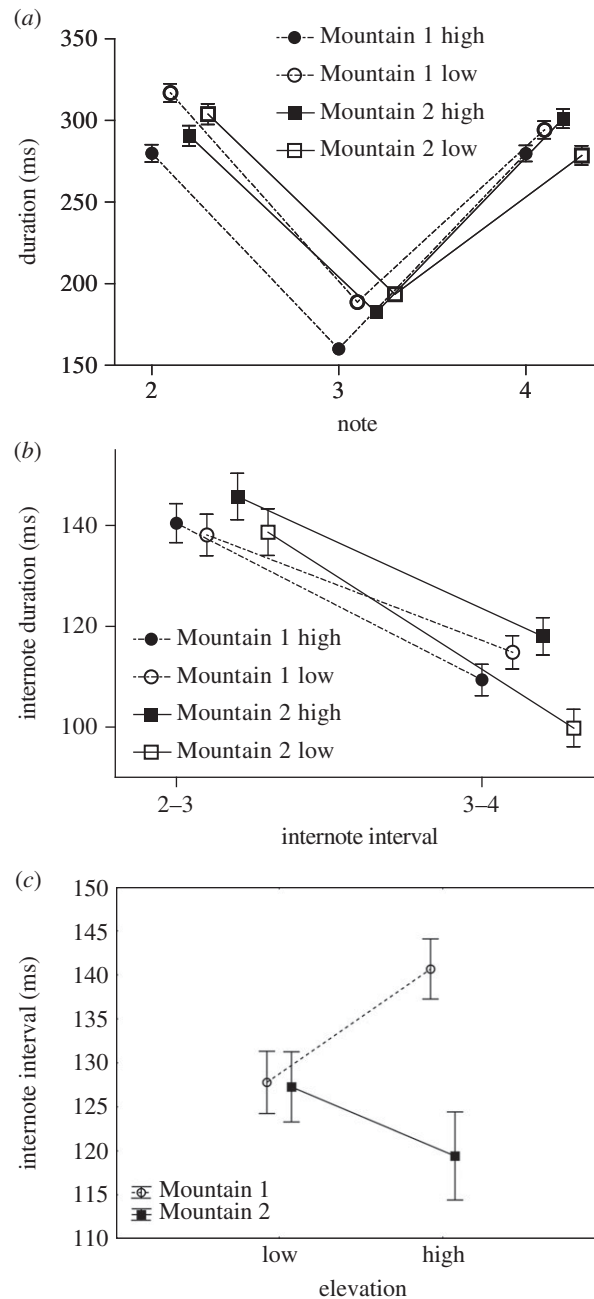


Figure 2. Means and s.e.m. for the three temporal measures that showed significant differences using Tukey post hoc comparisons. (a) ND for Notes 2, 3 and 4 from high and low elevation males sampled at Mountains 1 and 2, (b) II between Notes 2–3 and Notes 3–4 from high and low elevation males sampled at Mountains 1 and 2, (c) II between Notes 1–2 from high and low elevation males sampled at Mountains 1 and 2.

There was no effect of overall location ($F_{1,52} = 0.002$, $p = 0.967$) or elevation ($F_{1,52} = 2.66$, $p = 0.109$) on the II between Notes 2 and 3 or Notes 3 and 4, however there was a significant interaction between location and elevation ($F_{1,52} = 4.40$, $p = 0.041$; figure 2b). There were significant differences between the II of Notes 3 and 4 for high and low elevation males at Mountain 2, but no significant differences at Mountain 1 (table 1). No other pairwise comparisons were significant between mountains.

There was a significant effect of overall location ($F_{1,52} = 4.66$, $p = 0.036$) and elevation ($F_{1,52} = 7.06$, $p = 0.010$) on SD, however the interaction between location and elevation was not significant ($F_{1,52} = 0.32$, $p = 0.575$). There were no significant differences between high and low elevation males' SD at Mountain 1 or Mountain 2 (table 1). However, there was a significant difference between the SD at Mountain 1 low and Mountain 2 high ($p = 0.008$).

Further analyses of individuals with at least one song with the introductory note (Mountain 1 low $n = 14$; Mountain 1 high $n = 15$; Mountain 2 low $n = 11$; Mountain 2 high $n = 7$) showed an effect of overall location ($F_{1,43} = 4.35, p = 0.043$) on Note 1 ND, however there was no significant effect of elevation ($F_{1,43} = 2.184, p = 0.147$), and the interaction between location and elevation was not significant ($F_{1,43} = 0.16, p = 0.688$). There were no significant differences between high and low elevation male Note 1 ND at Mountain 1 or Mountain 2 (table 1) or between mountain comparisons.

There was a significant effect of overall location ($F_{1,43} = 7.29, p = 0.01$), but no effect of elevation ($F_{1,43} = 0.39, p = 0.535$) on II between Notes 1 and 2, however there was a significant interaction between location and elevation ($F_{1,43} = 6.60, p = 0.014$; figure 2c). The difference in Note 1 II between high and low elevation birds at Mountain 1 approached significance, but there was no difference at Mountain 2 (table 1). There was a significant difference in Note 1 II between Mountain 1 high and Mountain 2 high ($p = 0.006$).

4.1.2. Spectral

There was no effect for overall location (Mountain 1 versus Mountain 2) ($F_{1,52} = 0.3, p = 0.578$) on SF for Notes 2, 3 and 4, however there was a significant difference between elevations (high and low; $F_{1,52} = 5.8, p = 0.02$) and a significant interaction between location and elevation ($F_{1,52} = 8.3, p = 0.006$; figure 3a). The SFs for Notes 3 and 4 between males from high and low elevation were significantly different at Mountain 1, but not at Mountain 2 (table 1). Furthermore, for Note 3 SF, there was a significant difference between Mountain 1 low and Mountain 2 high ($p = 0.043$) as well as a trend for difference between Mountain 1 low and Mountain 2 low ($p = 0.054$).

There was an effect of overall location ($F_{1,52} = 8.3, p = 0.006$), however there was no significant effect of elevation ($F_{1,52} = 1.6, p = 0.212$) on the NG of Notes 2, 3 and 4—the interaction between location and elevation was also not significant ($F_{1,52} = 0.6, p = 0.434$). There were no significant differences in NG for Notes 2, 3 or 4 between high and low elevations at Mountain 1 or Mountain 2 (table 1), however there was a significant difference in NG for Note 4 between Mountain 1 high and Mountain 2 high ($p = 0.002$).

There was no significant effect of overall location ($F_{1,52} = 0.84, p = 0.364$), however there was a significant effect of elevation ($F_{1,52} = 8.75, p = 0.005$) on FR. The interaction between location and elevation was not significant ($F_{1,52} = 2.73, p = 0.105$; figure 3b). FRs between males from high and low elevation were significantly different at Mountain 1, but not at Mountain 2 (table 1). There was a significant difference in FR for Mountain 1 low and Mountain 2 high ($p = 0.044$).

There was no significant effect of overall location ($F_{1,43} = 0.67, p = 0.419$) or elevation ($F_{1,43} = 0.72, p = 0.399$) on the SF of Note 1, nor was the interaction between location and elevation significant ($F_{1,43} = 0.04, p = 0.849$). There were no significant differences in Note 1 SF for high and low elevation males from either Mountain 1 or Mountain 2 (table 1), and all other comparisons between mountains were not statistically significant.

There was no significant effect of overall location ($F_{1,43} = 1.0, p = 0.319$) on the NG of Note 1, however there was a significant effect of elevation ($F_{1,43} = 5.6, p = 0.022$) and a significant interaction between location and elevation ($F_{1,43} = 4.2, p = 0.047$; figure 3c). NG of Note 1 was significantly different between high and low elevation male song from Mountain 2 but not from Mountain 1 (table 1). Differences in NG for Note 1 approached significance between Mountain 1 high and Mountain 2 low ($p = 0.059$).

There was a significant effect of overall location ($F_{1,52} = 8.11, p = 0.006$) and elevation ($F_{1,52} = 6.02, p = 0.017$) on NN, with a significant interaction between location and elevation ($F_{1,52} = 5.89, p = 0.019$; figure 4). There was a significant difference in NN between males at high and low elevations from Mountain 2 but not from Mountain 1 (table 1). There was also a significant difference in NN between Mountain 1 low and Mountain 2 high ($p = 0.003$) and Mountain 1 high and Mountain 2 high ($p = 0.002$).

4.1.3. Amplitude

An analysis of the RA, a ratio of the amplitude of Note 3 over Note 2, showed no significant effect of overall location ($F_{1,52} = 1.12, p = 0.295$), however there was a significant effect of elevation ($F_{1,52} = 11.32, p = 0.001$) and a significant interaction between location and elevation ($F_{1,52} = 8.36, p = 0.006$; figure 5). RA for male song from high versus low elevation was significantly different at Mountain 2, but not at Mountain 1 (table 1). However, there was a significant difference between Mountain 1 low and Mountain 2 high ($p = 0.017$), as well as a difference between Mountain 1 high and Mountain 2 high ($p = 0.03$).

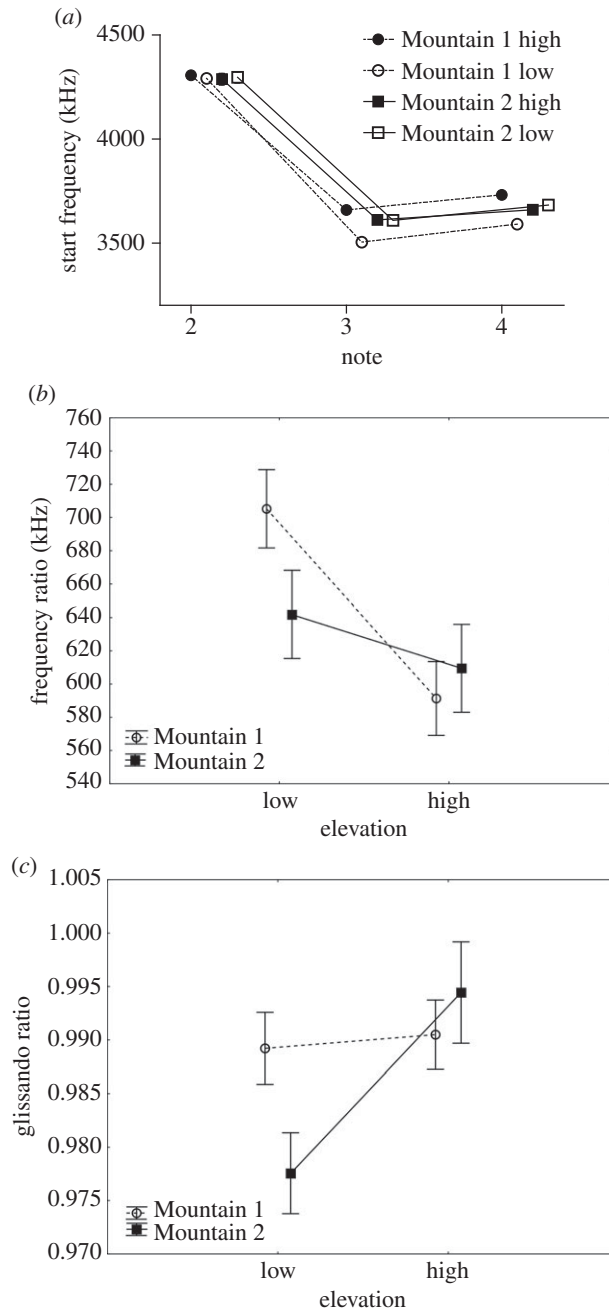


Figure 3. Means and s.e.m. for the three spectral measures that showed significant differences using Tukey post hoc comparisons. (a) SF for Notes 2, 3 and 4 from high and low elevation males sampled at Mountains 1 and 2 (note that s.e.m. are present but are very small and therefore do not always appear on the figure), (b) FR from high and low elevation males sampled at Mountains 1 and 2, and (c) NG for Note 1 from males at high and low elevations sampled from Mountains 1 and 2.

4.1.4. Location-specific individual variation

Only four acoustic measure CVs revealed significant differences between elevations and/or locations. (i) There was an effect of overall location ($F_{1,52} = 5.49$, $p = 0.023$) on NN CV, but no effect of elevation ($F_{1,52} = 0.48$, $p = 0.49$) and no significant interaction between location and elevation ($F_{1,52} = 0.13$, $p = 0.724$). There were no significant differences between high and low elevation in NN CVs at Mountain 1 or Mountain 2, or between mountains (all $ps > 0.05$). (ii) There was no significant effect of overall location ($F_{1,52} = 0.13$, $p = 0.72$) on the CV of SF, however there was an effect of elevation ($F_{1,52} = 4.11$, $p = 0.048$), but no significant interaction between location and elevation ($F_{1,52} = 1.24$, $p = 0.271$). There were no significant differences in Notes 2, 3 and 4 SF CVs between high and low elevations from

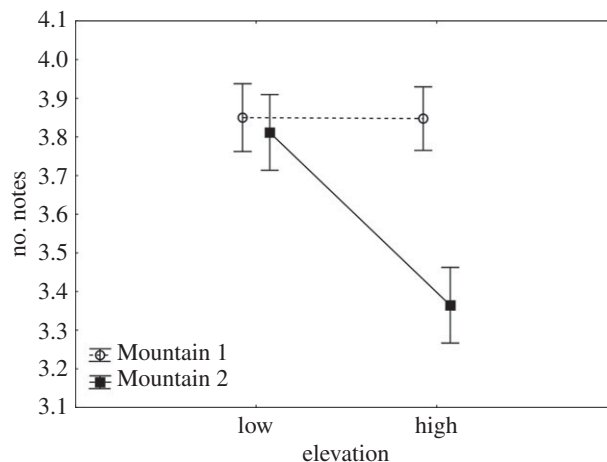


Figure 4. Mean and s.e.m. for NN in songs from high and low elevation males sampled at Mountains 1 and 2.

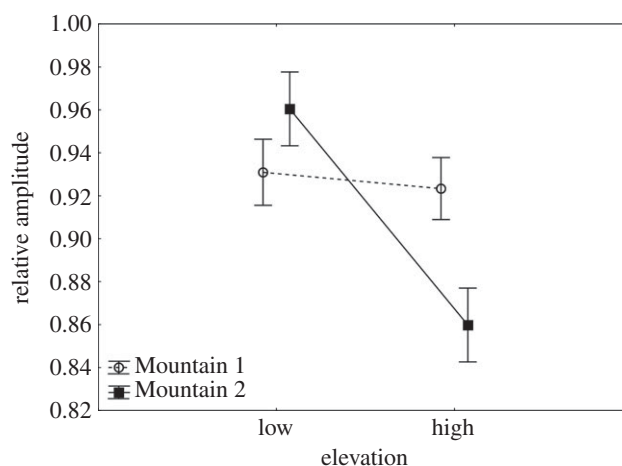


Figure 5. Mean and s.e.m. for RA of male song from high and low elevations sampled at Mountains 1 and 2.

Mountain 1 or Mountain 2, or between mountains (all $p > 0.05$). (iii) There was no effect of overall location ($F_{1,43} = 0.36$, $p = 0.554$) on the CV of NG, however there was an effect of elevation ($F_{1,43} = 4.68$, $p = 0.036$), but no significant interaction between location and elevation ($F_{1,43} = 1.70$, $p = 0.199$). Again, there were no significant differences in NG CV for Note 1 between high and low elevation males from Mountain 1 or Mountain 2, or between elevations (all $p > 0.05$). (4) Finally, there was no overall effect of location ($F_{1,52} = 1.44$, $p = 0.236$) on the CV of FR, however there was an effect of elevation ($F_{1,52} = 11.28$, $p = 0.001$), with a significant interaction between location and elevation ($F_{1,52} = 4.11$, $p = 0.048$). There was a significant difference in FR CV for males from Mountain 2, such that males from high elevation had a significantly higher FR CV than males from low elevation ($p = 0.004$). There were no significant differences in FR CV between high and low elevation males at Mountain 1 ($p > 0.05$), however there was a significant difference between Mountain 1 low and Mountain 2 high ($p = 0.013$).

4.2. Discriminant function analysis

We conducted two discriminant function analyses to classify male song based on location (Mountain 1 low and high, Mountain 2 low and high). The first discriminant function analysis classified male songs to a location using the means of 11 acoustic variables (NN, Note 3 SF, FR, Note 2 D, Note 3 D, Note 4 D, Note 3–4 II, Note 2 G, Note 3 G, Note 4 G and RA). This analysis was able to significantly discriminate between songs from the four locations using the 11 acoustic variables ($F_{33,124} = 7.94$, $p < 0.001$). Using the ‘hold-out-sample’ cross-validation method, this DFA correctly assigned 91.67% of the 12 cases (11/12, significant using a binomial test with chance set at 0.50, $p = 0.003$; figure 6a). The second discriminant

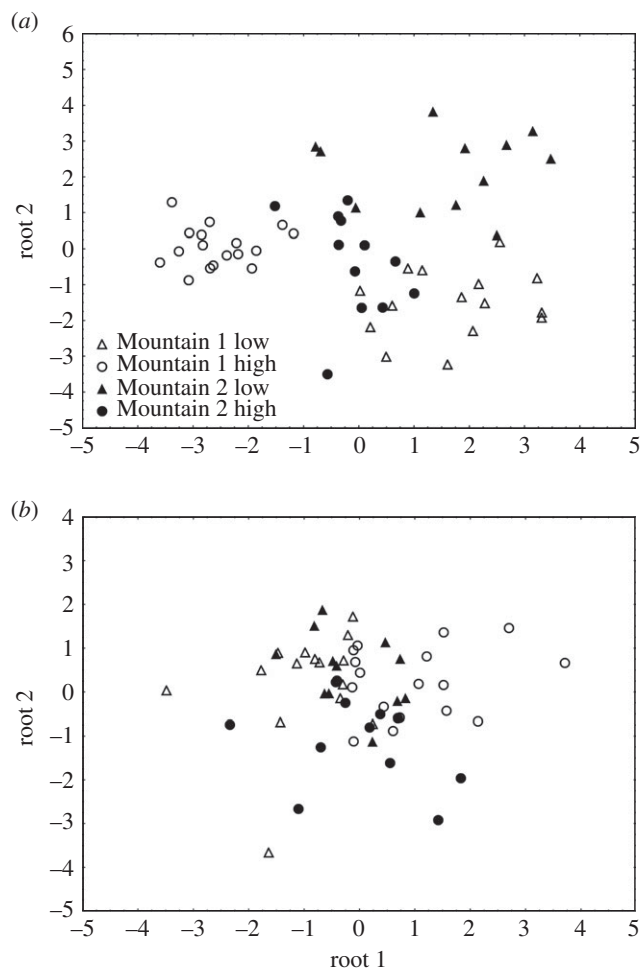


Figure 6. Discriminate function models of 11 variables for high and low elevations sampled at both Mountain 1 and Mountain 2. (a) DFA calculated from means, (b) DFA calculated from CVs.

Table 2. The original and cross-validated percentage of correct classifications by group using two ‘all-variables-together’ DFAs, one for means and one for CVs of acoustic parameters measured. The eigenvalue and canonical correlation coefficient, as well as the two acoustic features with the two largest (respectively) standardized and raw coefficients for the first discriminant function are listed for both DFAs, means and CVs.

group	original	cross-validated	eigenvalue	r_c	std. coeff	raw coeff
means	94.64	91.67	3.53	0.88	FR, ND of Note 3	NG of Notes 2 and 3
CVs	62.5	66.67	0.59	0.61	NG of Note 2, FR	NG of Notes 2 and 4

function analysis classified male song to a location using the acoustic measure CVs of the same 11 variables listed above; however, this analysis did not discriminate between songs from the four locations ($F_{33,124} = 1.47$, $p = 0.069$). This analysis correctly assigned 66.67% of the 12 cases (8/12, not significant using a binomial test with chance set at 0.50, $p = 0.121$) used for cross-validation (figure 6b). For the binomial tests, we used a more conservative approach by using a chance level of 0.50 to represent the dichotomy of correct versus incorrect classification; however, we also want to report the binomial test with chance set at 0.25 as there are four possible populations for which a male’s song can be classified (both classifications become significant, DFA 1, $p = 0.000$ and DFA 2, $p = 0.002$). We would, however, like to note that significant classification in DFA 2 at the 0.25 level is driven by one variable, the FR, which differed between Mountain 2 high and low and between Mountain 1 low and Mountain 2 high. See table 2 for eigenvalue and canonical correlation coefficient, as well as the standardized and raw coefficients for the first discriminant function for the discriminant function analyses conducted for means and the CVs.

5. Discussion

Our study showed that mountain chickadees living at high and low elevations from two different mountain locations differ in their song structure. These differences were unique at each of the four locations, and there were no general differences within elevations between the two mountains (see figure 7*a, b* for population comparisons). In addition, we found no significant differences in degree of individual variation (CV) among our four sites. Male song did not differ significantly in consistency of production within or between elevations. This finding is inconsistent with the idea that males with superior cognitive abilities are able to produce more consistent songs and therefore may be of higher quality [36–38]. Our high elevation birds have superior cognitive abilities compared with low elevation birds [23,42]; however, unlike previous work suggesting that better quality males sing more consistent songs [36–38], we do not find support for this, at least on a population level.

Several hypotheses could explain differences in male song structure from high and low elevations: (1) the acoustic adaptation hypothesis [10], (2) temporal variation [11] or (3) local adaptation [7,12–14,43] (see Introduction for detailed description). Song in songbirds has been shown to change along an elevation gradient as selection pressures change, including variation in vegetation and/or abundance of heterospecifics, both of which may interfere with signal transmission [44–46]. If song was acoustically adapted (hypothesis 1) to either altitude or elevation-specific habitat, birds at similar elevations should sing similar dialects, while birds at differing elevations should sing different dialects. Both the high and low elevation sites used in this study are found in the Sierra Nevada less than 35 km from each other, and low elevation sites exhibit comparable mixed-conifer forest species composition, and species abundance to each other, as do the two high elevation sites [30]. Despite similarities in within elevation environments, we found no consistent song differences based on differences in elevation across mountain sites; therefore, our results are inconsistent with predictions based on the acoustic adaptation hypothesis. Although we think it is unlikely, it may be possible that some minor specific local differences (besides general forest species and age composition) could contribute to the differences we see between populations.

We did, however, find some support for both hypotheses (2) and (3), as under both hypotheses we predict differences among song collected from each location. However, when combined with previous research, the third hypothesis seems to be the most consistent with our data. Specifically, previous research has shown that mountain chickadees living at high elevations, that experience harsher winter conditions, have superior spatial memory and related brain regions associated with more intense food caching compared with low elevation birds (these results found using different birds from the same Mountain 1 populations at exactly the same elevations used here [23–25]). Given that these birds rely heavily on food caches to survive winter, it seems likely that low elevation birds would be less successful at high elevations due to their lower food caching propensity and inferior memory abilities. Furthermore, high elevation birds are socially subordinate to low elevation birds [26], which may limit high elevation birds from penetrating low elevation, because subordinate birds would experience reduced fitness [47]. The fact that significant differences in song structure are produced on separate mountains along a similar elevation gradient suggests similar selection pressure for locally adapted individuals may lead to such differences. Considering that females might be a driving factor for the evolution of elevation-related song dialects, differences might be expected to evolve independently on different mountains assuming dispersal may occur along the slope of each mountain and not between mountains—chickadees have one dispersal event as juveniles and are thought to disperse rather short distances (0.4–11 km [48]). If this were the case, females at high elevation on each mountain are more likely to encounter a male from low elevation from the same mountain rather than a male from high elevation from a different mountain.

