A MULTI-DISCIPLINARY ANALYSIS OF A TITMOUSE (*BAEOLOPHUS*) HYBRID ZONE IN TEXAS

A Dissertation

by

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ABSTRACT

Hybridization, or interbreeding between two distinct species, can result in a variety of evolutionary consequences. To understand genetic introgression and stability of a hybrid zone, it is crucial to investigate multiple biological facets including behavior, communication, habitat, genetics, and even physiology. This study aims to better understand hybrid zone dynamics between the Black-crested and Tufted Titmouse by investigating differences in physiology, song structure, behavior, morphology, plumage, and genetic assignment across a hybrid zone in central Texas. Located along the Balcones Escarpment, an inactive fault zone, this hybrid zone occurs at a strong ecotone between the semi-arid scrub habitat of west Texas and the eastern mesic hardwood forests. By collecting individuals outside (both east and west) and within the hybrid zone, I was able to analyze blood analytes for physiological differences, compare morphological differences, and extract DNA for mitochondrial and microsatellite analyses. Furthermore, I conducted playback experiments and recorded songs to understand more about interspecific communication. Overall, species differed in physiology, song structures, aggression levels, body size, and mitochondrial haplotypes. Birds west of the Balcones Escarpment (including the hybrid zone) have higher glucose values that increase following rainfall events indicating Black-crested Titmice are physiologically adapted to the scrub habitat with intermittent rainfall. Songs of species are easily distinguished by note number and tempo and Tufted Titmice appears a more aggressive species. Tufted Titmice are large in body size compared to Black-cresteds with differences linked to habitat characteristics. In general, birds within the hybrid zone are more similar to Black-crested Titmice than

Tufteds. This was shown by similarities in body size, physiology, song structure, plumage, mitochondrial haplotypes, and microsatellite cluster assignment. Nuclear admixture (hybridization) was observed in low numbers, primarily within the hybrid zone, but analyses did not reveal a distinct cluster of genetic hybrids. Most importantly, genetic assignment did not match with a commonly used hybrid index such that crest plumage is not a reliable indicator of hybrid individuals or even species. I recommend that future research include a greater diversity of molecular markers, increased site collection for clinal analysis, and more behavioral research to better understand mate selection and hybrid fitness costs.

DEDICATION

A 10-year journey lends itself to multiple dedications

For my *parents* who sparked my passion for learning and the natural world and instilled in me the belief that my learning disability was not a reason to give up, rather a reason to try harder.

For my *family and friends*, especially my husband, who support my love of learning and encourage my natural curiosity.

For the *women* in my life who have struggled with and/or lost their battle to cancer or heart disease. These women have cheered on my love of science and their strength spurred my perseverance to complete this journey.

This is in memory of you

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CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a dissertation committee consisting of Drs. Gary Voelker (committee chair), Jessica Light, and Michael Morrison of the Department of Rangeland, Wildlife, and Fisheries Management as well as Dr. Gil Rosenthal of the Department of Biology. Dr. J. Jill Heatley, of Texas A&M's College of Veterinary Medicine, provided financial and editing assistance for Chapter 2.

Data collection, analysis, and all other work conducted for this dissertation were completed by the student with occasional assistance from undergraduate students as field technicians and graduate students during laboratory analysis.

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NOMENCLATURE

ANOVA	Analysis of Variance
BCTI	Black-crested Titmouse (*Baeolophus atricristatus*)
BE	Balcones Escarpment
BEecf	Base excess
BUN	Blood urea nitrogen
Cl-	Chloride
EP	Edwards Plateau
GC	Glucocorticoids
Hb	Hemoglobin
HCO_3	Bicarbonate
Hct	Hematocrit
iCA+	Ionized calcium
mtDNA	Mitochondrial DNA
Na+	Sodium
pCO_2	Partial pressure carbon dioxide
PCV	Packed cell volume
tCO_2	Total carbon dioxide
TUTI	Tufted Titmouse (*Baeolophus bicolor*)
TX	Texas
sO_2	Dissolved venous oxygen

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1. INTRODUCTION

Contact zones (or hybrid zones if hybridization confirmed), where two species potentially interbred, have long fascinated scientists by acting as an in-situ laboratory to explore a host of ecological and evolutionary topics resulting from continual exchange of genetic material (Harrison, 1993). How contact/hybrid zones form, and the outcomes from repeated admixture, are impacted by a variety of factors (Abbott et al., 2013; Arnold, 1997; Arntzen et al., 2017; Harrison & Larson, 2014; Shurtliff, 2013). In vertebrates, the most common type of hybrid zone is a product of secondary contact which occurs when two species come into contact, often through range expansion of one or both species (Abbott et al., 2013; Arnold, 1997). In North America, many known terrestrial hybrid zones are a result of the oscillating glacial periods that occurred during the late Pleistocene epoch (1.8 million to 12,000 years BP; Avise & Walker, 1998; Hewitt, 1996; Klicka & Zink, 1999; Mila et al., 2000).

Once considered rare and unique in vertebrates, hybridization is increasingly being confirmed in numerous taxa with studies focusing on reproductive fitness, behavior, selection, systematics, and various other evolutionary processes (Abbott et al., 2013; Buggs, 2007; Shurtliff, 2013). Advances in technology are creating more opportunities to study the impact of hybridization. Taking advantage of new technologies, I am investigating an avian contact zone in central Texas along the Balcones Escarpment (BE) (Balcones Contact Zone), an inactive fault zone and natural ecotone (transition between two habitats) (Abbott & Woodruff Jr, 1986; Kostecke, 2008; Toomey III et al., 1993). The BE is the central, and largest, of three contact zones for the Black-crested Titmouse

(Paridae: *Baeolophus atricristatus*) and Tufted Titmouse (*B. bicolor*) (Dixon, 1955), with others occurring north and south of the Balcones Contact Zone.

Black-crested and Tufted Titmice are non-migratory songbirds that share many similarities but can be distinguished by their plumage and habitat preference. Black-cresteds are distinguishable by their black crest and white forehead and preferences for semi-arid scrub habitat east of the BE with their range continuing west across the Edwards Plateau (EP) and south into northern Mexico (Patten & Smith-Patten, 2008). Tufted Titmice, on the other hand, have a black forehead and all grey crest (Ritchison et al., 2015). They prefer mesic hardwood forests east of the BE; their range continues east across the United States and north into southern Canada. Although they appear similar in size, the Tufted Titmouse is statistically larger than the Black-crested (Dixon, 1955; Ritchison et al., 2015). Both species sing songs with the repeated phrase "Peter, Peter, Peter" but Black-cresteds are thought to sing more notes per phrase and at a higher frequency compared with Tufteds (Coldren, 1992). Both species are sexually monomorphic and are thought to share a generalist diet (Patten & Smith-Patten, 2008; Ritchison et al., 2015). Long suspected of hybridization, these two sister species have oscillated between full species status and subspecies status for the last 120 years (Allen, 1907; Banks et al., 2002). Early genetic work confirmed they are closely related but separate species, but evidence for hybridization has been suggested by morphology and plumage (Gill et al., 1989; Gill & Slikas, 1992). In 1955, Dixon published the first large scale work on the two (at the time) subspecies and created a hybrid index scale to identify each subspecies and hybrids (Dixon, 1955). Since this time, researchers have relied on this hybrid index for identification without confirmation from genetic analysis.

In this study, I aimed to utilize technological advances to advance the understanding of interactions between these species by focusing on physiology, communication (vocal and behavioral), morphology, plumage, and genetics. The results of this study should provide a stronger understanding of the relationship between Black-crested and Tufted Titmice as well as a broader understanding of hybrid/contact zone dynamics when a multi-disciplinary approach is employed.

1.1. Aim 1: Physiology and Habitat

An under-utilized tool in understanding contact zone interactions is physiology, a known limit to geographic ranges (Bozinovic & Naya, 2015). Since most hybrid zones occur at an ecotone, or sharp habitat transition (Harrison, 1993), interspecific interactions require exposure to different habitats of which they may not be physiologically adapted. Any physiological limitations to a habitat will likely impact the development and/or expansion of a hybrid zone. In this study, I used portable clinical analyzers to measure analytes and electrolytes of venous blood collected from both species to determine if there are physiological differences between the species as well as determine physiology divergence with the hybrid zone (Figure 1.1). Knowing Black-crested Titmice have long survived in semi-arid scrub habitat (Patten & Smith-Patten, 2008), but Tufted Titmice prefer mesic forest (Ritchison et al., 2015), I suspect there are physiological differences between the species and these differences may impact range distribution.

1.2. Aim 2: Avian Communication

If closely related species live sympatrically, and are possibly hybridizing, it is essential to understand communication between species (Catchpole & Slater, 2008; den Hartog et al., 2007; Wilkins et al., 2013). In birds, communication is not only essential in survival and territorial defense but is used in mate attraction (Courter & Ritchison, 2010; Demko et al., 2016; Gil & Gahr, 2002; Naguib & Riebel, 2014; Searcy et al., 2006). Therefore, hybridization would require heterospecific recognition. Utilizing song recording and playback experiments, I investigate differences in song structure and species recognition. I focus on understanding species differences outside the contact zone as well as assessing songs and behaviors within the contact zone (Figure 1.1). I expect to confirm previous research showing species-specific songs, but I also hypothesize birds within the contact zone will sing a mixed-species song. I expect similar findings with behaviors predicting only conspecific recognition outside the contact zone but heterospecific recognition within.

1.3. Aim 3: Morphology and Molecular Genetics

Prior to this study, Black-crested and Tufted Titmice were classified as separate species based on mitochondrial divergence (Gill et al., 2005). Despite no genetic evidence, Black-crested and Tufted Titmice hybrids have been reported based on intermediate body size or plumage, often using the Dixon Hybrid Index (DHI) (Allen, 1907; Curry & Patten, 2014; Dixon, 1955). In this study, I use a combination of approaches to assess differences across and within the contact zone including morphological measurements, plumage identification, and genetic analyses (Figure 1.1). I used microsatellite (nuclear) DNA to determine if hybridization is occurring and compared genetic DNA to plumage

identification based on the DHI. I also analyzed mitochondrial DNA, using a different gene than previously published, to compare genetic divergence rates with previously reported genetic distances. Finally, I used morphological measurements of birds across central Texas to assess morphological variation between species and determine if body size was intermediate within the Balcones Contact zone, compared to parental species.

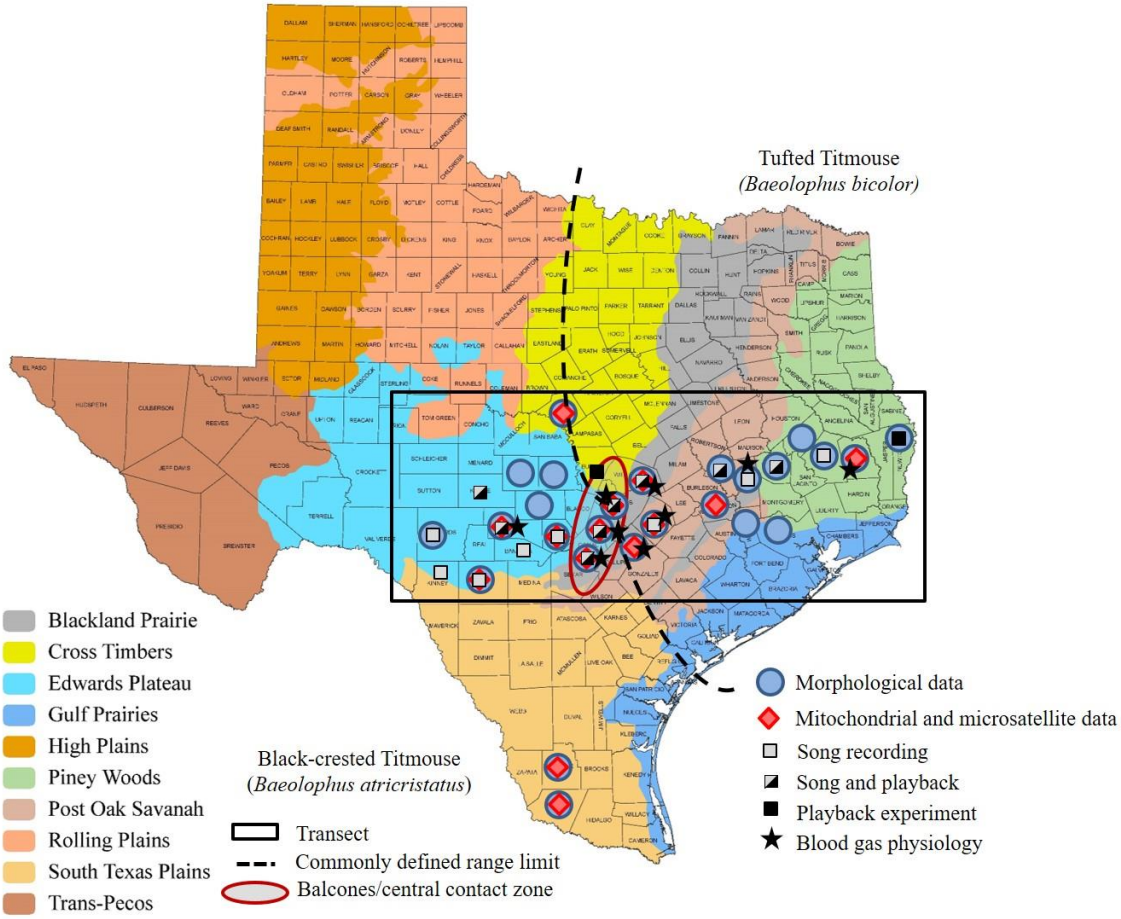


Figure 1.1 Map of collection locations across Texas. The black rectangle indicates the main research area referred to as the transect. The distribution range of the Titmice indicated by the dashed line with Black-crested occurring west of the line and Tufted Titmice east. The red oval indicates the Balcones Contact Zone. Morphological measurements taken from birds collected at sites indicated by blue circles. Red diamonds indicate sites where DNA was collected. Squares indicate song (gray) and playback (black) experiment locations. Black stars represent locations used in blood physiology analysis. Reprinted from Texas Parks and Wildlife modified map by Gould et al. (1960).

2. GLUCOSE CONCENTRATIONS IN CLOSELY RELATED TITMICE (*BAEOLOPHUS*) SPECIES LINKED TO REGIONAL HABITAT DIFFERENCES ACROSS AN AVIAN HYBRID ZONE[*]

2.1. Introduction

Geographic regions in which species interbreed or hybridize, commonly known as hybrid zones, are unique locations in which to examine speciation and adaptation (Barton & Hewitt, 1985). Hybrid zones, in animals, often form via secondary contact of lineages that initially diverged in allopatry (Swenson & Howard, 2005). Beyond genetics, morphology and behavior are classically often used to assess hybridization given their importance in species recognition and mate selection (Curry, 2015; Harrison & Larson, 2014; Miller et al., 2014; Semenov et al., 2017). However, during isolation, sister lineages may also develop physiological adaptations to different environmental conditions (Eme et al., 2014; Kotlik et al., 2014). Post-isolation, populations, and species expansion may be limited or affected by new adaptations. As secondary contact zones usually occur at sharp ecological boundaries (ecotones), the impact of physiology on distribution may be a critical component to understanding species or populations interactions, especially hybridization of species (Abbott et al., 2013; Endler, 1977).

Physiology is known to play a role in limiting geographic range, but some hybrid zone studies have supported physiological differences as limiting factors to distribution

[*]Reprinted with permission from "Glucose concentrations in closely related Titmice (*Baeolophus*) species linked to regional habitat differences across an avian hybrid zone" by Jennifer C. Vaughn, J. Jill Heatley, and Gary Voelker, 2020. *The Open Ornithology*, 13, 10-23, Copyright 2020 held by the authors.

(Bozinovic & Naya, 2015). Once range expansion or distribution is limited, genetic introgression, selection, and other evolutionary forces are impacted. For example, spatial distribution in two hybridizing swordtail fish was linked to differences in heat-tolerant gene expression in varying water temperatures (Culumber et al., 2012). In birds, hybrid tit-tyrant flycatchers in the Andes mountains are not found at the higher elevations preferred by one parental species because they are not physiologically adapted to high elevation living (Dubay & Witt, 2014). Thus, it is crucial that scientists consider physiological adaptations in hybridization research.

In this study we investigated physiological differences between two species of hybridizing songbirds in central Texas. Based on similar studies and new evidence, we suspect that differences are linked to regional habitats and/or climatic distinctions on either side of the hybrid zone.

2.1.1. **Texas hybrid zone**

In Texas, the Balcones Escarpment (BE) is a southwest-to-northcentral oriented inactive fault line that creates a strong west to east ecological boundary (Cadenasso et al., 2003). West of the BE, the Edwards Plateau ranges in elevation from 180 m to nearly 900 m with a semi-arid climate dominated by juniper-oak vegetation, receiving an average of 380-860 mm of rainfall per year, with the majority occurring in May or June (Griffith et al., 2007). East of the BE, lower elevation (0-180 m) and a more temperate climate characterize the more mesic habitats of the Blackland Prairie and Post-Oak forests nearer the BE, and the Piney Woods of the interior coastal plains in eastern-most Texas (Griffith et al., 2007; Webb, 1950). Rainfall in these ecoregions is fairly evenly distributed annually with an

average of 700-1000 mm in the Post-Oak and Blackland Prairie regions and 900-1300 mm in the Piney Woods (Griffith et al., 2007; Webb, 1950).

With this change in elevation, vegetation and rainfall regimes on and east of the BE coincides with a longitudinal distributional barrier for numerous vertebrate taxa, including birds (Goetze, 1995; Hibbits et al., 2019; Owen & Dixon, 1989). In addition to distributional range limits occurring at the escarpment, some species-pairs are thought to hybridize at this ecological interface (Owen & Dixon, 1989; Smith & Buechner, 1947). One avian species-pair recently confirmed to be hybridizing at this interface, is the Black-crested Titmouse (*Baeolophus atricristatus*) and Tufted Titmouse (*B. bicolor*) (Owen & Dixon, 1989). Black-crested Titmice are primarily found in the more arid-scrubby habitat to the west and south (to include northern Mexico) of the BE ecotone, but are occasionally found east of the BE (Patten & Smith-Patten, 2008; Ritchison et al., 2015). Tufted Titmice are found in the more mesic, deciduous forests east of the BE and throughout the eastern United States and Canada (Dixon, 1955; Ritchison et al., 2015).

While these non-migrating sister species share similar diet, breeding/nesting behavior, and general patterns of intraspecific communication, they differ in plumage, song, morphology and habitat preference (Patten & Smith-Patten, 2008; Ritchison et al., 2015). Currently ranked as separate species, their taxonomic status has fluctuated between species and subspecies because ornithologists have postulated hybridization (Patten & Smith-Patten, 2008; Ritchison et al., 2015). Using morphological measurements and crest plumage, Dixon identified several hybrid zones, with the primary one located in central Texas (Dixon, 1955). He estimated this zone to be approximately 50 km wide and 175 km long (Figure 2.1) (Dixon, 1955, 1989). Curry and Patten (2014) recently investigated

plumage and morphological variation within this central hybrid zone and their findings support Dixon's assessments of hybridization. In a related study, Vaughn and Voelker (unpublished) provide genetic evidence of hybridization in birds captured near the BE.

Since this hybrid zone occurs at a strong ecotone, we aim to investigate which blood gases and electrolytes differ between Tufted and Black-crested Titmice and assess if the differences are linked to climate and/or habitat. In a previous study of a suite of passerines, including titmice, we observed differences in venous blood analytes between individuals sampled east of the BE, relative to those sampled west of the BE (Heatley et al., 2015; Heatley et al., 2013). We hypothesize that, between species, differences in blood analyte concentrations will be strongly correlated with habitat or climatic differences associated with the different ecoregions east and west of the BE. We also aim to report the role other non-habitat variables (age, sex, and methodology) have on blood gas and electrolytes within two closely related species. Our hope is to expand the knowledge of the variability of blood gas and electrolytes in wild passerines as well as provide a better understanding of ecological impacts on physiological differences between closely related, hybridizing species.

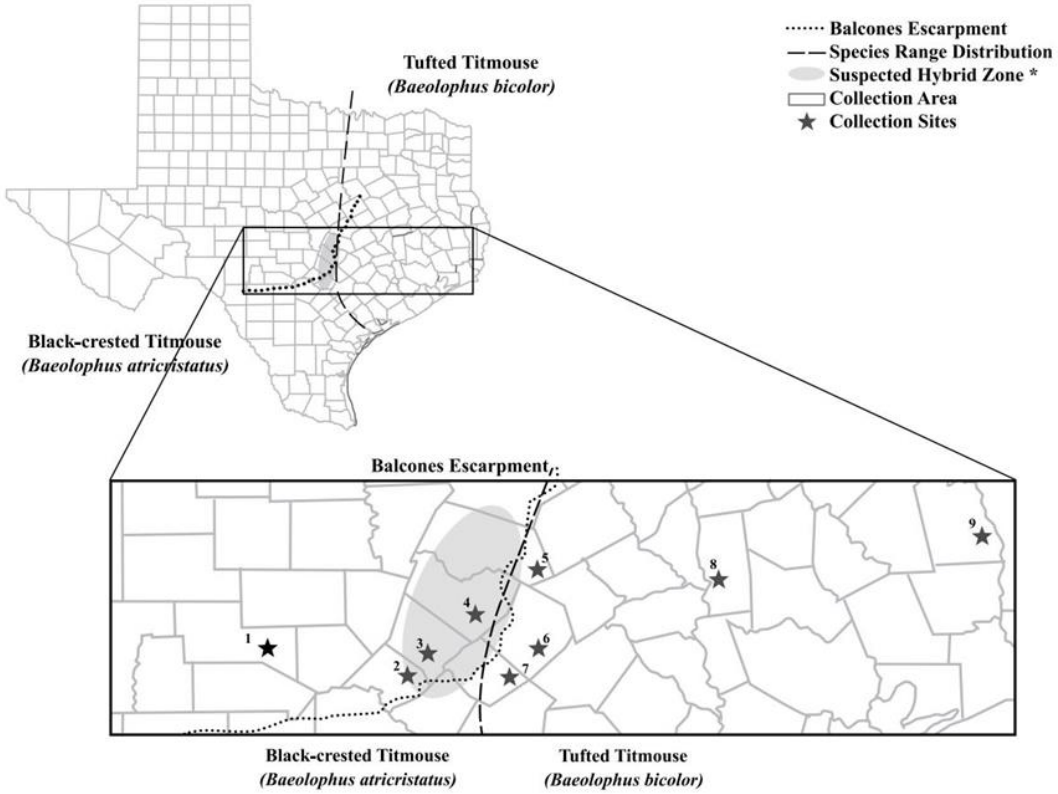


Figure 2.1 Map of locations for sampling (square) of Tufted and Black-crested Titmice. Counties west of the Balcones Escarpment (dashed line) include Kerr (1), Comal (2), Hays (3), and Travis (4). Counties east of the Escarpment are Williamson (5), Bastrop (6), Caldwell (7), Grimes (8), and Tyler (9). The extent of Titmouse species' distribution range in Texas (dotted line) and central contact zone (Dixon, 1955) (shaded oval) are also shown. Map modified and reprinted from WaterProofPaper.com (2020).

2.2. Materials and Methods

2.2.1. **Capture and sampling**

From March–August in 2010-2012, we captured 54 titmice (*Baeolophus* spp.) on private properties in central Texas (Figure 2.1, Appendix A). Most birds were collected via mist nests from 0630-1200 CST; two were caught between 1700-2000 CST. We collected 26 titmice west of the Balcones Escarpment; two of these were captured west of the contact zone (Table 2.1) (Curry & Patten, 2014; Dixon, 1955). East of the BE, we collected 28 individuals. In a related project, we determined that plumage is not a reliable indicator of hybridization or species (Vaughn and Voelker, unpublished). Therefore, for this study, we used mitochondrial DNA for species assignment for all individuals in this study.

Table 2.1 Titmice (*Baeolophus* spp) based on species (mtDNA clade), age, sex, and capture location. Balcones Escarpment consists of "West" and "Contact zone."

	West	Contact zone	East	**Total**
Juveniles	0	11	11	**22**
Adults	2	13	17	**32**
Adult Male	1	10	16	**27**
Adult Female	1	3	1	**5**
Black-crested Titmouse	2	24	0	**26**
Tufted Titmouse	0	0	28	**28**

After removal from the mist net, titmice were held in cloth bags for 5-15 min before blood collection. As part of a larger study, Titmice captured in 2012 (n = 26) were administered 0.01-0.03 mL midazolam (3.9+/- 1.8 mg kg^{-1}; 5 mg ml^{-1} Injection USP

Hospira, Inc.) intra-nasally prior to blood collection for sedation (Heatley et al., 2015). Midazolam is a safe and effective sedative of wild birds (Bigham & Moghaddam, 2013; Mans et al., 2012; Schaffer et al., 2017). Clinical effects of sedation were observed and recorded on a sedation scale (Heatley et al., 2015). Following rest or sedation plus rest, we obtained blood samples (200-500 µL) via jugular venipuncture and immediately transferred to lithium heparin Microtainer tubes (Sarstedt Inc., USA) for anticoagulation prior to analysis.

Samples were analyzed in the field using an i-STAT 1 system (Abbott Laboratories, USA), following standard field protocols (Heatley et al., 2015). Cartridges that evaluated blood gases were used first to minimize change in analytes based on time lapse from sampling (Heatley et al., 2015). Remaining cartridges were analyzed within 5 min of blood collection. Venous blood analytes measured by the i-STAT 1 system were pH, carbon dioxide partial pressure (pCO_2), oxygen partial pressure (pO_2), lactate, ionized calcium (iCa^+), glucose, sodium (Na^+), potassium (K^+), chloride (Cl^-), blood urea nitrogen (BUN), and hematocrit (Hct). The i-STAT 1 system also determined, via calculations, bicarbonate (HCO_3), total carbon dioxide (tCO_2), base excess (BEecf), dissolved venous oxygen (sO_2), and hemoglobin (Hb) (Abbott Point of Care, 2016, 2017). Packed red blood cell volume (PCV) was also determined via standard centrifugal methods (Heatley et al., 2013).

Following sampling, we humanely euthanized and deposited birds as museum specimens at the Biodiversity Research and Teaching Collections (BRTC) at Texas A&M University (College Station, TX). Morphological and genetic information from these birds will be used in future studies. This research was conducted under required Texas Parks and

Wildlife (permit# SPR-0209-016) and United States Fish and Wildlife Permits (permit #188 MB205752) and with approval of the Texas A&M University Institutional Animal Care and Use Committee (AUP# 2012-6).

2.2.2. **Statistical analysis**

All venous blood analytes (n=16) were assessed for normality using a Shapiro-Wilk Goodness of Fit Test (α=0.05). We transformed analytes, which were not normally distributed as well as tested for, and removed, any outliers. Thus, for all subsequent analyses, we used the log of lactate and tCO_2, the reciprocal of Hct, and the reciprocal of the exponent of pH. As obtaining the reciprocal square root failed to normalize BUN data, we categorized this analyte as less than 3 mg/dL or greater or equal to 3 mg/dL or greater. We performed non- parametric tests for further statistical analysis of this analyte.

We further explored possible confounding factors. We tested all analytes against age (juvenile, adult), sex (male, female), and administration of midazolam (yes, no) as well as differences between populations based upon capture locality (west, contact Zone, east) and location relative to BE (east, west) (Figure 2.1, Table 2.1). For each analyte and factor, heteroscedasticity was tested using a 2-sided, 2 factor F Test and the 3 factor Bartlett test. If variances were unequal, as in Hct, we utilized Welch's test to assess significant differences between means, and we weighted models to account for heteroscedasticity. For variables with equal variance, we used one-way analysis of variance (ANOVA) tests and paired T-tests to compare population differences and effect of sample size (α=0.05).

We further tested related or similar variables using a correlation analysis and Bland Altman plots to assess interactions between multiple variables for those analytes showing

significance. The i-STAT 1 system calculates sO_2 from measurements of pO_2, pH, and HCO_3. When tested with our data using matched-paired analysis, results confirmed a correlation between these sO_2 and pO_2, as well as sO_2 with pH, and HCO_3 (Abbott Point of Care, 2017). Therefore, we opted not to analyze sO_2 in future analyses, and focus on the measured value of pO_2. The i-STAT 1 system calculates hemoglobin by using the measured hematocrit and multiplying by a factor of 0.34, creating a manufactured value (Abbott Point of Care, 2016). We confirmed correlation of Hct and Hgb (0.925, $P < 0.001$) and then focused on Hct differences across variables. Hematocrit (Hct) from the i-STAT-1 system and PCV determined via centrifuge, were compared via Tukey Mean Difference plot. We incorporated rainfall data and geographic location in two-way ANOVAs or linear regressions to determine if there was a biological reason for those analytes showing statistical significance. All values in the results section are means with standard error, unless reported otherwise. All significance tests set alpha at 0.05.

2.3. Results

2.3.1. **Sample collection**

We analyzed venous blood samples from 54 titmice (*Baeolophus* spp.) (22 juveniles, 32 adults) (Table 2.1). In the field, using plumage, 27 were identified as Black-crested Titmice (*Baeolophus atricristatus*), 23 as Tufted Titmice (*B. bicolor*), and four as hybrids. Mitochondrial DNA, from a related genetic study, identified the four phenotypic hybrids as one Black-crested and three Tufted Titmice (Vaughn and Voelker, unpublished). It is also of importance to note that two phenotypic Black-crested Titmice captured east of the BE

(Grimes and Williamson counties) actually classified as Tufted Titmice from mtDNA (Patten & Smith-Patten, 2008).

2.3.2. **Age, sex, and midazolam**

Sex and age of titmice affected relatively few analytes. Females had increased potassium concentrations compared to males (ANOVA, F_2=4.77, P=0.03, Table 2.2). Hematocrit, as measured with an i-STAT 1 system (Hct), failed to agree with traditional centrifuge method (PCV) (Tukey Mean Difference=-12.6, SE=0.9, P<0.001) (Table 2.2). We confirmed correlation of Hct and Hb (0.93, P<0.001) and thus focused on Hct differences based on age and sex. Hematocrit (Hct) was higher in adult titmice compared to juveniles (Transformed 1/Hct, Welch's Test, t_1=2.03, P=0.05) and concentrations of hemoglobin (Hb) showed a similar trend (Welch's Test, t_1=1.97, P=0.05) (means, SE in Table 2.2).

Table 2.2 Venous blood analyte values from collected Titmice based on age (a), sex (b), and midazolam administration (c). Means, standard error, and sample size of untransformed values reported for reference purposes. Probability values from means' significance testing provided with two degrees of freedom. Significant values ($\alpha \leq 0.05$) are shown in bold.

a. Age

Analyte	Units	n	Juvenile	n	Adult	P
H^{NT}	at 37°C	21	7.56 ±0.01	29	7.59 ±0.01	0.09
pO_2	mmHg	21	34.5 ±1.1	30	35.2 ±0.8	0.61
sO_2	%	21	75 ±2	29	78 ±1	0.13
pCO_2	mmHg	21	28.7 ±1.2	29	28.2 ±1.1	0.75
HCO_3	mmol L^{-1}	21	25.0 ±0.9	29	27.0 ±0.8	0.09
tCO_2^{NT}	mmol L^{-1}	21	26.1 ±0.8	29	27.8 ±0.8	0.16
BEecf	mmol L^{-1}	21	3.2 ±0.9	29	5.3 ±0.9	0.10
$Lactate^{NT}$	mmol L^{-1}	16	3.8 ±0.3	26	3.8 ±0.2	0.98
Glucose	mg dL^{-1}	20	339.4 ±13.5	28	322.3 ±12.2	0.36
Na^+	mmol L^{-1}	20	158.5 ±1.0	28	158.7 ±0.7	0.80
K^+	mmol L^{-1}	20	3.7 ±0.1, 20	28	3.7 ±0.1	0.99
Cl^-	mmol L^{-1}	15	119.6 ±1.1	25	119.0 ±0.9	0.67
BUN^T	mg dL^{-1}	15	1.8 ±0.2	25	1.6 ±0.2	0.61^M
PCV	%	17	58 ±2	31	58 ±1	0.87
Hct^{NT}	% PCV	20	42 ±1	28	44 ±1	0.07^W
Hb^{NT}	g dL^{-1}	20	14.0 ±0.2	28	15.0 ±0.3	0.06^W

b. Sex

Analyte	Units	n	Female	n	Male	P
pH^{NT}	at 37°C	18	7.60 ±0.01	31	7.59 ±0.01	0.15
pO_2	mmHg	18	33.6 ±1.0	31	35.5 ±0.8	0.16
sO_2	%	18	74 ±2	31	78 ±1	0.06
pCO_2	mmHg	18	28.6 ±1.1	31	28.9 ±1.1	0.95
HCO_3	mmol L^{-1}	18	25.5 ±0.8	31	27.4 ±0.9	0.23
tCO_2^{NT}	mmol L^{-1}	18	25.8 ±1.0	31	27.7 ±0.8	0.13
BEecf	mmol L^{-1}	18	3.0 ±0.8	31	5.2 ±0.9	0.10
$Lactate^{NT}$	mmol L^{-1}	13	3.9 ±0.3	28	3.8 ±0.2	0.77
Glucose	mg dL^{-1}	16	349.6 ±15.4	31	317.4 ±11.1	0.10
Na^+	mmol L^{-1}	16	157.8 ±1.1	31	159.1 ±0.6	0.25
K^+	mmol L^{-1}	16	**3.9 ±0.2**	31	**3.6 ±0.1**	**0.03**
Cl^-	mmol L^{-1}	12	120.8 ±1.1	27	118.7 ±0.8	0.14
BUN^T	mg dL^{-1}	12	2.4 ±0.4	27	1.5 ±0.1	0.14^M
PCV	%	15	58 ±2	32	59 ±1	0.77
Hct^{NT}	% PCV	16	**42 ±1**	31	**44 ±1**	**0.04**
Hb^{NT}	g dL^{-1}	16	14.3 ±0.2	31	14.97 ±0.2	0.06

Table 2.2 continued
c. Midazolam

Analyte	Units	n	Not Administered	n	Administered	P
pH^{NT}	at 37°C	28	7.59 ±0.02	22	7.56 ±0.01	0.14^{W}
pO_2	mmHg	28	**36.4 ±0.8**	23	**33.2 ±0.9**	**0.01**
sO_2	%	28	**80 ±1**	22	**72.9 ±1.7**	**0.00**
pCO_2	mmHg	30	**25.8 ±1.1**	22	**31.9 ±0.7**	**<0.001^{W}**
HCO_3	mmol L^{-1}	28	**24.3 ±0.7**	22	**28.5 ±0.8**	**<0.001**
tCO_2^{NT}	mmol L^{-1}	28	**25.3 ±0.7**	22	**29.4 ±0.8**	**<0.001**
BEecf	mmol L^{-1}	28	**3.0 ±0.8**	22	**6.3 ±1.0**	**0.01**
$Lactate^{NT}$	mmol L^{-1}	19	**4.8 ±0.2**	23	**3.0 ±0.1**	**<0.001**
Glucose	mg dL^{-1}	25	**310.0 ±11.6**	23	**350.4 ±12.8**	**0.02**
Na^{+}	mmol L^{-1}	25	**157.5 ±0.7**	23	**159.8 ±0.7**	**0.02**
K^{+}	mmol L^{-1}	25	**3.8 ±0.1**	23	**3.5 ±0.1**	**0.02**
Cl^{-}	mmol L^{-1}	17	**121.3 ±1.0**	23	**117.7 ±0.8**	**0.01**
BUN^{T}	mg dL^{-1}	17	**2.3 ±0.2**	23	**1.3 ±0.2**	**<0.001^{M}**
PCV	%	30	58 ±1	18	58 ±1	0.92
Hct^{NT}	% PCV	25	43 ±1	23	44.0 ±0.9	0.49
Hb^{NT}	g dL^{-1}	25	14.6 ±0.2	23	14.8 ±0.3	0.61

^{N}Variable not normally distributed, tested with Shapiro-Wilk Goodness of Fit test for normality
^{T}Variable transformed to normalize data. 1/exp(x): pH, Log(x): tCO2, Lactate, 1/sqrt(x): BUN, 1/x: Hct, Hb
^{W}Means have unequal variance, p-value provided from Welch's 2 sided F-test
^{M}Variable unable to reach normal distribution, Mann-Whitney ranked score test performed (Mean scores and Z probability provided)

Midazolam administration affected all blood analytes except pH, Hct, PCV, and Hb (Table 2.3). Midazolam administration was associated with increased values of pCO_2, bicarbonate, tCO_2, base excess, glucose, and sodium but lower values of pO_2, sO_2, lactate, potassium, chloride, and blood urea nitrogen. Midazolam administration was associated with relatively decreased pO_2 in juveniles compared with adults receiving midazolam, while pO_2 was comparably lower in adults than juveniles, regardless of administration of midazolam (ANOVA, F_3=6.47, P<0.001, Table 2.4, Figure 2.2).

