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THE EVOLUTION OF REPRODUCTIVE ISOLATION IN A TEMPORALLY COMPLEX PASSERINE HYBRID ZONE

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THE EVOLUTION OF REPRODUCTIVE ISOLATION IN A TEMPORALLY COMPLEX PASSERINE HYBRID ZONE

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF BIOLOGY

BY

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Dedication

To the birds:

Color-banded titmice,
With vengeance on their minds,
Are coming out to get me;
I've wronged against their kind.
They don't like to be caught
Or have blood samples taken.
So I'd best now sleep lightly
Or I might never waken.

And to all of my favorite people (you know who you are) and

to all of my favorite non-human mammals (if you could read you'd know who you are).

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Abstract

Understanding the relative importance of reproductive isolating barriers between populations allows us learn what processes are most prevalent in causing speciation. Hybrid zones, where distinguishable populations interbreed, are particularly good systems in which to study how isolating barriers evolve because of the interaction between populations with incomplete reproductive isolation. Examining a hybrid zone over time or with contacts of different ages allows us to sort out which comes first—selection against hybrids, innate preferences for hybrid or parental types, or if one barrier type additionally evolves as a result of the other. One such temporally complex hybrid zone is that of two oscine songbirds, the Black-crested (*Baeolophus atricristatus*) and Tufted (*B. bicolor*) Titmice (family Paridae) in the southern Great Plains of North America; they differ in song, plumage, and genetics. In Texas, the two populations have been interbreeding for several thousands of years across a natural ecotone, while in Oklahoma the two species have contacted within the past century. Few studies examine multiple contacts within one species complex to compare how selection has changed their extent and behavioral interactions over time. I first discuss general patterns of selection in hybrid zones and then examine in titmice (1) how morphology and plumage change across the hybrid zone in the younger and older regions; (2) patterns of and potential mechanisms causing song variation; (3) genetic introgression and genetic signatures of recent range expansion; and (4) sexual selection on males and females and reproductive fitness of hybrids. My data suggest that ongoing interactions have resulted in a stable older zone where parental species prefer conspecific song and plumage and a younger zone with the potential for continuing

introgression of plumage and genes, as there are currently few preferences by males or females for song or plumage. These data best match a tension zone model (selection against hybrids), but intrinsic postmating isolation appears to be absent even in the older part of this system. Future studies should focus on potential ecological or behavioral post-mating isolation barriers that prevent the region of hybridization from spreading and that could cause the younger zone to evolve increased pre-mating isolation barriers such as those found in the older zone.

Chapter 1: Hybridization across space and time

Abstract

Hybridization, where distinguishable populations meet and interbreed, is useful for understanding the mechanisms involved in the evolution of reproductive isolation. Hybrid zones can range from interactions with near-complete reproductive isolation to zones that are stable in extent for long periods of time. Several models exist to explain how stable hybrid zones are maintained, but some zones fit more than one model, and these models do not emphasize moving hybrid zones or occasional hybridization events that add genetic variation to the parental populations. I propose that hybridization, instead of being fitted to these discrete models, instead be thought of as a continuum of selection pressures across geography. Hybrid zones produce a range of genotypes with a range of fitnesses; with genotype-by-environment interactions well-known, one expects hybrid fitness to vary by location. As such, hybrids that are fit within the zone may be unfit outside it or vice versa. The stage of speciation at which the populations have contacted, whether early with few genetic incompatibilities and only ecological or behavioral differences, or strongly diverged with intrinsic hybrid unfitness, also affects where along the selection pressure continuum a given interaction will fall. Each of the current hybrid zone models assumes a particular fitness within a part of the hybrid zone (within it or outside of it). These models are thus special cases along a continuum of selection pressures on hybrids within and outside of the hybrid zone.