Some research investigating geographical variation in black-capped chickadees focuses on how new dialects arise, via slight alterations or imperfect imitations of song leading to multiple song dialects in newly colonized areas, where individuals are geographically isolated [49,50]. Mountain chickadees in the Sierra Nevada, however, inhabit a continuous gradient of habitat, with no geographical barriers to movement. Despite this continuous distribution, we see significant differences in male song structure. Because song in songbirds is phenotypically plastic, it may be possible for males to move between elevations within a mountain and shift their song to the local structure (vocal fluctuation in response to noise [51–53]); however, this is unlikely because: (i) song in chickadees is learned from the natal habitat from other local males; once males disperse they may prune their repertoire, however once it is pruned it becomes crystallized [54]. (ii) If birds are able to move to another elevation or location and shift their songs to the local dialect it would not serve as an honest cue [55] of location and therefore females, as a general rule, should have no preference for local dialects [7,8]. Finally (iii) if males were moving between

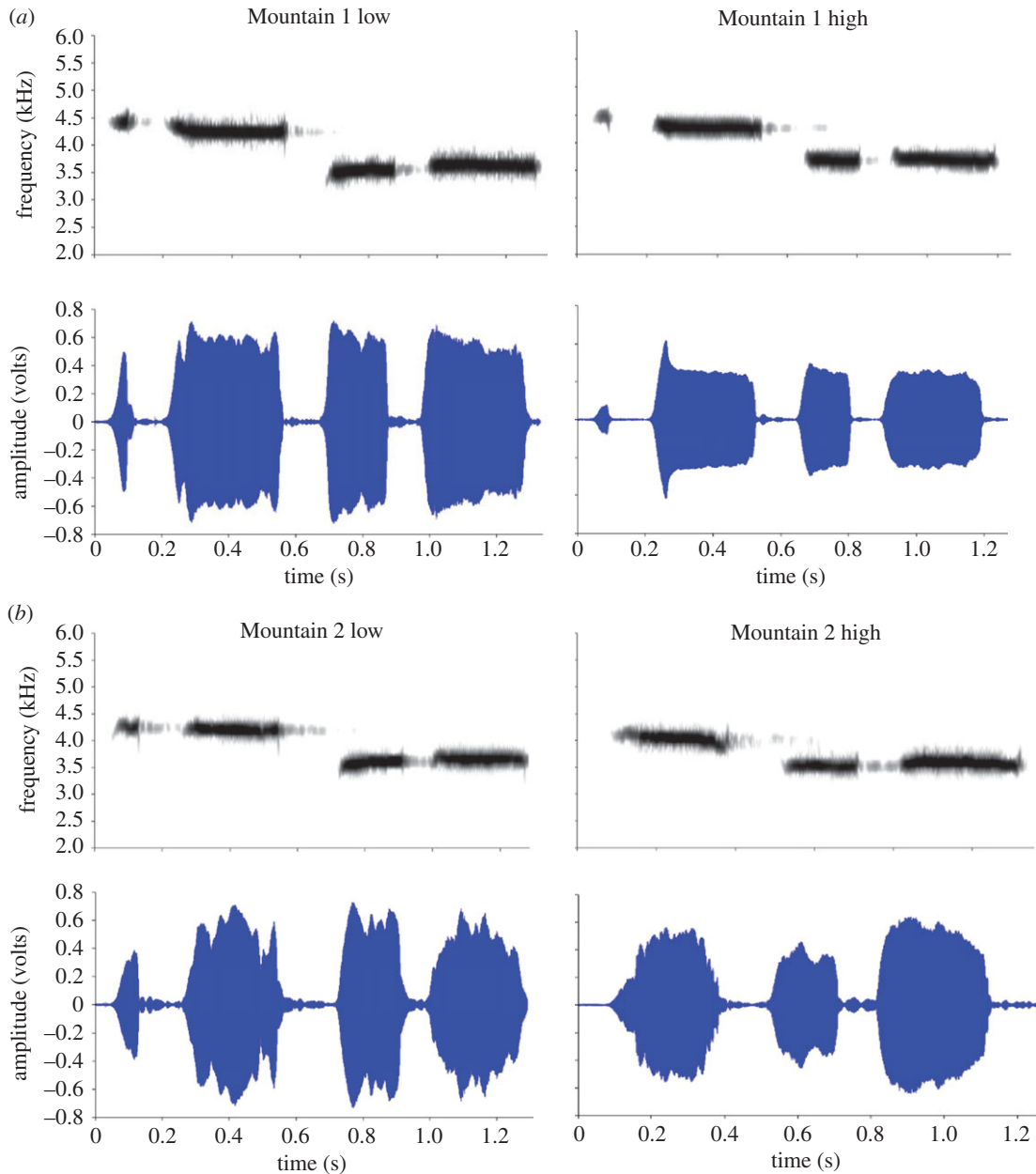


Figure 7. Example of male song from each of the four locations. (a) Left, spectrogram and waveform from Mountain 1 low; right, spectrogram and waveform from Mountain 1 high. (b) Left, spectrogram and waveform from Mountain 2 low; right, spectrogram and waveform from Mountain 2 high.

elevations or across mountains and shifting their song, we would not have detected the differences in song structure between high and low elevation male song across the two mountains sampled here.

If high and low elevation birds are locally adapted to their respective elevations (differences in spatial memory, food caching and exploration rates), it would benefit females to be able to discriminate between males from different elevations in order to mate with those from their respective elevation to produce the most fit offspring. Our recent work has shown that in a pairwise choice of high and low elevation males, high elevation females prefer high elevation males to low elevation males [29], despite their socially subordinate status (a feature known to be important in mate acquisition and fitness [47]). It is unknown what cue or proxy females may use to discriminate between males from high and low elevations; however, the plethora of differences seen here in song structure suggest that song may provide a salient signal of location of origin, which could be used in discrimination in the wild.

Future research will address females' ability to discriminate between male song from high and low elevations and their potential preference for these different song types.

In order to show support for hypothesis (2), we would need to show that there is genetic population structure between the four locations sampled here. Preliminary data from our laboratory has sampled these exact same populations and suggests no genetic population structure. However, even without this preliminary data, it would seem unlikely that there is absolutely no gene flow or movement between these four locations because there is a continuous distribution of chickadees along each mountain, with only a few kilometres between the elevations sampled. In addition, the two mountains sampled are less than 35 km away from one another, and there is no geographical barrier preventing movement, granted chickadees are known to disperse rather short distances [48]. Movements between elevations are probably somewhat restricted due to local adaptations; however, variation in climate severity among years may allow some movement between elevations, especially in years with mild winters. Even a low amount of movement between elevations might be sufficient to prevent genetic population structure [56], moreover local adaptations are known to evolve even in the face of gene flow [20,57].

Future work will address song structure across a gradient of elevations using these same mountains in order to elucidate if the differences in song observed here are a result of clinal variation or if they represent true 'high' and 'low' elevation dialects. An abrupt change in song structure would be suggestive of song dialects and would further suggest limited gene flow between elevations. If there is an elevation location where one dialects ends and the other begins, that location will inform future research related to high and low elevation contact zones and change in spatial memory ability needed to retrieve food caches. This information may help us answer whether these groups of individuals are shifting into separate populations or if there is continuous mixing along the high and low elevation gradient. This question is particularly interesting given the data correlating song dialects and gene flow. While some research suggests that song dialects represent a reduction in gene flow [14,58], an abundance of work has found differences in song structure despite gene flow [59–61]. Given the local adaptations we see in mountain chickadees and differences in song structure found here, it is critical to understand the levels of gene flow between high and low elevation as well as between mountains to identify the specific mechanism generating this variation in song structure.

Ethics statement. The study was conducted in accordance with University of Nevada ACUC Protocol (00576).

Data accessibility. All data are available in the electronic supplementary information.

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Author contributions. C.L.B. recorded songs in the field and collected song measurements using SIGNAL v. 5. C.L.B. and V.V.P. conceived the study, designed the experiment, conducted the statistical analysis, drafted the manuscript, and both gave final approval of the manuscript.

Conflict of interests. The authors have no competing interests.

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Do Male Mountain Chickadees Discriminate between Local and Non-Local Elevation Intruders?

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Abstract

Song in temperate songbirds is typically learned from local males and commonly thought to function in mate choice and male–male competition. Because male song is learned locally and is phenotypically plastic, it often varies geographically and may serve as a cue for an individual's location of origin. In montane environments, environmental heterogeneity occurs across the elevation gradient, potentially leading to local adaptations on rather small spatial scales. If local adaptations are associated with restricted movements between elevations, this leads to the question of whether individuals can discriminate between birds from the same vs. different elevations. Mountain chickadees inhabiting high and low elevations are known to differ in numerous phenotypic traits, including male song structure. In addition, we previously reported that female high-elevation mountain chickadees can discriminate between local and non-local elevation males and here, we tested whether males also respond differentially to high- vs. low-elevation male territory intruders. Playback stimuli were presented to resident high- ($N = 24$) and low- ($N = 20$) elevation males at our previously established study sites during spring to compare the males' behavioral responses to high- and low-elevation male song playbacks. Both high- and low-elevation males responded similarly to both types of playback stimuli, suggesting that they do not differentially respond to male intruders from their location of origin compared to non-local males. These results are considered in the framework of our previous findings and the larger body of literature, which suggests that male songbirds readily discriminate between local and non-local male intruders.

Background

Song in temperate songbirds is typically learned from other local males (Nelson et al. 2001) and frequently functions in female mate choice and male–male territory defense (Krebs et al. 1978; Searcy 1984). Because song is learned locally and is phenotypically plastic (Slabbekoorn & Ripmeester 2007), it tends to vary geographically, creating different song structures and dialects among conspecific populations (Marler & Tamura 1962; Krebs & Kroodsma 1980; Kroodsma et al. 1999; Rowell & Servedio 2012). This geographic variation may arise due to changes in song structure occurring over time between spatially separated individuals with no initial adaptive significance (cultural

drift or temporal variation, Derryberry 2011), due to variation in foliage composition effecting signal transmission (i.e., the acoustic adaptation hypothesis, Wilkins et al. 2013), or as a result of reduced movement between individuals due to local adaptation (Grant & Grant 2007; Caro et al. 2013). Regardless, geographic variation in song structure may enable males and females to discriminate between local and non-local male conspecifics. Indeed, an abundance of literature suggests that both female and male songbirds respond more strongly to male song from their respective locality (local song) than to male song from different localities (non-local song) (e.g., Lemon 1967; Milligan & Verner 1971; Harris & Lemon 1974; Pleszczynska 1980; Baker et al. 1981; Becker 1982;

Ratcliffe & Grant 1985; Lampe & Baker 1994; O'Loughlen & Rothstein 1995; Baker 2001; Derryberry 2011). Discrimination and mate preference for local males may benefit females because males occupying the same local habitat may possess genetic material beneficial for survival at that specific location (e.g., Nottebohm 1972; Becker 1982; MacDougall-Shackleton et al. 2002). As such, resident males may also respond more strongly to male song from the local population because females tend to prefer those males, and therefore, they may pose a greater threat to a resident male's territory and mating success.

Geographic variation in song structure may be associated with geographic barriers or large distances separating populations (Baptista 1977; Kroodsma et al. 1999). Likewise, heterogeneous environments are often associated with differential selection pressures and may lead to local adaptations among conspecific individuals, even in the presence of gene flow (Holt & Gaines 1992; Emelianov et al. 2004; Kawecki & Ebert 2004; Niemiller et al. 2008; Nosil 2008; Gonzalo-Turpin & Hazard 2009; McCracken et al. 2009; De Leon et al. 2010; Pravosudov et al. 2012). These local adaptations could result in the evolution of geographic variation in song structure in the absence of clear geographic barriers or large distances (e.g., Caro et al. 2013; Branch & Pravosudov 2015), specifically, if differences in song structure allow female discrimination among males, leading to increased fitness. Montane environments present rapid and predictable environmental change along an elevation gradient, and individuals living along these elevation gradients often vary phenotypically (Miaud & Merila 2001; Gimenez-Benavides et al. 2007; Gonzalo-Turpin & Hazard 2009; Pravosudov & Roth 2013). Likewise, song and call structure in songbirds has been shown to vary across rather small spatial scales, lacking geographic barriers, and in some cases may vary along with elevation-specific phenotypic variation (e.g., Mundinger 1975; Miyasato & Baker 1999; Moore et al. 2005; Caro et al. 2013; Branch & Pravosudov 2015).

As environmental conditions change rapidly over short distances in montane habitats, the individuals inhabiting these locations experience differential selection pressures on ecologically relevant traits. Mountain chickadees (*Poecile gambeli*, hereafter chickadees), for example, are resident, food-caching birds inhabiting a continuous elevation gradient throughout western North America (Mccallum et al. 1999). Individuals occupying higher elevations experience harsher winter conditions (lower temperature, more snow, extended periods of snow cover; Barbour & Minnich 2000). It has been hypothesized that

selection pressure on food-caching and cache retrieval mechanisms, including spatial memory and its underlying neural mechanisms, should be higher at higher elevations, resulting in the evolution of local adaptations (Freas et al. 2012). Indeed, chickadees have previously been shown to exhibit large elevation-related differences in caching propensity, spatial memory abilities, and the underlying brain regions needed to retrieve caches (Freas et al. 2012, 2013a,b). In addition, chickadees from high vs. low elevations appear to maintain elevation-related behavioral types; high-elevation males are slower explorers, less aggressive, and socially subordinate to low-elevation males (Kozlovsky et al. 2014; Branch et al. 2015a). What is most striking is that these differences exist on a very small spatial scale—chickadees from high and low elevations are separated by just a few kilometers with only 600 m difference in elevation—in juvenile birds, prior to their first winter, and have been shown to persist in a 'common garden' environment (Freas et al. 2013a).

Taken together, this evidence, however indirectly, is consistent with the hypothesis of local adaptation and suggests that chickadees at higher elevations have evolved numerous phenotypic differences that do not appear to be simply due to phenotypic plasticity. More specifically, birds hatched at high elevations would be predicted to fare poorly at low elevations due to their social subordinate status (Kozlovsky et al. 2014) and low-elevation birds would be predicted to fare poorly at high elevations due to their lower caching propensity and inferior memory limiting cache retrieval (Freas et al. 2012). In addition, chickadees exhibit high postnatal dispersal site fidelity—when juvenile birds from high and low elevations were taken into the laboratory during early autumn and released at a third location after spending approximately 6 mo in captivity, 50% of those birds were resighted at their initial capture site, at their respective elevations and not a single individual was sighted at the opposite elevation (Branch et al. 2015b). While the level of movement between high- and low-elevation birds is still unclear, local adaptations and even speciation have been well documented to occur in the face of gene flow (Emelianov et al. 2004; Niemiller et al. 2008; Nosil 2008; Gonzalo-Turpin & Hazard 2009; McCracken et al. 2009; De Leon et al. 2010).

If chickadees are locally adapted to their respective elevations, females may benefit from selecting males from their elevation of origin. In fact, given a pairwise preference test of an actual high- or low-elevation male, high-elevation females spent more time near high-elevation males compared to low-elevation

males, while low-elevation females showed no detectable preference (Branch et al. 2015b). While it is unclear exactly how high-elevation females are able to discriminate between high- and low-elevation males, because males were not actively singing in the two-choice testing cage, our recent work showed significant differences in the song structure (e.g., differences in frequency ratio shift, note duration, and relative amplitude) of males from high vs. low elevations consistent with the local adaptation hypothesis (see Branch & Pravosudov 2015). As a result of hypothesized local adaptations in this system leading to differences in song structure, mountain chickadees are an ideal model for testing resident male responses to local and non-local male intruders on a small spatial scale. To address this question, we presented male mountain chickadees at both high and low elevations with playbacks of both high- and low-elevation song to assess differential responses. If males were able to discriminate between high- and low-elevation male song, then they may respond more strongly toward a local elevation male than a non-local elevation male (Milligan & Verner 1971; Harris & Lemon 1974). A strong response to a playback stimulus will be indicated by increased rates of singing, closely approaching the playback speaker, and decreased latencies of both behaviors (Harris & Lemon 1974; Baker et al. 1981, 1984, 2012; Otter et al. 2002). In addition, countersinging, or singing over/with the playback, will also be quantified (Dabelsteen et al. 1997; Hyman 2003). Decreased latencies to countersinging and increased rates of countersinging would be indicative of a stronger response.

Methods

Study Site and Subjects

Playback stimuli were presented to high- (*c.* 2400–2500 m) and low- (*c.* 1900–2000 m) elevation male mountain chickadees at Sagehen Experimental Forest, CA, from May 03 to June 08, 2014, between 0800 and 1400 h PST. These exact locations have been used in our previous studies, which show numerous elevation-related phenotypic differences including differences in song structure (Freas et al. 2012, 2013a,b; Kozlovsky et al. 2014, 2015a,b; Branch & Pravosudov 2015; Branch et al. 2015a,b).

Playback Stimuli and Field Experiment

Playback stimuli were created from a sample of male mountain chickadee song collected in spring 2013 at

our established high- and low-elevation sites in Sagehen Experimental Forest, CA; these males were included in the analyses published in Branch & Pravosudov (2015), showing significant differences in song structure. Songs of five males from low elevation and five males from high elevation were chosen at random based on minimal noise in the sound file. All songs used consisted of four notes, including the short introductory note, as this is the most common song structure in our chickadee populations (Branch & Pravosudov 2015). Cool Edit Pro. 2.0 acoustic software was used to create five unique sets of playbacks, consisting of two separate playback stimuli, one from a high-elevation male and one from a low-elevation male. Each set included different high-elevation male and low-elevation male songs. Each individual playback consisted of three high-quality songs from one male, repeated three times for a total of nine songs in each playback. Brown noise was generated for 2-s at the beginning and end of the playback and for 5-s following each of the nine songs, resulting in 9 songs at fixed intervals of 5-s. The length of each playback stimulus was 57–58 s. Acoustic parameters from the three songs of each male used as playback stimuli were re-extracted using SIGNAL 5.0 digital signal analysis system (Engineering Design, Berkeley, CA) and analyzed to ensure they reflected the differences in song structure previously documented between high- and low-elevation populations (Branch & Pravosudov 2015). Brown noise was incorporated into the playback stimuli to soften the transition from a male song to ‘silence’ within the stimulus, as some wind noise remained in the songs used. The amplitude of each playback was standardized at 78–80 dB, based on the natural amplitude of mountain chickadee song, measured one meter from the speaker in the field using a RadioShack sound pressure level meter and the brown noise was always lower in amplitude than the male song, at approximately 72 dB.

Resident males were targeted using known nestbox locations between chick’s hatching day 1 and fledging. A FOXPRO Fury[®] playback speaker (remotely controlled by observer 10–15 m away from playback speaker and nestbox. Note: The FOXPRO Fury[®] playback speaker contains two speakers; however, only one was used to emit the playback stimuli) was hidden in shrubbery on the ground and a Sennheiser ME66 unidirectional microphone on a 1.6-m microphone stand was set up. In all cases, both the speaker and the microphone were placed approximately 10–15 m from the nestbox. Once chickadees were in the area (visual and/or acoustic assessment, within approximately 30 m of the nestbox), the playback

commenced. A full playback trial consisted of the following: 5 min baseline, playback stimulus (~58 s), 5 min post-playback silence, playback stimulus (~58 s), and 5 min post-playback silence. The same playback stimulus was broadcast twice. If a bird did not respond (e.g., if the bird did not approach the speaker or sing but generally continued foraging) to either of the first two playbacks, then a third playback was presented. All resident males received one playback trial per day, with the second/alternate elevation playback trial presented on the following day, at the same time of day. Playback stimuli (high or low playback stimulus) were counterbalanced across location (high- or low-elevation resident male) and playback set (1–5). During the entire playback trial, a Marantz PMD660 Compact Flash Card digital recorder with a sampling rate of 44 000 Hz and 16-bit resolution was used to annotate all of the resident male's responses, including closest approach to the playback speaker, latency to closest approach, initial approach to playback speaker, latency to initial approach, song rate, latency to sing, and rates of countersinging.

Mean initial approach was defined as the distance of the first approach into the playback area (30 m diameter around targeted nestbox), in meters, to the playback speaker once an individual playback commenced (averaged over the two or three times the stimulus was played within an elevation playback trial), and the mean latency to initial approach was simply the latency, in seconds, for an individual male to make the first approach once an individual playback commenced. Closest approach was defined as the closest distance, estimated in meters, a male came to the playback speaker within an entire playback trial, and the latency to closest approach was determined as the latency, in seconds, to the closest approach to the playback speaker once an individual playback commenced. As such, a male's initial approach could be equivalent to their closest approach. If a male did not approach the playback speaker during an individual playback stimulus, ceiling values were assigned to the missing data; 30 m for approach distances and 360 s for latency to approach. These values were chosen because the furthest actual approach distance by a male was 20 m and the total time the males had to approach was 360 s. Mean song rate was defined as the number of songs a male sang after an individual playback stimulus and the mean latency to sing was simply the latency, in seconds, of a male to sing the first song once an individual playback commenced. Latency to sing ceiling value was also 360 s. Countersinging latencies were calculated by measuring the latency, in seconds, for a male to sing

after one of the songs in the playback (Fig. 1). If a male sang over a playback song, the latency was recorded as a negative value. There were 5 s of brown noise between each song in the playback stimuli; therefore, if a male only countersang to one playback stimulus, the countersinging latency ceiling value was 5 s. Dependent measures for each playback trial were extracted from .wav files using Cool Edit Pro 2.0.

A stronger response to one playback stimulus vs. another would be indicated by an increased rate of singing and shorter approach to the speaker with a shorter latency (Harris & Lemon 1974; Baker et al. 1981, 1984, 2012; Dabelsteen et al. 1997; Hyman 2003).

Statistical Analysis

All data were normalized using log transformation and a principal components analysis was conducted in R 3.1.1 (R Core Team 2014) to reduce 6 response variables (closest approach, latency to closest approach, mean initial approach, latency to mean initial approach, song rate, and latency to sing) to two principal components. Generalized linear mixed models (GLMM) were run (R 3.1.1 using packages lme4, lmerTest, and MuMIn (Bates et al. 2015; Barton 2015; Kuznetsova et al. 2015)) on the first two principal components, with the elevation of the receiver bird and elevation of the playback stimulus as fixed factors and bird identity and stimulus set as random factors. Not all males countersang with the playback stimuli; therefore, only birds that countersang with at least one playback stimulus were included in the countersinging analysis (Low, $N = 9$; High, $N = 15$). Countersinging was analyzed in a separate GLMM, again with the elevation of the receiver bird and the elevation of the playback stimulus as fixed factors and male identity and stimulus set as random factors. Ceiling values for countersinging latencies were set to 5 s, as that was the amount of time in between each song in a playback stimulus.

For all dependent measures, except for closest approach and latency to closest approach, the mean response over the two or three playback stimuli for a given condition (high- or low-elevation playback) were calculated and used in the analyses. Only males that responded to at least one of the two playback stimuli were included in the analysis (Low, $N = 20$; High, $N = 24$).

Ethical Approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were

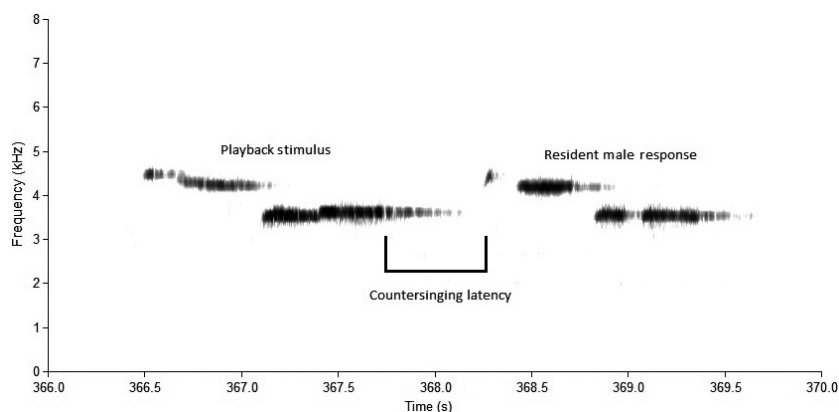


Fig. 1: Schematic of sound spectrogram (transform length of 512 points, time resolution of 11.6 ms, and frequency resolution of 86.1 Hz) showing how countersinging latencies were collected.

followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the University of Nevada Animal Care and Use Protocol (00576).