Table 2.3 Venous blood analyte values from collected Titmice based on capture location (a) and location relative to Balcones Escarpment (b). Mean, standard error, and sample size provided using untransformed values for reference purposes. Probability values from means' significance testing provided with 3 degrees of freedom for capture location and 2 for location relative to Balcones Escarpment. Significant values ($\alpha \leq 0.05$) are shown in bold.

a. Capture Location

Analyte	Units	n	West	n	Contact zone	n	East	P
pH^{NT}	at 37°C	2	7.59 ±0.12	23	7.58 ±0.02	25	7.58 ±0.01	0.98
pO_2	mmHg	2	36.5 ±6.5	23	**33.2† ±0.1**	26	**36.3† ±0.8**	**0.06**
sO_2	%	2	80 ±3	23	**74† ±1**	25	**79† ±1**	0.10
pCO_2	mmHg	2	28.9 ±0.8	23	28.6 ±1.4	25	28.2 ±1.0	0.97
HCO_3	mmol L^{-1}	2	28.5 ±6.75	23	26.3 ±0.8	25	25.8 ±0.9	0.68
tCO_2^{NT}	mmol L^{-1}	2	29.5 ±6.5	23	27.0 ±0.8	25	26.9 ±0.8	0.71
BEecf	mmol L^{-1}	2	6.5 ±8.5	23	4.4 ±0.8	25	4.2 ±0.9	0.80
$Lactate^{NT}$	mmol L^{-1}	1	3.5 ±1	19	3.5 ±0.3	22	4.7 ±0.3	0.41
Glucose	mg dL^{-1}	2	**348.0 ±25.0**	21	**369.0† ±13.6**	25	**294.7† ±8.3**	**<0.001**
Na^+	mmol L^{-1}	2	156.0 ±3.0	21	158.4 ±0.8	25	159.0 ±0.7	0.52
K^+	mmol L^{-1}	2	4.1 ±0.6	21	3.7 ±0.1	25	3.6 ±0.1	0.39
Cl^-	mmol L^{-1}	2	118.5 ±1.5	17	119.4 ±1.2	21	119.1 ±0.9	0.95
BUN^T	mg dL^{-1}	2	1.5 ±0.5	17	1.8 ±0.3	21	1.7 ±0.1	0.70^C
PCV	%	2	64 ±4	19	56 ±2	27	59 ±1	0.22
Hct^{NT}	%PCV	2	41 ±2	21	44 ±1	25	44 ±1	0.47
Hb^{NT}	g dL^{-1}	2	13.8 ±0.5	21	14.9 ±0.3	25	14.7 ±0.3	0.42

b. Location relative to Balcones Escarpment

Analyte	Units	n	West	n	East	P
pH^{NT}	at 37°C	25	7.58 ±0.02	25	7.58 ±0.01	0.87
pO_2	mmHg	25	**33.5 ±0.9**	26	**36.3 ±0.8**	**0.03**
sO_2	%	25	**75 ±2**	25	**79 ±1**	**0.06**
pCO_2	mmHg	25	28.6 ±1.3	25	28.2 ±1.0	0.81
HCO_3	mmol L^{-1}	25	26.5 ±0.9	25	25.8 ±0.9	0.59
tCO_2^{NT}	mmol L^{-1}	25	27.2 ±0.9	25	26.9 ±0.8	0.79
BEecf	mmol L^{-1}	25	4.6 ±0.9	25	4.2 ±0.9	0.78
$Lactate^{NT}$	mmol L^{-1}	20	3.5 ±0.3	22	4.1 ±0.3	0.18
Glucose	mg dL^{-1}	23	**367.1 ±12.6**	25	**294.7 ±8.3**	**<0.001**
Na^+	mmol L^{-1}	23	158.3 ±0.8	25	159.0 ±0.7	0.48
K^+	mmol L^{-1}	23	3.7 ±0.1	25	3.6 ±0.1	0.37
Cl^-	mmol L^{-1}	19	119.3 ±1.1	21	119.1 ±0.9	0.90
BUN^T	mg dL^{-1}	19	1.7 ±0.3	21	1.7 ±0.1	0.42^M
PCV	%	21	57 ±2	27	59 ±1	0.40
Hct^{NT}	%PCV	23	44 ±1	25	44 ±0.8	0.95
Hb^{NT}	g dL^{-1}	23	14.8 ±0.2	25	14.7 ±0.3	0.67

^{N}Variable not normally distributed, tested with Shapiro-Wilk Goodness of Fit test for normality
^{T}Variable transformed to normalize data. 1/exp(x): pH, Log(x): tCO2, Lactate, 1/sqrt(x): BUN, 1/x: Hct, Hb
^{W}Means have unequal variance, p-value provided from Welch's 2 sided F-test
^{M}Variable unable to reach normal distribution, Mann-Whitney ranked score test performed (Mean scores and Z probability provided)

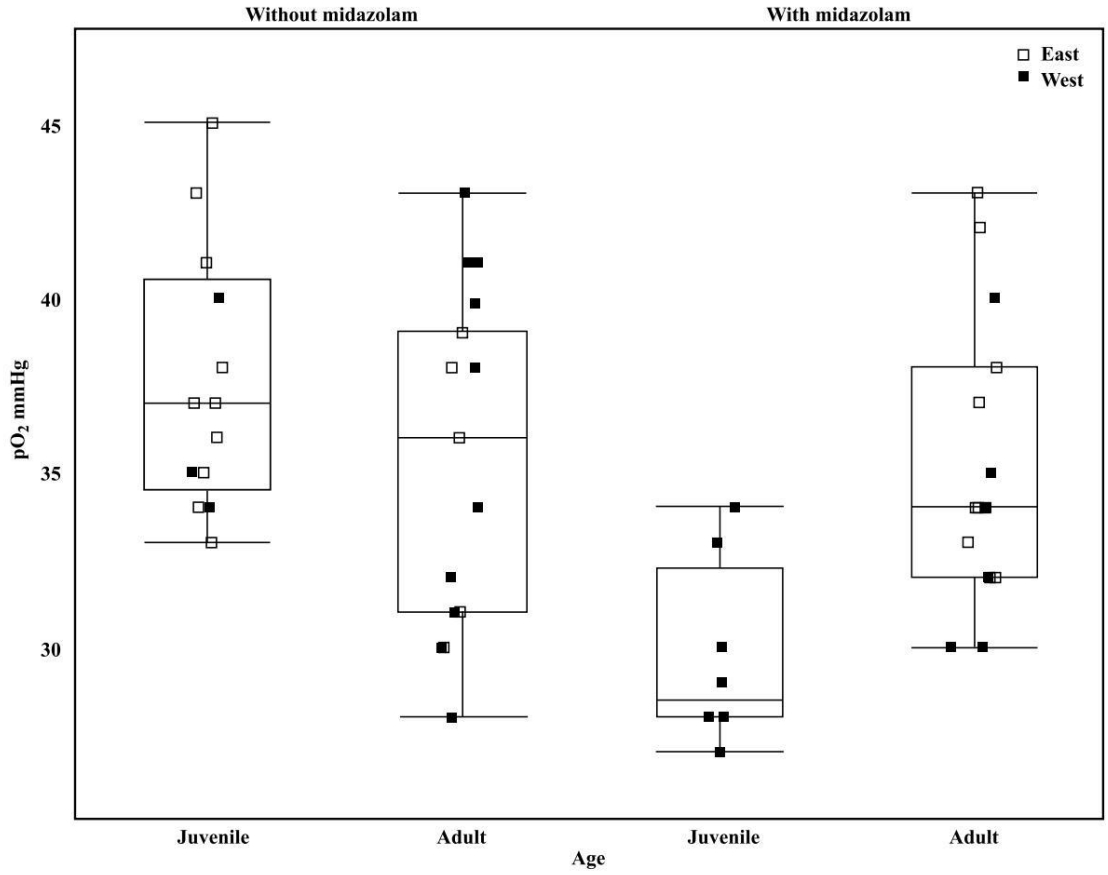


Figure 2.2 Venous partial pressure of oxygen (pO_2) from different ages of Titmice (*Baeolophus* spp.) both east (open squares) and west (filled squares) of the Balcones Escarpment and with and without administration of Midazolam. Juvenile titmice had lower pO_2 values after administration of midazolam (P=0.01), based on an interaction of midazolam and age (P=0.01, adj R^2=0.23).

2.3.3. **Population and Balcones Escarpment**

Blood glucose concentrations of titmice differed significantly based on population (west, contact zone, east) (Table 2.3). Titmice captured within the contact zone had the highest glucose concentrations, followed by western and then eastern populations (Table 2.3). Administration of the sedative midazolam also increased glucose concentrations, but

location of capture was a strong influence of glucose than sedative (east vs. west of BE) (2-way ANOVA, Table 2.4).

Blood glucose concentrations were related to relative rainfall (higher or lower) and "days since last rain" (Table 2.4). For titmice captured west of the BE, blood glucose concentrations were increased in months of high rainfall. However, for birds captured east of the BE no difference of blood glucose was noted in association with levels of monthly rainfall (Figure 2.3). In titmice captured west of the BE, length of time since rainfall was positively correlated with higher blood glucose concentrations occurring after 12-25 days after rainfall (Linear Regression R^2=0.40, ANOVA, F_3=9.83, P <0.001) (Table 2.4, Figure 2.4).

Other venous blood analytes that varied significantly based on population, primarily between the contact zone and eastern populations included pO_2, sO_2, pH and HCO_3. Both pO_2 and sO_2 values were significantly lower in individuals within the contact zone compared to the eastern population (Table 2.3). Although the western population had the highest values for pO_2 and sO_2, these did not differ significantly from contact zone or eastern population values (Table 2.3). Midazolam administration resulted in decreased pO_2 values, with more pronounced effect in Titmice west of the BE (ANOVA, F_1=5.10, P=0.03, Table 2.3). No interaction of capture location and midazolam administration was apparent (ANOVA, t_2=-0.99, P=0.33, Table 2.4).

Table 2.4 Effect of multiple variables on blood venous analytes collected from Tufted and Black-crested Titmice using Analysis of Variance (ANOVA), Analysis of Covariance (ANCOVA), and linear regression. Variable interactions represented by asterisk (*) and bold values indicate single variables or full models that showed significance (P<0.05).

Model Components			Full Model Results	
Analytes	**Factors**	**P-value**	**adj R^2**	**Prob>F**
Hct	Age	0.60		
	Sex	0.15		
	Age*Sex	0.53	0.04	0.19
Potassium (K^+)	Sex	0.09		
	Midazolam	0.11		
	Sex*Midazolam	0.94	0.10	0.06
K^+ With Midazolam	Sex		0.03	0.20
K^+ No Midazolam	Sex		0.02	0.24
pO_2	Midazolam	**0.02**		
	Balcones Escarpment (BE)	**0.04**		
	Midazolam*BE	0.33	0.15	**0.01**
pO_2	Midazolam	**0.00**		
	Age	0.17		
	Midazolam*Age	**0.00**	0.25	**<0.001**
Glucose	Midazolam	0.05		
	Balcones Escarpment (BE)	**<0.001**		
	Midazolam*BE	0.54	0.36	**<0.001**
Glucose	Balcones Escarpment	**<0.001**		
	Relative Rainfall	0.34		
	Relative Rainfall*BE	**0.03**	0.38	**<0.001**
Glucose	Balcones Escarpment	**<0.001**		
	Days since last rain	**0.02**	0.39	**<0.001**

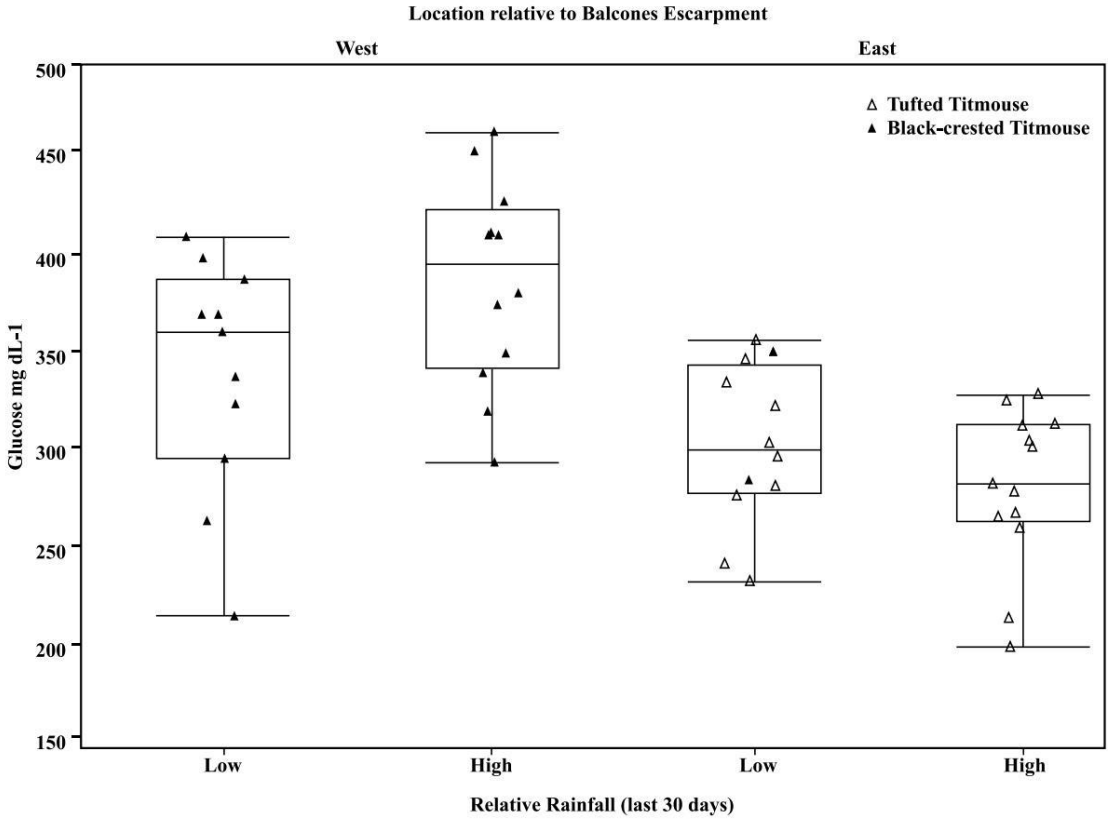


Figure 2.3 Venous blood glucose concentrations (mg dL^{-1}) of Titmice captured west and east of the Balcones Escarpment (BE) based on relative monthly rainfall data. Black-crested Titmice are represented by solid triangles and Tufted Titmice represented with open triangles. Titmice captured west of the BE had higher concentrations of glucose than those captured east (R^2=0.33, P<0.001). Concentrations were further elevated west of the escarpment during months of relatively high rainfall (BE P<0.001, relative rainfall P=0.34, BE*relative rainfall P=0.03; whole model R^2=0.38, P<0.001; Table 2.4).

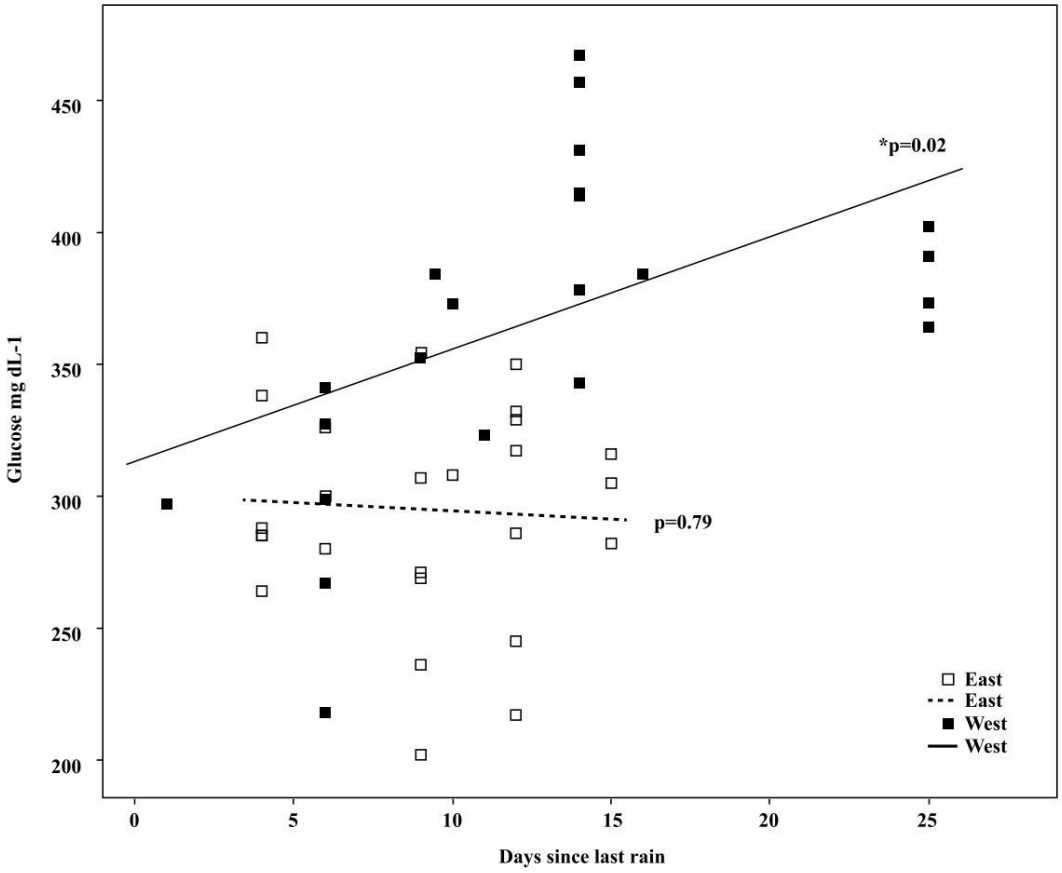


Figure 2.4 Venous blood glucose concentrations (mg dL^{-1}) of Titmice captured east (open square, dashed fit line) and west (filled square, solid fit line) of the Balcones Escarpment and the number of days since the last rain event. Titmice captured west of the Balcones Escarpment had higher blood glucose concentrations during periods of prolonged dryness (Adj. R^2 east=0.00, Adj. R^2 west=0.24; whole model Adj. R^2=0.30, P<0.001).

2.4. Discussion

Venous blood glucose concentrations of titmice differed east and west of the BE and were also higher in birds captured west of the BE especially during months with higher rainfall.

2.4.1. **Location and rainfall increase venous blood glucose concentrations**

Of 16 blood venous blood analytes assessed in two parapatric species of titmice, only blood glucose concentrations were statistically significantly different east and west of the BE, with higher values found west of the BE. Small sample size from the western population and inherent analyte variability likely prevented statistical distinctions between the western and hybrid zone populations (Table 2.3). The difference in glucose east and west of the BE was also observed in our related studies of multiple Texas passerine species (Heatley et al., 2015; Heatley et al., 2013). Although pO_2 and sO_2 also differed between titmice east and west of the BE, but differences resulted from age, sex, and/or midazolam administration.

In general, within bird species, glucose can vary by species, age, time of year, or can be elevated by physiological or psychological stress (Jimeno et al., 2018; Kaliński et al., 2015; Lill, 2011; Tomasek et al., 2019). In birds, blood glucose levels are modulated by glucocorticoid (GC) production pathways via two different receptors. Baseline GC production is mediated by the mineralocorticoid receptor from which increased production can occur following an increase in energetic demands, thermoregulatory changes, or even a period of low food abundance (Hau et al., 2016; Tomasek et al., 2019). Stress-induced GC production, however, is mediated through glucocorticoid receptors that upregulate gluconeogenesis based on 2-10 fold higher concentration of glucocorticoid than baseline.

Titmice captured west of the Balcones Escarpment have higher glucose values compared with those east of the BE (Tufted Titmice), irrespective of sedative administration. Black-crested and Tufted Titmice share many life history similarities, but differ in their habitat preference (Patten & Smith-Patten, 2008; Ritchison et al., 2015).

Black-cresteds reside in semi-arid scrub habitat of west Texas where precipitation is more sporadic, a factor that can lead to unpredictable food resources and induce physiological stress (Griffith et al., 2007; Heilman et al., 2009; Nielsen-Gammon, 2011; Yang et al., 2008). Low food abundance can increase baseline glucose levels. Survival and adaptation to a continually altered physiological state, due to constant stress, is known as the Chronic Stress Hypothesis (Cherel et al., 1988; Fokidis et al., 2012; Kitaysky et al., 2007). In an arid habitat, chronic stress may be due to scarce or unpredictable food or water sources and, over the long term, such stresses can induce adaptations to increase survivability (Cherel et al., 1988; Fokidis et al., 2012; Williams & Tieleman, 2001).

Evidence for a higher natural glucose level based on intermittent food/water resources is shown by the further increase in Black-crested glucose levels following rain events, a pattern not observed in Tufted Titmice. In arid and semi-arid regions, rainfall amplifies primary productivity and triggers an increase in insect and fruit abundance, stimulating foraging by secondary and tertiary consumers (Ostfeld & Keesing, 2000; Wenninger & Inouye, 2008; Yang et al., 2008). We suspect that sporadic rain showers temporarily increase food resources causing hyperphagia or an increased in metabolic activity (a known correlate of glucose levels). A rise in blood glucose has been observed in organisms exposed to unpredictable or pulse food resources as a result of a change in diet (*e.g.*, sugar filled berries) or a product of hyperphagia (Fokidis et al., 2012). We observed blood glucose concentrations were highest in birds captured 12-25 days following rain, likely a result from the time lag between rainfall and pulse food resources from increased primary productivity (Jimeno et al., 2018; Ostfeld & Keesing, 2000; Williams & Tieleman, 2001). Glucose levels in Tufted Titmice, on the other hand, did not increase following rain

events lending support to the idea that this species, living in mesic habitats, have a continual food source year around and, thus are not prone to infrequent bouts of low food sources. It is interesting to note that the two phenotypically (plumage) Black-crested Titmice captured east of the BE did not express similar glucose levels to those captured west. The adult male Black-crested Titmouse (Williamson county) had a glucose level slightly less than the average of birds west of the BE (354 mg/dL, x=367 mg/dL), but the juvenile male Black-crested (Grimes county) had a glucose value less than the average of birds east of the BE (288 mg/dL, x=294 mg/dL). Both birds received a sedative and were captured in late spring; however, the adult male was captured just east of the BE (closer to the hybrid zone). Therefore, though these birds look Black-crested and have Tufted Titmouse DNA, their glucose values appear to match with longitude/habitat.

If habitat drives glucose, as our data suggests, it may play an important role in species distribution and reduce genetic introgression across the hybrid zone. Black-crested Titmice have been observed east of the BE, but Tufted Titmice are rarely observed west of the BE (Patten & Smith-Patten, 2008; Ritchison et al., 2015). Tufted Titmice may have low survival west of the BE due to physiological intolerance to unpredictable food and water resources. Therefore, the physiology of glucose metabolism could act as a limiting factor to the colonization west of the BE by the Tufted Titmouse.

2.4.2. **Influence of age and sex**

Other venous blood analytes were influenced by age, sex, season, and sedative administration. Although saturated oxygen and base excess were higher in males,

especially in those administered midazolam, we suspect the sex difference is an artificial finding based on sample size and influence of midazolam.

Both sex and midazolam administration influenced venous blood potassium concentrations. Adult females showed relatively increased venous blood potassium concentrations compared to other groups. The most common cause of hyperkalemia as a laboratory error in human and veterinary medicine is red blood cell lysis (Asirvatham et al., 2013). However, in avian species, the effect of hemolysis on potassium concentrations has not been evaluated to our knowledge. In this study, however, no adult female received the sedative midazolam. Thus, adult females may have undergone more stress and struggling during sampling resulting in a sample with relatively increased cellular lysis prior to analysis. During stress or strenuous exercise, increased blood pressure and muscular contractions trigger the kidneys to release potassium to ensure adequate sodium-potassium pump activity (Siegel, 1980; Stranahan et al., 2008). Furthermore, as all females were captured during nesting or breeding season, females could have experienced greater energetic demands, and handling and blood sampling may have been more problematic without sedation. The capture of both sexes outside the breeding season could confirm if the increased potassium concentrations in female Titmice are consistently present or they result from increased energetic demands related to breeding. Recording a hemolysis score for the blood samples could have facilitated the determination of the effect of hemolysis, if any, upon potassium concentrations in our samples (Yoo et al., 2015).

As found in our study, juvenile passerine birds of many species often have a relatively decreased hematocrit compared to adults (Potti, 2007). Hematocrit's difference between ages is likely a product of body size (Senar & Pascual, 1997). The relationship

was strongest with males, as expected in Titmice, since males are larger than females (Patten & Smith-Patten, 2008; Ritchison et al., 2015). Since hematocrit varies greatly between individuals due to health, hydration, life history, and more, the unexplained high variance is unsurprising. The observed difference of Hct, between sexes, might have been stronger with a larger sample size of adult females and less disparity of the ratio of juveniles and adults.

Similar to other studies, we also found a discrepancy between PCV and measured Hct with PCV values consistently greater than Hct (Abbott Point of Care, 2016; Heatley et al., 2013). The discrepancy of methods for determination avian hematocrit is likely explained by the relative difference in the avian red blood cell shape, volume and retained nucleus, compared to the human red blood cell (Heatley et al., 2015; Pistone et al., 2017; Yaw et al., 2019). Packed cell volume (as determined by centrifugation), rather than Hct (as determined by electrical resistance via the i-STAT 1) providing consistently lower results, should remain the gold standard for use in passerine species. Packed cell volume (PCV or Hct), the ratio of red blood cells to total blood volume expressed as a percent, can indicate dehydration or fitness based on an individual's ability to transport oxygen (Abbott Point of Care, 2016). Larger organisms usually have increased metabolic demands based on increased body and organ size and greater demand for oxygen transport (Glazier, 2008; Minias, 2015). In addition, adult birds and those with androgens, rather than estrogens also tend toward relatively increased red blood cell mass and with resulting increased PCV (Johnstone et al., 2017; Scanes, 2016). However, Hct should be further evaluated in these species with a larger adult sample size and with individuals from across the full

distribution of the species to ensure other factors, such as latitude or elevation, are not influencing Hct or PCV.

2.4.3. **Effect of midazolam and handling upon venous blood analytes**

Administration of midazolam affected many blood gas and electrolyte values often used to determine health in wild birds. Interpretation of avian health in wild birds is inherently complicated as capture, handling, and blood collection are stressful events for free-living birds (Carere & van Oers, 2004; Le Maho et al., 1992; Newman et al., 2005). Physicians and dentists have used midazolam for decades to reduce procedural stress in children (Kupietzky & Houpt, 1993; Lee-Kim et al., 2004). Midazolam is a benzodiazepine that boosts inhibitory neurotransmitters, decreases neuron excitability and results sedation, anxiolysis, and/or hypnosis depending upon the dose administered (Kupietzky & Houpt, 1993). However, hypoxia, reduced respiration, reduced memory, and stimulation of a stress endocrine response may also occur (Heatley et al., 2015; Kupietzky & Houpt, 1993). In multiple avian species, intranasal administration of midazolam has been shown to reduce the stress of handling and venipuncture (Vesal & Eskandari, 2006).

In our study, midazolam administration influenced all analytes we measured in Titmice except pH, Hct, and Hb. For both species, irrespective of geographic location, Titmice given midazolam had, on average, higher levels of pCO_2, HCO_3, tCO_2, base excess, glucose, and sodium values. Midazolam lowered the venous partial pressure of oxygen (pO_2), and oxygen saturation (sO_2), as well as concentrations of lactate, potassium, chloride, and BUN. Except for glucose, changes in all analytes paralleled those seen in human following administration of midazolam to humans (Tucker et al., 1986).

While location of capture was a stronger predictor of blood glucose concentrations than sedative administration, glucose concentrations were unexpectedly higher in birds receiving midazolam. Gluconeogenesis occurs near the end of the stress response pathway to meet increased energetic demands of the stressor (Blas, 2015; Siegel, 1980). Production of glucose is a vital component of the negative feedback mechanism regulating the sympathetic (aka "fight or flight") nervous system. Even after a stress stimulus is removed, glucose concentrations may continue to rise based on the time lag between neuroendocrine communication and the hepatic metabolic response (Braun & Sweazea, 2008; Siegel, 1980). Mist-net capture, handling, and blood sampling each induce stress on wild birds so increased glucose concentrations were expected in birds (Harms & Harms, 2012; Harms et al., 2016; Newman et al., 2005). The apparent ineffectiveness of midazolam to reduce glucose concentrations is likely a product of the lag time between capture until midazolam administration. Although we obtained blood 0-5 min following sedation, a stressful event (mist net capture) has already occurred prior to anxiolytic administration. Further stress, from handling, during sedative administration could also cause blood glucose concentrations to continue increasing during blood collection despite sedative administration. To better assess the efficacy of midazolam for stress reduction in captured birds, future studies could measure blood glucose concentrations and or corticosterone at various time intervals (e.g., 15-60 min) following capture handling and midazolam administration. Although midazolam changed multiple venous blood analytes similarly to humans experiencing anxiolysis, this sedative's impact on blood gases and electrolytes in passerine birds needs further investigation.

We administrated midazolam to titmice in 2012 to reduce stress and facilitate blood collection while assessing health. Providing a rest period after capture and before blood collection may also reduce stress, as indicated by blood gases and other variables (Harms & Harms, 2012; Harms et al., 2016). Blood samples from Mourning Doves (*Zenaida macroura*), Boat-tailed Grackles (*Quiscalus major*), and House Sparrows (*Passer domesticus*), immediately after capture or after a 45-60 min delay, lacked differences in pH for any species, while blood gas values (pCO_2, pO_2, and HCO_3^-) and PCV varied based on species. However, lactate significantly decreased based on a rest period in all species. We found similar results in Titmice when comparing birds receiving a sedative to those without. However, this comparison should be reviewed with caution as the authors studied two Passeriformes species and a Columbiformes species that were larger than the Titmice of this study (Harms et al., 2016).

2.5. Summary

Titmice living west of the Balcones Escarpment have higher venous blood glucose concentrations than individuals east of the BE. Glucose values were further elevated following rainfall west of the BE. We suspect the naturally increased glucose values are due to chronic stress from unpredictable food sources in a semi-arid environment. Naturally higher glucose levels, in western birds, are supported by a further increase in their glucose levels following rainfall, an event representing a change in their environment. In arid and semi- arid environments, such as the BE, sporadic rainfall stimulates primary productivity, which can induce hyperphagia and/or increase metabolic activity, both of which are known causes of increased glucose. We recommend controlled experiments to

investigate behavior, physiology, and fitness of both species in opposing natural habitats. Such studies could provide evidence of hybrid zone reinforcement and possible hybrid zone movement or range expansion. Until data collection and knowledge advance to provide more complete physiological reference data for appropriate indicator species of study, we as wildlife biologists must strive to continue increasing our understanding of avian physiology through comparative studies between species, updating handling protocols to include use of sedatives, understand and include environmental conditions in our study design, as well as investigating the impact of physiological differences between populations and species, especially as it relates to habitat effects.

3. SPECIES-SPECIFIC SONG CHARACTERSTICS AND ASYMEMETRICAL AGGRESSION WITHIN A CENTRAL TEXAS TITMOUSE CONTACT ZONE

3.1. Introduction

As with other organisms, avian communication can greatly impact gene flow as individual and species recognition relate to territorial defense and mate selection (Catchpole & Slater, 2008; Podos, 2004; Searcy et al., 2006; Slabbekoorn & Smith, 2002). In passerines, bird songs (and subsequent responses to song) play a role in development of reproductive barriers. As such, male songs used for mate attraction act as sexual traits, and can become pre-mating isolating barriers (Kroodsma, 2005; Verzijden et al., 2012). However, these isolating barriers can be fractured in areas where closely related species come into contact. Lack of distinct species-specific traits or development of female preference for heterospecific songs can result in hybridization, which could lead to various fitness and evolutionary consequences (Qvarnstrom et al., 2006; Sattler et al., 2007; Secondi et al., 2011; Toews, 2017). Therefore, contact zones (areas of potential hybridization) between closely related species can provide a model for understanding the interplay between songs and gene flow.

Song development, including its structure and complexity, is a product of genetics, learning, and environmental factors. Several studies have shown some passerine species raised in isolation will sing a rudimentary song with components common to its species (Catchpole & Slater, 2008; Päckert, 2018). However, learning from fathers and neighbors helps produce melodic songs that will enable individual and species recognition in the wild (Beecher, 2017; Searcy & Nowicki, 2019). This learning process combining familial and

neighboring song components (cultural transmission) promotes population or regional dialects (Aplin, 2019; Hamao et al., 2016; Koetz et al., 2007; Mason et al., 2017; Planqué et al., 2014). However, bird song is influenced by more than just genetics and cultural transmission; environmental factors and body size also impact song structure (Catchpole & Slater, 2008; Derryberry et al., 2018). Many studies have shown that birds occupying denser forests sing a lower frequency than in open areas because dense vegetation can distort song transmission, a framework known as the acoustic adaptation hypothesis (Ey & Fischer, 2009; Patten et al., 2004). Birds with larger bodies often sing at a lower frequency, usually explained by a greater lung capacity (Hall et al., 2013; Mason & Burns, 2015; Riede & Goller, 2014). These additional factors might complicate contact zone research as zones of contact often occur at ecotones (transition between habitat types) involving different species likely varying in body size (Abbott et al., 2013; Harrison & Larson, 2014).

Behavioral responses, such as asymmetrical male aggression and female mate choice, can also act as a reproductive barrier and alter dynamics near or within a contact zone. Asymmetrical aggression occurs when one species outcompetes the other for resources or mates. An example of asymmetrical aggression impacts on songs was observed in New Guinea robins (Slaty and Ashy, *Peneothello cyanus* and *Heteromyias albispecularis*, respectively). In these species, researchers noted hybrid song asymmetry in sympatry was a result of the Ashy robins being dominantly aggressive with respect to Slaty robins (Freeman, 2016). Most studies note female preference for species-specific songs (Halfwerk et al., 2016; Lipshutz, 2018; Wheatcroft & Qvarnstrom, 2017). However,

understanding behavioral discrimination of heterospecific songs is necessary to elucidate any biological significance between song variations (Freeman & Montgomery, 2017).