Introduction

Hybrid zones, areas where populations with distinguishable, heritable differences meet and interbreed, are important systems (Barton and Hewitt 1985, 1989;

Arnold 1997) in which to study speciation. These zones contain ongoing interactions between populations with varying degrees of incomplete reproductive isolation whether at secondary or primary contact (as for parapatric or sympatric speciation). Thus hybridization, which can range from rare interbreeding that results in sterile or infertile offspring to extensive but stable zones of integradation (Arnold 1997) to complete intergradation of populations (Kleindorfer et al. 2014), provides the opportunity examine how populations at different stages of speciation interact. Extensive work in these systems has resulted in considerable knowledge about the types of isolating barriers that exist or evolve in the face of gene flow (Abbott et al. 2013). Such barriers include male-male competition (Rosenfield and Kodric-Brown 2003), mate preferences (Hughes et al. 2011; Merrill et al. 2011; Hood et al. 2012), intrinsic incompatibilities resulting in infertility or inviability (Brothers and Delph 2010; Bracewell et al. 2011), ecological adaptation (Raeymaekers et al. 2010; Rodríguez-Gómez et al. 2013), and combinations of these factors (Moriarty Lemmon and Lemmon 2010). Four models (Table 1) have been developed to describe mechanisms that maintain hybrid zones that are stable in extent over time. Selection can act against hybrids (tension zone), can vary with the environment and the patchiness of the habitat (mosaic), can favor hybrids based on environmental adaptation but only within the zone itself (bounded hybrid superiority), or can favor only hybrids with certain genotypes or in certain environments (evolutionary novelty).

The tension zone model (Barton and Hewitt 1985, 1989) is one of the most widely invoked models (Arnold 1997). It assumes a balance between intrinsic selection against hybrids and dispersal of parental individuals into the hybrid zone. This model

assumes intrinsic hybrid unfitness, i.e., fitness of hybrids is independent of the environment, regardless of whether low fitness of hybrids is a result of premating or postmating isolating barriers. One potential outcome of a tension zone is reinforcement, selection for increased reproductive isolation between sympatric taxa as a result of hybrid unfitness, whether pre- or postmating (Howard 1993; Noor 1999; Marshall et al. 2002; Coyne and Orr 2004; Shaw and Mendelson 2013). In the past, animal hybrids have been assumed to be unfit (Harrison, 1993), as in the tension zone model, although disadvantages to hybridization can differ for each parental species (Howard et al. 1993). Hybrid fitness is now recognized to vary in space and over time in some systems (Grant and Grant 2002; Arnold and Martin 2010; Marques et al. 2011), which better fits with models that involve environmental selection on hybrids. In the tension zone model, fitness is independent of the environment.

Three additional models involve environmental-based selection. The bounded hybrid superiority model (Moore 1977) posits extrinsic (environmental) selection for hybrids but only within intermediate habitat found in the hybrid zone, preventing the outward spread of hybrid phenotypes and genotypes. As their name implies relative to a clinal hybrid zone, a mosaic hybrid zone (Rand and Harrison 1989; Howard et al. 1993) shows a patchwork of contacts, each occurring where environmental conditions favor hybridization. The evolutionary novelty hypothesis (Arnold 1997) describes how the meeting of divergent taxa may result in the exchange of genes between them, resulting in offspring with diverse genotypes, some of which experience positive selection and others negative. Those offspring under positive selection can result in hybrid speciation or in transfer of adaptive genetic variation among species (Grant and Grant 2002;

Lepais and Gerber 2011). Historically, plant examples for both polyploid and homoploid speciation have been most well-known (Arnold 1997; Rieseberg et al. 2003), but hybrid speciation has been invoked for several animals (Schwarz et al. 2005; Mavárez and Linares 2008; Brelsford et al. 2011; Elgvin et al. 2011; Hermansen et al. 2011; Kunte et al. 2011), although some examples have been controversial (Wayne and Jenks 1991; Wilson et al. 2012).