Results

Playback Stimuli Comparison

Acoustic parameters from the three songs of each male used as playback stimuli were re-extracted using SIGNAL 5.0 digital signal analysis system (Engineering Design, Berkeley, CA) and analyzed to ensure they reflected the differences in song structure previously documented between high- and low-elevation populations (Branch & Pravosudov 2015). Branch & Pravosudov (2015) found five acoustic parameters that were significantly different between high- and low-elevation birds sampled at Sagehen Experimental Forest, CA, including the start frequency of notes 3 and 4, the frequency ratio shift between notes 2 and 3, and the duration of notes 2 and 3. The direction of all five parameters were consistent with Branch & Pravosudov (2015), however, likely due to lack of power, only start frequency of note 3, and duration of notes 2 and 3 was significantly different between the high- and low-elevation stimuli used as playbacks in this study ($p < 0.05$).

Principal Components Analysis and Generalized Linear Mixed Models

Six response variables (mean initial approach, Fig. 2a; latency to mean initial approach, Fig. 2b; closest approach, Fig. 3a; latency to closest approach, Fig. 3b; song rate, Fig. 4a; and latency to sing, Fig. 4b) were reduced to two principal components explaining 77.29% of the variation in high- and low-elevation

resident males' response to high- and low-elevation playbacks (Fig. 5). Mean latency to initial approach, mean latency to sing, and mean song rate contributed most to PC1, explaining 57.74% of the variation. Closest approach, latency to closest approach, and mean song rate contributed most to PC2, explaining 19.55% of the variation.

Generalized linear mixed models on PC1 revealed no significant effect of resident bird elevation ($F_{1,84} = 0.92$, $p = 0.34$) or elevation of the playback stimulus ($F_{1,84} = 1.15$, $p = 0.29$) and no significant interaction between elevation of resident bird and elevation of the playback stimulus ($F_{1,84} = 0.71$, $p = 0.40$).

Generalized linear mixed models on PC2 revealed no significant effect of resident bird elevation ($F_{1,42} = 0.25$, $p = 0.62$) or elevation of the playback stimulus ($F_{1,42} = 1.09$, $p = 0.30$), and no significant interaction between elevation of resident bird and elevation of the playback stimulus ($F_{1,42} = 0.02$, $p = 0.89$).

The random effects of bird identity and stimulus set did not explain a significant portion of the variation seen in male responses and therefore are not significant predictors of a male bird's response to a particular elevation stimulus (see Table 1).

Countersinging

There were no significant differences in the number of males from low elevation ($N = 9$) that countersang with at least one playback stimulus and the number of males from high elevation ($N = 15$) that countersang with at least one playback stimulus (Fisher Exact test, $p > 0.05$). Of the males that did countersing with at least one playback stimulus, there were no significant differences between high- and low-elevation resident males in the mean latency to sing after a single

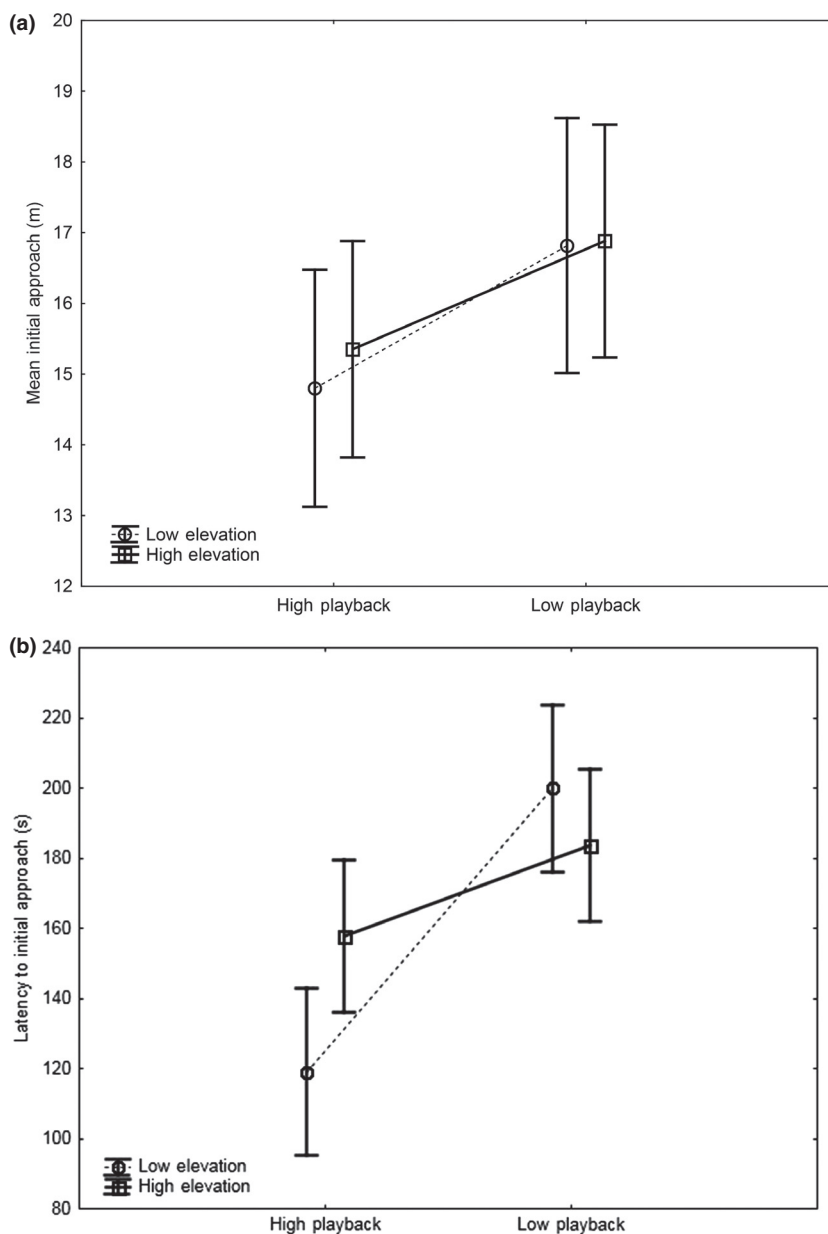


Fig. 2: Mean and SEM of (a) initial approach and (b) latency to initial approach for resident high- and low-elevation males presented with high- and low-elevation male playback stimuli.

playback song within a playback ($F_{1,44} = 0.14$, $p = 0.71$), nor were there significant differences in mean countersinging latencies to a high- vs. low-elevation playback ($F_{1,44} = 0.50$, $p = 0.48$). The interaction between the elevation of the resident male and the playback stimulus was also not significantly different ($F_{1,44} = 1.04$, $p = 0.31$, Fig. 6).

Discussion

Resident male mountain chickadees from high and low elevations responded similarly to male intruder song from high and low elevations, suggesting that

they do not respond differentially to local vs. non-local elevation male intruders. This is not to say that males *cannot* discriminate between high- and low-elevation conspecifics, but that they did not respond more strongly to one vs. another. We feel confident that our results reflect high- and low-elevation male responses to high- and low-elevation male intruders because we showed that the sets of high- and low-elevation playback stimuli were structurally different from each other.

An abundance of previous literature, primarily in sparrows, suggests that male songbirds respond to local male song more strongly than non-local male

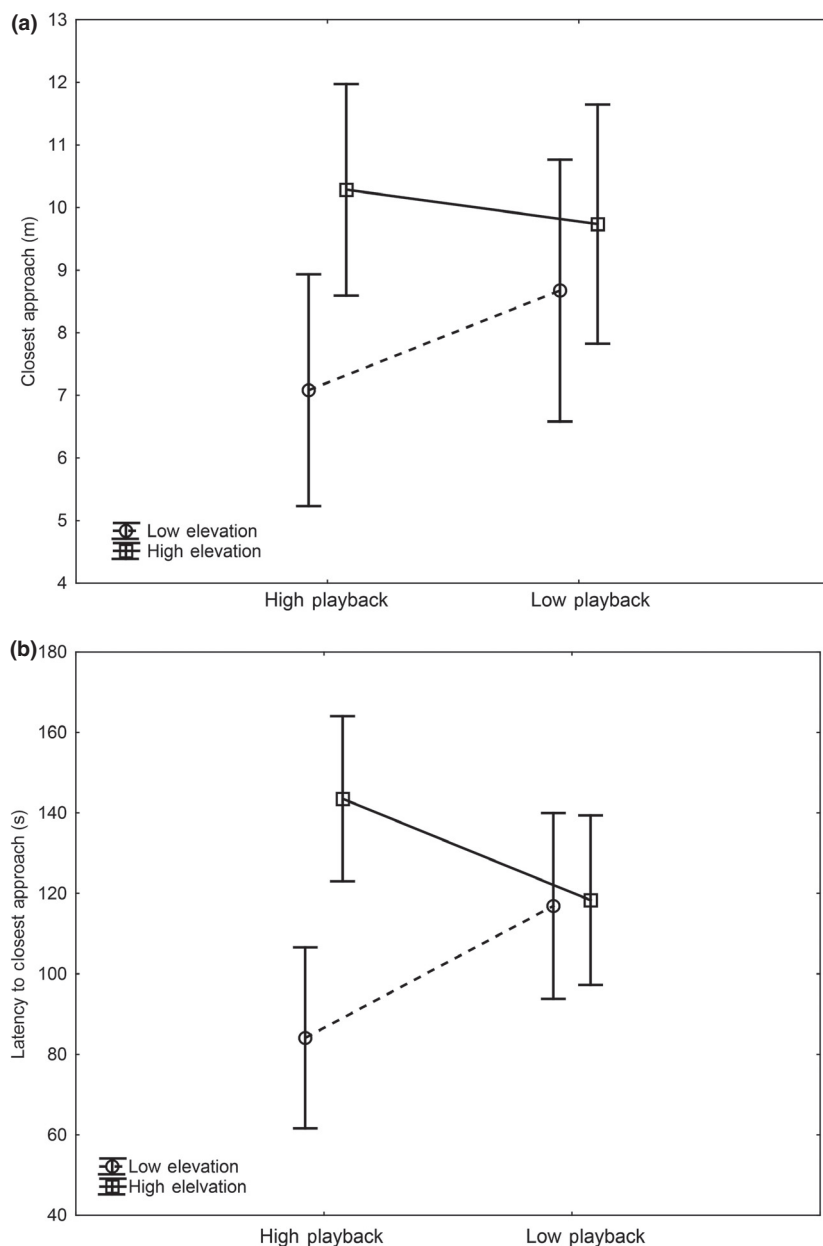


Fig. 3: (a) Closest approach and (b) latency to closest approach of resident high- and low-elevation males presented with high- and low-elevation male playback stimuli. Error bars represent SEM.

song (e.g., Lemon 1967; Milligan & Verner 1971; Harris & Lemon 1974; Ratcliffe & Grant 1985; Baker et al. 1987; Lampe & Baker 1994; O’Loughlen & Rothstein 1995; Nelson 1998; Baker 2001; Nelson & Soha 2004; Derryberry 2011). Indeed, there is little evidence suggesting that male songbirds respond to local and non-local intruders similarly (Colbeck et al. 2010; Danner et al. 2011). In contrast to the abundance of previous work, we found that males from high and low elevations respond similarly to local and non-local elevation males. This finding is particularly interesting given previous work with females from the exact

same high- and low-elevation locations showing that high-elevation females prefer local elevation males over non-local elevation males in a pair-wise preference test (Branch et al. 2015b). Theoretically, differences in discriminability (evidenced by behavioral responses) between male and female songbirds may not be surprising, due to the differential costs and benefits of discriminating between local and non-local dialects in males and females, as mistakes by females to identify a locally suited male may result in unfit offspring (Searcy & Brenowitz 1988; Searcy et al. 2002; Nelson & Soha 2004; Danner et al. 2011).

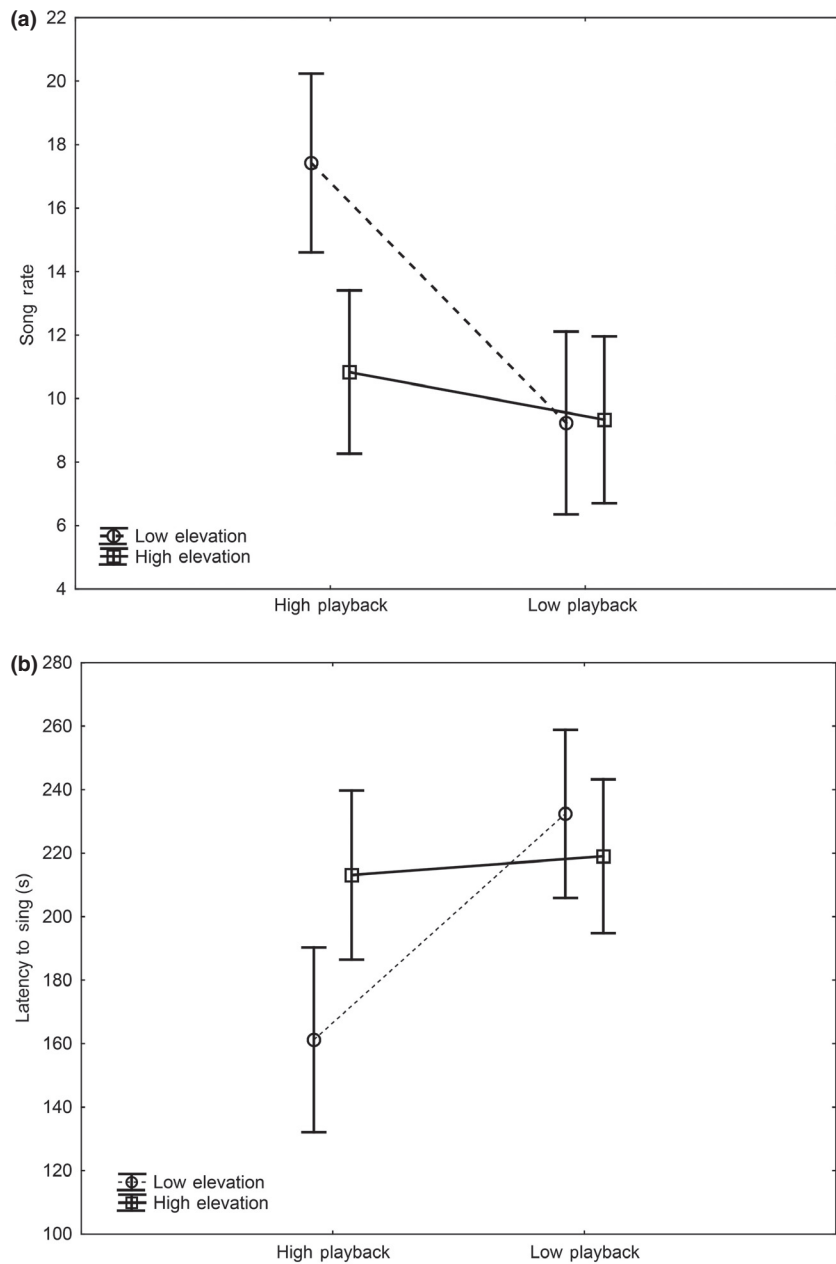


Fig. 4: Mean and SEM of (a) song rate and (b) latency to sing for resident high- and low-elevation males presented with high- and low-elevation male playback stimuli.

Indeed, it may be that males ‘hedge their bets’ and generalize their territorial responses to any conspecific male intruder, regardless of its origin—however, this hypothesis is in contrast to the previous work listed above. What is more perplexing is that male song structure differs between high and low elevations (Branch & Pravosudov 2015), which could allow males to discriminate between local and non-local male intruders.

It must be emphasized that while high- and low-elevation males did not respond differentially toward

high- and low-elevation male intruders, this is not definitive evidence that they are unable to discriminate between them. Future work on the structural parts of male song females and males attend to may shed light on how males and females may discriminate between high- and low-elevation males in this system. This is particularly interesting given that the same vocalization in songbirds is used by both sexes with disparate functional significance (Krebs et al. 1978; Kroodsma & Byers 1991) and, therefore, may have very different implications for population

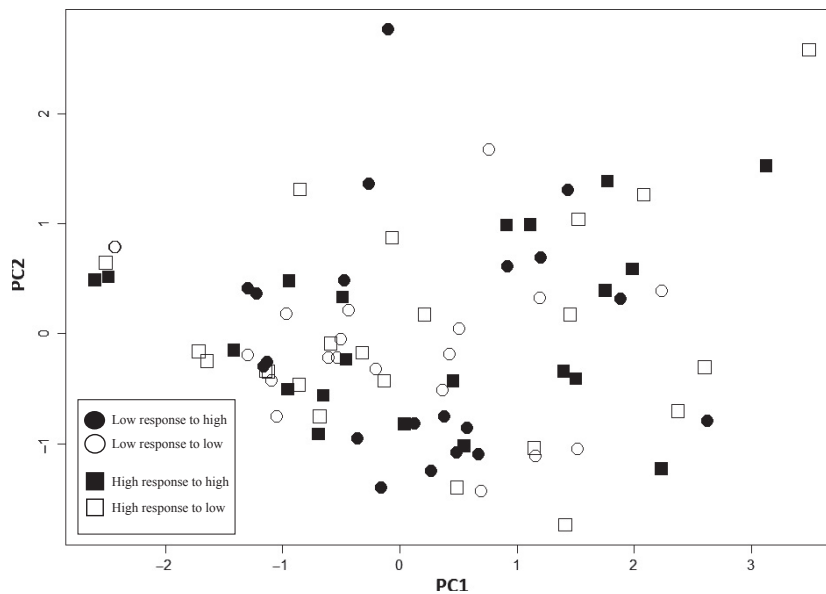


Fig. 5: High- and low-elevation resident males' responses to high- and low-elevation playback stimuli. Six response variables were reduced to two principal components, PC1 and PC2. Closed circles represent low-elevation, resident males' response to a high-elevation intruder, open circles represent low-elevation, resident males' response to low-elevation intruder, closed squares represent high-elevation, resident males' response to a high-elevation intruder, open squares represent high-elevation, resident males' response to a low-elevation intruder.

Table 1: Variance, standard deviation, and chi-squared values with corresponding p values for random effects of bird and stimulus set in GLMMs of response variables PC1 and PC2.

	Variance	SD	Chi-square, P
PC1			
Bird	0.00	0.00	0.00, 1.00
StimulusSet	0.00	0.00	0.00, 1.00
PC2			
Bird	0.00	0.00	3.11, 0.08
StimulusSet	0.00	0.00	0.00, 1.00

separation in what is hypothesized to be a locally adapted species (Danner et al. 2011).

Furthermore, given the small spatial scale along which high- and low-elevation birds were sampled in this study (approximately 6 km between low and high elevations), it may not be particularly surprising that males respond similarly to songs from both elevations. Indeed, previous work with sparrows shows local and non-local song discrimination on much larger spatial scales; in song sparrows, males will respond similarly to male song from 270 km away, with a decrease in responding to populations around 540 km away (Searcy et al. 2002). That being said, unlike sparrows, chickadees are highly sedentary once they have dispersed from their natal site, and on average, chickadees do not disperse particularly far, approximately 0.70 km in our population, and 4–11 km (median dispersal distance of 1.1 km) in black-capped chickadees (see Weise & Meyer 1979).

There are two potential limitations to the current study, including the number of playback stimuli used and the timing of presentation. First, only 5 different sets of high- and low-elevation playback stimuli were used. However, the songs used as playback stimuli were analyzed and compared between high- and low-elevation males, showing significant differences in song structure consistent with the previous work on the song structure of high- and low-elevation males from these exact same elevation locations (Branch & Pravosudov 2015). In addition, when stimulus set was included in the mixed model as a random factor, it did not explain the variation present in male responses (Table 1). Second, we presented playback stimuli to males at their nestboxes between hatch day 1 and fledging of offspring. Some research suggests that males may be more responsive to other male intruders prior to egg laying, when lost paternity still is a relevant concern (Langeman et al. 2000; Otter et al. 2002; Colbeck et al. 2010). However, we chose to present playback stimuli once chicks had hatched for two reasons: (1) Males are more predictably at the nestbox or in the area once chicks have hatched because they are actively feeding (personal observation), and (2) male chickadees appear to maintain their level of aggression toward intruder males even once chicks have hatched. To prevent an effect of feeding activity on approach to the playback speaker, we placed the speaker 10–15 m away from the nestbox. This placement ensured that visits to the nestbox for feeding were not considered as approaches to the playback speaker. In addition, the observer verified that in no

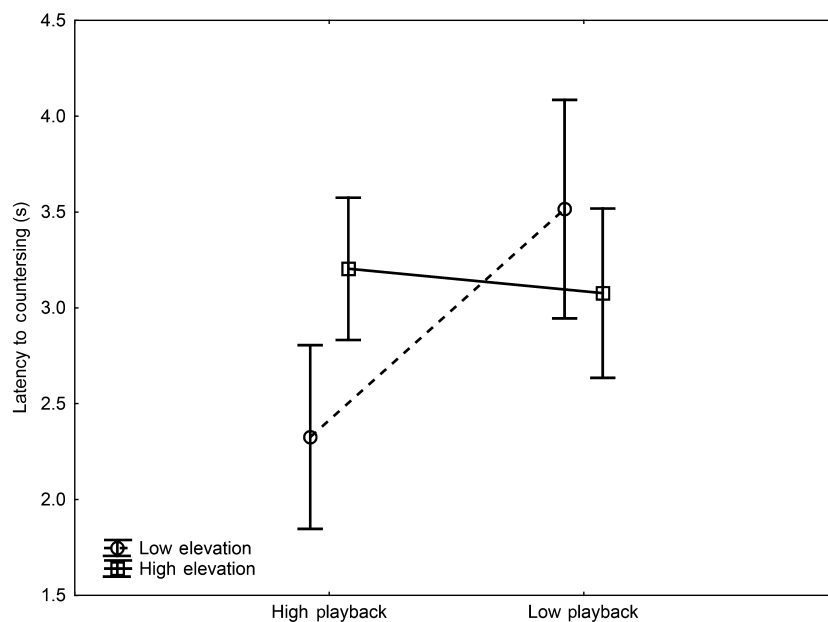


Fig. 6: Mean and SEM of countersinging latencies for resident high- and low-elevation males presented with high- and low-elevation male playback stimuli.

cases was the male recorded with a food item in its mouth when approaching the speaker. Lastly, high- and low-elevation males were sampled during the same breeding period; therefore, if there were any differences between their true responses to local vs. non-local elevation males, their responses, even if they were weaker than other times of the season, should still be representative of their perception of a particular male intruder.

Overall, our study suggests that male mountain chickadees from high and low elevations, hypothesized to be locally adapted, do not respond differentially to intruder males from their local vs. non-local elevation. This is in contrast to female mountain chickadees from the exact same elevation locations, which have been shown to prefer males from their local elevation (Branch et al. 2015b). Furthermore, these findings are inconsistent with previous research suggesting that male songbirds readily discriminate between male intruders from local and non-local populations. It is worth considering that the bulk of previous research investigating male responses to local and non-local male intruders has been conducted in several sparrow species, and to our knowledge, little to no work has investigated male Parid responses to local and non-local conspecific males. The song of Parids is considered to be rather simple (Hailman 1989; Kroodsma et al. 1999; Christie et al. 2004) compared to more complex sparrow and finch songs, and it may be that there is some functional variation in how male and female Parids utilize male song in comparison

with other passerine species with more complex repertoires.

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Funding

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Conflict of Interest

Carrie L Branch declares that she has no conflict of interest. Vladimir V Pravosudov declares that he has no conflict of interest.

Ethical Approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies

involving animals were in accordance with the ethical standards of the University of Nevada Animal Care and Use Protocol (00576).