In this study, we investigate songs and behavioral responses to further understand contact zone dynamics between Black-crested (*Baeolophus atricristatus*) and Tufted Titmice (*B. bicolor*). These two species have long been suspected of hybridization (Allen, 1907) based on morphological variation and plumage coloration (Braun et al., 1984; Curry & Patten, 2014; Dixon, 1955). This species-pair has several contact zones in Texas occurring in areas with strong ecotones. Dixon (1955) described three suspected contact zones in Texas with the most prominent located along the Balcones Escarpment in central Texas, our area of focus. The Balcones Escarpment is an inactive fault zone marking the eastern edge of the Edwards Plateau and producing an ecotone between the higher elevation semi-arid scrub habitats of west Texas and the lower elevation mesic grasslands and forested habitats of east Texas (Abbott & Woodruff Jr, 1986; Kostecke, 2008; Toomey III et al., 1993). Black-crested Titmice reside west of the Balcones Escarpment and south into northern Mexico. They are identified by having a black crest with a white forehead (Patten & Smith-Patten, 2008). Tufted Titmice, on the other hand, have a black forehead and grey crest and occupy mesic habitats at and to the east of the Balcones Escarpment with continuing distribution north and east across the United States (Ritchison et al., 2015).

Songs of both species are similar in structure with Tufted Titmice songs commonly characterized by a repeated three-note phrase, “peter, peter, peter” (Ritchison et al., 2015). Currently, only two studies have investigated song differences between the sister species. Coldren (1992), investigating Titmice in central Texas (Balcones contact zone), reported Black-crested Titmice songs have more notes per phrase and sing at a higher frequency

compared with Tufted Titmice. Coldren (1992) analyzed Black-crested Titmice songs recorded within and outside the contact zone but did not consider contact zone birds separately; therefore, it is unclear how much song variation exists between the parental species or if birds within the contact zone sing mixed-species (blended) songs. More recently, Curry and Patten (2019), focusing on younger contact zones in north Texas and Oklahoma, noted species-specific song differences (similar to the 1992 study) while comparing song differences across multiple contact zones, vegetation types, and noise backgrounds. Curry and Patten (2019) emphasized differences between the two contact zones and reported that song phrase duration varied with vegetation structure in one zone but not another.

To date, limited research has investigated behavioral interactions between Black-crested Titmice and Tufted Titmice. Research on wintering behavior and interactions with other species suggest Tufted Titmice are an aggressive species (Ritchison et al., 2015). Curry and Patten (2016) reported preference of both species towards conspecific songs but no preference by birds within the contact zone. Although Curry and Patten (2016) defined species and hybrids based on a hybrid index (Dixon, 1955), recent work has shown this index to be an inaccurate method of determining genetic assignment (Vaughn and Voelker, unpublished data). Despite these studies, it is still unknown if mixed-species songs between Black-crested and Tufted Titmice are present, if the species have different behavioral responses, or how (or if) avian communication (song and behavior) varies within the central Texas contact zone.

Here, we have two main goals with the aim to better understand songs and behaviors within a known contact zone. Firstly, we use song recordings to a) determine

song structure differences at the species level and across the contact zone and b) establish species-specific song characteristics and compare them with songs within the contact zone. Secondly, using playback experiments, we analyzed territorial aggression as a measure of species recognition to a) determine if species vary in types of behavioral response and b) determine if birds within the contact zone respond differently to parental species songs.

3.2. Methods

3.2.1. Song differentiation

3.2.1.1. Song recording and collecting

To assess geographic and species differences, we recorded a total of 32 songs from Tufted and Black-crested Titmice across central Texas. Of these, 29 were recorded by JCV between 2011 and 2014, between March and August each year. An additional three songs were downloaded from online databases (Harrison, 2012; Keller, 1993a, 1993b). Songs were collected from sites along a longitudinal transect across central Texas encompassing the main contact zone along the Balcones Escarpment (Figure 3.1, Appendix B). In total, 15 songs were from the eastern region (Tufted Titmouse), six songs from within the contact zone, and 11 from west of the contact zone (Black-crested Titmouse).

To understand results from within the contact zone, we investigated song recordings by two levels: expected species and transect region. As species identification could not be confirmed, we assigned expected species based on current geographic distributions with the Balcones Escarpment as the edge of the distributional range for each species (Patten & Smith-Patten, 2008; Ritchison et al., 2015). Expected species analyses excluded contact zone songs to avoid the effects of possible song mixing that often occurs

in contact zones (Qvarnstrom et al., 2006; Secondi et al., 2003). For transect region analyses, we used three regions: west of the contact zone, within the contact zone, and east of the contact zone. For the purposes of this study, the contact zone region lies along the eastern edge of the Edwards Plateau with the Balcones Escarpment as the center point, as commonly reported in other studies (Curry & Patten, 2014; Dixon, 1990). We chose to use a conservative width of 75 km which includes most of Travis and Hays counties, northern Comal county, and western Williamson county. This contact zone is centered at the edge of an elevation gradient, located near Onion Creek (in southwest Austin, TX) where the zone of contact was previously reported by Dixon (1955).

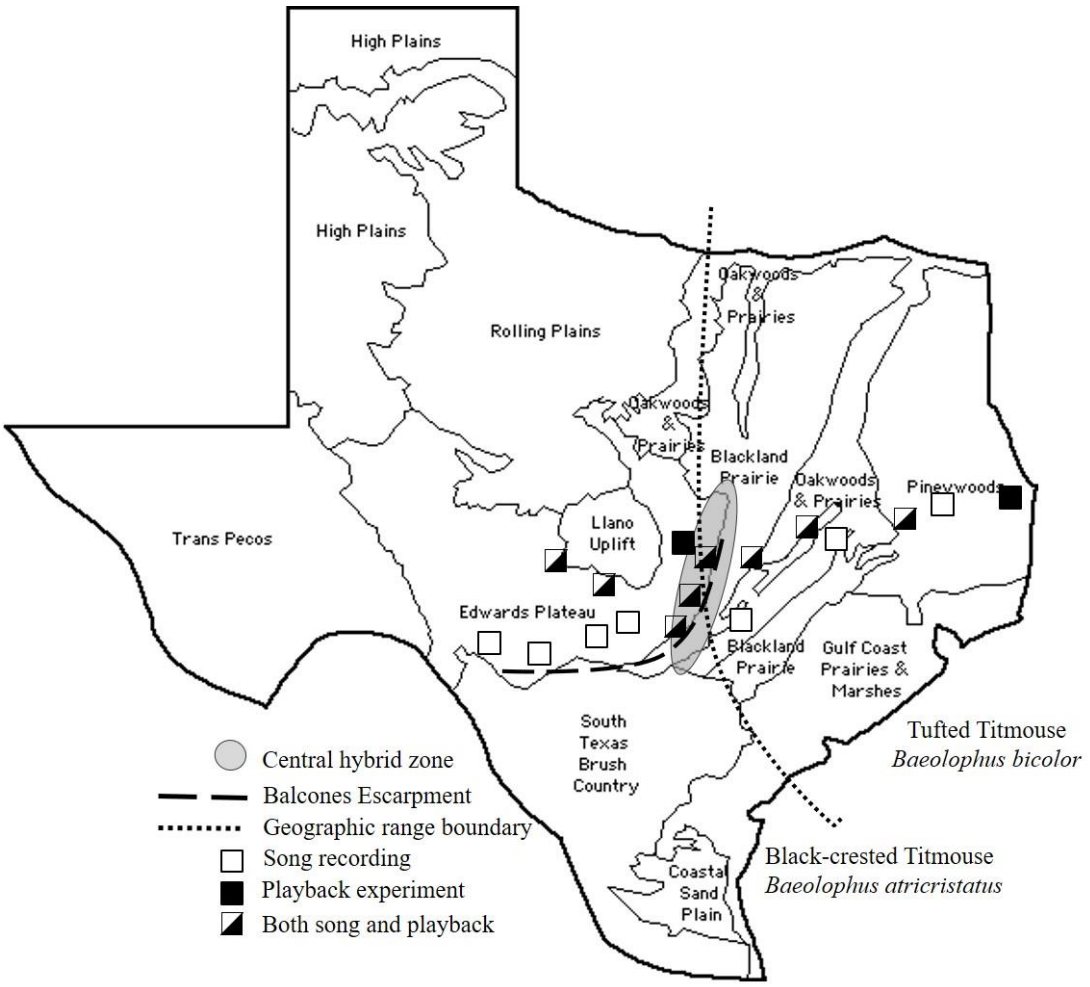


Figure 3.1 Map of Texas indicating song recording (open squares) and playback experiment (filled in squares) locations across central Texas. Site location was spread across central Texas to include areas where Tufted Titmice (*Baeolophus bicolor*) and Black-crested Titmice (*B. atricristatus*) live, including a zone of contact across the Balcones Escarpment (shaded oval). Geographic range boundary for the species (dotted line) and the eastern edge of the Edwards Plateau (dashed line) are also indicated. Map modified and reprinted from Enchanted Learning (2018).

3.2.1.2. Song measurements

To define the terminology used in this study, a song refers to the entire period of singing that was recorded and analyzed (Figure 3.2). Each song consists of repeated phrases, which are a reported number of syllables. In both Titmice species, the mnemonic "peter-peter-

peter" is often applied to a phrase, with each 'peter' being one-note. In some cases, a syllable can be made with two or more inflections, called notes (Berwick et al., 2011). Differences in the number of syllables and note production create unique phrase types that individuals utilize in communication (Mischler et al., 2017; Slabbekoorn & Smith, 2002).

Before analysis, each song bout was edited to remove the majority of extraneous noise (Audacity, 2018) and then digitalized before we measured characteristics using SonoBird^{TM} v1.6.5 (DNDesign, Arcata, CA). For each phrase within a song, we calculated averages of phrase duration (sec), number of notes per phrase, note tempo (number of notes per phrase duration), note length (sec), maximum phrase frequency (kHz), bandwidth (frequency range), and upper and lower bandwidth cutoffs (Catchpole & Slater, 2008; Nelson, 2000; Zollinger et al., 2012). To determine differences in song structure between species, we assigned songs a phrase type based on similar phrase/note patterns across both species.

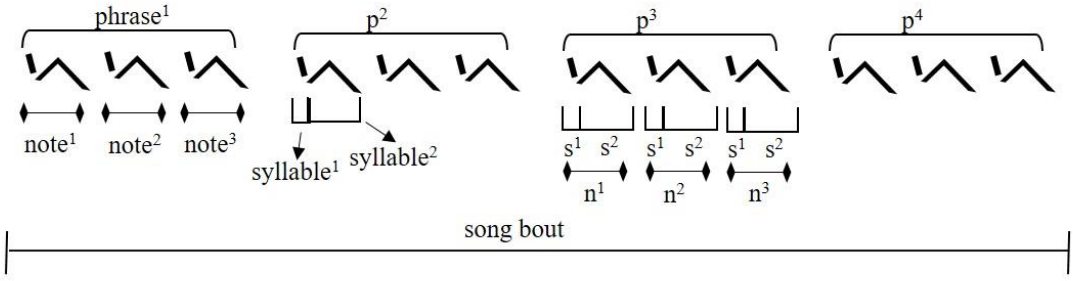


Figure 3.2 Graphic visual of terminology used to describe song components. A song bout is the length of a bird song recorded and analyzed. In Titmice, each song bout consists of multiple repeated phrases (p) made up of two or more syllables (s). In some Titmice phrases, syllables are comprised of distinct (and separated) notes (n).

3.2.1.3. Statistical analysis of songs

To reduce extraneous variation caused by different song-bout lengths, we tested within-song phrase rate and frequency variation and determined that variance was negligible (less than 0.001) across six phrases. Therefore, we took song measurements on six phrases from each song, except for one online song (Kendall county) which had only two useable phrases. For this online song, within phrase variance was less than 0.01, such that individual variation has minimal impact on results; we, therefore, chose to retain this song, especially as it was recorded in an important region of the transect with no other.

We assessed variation of raw measurements for all eight song characteristics (averages of phrase duration, number of notes per phrase, note tempo, note length, maximum phrase frequency, bandwidth, and upper and lower bandwidth cutoffs). We used non-parametric Mann-Whitney tests to test for song characteristic differences between expected species and Kruskal-Wallis tests to test for song characteristic differences between transect region(s).

To improve classification analysis through dimensional reduction, we first log-transformed all variables. Using log-transformed variables, we collapsed variables using a covariance principal component analysis (PCA) to determine which song characteristics explained the most variance between the expected species. To determine if songs could be correctly classified by transect region, we used log-transformed variables with the greatest eigenvalues (explained the most variance) in a quadratic discriminant function analysis (DFA). Each value was tested for homogeneity of variance using Levene's test of analysis of means for variances (McGarigal et al., 2000). Only variables with F-test ratios that were insignificant were included in DFA. Finally, we assessed the predominance of phrase types

across each transect region and expected species using correlation analysis. All statistical tests were performed in JMP 14 using an alpha of 0.05 for significance testing (JMP, 2018).

3.2.2. **Behavioral differential response**

3.2.2.1. Playback methods

We conducted 66 playback experiments, including controls, at 14 sites across the transect, between March and August (2012-2013), to measure behavioral response to songs of both species (Figure 3.1, Appendix C). Experiments were restricted to morning hours (before 1200 hours) and never during inclement weather to avoid impact on bird behavior. If multiple experiments were conducted at a locality, we avoided recording repeat individuals by ensuring experiment sites were further than sound could travel in the environment. When in doubt, we chose to record at opposite ends of the site location.

For each playback experiment, we played one of three songs: Tufted Titmice, Black-crested Titmice, or a control, Carolina Wren (*Thryothorus ludovivianus*). We used Carolina Wren because they are known to occupy the same habitats of both Titmice species and interact during mixed species winter flocks; thus, they should not illicit a threat response by male Titmice (Ritchison et al., 2015). Controls were played once at each site to ensure Titmice were not responding to noise/playbacks in general. To avoid a behavioral response to a specific song (or phrase type), we created a 3-minute digital compilation of pure species song compromised of 2-3 repeated phrases (Douglas & Mennill, 2010; Moseley et al., 2013). Using Audacity 2.0, we created these pure-species compilations by combining previously recorded songs (by JCV) of either Black-crested Titmouse, Tufted

Titmouse, and Carolina Wren (outside the contact zone.) Before and after the birdsong compilation, we inserted 3-minutes of silence to allow birds to adjust to our presence as well as provide time to monitor behavior after playback ended.

For each playback experiment, we placed a Radio Shack™ mini amplifier and portable mini MP3 player roughly at chest height in a visible area. Once we started the recording, we retreated to a semi-hidden location to observe responses. Using a stopwatch, we recorded time and distance (height/length) to the speaker for all non-vocal and vocal behaviors (see below) throughout the nine minutes. We also noted weather conditions, date/time, latitude/longitude, and habitat type. Two observers were always present during each experiment to increase the observation range and allow one observer to monitor the stopwatch and record the data.

3.2.2.2. Measuring and scoring behaviors

During each playback experiment, we recorded time and location of each behavior observed. Visual behaviors included: appearing, feather fluffing, rapidly switching perch, and approach to speaker. Audible behaviors included: call, song, chirp, and distress call. We later converted behavioral responses, for analysis, from raw data to modified scaled responses employed by Emlen (1972). These scales focused on intensity of the behavior and speed of behavioral response. Maximum intensity displayed was indicated by categorical rank scale from 0 (no change in behavior) to 6 (feather fluffing) (Table 3.1). Intensity scale was based on previous experiments reporting territorial aggression responses to playbacks (Emlen, 1972; Freeman & Montgomery, 2017; Martin & Martin, 2001). Time until first response has also been shown to be an indicator of aggression

(Emlen, 1972). Therefore, we categorized response time as: no response, fast response (within the first minute), slow response (after one minute) (Table 3.1b).

Table 3.1 Scoring intensity and behavioral response of each playback. a) Intensity scale used to compare behavioral responses. b) Speed of response. All values were then summed to determine a level of response. Scoring and ranking based upon modified ranking by Emlen (1972).

a) Maximum Intensity Behavior

Category	Ranked Scale
No Response	0: No change in behavior
	1: Appear/head tilt
Vocal	2: Song
	3: Call/trill
	4: Approach speaker within 3 m
Visual	5: Attack Speaker
	6: Feather fluffing

b) Speed of Response

Category	Time range
No Response	No behavior observed
Slow Response	Behavior observed after 1 min
Fast Response	Behavior observed within 1 min

3.2.2.3. Statistical analysis of playbacks

To determine if species varied in their response, we first assessed responsiveness to playbacks based on species present and then further distinguishing by the species' song played (heterospecific vs conspecific songs). These tests used only playbacks conducted outside the contact zone. Analysis utilized contingency tables, which reported Pearson's chi-square and fisher's 2-tailed exact test. To determine if the species responded differently to other species, we used only playbacks in which we recorded a response. Using

contingency tables, we compared behavioral speed (fast vs. slow) and maximum intensity (vocal vs. behavior) between the expected species. All statistical tests were performed in JMP 14 using an alpha of 0.05 for significance testing (JMP, 2018).

3.3. Results

3.3.1. Song characteristic differences

Tufted Titmice and Black-crested Titmice were significantly different in five song characteristics (Table 3.2). Tufted Titmice songs contained fewer notes per phrase, shorter phrase length, and lower frequency compared to Black-crested Titmice. Black-crested Titmice songs had shorter note lengths with a faster note tempo. Bandwidth and lower and upper cutoffs were not significantly different between the songs of the two species.

In comparing song characteristics across the transect regions (west of contact zone, contact zone, or east of contact zone), bandwidth and cutoffs were not significant across all transects regions (Table 3.2). No characteristic was unique (significant compared to other regions) in the contact zone; thus, no characteristics could be used to distinguish each transect region. Contact zone songs were not significantly different from western songs in regard to number of notes, note length, and phrase duration. On the other hand, contact zone songs were not significantly different compared to eastern songs in terms of note tempo, maximum frequency, upper bandwidth cutoff, and lower bandwidth cutoff.

Table 3.2 Means of raw data for average song characteristics within and between each transect region along with standard deviations (sd). Between transect region z-scores (Z) and P-values are provided along with overall chi-square (X^2) statistical differences (alpha=0.05). Significant P-values are indicated by an asterisk.

	West n=10		West vs. Contact zone		**Contact zone** n=7		Contact zone vs. East		**East** n=15		East vs. West		Overall		
Song Variable	Mean	sd	z	P	Mean	sd	z	P	Mean	sd	z	P	X^2	d.f.	P
Notes/phrase	7.82	2.99	0.00	1.000	7.45	2.53	2.89	0.004*	4.04	1.33	3.36	0.001*	14.99	2.0	0.001*
Note length (sec)	0.12	0.02	-0.63	0.526	0.14	0.05	-1.97	0.048*	0.18	0.04	-3.63	0.000*	13.57	2.0	0.001*
Note tempo	0.18	0.03	0.15	0.884	0.20	0.07	-1.69	0.091	0.24	0.05	-2.69	0.007*	7.74	2.0	0.021*
Phrase duration	1.34	0.31	-0.44	0.661	1.35	0.24	2.82	0.005*	0.92	0.25	3.19	0.001*	13.90	2.0	0.001*
Maximum frequency (kHz)	2.84	0.22	2.49	0.013*	2.63	0.17	-0.35	0.725	2.66	0.16	2.02	0.043*	6.87	2.0	0.032*
Bandwidth	0.73	0.21	0.63	0.526	0.64	0.18	-1.62	0.105	0.72	0.15	-0.64	0.524	2.49	2.0	0.288
Upper cutoff	3.14	0.25	2.29	0.022*	2.91	0.18	-1.13	0.259	2.99	0.17	1.58	0.114	5.97	2.0	0.051
Lower cutoff	2.41	0.18	2.09	0.036*	2.27	0.05	0.35	0.725	2.26	0.13	1.91	0.056	5.38	2.0	0.068

Principal components analyses indicated five characteristics produced the bulk of the variance in calls between expected species: phrase length, number of notes, note tempo, note length, and bandwidth (Table 3.3). For both species, at least 80% of the variation could be explained by the first two principal components. In Tufted Titmice, significant loadings (greater than 30%) for the first component (58.9 %) included phrase length and note number. The second component (additional 26.2%) included phrase length, note tempo, and note length. Black-crested songs differed by the inclusion of bandwidth, such that the first principal component was explained by phrase duration, note number, note tempo, note length, and bandwidth (70.3%). Notably, note tempo and note length were negatively correlated to other variables. The second principal component for Black-crested Titmice was primarily impacted by note tempo and bandwidth (additional 19.2%).

Following the result of principal component analysis, canonical discriminant analysis used the log-transformed averages of the influential variables: phrase duration, note number, note tempo, note length, and bandwidth. All variables met assumptions for homogeneity of variance. Analysis resulted in variance being explained by the two canonical functions (1: explaining 93.4% and 2: explaining 6.57%). Probability of F ratio was only significant for canonical function 1 (correlation=0.784, d.f.=10, P=0.002). Standardized coefficients for canonical function 1 indicated the most discriminating variables were note number (22.49) followed by note tempo (14.94) and phrase duration (-14.75). Bandwidth (-0.59) and note length (-9.33) had minimal explanatory influence. Classification based on the five prominent song characteristics did not misclassify any songs when only songs outside the contact zone were considered (expected species) and only misclassified two songs (~6.3%) when all songs were included. One of these

misclassified songs was recorded in the eastern region (Grimes county) but was predicted to be a contact zone song (probability of 66.7%). The other misclassified song was recorded in the contact zone (Hays County) but was predicted to be a western song (probability 72.9%). The prominent canonical coefficient scores from discriminant analysis were statistically different across the transect regions (d.f.=2, F ratio=23.17, r^2=0.615, P<0.001), with similar western and contact zone scores. However, the second set of scores were not significantly different (d.f.=2, F ratio=1.63, r^2=0.101, P=0.213).

Table 3.3 Eigenvalues and eigenvectors from principal components analysis using log-transformed song variables. Bolded PC values represent variables whose variance most greatly influences data transformation.

	Black-crested Titmice		Tufted Titmice	
Song Variables*	PC1	PC2	PC1	PC2
Phrase length (sec)	**0.405**	0.085	**0.506**	**0.542**
Number of notes/phrase	**0.719**	-0.263	**0.763**	-0.020
Note tempo (note rate/phrase)	**-0.314**	**0.351**	-0.252	**0.558**
Note length (sec)	**-0.316**	0.116	-0.278	**0.565**
Maximum frequency (Hz)	0.073	0.008	0.016	0.061
Bandwidth	**0.332**	**0.870**	0.134	0.261
Upper cutoff	0.070	0.157	0.040	0.064
Lower cutoff	-0.012	-0.069	0.001	0.006
Eigenvalue	0.05	0.01	0.04	0.02
% Explained	70.3	19.2	58.9	26.2
Cumulative %	70.3	89.5	58.9	85.2

*all variables are log-transformed

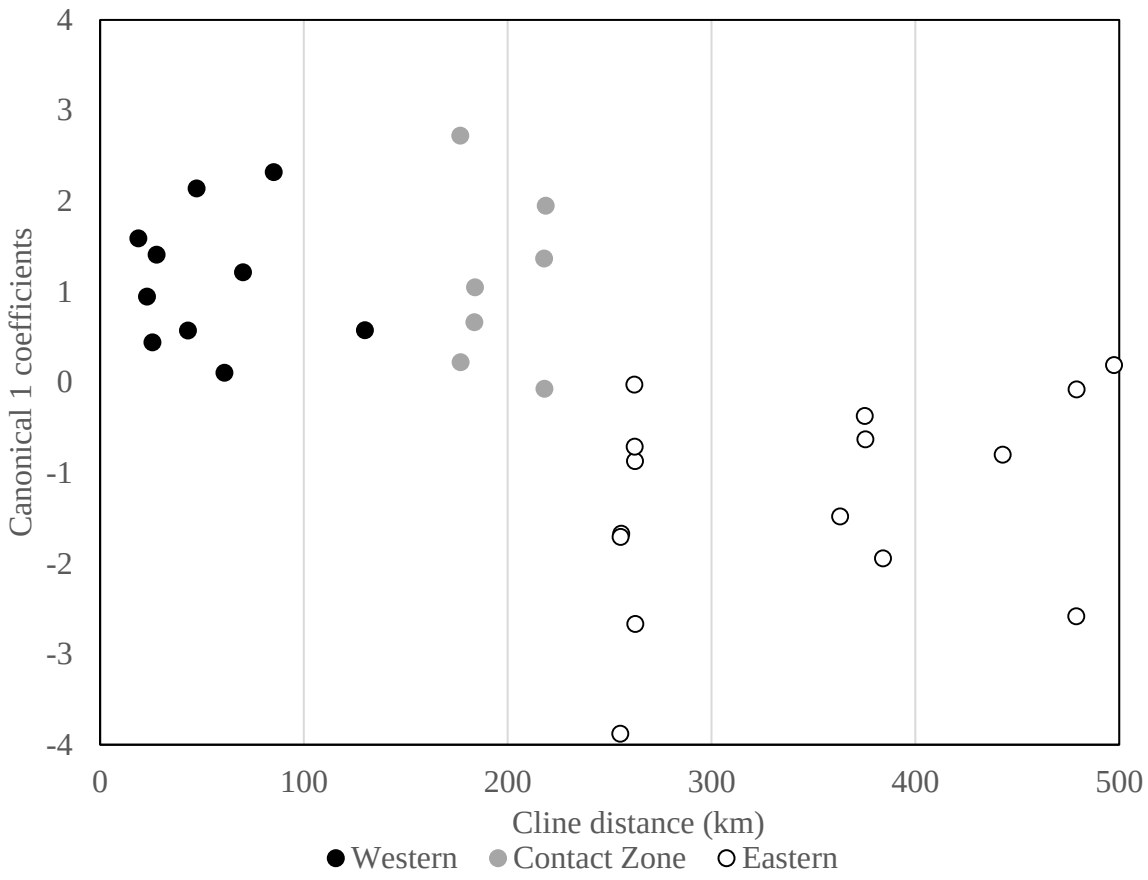


Figure 3.3 Canonical coefficient scores from first dimension. Scores taken from the five strongest song characteristics (as determined from PCA). Bird songs recorded east of the contact zone are represented by open circles, birds within the contact zone birds are grey circles, and birds west of the contact zone are closed circles.

3.3.1.1. Phrases

We identified seven phrase types across both species (Phrases A-G; Figure 3.4). Tufted Titmice and Black-crested Titmice (as expected species) were significantly different in phrase type (d.f.=5, r^2=0.23, Pearson P=0.03). At the species level, songs recorded east of the contact zone (Tufted Titmice) included all phrase types identified with the most common phrases being type E (~33%) and B (~20%). Songs recorded west of the contact

zone (Black-crested Titmice) included primarily phrase type D (~70%), but occasionally type A (24%) and E (10%). Songs within the contact zone included phrase D (57%), type A (29%), and type B (14%).

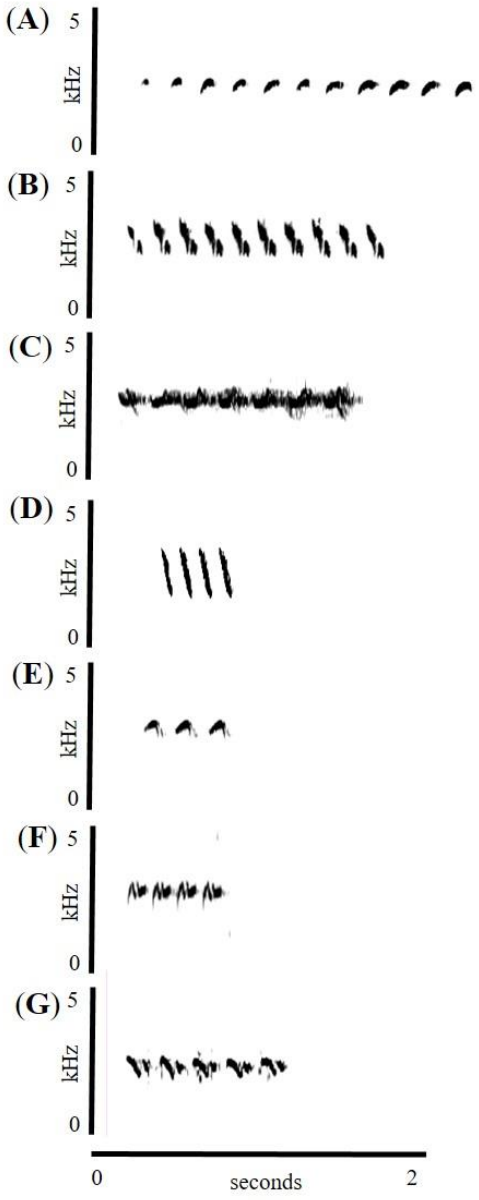


Figure 3.4 Spectrogram of seven different phrase types (arbitrarily named Phrase A-G) of Black-crested and Tufted Titmice identified from recordings used in this study. Phrases considered unique based on characteristic repeated notes and syllables.

3.3.2. **Behavioral differentiation**

3.3.2.1. Expected species differences

No Titmouse responded to the control song (Carolina Wren); these experiments were excluded from statistical analyses. Outside the contact zone, Black-crested Titmice responded to nine of the 19 playback experiments (47%), whereas Tufted Titmice responded to all 18 playbacks (100%) (Figure 3.5). Tufted Titmice did not distinguish (based on responsiveness) among songs played from different species; however, Black-crested Titmice responded significantly more often to conspecific songs (n=19, d.f.=1, X^2=6.74, 2-tailed Fisher's exact test=0.012). From those playbacks with a recorded response, Black-crested and Tufted Titmice did not differ in speed of behavior (n=29, d.f.=1, X^2=10.94, 2-tailed Fisher's exact test=0.432), despite Tufted Titmice responding slower more often (68%) (Figure 3.6). Although Tufted Titmice appeared to respond with vocal behavior more than Black-crested (57% Tufted, 30% Black-crested), the species did not vary in their maximum intensity behavior (n=29, d.f.=1, X^2=2.04, 2-tailed Fisher's exact test=0.245) (Figure 3.7).

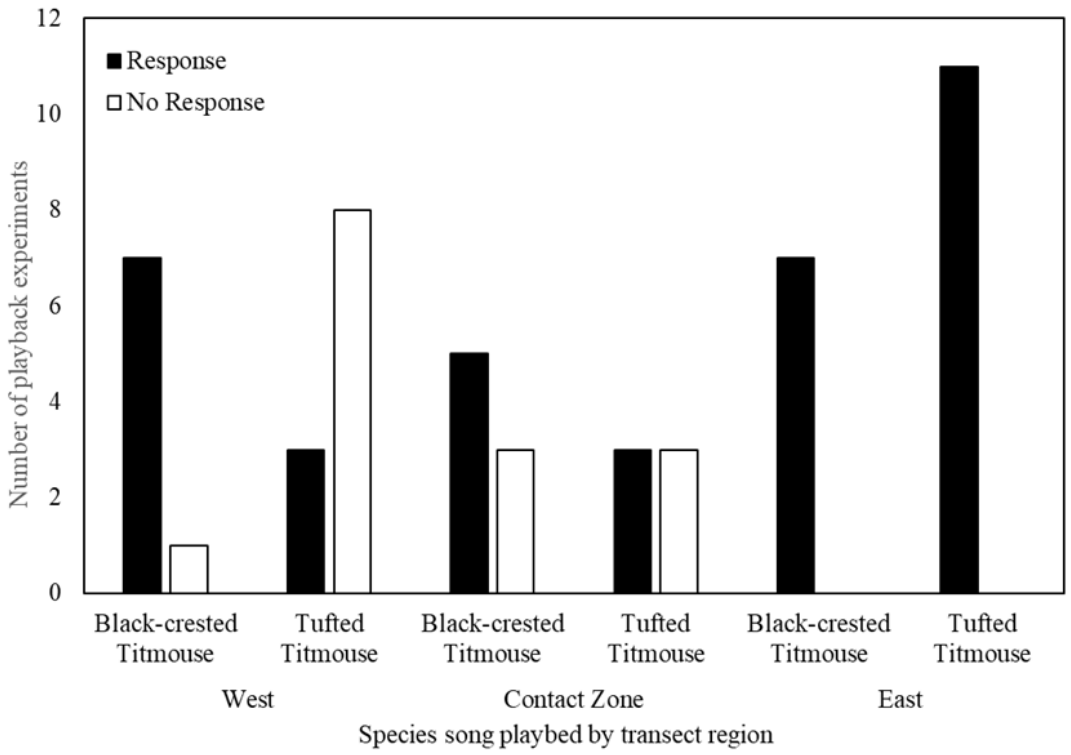


Figure 3.5 Number of responses based on species song played in each transect region. Black bars indicate number of responses to playback and white bars indicate number of no responses.

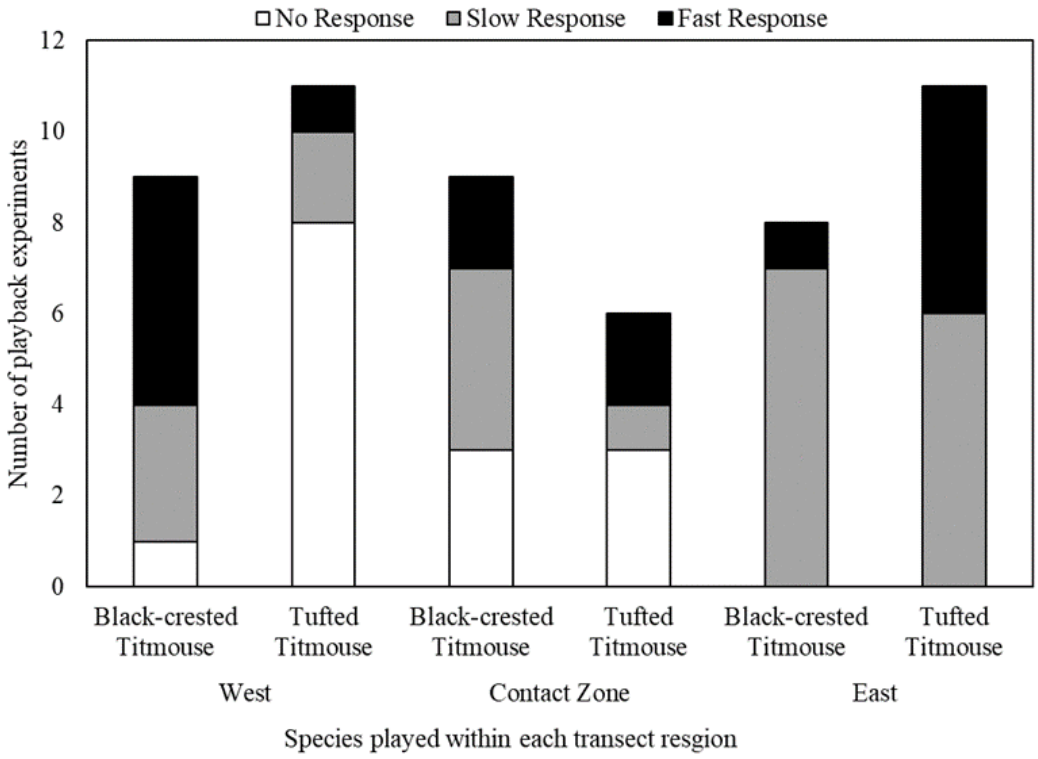


Figure 3.6 Playback response numbers based on speed of behavioral response related to species song played within each transect region. White indicates no response to playback, gray indicates responses occurring after one minute of playback, and black indicates responses within the first minute of playback recording.

3.3.2.2. Contact zone responses

Birds within the contact zone responded equally to Black-crested or Tufted Titmice songs (n=14, d.f.=1, X^2=0.219, 2-tailed Fischer's exact test=1.000) (Figure 3.5). Using only playbacks with a response recorded, we observed no difference in speed of behavioral response when comparing songs from different species (n=8, d.f.=1, X^2=0.53, 2-tailed Fisher's exact test=1.000) (Figure 3.6). We also note no difference in intensity of behavior based on species' song played (n=8, d.f.=1, X^2=0.04, 2-tailed Fisher's exact test=1.000) (Figure 3.7).