Each model, then, assumes a particular type of selection on hybrids, but even well-studied zones may be controversial with respect to which models they best fit (see Arnold 1997 for several examples), whereas other studies recognize that their system may be consistent with multiple models (Arntzen and Wallis 1991; Bert and Arnold 1995). Yet other zones do not fit as easily within any of these models. For example, whereas the tension zone model allows for movement of zone extent, most studies focus on stable zones (but see Dasmahapatra et al. 2002; Kawakami et al. 2009). One of the models, evolutionary novelty, is a special case that does not apply to those zones where hybrids are selected against (Davies et al. 1997; Dasmahapatra et al. 2002). Some instances of hybridization result in only occasional hybrids (Grant 1993)—i.e., most mating is assortative—but do not form either a typical geographically narrow zone or a mosaic zone. Clinal (tension zone and bounded hybrid superiority) and mosaic models specify different geographic arrangements of the meeting populations (as in Marshall et al. 2002), but the difference between the two classes of model is a matter of environmental grain size (see Figure 1 here and fig. 3 in Rand and Harrison 1989). In the mosaic model, clines simply occur within patches of each mosaic "tile" instead of

across the whole landscape. In clinal models, the transition between the two populations occurs at a landscape scale.

Because the models overlap in the assumed types of selection, and many zones match more than one model (depending on the trait examined), we can find that selection on hybrids is not relegated to distinct categories but is a continuum from strong to weak selection against hybrids to neutrality (resulting in a potentially slow spread of the hybrids) to weak or strong selection for hybrids (the latter resulting in hybrid speciation or merging of the species, depending on the geographic extent and nature of the selection for hybrids). Indeed, more than one selective pressure can act in a single zone (Barton and Hewitt 1989) and on different phases of the life cycle. However, the direction of the selection (i.e., for or against hybrids, regardless of whether selection on hybrids is intrinsic or extrinsic, as specified within the current models) only places a particular hybrid zone along the proposed continuum. Even reinforcement is a special case of sexual selection against hybrids (Shaw and Mendelson 2013) and as such does not require intrinsic hybrid unfitness in viability or sterility; unfitness can be premating and behavioral. Such a gradient between strong selection for or against hybrids thus suggests that each hybrid zone model is a special case of an overall continuum of selection on hybrids.

I propose that the study of hybridization will be aided by explicitly considering the geographic context of selection in all types of hybrid zones. Integrating these models on the basis of the geographic and temporal range of selection pressure experienced by hybridizing taxa and their offspring (Figure 2) will aid in our understanding of hybrid zones. Integrating the four models requires us to explicitly

consider what assumptions are being made about any given hybrid zone and the consequences of its place along the continuum of reproductive isolation from little isolation to almost complete isolation (Butlin et al. 2008; Mallet 2008). The overwhelming majority of studies focus on hybrid zones as clines (Barton and Hewitt 1985). We will gain the most value from our studies of hybridization if we realize that hybrid fitness can change across a landscape—not just in a clinal pattern but heterogenously as well—and its involvement in hybrid speciation, reinforcement, or merging of populations will vary accordingly.

Geographic context for selection

Hybridization by its very nature involves an overlap or contact in space between populations that are differentiated in some way, often by phenotype. Current models explain the stability and shape of hybrid zones but differ in the assumed fitness of hybrids and, where specified (not all models do so), the geographic form of the hybrid zone. However, hybrid fitness is not usually placed in an explicit geographic context. Hybrids are generally assumed to be fit or unfit in particular traits regardless of geographic location, although efforts are now made towards specifying the geographic landscape of fitness for hybrids (Bert and Arnold 1995; Abbott et al. 2013). This assumption is starting to change; for example, in *Drosophila* flies reinforcement has depends on the degree of sympatry (Nosil 2013). Because sexual selection can have a genotype-by-environment context (Ingleby et al. 2010; Narraway et al. 2010), one would expect fitness of hybrids to vary by environment.

Indeed, hybrid zones possess a range of genotypes (Arnold 1997; Senn et al. 2010) on which selection may act differentially (Burke et al. 1998). Some genotypes

may disperse to or be better adapted to new environments (Rieseberg et al. 2003). Studies of plants in the context of the evolutionary novelty concept have addressed this aspect of geography more explicitly (Donovan et al. 2010), particularly with regard to the potential need for escape from parental interactions for hybrid speciation to occur (Buerkle et al. 2000). Animal studies have not explicitly addressed this geographic context, likely because of the increased difficulty in creating controlled ranges of crosses (but see Martin and Wainwright 2013). The spatial scale at which heterogeneity in the environment causes occasional contact zones—typically between sister species putatively in secondary contact—is also important and is only specified by the mosaic model. Within small scale patches where hybridization occurs in the mosaic model, the dynamics can be described as for clinal tension zones (Rand and Harrison 1989; Arntzen and Wallis 1991), although other mosaic zones may be better described within the patches by the bounded hybrid superiority model (Cruzan and Arnold 1993). This geographic dependence means that interactions can be change (in genes exchanged, for example) at different regions of contact (Nolte et al. 2009; Davidson et al. 2013).