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RUNNING HEAD: Song dialects in mountain chickadees

Variation in song structure along a continuous montane gradient represents ‘high’ and ‘low’ elevation dialects in mountain chickadees (*Poecile gambeli*)

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Research Article

Keywords: song dialects, mountain chickadee, montane gradient, elevation, local adaptation

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ABSTRACT

Variation in the song of male songbirds has long been of interest because of its implications for mate choice, local adaptation, and speciation associated with environmental heterogeneity. Several hypotheses have proposed different mechanisms by which such variation may arise, and montane habitats are particularly useful for testing these hypotheses, as the environment, and subsequent selection pressures, change rapidly and predictably over rather small spatial scales. Here, we asked whether differences previously established in the song structure of mountain chickadees, at two elevational extremes that experience differential climatic harshness, are consistent with vocal dialects or gradual clinal variation by comparing song along the continuous elevation gradient at the same two previously sampled mountains. Acoustic analyses reveal steep clines with stepped variation, consistent with “high” and “low” elevation dialects, separated at the snow line, where critical shifts in winter precipitation occur. The findings of vocal dialects in mountain chickadees are especially interesting because these birds inhabit a continuous elevation gradient at both sampled mountain locations, yet winter environment changes abruptly rather than gradually. Finding support for vocal dialects in mountain chickadees at different elevations has important implications for the evolutionary trajectory of these birds as vocal dialects likely represent indicators of local adaptation associated with rather drastic changes in winter environment.

Keywords: song dialects, mountain chickadee, montane gradient, elevation, local adaptation

INTRODUCTION

Variation in the song of temperate male songbirds has long been of interest because of its potential implications for mate choice, local adaptation, and speciation (Salomon 1989; Irwin 2000; Slabbekoorn & Smith 2002; Edwards et al. 2005; Podos & Warren 2007). In temperate male songbirds, song is learned through the imitation of other local males (Slater 1989; Nelson et al. 2001), and serves a dual function, both in female mate choice (Searcy 1984; Byers & Kroodsma 2009) and conspecific territory defense (Krebs et al. 1978; Mennill et al. 2002). While bird song is phenotypically plastic and may be modulated in varying acoustic environments (Slabbekoorn & Ripmeester 2007), many oscine passerines are age-limited learners (Baker 2001) or closed learners, meaning they learn their song early in life and do not learn or produce new songs once their repertoire has been “crystalized” (Nelson 1999). Because song is learned, or culturally transmitted, it not only varies among species, but often varies between conspecifics experiencing distance or geographic barriers, forming geographic dialects within a species (Mundinger 1975; Krebs et al. 1980; Podos & Warren 2007; Caro et al. 2013). These geographic dialects appear to influence female mate choice; with females displaying a preference for dialects from their geographic location or population of origin (Baker et al. 1982; Baker et al. 1987; Slabbekoorn & Smith 2002; Rowell & Servedio 2012).

Several hypotheses have proposed different mechanisms by which such variation may arise:

(1) The acoustic adaptation hypothesis suggests that vocalizations have evolved to maximize transmission in a given environment, and as environments change (i.e. differences in plant density across forests), so will the structure of male song (Wilkins et al. 2012).

(2) The temporal variation hypothesis suggests that song structure may change or drift over time as individuals experience reduced movement and gene flow (Derryberry 2011). This is thought to occur primarily in newly colonized areas where populations are geographically isolated and males imperfectly imitate the song of male tutors (Gammon & Baker 2004; Gammon et al. 2005).

(3) Finally, the local adaptation hypothesis suggests that individuals within a species that inhabit different environments may experience differential selection pressures on ecologically relevant traits, potentially reducing movement between environments (King et al. 1980; Grant & Grant 2007; Caro et al. 2013; Branch & Pravosudov 2015). Over time, this reduced gene flow or movement may result in structural changes in male song that can now be used by females to mate assortatively with locally adapted males (MacDougall-Shackleton et al. 2002; Slabbekoorn & Smith 2002; Branch & Pravosudov 2015). This is interesting, as birds from different habitats may not actually be reproductively incompatible, but assortative mating for a local over nonlocal male may further enhance population-specific adaptations (Baker 1975; Kroodsma et al. 1999; Caro et al. 2013).

Montane habitats are particularly useful for testing such hypotheses as the environment, and subsequent selection pressures, change rapidly and predictably over rather small spatial scales. Mountain chickadees (*Poecile gambeli*) are oscine passerines that inhabit continuous montane gradients in western North America (McCallum et al. 1999). These are resident, food-caching birds that store food in the autumn when food is abundant, to use later, in winter, when food is scarce. Mountain chickadees use spatial memory in part to relocate their food caches (Roth & Pravosudov 2013), and previous research shows that birds inhabiting harsher climates with more and longer snow cover (high elevations) cache 3-4 times more food items, perform

better on spatial memory tasks, and have a larger hippocampus with more neurons, than birds inhabiting milder climates (low elevations) (Freas et al. 2012; Croston et al. 2016). In addition, birds inhabiting low elevations are socially dominant in pairwise interactions and more aggressive, than high elevation birds (Kozlovsky et al. 2014, Branch et al. 2015a). These birds live in flocks with stable dominance hierarchies outside of the breeding season, and dominant birds have better access to mates and food resources, therefore, dominance status has significant implications for an individual's fitness (Ratcliffe et al. 2007).

Indeed, the phenotypic variation between high and low elevation birds suggests that there may be negative fitness consequences for birds moving between elevations. For example, migrants from low to high elevations may experience decreased fitness due to their inferior spatial memory ability and reduced food caching propensity, while migrants from high to low elevations may experience decreased fitness due to their socially subordinate status. What is interesting in this system is that winter climate does not change gradually from low to high elevation extremes, but shifts rather abruptly around 2150 m, where precipitation frequently falls as snow above this line and rain below (Barbour & Minnich 2000; personal observations). While there is some level of gene flow between birds inhabiting high and low elevations (Branch et al. 2017), a high elevation bird has never been observed breeding at the low elevation site, or vice versa (personal observation). This is after banding more than 1,000 chickadees at established feeders and almost 2,000 nestlings at nests over the last 5 years.

If mountain chickadees experience decreased fitness when moving between elevations, it would benefit females to be able to discriminate between high and low elevation males and mate with males suited to their elevation of origin. Indeed, previous work in our system has shown that high elevation females have a preference for high elevation males over low elevations males,

while low elevation females show no elevation-related preference (Branch et al. 2015b). It is not clear what mechanism females use to discriminate between high and low elevation males, however, there is significant variation in the song structure of males inhabiting high versus low elevations consistent with the local adaptation hypothesis described above (Branch & Pravosudov 2015). The structural song variation at the two extreme ends of this montane gradient may be used by females to mate assortatively with males from their elevation of origin, however, these structural differences do not emphatically represent “high” and “low” elevation dialects, as many phenotypic characteristics, including male song, are known to change gradually along a cline, as distance between groups of individuals increases (e.g. Miyasato & Baker 1999; Sung & Handford 2006; Funk et al. 2015; Yandell et al. 2017). Whether or not the variation we see in mountain chickadee song is consistent with vocal dialects changing around the 2150 m snow line, where critical changes in climate occur in the Sierra Nevada (Barbour & Minnich 2000), or with gradual clinal variation, has important implications for the trajectory of this population of birds (Edwards et al. 2005; Podos & Warren 2007). If the differences in song structure represent indicators of local adaptation, as we have previously suggested (Branch & Pravosudov 2015), vocal dialects may result in further separation between birds inhabiting high versus low elevations in the Sierra Nevada. Alternatively, a gradual change along the elevation gradient may suggest that the differences between high and low elevation chickadee song are not necessarily related to environmental variation, but simply vary due to distance (Podos & Warren 2007). As the climate continues to warm (Fischer & Knutti 2015), further separation of these groups may have important implications for their success and genetic variation, as warming will likely increase the area that low elevation birds are suited to colonize, while high elevation birds, and their associated genetic variation, may be decimated.

As such, we asked whether differences found in the song structure of male mountain chickadees inhabiting high and low elevation locations are consistent with vocal dialects or gradual clinal variation. In order to address this question, we recorded song from males along a continuous elevation gradient from the original low elevation location up to the high elevation site at the same two mountain locations previously sampled in Branch & Pravosudov 2015. If variation in male song structure is consistent with clinal variation, having little functional implications, we would expect to see gradual changes or shifts in the song measures collected across the continuous elevation gradient, such that the largest differences between sampling locations are consistent with the largest differences in acoustic measures (Sung & Handford 2006; Podos & Warren 2007, see Figure 1). However, if these differences in song structure represent dialects associated with significant changes in winter climate, around 2150 m, we would expect to see structural variation consistent with steep changes in acoustic parameters with stepped variation (Podos & Warren 2007, see Figure 1).

METHODS

Subjects and Song Collection

Male mountain chickadee song was recorded along a continuous elevation gradient at two spatially distinct mountains separated by approximately 35 km; Sagehen, CA and Mount Rose, NV, USA. Birds were recorded between 0700 and 1400h PST from 18 April to 4 June 2015. Song was recorded along a continuous transect at each mountain (Sagehen, ~ 16 km transect; Mount Rose, ~ 13 km transect) and was binned into elevations by every 100 m. A total of 159 males were recorded (Sagehen, N=87 and Mount Rose, N=72). Songs were binned into six elevations at Sagehen (1900 m, N=13; 2000 m, N=10; 2100 m, N=26; 2200 m, N=10; 2300 m,

N=11; 2400 m, N=17) and at Mount Rose (1900 m, N=11; 2000 m, N=10; 2100 m, N=11; 2200 m, N=11; 2300 m, N=11; 2400 m, N=18). At all locations, a minimum of 20 songs were recorded from each male using a Marantz PMD661 Compact Flash Card digital recorder and Sennheiser ME-66 unidirectional microphone with a sampling rate of 44,000 Hz and 16-bit resolution. Males were located auditorily and approached with microphone and recorder in hand. Not every male along these montane gradients was individually color banded; therefore, 500 m were established between unbanded individuals (Hill & Lein 1989) to avoid recording the same males multiple times. Each male was recorded on one day.

Acoustic Analysis

Song recordings were first viewed in SIGNAL Real-Time Spectrogram (Engineering Design, Berkley, CA), and high-quality songs (i.e. with limited wind noise) were extracted and saved as individual .wav files. For each male, we analyzed between 6 – 30 songs (mean = 19.35). To obtain a comprehensive description of the song, we extracted 15 acoustic measures based on previous work on mountain and black-capped chickadees (see Table 1 and Figure S1) (Wiebe & Lein 1999; Gil & Gahr 2002; Hahn et al. 2013; Branch & Pravosudov 2015). Mountain chickadee song typically consists of either 3 or 4 notes, with variation in the presence/absence of an introductory note (Wiebe & Lein 1999; Branch & Pravosudov 2015, Figure S1a). We extracted measures from the entire song as well as the individual notes within each song; therefore, the number of measures per song was partially dependent on the presence/absence of the introductory note.

Temporal and spectral measures were taken from sound spectrograms: 8 temporal measures including (1) total song duration (SD), (2) note duration (3 or 4 depending on the song)

(ND), and (3) internote interval (2 or 3 depending on song) (II) and 9 spectral measures including (1) start frequency for each note (3 or 4 depending on song) (SF), (2) frequency ratio (FR), mountain chickadee songs have a frequency shift between notes 2 and 3, where the start frequency of note 3 drops (calculated by dividing the end frequency of note 2 by the start frequency of note 3). We also measured the number of notes (NN) in each song. The one amplitude measure, relative amplitude (RA), was taken from a power spectrum and calculated by dividing the maximum amplitude of Note 3 by the maximum amplitude of Note 2 (Figure S1c). Temporal and spectral measures were made using SIGNAL 5 and a spectrogram window size of 512 points, a time resolution of 11.6 ms, and a frequency resolution of 86.1 Hz (Figure S1a).

Statistical Analysis

For each individual bird, the mean and standard deviation of each acoustic parameter was used for analyses. Previous work investigated the song structure of high versus low elevation birds (high = 2400 m and low = 1900 m) at these exact same mountain locations (Branch & Pravosudov 2015), therefore, here we only used acoustic parameters that explained significant amounts of variation in song structure for the regression, mantel, and one-way ANOVA analyses. We used two additional parameters to enhance discriminant function analysis (DFA) classification (the number of variables used in a DFA are limited by the least number of objects in a particular group minus one; our lowest sample size was 10, therefore we used 9 predictor variables; Mundry & Sommer 2007). For both mountain locations this included, Number of Notes (NN), total Song Duration (SD), Frequency Ratio (FR), Note 2 Duration (N2 D), Note 3 Duration (N3 D), Note 3 Start Frequency (N3 SF), Note 4 Start Frequency (N4 SF), Note 3 to Note 4 Internote Interval (N3N4 II), and Relative Amplitude (RA). For principle component

analyses we used 12 acoustic parameters, as PCA is not limited in the number of variables that can be used; including those listed for the DFA as well as Note 2 Start Frequency, Note 2 to Note 3 Internote Interval, and Note 4 Duration. Sagehen and Mount Rose song structure at high and low elevations were found to differ based on different acoustic parameters (Branch & Pravosudov 2015), therefore, the two mountain locations were analyzed separately. In addition, “high” and “low” sites along this gradient were defined as; low = 1900 m, 2000 m, and 2100 m; high = 2200 m, 2300 m, and 2400 m, because there is an important ecological threshold at 2150 m in the Sierra Nevada, where precipitation falls as rain below this elevation and as snow above this elevation (Barbour & Minnich 2000). Statistical analyses such as linear regressions and mantel tests will often find significant patterns among two extreme points, and do not always accurately describe the pattern of the data within those two points, which is why we think it is important to separate high and low elevation locations. This approach still makes it very easy to compare the relationships between high and low elevation data, as if regression analyses or mantel tests are significant in the same direction across ‘high’ and ‘low’ elevation groupings, this will be revealed in the statistical results and the figures that display the relationships between data points.

Linear Regressions

Linear models were only run for the 7 acoustic parameters found to be significant in Branch & Pravosudov 2015, these included number of notes (NN), frequency ratio (FR), Note 2 Duration (N2 D), Note 3 duration (N3 D), Note 3 start frequency (N3 SF), Note 3 to Note 4 Internote Interval (N3N4 II), and relative amplitude (RA). For each linear model, elevation was the predictor variable and an acoustic parameter was the response variable (linear model (lm))

function in R Core Team 2017). Based on the ecology and precipitation patterns of the Sierra Nevada, high and low elevations were separated, therefore, we ran regressions separately for “high” and “low” elevation groupings, as described above.

Discriminant Function Analysis

We conducted separate “all-variables-together” discriminant function analyses (DFA) (STATISTICA 12) for Sagehen and Mount Rose, using the same nine acoustic parameters listed above. For each mountain location, we ran 4 separate DFAs, using the same response variables to categorize all six elevation bins (1900 m, 2000 m, 2100 m, 2200 m, 2300 m, and 2400 m). As such, a total of 8 DFAs were run; one DFA placed each of the six elevation locations into 2 groupings, either a “high” elevation category (2200 m, 2300 m, and 2400 m) or a “low” elevation category (1900 m, 2000 m, and 2100 m); one placed all six locations into 3 groups, “high” (2400 m and 2300 m), “medium” (2200 m and 2100 m), or “low” (2000 m and 1900 m); and the final DFA placed all six locations into 6 groups, with each elevation bin having its own group.

We used the ‘hold-out-sample’ method to cross-validate the location classifications, which simply involves running a DFA without a set of observations from each group and then using those results to classify the observations that were held out (Tabachnick & Fidell 2001; Mundry & Sommer 2007). We ran an additional DFA at each mountain location using the mid-elevation bins (2100 and 2200 m) as the “hold-out-samples.”

Mantel Tests

To test for the effects of geographical, or elevational, distance on variation in individual song parameters among elevation sampling sites, we used a multiple regression on distance matrices (using the *ecodist* package and *MRM* function in R Core Team 2017), with pairwise differences in each acoustic parameter as the response variable and pairwise geographical distance as the predictor variables.

Principle Component Analyses and Analysis of Variance

In order to assess whether the amount of variation within locations was less than the amount of variation between locations, we conducted ANOVAs (using *aov* function in R Core Team 2017) on reduced representations (principle components analysis, using *prcomp* function in R Core Team 2017) of 12 acoustic variables (Number of Notes, Song Duration, Frequency Ratio, Note 2 Start Frequency, Note 2 Duration, Note 2 to Note 3 Internote Interval, Note 3 Start Frequency, Note 3 Duration, Note 3 to Note 4 Internote Interval, Note 4 Start Frequency, Note 4 Duration, and Relative Amplitude) for high and low elevations, separately for Sagehen and Mount Rose. We also conducted ANOVAs for each individual acoustic parameter, again separately for Sagehen and Mount Rose. For each ANOVA, elevation (high or low) was the predictor variable and an acoustic parameter was the response variable. Unlike DFA, principle component analyses are not limited in the number of variables that can be included for discrimination; therefore we used all of the acoustic parameters that applied to every bird (i.e. we did not use any measures from the introductory note, or Note 1, because not all birds produce this note).

RESULTS

Linear Regressions

Linear regressions were run for each acoustic measure from both mountain locations (Sagehen and Mount Rose), and high and low elevations, run separately (see Table 1 for means and standard deviations of each acoustic measure).

Temporal Measures

Sagehen

For Note 2 Duration, there was a significant regression for low elevation sites ($F_{1,47} = 5.00$, $p = 0.03$, Figure 2a), however, the regression for high elevation sites was not significant ($F_{1,36} = 0.113$, $p = 0.739$, Figure 2a). For Note 3 Duration, the regression was not significant for low elevation sites ($F_{1,47} = 1.446$, $p = 0.235$, Figure 2b), but was significant for the high elevation sites ($F_{1,36} = 4.948$, $p = 0.032$, Figure 2b). For Note 3 to Note 4 Internote Interval, the regression was not significant across low elevation sites ($F_{1,47} = 0.000$, $p = 0.991$; Figure 2c), but was significant across high elevation sites ($F_{1,36} = 8.437$, $p = 0.006$; Figure 2c).

Mount Rose

For Note 2 Duration, the regression for low elevation sites ($F_{1,30} = 1.23$, $p = 0.277$) and high elevation sites was not significant ($F_{1,38} = 0.33$, $p = 0.569$). For Note 3 Duration, the regression was not significant for low elevation ($F_{1,30} = 0.64$, $p = 0.43$) or for high elevation sites ($F_{1,38} = 0.23$, $p = 0.636$). For Note 3 to Note 4 Internote Interval, the regression was not significant across low elevation sites ($F_{1,30} = 2.70$, $p = 0.111$; Figure 2d), but was significant across high elevation sites ($F_{1,38} = 16.98$, $p = 0.0002$; Figure 2d).

Spectral Measures

Sagehen

For Frequency Ratio, the regression for both low ($F_{1,36} = 16.25, p = 0.0003$, Figure 2e) and high ($F_{1,47} = 4.874, p = 0.032$, Figure 2e) elevation sites was significant. For Note 3 Start Frequency, the regression was not significant across low elevation sites ($F_{1,36} = 0.805, p = 0.376$, Figure 2f), however, it was significant across the high elevation sites ($F_{1,47} = 10.25, p = 0.002$, Figure 2f). In addition, the Number of Notes regression for low elevation sites was not significant ($F_{1,47} = 1.434, p = 0.237$), nor was the regression for the high elevation sites ($F_{1,36} = 0.76, p = 0.389$).

Mount Rose

For Frequency Ratio, the regression for both low ($F_{1,30} = 3.44, p = 0.074$) and high ($F_{1,38} = 0.67, p = 0.42$) elevation sites was not significant. For Note 3 Start Frequency, the regression was not significant across low elevation sites ($F_{1,30} = 2.28, p = 0.142$, Figure 2g), however, it was significant across the high elevation sites ($F_{1,38} = 8.99, p = 0.005$, Figure 2g). In addition, the Number of Notes regression for low elevation sites was not significant ($F_{1,30} = 1.4, p = 0.246$, Figure 2h), however, the regression for high elevation sites ($F_{1,38} = 9.95, p = 0.003$, Figure 2h) was significant.

Amplitude Measure

Sagehen

For Relative Amplitude, the regressions for both low elevation ($F_{1,47} = 0.243, p = 0.624$) and high elevation ($F_{1,36} = 0.007, p = 0.936$) sites were not statistically significant.

Mount Rose

For Relative Amplitude, the regression was significant for low elevation sites ($F_{1,30} = 8.81, p = 0.006$, Figure 9a), but not for the high elevation sites ($F_{1,38} = 0.39, p = 0.534$, Figure 2i).

Discriminate Function Analyses

We conducted 6 total DFAs, 3 for each mountain location; there was not a significant difference between 2, 3, or 6 groupings for Sagehen ($X^2 = 3.44, p > 0.05$), or for Mount Rose ($X^2 = 1.18, p > 0.05$) (chisq.test function, R Core Team), however, the classification percent correct did decrease at both locations as the number of groupings increased (Figure 3, Table 2 for grouping level comparisons and cross-validations), suggesting that two groups, consistent with a “high” and a “low” elevation dialect, best describes the data.

Sagehen

Using two grouping levels (low = 1900 m, 2000 m, and 2100 m; high = 2200 m, 2300 m, and 2400 m) and 9 acoustic measures (number of notes (NN), total song duration (SD), frequency ratio (FR), Note 2 Duration (N2 D), Note 3 duration (N3 D), Note 3 start frequency (N3 SF), Note 4 start frequency (N4 SF), Note 3 to Note 4 Internote Interval (N3N4 II), and relative amplitude (RA)), the DFA was able to significantly discriminate between songs from “low” and “high” elevation ($F_{9,77} = 10.84, p < 0.000$, Figure 4a). Using the ‘hold-out-sample’ cross-validation method, this DFA correctly assigned 11 of the 12 withheld cases ($p = 0.006$, significant using a binomial test with chance set to 0.50 for correct or incorrect classification) (Table 2).

Using three grouping levels (low = 1900 m and 2000 m; medium = 2100 m and 2200 m; and high = 2300 m and 2400 m) and the same 9 acoustic measures, the DFA was able to significantly discriminate between songs from “low”, “medium”, and “high” elevations ($F_{18,152} = 6.65, p < 0.000$). Using the ‘hold-out-sample’ cross-validation method, this DFA correctly assigned 7 of the 15 withheld cases ($p = 1.0$, not significant using a binomial test with chance set to 0.50 for correct or incorrect classification) (Table 2).

Using 6 grouping levels (each elevation bin on its own; 1900 m, 2000 m, 2100 m, 2200 m, 2300 m, and 2400 m) and the same 9 acoustic measures, the DFA was able to significantly discriminate between songs from all 6 elevations ($F_{45,329} = 3.60$, $p < 0.000$). Using the ‘hold-out-sample’ cross-validation method, this DFA correctly assigned 10 of the 18 withheld cases ($p = 0.815$, not significant using a binomial test with chance set to 0.50 for correct or incorrect classification) (Table 2).

Mount Rose

Using two grouping levels (low = 1900 m, 2000 m, and 2100 m; high = 2200 m, 2300 m, and 2400 m) and 9 acoustic measures (NN, SD, FR, N2 D, N3 D, N3 SF, N4 SF, N3N4 II, and RA) the DFA was able to significantly discriminate between songs from “low” and “high” elevation ($F_{9,62} = 4.08$, $p < 0.004$, Figure 4b). Using the ‘hold-out-sample’ cross-validation method, this DFA correctly assigned 11 of the 12 withheld cases ($p = 0.006$, significant using a binomial test with chance set to 0.50 for correct or incorrect classification) (Table 2).