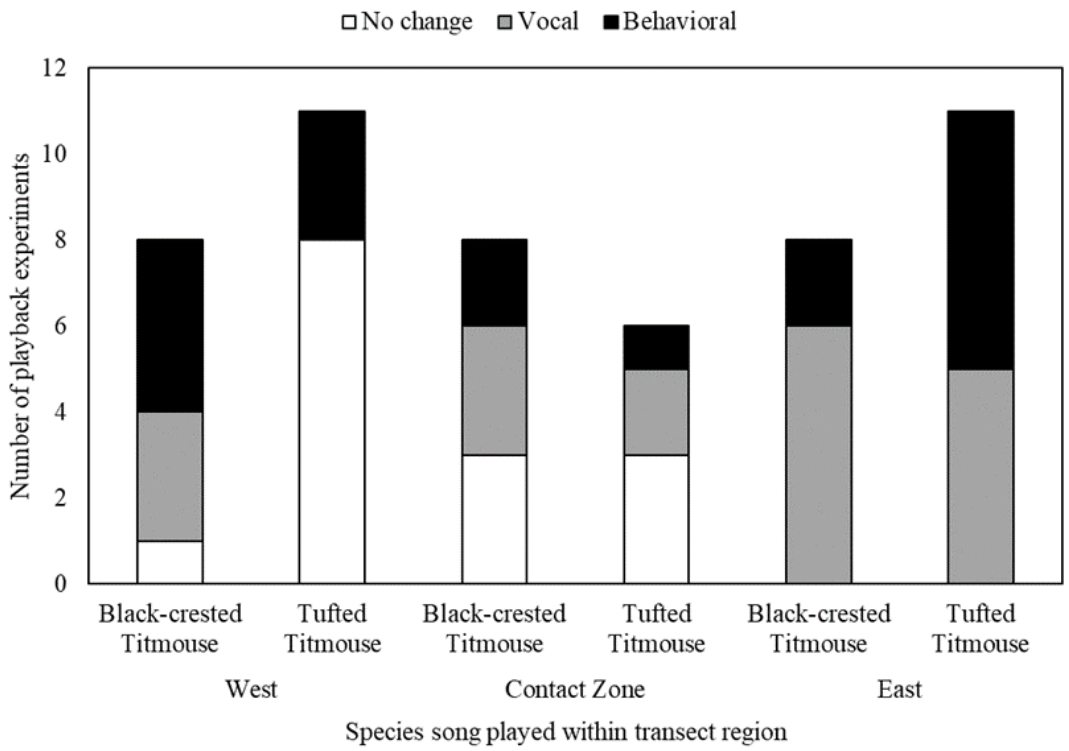


Figure 3.7 Playback response numbers based on maximum intensity behavior displayed for different species song within transect regions. White indicates no change in behavior from the start of playback recording (i.e., no response), gray indicates the maximum intensity displayed to be vocal (song or calling), and black indicates a maximum intensity response with behavioral displays (feather fluffing, switching perch, etc.)

3.4. Discussion

Black-crested and Tufted Titmice differ in several song characteristics, phrase preferences, and levels of aggression in central Texas. Within the Balcones contact zone, song structure is more similar to Black-crested songs, but song frequency is comparable to Tufted Titmice. It is possible that frequency is related to signal transmission in denser vegetation within and east of the contact zone. In this particular contact zone in central Texas, Titmice species appear to exhibit heterospecific behavioral recognition. Despite the continual presence of a contact zone, it seems each species is maintaining species-specific songs and behaviors outside the contact zone.

3.4.1. **Song variation**

Within and between species song variation is common and reportedly caused by a) species or genetic differences, b) cultural transmission (learning), or c) environmental conditions (Brumm & Naguib, 2009; Derryberry et al., 2018; Ey & Fischer, 2009). In the contact zone explored here, all three factors have the potential to impact song structure as these species likely diverged during Pleistocene glaciation (Gill et al., 2005), currently live in close proximity. Thus it is likely juveniles could learn song variation from neighbors or cultural transmission (Ritchison et al., 2015) as the hybrid zone occurs at a strong ecotone between different ecoregions (Griffith et al., 2007). Given the multitude of factors potentially impacting song variability, we focused on identifying species differences outside of the contact zone to assess the current song structure from birds within the contact zone and to provide a foundation for future studies.

Tufted and Black-crested Titmice are most easily distinguished by the number of notes per phrase, song rate (note length, tempo, and phrase duration), and frequency. Tufted Titmice sing fewer notes at a slower rate and lower frequency compared to Black-crested Titmice. The basic song pattern is relatively easy to distinguish in the field as Tufteds sing only 3-4 notes ("peters") in a phrase but Black-cresteds sing, on average, 7-8 notes per phrase. The rate and tempo of notes are likely connected to increasing signal transmission. By speeding up the rate and tempo of a greater number of notes, Black-cresteds increase signal transmission and increase clarity for the receiver (i.e., other birds) in an environment with fewer trees and greater distance between territories (Catchpole & Slater, 2008). Principal component analysis and subsequent discrimination classification showed species songs were primarily explained by note number and phrase length.

Furthermore, note number and phrase length of songs within the contact zone were similar to Black-crested songs west of the contact zone. The contact zone occurs at the western edge of Black-crested distribution and molecular studies report birds in this area share mitochondrial haplotypes with Tufted Titmice (Vaughn and Voelker, unpublished; Gill et al., 2005). From this, it is clear that basic song structure (note number, phrase length) is a species-specific trait, which is known to have a strong genetic component (Mason et al., 2017; Wheatcroft & Qvarnström, 2017).

Frequency was also different between species, yet it failed to be a distinguishing factor in principal components analysis. However, when comparing frequency between transect regions, frequency values were shared between eastern and contact zone songs. Frequency has long been associated with signal transmission across the environment (Henry & Lucas, 2010; Wilkins et al., 2013) and is often cited in support of the acoustic

adaptation hypothesis (Ey & Fischer, 2009; Patten et al., 2004). Most researchers agree that lower frequencies are associated with denser vegetation habitats (e.g., forests) to minimize signal transmission interference. This supports our finding of songs within the contact zone sharing similar frequencies to Tufted Titmice songs as the contact zone lies at transition between open scrub and hardwood forests (Griffith et al., 2007); thus, contact zone birds are more likely to encounter forested regions than birds west of the contact zone.

However, recent studies are reporting many songbirds exhibit vocal plasticity across different environments or in the presence of human-induced noise disturbances (Hamao et al., 2016; Lee & Park, 2019; Nemeth et al., 2013; Tolentino et al., 2018). Birds appear to be altering frequency, bandwidth, and even amplitude as their environment changes (at both the population and individual level) (Roca et al., 2016; Zollinger et al., 2012). Multiple species of Parids (the same Passeriformes family to which Titmice belong), including Great Tits and Mountain Chickadees, exhibit vocal plasticity (LaZerte et al., 2017; Lee & Park, 2019; Zollinger et al., 2017), so it is possible Black-crested and Tufted Titmice are capable of altering their frequency or, at minimum, their frequency is correlated with their environment. Both species utilize trees for shelter, nesting, and advertising songs, but tree density and tree species greatly varied across the transect (Griffith et al., 2007; Patten & Smith-Patten, 2008; Ritchison et al., 2015). As such, we recommend future research measure vegetation density as well as record environment noises if songs are recorded in urban areas. Such information will be valuable as this central contact zone is located in an area of rapid population growth as it includes Austin, Texas (state capitol), and its suburbs.

3.4.2. **Species recognition behaviors**

Territorial defense in males is often used as a proxy for species recognition in songbirds (Freeman & Montgomery, 2017; Martin & Martin, 2001; Searcy et al., 2006) as defense behaviors are energetically costly and thus only advantageous if the perceived threat is a real (conspecific) threat. We first compared male responses, outside the contact zone, to a perceived threat (song recordings of conspecifics and heterospecific Titmice) to acquire a bassline for behavioral differences in species. Our results show that Tufted Titmice are a more aggressive species, responding to conspecific and heterospecific songs. Their lack of species discrimination indicates Black-crested songs are similar enough to Tufteds to represent a territorial threat. Black-cresteds, on the other hand, were not only selective in responding to recordings in general, but could discriminate between species songs, responding more often to conspecific songs (Figure 3.5). Notably, we did not observe a response to all conspecific songs west of the contact zone. A lack of response could be a result of observational bias (we failed to observe a response) or prior presence of Titmice. However, we conducted playback experiments using two observers and only in areas where we heard Titmice singing prior to the experiment. Therefore, a lack of response may be a result of highly discerning or secretive males. Such behaviors further exemplify disparity from Tufted Titmice who have been reported as “aggressive” and “gregarious” (Ritchison et al., 2015), a description supported by personal observations by JCV. In a related study, it was difficult to locate or attract Black-crested Titmice with speakers compared to the curious and aggressive Tufted Titmice (JCV, personal observation).

Despite observing a difference in responsiveness between species, we found no difference in behavioral responses for individuals that did respond (either type of behavior

or speed). The similarity in maximum intensity behavior (vocal or behavior) is unsurprising as these species only recently diverged and likely share many other behaviors (Vaughn and Voelker, unpublished data; Gill et al., 2005; Ritchison et al., 2015). However, we were surprised by the lack of difference in the speed of the response as Tufteds are a more aggressive species. Population density, time of day, and season can impact territorial aggression in songbirds (Fletcher, 2007; Hyman, 2005; Nowicki et al., 2002), so it is possible there is a difference in speed of response, but we failed to observe the difference with our sample size. Differences in aggression between hybridizing species, known as asymmetrical aggression, can greatly impact contact zone dynamics (Freeman, 2016; Pearson, 2000; Pearson & Rohwer, 2000). Without alternative barriers in place, asymmetrical aggression can allow the dominant species (in this case, Tufted Titmouse) to outcompete and wipe out the other species. Therefore, we recommend future studies investigate the impact of asymmetrical aggression in these Titmice species.

Within the Balcones Escarpment contact zone, we observed similar responsiveness (response or no response) to either species song played and no differences in speed or intensity behaviors when comparing between species song played. These findings indicate contact zone birds are failing to discriminate between songs of Black-crested and Tufted Titmice. As mentioned previously, the contact zone occurs at the edge of the Black-crested distribution, so if the contact zone is occupied primarily by Black-crested Titmice and species recognition acts as a reproductive barrier, we should have seen contact zone birds showing similar discriminatory ability as Black-crested Titmice to heterospecific songs. This lack of discrimination could be a result of the small sample size within the contact zone, but it could also support cultural transmission in songs such that contact zone birds

do not perceive Tufted Titmice songs as a territorial threat. Notably, song characteristics showed no strong evidence of mixed-species songs (from cultural transmission) as is often reported in contact zone studies (Akçay et al., 2014; Kenyon et al., 2011; Price, 2008; Vokurková et al., 2013). It is possible this disparity between song and behavior could be result of song structure we are unable to distinguish. We did observe contact zone songs consisted of a wider variety of phrase types compared to Black-crested songs. Tufted Titmice showed no preference to phrase type, so the increased phrase type variety may be a product of cultural transmission. We recommend studies investigating the disparity between species-specific songs despite a lack of species recognition, either in Titmice or similar species.

3.4.3. **Communication implications**

Within a contact zone, bird song, as a sexual trait, often acts as a reproductive barrier if females prefer conspecific male song over mixed-species songs (Derryberry, 2007; Podos, 2004; Rowell & Servedio, 2012). In such cases, female preference maintains reproductive isolation as contact songs are selected against. Moreover, female mate choice can impact gene flow when they display a preference towards territorially aggressive males (Baldassarre et al., 2014; Freeman & Montgomery, 2017; Mennill et al., 2002). A recent study on mate choice in another Titmouse contact zone, in northern Texas and Oklahoma, reported a slight preference of females (of either species) towards male Tufted Titmice (Curry & Patten, 2019). The dominant behavior of Tufteds may explain the northward (not eastward) expansion of Black-crested Titmice, as Tufteds may outcompete Black-cresteds

for territories and mate selection in their (Tufted) preferred habitat type of mesic hardwood forests.

Given the dominant behavior of Tufted Titmice and the lack of heterospecific recognition of contact zone males, one could expect Tufted Titmice to outcompete Black-crested Titmice and expand their range westward, as they have continually done eastward across North America over the last century (Ritchison et al., 2015). However, physiological adaptations to ecoregions may be acting as a barrier. Recently, Vaughn et al. (unpublished) noted physiological differences between Titmice east and west of the Balcones Escarpment tied to precipitation. They suggest that Black-crested Titmice are physiologically adapted to the semi-arid climate of their distribution range, but Tufteds are ill-adapted to survive in such areas with sporadic precipitation.

The contact zone between Tufted and Black-crested Titmice along the Balcones Escarpment has been reported as stable with no discernable longitudinal shift (Dixon, 1990). Although genetic (as opposed to morphology/plumage) hybridization was only recently confirmed (Vaughn and Voelker, unpublished), results from this, and other recent studies (Curry & Patten, 2014, 2016), suggest a narrow and stable contact zone. Much more research is needed to understand contact zone dynamics and we recommend future research focus on female mate choice, contact fitness, and the impacts of asymmetrical aggression on gene flow.

3.5. Summary

Our results indicate that outside the contact zone (in allopatry) the species have distinctive songs and Tufted Titmice behavior is more aggressive than Black-crested

Titmice. Within the contact zone, song structure (number of notes and phrase length) is more similar to songs of Black-crested Titmice, with more notes per phrase; however, songs within the contact zone are lower in frequency, similar to that of Tufted Titmice songs. The contact zone lies on the eastern edge of Black-crested range; however, it occurs at a transitional ecotone. Song frequency often varies with vegetation density, so the lower frequency songs recorded in the contact zone are likely due to vegetation density rather than to genetic influence. Behaviorally, within the contact zone birds respond equally to Black-crested or Tufted Titmouse songs. Overall, we believe birds within the contact zone maintain species-specific song and behavioral characteristics but that environmental conditions may be impacting song frequency.

4. GENETIC ANALYSIS CONFIRMS HYBRIDIZATION BUT OVERTURNS PLUMAGE HYBRID INDEX IN A TEXAS CONTACT ZONE

4.1. Introduction

Technological advances in genetic analyses are expanding our understanding of the natural world, but they are also allowing scientists to re-evaluate past research (Hewitt, 2004). These advances are shedding light on the complexity of nature, including extra-pair paternity, interspecific mating, and cryptic admixture, among others (Reudink et al., 2006; Ribot et al., 2012). For example, genomic data are revealing that interspecific mating and cryptic genetic admixture are much more common than previously thought (Coster et al., 2018; Saitoh et al., 2015; Trigo et al., 2013). These discoveries can challenge taxonomists in their attempts to classify organisms (Abbott et al., 2013; Krosby & Rohwer, 2010; Mallet, 2005). Notably, genetic studies of contact zones (where genetic introgression may occur) often provide an in-situ platform to investigate a wide variety of evolutionary processes because hybridization can alter genetic diversity at a much faster rate than natural selection and recombination (Abbott et al., 2013; Mallet, 2005; Rheindt & Edwards, 2011). Therefore, contact zones can act as natural laboratories to investigate evolutionary concepts such as selection, gene flow, and environmental adaptations.

Avian contact zones are particularly convenient systems because birds use an array of auditory and visual cues (e.g., songs, plumage, and body size) to recognize conspecifics and assess mate choice which provide researchers with observable in-situ behaviors to gain a greater understanding of species interactions in contact zones (Catchpole & Slater, 2008; Kroodsma, 2005; Rowell & Servedio, 2012). Using genetic analyses in avian contact

zones, researchers are unearthing a myriad of findings about behaviors, mate preferences, and how birds maintain separate species. The earliest research was quick to determine hybridization and extra-pair paternity occurs more often than suspected, but as technology advanced, researchers have also discovered a) development of secondary sex ornamentation due to hybridization (Barrera-Guzmán et al., 2018), b) nearly identical species maintain species status based on mate preference of plumage color (Knief et al., 2019), and c) hybrids can have reduced capability of learning and memory (Rice & McQuillan, 2018). These findings, and more, signify the importance of utilizing contact zones to not only learn more about particular species, but also to further our understanding of evolutionary processes across the animal kingdom.

The Black-crested Titmouse (*Baeolophus atricristatus*) and Tufted Titmouse (*B. bicolor*) have long been suspected of interbreeding in Texas based on crest plumage, morphology, and small mitochondrial (mtDNA) genetic distance (Allen, 1907; Dixon, 1955; Gill et al., 2005). Black-crested Titmice reside in the semi-arid scrub vegetative region of west Texas south into northern Mexico, particularly in regions dominated by Ashe Juniper (*Juniperus ashei*) and Texas Live Oaks (*Quercus fusiformis*) (Patten & Smith-Patten, 2008). Tufted Titmice prefer mesic hardwood forests and their geographic range extends north and east from Texas across the eastern U.S. and north into Maine (Ritchison et al., 2015).

Dixon (1955) proposed three zones of contact between Black-crested and Tufted Titmice in areas of ecotones: 1) a small southern zone around Bee County, Texas, near the coast of the Gulf of Mexico (Gulf Contact Zone), 2) a presumably recent zone in north-central Texas and Oklahoma (TX/OK Contact Zone), and 3) a large and prominent zone in

central Texas along an inactive fault line known as the Balcones Escarpment (Balcones Contact Zone), which creates a geographic barrier for many vertebrate species and subspecies due to the differences in elevation, climate, and soil types on either side of the fault (Dixon, 1961, 1989; Goetze, 1995; Hafner, 1993; Smith & Buechner, 1947). Most research between the species has focused on the Balcones Contact Zone, including a large study on morphology and plumage differences in 1955, when Black-crested and Tufted Titmice were classified as subspecies (Dixon, 1955). In that study, Dixon reported Tufted Titmice are larger, in size, compared to Black-crested. Also, using a self-created hybrid index (Appendix D), Dixon reported the presence of hybrid birds based on crest and forehead plumage coloration. Following the advancement of genetic analyses, protein and mtDNA studies determined the genetic distance was great enough to warrant species status (0.4%-0.6%) and indicated the species diverged around 200,000 years ago (late Pleistocene glaciation) (Abbott & Woodruff Jr, 1986; Avise & Walker, 1998; Avise & Zink, 1988; Dixon, 1990).

To date, no study has confirmed hybridization between Black-crested and Tufted Titmice using genetic analysis. Furthermore, since Dixon's 1955 study, birdwatchers and ornithologists have reported the presence of hybrids, based plumage coloration, despite the lack of studies confirming interbreeding. Therefore, in this study, we utilized microsatellite analysis to determine if interbreeding is occurring within the Balcones Contact Zone. Also, using Dixon's hybrid index (DHI), we compared genetic identification with plumage coloration to assess the accuracy of identifying species and/or hybrids based on plumage coloration. Also, we analyzed the mtDNA NADH dehydrogenase subunit 2 gene (ND2) to provide a comparison of genetic divergence rates (previous studies used the Cytochrome B

mtDNA gene). Finally, we employed traditional museum techniques to measure body size (tarsus, tail, wing, and bill) of birds to determine if body size varied across the Balcones Contact Zone, particularly if birds within the contact zone were intermediate in size of parental species.

4.2. Material and Methods

For this study, we focused on the contact zone located along the Balcones Escarpment in central Texas (Figure 4.1). Originally, Dixon (1955) published the width of the contact zone to be about 18 km wide (east/west) across the Balcones Escarpment. Later, he reported a much wider contact zone with a range of 50-100 km (Dixon, 1990). For this study, we conservatively estimated the contact zone to be 75 km in width. Following Dixon's research, we placed the center of the contact zone on the Balcones Escarpment. Although the escarpment curves and lacks distinctive boundaries, we identify the center of the contact zone to be at 30.1183 (latitude) and -97.7994 (longitude) for reference purposes. Study sites were scattered across central Texas, both east and west of the contact zone; therefore, we refer to our study area as a transect. We labeled all study sites located within the 75 km contact zone width as being within the Balcones Escarpment contact zone. Sites outside this region (37.5 km east/west of the center) were labeled as either west or east.

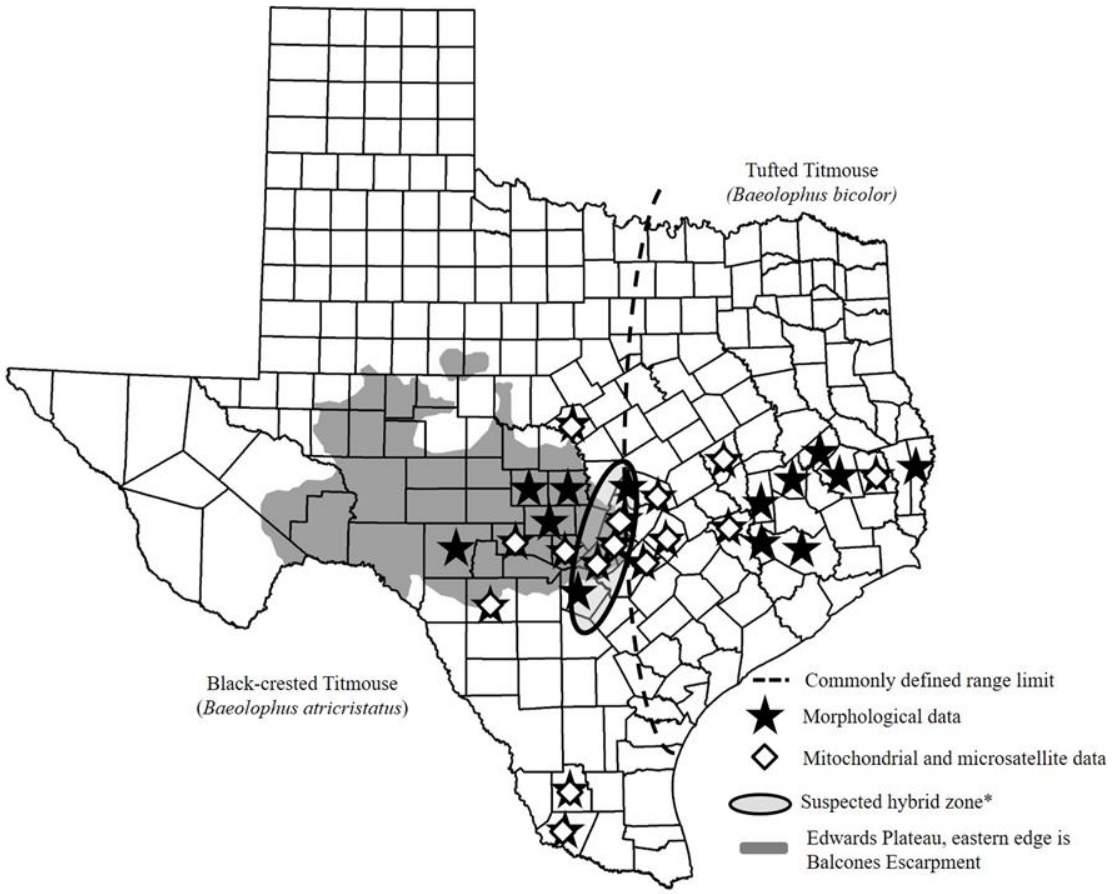


Figure 4.1 Map of collection sites for morphological (black stars) and genetic (open diamonds) data. Number of birds per site varied (Appendix A). The dashed line indicates the recognized species distribution boundaries in Texas: Black-crested Titmouse west of the line and Tufted Titmouse east of the line. The described contact zone is shown by an oval. Modified and reprinted from Texas Parks and Wildlife (2020).

4.2.1. **Data collection**

4.2.1.1. Genetic data collection.

During 2009-2013, we collected a total of 97 Black-crested and Tufted Titmice individuals at various private properties across central Texas (Figure 1, Appendix A, E). Birds were either mist-netted (with and without playback; see below) and humanely euthanized or shot. Pectoral muscle was removed and frozen for DNA extraction and skins were prepared and stored at the Biodiversity Research and Teaching Collections at Texas A&M University (Appendix A). All collection protocols and sample numbers were approved by Texas A&M University's IACUC (AUP #2012-6), the U.S. Fish and Wildlife Service (permit #MB205752), and the Texas Parks and Wildlife Department (permit #SPR-0209-016).

Ninety-four of the 97 birds were collected within the transect (west n=24, contact zone n=30, east n=40). The remaining three birds were obtained from south of the transect and used as a reference for birds well outside the contact zone (Avise & Walker, 1998; Dixon, 1978). We included these birds to compare genetic differences between Black-crested Titmice within and outside the transect. We included Carolina Chickadee (*Poecile carolinensis*) in our study as they belong to the same family as Titmice (Paridae), thus acting as a suitable outgroup for rooting phylogenetic trees. In total, we extracted DNA from 104 samples (Titmice=97, Carolina Chickadee=7) with the DNAeasy Tissue Kit following the manufacturer's protocols (QIAGEN Inc., Valencia, California).

4.2.1.2. Plumage and morphology

In addition to the birds captured for this study, we had access to previously collected Black-crested and Tufted Titmice housed as museum specimens in the Biodiversity Research and Teaching Collections. To compare plumage with genetic identification, we used the Dixon Hybrid Index (DHI) to calculate a hybrid index value (range 0-6) for 168 Titmice museum specimens, which included those captured in the course of this study. According to the DHI (based on the sum scores crest and forehead) pure Tufted Titmice range from 0-1, hybrids from 2-4, and pure Black-cresteds from 5-6 (scoring table Appendix D; Dixon, 1955). Based on this scale, a "hybrid" has a blend of plumage (between the two species), often with an all grey crest/forehead or grey crest with brown forehead, both of which are used by birdwatchers to report sightings of hybrids.

To compare morphological measurements of contact zone birds to birds outside the contact zone, we measured the length of the bill (exposed culmen), right wing, right tarsus, and tail on the 168 Titmice museum specimens (Figure 1; Appendix A). Using digital calipers (+/- 0.01 mm) and wing cord rulers, each morphological variable was measured twice (by the same person), and the average was used for statistical analysis. Additionally, using specimen labels, we recorded sex, age (based on ossification), and capture location information (county, latitude, and longitude).

4.2.2. **Laboratory methods**

4.2.2.1. Microsatellite DNA methods

We selected a set of 29 previously published loci for closely related Paridae species and initially tested them for amplification using eight individuals collected east of the contact

zone (pure Tufted) (Appendix F). As described by Boutin-Ganache et al. (2001), we amplified microsatellites using the 'tail' methodology which fluorescently labels the tail primer to allow for higher throughput through the analyzer. Each PCR was performed in 10 µl reactions containing 4.85 µL water, 0.5 µl forward primer (10 µM), 0.5 µL fluorescently dye-labeled "tail" primer (10 µM), 0.5 µL reverse primer (10 µM), 2 µL buffer, 0.8 µL $MgCl_2$, 0.2 dNTP, 0.1 µLTaq, and 1 µL DNA. We used a touchdown PCR protocol to amplify all loci: 3 min at 95°C; 2 cycles of 94°C for 30 sec, 58°C for 45 sec, and 72°C for 1 min; 2 cycles of 95°C for 30 sec, 55°C for 45 sec, and 72°C for 1 min; 2 cycles of 95°C for 30 sec, 52°C for 45 sec, 72°C for 1 min; 38 cycles of 95°C for 30 sec, 50°C for 45 sec, and 72°C for 1 min; and a final extension at 72°C for 10 min. Using an ABI Prism 377 DNA Sequencer, we visualized samples and then performed analyses and assessed allelic identities using GeneScan and Genotyper software (Applied Biosystems, Foster City, CA). Of the 29 primer sets tested, 14 were polymorphic and were subsequently used on the 97 Titmice samples (Appendix F).

4.2.2.2. Mitochondrial DNA.

We amplified the NADH dehydrogenase subunit 2 (ND2) gene (1042 base pairs) using polymerase chain reaction (PCR) with primer pairs L5215 (Hackett, 1996) and H6313 (Johnson & Sorenson, 1998) and LMNPQAKL (Lerner et al. 2011) and H6313 (Johnson & Sorenson, 1998; Lerner et al., 2011). Each PCR was performed in 25 µl reactions containing 2.5 µL 5x buffer, 2 µL 10µM deoxynucleoside triphosphate (dNTP), 1µL of 10µM forward and reverse primer (each), 0.125 µL 5 µM Taq polymerase (TaKaRa, Mountain View, CA and GoTaq, Promega, Madison, WI), 17.375 µl H_2O, and 1 µl DNA

template. We used the following thermocycler protocol, with ranges depending on different Taqs and sample variation: 2-4 min at 95°C, 35-45 cycles of 95°C (45 sec), 50-52°C (30-45 sec), and 72°C (1 min), followed by a final extension of 72°C (8-10 min). All successfully amplified products (verified with gel electrophoresis) were purified using EXOSap-IT (USB Corporation, Cleveland, OH) and all sequencing reactions were performed at the University of Florida DNA Sequencing Core Laboratory (Gainesville, Florida) using ABI Prism BigDye Terminator cycle sequencing protocols (described by Light and Reed (2009). Sequencing edits were completed using Sequencher 4.9 (Gene Codes Co., Ann Arbor, MI), primers trimmed and sequenced aligned, by eye, using a Tufted Titmouse reference sample (GenBank accession number AY825995.1). Further alignment, for phylogenetic analysis, was completed in MEGA 7.0 (Kumar et al., 2016), including the alignment of Carolina Chickadees.

4.2.3. **Statistical analysis**

4.2.3.1. Morphological data.

Knowing titmice morphological variables can vary by age, we used only adult birds, within the transect region, for morphological comparison (n=71). First, we determined normality for each morphological variable using a continuous fit (normal) and goodness of fit test of probability and found all variables to be normally distributed. We used histogram distributions and outlier plots (based on Tukey's rule) to determine the presence of outliers (Aggarwal, 2017). To assess overall morphological differences and to provide body measurements for reference in other studies across transect regions (east, contact zone, west), we performed one-way ANOVAs on each morphological variable (bill, wing, tarsus,

tail). To determine differences between transect regions, while reducing the chance of type I error, we used an all pair Tukey HSD (honest significance difference) test (Urdan, 2011). To compare variable measurements between sexes, we repeated ANOVAs for males (n=55) and females (n=16) separately. Next, we used covariance principal components analysis to understand which variables explain the most variance between transect regions. For this analysis, we used only adult males and a covariance PCA was used as all variables are body length variables measured using the same units (mm) and to ensure we retained maximum variance (Jolliffe, 2002). Only birds with measurements for all four variables were used in PC analysis. We then compared principal component values across the transect region using one-way ANOVAs and all pair Tukey HSD between transect regions (Urdan, 2011). All statistical analyses were performed using JMP 14 (JMP, 2018).

4.2.3.2. Microsatellite analysis

To minimize the impact of sibling relatedness (an assumption of some analyses), we omitted juveniles caught at the same time as adults for microsatellite analyses (n=67 for analysis) (Porras-Hurtado et al., 2013). We tested for random associations between genotypes at each locus with linkage disequilibrium tests using the commonly employed Markov chain algorithm in the program GENEPOP (Raymond & Rousset, 1995). Deviations from Hardy-Weinberg equilibrium (HWE) were also tested at each locus using exact tests in GENEPOP. To ensure deviations from HWE were not due to genotyping errors, we checked for the presence of null alleles using Micro-checker (Van Oosterhout et al., 2004). We collected basic diversity measurements including the number of alleles, allelic richness (number of alleles per locus), expected heterozygosity (gene diversity), and

Nei's genetic distance using Arlequin (Excoffier & Lischer, 2010). To compare nuclear genetic variance with mtDNA variance, we created pairwise F_{ST} estimates using a hierarchical analysis of molecular variance in Arlequin.

Potential hybrid individuals were identified using the Markov chain Monte Carlo (MCMC) and Bayesian clustering algorithms implemented in the program STRUCTURE (Porras-Hurtado et al., 2013; Pritchard et al., 2000). This program attempts to assign genetic individuals to different clusters while minimizing linkage disequilibrium. Beyond determining if genetic admixture (hybridization) is present, we aimed to see if the geographic locations of captured birds were unique genetic populations (southern, west, contact zone, or east). Using STRUCTURE, we ran three different models varying in admixture and parameters. The models were 1) no admixture model with local priors (capture location) 2) MCMC admixture model of ancestry, allowing admixed individuals to be assigned to more than one cluster (assumes admixture is occurring) and 3) admixture with local priors. Using local prior (sampling) information is recommended for stronger assignment, especially if admixture is suspected (Porras-Hurtado et al., 2013; Pritchard et al., 2000). For all models, we employed a burn-in of 10,000 replicates, followed by 10,000 MCMC replications, and set the maximum number of clusters to five, a number of genetic populations we did not believe existed but would capture multiple populations if they were present. To ensure we captured the correct number of clusters we conducted 30 iterations (number of runs). We determined the number of genetic clusters by employing the Evanno methods in STRUCTURE harvester (Earl & von Holdt, 2012; Evanno et al., 2005). Using the data from the cluster with the lowest log-likelihood, we used CLUMPP to independently reassess individual assignment to each cluster (Jakobsson & Rosenberg,

2007). Individuals are assigned a cluster based on estimated membership coefficient (q). We used a conservative 80/20 cutoff to identify pure and hybrid individuals with hybrids having a value of $0.2 < q > 0.8$ (Vähä & Primmer, 2006). Lastly, we used Distruct to create a visual representation of the cluster assignments across each population (Rosenberg, 2007).

4.2.3.3. Mitochondrial phylogenetic and haplotype network analysis

We first conducted a preliminary analysis of mtDNA sequences (n=97) to gain a baseline of relatedness between the expected species using a neighbor-joining tree in MEGA 7 (Kumar et al., 2016). Using the model selection tool in MEGA 7, the best-fitting substitution model (based on lowest BIC value) was HKY+G (Hasewa-Kishino-Yano, plus gamma). Using this model, we created a maximum likelihood tree using the bootstrap method (500 replications) with five gamma categories in the program MEGA 7.0 (Kumar et al., 2016; Tamura et al., 2011). The tree was rooted using Carolina Chickadees as an outgroup to help assess the magnitude of genetic distance between species. Visualization of a Newick tree was created using a web-based Interactive Tree of Life (iTOL) (Letunic & Bork, 2019). Pairwise and maximum likelihood between-group distances were calculated between Carolina Chickadee, Tufted Titmice, Contact zone, Black-crested Titmice, and Black-crested Titmice from south Texas using MEGA 7.0 (Kumar et al., 2016). We calculated haplotype diversity (Hd) and nucleotide diversity (P_i) to measure within-transect region variation using DNAsp (Librado & Rozas, 2009; Rozas et al., 2017). We used PopArt (Population Analysis with Articulate Trees) to develop haplotype cluster network graphics using an integral neighbor-joining method (Leigh & Bryant, 2015).

4.3. Results

4.3.1. Microsatellite analysis

Following initial analysis, four loci (Pma28, Pma86, Pmo02, and Pma42) showed the presence of null alleles and were therefore removed from further analysis (Appendix F). Nine of the 10 loci were polymorphic for Black-crested Titmice, with allelic richness of 6.5 ±4.1; however, for birds east of the contact zone (Tufted Titmice) only six loci were polymorphic with allelic richness of 10.50 ±4.5 (Table 4.1). Diversity analysis showed all transect regions (south, west, contact zone, and east) having greater expected (H_E) than observed (H_O) heterozygosity, although none of the differences were statistically significant (Table 4.1). Tufted Titmice had greater H_O and H_E than Black-crested Titmice (birds south, west, and within the contact zone). Within population F_{IS} ranged from 0.145 (east) to 0.208 (contact zone) with the western birds having a coefficient 0.154 and southern birds 0.167. F_{IS} values for east, west, and contact birds were significant; however, the south was not significant (P=0.193). F_{ST} values were highest between the east and west and east and contact zone (Table 4.2), with significant differences between all transect regions except west and contact zone (F_{ST} 0.014, P=0.189).