Balance of selection and dispersal in all types of hybrid zones

The geography of selection will interact with dispersal of hybrids and parental individuals to determine the width of the zone, as in a tension zone, but the scale will be taxon-dependent (Kisel and Barraclough 2010). Nevertheless, even a bounded hybrid superiority zone, which is currently considered "dispersal independent" (Barton and Hewitt 1985), depends on selection balancing dispersal at the edges of the zone. Hybrid genes would continue to introgress into one or both parental populations (Buggs 2007) if not for the extrinsic selection against hybrids outside the zone (Moore 1977). Parental

individuals disperse into a tension zone, but there is no a priori reason why viable hybrids should not disperse out of a zone as well. When inviability or sterility occurs, hybrid offspring may not survive to disperse or the heterogametic sex may suffer disproportionate problems, as encapsulated by Haldane's rule (Coyne and Orr 2004). Once viable hybrids disperse into a population outside the zone, selection continues to act on survival and reproduction, with high-fitness genotypes spreading (Martin and Cruzan 1999). Additionally, changes in genetic variance in a population should affect the evolution of niche width and could allow expansion or contraction of the niche available to hybrids (Aguilée et al. 2013). Dispersal (colonization of patches) followed by selection maintains mosaic hybrid zones as well (Rand and Harrison 1989).

The models in the preceding paragraph generally focus on stable hybrid zones. Differential dispersal (or fitness) can also result in moving zones (Buggs 2007). Detection of zone movement can be difficult without repeated sampling (Krosby and Rohwer 2010), so determination of movements is sometimes based on genetic signatures of introgression (Martin and Cruzan 1999). Many investigators are re-sampling zones studied in the past (Blum 2002; Brelsford and Irwin 2009; Mettler and Spellman 2009; Krosby and Rohwer 2010; Senn et al. 2010; Carling and Zuckerberg 2011; Smith et al. 2013; Curry and Patten 2014), allowing for better evaluations of when, how often, and how far hybrid zones move. Zones can change in many permutations, varying from introgression of single traits (Parsons et al. 1993; Brumfield et al. 2001) to shifts that entail replacement of one species (Krosby and Rohwer 2009, 2010). Hybrid zones where both species are common species can also shift their zones short (Blum 2002; Carling and Zuckerberg 2011; Smith et al. 2013). A more common

taxon may swamp a rarer taxon (Patten and Campbell 2000; Perry et al. 2001) or even a previously widespread taxon (Vallender et al. 2007). One might assume that the hybrids or the more common species is more fit in that case, but in principle population pressure from a more common species can shift the zone in the direction of the rarer species even when hybrids are selected against (Barton 1992). Hybrids with a selective advantage under at least some conditions will also spread (Ryan et al. 2013), assuming suitable conditions for the hybrid exist away from the hybrid zone. Either situation is of conservation concern (Rhymer and Simberloff 1996). In addition to movement caused by changes in local population structure (Barton and Hewitt 1989), movement can be precipitated by environmental change (Kohlmann and Shaw 1991; Blum 2002) or with introduction of invasive species (Perry et al. 2002; Fitzpatrick and Shaffer 2004; Ryan et al. 2013). Additionally, hybridization can promote natural invasions or dispersal (Potts and Reid 1988; Petit et al. 2004).