Using three grouping levels (low = 1900 m and 2000 m; medium = 2100 m and 2200 m; and high = 2300 m and 2400 m) and the same 9 acoustic measures, the DFA was able to significantly discriminate between songs from “low”, “medium”, and “high” elevations ($F_{18,122} = 4.01$, $p < 0.000$). Using the ‘hold-out-sample’ cross-validation method, this DFA correctly assigned 11 of the 15 withheld cases ($p = 0.119$, not significant using a binomial test with chance set to 0.50 for correct or incorrect classification) (Table 2).

Using 6 grouping levels (each elevation bin as a level; 1900 m, 2000 m, 2100 m, 2200 m, 2300 m, and 2400 m) and the same 9 acoustic measures, the DFA was able to significantly discriminate between songs from all 6 elevations ($F_{45,262} = 2.84$, $p < 0.000$). Using the ‘hold-out-sample’ cross-validation method, this DFA correctly assigned 11 of the 18 withheld cases ($p =$

0.481, not significant using a binomial test with chance set to 0.50 for correct or incorrect classification) (Table 2).

Mantel Tests

Sagehen

At Sagehen, geographical, or elevational, distance between all six sampled elevations did not consistently explain the variation in individual acoustic parameters, however, for low elevation sites, there were significant effects of distance for Frequency Ratio and Note 2 Duration. While for high elevation sites, there were significant effects of distance for Note 3 Start Frequency, Note 3 to Note 4 Internote Interval, and Relative Amplitude (see Table 3 for corresponding R^2 and P values).

Mount Rose

Similarly, at Mount Rose, distance between all six sampled elevations did not consistently explain the variation in individual acoustic parameters, however, for low elevation sites, there was a significant effect of distance for Relative Amplitude. While for high elevation sites, there were significant effects of distance for Number of Notes and Note 3 to Note 4 Internote Interval (see Table 3 for corresponding R^2 and P values).

Principle Component Analyses and Analysis of Variance

Sagehen

Twelve acoustic parameters (Number of Notes, Song Duration, Frequency Ratio, Note 2 Start Frequency, Note 2 Duration, Note 2 to Note 3 Internote Interval, Note 3 Start Frequency, Note 3 Duration, Note 3 to Note 4 Internote Interval, Note 4 Start Frequency, Note 4 Duration, and

Relative Amplitude) were reduced to two principle components explaining 60.18% of the variation in high and low elevation song at Sagehen. Note 2 to Note 3 Internote Interval and Note 3 to Note 4 Internote Interval contributed most to PC1, explaining 39.56% of the variation. Number of Notes, Song Duration, Note 2 and Note 3 Duration contributed most to PC2, explaining 20.63% of the variation.

Analysis of variance on PC1 revealed no significant difference between high and low elevation song at Sagehen ($F_{1,85}=1.12, p = 0.293$). However, there was a significant difference between high and low elevation song using PC2 ($F_{1,85}=7.67, p = 0.007$) (Figure 5a).

Additional ANOVAs conducted on individual acoustic parameters revealed a significant difference between high and low elevation song for Note 3 Start Frequency, Frequency Ratio, Note 3 Duration, and Relative Amplitude at Sagehen ($p < 0.05$, see Table 4).

Mount Rose

The same twelve acoustic parameters were reduced to two principle components explaining 65.84% of the variation in high and low elevation song at Mount Rose. Number of Notes, Song Duration, Note 2 to Note 3 Internote Interval and Note 3 to Note 4 Internote Interval contributed most to PC1, explaining 40.24% of the variation. The same parameters in PC1 and Note 3 Duration contributed most to PC2, explaining 25.60% of the variation.

Analysis of variance on PC1 revealed a significant difference between high and low elevation song at Mount Rose ($F_{1,70}=4.82, P = 0.032$). However, there was not a significant difference between high and low elevation song using PC2 ($F_{1,70}=0.781, P = 0.38$) (Figure 5b).

Additional ANOVAs conducted on individual acoustic parameters revealed a significant difference between high and low elevation song for Number of Notes and Note 3 Start Frequency at Mount Rose ($p < 0.05$, see Table 4).

DISCUSSION

By recording male song along two continuous elevation gradients, we have confirmed the structural differences in song produced by males recorded at high versus low elevations previously reported in Branch & Pravosudov (2015), and shown that this structural song variation is consistent with patterns of vocal dialects. Regression analyses show that each acoustic parameter varies differentially, with the majority of the acoustic parameters showing steep clines with stepped variation, and not gradual clines (e.g. Comparing Figure 1 with Note 3 to Note 4 Internote Interval, Figure 2c, d for both mountain locations). In addition, the summary of each acoustic parameter measured (Table 1), and the mantel tests (Table 3), do not support gradual clinal variation, as the largest differences in these measures are rarely consistent with the furthest geographic or elevational distance (Sung & Handford 2006; Yandell et al. 2017). Analyses were split into “high” and “low” groups due to *a priori* information suggesting a significant shift in winter climate around 2150 m in the Sierra Nevada (Barbour & Minnich 2000). There are several instances where the acoustic parameter changes gradually or clinally for one elevation, but changes in the opposite direction for the other elevation (e.g. Note 3 Start Frequency, Figure 2f, g for both mountain locations), which is consistent with patterns of vocal dialects.

Separate DFAs for Sagehen and Mount Rose show that the best model fits are those with two groupings, divided into “high” and “low” elevation dialects. Although the other grouping DFAs (3 or 6 groups) were able to significantly categorize songs by location, the classification matrix performed worse (Table 2) and the F values decreased (i.e. lower significance of categorization) as the number of groupings increased. In addition, the hold-out-sample cross-validations for 2 groupings were significantly able to place withheld individuals in the correct

elevation groups, while the cross-validations for 3 and 6 groupings were not. This was the case at both mountain locations, again, suggesting that the most appropriate grouping classification for these data are for 2 groups that represent “high” and “low” elevation dialects. Finally, ANOVAs conducted on reduced representations of the data, or principle components, reveal that the variation within high and low elevations is lower than the variation between high and low elevations.

It is clear from the literature that chickadees exhibit the characteristics that facilitate the evolution of song dialects, (1) chickadees have one main song type that they produce and lack repertoires (Wiebe & Lein 1999), increasing homogeneity of transmission, (2) songs are culturally transmitted, or learned, and therefore errors may occur which would then be passed on to the next generation (Gammon et al. 2005), and (3) chickadees exhibit rather short post-natal dispersal distances (Weise & Meyer 1979), thus maintaining dialect neighborhoods (Marler & Tamura 1962; 1964; Podos & Warren 2007). And indeed, our analyses are consistent with vocal dialects, however, our ability to quantify dialects compared with previous work in this area, specifically in sparrows, may not be as definitive. For example, Marler and Tamura (1962; 1964) were able to show stark visual differences in the songs of white crowned sparrow populations using spectrograms, which is not quite the case for our mountain chickadees. When comparing chickadee song variation with work in sparrows it is important to consider that the vocal repertoire and general complexity of their songs is much higher. Various sparrow species show a diverse production of syllables and note types (repertoire size), as well as trills in their songs, while chickadee song is rather simple, consisting of several whistles. As such, it is likely more nuanced shifts in frequency or song duration that allow females to discriminate between male chickadee songs.

Furthermore, in a thorough review of geographic variation in bird song, Podos & Warren (2007) describe several predictions of the local adaptation hypothesis; Prediction 1, birds learn their song early, prior to natal dispersal; Prediction 2, birds will settle where their natal dialect is sung; Prediction 3, dialect groups should become genetically differentiated; and Prediction 4, females should evolve preferences for males that sing their local dialect. All of these predictions are met by our mountain chickadee system, except for Prediction 3, which is now dated, as recent work suggests that local adaptation commonly occurs in the face of gene flow (Gonzalo-Turpin & Hazard 2009; McCracken et al. 2009; de Leon et al. 2010; Oliveira et al. 2015; Tigano & Friesen 2016; Branch et al. 2017). Furthermore, a whole-genome comparison may be necessary to detect nuanced genetic variation in continuous or hybridizing populations, as even established species have shown a lack of genetic structure using traditional microsatellite and genotype-by-sequencing approaches (e.g. golden and blue-winged warblers, Toews et al. 2016).

Previous work investigating song structure at the elevation extremes (1900 vs 2400 m) of these two montane gradients showed that variation in song may serve as an indicator of local adaptation in mountain chickadees (Branch & Pravosudov 2015). The current findings supporting vocal dialects along these two montane gradients in combination with the previous work on this system, further support the local adaptation hypothesis. Indeed, chickadees are food-caching birds that rely on spatial memory to retrieve their scattered food caches, which is critical for winter survival in these resident birds (Pravosudov & Roth 2013). Previous work in our lab has shown that birds inhabiting harsher, higher elevations have better spatial memory with more underlying neural support compared to birds inhabiting milder, lower elevations (Freas et al 2012). In addition, chickadees have only one, rather short, post-natal dispersal event and remain in their post-natal dispersal area for the rest of their lives (Weise & Meyer 1979;

Branch et al. 2016). And finally, females inhabiting high elevations show a preference for males from high elevations (Branch et al. 2015b).

Finding support for vocal dialects in mountain chickadees at different elevations has important implications for the evolutionary trajectory of these birds (Edwards et al. 2005; Podos & Warren 2007), as these differences in song structure likely represent indicators of local adaptation (Branch & Pravosudov 2015), and vocal dialects may result in further separation between these groups of birds inhabiting high versus low elevations in the Sierra Nevada. As the climate continues to change (Fischer & Knutti 2015), further separation of these groups may have important implications for their success and genetic variation, as warming will likely increase the area that low elevation birds are suited to colonize, while high elevation birds, and their associated genetic variation, may decline. Fortunately, these birds inhabit a continuous elevation gradient, and there is some level of gene flow between both high and low groups, and between birds inhabiting Sagehen and Mount Rose (Branch et al. 2017). However, recent work in our lab shows that large variation in annual precipitation events, such as severe drought and heavy snow fall, has significant effects on the breeding patterns and reproductive output of these birds (Kozlovsky et al. 2018). The effects of global climate change on breeding success and the fact that these birds exhibit vocal dialects further highlight the importance understanding how large climatic events may affect least concern species. At this stage, mountain chickadees are abundant and of least concern, which makes them a prime study system for addressing potential issues related to the effects of global climate change and shed light on the fact that even our most common species may be at risk as severe weather events increase in frequency (Peterson et al. 2013; Kunkel et al. 2016).

To the best of our knowledge, this is the first evidence of song dialects within a single, continuously distributed population of birds. The findings of vocal dialects in mountain chickadees are rather astounding for several reasons, (1) these birds inhabit a continuous elevation gradient at both mountain locations assessed, (2) the differences seen in song structure exist along a *ca* 15 km transect, and (3) unsurprisingly, there is some level of gene flow between all of these locations, including within the elevation scale and between the two mountain locations. It has been well established that local adaptation occurs along elevation gradients and when selection is strong enough can result in adaptation in the presence of gene flow (Endler 1977; Miyasato & Baker 1999; Maud & Merila 2001; Ottvall et al. 2002; Gimenez-Benavides et al. 2007; Gonzalo-Turpin & Hazard 2009; McCracken et al. 2009; de Leon et al. 2010; Porlier et al. 2012; Wilson et al. 2012; Caro et al. 2013; Oliveira et al. 2015; Bertrand et al 2016; Szulkin et al. 2016; Tigano & Friesen 2016; Branch et al. 2017). However, previous work investigating song dialects along continuous habitat distributions has either shown distinct genetic variation between populations living in parapatry (Irwin et al. 2008; Caro et al. 2013) or has found support for gradual clinal variation and not for song dialects (Irwin 2000; Sung & Handford 2006; Irwin et al. 2008). These results suggest that selection on chickadees inhabiting high versus low elevations is likely strong and may be facilitated by “high” and “low” elevation song dialects. Investigating song variation among continuously distributed populations experiencing differential selection on ecologically relevant traits may be a fruitful future avenue for understanding the evolution of song dialects.

Ethics

To the best of our knowledge, no birds were harmed by the collection of this data. All procedures were in accordance with the UNR IACUC protocol (00046) and with Federal Fish and Wildlife Permit no. MB022532.

Data Accessibility

Song files will be made available through Xeno-Canto upon publication.

Competing Interests

The authors have no competing interests to report.

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Figure Captions:

Figure 1. Schematic of three patterns of geographic variation that may arise in male songbird song. Redrawn from Podos & Warren 2007.

Figure 2. Linear regressions with acoustic parameter on the y-axis and elevation on the x-axis. Left panels are low elevation bins and right panels are high elevation bins. Black circles represent Sagehen and grey squares represent Mount Rose. Acoustic measure on y-axis for all figures; (a) Note 2 Duration, (b) Note 3 Durations, (c) and (d) Note 3 to Note 4 Internote Interval, (e) Frequency Ratio, (f) and (g) Note 3 Start Frequency, (h) Number of Notes, (i) Relative Amplitude.

Figure 3. Comparison of DFA percent correct categorization using 2, 3, or 6 groups. Black circles represent Sagehen and grey squares represent Mount Rose.

Figure 4. Discriminant function models of 9 variable for continuous elevation locations sampled and categorized into high versus low elevation groups at (a) Sagehen low (black circles) and Sagehen high (grey circles) and (b) Mount Rose low (black squares) and Mount Rose high (grey squares).

Figure 5. Principle component 1 and principle component 2 plotted for (a) Sagehen and (b) Mount Rose. Principle component analyses were conducted using 12 acoustic variables (Number of Notes, Song Duration, Frequency Ratio, Note 2 Start Frequency, Note 2 Duration, Note 2 to Note 3 Internote Interval, Note 3 Start Frequency, Note 3 Duration, Note 3 to Note 4 Internote

Interval, Note 4 Start Frequency, Note 4 Duration, and Relative Amplitude). For Sagehen and Mount Rose, black symbols represent low elevation (1900, 2000, and 2100 m sites lumped) and grey symbols represent high elevation (2200, 2300, 2400 m sites lumped).

- 1 Table 1. Means and standard deviations for each acoustic parameter measured across all six elevations at (a) Sagehen and (b) Mount
 2 Rose. Bold values represent significant regression analyses determined by P values, and corresponding R^2 values. All 7 acoustic
 3 measures listed in the table were used in the DFA.

(a) Sagehen										
	<i>low</i>					<i>high</i>				
Acoustic Measure	<i>1900</i>	<i>2000</i>	<i>2100</i>	<i>P</i>	<i>R²</i>	<i>2200</i>	<i>2300</i>	<i>2400</i>	<i>P</i>	<i>R²</i>
NN	3.85 ± 0.33	3.88 ± 0.31	3.98 ± 0.35	0.237	0.009	3.96 ± 0.14	3.69 ± 0.57	3.78 ± 0.43	0.389	-0.007
N3 SF	3460.95 ± 82.36	3544.74 ± 59.62	3518.56 ± 114.48	0.376	-0.005	3533.12 ± 57.15	3534.39 ± 87.38	3658.2 ± 87.49	0.002	0.162
FR	1.22 ± 0.02	1.21 ± 0.02	1.18 ± 0.02	0.000	0.34	1.17 ± 0.02	1.18 ± 0.03	1.16 ± 0.03	0.115	0.042
N2 D	334.2 ± 28.96	316.73 ± 21.67	315.76 ± 19.89	0.03	0.077	310 ± 15.19	312.83 ± 32.08	313.2 ± 19.70	0.739	-0.025
N3 D	198.08 ± 7.06	188.3 ± 7.96	192.72 ± 12.31	0.235	0.009	179.11 ± 7.97	178.54 ± 6.77	171.74 ± 10.56	0.032	0.096
N3-N4 II	95.31 ± 13.56	114.73 ± 16.44	97.96 ± 11.95	0.991	-0.021	97.19 ± 10.29	94.53 ± 7.42	110.92 ± 16.13	0.006	0.167
RA	0.95 ± 0.05	0.95 ± 0.06	0.94 ± 0.05	0.624	-0.016	0.91 ± 0.02	0.87 ± 0.09	0.91 ± 0.07	0.936	-0.028

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(b) Mount Rose										
	<i>low</i>					<i>high</i>				
Acoustic Measure	<i>1900</i>	<i>2000</i>	<i>2100</i>	<i>P</i>	<i>R²</i>	<i>2200</i>	<i>2300</i>	<i>2400</i>	<i>P</i>	<i>R²</i>
NN	3.59 ± 0.44	3.45 ± 0.82	3.30 ± 0.46	0.246	0.013	3.87 ± 0.30	3.95 ± 0.35	3.42 ± 0.45	0.003	0.187
N3 SF	3556.53 ± 68.45	3644.68 ± 152.18	3631.46 ± 115.49	0.142	0.04	3585.11 ± 54.02	3512.87 ± 73.61	3497.94 ± 81.71	0.005	0.17
FR	1.19 ± 0.03	1.16 ± 0.05	1.16 ± 0.02	0.074	0.073	1.17 ± 0.02	1.18 ± 0.05	1.16 ± 0.04	0.42	-0.009
N2 D	313.22 ± 18.85	314.12 ± 28.81	301.09 ± 29.10	0.277	0.007	322.61 ± 18.40	301.30 ± 18.72	324.55 ± 23.62	0.569	-0.017
N3 D	196.96 ± 9.15	200.29 ± 26.71	191.43 ± 6.09	0.43	-0.012	197.71 ± 10.23	186.74 ± 10.83	193.49 ± 21.07	0.636	-0.02
N3-N4 II	96.43 ± 12.81	97.42 ± 8.47	88.72 ± 10.86	0.111	0.052	91.01 ± 7.28	92.46 ± 7.54	110.91 ± 17.56	0.000	0.291
RA	0.93 ± 0.08	0.88 ± 0.05	0.84 ± 0.06	0.006	0.201	0.92 ± 0.04	0.88 ± 0.07	0.90 ± 0.06	0.534	-0.016

5 Table 2. The original and cross-validated percentage of correct classifications by grouping level
 6 using ‘all-variables-together’ DFAs. The eigenvalue and canonical correlation coefficient, as
 7 well as the two acoustic features with the two largest (respectively) standardized coefficients for
 8 the first discriminant function are listed for all DFAs.

Group	Original	Cross-Validated	Eigenvalue	Cononical R	Stand. Coeff.
<u>Sagehen</u>					
2 Groups	88.51%	91.67%	1.27	0.75	N3Dur, RA
3 Groups	79.31%	46.67%	1.478	0.77	FR, RA
6 Groups	65.52%	55.56%	1.99	0.82	FR, N3Dur
<u>Mount Rose</u>					
2 Groups	80.56%	91.67%	0.59	0.61	NN, N3SF
3 Groups	70.83%	73.33%	0.94	0.7	SD, N3SF
6 Groups	68.06%	61.11%	1.19	0.74	SD, N3SF

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23 Table 3. Results of mantel distance analyses for individual acoustic parameters at (a) Sagehen
 24 and (b) Mount Rose. Bold values represent significant regression analyses determined by P
 25 values at 0.05, and corresponding R^2 values.

(a) Sagehen				
	Low		High	
Acoustic Measure	R^2	P	R^2	P
NN	0.000	0.760	0.007	0.060
N3 SF	0.002	0.375	0.047	0.001
FR	0.105	0.001	0.0002	0.852
N2 D	0.016	0.019	0.008	0.072
N3 D	0.001	0.552	0.003	0.199
N3-N4 II	0.119	0.862	0.009	0.034
RA	0.001	0.592	0.013	0.032

(b) Mount Rose				
	Low		High	
Acoustic Measure	R^2	P	R^2	P
NN	0.0002	0.605	0.016	0.009
N3 SF	0.001	0.505	0.007	0.079
FR	0.001	0.62	0.002	0.453
N2 D	0.029	0.868	0.0002	0.784
N3 D	0.006	0.119	0.0001	0.85
N3-N4 II	0.005	0.143	0.029	0.009
RA	0.024	0.005	0.002	0.292

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35 Table 4. Means and standard deviations for individual acoustic parameter by low (1900, 2000,
 36 and 2100 m sites lumped) and high elevation (2200, 2300, 2400 m sites lumped) sites at (a)
 37 Sagehen and (b) Mount Rose. Bold values represent significant ANOVA analyses determined by
 38 *P* values and corresponding *F* values.

(a) Sagehen				
Acoustic Measure	<i>Low</i>	<i>High</i>	<i>F</i>	<i>P</i>
NN	3.93 ± 0.34	3.80 ± 0.43	2.42	0.123
N3 SF	3508.62 ± 100.64	3589.44 ± 100.49	13.82	0.0004
FR	1.20 ± 0.03	1.17 ± 0.03	23.21	0.0001
N2 D	312.25 ± 22.45	320.85 ± 23.87	2.95	0.091
N3 D	175.65 ± 9.42	193.24 ± 10.72	63.99	0.0001
N3-N4 II	100.68 ± 14.97	102.56 ± 14.56	0.347	0.558
RA	0.94 ± 0.05	0.90 ± 0.07	10.96	0.0014

(b) Mount Rose				
Acoustic Measure	<i>Low</i>	<i>High</i>	<i>F</i>	<i>P</i>
NN	3.45 ± 0.58	3.69 ± 0.45	3.87	0.053
N3 SF	3609.83 ± 118.76	3526.01 ± 80.28	12.7	0.0007
FR	1.17 ± 0.04	1.17 ± 0.03	0.163	0.688
N2 D	309.33 ± 25.80	317.62 ± 22.89	2.082	0.154
N3 D	196.10 ± 16.12	192.79 ± 16.36	0.735	0.394
N3-N4 II	94.09 ± 11.29	100.37 ± 16.01	3.514	0.065
RA	0.883 ± 0.07	0.901 ± 0.06	1.277	0.262

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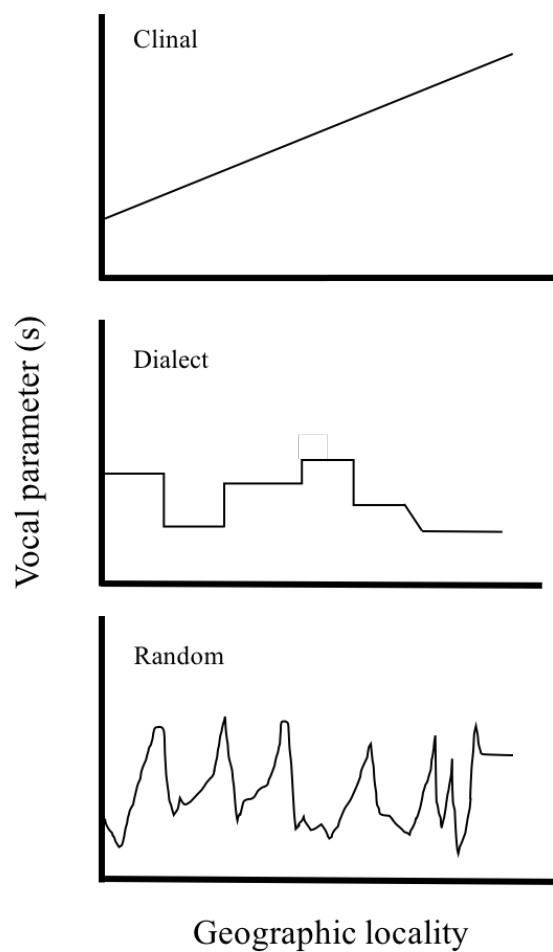
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49 Figure 1.