Table 4.1 Diversity statistics from mitochondrial (n=97) and microsatellite (juveniles removed, n=64) analysis. For mitochondrial data, n=number of individuals, N_{haplo}=number of haplotypes, H, No.=haplotype number, HD=haplotype diversity, π=nucleotide diversity, D=Tajima's D neutrality test. For microsatellite data, n=number of individuals, N_{poly}=number of polymorphic loci, a=mean number of alleles with standard deviation (sd), H_O=mean observed heterozygosity, H_E=mean expected heterozygosity, F_{IS}=inbreeding coefficient, F_{ST}=genetic distant coefficient. Asterisk (*) indicates significance P<0.05.

	Mitochondrial DNA, ND2						Microsatellite					
	N	N_{haplo}	H, No.	HD	π	D	N	N_{poly}	a, s.d	H_O	H_E	F_{IS}
East	40	18	13-30	0.879	0.002	-1.99*	27	6	10.5, +/-4.5	0.67	0.81	*0.145
Contact zone	30	8	1-5, 10-11	0.703	0.001	-1.10	18	9	6, +/-3.9	0.51	0.64	*0.208
West	24	9	1-9	0.855	0.002	-0.96	19	8	4.7, +/-1.8	0.46	0.51	*0.154
South	3	2	2, 12	0.667	0.001	n/a	3	6	2.3, +/-1.6	0.44	0.66	0.167
Tufted Titmouse	40	18	13-30	0.879	0.002	-1.99*	27	6	10.5, +/-4.5	0.65	0.81	*0.164
Black-crested Titmouse	57	12	1-12	*0.830	0.001	-1.24	37	9	6.5, +/-4.1	0.47	0.52	*0.121

Table 4.2 Pairwise F_{ST} values between transect regions. Bottom values indicate F_{ST} values, Top values indicate p-values. Bold indicates significance, alpha=0.05.

	South	West	Contact zone	East
South		**0.054**	**0.045**	**0.000**
West	0.102		0.189	**0.000**
Contact zone	0.100	0.014		**0.000**
East	0.268	0.217	0.134	

Microsatellite cluster analysis indicated two genetic populations (K=2) no matter which model or parameters were used (Figure 4.2), indicating the contact zone is not a distinct genetic cluster. All genetically pure individuals (non-hybrids) were located in the cluster with other birds captured from the same area; that is one cluster included birds captured regions where Black-crested Titmice reside (west, south, and contact zone) and another cluster included birds captured east of the contact zone (Tufted Titmouse range). When a no-admixture model was employed, only one individual was determined to be a hybrid (Appendix A): an adult female from Comal County who was classified as Black-crested using the DHI and had mtDNA similar to other Black-crested Titmice (Figure 4.3). Twelve birds were classified as admixed (hybrids) using a 20% admixture cutoff ($0.2<q>0.8$), yet only five of these birds were classified as hybrids using DHI (index score 2-4) (Figure 4.4). Of these 12 birds, three individuals were captured east of the Balcones Escarpment (Williamson and Bastrop Counties) and are in the Tufted mtDNA clade based on maximum likelihood phylogenetic tree (Figure 4.4). The remaining nine birds fell out in the Black-crested clade with three captured west of the Balcones Escarpment (Uvalde, Kendall, and Mills Counties) and six within the contact zone (Hays, Comal, and Travis Counties; Figure 4.4). Only one of the 12 hybrid birds was a juvenile and collectively all of these potential hybrid birds reflected a wide variety of DHI scores (1-6) (Appendix A).

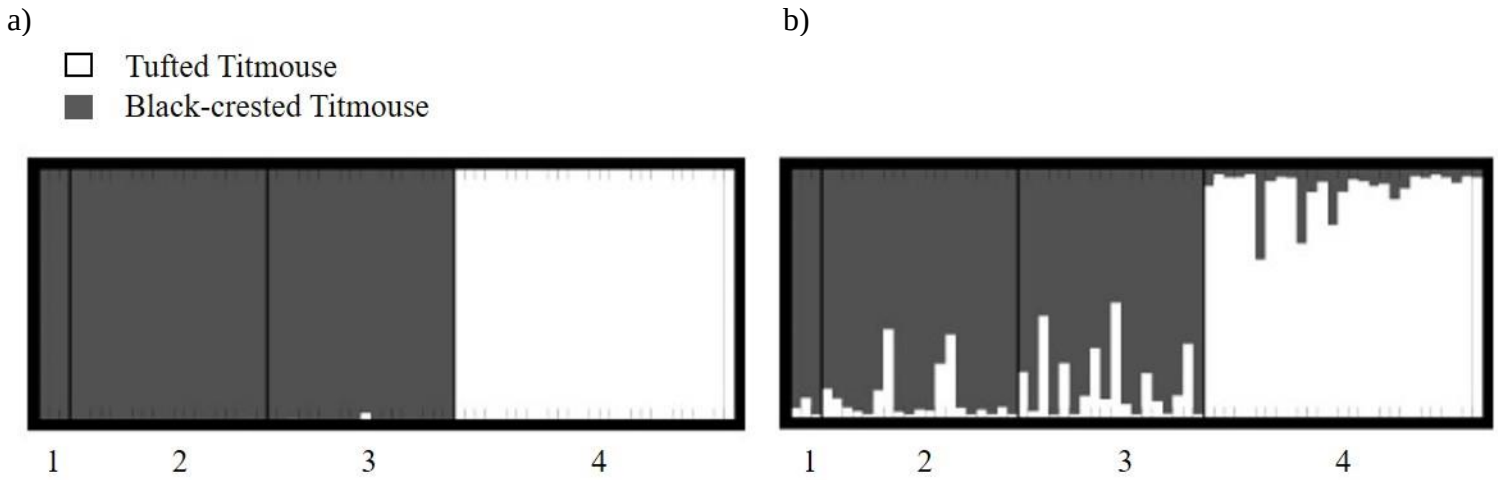


Figure 4.2 Results from microsatellite analysis from Texas Titmice, with potential siblings removed (n=67) (K=2). Numbers below figures represent potential populations: 1: south (of Balcones Escarpment), 2: west of contact zone, 3: contact zone, 4: east of contact zone. a) Analysis conducted using the basic model (no admixture) and b) using the admixture model.

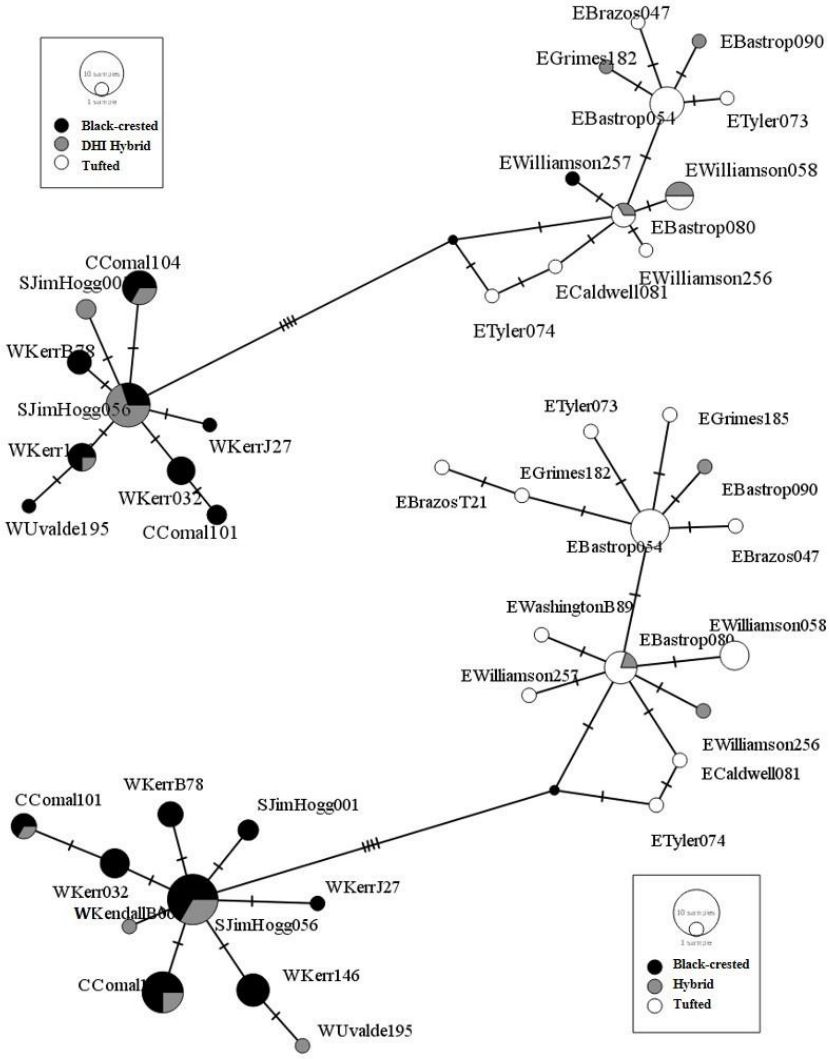


Figure 4.3 Top: Cluster network map of mtDNA haplotypes for birds captured within the transect (centered around the Balcones Contact Zone) with potential siblings removed (n=67) and color-coded according to the Dixon Hybrid Index. Black represents birds identified as adult Black-crested Titmice (DHI 5-6), grey represents "hybrids" (2-4), and white represents Tufted Titmice (0-1). Bottom: Individuals labeled by microsatellite assignment. Black represents Black-crested Titmice (q value greater than 80%), grey are hybrid birds (q values between 20-80%), and white are Tufted Titmice (q less than 20%).

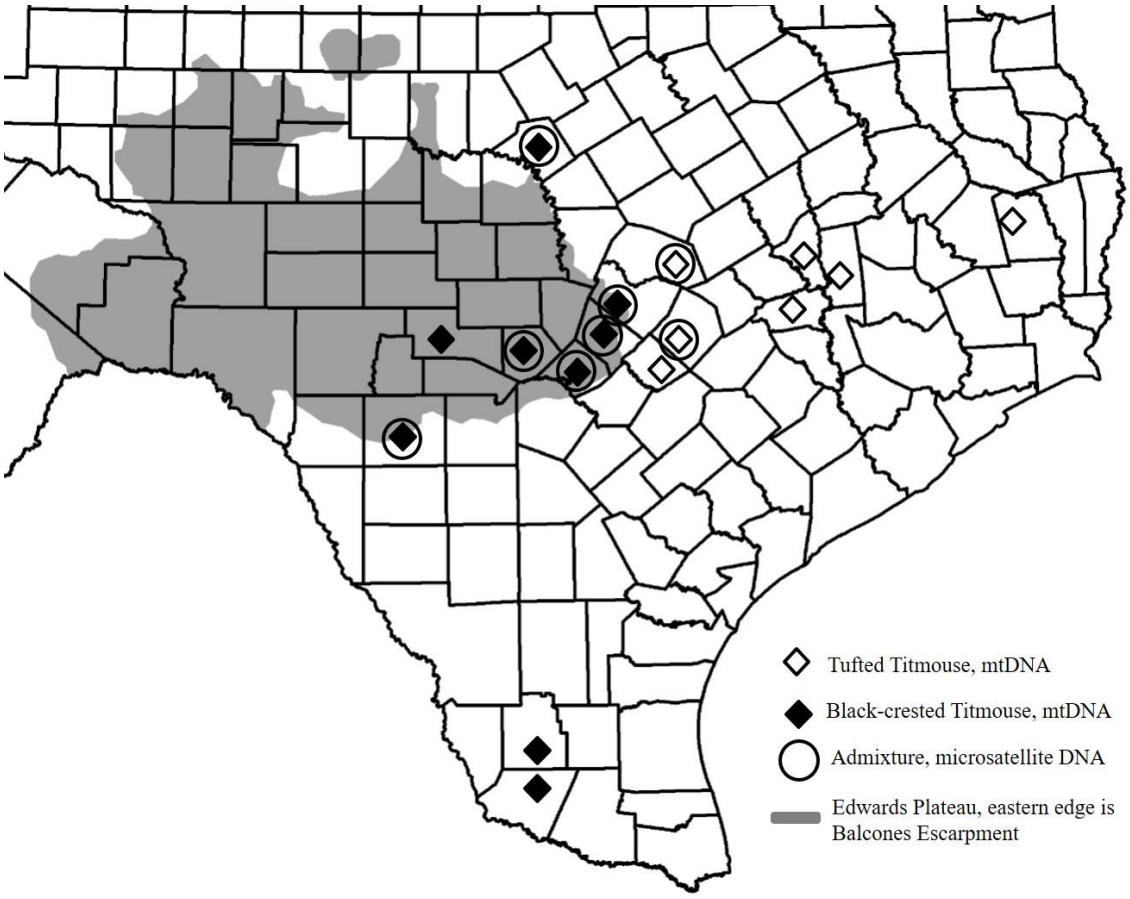


Figure 4.4 Map of site capture locations and indicating the mtDNA clade that individuals belong within. Black diamonds indicate Tufted Titmice, white diamonds represent Black-crested Titmice. A black circle indicated at least one individual is considered a hybrid based on microsatellite assignment.

4.3.2. **mtDNA clades, phylogenetic tree, and genetic distances**

Using all mtDNA birds (n=97), birds captured east of the contact zone (Tufted Titmouse range) had more haplotypes (N_{haplo}=18), greater haplotype diversity (0.879), and larger Tajima's D (-1.99) compared to birds captured west of the contact zone (Black-crested Titmouse range) (Table 4.1). Tajima's D test was significant for the Tufted clade (-1.99) but not for Black-crested (-0.96) (Table 4.1). Birds captured south of the Balcones Contact Zone (Black-crested Titmouse range) (n=3) have fewer mitochondrial haplotypes (N_{haplo}=2) and lower haplotype diversity (0.667) compared to birds across the Balcones contact zone transect. Sample size for the southern birds was too small for a Tajima's D test for significance. Within the contact zone, diversity statistics were similar to birds captured west of the contact zone (Table 4.1). Nucleotide diversity was the same for birds west and east of the contact zone (0.002) but for birds within the contact zone and birds south of the contact zone the haplotype diversity was 0.001.

Maximum likelihood analysis produced two distinct clades with birds from east of the Balcones Escarpment falling into one clade (Tufted Titmouse), and all birds from west of the Balcones Escarpment (including south and contact zone) in the second clade (Figure 4.5). Average uncorrected pairwise distance between Tufted Titmice and Black-crested Titmice was 0.007 with maximum likelihood distance of 0.004 (Table 4.3). Genetic distances between Black-crested Titmice and birds within the contact zone (0.001) and south Texas birds (0.002) was minimal. In comparison, average pairwise distance between Carolina Chickadee and both Tufted and Black-crested Titmice was 0.111 with a maximum likelihood distance of 0.074.

Mitochondrial haplotype cluster analysis, using only birds for which we had both mtDNA and microsatellite data (n=67), produced two distinct groups separated by five steps (Figure 4.3). One group contained birds captured east of the contact zone and the other group included birds captured west and within the contact zone.

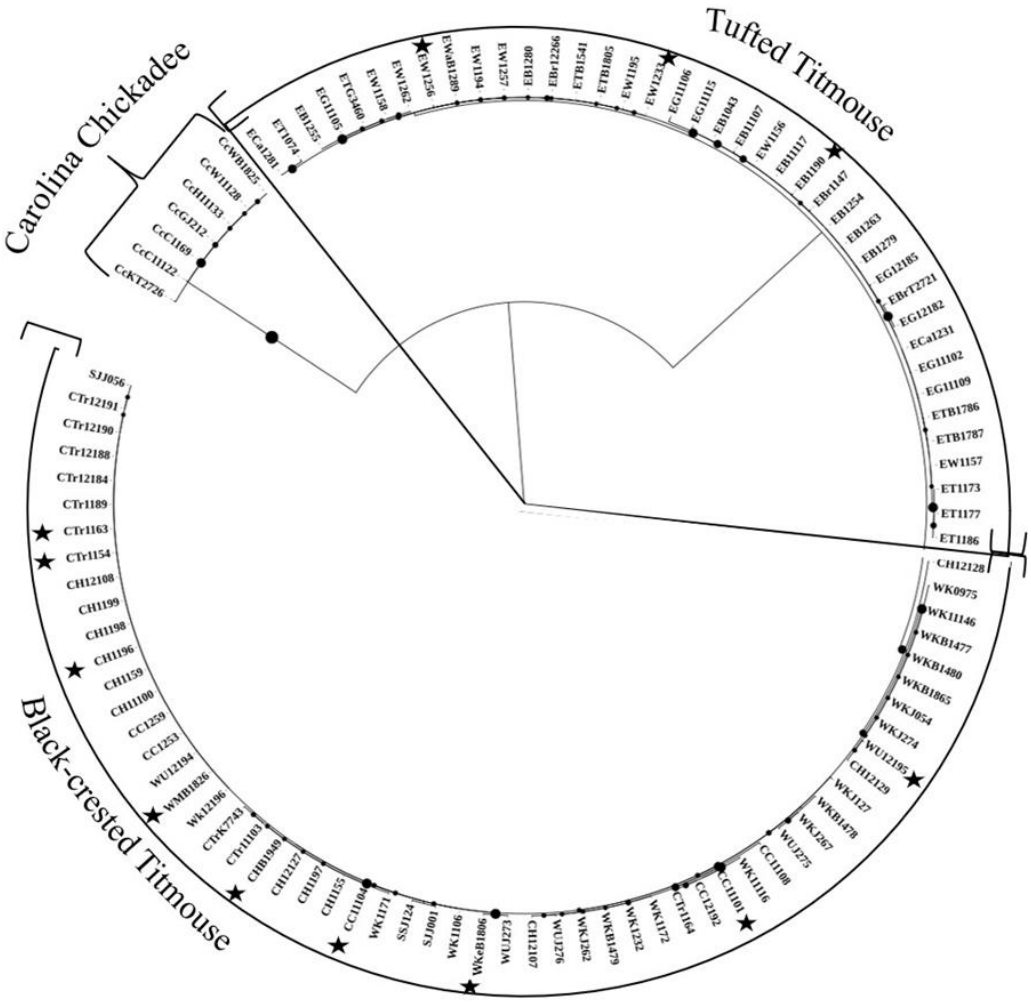


Figure 4.5 Maximum likelihood phylogenetic tree from mitochondrial DNA (ND2) of Black-crested and Tufted Titmice. Black-crested Titmice sequences starting with "C" were captured in the contact zone, those starting with "S" are from south Texas, all others are labeled "W." The tree is rooted with closely related Carolina Chickadee. Stars indicate hybrid birds based on microsatellite analysis.

Table 4.3 Pairwise distances genetic distances between each group (bottom) and maximum composite likelihood (top) of Tufted and Black-crested Titmice as well as the other species in the genera Baeolophus (Family: Paridae) and their sister species Carolina Chickadee (Family: Paridae, *Poecile*). All standard error values were less than or equal to 0.01.

	Carolina Chickadee	Tufted Titmouse	Contact zone	Black-crested Titmouse	Black-crested Titmouse, south
Carolina Chickadee		*0.074*	*0.074*	*0.074*	*0.074*
Tufted Titmouse	0.111		*0.004*	*0.004*	*0.004*
Contact zone	0.111	0.007		*0.001*	*0.001*
Black-crested Titmouse	0.111	0.007	0.001		*0.001*
Black-crested Titmouse, south	0.111	0.007	0.001	0.002	

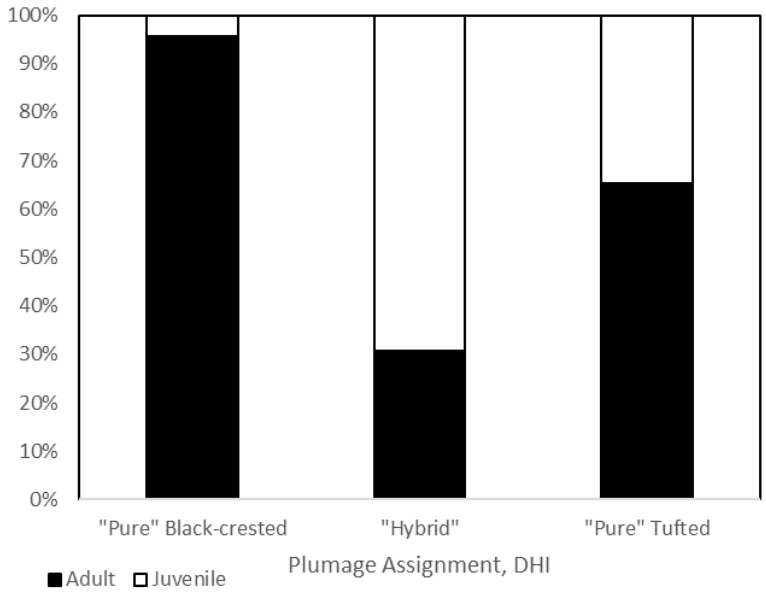
4.3.3. **Plumage and DHI**

Using all Titmice from genetic analysis (n=97), 69% of birds who appear "hybrid" (via DHI 2-4) were juveniles (Figure 4.6). For "pure" Black-cresteds (DHI 5-6), only 4% were juveniles and 35% of "pure" Tufteds were juveniles. Microsatellite analysis, which removed many juveniles, showed that of those assigned as admixed individuals (n=12), 42% appeared "hybrid" according to DHI; another 43% appeared "pure" Black-crested, and only 4% appeared "pure" Tufted (Figure 4.6). Tufted Titmice had the fewest birds that appeared as "hybrids" at 30% compared with 45% Black-crested Titmice.

In comparing plumage to maximum likelihood trees, their mtDNA clade, irrespective of their plumage, DHI hybrids (hybrid index based on plumage) were present in both haplotype clusters (Figure 4.3). Haplotype cluster analyses provide a visual representation of the incongruence between plumage (using DHI) and microsatellite assignment (Figure 4.3, Appendix A, E). The overall pattern of the clusters is similar but

associations between individuals vary as plumage does not always equate to genetic assignment.

a)



b)

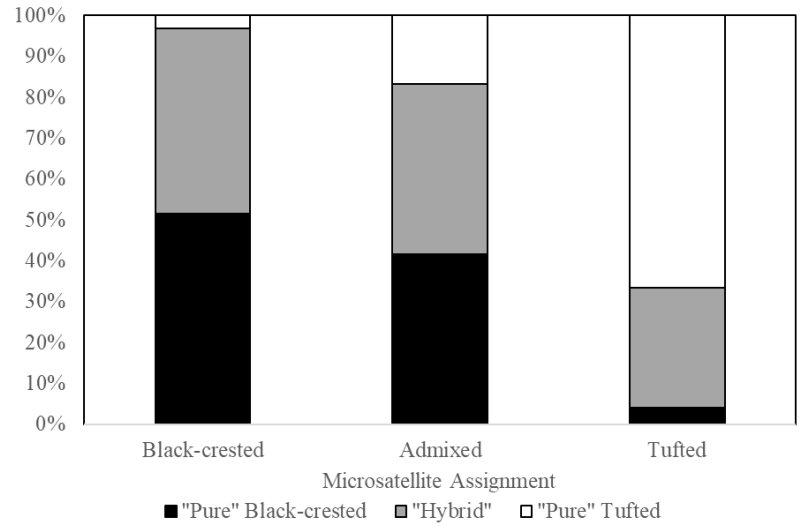


Figure 4.6 Dixon Hybrid Index. a) Plumage assignment based on age using birds used in mitochondrial analysis (n=97), b) Plumage assignment for each microsatellite assignment (n=67).

4.3.4. **Morphology**

In reviewing the distribution of each morphological variable for transect-only birds, there were two outliers (Appendix A). To understand true variation for each morphological variable, we only removed the outlying value, not all variables for that bird. Using all birds, females have shorter bills, wings, and tarsi compared to males (Appendix H; Table 4.2); however, when considering each transect regions separately, only wing size was smaller in females than males. Across the transect, all measurements were similar in size in birds captured west and within the contact zone (Table 4.4). Bill and wing length were larger in birds east of the contact for adult male birds, but only wing length was significantly larger for female birds captured east of the contact zone.

Principal component (PC) analysis, conducted on adult males, indicated the first PC was weighted heaviest for tail measurements and PC2 weighted strongest with wing variance (Table 4.5). Across transect regions, both PC values varied, overall, between regions; however, PC1 values were significantly different between birds captured east and west of the contact zone, whereas PC2 values were significantly different between birds captured east of the contact zone and birds captured within the contact zone (Table 4.6).

Table 4.4 Sample size (n), mean (x), and standard deviation (sd) of morphological measurements (mm) from only birds collected across central Texas for a) all adult birds, b) only adult males, c) adult females. Each pair t-tests between-group p-values provided for morphological differences between regions. [d.f=degrees of freedom, F-statistic (sample mean: sample variation), adjusted r^2 value, and P-value (alpha=0.05)] Bold indicates significant p-value with alpha set to p<0.05.

a) all adult birds

	West			Contact zone: West	Contact zone			East: Contact zone	East			East: West	Overall Comparison			
(mm)	n	x	sd	p-value	n	x	sd	p-value	n	x	sd	p-value	d.f.	F	R^2 adj	p-value
Bill	22	12.0	0.55	0.250	16	12.3	0.38	**0.03**	32	12.7	0.52	**<0.001**	2	11.90	0.24	**<0.000**
Wing	23	73.8	1.88	0.967	16	73.6	2.94	**<0.001**	32	77.4	2.59	**<0.001**	2	19.95	0.35	**<0.001**
Tarsus	23	20.4	0.89	0.072	16	21.0	0.82	0.819	32	20.8	0.86	0.122	2	3.04	0.06	0.054
Tail	23	73.2	3.00	0.925	15	73.7	4.13	0.124	30	76.4	5.14	**0.023**	2	4.21	0.09	**0.019**

b) adult males

	West			Contact zone: West	Contact zone			East: Contact zone	East			East: West	Overall Comparison			
(mm)	n	x	sd	p-value	n	x	sd	p-value	n	x	sd	p-value	d.f.	F	R^2 adj	p-value
Bill	14	12.0	0.58	0.288	12	12.3	0.38	**0.081**	28	12.7	0.51	**<0.000**	2	9.06	0.23	**0.000**
Wing	15	74.6	1.54	0.993	12	74.5	2.71	**<0.000**	28	77.8	2.40	**<0.000**	2	13.89	0.32	**<0.000**
Tarsus	15	21.0	0.75	0.120	12	21.2	0.78	0.611	28	21.0	0.84	0.345	2	2.09	0.04	0.134
Tail	15	73.3	3.49	0.784	11	74.5	4.59	0.459	27	76.5	5.21	0.089	2	2.46	0.05	0.096

c) adult females

	West			Contact zone: West	Contact zone			East: Contact zone	East			East: West	Overall Comparison			
(mm)	n	x	sd	p-value	n	x	sd	p-value	n	x	sd	p-value	d.f.	F	R^2 adj	p-value
Bill	8	11.9	0.52	0.508	4	12.0	0.30	0.788	4	12.3	0.37	0.508	2	0.65	0.09	0.539
Wing	8	72.2	1.42	0.374	4	70.8	1.50	**0.016**	4	74.6	2.32	0.083	2	5.45	-0.05	**0.019**
Tarsus	8	20.0	1.02	0.748	4	20.4	0.72	0.891	4	20.1	0.59	0.977	2	0.27	0.04	0.767
Tail	8	73.1	1.99	0.681	4	71.6	1.03	0.156	3	75.8	5.51	0.334	2	2.02	0.13	0.175

Table 4.5 Principal component (PC) differences of adult males only by a) expected species and b) transect region. Bold indicates statistical significance with alpha <0.05.

Variable	PC1	PC2
Bill	0.017	0.106
Wing	**0.422**	**0.900**
Tarsus	0.032	0.002
Tail	**0.906**	**-0.422**
Cumulative Eigenvalue	86.23	97.16

Table 4.6 ANOVA and linear regression results from principal component values and various variables by transect region. Statistical values provided including n=sample size, x=mean, sd=standard deviation, d.f=degrees of freedom, F-statistic (sample mean: sample variation), adjusted r^2 value, and P-value (alpha=0.05).

	West			Contact zone: West	Contact zone			East: Contact zone	East			East: West	Overall			
	n	x	sd	p-value	n	x	sd	p-value	n	x	sd	p-value	d.f.	F	R^2 adj	P-value
PC1	14	0.37	3.13	0.943	11	1.01	5.17	0.143	27	4.38	5.49	**0.043**	2	3.81	0.10	**0.029**
PC2	14	-3.05	1.96	0.558	11	-1.03	1.37	**0.005**	27	1.00	1.73	0.066	2	6.26	0.17	**0.004**

4.4. Discussion

In this study, we confirmed the presence of genetic hybrids, though in low numbers, between the Tufted and Black-crested Titmouse in central Texas. In comparing genetic assignment to a commonly employed plumage hybrid index scale, we discovered that plumage is not indicative of hybridization. Many of the birds that appeared to be hybrids, based on crest and forehead plumage are actually juveniles. Maximum likelihood analysis, from the ND2 mtDNA gene, produced two separate clades with genetic difference of 0.7%, as similarly reported previously with other mtDNA markers. Mitochondrial clades were divided based on capture location, with birds captured within the contact zone having mtDNA more similar to birds captured west of the zone (Black-crested Titmouse range). Morphological analysis indicate that birds captured within the hybrid zone are more similar in body size to Black-crested Titmice (birds captured west of the contact zone) than to Tufted Titmice (east of the contact zone). Overall, genetic analysis (using both mtDNA and microsatellites) and morphology indicate that birds within the contact zone are more similar to Black-crested Titmice. With the contact zone occurring at an elevation gradient and ecotone, we suspect habitat preferences (or adaptations to environmental conditions) play a strong role in minimizing range expansion and thus encourage a stable hybrid zone.

4.4.1. **Genetic introgression and species divergence**

Our microsatellite results provide the first genetic evidence of hybridization between Black-crested and Tufted Titmice in central Texas. Using 9 microsatellite loci, we observed 12 admixed individuals (19% of those captured). Of these, five birds were captured within the contact zone, with another five captured west of the contact zone, and

two captured east of the Balcones Escarpment. Our finding confirms the presence of genetic admixture proposed over 100 years ago (Allen, 1907). However, with the low number of polymorphic alleles and the use of only 10 loci, we are cautious to discuss the amount of hybridization occurring. We are confident in the presence of hybrid individuals because used a conservative 80% cutoff (0.2<q>0.8), unlike other avian hybrid studies that often use a 90% cutoff, to ensure that we more accurately captured individuals with enough genetic admixture to be considered hybrids (Päckert et al., 2019; Taylor et al., 2012; Väli et al., 2010). However, our study has fewer hybrid individuals than would be expected if extensive hybridization is occurring. Further, when a no admixture model was employed, only one individual was identified as a hybrid. Therefore, more research is needed to understand the extent of gene flow occurring between the two species, the impact of hybridization across Texas, and how such an impact might impact the species in the coming decades as climates change.

Tufted Titmice showed a higher level of genetic diversity compared with Black-crested using both mitochondrial and microsatellite data. This finding is unsurprising given the historical movement and current geographic distribution of Tufted Titmice as dispersal, range expansion, and population density are known impactors of genetic density (Curry, 2015; Rheindt & Edwards, 2011; Shurtliff, 2013). Tufted Titmice are an aggressive species and it is continually expanding its range north and east across the U.S. and southern Canada (Ritchison et al., 2015; Vaughn and Voelker, unpublished). This contrasts with Black-cresteds timid behavior and preferred habitats (limited to semi-arid scrub habitats found primarily in the western U.S.) (Patten & Smith-Patten, 2008). More information on dispersal rates, range expansion, and population density of both species is needed to

understand the main driving forces behind the higher genetic diversity in Tufted Titmice. We also observed that birds within the contact zone have lower levels of haplotype (mtDNA) diversity and more shared alleles with Black-crested Titmice. This low diversity could simply be a product of the small geographic area where we collected specimens. It is interesting to note that the pattern of shared alleles between Black-crested in the west and those within the contact zone parallels our results of shared morphological values between those regions.

Mitochondrial analysis produced two distinct clades that matched with geographic location (east or west of the Balcones Escarpment). By comparing plumage and mtDNA, we found that all birds captured east of the Balcones Escarpment were in the Tufted clade even if their plumage would suggest hybridization or Black-crested species. Both species are known to have distinct habitat preferences of which the ecological barrier between regions is the Balcones Escarpment. The presence of two clades, with recent divergence, is similar to previous studies. Our maximum likelihood distance of 0.4% is similar to previously reported distances (0.4%-0.6%) (Braun et al., 1984; Gill et al., 1989; Gill & Slikas, 1992; Gill et al., 2005). Although this small distance was suspect for species classification, a study on multiple sister species reported similar genetic distances (Johnson & Cicero, 2004). Similar to morphological and microsatellite diversity results, genetic distance is the same between Tufted Titmice and Black-crested Titmice in the west and those in the contact zone. Therefore, it appears there is continual gene flow between birds in the western transect and those within the contact zone.

4.4.2. **DHI unrelated to genetic assignment**

In comparing Dixon's Hybrid Index (DHI) with genetic assignment, we found that genetic admixture is not linked to species-specific crest plumage or the DHI. Of the 12 admixed birds, only five possessed crest plumage indicative of phenotypic hybrids according to the DHI (DHI 2-4). Additionally, two individuals that appeared phenotypically Black-crested according to the DHI captured within Tufted Titmice range (east of the Balcones Escarpment) showed no evidence of genetic admixture and fell within the Tufted Titmouse mitochondrial clade. More importantly, using the DHI, 21 birds would be labeled as "hybrids" even though they have no genetic evidence of hybridization. Given these results, we suggest that the DHI fails to be a reliable indicator of admixture and should not be used to characterize putative hybrids.

DHI assignment can be problematic, especially in the field, because subtle coloration differences can be subjective and harsh lighting in natural environments can also impact color determination. Titmice are known to be primarily sexually monochromatic (males and females appear the same); however, some studies (but not all) state that males have a deeper black forehead or crest compared with the dark brown observed in some females (Dixon, 1955; Ritchison et al., 2015). Recently, Curry and Patten (2014) used a colorimeter on Black-crested and Tufted Titmice from a more recent contact zone in north Texas and Oklahoma to assess DHI. Curry and Patten (2014) found a correlation between DHI score and population specific color pattern, but they only used adult males. Thus, it is unknown if either species of Titmice exhibits cryptic sexual dichromatism or if age plays role in plumage.

In our study, approximately 67% of the phenotypically designated hybrids (DHI 2-4) were juveniles. The literature on juvenile plumage of both species seems to be inconsistent. Dixon (1955) found little difference between adults and first-year juveniles as related to their molt patterns; he made no mention of their crest coloring. Pyle (1997) made note that in Black-crested Titmice it might be difficult to distinguish between females and juveniles. However, other field guides and sources indicate that juveniles of both species lack the black crest or forehead, instead of having an all gray crest or forehead (Grubb, 1998; Sibley, 2000). In this study, the large numbers of juveniles designated as hybrids, based on DHI, leads us to suspect that age or age-related factors may be impacting plumage coloration in Black-crested and Tufted Titmice. Many of the past Titmice studies have been conducted during the breeding season when both young and adults are present. As in many songbirds, age is best determined by skull ossification, which is not practical in observational studies. It is possible that previous studies have overestimated the number (and location) of hybrid individuals. Until more is understood about Titmouse contact zones, we recommend discontinuation of the DHI to identify hybrid individuals (especially between March and May) and urge caution when identifying species based on plumage, especially within or adjacent to the central Texas contact zone across the Balcones Escarpment.