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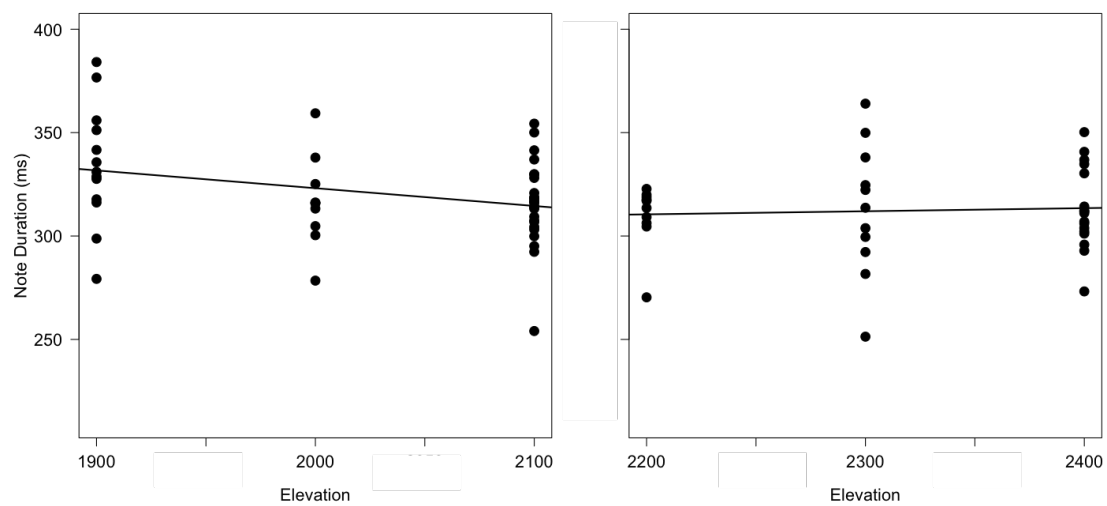
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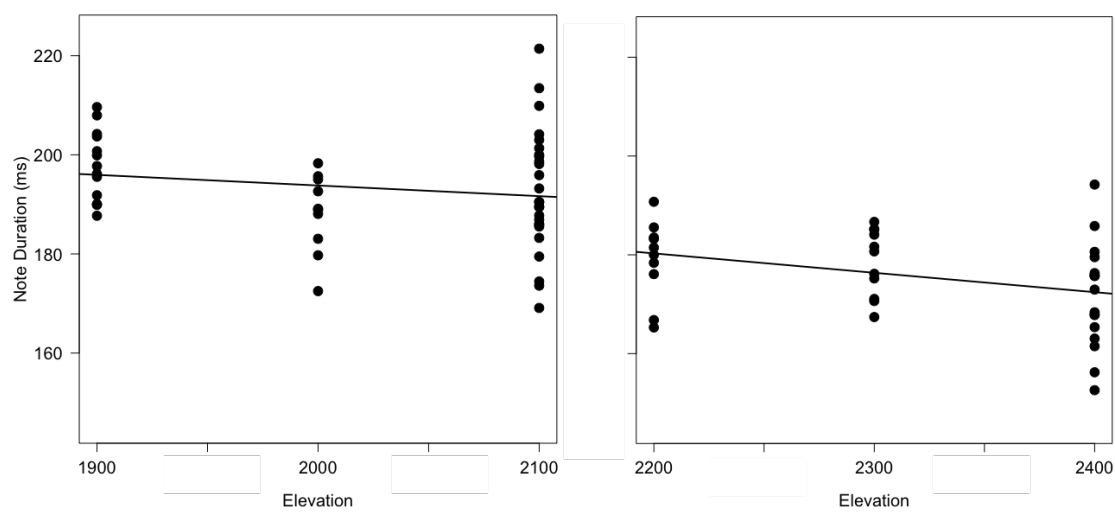
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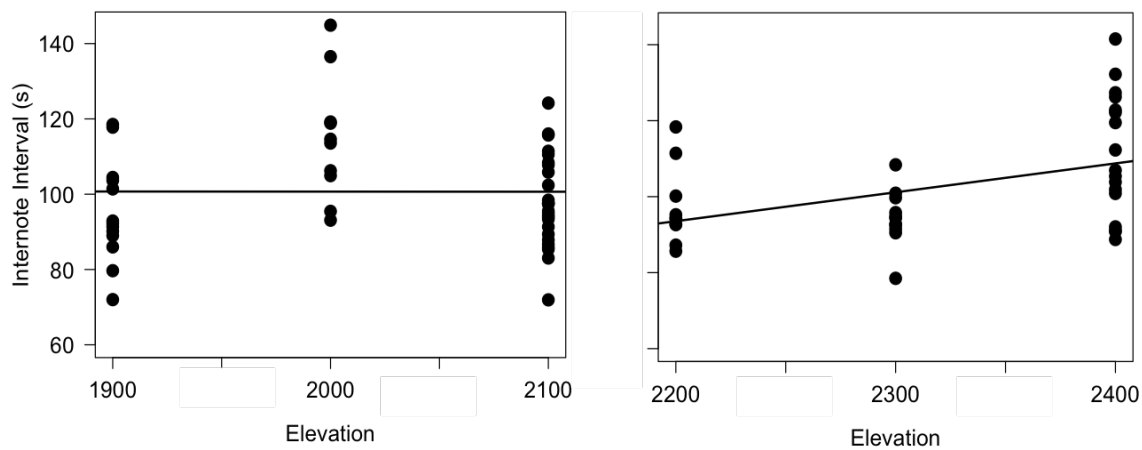
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58 Figure 2a.



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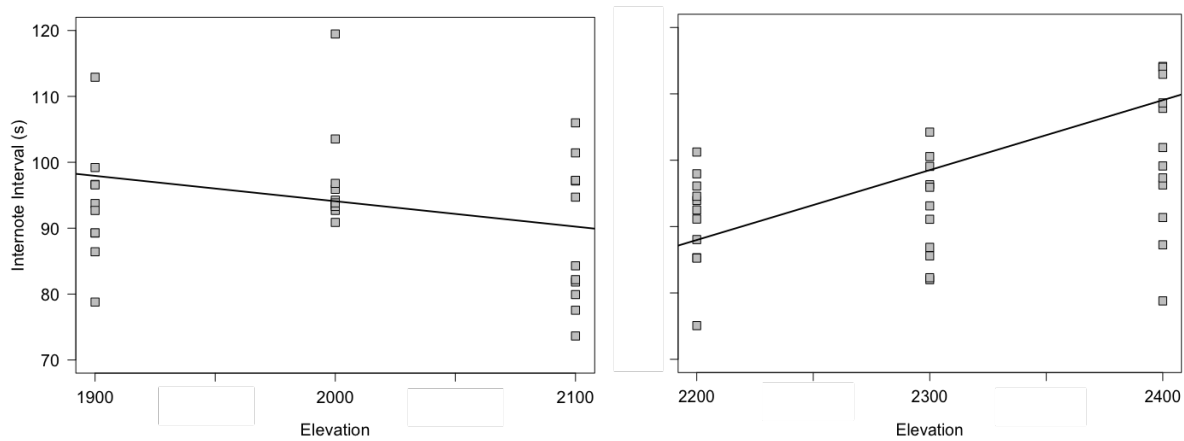
60 Figure 2b.



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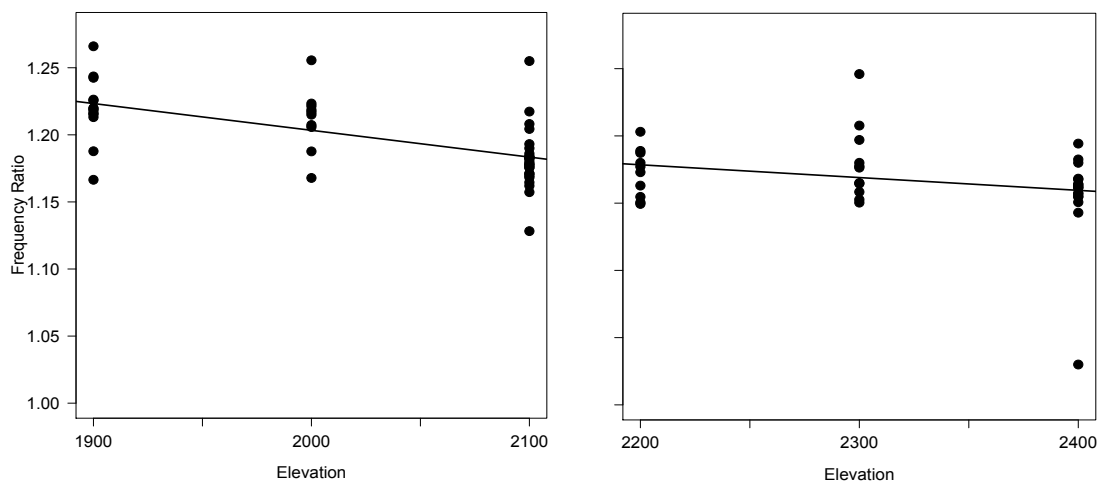
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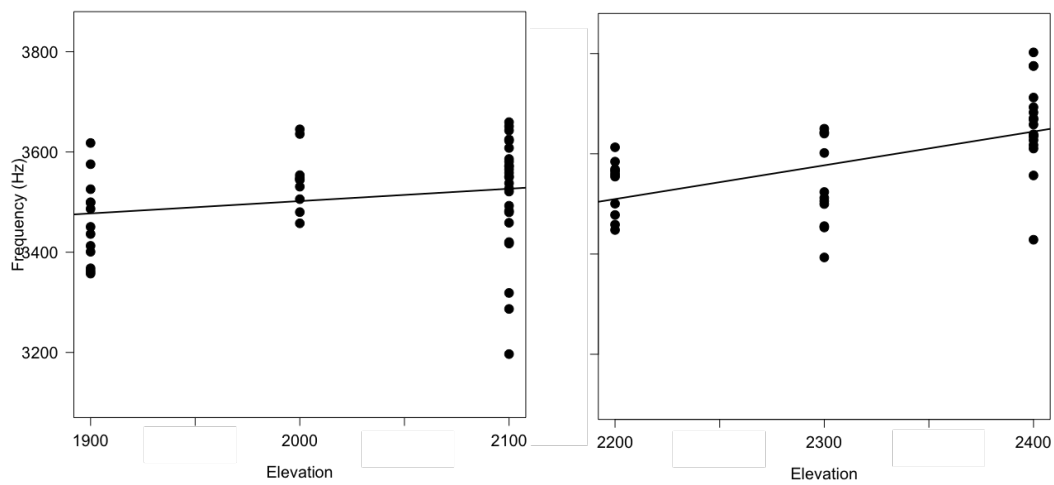
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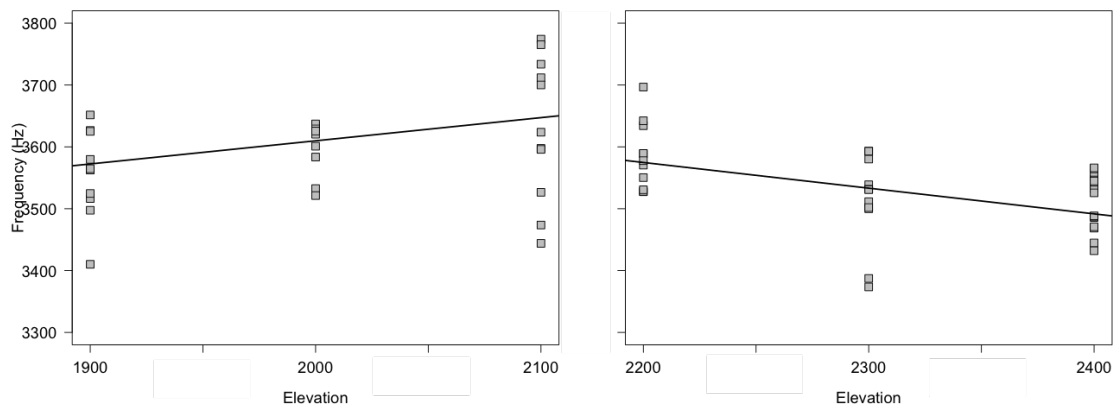
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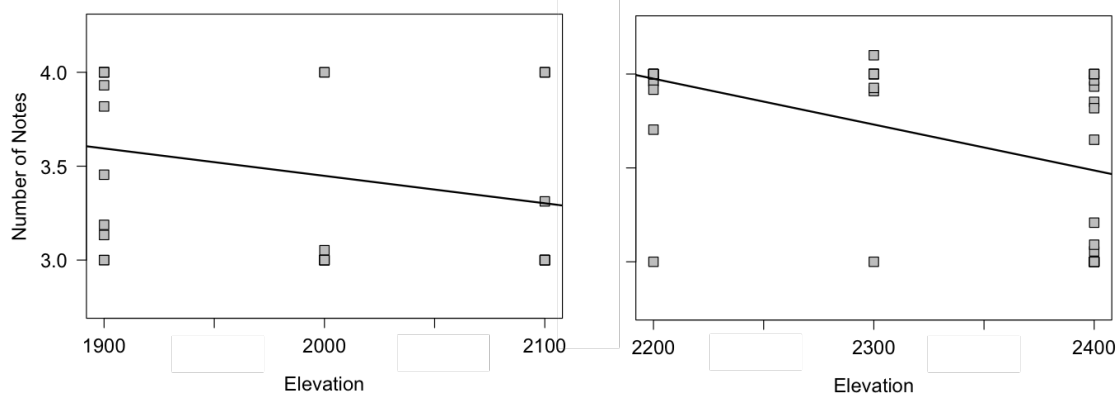
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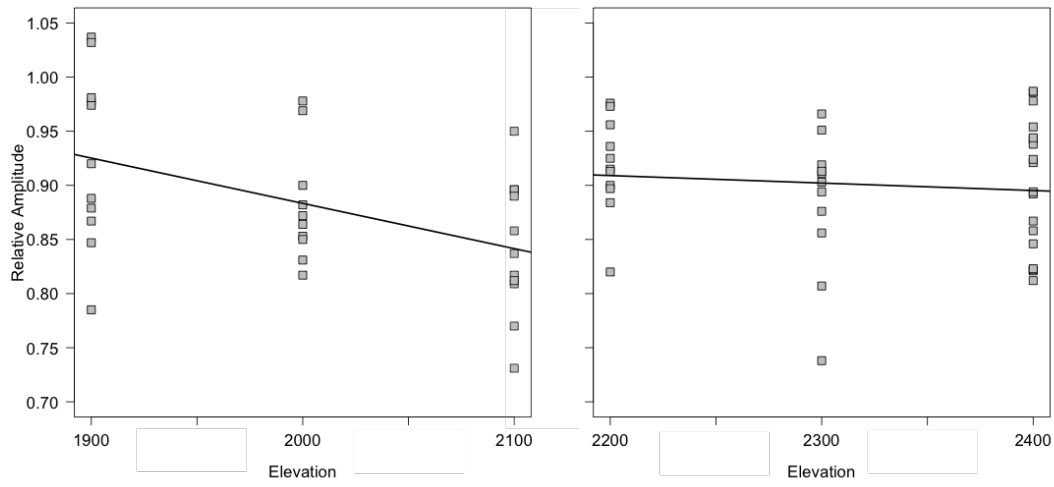


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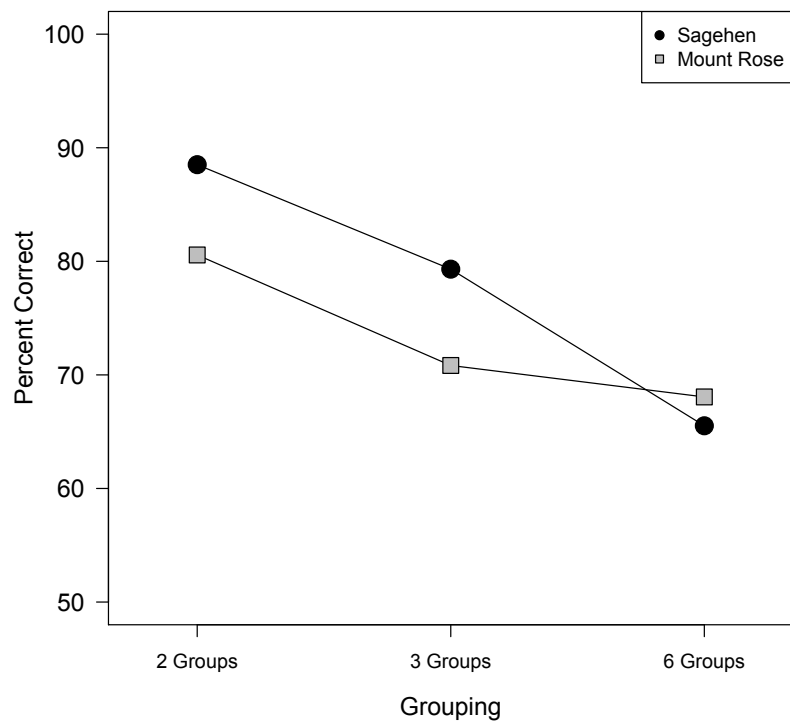
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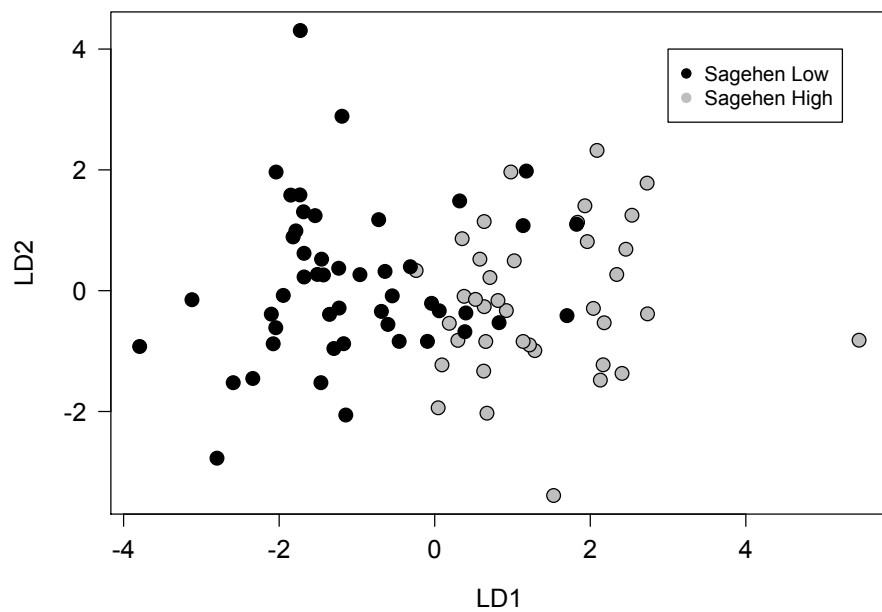
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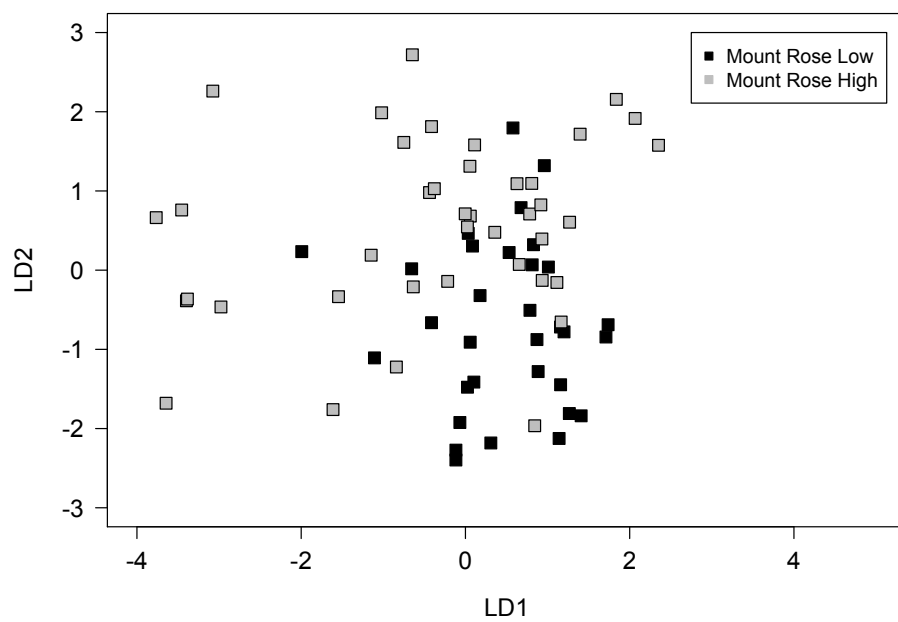
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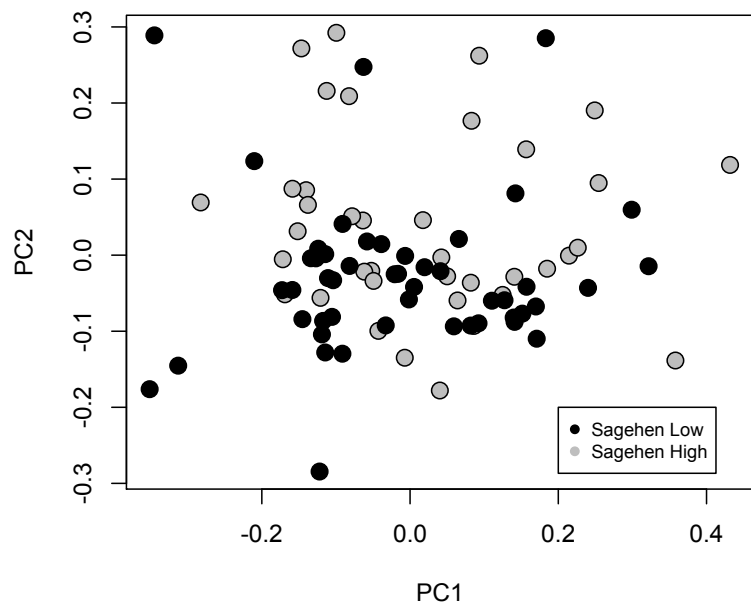
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95 Figure 4a.



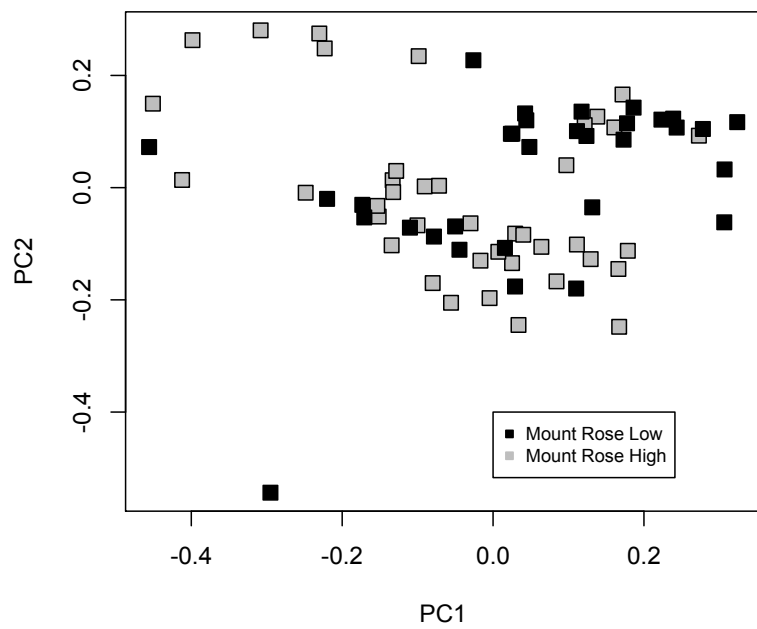
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97 Figure 4b.



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99 Figure 5a.



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101 Figure 5b.

102 Supplemental Materials

103 Figure S1. Schematic of sound spectrogram and power spectrum showing acoustic measurements
104 used for analysis. Other measurements not physically shown in diagram were calculated using
105 the measurements depicted. (a) Sound spectrogram (transform length of 512 points, time
106 resolution of 11.6 ms, and frequency resolution of 86.1 Hz); measurements include temporal:
107 total song duration (SD), note duration (ND), internote interval (II) and spectral: start frequency
108 (SF), end frequency (used with SF to calculate note glissando (NG)), change in frequency from
109 end of Note 2 to start of Note 3 used to calculate frequency ratio (FR), also the number of notes
110 can be counted from the spectrogram, there are 4 notes in this example. (b) Waveform of 1a. (c)
111 Power spectrum (FFT window = 65,536 points; 88 Hz smoothing) showing amplitude 1 and 2
112 used to calculate relative amplitude (RA). (d) Waveform of 1c. Redrawn from Branch &
113 Pravosudov 2015.

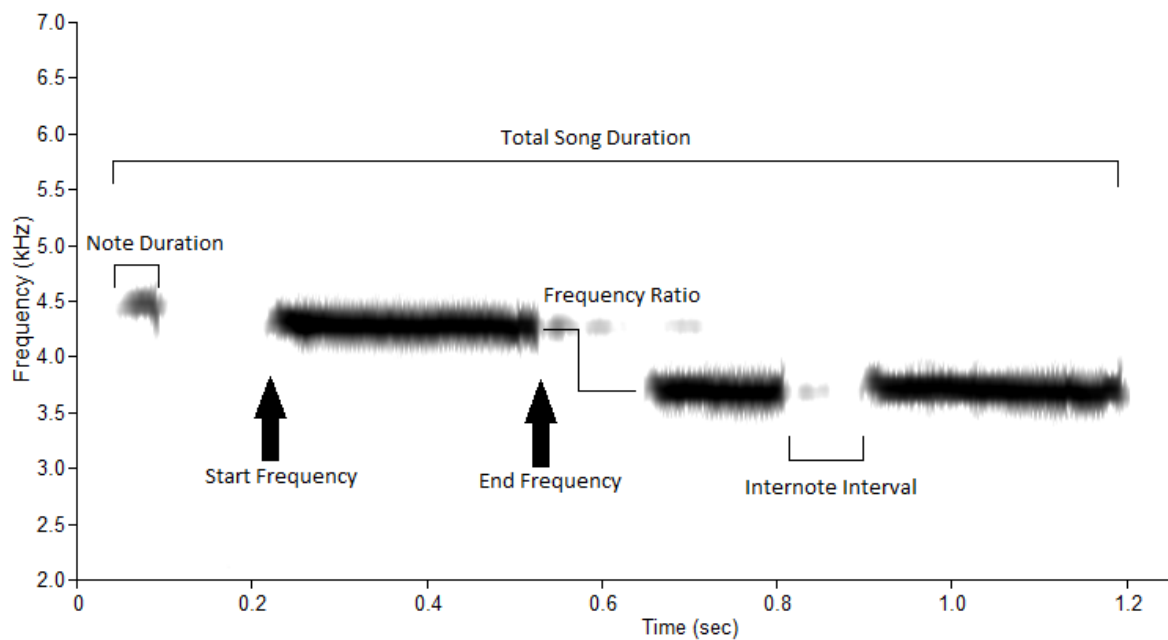
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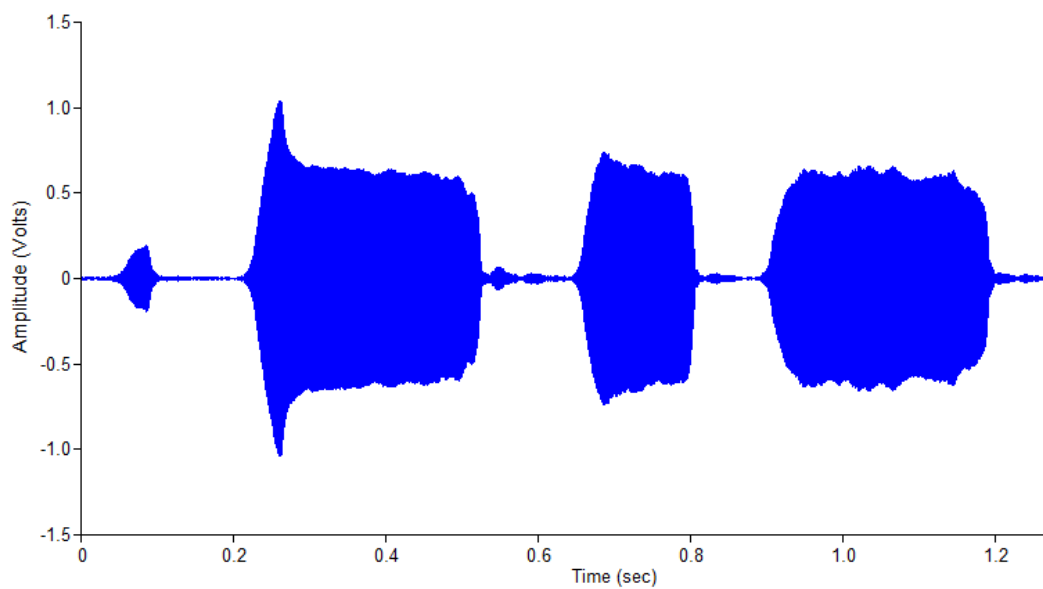
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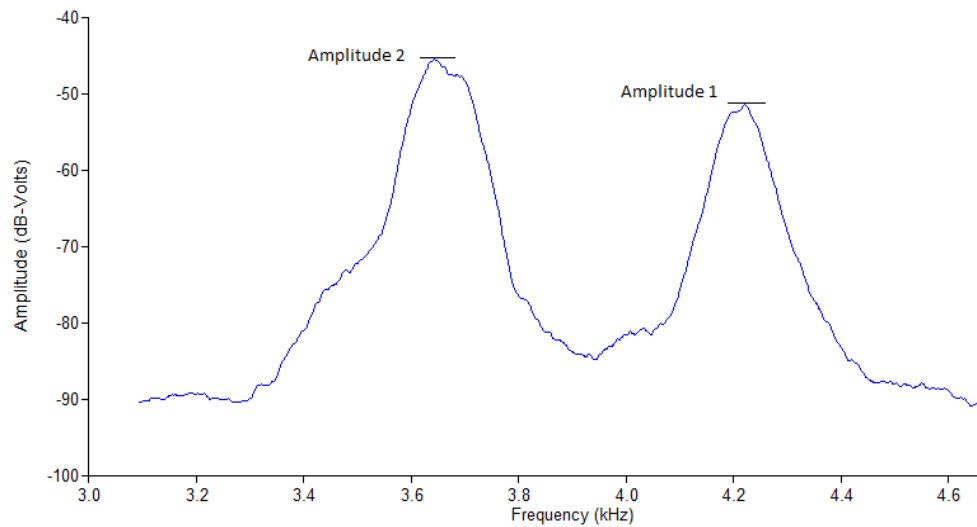
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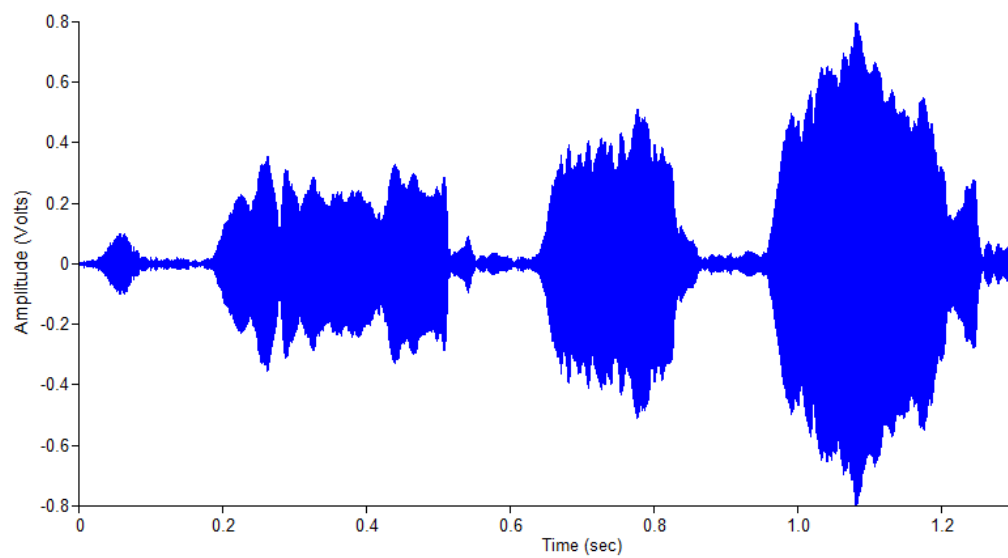
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126 Figure S1c.

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129 Figure S1d.

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Absence of population structure across elevational gradients despite large phenotypic variation in mountain chickadees (*Poecile gambeli*)

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Montane habitats are characterized by predictably rapid heterogeneity along elevational gradients and are useful for investigating the consequences of environmental heterogeneity for local adaptation and population genetic structure. Food-caching mountain chickadees inhabit a continuous elevation gradient in the Sierra Nevada, and birds living at harsher, high elevations have better spatial memory ability and exhibit differences in male song structure and female mate preference compared to birds inhabiting milder, low elevations. While high elevation birds breed, on average, two weeks later than low elevation birds, the extent of gene flow between elevations is unknown. Despite phenotypic variation and indirect evidence for local adaptation, population genetic analyses based on 18 073 single nucleotide polymorphisms across three transects of high and low elevation populations provided no evidence for genetic differentiation. Analyses based on individual genotypes revealed no patterns of clustering, pairwise estimates of genetic differentiation (F_{ST} , Nei's D) were very low, and AMOVA revealed no evidence for genetic variation structured by transect or by low and high elevation sites within transects. In addition, we found no consistent evidence for strong parallel allele frequency divergence between low and high elevation sites within the three transects. Large elevation-related phenotypic variation

may be maintained by strong selection despite gene flow and future work should focus on the mechanisms underlying such variation.

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1. Introduction

Multiple evolutionary and environmental factors influence variation in local adaptation and population genetic structure across the landscape. Geographical isolation can lead to spatial genetic structure by directly limiting gene flow and allowing allele frequencies to differ as a result of genetic drift (isolation by distance [1]). In addition, ecological isolation of populations experiencing divergent selection can indirectly reduce gene flow as a result of selection against immigrants or by individual preferences to remain in different habitats [2–4]. Thus, the extent to which geography and local adaptation shape patterns of landscape genetic structure is jointly influenced by the strength of geographically based divergent selection and by geographical distribution, dispersal, and life history [5,6]. In addition, when adaptation occurs in the face of gene flow, genetic differentiation can be heterogeneous across the genome, with genetic regions involved in local adaptation restricted to small portions of the genome [7–9]. As a result, extensive genomic sampling may be required to detect fine-scale geographical genetic structure arising either directly or indirectly from spatially variable selection.

Spatially variable environments play a major role in ecological and evolutionary processes, and offer the opportunity for understanding the factors that shape phenotypic and population genetic variation. Habitats exhibiting environmental variation on relatively small spatial scales could improve our understanding of the relationship among environmental variation, local adaptation and population genetic structure. Montane habitats present a particularly useful setting to investigate these issues, because heterogeneity often occurs rapidly and predictably along elevation gradients [10,11]. Montane heterogeneity is manifested through predictable climatic and environmental variation, often resulting in variation between populations inhabiting different elevations. Elevation-related variation may be exhibited through differences in morphology, physiology, development and behaviour [11–25]. In some cases, elevation associated phenotypic differentiation coincides with population genetic structure [11,12,14,16,18,20,21,26,27], illustrating how divergent selection can shape phenotypic variation across small spatial scales when sharp ecological gradients reduce gene flow and lead to isolation by environment [5].

Mountain chickadees (*Poecile gambeli*) are resident, food-caching passerines that inhabit a range of elevations along the Western montane regions of North America [28]. These birds are scatter hoarders, meaning they cache (store) food items in multiple locations when food is abundant (summer/autumn) to use later when food is scarce (winter), and use spatial memory, at least in part, to relocate these caches [29]. Birds inhabiting higher elevations experience harsher winter conditions (lower temperature, more snow, extended periods of snow cover [30]) and probably have a higher reliance on previously cached food to survive winter compared to birds inhabiting lower, milder elevations [19].

Mountain chickadees, like other parids, remain in flocks of unrelated individuals throughout the winter and experience approximately 50% winter mortality [28]. As their winter survival is affected by the local environmental conditions, phenotypes enhancing winter survival would probably be favoured by selection (e.g. enhanced spatial memory for cache recovery). Indeed, high elevation birds (approx. 2400 m) cache more food items (3–4 times more than low elevation birds) [19,31] and exhibit superior spatial memory ability (high elevation birds perform twice as well on spatial memory tasks) [19] associated with significant differences in morphology of the hippocampus (high elevation birds have up to 20% larger hippocampal volume and up to 50% more hippocampal neurons) [19,32], the brain region underlying memory abilities, compared to low elevation birds (approx. 1900 m) [19,31,32]. While there are no direct estimates of heritability for such cognitive phenotypes, work in mammals suggests that spatial memory ($h^2 = 0.32\text{--}0.54$) [33–36] and hippocampal volume ($h^2 = 0.29\text{--}0.95$) [37,38] are moderately to highly heritable. In addition, the elevation-related differences we have documented in mountain chickadees have been found in juvenile birds prior to their first winter and, therefore, prior to the largest climate-related mortality event, further suggesting that differences found in spatial ability and hippocampus morphology may be heritable and capable of responding to selection [32].

In addition to elevation-related variation in spatial memory and neural morphology, these same populations of high and low elevation birds exhibit differences in social dominance. Low elevation males were dominant to high elevation males in pairwise interactions [39]. Differences in social dominance are particularly interesting given that dominant individuals tend to experience higher fitness (e.g. better

access to food resources, territories and mates) compared to their subordinate counterparts (e.g. [40]). Taken together, existing evidence suggests that there are fitness consequences of moving between elevations. High elevation birds may have lower fitness at low elevations, owing to their socially subordinate status, while low elevation birds may have lower fitness at high elevations owing to their lower food caching propensity and inferior memory ability for retrieving caches, which may be required to survive in harsher high elevation environment. Furthermore, female mate choice could limit gene flow between high and low elevation populations. High elevation females prefer high elevation males over low elevation males in pairwise choice trials [22], and potentially use differences in song structure to discriminate males from high versus low elevations [23]. Finally, on average, low elevation birds begin breeding two weeks earlier than high elevation birds [41], providing yet another potential premating isolation mechanism.

Highly vagile organisms, such as birds, are often expected to show limited population structure, even over large geographical regions. Indeed, some formally recognized and phenotypically differentiated bird subspecies often show little or no evidence of genome-wide differentiation despite marked phenotypic differentiation [9,42,43]. Previous studies of mtDNA variation in mountain chickadees have indicated genetic structure of populations at broad geographical scales corresponding to eastern (Rocky Mountain) and western (Sierra Nevada and Cascades) clades but revealed little evidence for genetic structure across smaller geographical scales within these clades [44]. This is perhaps not surprising, as mountain chickadees are continuously distributed across the coniferous forests of the Sierra Nevada. However chickadees and tits from the genus *Poecile* are well known to be highly sedentary with rather short post-natal dispersal distances and limited to no movements once juveniles settle (e.g. [28,45]). The previous study of genetic variation in mountain chickadees sampled small numbers of molecular markers with limited resolution for understanding fine-scale population structure [44]. Indeed, some recent studies have documented fine-scale genetic structure within other avian populations across small geographical regions, which appear to be driven by strong ecological variation [20,21,46,47].

Recent innovations in DNA sequencing technology have drastically improved our ability to quantify fine-scale population genetic variation in ecologically significant but genomically understudied organisms [48–51]. Population genomic approaches can query thousands of sites across the genomes of many individuals and may reveal previously unrecognized genetic structure across environmental gradients over fine geographical scales [52,53]. Despite the accumulation of evidence indirectly suggesting that high and low elevation mountain chickadees may be locally adapted to their respective elevations (e.g. [54]), it remains unknown whether or not local adaptation and selection against migrants has generated ecological isolation across the elevation gradient. Here we use a genotyping-by-sequencing approach [49,55–57] to generate thousands of single nucleotide polymorphisms (SNPs) to assess genetic differentiation and population structure across six sampling sites; birds were sampled at high and low elevation across three transects. Our goal was to assess if high and low elevation birds exhibit fine-scale genetic structure that could potentially arise from divergent selection between high and low elevation habitats.

2. Methods

2.1. Tissue sample collection

In the autumn of 2013, we sampled 167 birds from three pairs of high (2535–2590 m) and low elevation sites (1891–2122 m) in the Sierra Nevada (table 1); Sagehen Experimental Forest, CA (exact same locations sampled as those used in all phenotypic variation studies [19,22,23,31,32,39]), Mount Rose, NV (exact same locations sampled as those used to show male song structure variation [23]), and Red Lake Peak, CA (no phenotypic variation data have been collected from these locations) (figure 1). Birds were captured using mistnets at established feeders at Sagehen Experimental Forest; the other locations do not have a feeder system in place. At Mount Rose and Red Lake Peak, a mistnet was set and mountain chickadee song was broadcast from the centre of the net using a FOXPRO Fury© playback speaker. Upon capture, approximately 100 µl of blood was collected from the basilic vein with a capillary tube and stored directly in Queen's lysis buffer (1 mM NaCl, 1 mM EDTA, 1 mM Tris).

2.2. Population genetic analyses

DNA was extracted from blood samples using a Qiagen DNeasy Blood and Tissue kit (Qiagen Inc., Valencia, CA, USA) and quantified using spectrophotometry on a QIAxpert machine (Qiagen, Inc).

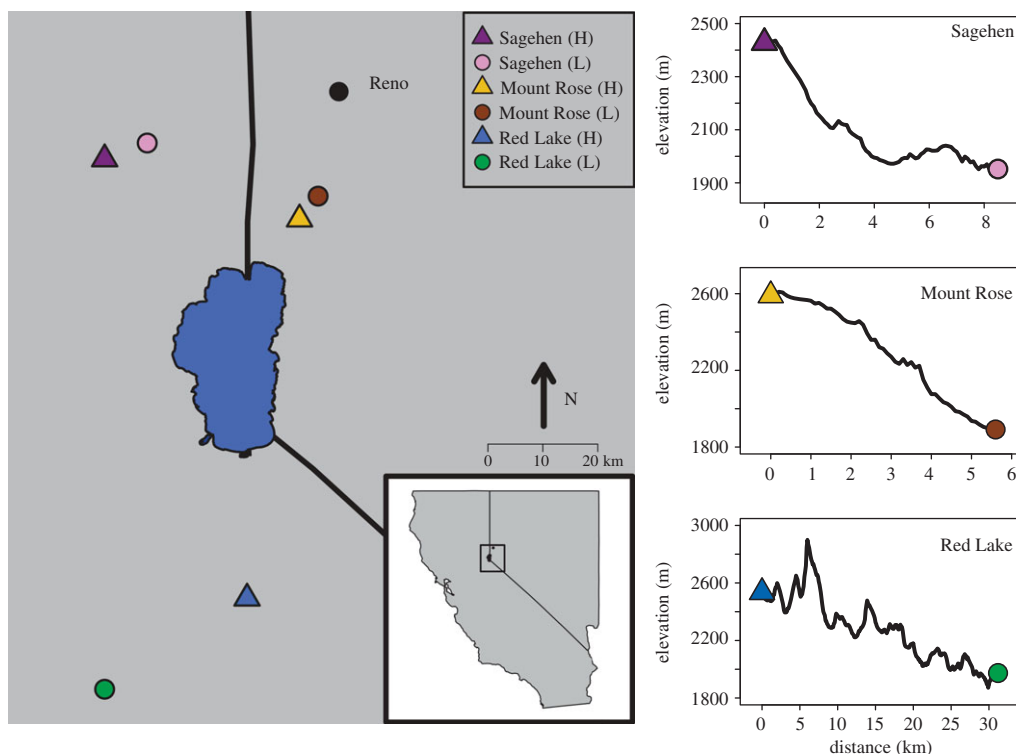


Figure 1. Map and elevation profiles of the six sites where mountain chickadees were sampled (created using R 3.3.0, R Core Team 2015). Inset shows region of California and Nevada where sites were sampled. Large blue shape in the middle of the inset represents Lake Tahoe. Panels show the elevation profiles found between the three pairs of elevational transects (points were sampled every 30 m as the duck flies between pairs of sites in ArcGIS). Triangles represent high elevation locations and circles represent low elevation locations.

Table 1. Sample sizes (N), latitude and longitude coordinates, and elevation (m) for each of the six locations where blood samples were collected for genomic analyses. (High (H) and low (L) elevation localities are delineated parenthetically.)

location	latitude	longitude	elevation (m)
Sagehen (H)	39° 25' 7.73"	120° 18' 24.72"	2428
Sagehen (L)	39° 26' 42.36"	120° 13' 02.01"	1952
Mount Rose (H)	39° 19' 11.72"	119° 53' 47.57"	2590.8
Mount Rose (L)	39° 21' 27.99"	119° 51' 26.95"	1891.59
Red Lake (H)	38° 41' 47.09"	120° 00' 25.35"	2535.33
Red Lake (L)	38° 32' 48.59"	120° 18' 24.97"	1973.58

Reduced representation libraries for Illumina sequencing were constructed using a genotyping-by-sequencing (GBS) approach used in multiple previous studies [49,55–57]. Specifically, DNA fragments were cut using two restriction enzymes, EcoRI and MseI, which have cut sites evenly distributed throughout avian genomes [58]. Barcoded adaptors were ligated to the EcoRI cut site, which included an Illumina adaptor, an 8–10 bp barcode unique to each individual bird, and the bases matching the cut site. An MseI adaptor consisting of the opposite Illumina adaptor was ligated to the opposite ends (MseI cutsites) of the fragments. DNA from all individual reactions were pooled and PCR-amplified using Illumina primers. Finally, fragments between 350 and 450 bases in length were size selected using a BluePippin quantitative electrophoresis unit (Sage Science, Beverly, MA, USA). Single end, 100 base reads were generated on one lane of an Illumina HiSeq 2500 at the University of Texas Genomic Sequencing and Analysis Facility (UTGSAF, Austin, TX, USA).

The 100bp reads generated on the HiSeq run were first filtered to remove contaminant DNA (e.g. *Escherichia coli*; PhiX) and low quality reads. A perl script was then used to identify individual barcodes, correct barcodes with errors, and remove reads containing sequences associated with Illumina adaptors or PCR primers. After this step, fragments were 86–88 bases in length. A random subset of

25 million reads was assembled *de novo* using the SeqMan ngen software (DNASTAR Inc.), specifying a minimum match percentage of 95 and a gap penalty of 30 (full details of parameter settings are available from the authors by request). Contigs were removed from the reference if they contained fewer than 10 reads, were over-assembled, or were not 84–90 bp in length. This step produced a reference of genomic regions sampled with our GBS approach, providing a template for subsequent reference guided assembly. DNA sequences from each chickadee were subsequently aligned to the reference with `bwa v7.5` [59] using the `aln` and `samse` algorithms and an edit distance of 4. Because all sampled genomic regions begin with the EcoRI cut site and all HiSeq reads contained 100 bases of sequence, these alignments produced consistently rectangular contigs with even positional coverage.

Variant sites (i.e. SNPs) were called and quantified using `samtools v.0.1.19` and `bcftools v.0.1.19` [59,60]. SNPs were considered if at least 90% of individual birds had at least one read at the position, the site was biallelic, and the minor allele frequency was greater than 5%. For reference contigs containing multiple SNPs, a single SNP was randomly selected to increase independence of SNPs and to decrease the effect of linkage disequilibrium on subsequent analyses. For each bird, genotype likelihoods were calculated for each SNP using `bcftools`. Genotype likelihoods were initially stored in Variant Call Format (.vcf) and then converted to a composite genotype likelihood format. Genotype likelihood matrices and assembly related files are available at Dryad and additional information regarding parameter settings is available from the authors upon request.

To account for uncertainty associated with variation in coverage depth, a hierarchical Bayesian model [55] was employed to estimate genotype probabilities based on the genotype likelihoods estimated above. This model treats population allele frequencies as priors and simultaneously estimates both allele frequencies and genotype probabilities after accounting for variation in coverage. Essentially, individuals with low coverage for a given locus will have genotype probabilities more heavily informed by the prior (i.e. the allele frequency), while high coverage loci will have genotype probabilities with higher certainty of the homozygous or heterozygous genotypes. The model was run for 10 000 Markov chain Monte Carlo steps (thinning every other step) with a 6000 step burn-in to obtain the posterior estimates of genotype probabilities, which were subsequently stored as convenient composite genotype values (ranging from 0 to 2; 0 and 2 for homozygous and 1 for heterozygous genotypes) for use in all downstream analyses.