4.4.3. **Morphology across the Balcones Contact Zone**

Titmice within the contact zone are morphologically more similar to Black-crested Titmice with no signs of intermediate body size. Of the four morphological variables (bill, wing, tarsus, tail), wing length varied the greatest between birds captured east and west of the

contact zone (different species). Wing length is a common predictor of body size in many bird taxa (Gosler et al., 1998; Nudds, 2007) but in non-migratory birds, it has also been shown to vary with climate, altitude, and vegetation (Sun et al., 2017). The contact zone occurs on the eastern edge of the distributional boundary of Black-crested Titmice (same geographic region). With distinctively different vegetation densities and climate on either side of the Balcones Escarpment, further investigations are needed to identify if climate or vegetation density is playing a more significant role in tail length variation. This information should be considered in future research on the contact zone as climate change and urbanization of the region may impact body size. The impacts of climate change on body size (and wing length) varies depending on location. For example, in the eastern U.S., a study showed decreasing avian body size due to climate change (Van Buskirk et al., 2010) but in the western U.S., a study showed increasing body size due to climatic variability (Goodman et al., 2012). Furthermore, the Balcones Contact zone lies in a previous rural environment that is experiencing rapid urbanization due to development around the Texas capital city of Austin (Lai & Kreuter, 2012). With urbanization comes changes in landscape ecology and available resources for wildlife. Urbanization has already shown to impact avian body size due to food availability and changes in vegetation (Meillère et al., 2015; Miller et al., 2001). Therefore, at this time, wing length is the best predictor of each species; however, vegetative structure and changes in climate should be considered in future studies.

4.4.4. **Factors contributing to a stable contact zone**

The distinctive mtDNA clades on either side of the Balcones Escarpment ecotone indicate that differences in habitat preference may impede mitochondrial gene flow between Black-crested and Tufted Titmice. The location of the contact zone has not shifted since it was first described in 1887 (Allen, 1907; Curry & Patten, 2016) and we suspect that differences in behavior, physiological adaptations, and habitat preferences are maintaining a stable contact zone between Black-crested and Tufted Titmice.

A recent study on Texas Titmice suggested that physiological adaptions to ecoregions are limiting westward expansion of Tufted Titmice (Vaughn et al., 2020). In this study, Vaughn et al. (2020) showed Black-crested Titmice have naturally higher levels of glucose (compared to Tufted Titmice), which may be an adaptation to living in a chronically stressed state due to sporadic food resources driven by lower rainfall regimes in semi-arid environments (Fokidis et al., 2012; Nielsen-Gammon, 2011; Yang et al., 2008). Semi-arid habitats have been shown to produce a physiological stress response in animal populations compared with populations in mesic environments (Champagne et al., 2012; Fokidis et al., 2012; Guillette et al., 1997). Tufted Titmice evolving in a mesic habitat with continual food resources may be unable to survive in semi-arid conditions and thus, are prevented from westward territory expansion. Data from this study lend support to the stability (no longitudinal shift) of the contact zone as we captured no genetic Tufteds (mtDNA or nuDNA) on or west of the Balcones Escarpment. On the other hand, we captured two birds that appear Black-crested individuals east of the Balcones Escarpment in Tufted Titmouse range. If physiology is a major limiting factor for westward expansion, Black-cresteds should be able to survive the mesic forests east of the Balcones Escarpment

as the continual rainfall would pose less physiological stress. However, both individuals were genetically Tufted (mtDNA and nuDNA) and showed no evidence of genetic admixture. The presence of genetic hybrids and Black-crested plumage east of the Balcones Escarpment support some amount of eastward gene flow, but, at this time, there appears to be no expansion of the Black-crested distribution range. This finding suggests that other limitations might be in place such as competition between species and/or mitonuclear incompatibility.

In a recent behavioral and communication study, Vaughn and Voelker (unpublished, chapter 3) determined that Tufted Titmice are more aggressive than Black-crested Titmice and may be outcompeting Black-crested Titmice for territory acquisition and mate selection in areas of overlap on the Balcones Escarpment. Competitive asymmetry is known to cause reproductive isolation between species (Freeman, 2016; Lipshutz, 2018; Martin et al., 2017). In fact, Curry and Patten (2016) observed asymmetric mate preferences between an older Titmouse contact zone (our study area in central Texas) and a recently established contact zone (north Texas, Oklahoma). Their results indicate that, in the older zone, males (of either species) showed a stronger response to conspecifics compared with the younger zone, but overall females preferred male Tufted Titmice. This preference for male Tufteds might be due to their more aggressive behavior. A preference towards aggressive male Tufted Titmice would certainly limit range expansion of Black-crested Titmice.

Knowing physiological environmental adaptations may be playing a role in limiting range expansions, it would also be prudent to consider the potential that mitonuclear incompatibility may be acting as a barrier to increased genetic admixture. Recently, Hill

(2017) proposed a new species concept, known as mitonuclear incompatibility, to explain recent genetic introgression despite clear species delimitation in avian species. Hill explains that avian hybrids likely have reduced fitness because they lack coadapted nuclear and mitochondrial genes responsible for energy production (via oxidative phosphorylation) (Hill, 2019). The physiological adaptations that enable Black-crested Titmice to survive in chronically stressful environments may be a product of mitochondrial genes suited for semi-arid environments. If so, the lack of these genes in Tufted Titmice explains why, despite their aggressive nature, they have not expanded into Black-crested Titmice range. Further, mitonuclear incompatibility supports the low number of hybrids observed within the contact zone as admixed individuals would have mitochondrial and nuclear genes that have not co-evolved together resulting in lower fitness due to inefficient oxidative phosphorylation. Therefore, the combination of physiological adaptations, behavior, and mitonuclear incompatibility may play an important role in the stability of the contact zone between Black-crested and Tufted Titmice in central Texas.

4.5. Summary

By using genetic techniques, we provide evidence of some genetic introgression between the Black-crested and Tufted Titmice in central Texas. Our study confirms recent divergence between the sister species with mtDNA haplotypes linked to different ecoregions relative to their distributional ranges. Importantly, our data refutes the use of Dixon's Hybrid Index in determining hybrids as plumage does not always match genetic assignment. Our results indicate plumage is related to age as most birds identified as hybrids using the DHI were most often juveniles. Our most reliable factor at determining

species assignment is the location of capture, east or west of the Balcones Escarpment; however, this does not apply to hybrid birds captured primarily within the contact zone. A greater diversity of molecular markers is necessary to confirm the extent of hybridization, but our study indicates gene flow is occurring and that the contact zone is likely stabilized by behavioral differences and environmentally dependent physiological adaptations.

5. CONCLUSIONS

After a century of speculation, this study provides the first genetic evidence of hybridization between Black-crested and Tufted Titmice in the central Texas hybrid zone. Hybridization was confirmed using nuclear DNA (microsatellites) (Allen, 1907; Patten & Smith-Patten, 2008; Ritchison et al., 2015). Mitochondrial DNA (ND2) showed no evidence of hybridization, instead supporting two distinct clades separated by a small genetic distance. Previously, researchers suspected hybridization based on the presence of individuals with intermediate plumage, often utilizing the Dixon Hybrid Index (Curry & Patten, 2014; Dixon, 1955). This study shows that plumage is not a reliable indicator of hybridization or even species assignment. The majority of individuals with hybrid plumage were juveniles. The strongest predictor of species assignment was habitat/geographic location. All birds captured on or west of the Balcones Escarpment were genetically Black-crested Titmice and those captured east were Tufted Titmice, irrespective of plumage. Although most hybrid birds were captured within the hybrid zone, a few were captured further west/east of the hybrid zone, indicating gene flow extends beyond the narrow hybrid zone.

Habitat type was also a large driving force on physiology. Glucose values were higher in birds captured west of the Balcones Escarpment (Black-crested Titmice) and further increased following rain events. Since west of Balcones Escarpment is scrub, semi-arid habitat, it is probable that Black-cresteds have elevated glucose due to chronic physiological stress from sporadic rain and food resources (Fokidis et al., 2012; Griffith et al., 2007; Kitaysky et al., 2007; Williams & Tieleman, 2001). All other signs of avian

health appear normal, so Black-crested Titmice have likely adapted to elevated glucose levels. Increased glucose following rain events is likely a product of dietary changes or increased metabolism due to increased or varied food resources that appear following rain events in a semi-arid habitat (Ostfeld & Keesing, 2000; Wenninger & Inouye, 2008; Yang et al., 2008). Evidence to support this is the lack of relationship to glucose and rainfall in Tufted Titmice as their preferred mesic forested habitat provides regular rainfall and food resources throughout the year.

Morphologically, data in this study match previous studies with Black-cresteds being statistically smaller than Tufted Titmice (Patten & Smith-Patten, 2008; Ritchison et al., 2015). Within arid habitats, smaller body sizes provide an advantage by reducing evaporative water loss (Salewski & Watt, 2017; Williams & Tieleman, 2001). The hybrid zone is at the edge of the semi-arid habitat preferred by Black-cresteds, which explains why birds in the hybrid zone were more similar to Black-crested than Tufted Titmice or being intermediate in value.

Song analysis between the two species confirms specifies-specific differences. Black-crested Titmice sing songs at a higher frequency with more notes per phrase at a faster tempo. This pattern has been observed in other species residing in open habitats, such as the semi-arid scrub habitat of west Texas. Higher frequency and more notes ensure longer range transmission (Boncoraglio & Saino, 2007; Ey & Fischer, 2009). Tufted Titmice, on the other hand, sing few notes per phrase at a lower frequency to minimize disturbance and reverberations from the larger number of trees in their habitat. Similar to morphology and physiology, birds within the hybrid zone sing songs with structure similar to Black-crested Titmice but with frequency similar to Tufted Titmice. Frequency is

usually tied to environmental differences (Boncoraglio & Saino, 2007; Zollinger et al., 2012) supporting similarity in frequency between Tufted and hybrid songs as the hybrid zone occurs at an ecotone where habitat types overlap. Unlike songs, birds within the hybrid zone failed to discriminate between Tufted and Black-crested songs, indicating recognition of both species. The presence of heterospecific recognition within the hybrid zone is likely a product of cultural transmission but raises questions related to reproductive isolating barriers. Distinct songs may act as a reproductive barrier limiting gene flow but if hybrid zone birds fail to discriminate, this weakens the barrier.

Overall, the use of a multi-disciplinary approach highlights the complex relationship between hybridizing Tufted and Black-crested Titmice in central Texas. In isolation, each variable might tell contradictory results, but as a whole, it becomes clear that multiple factors are contributing to maintaining a narrow hybrid zone. This hybrid zone has been discussed for over a century with limited range expansion of either species, despite evidence that both species have expanded their ranges elsewhere (Dixon, 1990; Patten & Smith-Patten, 2008; Ritchison et al., 2015). Results from this study strongly point to physiological adaptations and behavior as mechanisms towards stability. Birds, like most organisms, are sensitive to changes in blood and electrolyte balances (McWilliams et al., 2016). Being an aggressive species, it likely that Tufted Titmice would have expanded their range westward if they could maintain survival and fitness in the semi-arid environment. However, the stress of sporadic rain and food resources may prevent them from outcompeting Black-crested Titmice west of the Edwards Plateau. Therefore, the less stressful mesic forests of east Texas would appear ideal for Black-crested Titmice and, in fact, Black-cresteds have been observed east of the Balcones Escarpment (including this

study) but 1) their numbers are few and 2) the individuals may actually be hybrids or genetically Tufted Titmice (as was the case in this study). The lack of eastward expansion by Black-cresteds is possibly a result of the dominant, aggressive behavior of Tufted Titmice. Behavioral results show that Tufted Titmice perceive songs of both species as a territorial threat, unlike Black-cresteds.

Although this study raises many more questions, the data are groundbreaking in 1) providing the first confirmed evidence of genetic hybridization in central Texas, 2) overturning the Dixon hybrid index, 3) showing the importance of physiology in hybrid zone movement, and 4) demonstrating the strong impact habitat plays in Tufted and Black-crested Titmice morphology, physiology, and possibly song structure.

REFERENCES

Abbott, P., & Woodruff Jr, C. M. (1986). *The Balcones Escarpment: Geology, hydrology, ecology, and social development in Central Texas*. San Antonio, TX: Geological Society of America.

Abbott Point of Care. (2016). i-STAT System Manual: Hematocrit/HCT and calculated hemogloblin/HB. In (Vol. Art: 714178-00). Abbott Park, IL: Abbott Point of Care, Inc.

Abbott Point of Care. (2017). i-STAT System Manual: PCO2 and calculated values for HCO3, base excess and anion gap. In (Vol. Art: 714182-00U). Abbott Park, IL: Abbot Point of Care, Inc.

Abbott, R., Albach, D., Ansell, S., Arntzen, J. W., Baird, S. J. E., et al. (2013). Hybridization and speciation. *Journal of Evolutionary Biology, 26*(2), 229-246.

Aggarwal, C. C. (2017). Probabilistic and Statistical Models for Outlier Detection. In C. C. Aggarwal (Ed.), *Outlier Analysis* (pp. 35-64). Cham, Switzerland: Springer International Publishing.

Akçay, Ç., Hambury, K. L., Arnold, J. A., Nevins, A. M., & Dickinson, J. L. (2014). Song sharing with neighbours and relatives in a cooperatively breeding songbird. *Animal Behaviour, 92*, 55-62.

Allen, J. A. (1907). The *Baeolophus bicolor-atricristatus* group. *Bulletin of the American Museum of Natural History, 23*, 467-481.

Aplin, L. M. (2019). Culture and cultural evolution in birds: a review of the evidence. *Animal Behaviour, 147*, 179-187.

Arnold, M. L. (1997). *Natural Hybyridization and Evolution*. New York, NY: Oxford University Press.

Arntzen, J. W., de Vries, W., Canestrelli, D., & Martínez-Solano, I. (2017). Hybrid zone formation and contrasting outcomes of secondary contact over transects in common toads. *Molecular Ecology, 26*(20), 5663-5675.

Asirvatham, J. R., Moses, V., & Bjornson, L. (2013). Errors in potassium measurement: a laboratory perspective for the clinician. *North American Journal of Medical Sciences, 5*(4), 255-259.

Audacity. (2018). Audacity 2.0: Audacity Team. Retrieved from https://audacityteam.org/

Avise, J. C., & Walker, D. (1998). Pleistocene phylogeographic effects on avian populations and the speciation process. *Proceedings of the Royal Society of London - Series B: Biological Sciences, 265*, 457-463.

Avise, J. C., & Zink, R. M. (1988). Molecular genetic divergence between avian sibling species: King and Clapper Rails, Long-billed and Short-billed Dowitchers, Boat-tailed and Great-tailed Grackles, and Tufted and Black-crested Titmice. *The Auk, 105*(3), 516-528.

Baldassarre, D. T., White, T. A., Karubian, J., & Webster, M. S. (2014). Genomic and morphological analysis of a semipermeable avian hybrid zone suggests asymmetrical introgression of a sexual signal. *Evolution, 68*(9), 2644-2657.

Banks, R. C., Cicero, C., Dunn, J. L., Kratter, A. W., Rasmussen, P. C., et al. (2002). Forty-third supplement to the American Ornithologists' Union check-list of North American birds. *The Auk, 119*(3), 897-906.

Barrera-Guzmán, A. O., Aleixo, A., Shawkey, M. D., & Weir, J. T. (2018). Hybrid speciation leads to novel male secondary sexual ornamentation of an Amazonian bird. *Proceedings of the National Academy of Sciences, 115*(2), E218.

Barton, N. H., & Hewitt, G. M. (1985). Analysis of hybrid zones. *Annual Review of Ecology and Systematics, 16*, 113-148.

Beecher, M. D. (2017). Birdsong learning as a social process. *Animal Behaviour, 124*, 233-246.

Berwick, R. C., Okanoya, K., Beckers, G. J. L., & Bolhuis, J. J. (2011). Songs to syntax: the linguistics of birdsong. *Trends in Cognitive Sciences, 15*(3), 113-121.

Bigham, A. S., & Moghaddam, A. K. Z. (2013). Finch (*Taeneopygia guttata*) sedation with intranasal administration of diazepam, midazolam or xylazine. *Journal of Veterinary Pharmacology and Therapeutics, 36*(1), 102-104.

Blank Texas County Map. (2017). *WaterProofPaper.com*. Retrieved from https://www.waterproofpaper.com/printable-maps/texas/printable-texas-county-map.pdf

Blas, J. (2015). Chapter 33 - Stress in Birds. In C. G. Scanes (Ed.), *Sturkie's Avian Physiology (Sixth Edition)* (pp. 769-810). San Diego, CA: Academic Press.

Boncoraglio, G., & Saino, N. (2007). Habitat structure and the evolution of bird song: a meta-analysis of the evidence for the acoustic adaptation hypothesis. *Functional Ecology, 21*(1), 134-142.

Boutin-Ganache, I., Raposo, M., Raymond, M., & Deschepper, C. F. (2001). M13-Tailed Primers improve the readability and usability of microsatellite analyses performed with two different allele-sizing methods. *BioTechniques, 31*(1), 25-28.

Bozinovic, F., & Naya, D. E. (2015). Linking Physiology, Climate, and Species Distributional Ranges. In L. B. Martin, C. K. Ghalambor, & H. A. Woods (Eds.), *Integrative Organismal Biology* (First ed., pp. 14). Hoboken, NJ: John Wiley & Sons.

Braun, D., Kitto, G. B., & Braun, M. J. (1984). Molecular population genetics of Tufted and Black-crested forms of *Parus bicolor*. *The Auk, 101*(1), 170-173.

Braun, E. J., & Sweazea, K. L. (2008). Glucose regulation in birds. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology, 151*(1), 1-9.

Brumm, H., & Naguib, M. (2009). Chapter 1 Environmental Acoustics and the Evolution of Bird Song. In *Advances in the Study of Behavior* (Vol. 40, pp. 1-33). New York, NY: Academic Press.

Buggs, R. J. A. (2007). Empirical study of hybrid zone movement. *Heredity, 99*(3), 301-312.

Cadenasso, M. L., Pickett, S. T. A., Weathers, K. C., Bell, S. S., Benning, T. L., et al. (2003). An interdisciplinary and synthetic approach to ecological boundaries. *Bioscience, 53*(8), 717-722.

Carere, C., & van Oers, K. (2004). Shy and bold Great Tits (*Parus major*): body temperature and breath rate in response to handling stress. *Physiology and Behavior, 82*, 905-912.

Catchpole, C. K., & Slater, P. J. B. (2008). *Bird Song* (2nd ed.). New York, NY: Cambridge University Press.

Champagne, C. D., Houser, D. S., Costa, D. P., & Crocker, D. E. (2012). The effects of handling and anesthetic agents on the stress response and carbohydrate metabolism in northern elephant seals. *Plos One, 7*(5).

Cherel, Y., Robin, J.-P., & Maho, Y. L. (1988). Physiology and biochemistry of long-term fasting in birds. *Canadian Journal of Zoology, 66*(1), 159-166.

Coldren, C. L. (1992). *A comparison of the songs of the Tufted and Black-crested Titmice in Texas.* (M. Sc. thesis). Texas A & M Univ., College Station, TX.

Coster, S. S., Welsh, A. B., Costanzo, G., Harding, S. R., Anderson, J. T., et al. (2018). Genetic analyses reveal cryptic introgression in secretive marsh bird populations. *Ecology and Evolution, 8*(19), 9870-9879.

Courter, J. R., & Ritchison, G. (2010). Alarm calls of Tufted Titmice convey information about predator size and threat. *Behavioral Ecology, 21*(5), 936-942.

Culumber, Z. W., Shepard, D. B., Coleman, S. W., Rosenthal, G. G., & Tobler, M. (2012). Physiological adaptation along environmental gradients and replicated hybrid zone structure in swordtails (Teleostei: *Xiphophorus*). *Journal of Evolutionary Biology, 25*(9), 1800-1814.

Curry, C. M. (2015). An integrated framework for hybrid zone models. *Evolutionary Biology, 42*(3), 359-365.

Curry, C. M., & Patten, M. A. (2014). Current and historical extent of phenotypic variation in the Tufted and Black-crested titmouse (Paridae) hybrid zone in the southern Great Plains. *American Midland Naturalist, 171*(2), 271-300.

Curry, C. M., & Patten, M. A. (2016). Shadow of a doubt: premating and postmating isolating barriers in a temporally complex songbird (Passeriformes: Paridae) hybrid zone. *Behavioral Ecology and Sociobiology, 70*(8), 1171-1186.

Curry, C. M., & Patten, M. A. (2019). Complex spatiotemporal variation in processes shaping song variation. *Behaviour, 156*(10), 1057-1082.

Dawson, D. A., Hanotte, O., Greig, C., Stewart, I. R. K., & Burke, T. (2000). Polymorphic microsatellites in the Blue Tit (*Parus caeruleus*) and their cross-species utility in 20 songbird families. *Molecular Ecology, 9*(11), 1941-1944.

Demko, A. D., Reitsma, L. R., & Staicer, C. A. (2016). Repertoire structure, song sharing, reproductive success, and territory tenure in a population of Canada Warblers (*Cardellina canadensis*) in central New Hampshire. *Canadian Journal of Zoology, 94*(4), 283-290.

den Hartog, P. M., de Kort, S. R., & ten Cate, C. (2007). Hybrid vocalizations are effective within, but not outside, an avian hybrid zone. *Behavioral Ecology, 18*(3), 608-614.

Derryberry, E. P. (2007). Evolution of bird song affects signal efficacy: An experimental test using historical and current signals. *Evolution, 61*(8), 1938-1945.

Derryberry, E. P., Seddon, N., Derryberry, G. E., Claramunt, S., Seeholzer, G. F., et al. (2018). Ecological drivers of song evolution in birds: Disentangling the effects of habitat and morphology. *Ecology and Evolution, 8*(3), 1890-1905.

Dixon, K. L. (1955). An ecological analysis of the inter-breeding of crested titmice in Texas. *University California Publications in Zoology, 54*(3), 125-206.

Dixon, K. L. (1961). Habitat distribution and niche relationships in North American species of *Parus*. In W. F. Blair (Ed.), *Vertebrate Speciation* (pp. 179-216). Austin, TX: Univ. Texas Press.

Dixon, K. L. (1978). A distributional history of the Black-Crested Titmouse. *American Midland Naturalist, 100*(1), 29-42.

Dixon, K. L. (1989). Contact zones of avian congeners on the southern Great Plains. *Condor, 91*(1), 15-22.

Dixon, K. L. (1990). Constancy of margins of the hybrid zone in titmice of the (*Parus bicolor*) complex in coastal Texas. *The Auk, 107*(1), 184-188.

Douglas, S. B., & Mennill, D. J. (2010). A review of acoustic playback techniques for studying avian vocal duets. *Journal of Field Ornithology, 81*(2), 115-129.

Dubay, S. G., & Witt, C. C. (2014). Differential high-altitude adaptation and restricted gene flow across a mid-elevation hybrid zone in Andean tit-tyrant flycatchers. *Molecular Ecology, 23*, 3551-3565.

Earl, D. A., & von Holdt, B. M. (2012). STRUCTURE HARVESTER: A website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetics Resources, 4*, 359-361.

Eme, D., Malard, F., Colson-Proch, C., Jean, P., Calvignac, S., et al. (2014). Integrating phylogeography, physiology and habitat modelling to explore species range determinants. *Journal of Biogeography, 41*(4), 687-699.

Emlen, S. T. (1972). An experimental analysis of the parameters of bird song eliciting species recognition. *Behaviour, 41*(1/2), 130-171.

EnchantedLearning.com. (2018). Natural Features of Texas-Labeled. . *Enchanted Learning, LLC*. Retrieved from http://www.enchantedlearning.com/usa/states/texas/naturalfeatures/labeled.shtml.

Endler, J. A. (1977). *Geographic Variation, Speciation, and Clines*. Princeton, NJ: Princeton University Press.

Evanno, G., Regnaut, S., & Goudet, J. (2005). Detecting the number of clusters of individuals using the software structure: a simulation study. *Molecular Ecology, 14*(8), 2611-2620.

Excoffier, L., & Lischer, H. E. L. (2010). Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources, 10*(3), 564-567.

Ey, E., & Fischer, J. (2009). The "Acoustic Adaptation Hypothesis" - A review of the evidence from birds, anurans, and mammals. *Bioacoustics, 19*(1-2), 21-48.

Fletcher, R. (2007). Species interactions and population density mediate the use of social cues for habitat selection. *The Journal of Animal Ecology, 76*, 598-606.

Fokidis, H. B., Roziers, M. B. D., Sparr, R., Rogowski, C., Sweazea, K., et al. (2012). Unpredictable food availability induces metabolic and hormonal changes independent of food intake in a sedentary songbird. *Journal of Experimental Biology, 215*(16), 2920-2930.

Freeman, B. G. (2016). Strong asymmetric interspecific aggression between two sympatric New Guinean robins. *Ibis, 158*(1), 75-81.

Freeman, B. G., & Montgomery, G. A. (2017). Using song playback experiments to measure species recognition between geographically isolated populations: A comparison with acoustic trait analyses. *The Auk, 134*(4), 857-870.

Gil, D., & Gahr, M. (2002). The honesty of bird song: multiple constraints for multiple traits. *Trends in Ecology & Evolution, 17*(3), 133-141.

Gill, F. B., Funk, D. H., & Silverin, B. (1989). Protein relationships among titmice *Parus*. *Wilson Bulletin, 101*(2), 182-197.

Gill, F. B., & Slikas, B. (1992). Patterns of mitochondrial DNA divergence in North American crested Titmice. *Condor, 94*(1), 20-28.

Gill, F. B., Slikas, B., & Sheldon, F. H. (2005). Phylogeny of titmice (Paridae): II. Species relationships based on sequences of the mitochondrial cytochrome-*b* gene. *The Auk, 122*(1), 121-143.

Glazier, D. S. (2008). Effects of metabolic level on the body size scaling of metabolic rate in birds and mammals. *Proceedings of the Royal Society B: Biological Sciences, 275*(1641), 1405-1410.

Goetze, J. R. (1995). *Distribution, natural history, and biogeographic relationships of mammals on the Edwards Plateau of Texas.* (Dissertation). Texas Tech University, Lubbock, TX.

Goodman, R. E., Lebuhn, G., Seavy, N. E., Gardali, T., & Bluso-Demers, J. D. (2012). Avian body size changes and climate change: warming or increasing variability? *Global Change Biology, 18*(1), 63-73.

Gosler, A. G., Greenwood, J. J. D., Baker, J. K., & Davidson, N. C. (1998). The field determination of body size and condition in passerines: a report to the British Ringing Committee. *Bird Study, 45*(1), 92-103.

Gould, F. W., Hoffman, G. O., & Rechenthin, C. A. (1960). Vegetation Areas of Texas. In T. A. M. University (Ed.), (Vol. Leaflet No., 492). Texas Parks and Wildlife: Texas Agricultural Experiment Station.

Griffith, G., Bryce, S., Omernick, J., & Rogers, A. (2007). *Ecoregions of Texas*. Retrieved from Austin, TX:

Grubb, J., T. C. (1998). *Tufted Titmouse*. Mechanicsburg, PA: Stackpole Books.

Guillette, L. J., Crain, D. A., Rooney, A. A., & Woodward, A. R. (1997). Effect of acute stress on plasma concentrations of sex and stress hormones in juvenile alligators living in control and contaminated lakes. *Journal of Herpetology, 31*(3), 347-353.

Hackett, S. J. (1996). Molecular phylogenetics and biogeography of Tanagers in the genus *Ramphocelus* (Aves). *Molecular Phylogenetics and Evolution, 5*(2), 368-382.

Hafner, D. J. (1993). Reinterpretation of the Wisconsinan mammalian fauna and paleoenvironment of the Edwards Plateau, Texas. *Journal of Mammalogy, 74*(1), 162-167.

Halfwerk, W., Dingle, C., Brinkhuizen, D. M., Poelstra, J. W., Komdeur, J., et al. (2016). Sharp acoustic boundaries across an altitudinal avian hybrid zone despite asymmetric introgression. *Journal of Evolutionary Biology, 29*(7), 1356-1367.

Hall, M. L., Kingma, S. A., & Peters, A. (2013). Male songbird indicates body size with low-pitched advertising songs. *Plos One, 8*(2).

Hamao, S., Sugita, N., & Nishiumi, I. (2016). Geographic variation in bird songs: examination of the effects of sympatric related species on the acoustic structure of songs. *acta ethologica, 19*(1), 81-90.

Harms, C. A., & Harms, R. V. (2012). Venous blood gas and lactate values of Mourning Doves (*Zenaida macroura*), Boat-Tailed Grackles (*Quiscalus major*), and House Sparrows (*Passer domesticus*) after capture by mist net, banding, and venipuncture. *Journal of Zoo and Wildlife Medicine, 43*(1), 77-84.

Harms, C. A., Jinks, M. R., & Harms, R. V. (2016). Blood gas, lactate, and hematology effects of venipuncture timing and location after mist-net capture of Mourning Doves (*Zenaida macroura*), Boat-tailed Grackles (*Quiscalus major*), and House Sparrows (*Passer domesticus*). *Journal of Wildlife Diseases, 52*(2s), S54-S64.

Harrison, C. (Producer). (2012, 2015). Black-crested Titmouse-*Baeolophus atricristatus*: XC122454. [Audio Recordings] Retrieved from www.xeno-canto.org/122454.

Harrison, R. G. (1993). Hybrids and hybrid zones: historical perspective. In R. G. Harrison (Ed.), *Hybrid Zones and the Evolutionary process* (pp. 3-12). New York, NY: Oxford University Press.

Harrison, R. G., & Larson, E. L. (2014). Hybridization, introgression, and the nature of species boundaries. *Journal of Heredity, 105*(S1), 795-809.

Hau, M., Casagrande, S., Ouyang, J. Q., & Baugh, A. T. (2016). Chapter Two - Glucocorticoid-Mediated Phenotypes in Vertebrates: Multilevel Variation and

Evolution. In *Advances in the Study of Behavior* (Vol. 48, pp. 41 - 115). Cambridge, MA: Academic Press.

Heatley, J. J., Cary, J., Kingsley, L., Beaufrere, H., Russell, K. E., et al. (2015). Midazolam sedates Passeriformes for field sampling but affects multiple venous blood analytes. *Veterinary Medicine: Research and Reports, 6*, 61-69.

Heatley, J. J., Cary, J., Russell, K. E., & Voelker, G. (2013). Clinicopathological analysis of Passeriform venous blood reflects transitions in elevation and habitat. *Veterinary Medicine: Research and Reports, 4*, 21-29.

Heilman, J. L., McInnes, K. J., Kjelgaard, J. F., Keith Owens, M., & Schwinning, S. (2009). Energy balance and water use in a subtropical karst woodland on the Edwards Plateau, Texas. *Journal of Hydrology, 373*(3), 426-435.

Henry, K. S., & Lucas, J. R. (2010). Habitat-related differences in the frequency selectivity of auditory filters in songbirds: Songbird auditory filters. *Functional Ecology, 24*(3), 614-624.

Hewitt, G. (1996). Some genetic consequences of ice ages, and their role in divergence and speciation. *Biological Journal of the Linnean Society, 58*(3), 247-276.

Hewitt, G. M. (2004). The structure of biodiversity – insights from molecular phylogeography. *Frontiers in Zoology, 1*(1), 4.

Hibbits, T. J., Ryberg, W. A., Harvey, J. A., Voelker, G., Lawing, A. M., et al. (2019). Phylogenetic structure of *Holbrookia lacerata* (Cope 1880) (Squamata: Phrynosomatidae): one species or two? *Zootaxa, 4619*(1), 139-154.

Hill, G. E. (2017). The mitonuclear compatibility species concept. *The Auk, 134*(2), 393-410.

Hill, G. E. (2019). *Mitonuclear Ecology*. In J. A. Dunne, H. C. J. Godrey, & B. Sheldon (Eds.). Oxford Scholarship Online.

Hyman, J. (2005). Seasonal variation in response to neighbors and strangers by a territorial songbird. *Ethology, 111*(10), 951-961.

Jakobsson, M., & Rosenberg, N. A. (2007). CLUMPP: A cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics, 23*(14), 1801-1806.

Jimeno, B., Hau, M., & Verhulst, S. (2018). Corticosterone levels reflect variation in metabolic rate, independent of 'stress'. *Scientific Reports, 8*(1), 13020.

JMP. (2018). JMP. Cary, NC: SAS Institute, Inc.

Johannessen, L. E., Ke, D. H., Lu, X., & Lifjeld, J. T. (2011). Geographical variation in patterns of parentage and relatedness in the co-operatively breeding Ground Tit *Parus humilis*. *Ibis, 153*(2), 373-383.

Johnson, K. P., & Sorenson, M. D. (1998). Comparing molecular evolution in two mitochondrial protein coding genes (cytochrome b and ND2) in the dabbling ducks (Tribe: Anatini). *Molecular Phylogenetics and Evolution, 10*(1), 82-94.

Johnson, N., & Cicero, C. (2004). New mitochondrial DNA data affirm the importance of Pleistocene speciation in North American birds. *Evolution, 58*(5), 1122-1130.

Johnstone, C. P., Lill, A., & Reina, R. D. (2017). Use of erythrocyte indicators of health and condition in vertebrate ecophysiology: a review and appraisal. *Biological Reviews, 92*(1), 150-168.

Jolliffe, I. T. (2002). *Principal Component Analysis* (Second ed.). New York, NY: Springer-Verlag.

Kaliński, A., Bańbura, M., Glądalski, M., Markowskic, M., Skwarskac, J., et al. (2015). Long-term variation in blood glucose concentration in nestling Great Tits (*Parus major*). *Avian Biology Research, 8*(3), 129-137.

Kawano, K. M. (2003). Isolation of polymorphic microsatellite markers in the Great Tit (*Parus major minor*). *Molecular Ecology Notes, 3*(2), 314-315.

Keller, G. A. (Producer). (1993a). Black-crested Titmouse - *Baeolophus atricristatus*: ML 105232. [Audio Worker] Retrieved from https://macaulaylibrary.org/asset/105232.

Keller, G. A. (Producer). (1993b). Tufted Titmouse - *Baeolophus bicolor*: ML 105260. [Audio Recording] Retrieved from http://macaulaylibrary.org/audio/105260.

Kenyon, H. L., Toews, D. P. L., & Irwin, D. E. (2011). Can song discriminate between Macgillivray's and Mourning Warblers in a narrow hybrid zone? *Condor, 113*(3), 655-663.

Kitaysky, A. S., Piatt, J. F., & Wingfield, J. C. (2007). Stress hormones link food availability and population processes in seabirds. *Marine Ecology Progress Series, 352*, 245-258.

Klicka, J., & Zink, R. M. (1999). Pleistocene effects on North American songbird evolution. *Proceedings of the Royal Society of London. Series B: Biological Sciences, 266*(1420), 695-700.

Knief, U., Bossu, C. M., Saino, N., Hansson, B., Poelstra, J., et al. (2019). Epistatic mutations under divergent selection govern phenotypic variation in the crow hybrid zone. *Nature Ecology & Evolution, 3*(4), 570-576.

Koetz, A. H., Westcott, D. A., & Congdon, B. C. (2007). Geographical variation in song frequency and structure: the effects of vicariant isolation, habitat type and body size. *Animal Behaviour, 74*(5), 1573-1583.

Kostecke, R. M. (2008). Population trends of breeding birds on the Edwards Plateau, Texas: Local versus regional patterns. *The Southwestern Naturalist, 53*(4), 466-471.

Kotlik, P., Markova, S., Vojtek, L., Stratil, A., lechta, V., et al. (2014). Adaptive phylogeography: functional divergence between haemoglobins derived from different glacial refugia in the bank vole. *Proceedings of the Royal Society B: Biological Sciences, 281*(1786), 20140021-20140021.

Kroodsma, D. E. (2005). *The Singing Life of Birds: The art and science of listening to birdsong*. Boston, MA: Houghton Mifflin.

Krosby, M., & Rohwer, S. (2010). Ongoing movement of the Hermit Warbler x Townsend's Warbler hybrid zone. *Plos One, 5*(11), e14164.