The distribution of genetic variation among individuals was first summarized with principal component analysis (PCA) using the `prcomp` function in R [57]. As a complementary approach to PCA, we performed discriminant analysis of principle components (DAPC) [61] using the `adegenet` package [62,63] in R. While PCA is inherently constrained to maximize the total variance explained in the data, DAPC maximizes the proportion of variance explained among groups of individuals [64]. Additionally, DAPC is useful for GBS datasets because it finds the most likely number of clusters within a dataset using k-means clustering and calculates individual assignment probabilities much faster than more computationally intensive Bayesian clustering algorithms commonly used in molecular ecology studies (e.g. STRUCTURE [65]). The most likely number of clusters for DAPC was calculated using the `find.clusters` function and the appropriate number of retained PCs was estimated using the `optim.a.score` function. For both PCA and DAPC, permutational multivariate analysis of variance (PERMANOVA [66]) implemented in the `vegan` package [67] of R was used as a post hoc test for genetic differentiation among sampling sites based on Euclidean distances of the first two ordination axes.

We summarized the degree of genetic differentiation among sampling sites by calculating Nei's genetic distance (D [68]) for each pairwise combination of sites. We also calculated pairwise genome-wide F_{ST} using Hudson's estimator [69] as an additional metric of differentiation among sites, and tested the significance of these estimates using a permutation-based approach. To test for the effects of geographical distance and elevational distance on genetic distances among sampling sites, we used a multiple regression on distance matrices (MRM [70]) using the `ecodist` package [71] in R, with pairwise genetic distance (D) as the response variable and pairwise elevational and geographical distance as the predictor variables. We estimated geographical distances among sampling sites by calculating Haversine distances from latitude and longitude coordinates using the `fossil` package in R [72]. Finally, we examined how hierarchical variation was distributed among individuals, among transects, and among the high and low elevation sites within each transect using AMOVA [73] as implemented in the R `poppr` package [74,75]. For this analysis we only used genotype probabilities with relatively high certainty of either the homozygous or the heterozygous genotype (point estimates within 0.1 from 0, 1 or 2), and treated others as missing data. We tested for significance of ϕ statistics with a permutation-based approach using the `randtest` function in the `ade4` R package [76].

To test for patterns of parallel locus specific allele frequency shifts across low and high elevation groups, we quantified locus specific F_{ST} estimates for all loci for the high and low elevation contrasts at

each of the three geographical areas. We considered loci residing above the 97th, 98th and 99th quantiles of the F_{ST} distribution for each elevational contrast as outliers potentially residing in genomic regions experiencing directional selection. We evaluated potential parallel differentiation using a permutation-based approach to assess significance of the counts of loci that had extreme F_{ST} values in more than one elevational contrast. More specifically, the loci in all three elevational comparisons were permuted and then we counted the number of loci that resided in the upper quantile in multiple elevational comparisons; permutations were conducted 10 000 times to create a null distribution.

3. Results

3.1. Population genetic structure

One lane of sequencing on the Illumina HiSeq generated 225 302 911 reads. After cleaning contaminants, parsing bar codes, and removing sequences containing pieces of the Illumina adaptors or primers, 185 281 261 reads from 167 individual birds were retained for analyses. *De novo* assembly of a subset of 25 million reads aligned 21 269 517 reads into 313 976 contigs with a coverage depth greater than 10. The consensus sequences of these contigs were retained as a GBS reference onto which we assembled reads from each individual bird. The final reference based assemblies (executed using *bwa*) placed 143 928 656 reads across all individuals into alignments. Thus, the final alignments contained 77% of the cleaned reads. After removing loci with minor allele frequency < 0.05 , we retained genotype estimates for 37 252 loci in the 167 individual birds. We further filtered for SNPs with a minimum coverage depth of $4\times$ and maximum of $15\times$ per locus and a minimum of coverage depth of $3\times$ and a maximum $10\times$ per individual. After this filtering step we retained a final set of genotypes at 18 073 loci in 151 birds that passed our coverage criteria.

The first two principal components accounted for 5.77% of the genotypic variation and revealed no evidence that individuals from the six separate sampling sites were more genetically similar to each other than to those from other sites (PERMANOVA $R^2 = 0.025$; $F_{5,145} = 0.742$; $p = 0.653$) (figure 2). In addition, there was no evidence for overall genetic differentiation among high and low elevation sites (PERMANOVA $R^2 = 0.001$; $F_{1,149} = 0.201$; $p = 0.870$). Similarly, pairwise genome-wide F_{ST} and Nei's D estimates for samples of chickadees from different sampling sites were consistent with minimal genetic differentiation (mean $F_{ST} = 0.020$, range: 0.010–0.029). Groups of chickadees from high elevation sampling sites did not consistently exhibit significant genetic differentiation from neighbouring low elevation sites, and the genome-wide F_{ST} estimates for most contrasts were not significantly different from zero (table 2). Although some F_{ST} estimates were statistically larger than zero, the values of these estimates were very small (table 2). Consistent with the absence of genetic structure among sampling sites, geographical distance was unrelated to genetic distance (Mantel $R^2 = 0.053$; $p = 0.520$; figure 3*a*) and elevational distance was unrelated to genetic distance (Mantel $R^2 = 0.002$; $p = 0.713$; figure 3*b*). Hierarchical analysis of genetic variation using AMOVA partitioned nearly all of the variance within samples (99.5%), with minute proportions attributed to variation among transects (0.49%) and across elevations within transects (0.01%). Based on these analyses, there was no evidence for significant genetic structuring among transects ($\phi = 0.004$; $p = 0.10$) or among different elevations within transects ($\phi = -0.001$; $p = 0.59$). One PC was retained for the DAPC analysis, which found $K = 2$ as the most likely number of clusters in explaining the data (figure 4). Individuals with high assignment probabilities for each of the two clusters were found at each site and across elevations (figure 4), consistent with a lack of any discernable population structure.

Although there was little evidence for genome-wide genetic differentiation among populations, we examined the distributions of locus specific F_{ST} in order to evaluate whether individual loci exhibited elevated differentiation in multiple elevational contrasts, which would be consistent with such loci tagging genomic regions responding to elevation-related selection. There were a small number of loci that were outside the 97th, 98th and 99th quantiles of the genome-wide F_{ST} distribution in two elevational contrasts ($N = 71$, 38 and 8, respectively), and few to zero loci that were outside these quantiles in all three elevational contrasts (table 3). For all three F_{ST} cut-offs, we observed more loci in the upper F_{ST} distributions for two transects than expected from permuted null simulations (table 3). Additionally, there were more loci in the 97th quantile for all three elevational transects ($N = 2$) than expected (table 3). Thus, there is subtle evidence for parallel allele frequency shifts in the same genomic regions. However, all of the loci that did exhibit parallel shifts had $F_{ST} \leq 0.3$, reflecting relatively weak allele frequency differences even for the very small number of loci that exhibited the most pronounced allele frequency differential.

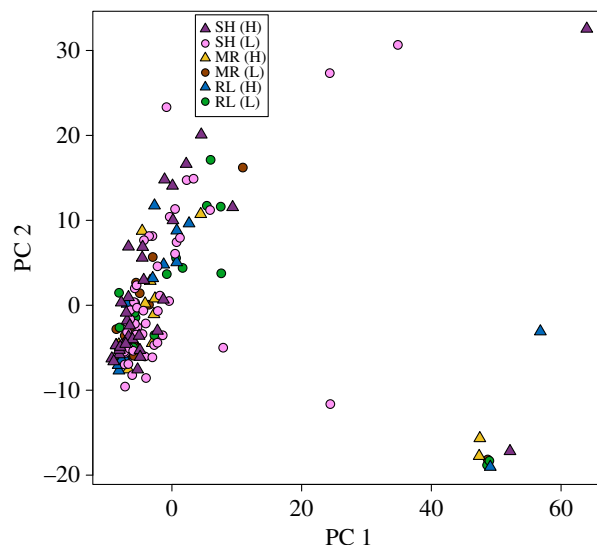


Figure 2. Genetic variation among individual mountain chickadees, as illustrated by the first two principal components from a PCA on the genotype covariance matrix. Individuals from different sampling sites are represented by different colours; high and low elevation sites are labelled in the legend with H and L and plotted as triangles and circles, respectively. MR, Mount Rose; RL, Red Lake; SH, Sagehen.

Table 2. For each pairwise site comparison, the observed mean F_{ST} [69] was compared to a null distribution of F_{ST} estimates constructed from 100 permutations of site identity. (Observed mean F_{ST} estimates residing outside of the null 95% confidence intervals were deemed significantly larger (*) or smaller (***) than expected by chance. Low (L) and high (H) populations are delineated parenthetically after population abbreviations (MR, Mount Rose; RL, Red Lake; SH, Sagehen).)

site 1	site 2	null mean	null s.d.	95% lower	95% upper	observed mean
MR (L)	RL (H)	0.0279	0.0027	0.0273	0.0284	0.0290*
MR (L)	RL (L)	0.0248	0.0024	0.0243	0.0253	0.0274*
MR (L)	MR (H)	0.0240	0.0019	0.0236	0.0244	0.0251*
MR (L)	SH (H)	0.0201	0.0018	0.0198	0.0205	0.0221*
MR (L)	SH (L)	0.0179	0.0007	0.0178	0.0181	0.0205*
RL (H)	RL (L)	0.0243	0.0021	0.0239	0.0247	0.0224***
RL (H)	MR (H)	0.0247	0.0028	0.0242	0.0253	0.0235***
RL (H)	SH (H)	0.0205	0.0022	0.0201	0.0209	0.0200***
RL (H)	SH (L)	0.0187	0.0011	0.0185	0.0189	0.0190*
RL (L)	MR (H)	0.0215	0.0024	0.0210	0.0219	0.0210
RL (L)	SH (H)	0.0173	0.0015	0.0170	0.0176	0.0173
RL (L)	SH (L)	0.0155	0.0009	0.0153	0.0157	0.0164*
MR (H)	SH (H)	0.0165	0.0013	0.0162	0.0168	0.0173*
MR (H)	SH (L)	0.0146	0.0007	0.0145	0.0148	0.0160*
SH (H)	SH (L)	0.0104	0.0007	0.0103	0.0106	0.0101***

4. Discussion

Despite evidence of large elevation-related phenotypic divergence at two of our sampling locations (Sagehen Experimental Forest and Mount Rose), we detected little evidence for genetic differentiation between high and low elevation locations or across space for the geographical region we sampled. There was no indication in PCA or DAPC analyses of any population structure (figures 2 and 4), and pairwise F_{ST} estimates were small, with many not statistically different from zero. Similarly, geographical

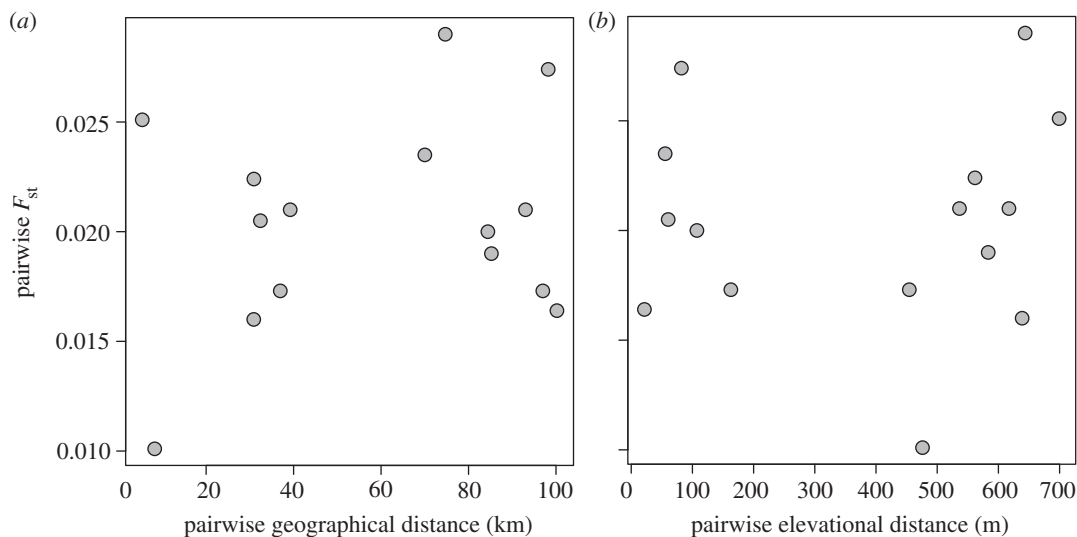


Figure 3. Geographical distance (a) and elevational distance (b) were not related to pairwise genome-wide F_{ST} estimates for each of the six sampling sites. Geography Mantel $R^2 = 0.053$; $p = 0.520$. Elevation Mantel $R^2 = 0.002$; $p = 0.713$.

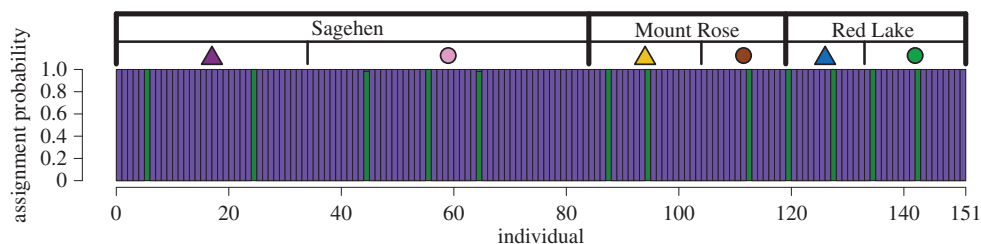


Figure 4. DAPC analyses found support for two genetic clusters of individuals. Individual assignment probabilities for each cluster are plotted, with individuals grouped by sampling locality. Sampling locations are indicated at the top of the plot, and separated by vertical bars. Individuals with high assignment probabilities for each cluster are found in every sampling locality, consistent with a pattern of no population genetic structure.

Table 3. Summary data from tests of parallel differentiation for individual loci across multiple transects. (The number of observed loci that were extreme (based on three F_{ST} cut-offs) in two or three elevational contrasts was compared to mean expected values based on 10 000 permutations of the loci (95% confidence intervals are listed parenthetically). Observed values greater than expected 95% confidence interval upper bounds are suggestive of parallel differentiation across elevational transects.)

F_{ST} quantile	observed		expected	
	2 transects	3 transects	2 transects	3 transects
99th	8	0	5.40 (4.96 to 5.84)	0.015 (−0.01 to 0.04)
98th	38	0	21.27 (20.40 to 22.13)	0.147 (0.07 to 0.22)
97th	71	2	47.33 (46.07 to 48.59)	0.493 (0.36 to 0.63)

and elevation distances were not related to genetic distances (figure 3). Finally, a hierarchical AMOVA revealed no evidence for genetic variation structured by transect or by high and low elevation sites within transects. Mountain chickadees inhabit a continuous gradient of habitat among and within the locations that were sampled, and experience no known geographical barriers to movement. A lack of geographical barriers and the fact that birds can be highly mobile, make the absence of population genetic structure at this geographical scale perhaps unsurprising [43,77,78].

All chickadee species are known to be resident birds that disperse short distances from their natal site and remain fairly sedentary for the rest of their lives [28,45,79]. Preliminary data on natal dispersal and movement in mountain chickadees inhabiting our Sagehen location agree with this previous work, and show that chickadees disperse on average between 0.04 and 2.4 km from their nest-boxes. Despite these

sedentary habits, there are few examples of population genetic structure, particularly on small spatial scales, in Paridae. These instances involve limited gene flow between populations as a result of habitat differentiation (e.g. urban park fragmentation in *Parus major* [80] and deciduous versus evergreen oak forests in *Cyanistes caeruleus* [81]). Unlike these examples, our mountain chickadee populations inhabit a continuous gradient of mixed conifer forests. As such, it is possible that even though juveniles in each generation disperse only a short distance, over multiple generations such movements may close the gap between high and low elevations, especially considering that one migrant per generation can abolish any population genetic structure [82,83]. While the conifer species differ as elevation changes, there is no true habitat fragmentation or differentiation, which probably allows enough gene flow to prevent population genetic structure. Given the low marker density our data represent (1 SNP per approximately 83 kb based on an expected genome size of approximately 1.5 Gb (e.g. *P. major* [84])), these SNPs could fail to sample regions of the genome containing variants influencing spatial memory ability between high and low elevation birds.

Instances of loci exhibiting exceptional divergence (outlier loci) across replicated environmental gradients could arise when loci are linked to genomic regions involved in local adaptation (e.g. [3,10,85,86]). Although the elevational gradient that our study populations inhabit could lead to such a pattern, we found little evidence for any of the 18073 loci we analysed exhibiting parallel patterns of exceptional divergence across high and low elevations. There are several reasons why the genetic variation in our data is unlikely to reflect local adaptation to high and low elevation environments. First, given the low marker density of our data, and expected patterns of linkage disequilibrium in avian genomes (e.g. [87,88]), GBS data may have limited ability to detect genetic regions responding to selection in many cases.

Second, variation in complex quantitative traits often involves large numbers of small effect loci [87,89–95]. Cognition and other behavioural phenotypes that exhibit divergence between low and high elevation chickadees are likely to have a strongly polygenic basis (see review in [94]). Polygenic adaptation resulting from many small effect loci has been notoriously difficult to detect and understand, especially when effective population size is large and linkage disequilibrium decays rapidly [92,95,96]. For example, recent genome-wide studies in birds have failed to detect any variants associated with polygenic phenotypes [97], even when using whole genome resequencing approaches [87]. Understanding the genetic basis of complex behavioural phenotypes such as cognition could require whole genome resequencing studies with very large numbers of individuals. Owing to the immense difficulties and uncertainties involved with quantitative trait mapping [96], it may be that assessing fitness consequences and heritability of cognition and other polygenic behavioural phenotypes may be a more promising approach for detecting evidence of local adaptation.

Previous work on this mountain chickadee system has shown that high and low elevation birds exhibit differences in spatial memory ability, hippocampal morphology, social dominance, novel environment exploration, problem solving and proactive aggression [19,31,39,98,99], as well as significant differences in mate preference [22], and male song structure [23]. In addition, several of these phenotypic differences between high and low elevation chickadees have been documented over multiple years despite large climatic variation among these years [32,54]. If these phenotypes have even moderate heritability, this variation would be consistent with local adaptation, as migrants moving from low to high elevations are likely to experience decreased fitness owing to inferior memory ability [19,32] and assortative mating [22], while migrants moving from high to low elevations are likely to experience decreased fitness owing to their low social dominance status [39].

Adaptation in the presence of gene flow is quite common in many species, including birds [11,14,15,20,81,100–104], however, we do not have definitive evidence that the phenotypic differences between high and low elevation birds are the result of local adaptation rather than phenotypic plasticity. It is likely that some of the variation we see across these numerous phenotypic traits is influenced by phenotypic plasticity resulting from climatic variation in high and low elevation environments. On the other hand, spatial memory ability and hippocampal morphology are moderately to highly heritable, at least in mammals, and therefore are capable of responding to selection [33–38] (also see [94] for in depth review). Assuming there are environment related differences in selection pressures on spatial memory at different elevations (e.g. [19,54]), these differences in selection pressure may result in variation in spatial memory and hippocampal morphology, even in the presence of gene flow.

Overall, our study detected no elevation or geographical related population genetic structure despite numerous phenotypic differences previously documented in mountain chickadees. Future work should now focus on identifying the mechanisms underlying elevation-related phenotypic variation in these

birds. In order to understand the role of local adaptation in driving these phenotypic differences, it might be more fruitful to concentrate on measurements of heritability and selection, as opposed to genome-wide association techniques, owing to the well-known difficulties associated with identifying the genetic basis of highly polygenic traits [92,95,96].

Ethics. All procedures were in accordance with the UNR IACUC protocol (00046) and with Federal Fish and Wildlife Permit no. MB022532, California Fish and Game Scientific Collecting Permit D-0011776516, and Nevada Department of Wildlife Scientific Collecting Permit no. MB022532.

Data accessibility. Genomic data: a directory with .bam files containing sequence alignment data for each individual, and a matrix of genotype probabilities used for population genetic analyses are deposited at Dryad: <http://dx.doi.org/10.5061/dryad.686rq> [105].

Authors' contributions. C.L.B. participated in the conception and design of the study, collected field data for molecular work, conducted DNA extractions, and drafted the manuscript. J.P.J. aided in analysis of genetic data and created the site map. D.Y.K. collected field data for molecular work. T.L.P. participated in the design of the study, generated and analysed all genetic data, drafted the manuscript, and provided funding for the project. V.V.P. participated in the conception and design of the study, collected field data for molecular data, drafted the manuscript, and provided funding for the project.

Competing interests. We have no competing interests.

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CONCLUSION

Throughout my dissertation, I have investigated premating mechanisms potentially associated with elevation-related variation in food-caching mountain chickadees (*Poecile gambeli*). Applying behavioral and genomic techniques to mountain chickadees in habiting a continuous elevation gradient in the Sierra Nevada, I have elucidated elevation-related female preference (chapter one), variation in male song structure consistent with vocal dialects and local adaptation (chapters two and four), no differential response to local versus nonlocal elevation male intruders (chapter three), and finally, no population genetic structure among high and low elevation birds (chapter five). Taken together, the findings from my dissertation along with the previous findings of elevation-related variation in spatial memory and hippocampal morphology (Freas et al. 2012; Freas et al. 2013a; Freas et al 2013b; Croston et al. 2016), suggest that mountain chickadees inhabiting an elevational gradient in the Sierra Nevada represent local adaptation in the presence of gene flow.

Arguably, what is most astounding about the findings presented throughout my dissertation, and on this chickadee system as a whole, is that these phenotypic differences exist along a rather small spatial scale in birds that are highly mobile and continuously distributed. Furthermore, to the best of my knowledge, this is the first evidence of song dialects within a single, continuously distributed population of birds (chapter four). Previous work investigating song dialects along continuous habitat distributions has either shown distinct genetic variation between populations living in parapatry (Irwin et

al. 2008; Caro et al. 2013) or has found support for gradual clinal variation and not for song dialects (Irwin 2000; Sung & Handford 2006; Irwin et al. 2008).

Finding support for vocal dialects in mountain chickadees at different elevations has important implications for the evolutionary trajectory of these birds (Edwards et al. 2005; Podos & Warren 2007), as these differences in song structure likely represent indicators of local adaptation, and vocal dialects may result in divergence between these birds. Ultimately, as the climate continues to change (Fischer & Knutti 2015), separation of these birds may have important implications for their success and genetic variation, as warming will likely increase the area that low elevation birds are suited to colonize, while high elevation birds, and their associated genetic variation, may decline. Recent work in this chickadee system shows that large variation in annual precipitation events, such as severe drought and heavy snow fall, has significant effects on the breeding patterns and reproductive output of these birds (Kozlovsky et al. 2018). Because mountain chickadees inhabit a gradient of climatic severity, and are of least concern from a conservation standpoint, they may be a prime study system for addressing effects of global climate change; particularly as severe weather events increase in frequency (Peterson et al. 2013; Kunkel et al. 2016).

FUTURE DIRECTIONS

Future research using this mountain chickadee system aims to address the relationship between male song production, female mate choice, and spatial memory ability. If females use song to assess male quality, and male fitness is linked to their ability to recover stored food using spatial memory, then it may be that song production and spatial

memory abilities are coupled or correlated. Females may not be able to directly assess the spatial memory abilities of a male; however, they can assess male song, and may use song as a cue to mate with male who have superior spatial memory abilities.

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