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology Evolution, 33*(7), 1870-1874.

Kupietzky, A., & Houpt, M. (1993). Midazolam: a review of its use for conscious sedation of children. *Pediatric Dentistry, 15*(4), 237-241.

Lai, P.-H., & Kreuter, U. P. (2012). Examining the direct and indirect effects of environmental change and place attachment on land management decisions in the Hill Country of Texas, USA. *Landscape and Urban Planning, 104*(3), 320-328.

LaZerte, S. E., Otter, K. A., & Slabbekoorn, H. (2017). Mountain chickadees adjust songs, calls and chorus composition with increasing ambient and experimental anthropogenic noise. *Urban Ecosystems, 20*(5), 989-1000.

Le Maho, Y., Karmann, H., Briot, D., Handrich, Y., Robin, J., et al. (1992). Stress in birds due to routine handling and a technique to avoid it. *American Journal of Physiology, 263*(4, Pt. 2), R775-781.

Lee-Kim, S. J., Fadavi, S., Punwani, I., & Koerber, A. (2004). Nasal versus oral midazolam sedation for pediatric dental patients. *Journal of Dentistry for Children, 71*(2), 126-130.

Lee, C., & Park, C. R. (2019). An increase in song pitch of eastern great tits (*Parus minor*) in response to urban noise at Seoul, Korea. *Urban Ecosystems, 22*(2), 227-233.

Leigh, J. W., & Bryant, D. (2015). PopArt: Full-feature software for haplotype network construction. *Methods in Ecology and Evolution, 6*(9), 1110-1116.

Lerner, H. R. L., Meyer, M., James, H. F., Hofreiter, M., & Fleischer, R. C. (2011). Multilocus resolution of phylogeny and timescale in the extant adaptive radiation of Hawaiian Honeycreepers. *Current Biology, 21*, 1838-1844.

Letunic, I., & Bork, P. (2019). Interactive Tree Of Life (iTOL) v4: recent updates and new developments. *Nucleic Acids Research, 47*(W1), W256-W259.

Librado, P., & Rozas, J. (2009). DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics, 25*(11), 1451-1452.

Light, J. E., & Reed, D. L. (2009). Multigene analysis of phylogenetic relationships and divergence times of primate sucking lice (Phthiraptera: Anoplura). *Molecular Phylogenetics and Evolution, 50*(2), 376-390.

Lill, A. (2011). Sources of variation in blood glucose concentrations of free-living birds. *Avian Biology Research, 4*(2), 78-86.

Lipshutz, S. E. (2018). Interspecific competition, hybridization, and reproductive isolation in secondary contact: missing perspectives on males and females. *Current Zoology, 64*(1), 75-88.

Mallet, J. (2005). Hybridization as an invasion of the genome. *Trends in Ecology & Evolution, 20*(5), 229-237.

Mans, C., Guzman, D. S. M., Lahner, L. L., Paul-Murphy, J., & Sladky, K. K. (2012). Sedation and physiologic response to manual restraint after intranasal administration of midazolam in Hispaniolan Amazon Parrots (*Amazona ventralis*). *Journal of Avian Medicine and Surgery, 26*(3), 130-139.

Martin, P. R., Freshwater, C., & Ghalambor, C. K. (2017). The outcomes of most aggressive interactions among closely related bird species are asymmetric. *PeerJ, 5*, e2847.

Martin, P. R., & Martin, T. E. (2001). Behavioral interactions between coexisting species: Song playback experiments with Wood Warblers. *Ecology, 82*(1), 207-218.

Mason, N. A., & Burns, K. J. (2015). The effect of habitat and body size on the evolution of vocal displays in Thraupidae (Tanagers), the largest family of songbirds. *Biological Journal of the Linnean Society, 114*(3), 538-551.

Mason, N. A., Burns, K. J., Tobias, J. A., Claramunt, S., Seddon, N., et al. (2017). Song evolution, speciation, and vocal learning in passerine birds. *Evolution, 71*(3), 786-796.

McGarigal, K., Cushman, S., & Stafford, S. (2000). *Multivariate Statistics for Wildlife and Ecology Research*. New York, NY: Springer.

McWilliams, S. R., Adkins-Regan, E., & Vleck, C. (2016). Bird Physiology. In Irby J. Lovette & J. W. Fitzpatrick (Eds.), *The Cornell Lab of Ornithology's Handbook of Bird Biology* (3 ed.). Chichester, UK: John Wiley & Sons.

Meillère, A., Brischoux, F., Parenteau, C., & Angelier, F. (2015). Influence of urbanization on body size, condition, and physiology in an urban exploiter: A multi-component approach. *Plos One, 10*(8), e0135685.

Mennill, D. J., Ratcliffe, L. M., & Boag, P. T. (2002). Female eavesdropping on male song contests in songbirds. *Science, 296*(5569), 873.

Mila, B., Girman, D. J., Kimura, M., & Smith, T. B. (2000). Genetic evidence for the effect of a postglacial population expansion on the phylogeography of a North American songbird. *Proceedings of the Royal Society B-Biological Sciences, 267*(1447), 1033-1040.

Miller, J. R., Fraterrigo, J. M., Hobbs, N. T., Theobald, D. M., & Wiens, J. A. (2001). Urbanization, avian communities, and landscape ecology. In J. M. Marzluff, R. Bowman, & R. Donnelly (Eds.), *Avian Ecology and Conservation in an Urbanizing World* (pp. 117-137). Boston, MA: Springer US.

Miller, M. J., Lipshutz, S. E., Smith, N. G., & Bermingham, E. (2014). Genetic and phenotypic characterization of a hybrid zone between polyandrous Northern and Wattled Jacanas in western Panama. *Bmc Evolutionary Biology, 14*(1).

Minias, P. (2015). The use of haemoglobin concentrations to assess physiological condition in birds: a review. *Conservation Physiology, 3*(1).

Mischler, S. K., Congdon, J. V., Scully, E. N., Campbell, K. A., & Sturdy, C. B. (2017). Passerine Vocal Communication. In J. Vonk & T. Shackelford (Eds.), *Encyclopedia of Animal Cognition and Behavior* (pp. 1-7). Cham, Switzerland: Springer International Publishing.

Moseley, D. L., Lahti, D. C., & Podos, J. (2013). Responses to song playback vary with the vocal performance of both signal senders and receivers. *Proceedings of the Royal Society B: Biological Sciences, 280*(1768), 20131401.

Naguib, M., & Riebel, K. (2014). Singing in Space and Time: The Biology of Birdsong. In G. Witzany (Ed.), *Biocommunication of Animals*. Berlin, Germany: Springer, Dorecht.

Nelson, D. A. (2000). Song overproduction, selective attrition and song dialects in the White-Crowned Sparrow. *Animal Behaviour, 60*, 887-898.

Nemeth, E., Pieretti, N., Zollinger, S. A., Geberzahn, N., Partecke, J., et al. (2013). Bird song and anthropogenic noise: vocal constraints may explain why birds sing

higher-frequency songs in cities. *Proceedings. Biological sciences, 280*(1754), 20122798-20122798.

Newman, S. H., Carter, H. R., Whitworth, D. L., & Zinkl, J. G. (2005). Health assessments and stress response of Xantus's murrelets to capture, handling, and radio-marking. *Marine Ornithology, 33*, 147-154.

Nielsen-Gammon, J. W. (2011). Changing Climate of Texas. In J. Schmandt, G. R. North, & J. Clarkson (Eds.), *Impact of Global Warming on Texas* (2nd ed., pp. 39-68). Austin, TX: University of Texas Press.

Nowicki, S., Searcy, W. A., Krueger, T., & Hughes, M. (2002). Individual variation in response to simulated territorial challenge among territory-holding song sparrows. *Journal of Avian Biology, 33*(3), 253-259.

Nudds, R. L. (2007). Wing-bone length allometry in birds. *Journal of Avian Biology, 38*(4), 515-519.

Ostfeld, R. S., & Keesing, F. (2000). Pulsed resources and community dynamics of consumers in terrestrial ecosystems. *Trends in Ecology & Evolution, 15*(6), 232-237.

Otter, K., Ratcliffe, L., Michaud, D., & Boag, P. T. (1998). Do female black-capped chickadees prefer high-ranking males as extra-pair partners? *Behavioral Ecology and Sociobiology, 43*(1), 25-36.

Owen, J. G., & Dixon, J. R. (1989). An ecogeographic analysis of the herpetofauna of Texas. *The Southwestern Naturalist, 34*(2), 165-180.

Päckert, M. (2018). Song: The Learned Language of Three Major Bird Clades. In D. T. Tietze (Ed.), *Bird Species: How They Arise, Modify and Vanish* (pp. 75-94). Cham, Switzerland: Springer International Publishing.

Päckert, M., Ait Belkacem, A., Wolfgramm, H., Gast, O., Canal, D., et al. (2019). Genetic admixture despite ecological segregation in a North African sparrow hybrid zone (Aves, Passeriformes, *Passer domesticus* × *Passer hispaniolensis*). *Ecology and Evolution, 9*(22), 12710-12726.

Patten, M. A., Rotenberry, J. T., & Zuk, M. (2004). Habitat selection, acoustic adaptation, and the evolution of reproductive isolation. *Evolution, 58*(10), 2144-2155.

Patten, M. A., & Smith-Patten, B. D. (2008). Black-crested Titmouse (*Baeolophus atricristatus*). from Cornell Lab of Ornithology http://bna.birds.cornell.edu/bna/species/717/articles/introduction

Pearson, S. F. (2000). Behavioral asymmetries in a moving hybrid zone. *Behavioral Ecology, 11*(1), 84-92.

Pearson, S. F., & Rohwer, S. (2000). Asymmetries in male aggression across an avian hybrid zone. *Behavioral Ecology, 11*(1), 93-101.

Pistone, J., Heatley, J. J., Campbell, T. A., & Voelker, G. (2017). Assessing Passeriformes health in south Texas via select venous analytes. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 210,* 64-71.

Planqué, R., Britton, N. F., & Slabbekoorn, H. (2014). On the maintenance of bird song dialects. *Journal of Mathematical Biology, 68*(1), 505-531.

Podos, J., Sarah K. Huber, and Benjamin Taft. (2004). Bird song: The interface of evolution and mechanism. *Annual Review of Ecology, Evolution, and Systematics, 34*, 55-87.

Porras-Hurtado, L., Ruiz, Y., Santos, C., Phillips, C., Carracedo, Á., et al. (2013). An overview of STRUCTURE: Applications, parameter settings, and supporting software. *Frontiers in Genetics, 4*(98), 1-13.

Potti, J. (2007). Variation in the hematocrit of a passerine bird across life stages is mainly of environmental origin. *Journal of Avian Biology, 38*(6), 726-730.

Price, T. (2008). *Speciation in Birds*. Greenwood Village, CO: Roberts and Company.

Pritchard, J. K., Stephens, M., & Donnelly, P. (2000). Inference of population structure using multilocus genotypes data. *Genetics, 155,* 945-959.

Pyle, P. (1997). *Identification guide to North American birds. Part 1: Columbidae through Ploceidae*. Point Reyes, CA: Slate Creek Press.

Qvarnstrom, A., Haavie, J., Saether, S. A., Eriksson, D., & Part, T. (2006). Song similarity predicts hybridization in flycatchers. *Journal of Evolutionary Biology, 19*(4), 1202-1209.

Raymond, M., & Rousset, F. (1995). Genepop (Version-1.2) - Population-genetics software for exact tests and ecumenicism. *Journal of Heredity, 86*(3), 248-249.

Reudink, M. W., Mech, S. G., & Curry, R. L. (2006). Extrapair paternity and mate choice in a chickadee hybrid zone. *Behavioral Ecology, 17*(1), 56-62.

Reudink, M. W., Mech, S. G., Mullen, S. P., & Curry, R. L. (2007). Structure and dynamics of the hybrid zone between Black-capped Chickadee (*Poecile atricapillus*) and Carolina Chickadee (*P. carolinensis*) in southeastern Pennsylvania. *The Auk, 124*(2), 463-478.

Rheindt, F. E., & Edwards, S. V. (2011). Genetic introgression: An integral but neglected component of speciation in birds. *The Auk, 128*(4), 620-632.

Ribot, R. F. H., Buchanan, K. L., Endler, J. A., Joseph, L., Bennett, A. T. D., et al. (2012). Learned vocal variation is associated with abrupt cryptic genetic change in a parrot species complex. *Plos One, 7*(12), e50484.

Rice, A. M., & McQuillan, M. A. (2018). Maladaptive learning and memory in hybrids as a reproductive isolating barrier. *Proceedings of the Royal Society B: Biological Sciences, 285*(1879), 20180542.

Riede, T., & Goller, F. (2014). Morphological basis for the evolution of acoustic diversity in oscine songbirds. *Proceedings of the Royal Society B: Biological Sciences, 281*(1779).

Ritchison, G., Grubb Jr., T. C., & Pravosudov, V. V. (2015). Tufted Titmouse (*Baeolophus bicolor*). from Cornell Lab of Ornithology http://bna.birds.cornell.edu/bna/species/086/articles/introduction

Roca, I. T., Desrochers, L., Giacomazzo, M., Bertolo, A., Bolduc, P., et al. (2016). Shifting song frequencies in response to anthropogenic noise: a meta-analysis on birds and anurans. *Behavioral Ecology, 27*(5), 1269-1274.

Rosenberg, N. (2007). Distruct: a program for the graphical display of population structure (Version 1.1): Center for Computational Medicine and Biology.

Rowell, J. T., & Servedio, M. R. (2012). Vocal communications and the maintenance of population specific songs in a contact zone. *Plos One, 7*(5).

Rozas, J., Ferrer-Mata, A., Sánchez-DelBarrio, J. C., Guirao-Rico, S., Librado, P., et al. (2017). DnaSP 6: DNA sequence polymorphism analysis of large data sets. *Molecular Biology and Evolution, 34*(12), 3299-3302.

Saitoh, T., Sugita, N., Someya, S., Iwami, Y., Kobayashi, S., et al. (2015). DNA barcoding reveals 24 distinct lineages as cryptic bird species candidates in and around the Japanese Archipelago. *Molecular Ecology Resources, 15*(1), 177-186.

Saladin, V., Bonfils, D., Binz, T., & Richner, H. (2003). Isolation and characterization of 16 microsatellite loci in the Great Tit (*Parus major)*. *Molecular Ecology Notes, 3*(4), 520-522.

Salewski, V., & Watt, C. (2017). Bergmann's rule: a biophysiological rule examined in birds. *Oikos, 126*(2).

Sattler, G. D., Sawaya, P., & Braun, M. J. (2007). An assessment of song admixture as an indicator of hybridization in Black-capped Chickadees (*Poecile atricapillus*) and Carolina Chickadees (*P. carolinensis*). *The Auk, 124*(3), 926-944.

Scanes, C. G. (2016). Allometric and phylogenic comparisons of hematological parameters between and within birds and mammals. *International Journal of Veterinary Health Science and Research, 4*(5), 123-129.

Schaffer, D. P. H., de Araujo, N. L. L. C., Raposo, A. C. S., Martins, E. F., Vieira, J. V. R., et al. (2017). Sedative effects of intranasal midazolam administration in wild caught Blue-fronted Amazon (*Amazona aestiva*) and Orange-winged Amazon (*Amazona amazonica*) Parrots. *Journal of Avian Medicine and Surgery, 31*(3), 213-218.

Searcy, W. A., Anderson, R. C., & Nowicki, S. (2006). Bird song as a signal of aggressive intent. *Behavioral Ecology and Sociobiology, 60*(2), 234-241.

Searcy, W. A., & Nowicki, S. (2019). Birdsong learning, avian cognition and the evolution of language. *Animal Behaviour, 151*, 217-227.

Secondi, J., Bordas, P., Hipsley, C. A., & Bensch, S. (2011). Bilateral song convergence in a passerine hybrid zone: Genetics contribute in one species only. *Evolutionary Biology, 38*(4), 441-452.

Secondi, J., Bretagnolle, V., Compagnon, C., & Faivre, B. (2003). Species-specific song convergence in a moving hybrid zone between two passerines. *Biological Journal of the Linnean Society, 80*(3), 507-517.

Semenov, G. A., Scordato, E. S. C., Khaydarov, D. R., Smith, Chris C. R., Kane, N. C., et al. (2017). Effects of assortative mate choice on the genomic and morphological structure of a hybrid zone between two bird subspecies. *Molecular Ecology, 26*(22), 6430-6444.

Senar, J. C., & Pascual, J. (1997). Keel and tarsus length may provide a good predictor of avian body size. *Ardea, 85*(2), 269-274.

Shurtliff, Q. R. (2013). Mammalian hybrid zones: A review. *Mammal Review, 43*(1), 1-21.

Sibley, D. A. (2000). *The Sibley Guide to Birds*. New York, NY: Alfred A. Knopf.

Siegel, H. S. (1980). Physiological stress in birds. *Bioscience, 30*(8), 529-534.

Slabbekoorn, H., & Smith, T. B. (2002). Bird song, ecology and speciation. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences, 357*(1420), 493-503.

Slattery, S. J. (2008). *Influence of male song on extra-pair paternity in the Black-capped Chickadee (Poecile atricapillus) and Carolina Chickadee (P. carolinensis) hybrid zone.* (M.S). Villanova University, Villanova, PA.

Smith, H. M., & Buechner, H. K. (1947). The influence of the Balcones Escarpment on the distribution of amphibians and reptiles in Texas. *Bullentin of the Chicago Academy of Sciences, 8*(1), 1-16.

Stranahan, A. M., Lee, K., & Mattson, M. P. (2008). Central mechanisms of HPA axis regulation by voluntary exercise. *Neuromolecular Medicine, 10*(2), 118-127.

Sun, Y., Li, M., Song, G., Lei, F., Li, D., et al. (2017). The role of climate factors in geographic variation in body mass and wing length in a passerine bird. *Avian Research, 8*(1), 1.

Swenson, N. G., & Howard, D. J. (2005). Clustering of contact zones, hybrid zones, and phylogeographic breaks in North America. *American Naturalist, 166*(5), 581-591.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., et al. (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution, 28*, 2731-2739.

Target Species: Edwards Plateau Ecoregion. *Texas Parks and Wildlife*. Retrieved from https://tpwd.texas.gov/huntwild/wild/wildlife_diversity/texas_nature_trackers/target_species/edwards_plateau.phtml

Taylor, S. A., Anderson, D. J., Zavalaga and, C. B., & Friesen, V. L. (2012). Evidence for strong assortative mating, limited gene flow, and strong differentiation across the blue-footed/Peruvian booby hybrid zone in northern Peru. *Journal of Avian Biology, 43*(4), 311-324.

Toews, D. P. L. (2017). From song dialects to speciation in white-crowned sparrows. *Molecular Ecology, 26*(11), 2842-2844.

Tolentino, V. C. d. M., Baesse, C. Q., & de Melo, C. (2018). Dominant frequency of songs in tropical bird species is higher in sites with high noise pollution. *Environmental Pollution, 235*, 983-992.

Tomasek, O., Bobek, L., Kralova, T., Adamkova, M., & Albrecht, T. (2019). Fuel for the pace of life: Baseline blood glucose concentration co-evolves with life-history traits in songbirds. *Functional Ecology, 33*(2), 239-249.

Toomey III, R. S., Blum, M. D., & Valastro Jr., S. (1993). Late Quaternary climate and environments of the Edwards Plateau, Texas. *Global and Planetary Change, 7*, 299-320.

Trigo, Tatiane C., Schneider, A., de Oliveira, Tadeu G., Lehugeur, Livia M., Silveira, L., et al. (2013). Molecular data reveal complex hybridization and a cryptic species of neotropical wild cat. *Current Biology, 23*(24), 2528-2533.

Tucker, M. R., Ochs, M. W., & White, R. P. (1986). Arterial blood gas levels after midazolam or diazepam administered with or without fentanyl as an intravenous sedative for outpatient surgical procedures. *Journal of Oral and Maxillofacial Surgery, 44*(9), 693-697.

Urdan, T. C. (2011). *Statistics in Plain English, Third Edition*. Oxford, United Kingdom: Taylor & Francis Group.

Vähä, J.-P., & Primmer, C. R. (2006). Efficiency of model-based Bayesian methods for detecting hybrid individuals under different hybridization scenarios and with different numbers of loci. *Molecular Ecology, 15*(1), 63-72.

Väli, Ü., Saag, P., Dombrovski, V., Meyburg, B.-U., Maciorowski, G., et al. (2010). Microsatellites and single nucleotide polymorphisms in avian hybrid identification: a comparative case study. *Journal of Avian Biology, 41*(1), 34-49.

Van Buskirk, J., Mulvihill, R. S., & Leberman, R. C. (2010). Declining body sizes in North American birds associated with climate change. *Oikos, 119*(6), 1047-1055.

Van Oosterhout, C., Hutchinson, W. F., Wills, D. P. M., & Shipley, P. (2004). MICRO-CHECKER: Software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes, 4*(3), 535-538.

Vaughn, J. C., Voelker, G., & Heatley, J. J. (2020). Glucose concentrations in closely related Titmice (Paridae: *Baeolophus*) species linked to regional habitat differences within a suspected avian hybrid zone. *The Open Ornithology Journal, 13*, 10-23.

Verzijden, M., Ten Cate, C., Servedio, M., Kozak, G., Boughman, J., et al. (2012). The impact of learning on sexual selection and speciation. *Trends in Ecology & Evolution, 27*, 511-519.

Vesal, N., & Eskandari, M. H. (2006). Sedative effects of midazolam and xylazine with or without ketamine and detomidine alone following intranasal administration in Ring-necked Parakeets. *Journal of the American Veterinary Medical Association, 228*(3), 383-388.

Vokurková, J., Petrusková, T., Reifová, R., Kozman, A., Mořkovský, L., et al. (2013). The causes and evolutionary consequences of mixed singing in two hybridizing songbird species (*Luscinia* spp.). *Plos One, 8*(4), e60172-e60172.

Wang, M. T., Hsu, Y. C., Yao, C. T., & Li, S. H. (2005). Isolation and characterization of 12 tetranucleotide repeat microsatellite loci from the Green-backed Tit (*Parus monticolus*). *Molecular Ecology Notes, 5*(2), 439-442.

Webb, W. L. (1950). Biogeographic regions of Texas and Oklahoma. *Ecology, 31*(3), 426-433.

Wenninger, E. J., & Inouye, R. S. (2008). Insect community response to plant diversity and productivity in a sagebrush–steppe ecosystem. *Journal of Arid Environments, 72*(1), 24-33.

Wheatcroft, D., & Qvarnstrom, A. (2017). Reproductive character displacement of female, but not male song discrimination in an avian hybrid zone. *Evolution, 71*(7), 1776-1786.

Wheatcroft, D., & Qvarnström, A. (2017). Genetic divergence of early song discrimination between two young songbird species. *Nature Ecology & Evolution, 1*(7), 0192.

Wilkins, M. R., Seddon, N., & Safran, R. J. (2013). Evolutionary divergence in acoustic signals: causes and consequences. *Trends in Ecology & Evolution, 28*(3), 156-166.

Williams, J., & Tieleman, B. I. (2001). Physiological Ecology and Behavior of Desert Birds. In V. Nolan, Jr. & C. Thompson (Eds.), *Current Ornithology* (Vol. 16, pp. 299-353): Springer US.

Yang, L. H., Bastow, J. L., Spence, K. O., & Wright, A. N. (2008). What can we learn from resource pulses? *Ecology, 89*(3), 621-634.

Yaw, T. J., Gentry, J., Ratliff, C., Acierno, M., Schmalz, S., et al. (2019). Venous blood analytes and osmolality of rehabilitated juvenile Black-bellied Whistling Ducks (*Dendrocygna autumnalis*). *Journal of Avian Medicine and Surgery, 33*(2), 123-132.

Yoo, G., Kim, J., Uh, Y., Yoon, K. R., Park, S. D., et al. (2015). Scoring system for detecting spurious hemolysis in anticoagulated blood specimens. *Annuals of Laboratory Medicine, 35*(3), 341-347.

Zollinger, S. A., Podos, J., Nemeth, E., Goller, F., & Brumm, H. (2012). On the relationship between, and measurement of, amplitude and frequency in birdsong. *Animal Behaviour, 84*(4), e1-e9.

Zollinger, S. A., Slater, P. J. B., Nemeth, E., & Brumm, H. (2017). Higher songs of city birds may not be an individual response to noise. *Proceedings of the Royal Society B: Biological Sciences, 284*(1860), 20170602.

Appendix A Collection information, age (1=adult, 0=juvenile, U=unknown), sex (M=male, F=female, U=unknown), body measurements, Dixon Hybrid Index (DHI), and mitochondrial and microsatellite assignment for all birds used in this study. Expected Species based on geographic location of known distribution ranges. BCTI=Black-crested Titmouse, TUTI=Tufted Titmouse, CACH=Carolina Chickadee. Birds used in blood physiology analysis indicated by (*).

BRTC ID #	Month	Year	County	Expected Species	Transect Region	Latitude	Longitude	Sex	Age	Avg Bill (mm)	Avg Wing (mm)	Avg Tarsus (mm)	Avg Tail (mm)	DHI	DHI Species	mtDNA clade	q, admix	q, no admix	Nuclear Group
17232	June	2012	Uvalde	BCTI	West	29.402	-100.000	M	1	11.9	72.5	20.7	70.5	6	BCTI	BCTI	0.11	0.89	BCTI
17233	June	2012	Uvalde	BCTI	West	29.402	-100.000	M	1	11.4	72.0	20.1	74.5	6	BCTI	BCTI	0.30	0.70	Hybrid
17234	June	2012	Uvalde	BCTI	West	29.402	-100.000	F	0	12.8	70.5	20.0	72.5	3	unk	BCTI			
17235	June	2012	Uvalde	BCTI	West	29.402	-100.000	M	1	13.4	73.5	20.6	70.5	6	BCTI	BCTI	0.04	0.96	BCTI
17236	June	2012	Uvalde	BCTI	West	29.402	-100.000	M	1	11.8	75.0	19.8	69.0	6	BCTI	BCTI	0.02	0.98	BCTI
5838	May	1956	Kimble	BCTI	West	30.459	-99.822	M	1	12.5	74.3	20.5	77.5	6	BCTI				
5839	May	1956	Kimble	BCTI	West	30.459	-99.822	F	1	11.3	70.8	19.3	70.5	4	BCTI				
15518	Dec	1966	Kimble	BCTI	West	30.459	-99.822	M	U	12.4	71.5	20.4	76.5	5	BCTI				
15524	Dec	1966	Kimble	BCTI	West	30.459	-99.822	M	U	12.4	72.0	20.1	69.5	4	BCTI				
15525	Dec	1965	Kimble	BCTI	West	30.459	-99.822	F	U	12.4	72.3	20.3	70.0	4	BCTI				
15526	Nov	1966	Kimble	BCTI	West	30.459	-99.822	M	U	11.6	74.0	20.0	74.0	5	BCTI				
15527	Nov	1966	Kimble	BCTI	West	30.459	-99.822	M	U	11.7	77.0	21.0	72.5	5	BCTI				
15528	Dec	1966	Kimble	BCTI	West	30.459	-99.822	F	U	12.0	75.0	19.7	71.0	5	BCTI				
9136	March	1937	Uvalde	BCTI	West	29.305	-99.785	M	1	12.0	76.0	19.7	79.0	5	BCTI				
16175	Aug	2011	Kerr	BCTI	West	30.043	-99.397	F	0	11.8	75.5	22.0	77.5	4	BCTI	BCTI	0.07	0.93	BCTI
16289	Aug	2010	Kerr	BCTI	West	30.043	-99.397	M	0	11.6	75.0	19.8	71.5	3	unk	BCTI	0.04	0.96	BCTI
16290	Aug	2010	Kerr	BCTI	West	30.043	-99.397	M	0	11.7	74.5	19.7	67.0	4	BCTI	BCTI			
16292	Aug	2010	Kerr	BCTI	West	30.043	-99.397	M	0	12.5	71.5	18.3	67.0	4	BCTI	BCTI			
16454	Aug	2010	Kerr	BCTI	West	30.043	-99.397	F	0	11.4	72.8	20.4	72.5	5	BCTI	BCTI			
*16528	Aug	2011	Kerr	BCTI	West	30.043	-99.397	F	1	12.4	73.5	20.3	73.5	6	BCTI	BCTI	0.02	0.98	BCTI
*17164	March	2012	Kerr	BCTI	West	30.043	-99.397	M	1	11.3	73.5	19.8	73.5	6	BCTI	BCTI	0.01	0.99	BCTI
17221	June	2012	Kerr	BCTI	West	30.043	-99.397	M	1	12.8	74.5	20.7	70.5	6	BCTI	BCTI	0.01	0.99	BCTI
17222	June	2012	Kerr	BCTI	West	30.043	-99.397	F	0	12.0	69.5	20.2	68.5	3	unk	BCTI			

Appendix A: continued

BRTC ID #	Month	Year	County	Expected Species	Transect Region	Latitude	Longitude	Sex	Age	Avg Bill (mm)	Avg Wing (mm)	Avg Tarsus (mm)	Avg Tail (mm)	DHI	DHI Species	mtDNA clade	q, admix	q, no admix	Nuclear Group
17223	June	2012	Kerr	BCTI	West	30.043	-99.397	F	0	11.3	70.3	22.1	72.5	3	unk	BCTI			
17224	June	2012	Kerr	BCTI	West	30.043	-99.397	M	1	12.2	76.0	20.4	69.0	6	BCTI	BCTI	0.03	0.97	BCTI
18101	June	2013	Kerr	BCTI	West	30.043	-99.397	F	1	11.8	74.0	21.4	73.5	5	BCTI	BCTI	0.01	0.99	BCTI
402	June	1937	Kerr	BCTI	West	30.031	-99.395	M	U	12.5	75.0	20.2	72.5	3	unk				
403	June	1937	Kerr	BCTI	West	30.031	-99.395	F	U	13.0	73.5	18.2	67.5	4	BCTI				
404	June	1937	Kerr	BCTI	West	30.031	-99.395	M	U	13.2	73.8	18.8	64.5	6	BCTI				
405	June	1937	Kerr	BCTI	West	30.031	-99.395	M	U	13.0	72.8	19.5	75.5	3	unk				
406	June	1937	Kerr	BCTI	West	30.031	-99.395	F	U	12.9	70.0	19.0	65.5	5	BCTI				
407	June	1937	Kerr	BCTI	West	30.031	-99.395	M	U	13.1	76.3	20.3	75.5	3	unk				
408	June	1937	Kerr	BCTI	West	30.031	-99.395	F	U	12.9	70.0	20.8	75.0	5	BCTI				
409	June	1937	Kerr	BCTI	West	30.031	-99.395	M	U	13.0	72.0	19.7	68.0	3	unk				
410	July	1937	Kerr	BCTI	West	30.031	-99.395	M	U	12.9	74.5	19.2	72.3	4	BCTI				
411	July	1937	Kerr	BCTI	West	30.031	-99.395	M	U	12.2	74.0	19.7	77.5	5	BCTI				
412	July	1937	Kerr	BCTI	West	30.031	-99.395	M	U	12.0	75.0	19.3	70.0	3	unk				
413	Aug	1937	Kerr	BCTI	West	30.031	-99.395	M	U	12.4	80.0	19.8	80.0	5	BCTI				
1158	Dec	1937	Kerr	BCTI	West	30.031	-99.395	M	U	12.3	75.0	18.1	74.5	4	BCTI				
14934	July	2009	Kerr	BCTI	West	30.060	-99.225	M	1		73.0	20.5	68.5	4	unk	BCTI	0.10	0.90	BCTI
15462	Oct	2009	Kerr	BCTI	West	30.060	-99.225	M	1	11.4	75.5	21.7	73.5	5	BCTI	BCTI	0.02	0.98	BCTI
15463	Oct	2009	Kerr	BCTI	West	30.060	-99.225	F	1	11.2	73.5	19.4	71.5	5	BCTI	BCTI	0.01	0.99	BCTI
15464	Oct	2009	Kerr	BCTI	West	30.060	-99.225	M	1	12.0	77.8	21.0	73.5	5	BCTI	BCTI	0.03	0.97	BCTI
15465	Oct	2009	Kerr	BCTI	West	30.060	-99.225	M	1	12.2	76.0	22.6	75.0	5	BCTI	BCTI	0.03	0.97	BCTI
18085	June	2013	Jim Hogg	BCTI	South	26.911	-98.844	F	0	10.7	67.0	19.3	60.5	3	unk				
18086	June	2013	Jim Hogg	BCTI	South	26.911	-98.844	F	0	10.0	72.0	20.3	65.5	3	unk				
17725	May	2013	Jim Hogg	BCTI	South	26.982	-98.829	M	1	12.0	72.8	20.4	69.5	4	BCTI	BCTI	0.98	0.02	BCTI
18030	June	2013	Jim Hogg	BCTI	South	26.982	-98.829	F	1	12.1	65.0	18.7	65.5	4	BCTI	BCTI	0.98	0.02	BCTI
18098	July	2013	Starr	BCTI	South	26.757	-98.827	M	1	11.5	67.0	19.7	64.0	4	BCTI	BCTI	0.98	0.02	BCTI
16158	Spring	2009	Kendall	BCTI	West	29.795	-98.734	F	0	12.0	71.0	19.2	71.5	3	unk	BCTI	0.21	0.79	Hybrid
1121	March	1938	San Saba	BCTI	West	31.202	-98.734	M	U	12.3	78.0	21.0	78.5	6	BCTI				
5840	Sept	1956	Llano	BCTI	West	30.722	-98.717	F	1	11.9	70.0		76.5	5	BCTI				
5841	Sept	1956	Llano	BCTI	West	30.722	-98.717	F	1	12.0	72.3	20.8	74.5	5	BCTI				
5842	Sept	1956	Llano	BCTI	West	30.722	-98.717	M	1	12.0	74.5	20.6	76.5	5	BCTI				

Appendix A: continued

BRTC ID #	Month	Year	County	Expected Species	Transect Region	Latitude	Longitude	Sex	Age	Avg Bill (mm)	Avg Wing (mm)	Avg Tarsus (mm)	Avg Tail (mm)	DHI	DHI Species	mtDNA clade	q, admix	q, no admix	Nuclear Group
5843	Sept	1956	Llano	BCTI	West	30.722	-98.717	M	U	12.2	70.0	20.0	65.0	5	BCTI				
5844	Sept	1956	Llano	BCTI	West	30.722	-98.717	M	1	11.6	75.0	20.2	78.0	5	BCTI				
5846	Sept	1956	Llano	BCTI	West	30.722	-98.717	F	1	12.7	71.5	20.3	71.0	4	BCTI				
5847	Sept	1956	Llano	BCTI	West	30.722	-98.717	U	1	11.7	75.5	19.6	78.5	5	BCTI				
9655	Jan	1975	Llano	BCTI	West	30.722	-98.717	F	U	11.7	75.5	20.9	70.0	6	BCTI				
16202	April	2011	Mills	BCTI	West	31.465	-98.472	F	1	12.2	72.0	20.3	73.5	5	BCTI	BCTI	0.28	0.72	Hybrid
*16360	July	2011	Comal	BCTI	Contact	29.917	-98.156	M	1	12.6	76.5	20.9		5	BCTI	BCTI	0.27	0.73	Hybrid
*16362	July	2011	Comal	BCTI	Contact	29.917	-98.156	F	1	12.4	72.5	21.3	70.5	5	BCTI	BCTI	0.46	0.54	Hybrid
*16453	July	2011	Comal	BCTI	Contact	29.917	-98.156	F	0	11.3	70.5	19.7	68.5	3	unk	BCTI			
*17167	June	2012	Comal	BCTI	Contact	29.917	-98.156	M	0	11.6	75.0	20.9	71.5	3	unk	BCTI	0.08	0.92	BCTI
*17168	April	2012	Comal	BCTI	Contact	29.917	-98.156	M	1	12.8	78.5	21.7	79.5	3	unk	BCTI	0.01	0.99	BCTI
*17169	April	2012	Comal	BCTI	Contact	29.917	-98.156	M	1	12.3	71.0	22.4	72.5	4	BCTI	BCTI	0.05	0.95	BCTI
16249	May	2011	Hays	BCTI	Contact	29.962	-98.082	F	1	12.1	69.5	20.5	71.0	3	unk	BCTI	0.03	0.97	BCTI
*16295	May	2011	Hays	BCTI	Contact	29.962	-98.082	M	0	12.9	71.0	19.3	75.5	2	TUTI	BCTI			
16365	May	2011	Hays	BCTI	Contact	29.962	-98.082	M	1	12.0	74.5	20.9	78.5	5	BCTI	BCTI	0.01	0.99	BCTI
*16366	May	2011	Hays	BCTI	Contact	29.962	-98.082	M	1	12.5	70.5	20.4	70.0	3	unk	BCTI	0.21	0.79	Hybrid
16444	May	2011	Hays	BCTI	Contact	29.962	-98.082	M	0	11.7	72.0	19.7	77.5	3	unk	BCTI			
*16445	May	2011	Hays	BCTI	Contact	29.962	-98.082	F	1	12.0	71.5	19.7	72.5	4	BCTI	BCTI	0.07	0.93	BCTI
*16446	May	2011	Hays	BCTI	Contact	29.962	-98.082	F	0	12.1	70.3	20.1	74.5	3	unk	BCTI			
*16774	April	2012	Hays	BCTI	Contact	29.962	-98.082	M	1	13.0	72.0	21.3	66.0	5	BCTI	BCTI	0.25	0.75	Hybrid
*17157	May	2012	Hays	BCTI	Contact	29.962	-98.082	M	1	12.2	74.0	20.6	72.0	5	BCTI	BCTI	0.16	0.84	BCTI
*17158	May	2012	Hays	BCTI	Contact	29.962	-98.082	U	0	12.1	73.5	21.8	73.5	3	unk	BCTI			
17159	May	2012	Hays	BCTI	Contact	29.962	-98.082	M	0	12.0	69.5	21.1	72.0	3	unk	BCTI			
*17160	May	2012	Hays	BCTI	Contact	29.962	-98.082	M	1	12.7	72.5	20.7	70.5	6	BCTI	BCTI	0.06	0.94	BCTI
*17161	May	2012	Hays	BCTI	Contact	29.962	-98.082	F	0	12.0	71.5	20.3	70.0	3	unk	BCTI			
5356	April	1953	Hays	BCTI	Contact	29.961	-98.016	M	U	12.8	74.5	19.5	74.5	5	BCTI				
17681	Oct	1985	Hays	BCTI	Contact	29.961	-98.016	M	1	11.9	77.0	20.5	80.0	4	BCTI				
477	Feb	1963	Hays	BCTI	Contact	29.961	-98.003	U	U	12.2	75.0	20.0	65.5	5	BCTI				
478	Feb	1963	Hays	BCTI	Contact	29.961	-98.003	U	U	11.2	69.0	18.9	63.5	3	unk				
*16250	April	2011	Travis	BCTI	Contact	30.476	-97.959	F	1	11.7	69.5	19.9	72.5	4	BCTI	BCTI	0.22	0.78	Hybrid
*16294	April	2011	Travis	BCTI	Contact	30.476	-97.959	M	1	12.0	74.5	22.8	76.5	4	BCTI	BCTI	0.39	0.61	Hybrid
*16297	April	2011	Travis	BCTI	Contact	30.476	-97.959	M	1	11.9	78.5	21.1	78.5	5	BCTI	BCTI	0.01	0.99	BCTI

Appendix A: continued

BRTC ID #	Month	Year	County	Expected Species	Transect Region	Latitude	Longitude	Sex	Age	Avg Bill (mm)	Avg Wing (mm)	Avg Tarsus (mm)	Avg Tail (mm)	DHI	DHI Species	mtDNA clade	q, admix	q, no admix	Nuclear Group
*16359	June	2011	Travis	BCTI	Contact	30.476	-97.959	M	1	12.1	74.5	21.3	75.5	4	BCTI	BCTI	0.06	0.94	BCTI
*16451	June	2011	Travis	BCTI	Contact	30.476	-97.959	F	0	11.4	71.3	19.8	66.5	3	unk	BCTI			
*17174	June	2012	Travis	BCTI	Contact	30.476	-97.959	F	0	12.5	79.3	20.5	76.5	1	TUTI	BCTI			
*17175	June	2012	Travis	BCTI	Contact	30.476	-97.959	F	0	13.0	76.8	20.5	73.5	1	TUTI	BCTI	0.02	0.98	BCTI
*17176	June	2012	Travis	BCTI	Contact	30.476	-97.959	F	0	12.1	70.5	19.4	68.5	3	unk	BCTI			
*17177	June	2012	Travis	BCTI	Contact	30.476	-97.959	F	0	11.9	75.0	21.3	72.0	3	unk	BCTI			
17862	April	2012	Travis	BCTI	Contact	30.476	-97.959	U	0	11.7	72.3	20.9	64.5	3	unk	BCTI	0.01	0.99	BCTI
5357	Oct	1953	Williamson	BCTI	Contact	30.678	-97.627	F	0	10.6	70.3	19.7	74.5	4	BCTI				
*17196	May	2012	Caldwell	TUTI	East	29.785	-97.469	M	1	12.8	75.5	21.6	76.0	1	TUTI	TUTI	0.88	0.12	TUTI
*17200	March	2012	Caldwell	TUTI	East	29.785	-97.469	M	1	12.2	80.0	19.8	79.5	1	TUTI	TUTI	0.97	0.03	TUTI
*16448	June	2011	Bastrop	TUTI	East	29.958	-97.335	M	1	12.8	78.8	22.2	73.5	2	TUTI	TUTI	0.66	0.34	Hybrid
*16449	June	2011	Bastrop	TUTI	East	29.958	-97.335	M	0	12.6	72.5	20.5	68.5	1	TUTI	TUTI			
*16450	June	2011	Bastrop	TUTI	East	29.958	-97.335	M	0	11.9	76.3	20.2	73.5	2	TUTI	TUTI			
*17182	April	2012	Bastrop	TUTI	East	29.958	-97.335	M	1	12.6	80.0	20.3	79.5	0	TUTI	TUTI	0.96	0.04	TUTI
*17183	April	2012	Bastrop	TUTI	East	29.958	-97.335	M	1	12.5	78.8	21.7	78.3	1	TUTI	TUTI	0.96	0.04	TUTI
*17184	April	2012	Bastrop	TUTI	East	29.958	-97.335	M	1	12.6	80.5	21.9	80.0	1	TUTI	TUTI	0.92	0.08	TUTI
*17217	May	2012	Bastrop	TUTI	East	29.958	-97.335	M	1	12.1	77.0	20.7	79.5	1	TUTI	TUTI	0.94	0.06	TUTI
17218	May	2012	Bastrop	TUTI	East	29.958	-97.335	M	1	12.4	74.5	21.4	70.0	2	TUTI	TUTI	0.94	0.06	TUTI
15424	March	2010	Bastrop	TUTI	East	30.131	-97.287	F	1	12.4	75.5	20.6	70.5	1	TUTI	TUTI	0.94	0.06	TUTI
*16293	June	2011	Williamson	TUTI	East	30.467	-97.264	F	0	12.5	76.0	19.5	68.5	3	unk	TUTI			
*16296	June	2011	Williamson	TUTI	East	30.467	-97.264	M	0	12.7	76.5	22.0	79.5	3	unk	TUTI			
*16298	June	2011	Williamson	TUTI	East	30.467	-97.264	M	1	12.4	73.5	18.9	65.0	3	unk	TUTI	0.97	0.03	TUTI
16363	June	2011	Williamson	TUTI	East	30.467	-97.264	M	1	12.2	77.5	20.8	73.0	1	TUTI	TUTI	0.95	0.05	TUTI
*16447	June	2011	Williamson	TUTI	East	30.467	-97.264	F	0	13.0	70.0	19.2	71.5	3	unk	TUTI			
*17147	April	2012	Williamson	TUTI	East	30.467	-97.264	M	1	12.7	76.0	21.9	74.5	5	BCTI	TUTI	0.92	0.08	TUTI
*17148	April	2012	Williamson	TUTI	East	30.467	-97.264	M	1	12.6	73.0	20.5	66.5	1	TUTI	TUTI	0.79	0.21	Hybrid
*17149	April	2012	Williamson	TUTI	East	30.467	-97.264	M	1	13.0	80.0	21.4	72.5	3	unk	TUTI	0.96	0.04	TUTI
*17181	March	2012	Williamson	TUTI	East	30.467	-97.264	M	1	11.9	76.5	20.7	74.5	1	TUTI	TUTI	0.72	0.28	Hybrid
14864	June	2004	Washington	TUTI	East	30.183	-96.755	U	0	11.6	77.5	22.4	70.5	3	unk	TUTI	0.98	0.02	TUTI
1	March	1988	Jackson	TUTI	East	28.933	-96.591	M	U	11.5	78.5	21.0	80.0	1	TUTI				
1268	Feb	1938	Brazos	TUTI	East	30.666	-96.279	F	U	13.2	77.0	19.8	72.0	1	TUTI				
1447	Feb	1938	Brazos	TUTI	East	30.666	-96.279	M	1	13.3	76.0	20.3	75.0	1	TUTI				

Appendix A: continued

BRTC ID #	Month	Year	County	Expected Species	Transect Region	Latitude	Longitude	Sex	Age	Avg Bill (mm)	Avg Wing (mm)	Avg Tarsus (mm)	Avg Tail (mm)	DHI	DHI Species	mtDNA clade	q, admix	q, no admix	Nuclear Group
5816	Sept	1955	Brazos	TUTI	East	30.666	-96.279	M	1	13.7	82.0	20.6	85.0	0	TUTI				
5817	Sept	1955	Brazos	TUTI	East	30.666	-96.279	F	1	11.8	74.5	19.3	81.5	1	TUTI				
5818	Sept	1955	Brazos	TUTI	East	30.666	-96.279	M	1	13.4	80.3	21.3	83.5	0	TUTI				
5819	Sept	1955	Brazos	TUTI	East	30.666	-96.279	M	1	12.5	80.0	21.2	81.0	0	TUTI				
5820	Sept	1955	Brazos	TUTI	East	30.666	-96.279	M	0	13.1	76.5	19.5	80.5	0	TUTI				
5821	Sept	1955	Brazos	TUTI	East	30.666	-96.279	F	1	12.7	77.0	20.5	75.5	2	TUTI				
5822	Sept	1955	Brazos	TUTI	East	30.666	-96.279	U	1	13.5	77.3	20.6	84.5	0	TUTI				
5823	Sept	1955	Brazos	TUTI	East	30.666	-96.279	M	1	12.8	77.3	21.8	80.0	1	TUTI				
5824	Sept	1955	Brazos	TUTI	East	30.666	-96.279	U	0	13.1	78.0	20.5	80.0	0	TUTI				
5825	Sept	1956	Brazos	TUTI	East	30.666	-96.279	M	1	13.2	80.0	20.9	80.5	0	TUTI				
5826	Sept	1956	Brazos	TUTI	East	30.666	-96.279	M	1		80.0	21.8	84.0	1	TUTI				
5827	Sept	1956	Brazos	TUTI	East	30.666	-96.279	F	0	12.2	75.5	20.1	65.5	1	TUTI				
5828	Sept	1956	Brazos	TUTI	East	30.666	-96.279	F	0	13.5	72.0	20.6	76.5	1	TUTI				
6418	Sept	1955	Brazos	TUTI	East	30.666	-96.279	F	0	13.1	76.0	19.5	72.5	0	TUTI				
17809	Oct	1979	Brazos	TUTI	East	30.666	-96.279	U	1	12.5	75.0	19.3	70.5	0	TUTI				
15845	Aug	2010	Brazos	TUTI	East	30.513	-96.272	F	0	11.7	78.5	20.9	67.5	1	TUTI	TUTI	0.97	0.03	TUTI
16211	April	2011	Brazos	TUTI	East	30.513	-96.272	M	1	12.6	75.5	21.5	80.5	0	TUTI	TUTI	0.97	0.03	TUTI
17781	May	2012	Brazos	TUTI	East	30.513	-96.272	U	0	12.9	80.0	19.8	75.5	2	TUTI	TUTI	0.97	0.03	TUTI
*16361	July	2011	Grimes	TUTI	East	30.470	-96.082	F	0	12.9	74.0	19.9	75.5	3	unk	TUTI			
*16364	July	2011	Grimes	TUTI	East	30.470	-96.082	F	0	12.1	77.0	20.9	80.5	2	TUTI	TUTI			
*16441	July	2011	Grimes	TUTI	East	30.470	-96.082	M	1	12.8	76.5	20.5	80.5	0	TUTI	TUTI	0.97	0.03	TUTI
*16442	July	2011	Grimes	TUTI	East	30.470	-96.082	F	0	12.7	72.0	19.5	71.5	3	unk	TUTI			
*16443	July	2011	Grimes	TUTI	East	30.470	-96.082	M	U	12.3	79.5	20.7	79.5	3	unk	TUTI			
*17202	June	2012	Grimes	TUTI	East	30.470	-96.082	M	0	12.0	75.0	21.3	70.0	4	BCTI	TUTI	0.97	0.03	TUTI
*17203	June	2012	Grimes	TUTI	East	30.470	-96.082	M	1	12.4	74.0	20.0	69.5	3	unk	TUTI	0.93	0.07	TUTI
1450	May	1938	Waller	TUTI	East	30.003	-96.064	M	1	13.0	79.0	19.6	78.0	0	TUTI				
8823	July	1971	Grimes	TUTI	East	30.537	-95.993	M	U	13.6	76.5	20.4	76.5	1	TUTI				
181	March	1936	Walker	TUTI	East	30.710	-95.640	M	1	13.5	78.0	20.4	71.3	0	TUTI				
182	July	1936	Walker	TUTI	East	30.710	-95.640	M	U	13.0	73.5	20.0	65.5	3	unk				
1707	July	1938	Harris	TUTI	East	29.813	-95.294	F	U	12.8	73.8	19.4	66.0	3	unk				
1708	Aug	1938	Harris	TUTI	East	29.813	-95.294	M	U	12.9	80.0	20.7	75.5	1	TUTI				

Appendix A: continued

BRTC ID #	Month	Year	County	Expected Species	Transect Region	Latitude	Longitude	Sex	Age	Avg Bill (mm)	Avg Wing (mm)	Avg Tarsus (mm)	Avg Tail (mm)	DHI	DHI Species	mtDNA clade	q, admix	q, no admix	Nuclear Group
1709	Aug	1938	Harris	TUTI	East	29.813	-95.294	M	U	13.5	76.8	20.2	76.5	1	TUTI				
1709	Aug	1938	Harris	TUTI	East	29.813	-95.294	M	U	13.5	76.8	20.2	76.5	1	TUTI				
1710	Sept	1938	Harris	TUTI	East	29.813	-95.294	F	U	12.8	74.0	18.4	76.5	2	TUTI				
794	Jan	1937	Trinity	TUTI	East	31.082	-95.144	M	U	13.2	80.0	20.3	83.5	1	TUTI				
795	Jan	1937	Trinity	TUTI	East	31.082	-95.143	F	U	12.0	79.8	19.4	77.5	1	TUTI				
887	Sept	1937	Polk	TUTI	East	30.758	-94.809	M	U	12.7	81.5	19.9	79.5	0	TUTI				
888	Sept	1937	Polk	TUTI	East	30.758	-94.809	M	U	12.5	79.3	20.7	77.5	1	TUTI				
889	Sept	1937	Polk	TUTI	East	30.758	-94.809	M	U	11.8	81.5	18.8	80.5	0	TUTI				
*15843	April	2010	Tyler	TUTI	East	30.768	-94.443	M	1	12.5	78.5	22.5	74.5	1	TUTI	TUTI	0.98	0.02	TUTI
15844	March	2010	Tyler	TUTI	East	30.768	-94.443	M	0	12.7	78.0	20.9	72.5	1	TUTI	TUTI	0.97	0.03	TUTI
15880	Aug	2010	Tyler	TUTI	East	30.768	-94.443	M	0	11.8	78.8	20.7	72.5	1	TUTI	TUTI		1.00	
15881	Aug	2010	Tyler	TUTI	East	30.768	-94.443	M	0	11.9	81.8	20.3	72.5	1	TUTI	TUTI	0.95	0.05	TUTI
15905	April	2010	Tyler	TUTI	East	30.768	-94.443	M	1	11.8	79.8	20.6		0	TUTI	TUTI	0.97	0.03	TUTI
16160	Aug	2010	Tyler	TUTI	East	30.768	-94.443	F	U	12.0	78.0	19.6	77.5	0	TUTI	TUTI		1.00	
*16288	July	2010	Tyler	TUTI	East	30.768	-94.443	F	1	12.2	71.5	19.9		1	TUTI	TUTI	0.98	0.02	TUTI
*16291	July	2010	Tyler	TUTI	East	30.768	-94.443	F	0	11.9	76.5	22.2	69.0	3	unk	TUTI			
*16452	July	2010	Tyler	TUTI	East	30.768	-94.443	M	0	11.9	74.0	21.5	65.5	3	unk	TUTI			
1369	March	1938	Tyler	TUTI	East	30.764	-94.436	F	U	11.8	78.0	19.9	77.5	1	TUTI				
9563	April	1974	Newton	TUTI	East	30.869	-93.685	M	U	12.1	77.0	20.6	77.5	0	TUTI				

Carolina Chickadee

BRTC ID #	Month	Year	County	Expected Species	Transect Region	Latitude	Longitude
	June	2011	Comal	CACH	Contact	29.917	-98.156
	June	2011	Comal	CACH	Contact	29.917	-98.156
	April	2012	Grimes	CACH	East	30.470	-96.082
	May	2011	Hays	CACH	Contact	29.962	-98.082
	July	2009	Kerr	CACH	West	30.060	-99.225
	March	2011	Williamson	CACH	East	30.467	-97.264
	March	2011	Williamson	CACH	East	30.467	-97.264

APPENDIX B: SONG RECORDING LOCATIONS

Appendix B Locations and number of the 32 songs recorded. Expected species is based on known geographic range distribution for Black-crested Titmice (BCTI) and Tufted Titmice (TUTI). Song recording source noted by JCV recording or if downloaded from online with website and song number provided.

Site Location	Latitude	Longitude	Transect Region	Expected Species	County	Number of songs	Source
Bastrop Ecolab Property	29.9579	-97.3367	East	TUTI	Bastrop	3	JCV recording
Lick Creek Park	30.5670	-96.2113	East	TUTI	Brazos	1	JCV recording
Grimes Ecolab Property	30.5372	-95.9928	East	TUTI	Grimes	3	JCV recording
Lake Livingston State Park	30.7582	-94.8086	East	TUTI	Polk	3	JCV recording
Sam Houston National Forest	30.5353	-95.3801	East	TUTI	Walker	1	Macaulay Library, 105260
Williamson Ecolab Property	30.4674	-97.2615	East	TUTI	Williamson	4	JCV recording
Comal Ecolab Property	29.9187	-98.1573	Contact zone	BCTI	Comal	2	JCV recording
Private Residence, Wimberley	29.9642	-98.0839	Contact zone	BCTI	Hays	1	JCV recording
McKinney Falls State Park	30.1809	-97.7255	Contact zone	BCTI	Travis	3	JCV recording
TX-16, Robertson Creek road	29.8280	-99.2686	West	BCTI	Bandera	2	JCV recording
Guadalupe River near Boerne	29.8917	-98.6447	West	BCTI	Kendall	1	xeno-canto, XC122454
Kerr Ecolab Property, River	30.0188	-99.3640	West	BCTI	Kerr	2	JCV recording
Kerr Wildlife Management Area	30.0674	-99.5061	West	BCTI	Kerr	1	JCV recording
Schreiner Park, Junction	30.4898	-99.7601	West	BCTI	Kimble	1	JCV recording
South Llano River State Park	30.4507	-99.8055	West	BCTI	Kimble	1	JCV recording
Kickapoo Caverns State Park	29.6182	-100.4509	West	BCTI	Kinney	1	Macaulay Library, 105232
TX-127, Concan	29.4953	-99.7115	West	BCTI	Uvalde	2	JCV recording

Appendix C Playback Experiment site locations with number of playbacks for each species song played. Species played defined as BCTI (Black-crested Titmouse) and TUTI (Tufted Titmouse).

Site	Latitude Longitude	Transect Region	County	Species Played	Number of Playbacks
South Llano River State Park	30.4507 -99.8055	West	Kimble	BCTI TUTI	1 1
Kerr Wildlife Management Area	30.0674 -99.5061	West	Kerr	BCTI TUTI	3 6
Kerr Ecolab	30.0435 -99.3967	West	Kerr	BCTI TUTI	2 1
Balcones Canyonlands National Wildlife Refuge	30.6197 -98.0736	West	Burnet	BCTI TUTI	1 1
Inks Lake State Park	30.7375 -98.3704	West	Burnet	BCTI TUTI	1 2
Comal Ecolab	29.9187 -98.1573	Contact Zone	Comal	BCTI TUTI	0 2
Hays, private property	29.9642 -98.0839	Contact Zone	Hays	BCTI TUTI	3 2
Travis Ecolab	30.4761 -97.9590	Contact Zone	Travis	BCTI TUTI	1 0
McKinney Falls State Park	30.1809 -97.7255	Contact Zone	Travis	BCTI TUTI	4 2
Williamson Ecolab	30.4674 -97.2615	East	Williamson	BCTI TUTI	2 2
Lick Creek city park	30.5670 -96.2113	East	Brazos	BCTI TUTI	1 2
Huntsville State Park	30.6168 -95.5283	East	Walker	BCTI TUTI	3 3
Martin Dies Jr. State Park	30.8633 -94.1813	East	Tyler	BCTI TUTI	2 4

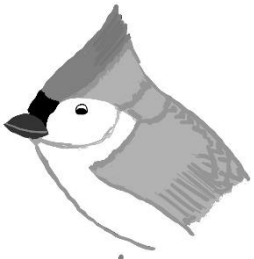
Appendix D Dixon hybrid index scale and scoring.

Dixon Hybrid Index: sum of forehead and crest scores

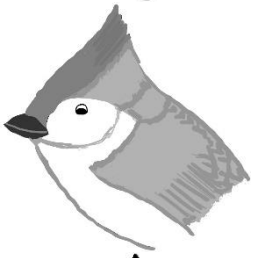
Forehead:
0: Black
1: Brown/black
2: Tan
3: White

Crest:
0: Gray
1: Dark gray
2: Mixed soft black
3: Black

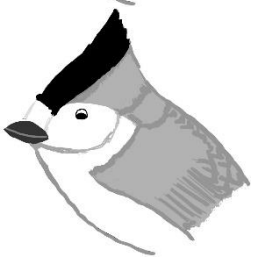
"Pure" Tufted Titmouse
DHI: 0-1



"Hybrid"
DHI: 2-4



"Pure" Black-crested Titmouse
DHI: 5-6



Appendix E Number of birds used for genetic analysis per location of capture. Transect region, county, latitude, longitude, sex, and age provided. Expected species (A, B) based on capture location relative to geographic distribution ranges.

A) Tufted Titmouse

				# juveniles			# adults			# unknown age		
Transect Region	County	Latitude	Longitude	female	male	unknown	female	male	unknown	female	male	**Total**
East	Bastrop1	29.958	-97.335		2			6				**8**
	Bastrop2	30.131	-97.287				1					**1**
	Brazos	30.513	-96.272	1		1		1				**3**
	Caldwell	29.785	-97.469					2				**2**
	Grimes	30.470	-96.082	3	1			2			1	**7**
	Tyler	30.768	-94.443	1	4		1	2		1		**9**
	Washington	30.183	-96.755			1						**1**
	Williamson	30.467	-97.264	2	1			6				**9**
	Total			**7**	**8**	**2**	**2**	**19**	**0**	**1**	**1**	**40**

B) Black-crested Titmouse

				# juveniles			# adults			# unknown age		
Transect Region	County	Latitude	Longitude	female	male	unknown	female	male	unknown	female	male	**Total**
Contact Zone	Comal	29.917	-98.156	1	1		1	3				**6**
	Hays	29.962	-98.082	2	3	1	2	5				**13**
West	Kendall	29.795	-98.734	1								**1**
	Kerr1	30.043	-99.397	5	3		2	3				**13**
	Kerr2	30.060	-99.225				1	4				**5**
	Mills	31.465	-98.472				1					**1**
	Travis	30.476	-97.959	5		1	1	3				**10**
	Uvalde	29.402	-100.000	1				4				**5**
South	Jim Hogg	26.982	-98.829				1	1				**2**
	Starr	26.757	-98.827					1				**1**
				15	**7**	**2**	**9**	**24**	**0**	**0**	**0**	**57**

Appendix F Microsatellite loci tested with *Baeolophus*. Locus names provided are those cited in previous papers, parentheses names indicate alternative names used in other studies.

Locus Name	Primer Sequence (5->3)	Allele size (range)*	Repeat size	Original species	Studies utilized	Successful
Pma69	F: CCCAGACAAAGCATCACTGG	214	Di	*Parus major*	Kawano 2003	Yes
(Pma69u)	R: GACAGTTCACATAGCCCTGG	(228-250)				
Pma179	F: GGAGGCTTAAACATTCTGTGTG	180	Di	*Parus major*	Kawano 2003	Yes
	R: GGGCTGAAGGAGTTTGCTAC	(198-236)				
PmaC25	F: CGTCCTGCTGTTTGTATTTCTG	323	Tri	*Parus major*	Saladin et al. 2003	Yes
	R: CCATGAACCATTTTTAGGGTG	(324-360)			Johannessen et al. 2011	
Pma30	F: GTTTCTGCCCAAATGGTGTC	305	Di	*Parus major*	Saladin et al. 2003	Yes
(PmaGAn30)	R: TCAGACCTTTCCAATGATGG	(314-326)				
Pma45	F: CCCCTGGCTCTTTCATATCC	307	Tri	*Parus major*	Saladin et al. 2003	Yes
(PmaTGAn45)	R: GACAGGTGTTGGCACAAGG	(306-342)				
Pca7	F: TGAGCATCGTAGCCCAGCAG	127	Di	*Parus caeruleus*	Dawson et al. 2000	Yes
	R: GGTTCAGGACACCTGCACAATG	(120-158)			Johannessen et al. 2011	
Pca9	F: ACCCACTGTCCAGAGCAGGG	131	Di	*Parus caeruleus*	Dawson et al. 2000	Yes
	R: AGGACTGCAGCAGTTTGTGGG	(124-174)			Reudink et al. 2005, 2007	
					Johannessen et al. 2011	
Pat2-14	F: GGACAGATAAAAGCCAAATTAC	155	Di	*Poecile atricapillus*	Otter et al. 1998	Yes
	R: TAGTGAATGCTTGATTTCTTTG	(1347-195)			Reudink et al. 2005, 2007	
					Johannessen et al. 2011	
Pmo39	F: CATGTATTTTCCAAAAGTAAATAT	216-232	Tetra	*Parus monticolus*	Wang et al. 2005	Yes
(Titgata39)	R: CTGCTATTCTGCAAACTTGTGG	(210-216)			Slattery 2008	
Pmo67	F: AACCAGTGATTCCCTGCAAA	296-352	Tetra	*Parus monticolus*	Wang et al. 2005	Yes
(Titgata67)	R: ACAGGTATATTTTGAGTGCCATAT	(279-343)			Slattery 2008	

Appendix F: continued

Locus Name	Primer Sequence (5->3)	Allele size (range)*	Repeat size	Original species	Studies utilized	Successful
Pmo02	F: ATTGCTTGATATTTGAAAGCATA	212-232	Tetra	*Parus monticolus*	Wang et al. 2005	No
(Titgata02)	R: TTGTCTTTTGGGTTGCCTGA	(244-310)			Slattery 2008	(null alleles)
Pma86	F: AAAACAAGGCCACTTAGAGCTG	196	Tetra	*Parus major*	Saladin et al. 2003	No
(PmaTAGAn86)	R: ACTCCTCCAGGTCACACAGG	(124-158)				(null alleles)
Pma28	F: GTTGGTGCAGCGGTCTACTC	199	Di	*Parus major*	Saladin et al. 2003	No
(PmaGAn28)	R: CATGTTGGGACAGCAGTTTG	(212-250)				(null alleles)
Pma42	F: ACTTCCACATGCCAGTTTCC	285	Tetra	*Parus major*	Saladin et al. 2003	No
(PmaTGAn42)	R: TGTTAAGGCAGAGAGGTGGG	(284-348)				(null alleles)
Pma11	F: GCTTCTGCCTCCATTAAGAGTC	105	Di	*Parus major*	Saladin et al. 2003	No
(PmaGAn11)	R: GAAAAATCACCCACTCAGCC					
Pma27	F: TATAAACCACAGCCACACGC	202	Tri	*Parus major*	Saladin et al. 2003	No
(PmaGAn27)	R: CACAACCACAGAGGCATGAG				Johannessen et al. 2011	
Pma31	F: TGTTCTAATATGGAAGCAAGGG	88	Di	*Parus major*	Saladin et al. 2003	No
(PmaGAn31)	R: TCATGCCAGAGAAAGCTGTG					
Pma40	F: CGTTCCTCCTTTGCTTTCTG	416	Di	*Parus major*	Saladin et al. 2003	No
(PmaGAn40)	R: AATGGCACAACACCTTCTCC				Johannessen et al. 2011	
Pma48	F: CACTCAGCCTCTCAGATCTG	192	Di	*Parus major*	Kawano 2003	No
(Pma48m)	R: CGGGCTGGTACTTATTGGGAG					
Pma49	F: CAGGAACACCCAAACCCAG	255	Di	*Parus major*	Kawano 2003	No
(Pma49m)	R: GCTGGGTGGTGAATGTGAGGG					
Pma130	F: TGAGTGGAAAGATGCTGGC	438	Tetra	*Parus major*	Saladin et al. 2003	No
(PmaD130)	R: CCCTATAAAAACCGAGGCTG					
Pma303	F: CCCACAGCAATCTCCCTCCA	159	Di	*Parus major*	Kawano 2003	No
	R: GGTGGCTTTTCTCTGCACAC					
Pca1	F: GATCGCTGTGCTCCTTGTCAG	125	Tri	*Parus caeruleus*	Dawson et al. 2000	No
	R: CTGGCCCATTTTGCTGTGC					

Appendix F: continued

Locus Name	Primer Sequence (5->3)	Allele size (range)*	Repeat size	Original species	Studies utilized	Successful
Pca2	F: GTTGGCCTTCTTGGCCCC R: TGTTGGAGGTTAGGAGGCCTCT	291	Tetra	*Parus caeruleus*	Dawson et al. 2000 Reudink et al. 2005, 2007	No
Pca5	F: TTGGCTGGGAGCAGAGCTG R: CCAGCCTGTCCTCAGCAGC	132	Di	*Parus caeruleus*	Dawson et al. 2000	No
Pat2-43	F: ACAGGTAGTCAGAAATGGAAAG R: GTATCCAGAGTCTTTGCTGATG	n/a	Di	*Poecile atricapillus*	Otter et al. 1998 Reudink et al. 2005, 2007	No
Pmo84 (Titgata84)	F: GCAAGGCTAGCCATTAAAAG R: GTGGCATACAAAATCTTCTGC	575-591	Tetra	*Parus monticolus*	Wang et al. 2005 Slattery 2008	No
Pmo88 (Titgata88)	F: CCAGCATGTTTCATTTAAGAA R: ACAAAGGGAGATATGGGCAG	172-242	Tetra	*Parus monticolus*	Wang et al. 2005 Slattery 2008	No
Pmo94 (Titgata94)	F: TCAAGAGGGCAACCAGCCTG R: TGCTTTGGCTTAAAATATACACAGT	317-333	Tetra	*Parus monticolus*	Wang et al. 2005 Slattery 2008	No

Appendix G Sample size (n), mean (x), standard deviation (sd) values for juvenile and adult Titmice within each transect region a) west of contact zone, b) contact zone, c) east. Analysis of variance results (comparison) showing degrees of freedom (d.f.), the F-ratio (F), r-squared value (r^2), and P-value. Significance represented by bold P-value. Alpha set at 0.05.

a) West

	Juveniles			Adults			Comparison			
	n	x	sd	n	x	sd	d.f.	F	adj R^2	P-value
Bill	10	11.9	0.44	23	12.0	0.54	1	0.23	-0.02	0.632
Wing	10	24.0	2.17	24	1.9	1.87	1	5.37	0.12	**0.027**
Tarsus	10	20.3	1.21	23	20.4	0.75	1	0.21	-0.02	0.647
Tail	10	70.8	3.33	24	73.4	3.13	1	4.76	0.10	**0.037**

b) Contact zone

	Juveniles			Adults			Comparison			
	n	x	sd	n	x	sd	d.f.	F	adj R^2	P-value
Bill	15	11.94	0.61	16	12.27	0.38	1	3.38	0.07	0.076
Wing	15	72.6	2.79	16	73.6	2.94	1	0.93	0.00	0.343
Tarsus	15	20.3	0.75	16	21.0	0.82	1	5.96	0.14	**0.021**
Tail	15	71.9	3.73	15	73.7	4.13	1	1.57	0.02	0.221

c) East

	Juveniles			Adults			Comparison			
	n	x	sd	n	x	sd	d.f.	F	adj R^2	P-value
Bill	22	12.4	0.57	33	12.7	0.49	1	2.04	0.02	0.159
Wing	22	76.0	2.80	34	77.3	2.54	1	3.29	0.04	0.752
Tarsus	22	20.5	0.91	34	20.8	0.87	1	1.38	0.00	0.291
Tail	22	72.7	4.61	33	76.5	5.29	1	7.43	0.11	**0.009**

Appendix H Sample size (n), mean (x), standard deviation (sd) values for juvenile and adult Titmice within each transect region a) west of contact zone, b) contact zone, c) east. Analysis of variance results (comparison) showing degrees of freedom (d.f.), the F-ratio (F), r-squared value (r^2), and P-value. Significance represented by bold P-value. Alpha set at 0.05.

a) West

	Females			Males			Comparison			
	n	x	sd	n	x	sd	d.f.	F	adj R^2	P-value
Bill	8	11.9	0.52	14	12.0	0.58	1	0.14	-0.04	0.710
Wing	8	72.2	1.42	15	74.6	1.54	1	13.48	0.36	**0.001**
Tarsus	7	20.3	0.72	15	20.6	0.75	1	1.04	0.00	0.319
Tail	8	73.1	1.99	15	73.3	3.49	1	0.02	-0.04	0.881

b) Contact zone

	Females			Males			Comparison			
	n	x	sd	n	x	sd	d.f.	F	adj R^2	P-value
Bill	4	12.0	0.18	12	12.3	0.11	1	1.99	0.06	0.180
Wing	4	70.8	1.50	12	74.5	2.71	1	6.73	0.28	**0.021**
Tarsus	4	20.4	0.72	12	21.2	0.76	1	3.89	0.16	0.069
Tail	4	71.6	1.03	11	74.5	4.59	1	1.47	0.03	0.247

c) East

	Females			Males			Comparison			
	n	x	sd	n	x	sd	d.f.	F	adj R^2	p-value
Bill	4	12.3	0.37	27	12.7	0.48	1	3.00	0.06	0.094
Wing	4	74.6	2.32	28	77.8	2.40	1	6.13	0.14	**0.019**
Tarsus	4	20.1	0.59	28	21.0	0.84	1	3.96	0.09	0.056
Tail	3	75.8	5.51	27	76.5	5.21	1	0.04	-0.03	0.836