Even if a hybrid zone does not move because of differential dispersal or population pressure, selection on hybrids can change over time within a particular region (Grant and Grant 2002). When a stable hybrid zone does not form, occasional introgression can add genetic diversity to parental populations (Grant and Grant 1994). Selection for or against hybrids can vary over a life cycle (Howard et al. 1993; Moriarty Lemmon and Lemmon 2010), although in such cases the net fitness loss or gain is what matters even if survival is elevated or depressed at a particular life stage. If overall fitness is disproportionately influenced by different life stages in different breeding seasons because of environmental variation (Pfennig 2007), temporal variation in

hybrid fitness could result. Such scenarios can cause hybrid zones to move irregularly (Roy et al. 2012) and fluctuate in genetic composition (Shaw et al. 1985).

Integration of hybrid zone models with speciation timelines

Ecological and sexual selection, whether in sympatry or allopatry, can drive populations to diverge and even speciate. The extent and cause of divergence (natural selection, sexual selection, or drift) and the ecological interactions of hybrids with the environment should be the focus of hybrid zone studies, akin to how speciation studies (ought to) have moved beyond simply whether speciation was allopatric or sympatric and more on the processes involved in divergence (Butlin et al. 2008; Fitzpatrick et al. 2009; Mallet et al. 2009). Namely, how does the degree of divergence before secondary contact affect the strength of selection on hybrids at secondary contact? Does the degree of divergence affect which isolating mechanisms stabilize or shift hybrid zones? The strength and direction of selection on hybrids may correlate with time since divergence (Merrill et al. 2011), just selection can with genetic distance (Bracewell et al. 2011). Early sympatric speciation may involve an ecological barrier and a few genes with ecological effects that create a disadvantage for hybrids (Feder 1998), whereas later stages might have more extensive genetic incompatibilities. If these differences between young and old species occur, then a region where hybrids are selected for (resulting in situations defined as bounded hybrid superiority zones) will be more likely to occur in species pairs that are more closely related and have only slight ecological differences (i.e., "niche conservatism"), regardless of whether divergence occurred in allopatry or sympatry. Selection strength in such cases will depend on the nature of ecological differences and divergence of intermediate habitat from the habitat found for either

parental species, as ecological divergence can vary but will still likely occur before strong genetic incompatibilities evolve. Where ecological selection is weak, other factors such as sexual selection may play a role in maintaining isolation. Or the two populations will merge (as in Kleindorfer et al. 2014). The fast-developing field of genomics will be useful to determine which isolating genes evolved first and where they evolved in the genome (Feder 1998; Rice et al. 2011; Ellegren et al. 2012; Parchman et al. 2013), aiding both our immediate understanding of how hybrid zones are maintained and how reproductive isolation, and thus speciation, both initiates and progresses (Stelkens et al. 2010). Applying these techniques to temporal replicates of the same hybrid zone by repeated sampling over time or in zones with young and old regions (e.g., Sætre et al. 1999; Curry and Patten 2014), or differing conditions of contact in more than one region (Nolte et al. 2009) may provide additional insights into contingencies that may alter the predicted progression of speciation.

In addition to considering the stage of speciation at which hybrid zones form, we should forecast the effects hybridization will have on speciation given the strength and direction of selection on hybrids. Hybridization can have three effects on the completion of reproductive isolation (i.e.,progress towards speciation because the two interacting populations are not currently completely isolated): enhance (reinforcement or hybrid speciation), impede (gene flow), or no effect (a stable hybrid zone) (Abbott et al. 2013; Shaw and Mendelson 2013). If selection against hybrids is strong enough (and against hybridization specifically), reproductive isolation can be increased by the process of reinforcement. If hybrids are favored enough and become isolated (temporally, behaviorally, or spatially) from parental species, a new hybrid species

forms. If selection favors hybrids in at least some regions, the hybrid zone expands either directionally (potentially swamping one species) or in both directions (merging the two species). Directional movement may add another combined category, currently restricted to plants, of enhance or no effect, resulting in range expansion with both parental species surviving after gene flow (Potts and Reid 1988; Petit et al. 2004). If merging of the two populations is prevented by a balance of selection and dispersal, there is no effect on speciation or a potentially positive effect of increasing genetic variation in one or both of the parental populations.

Future directions

Each of the current hybrid zones models is a special case along a continuum of selection pressures in varying geographic contexts. Although this means that the fate of hybrid zones cannot be predicted without detailed knowledge of conditions in a particular zone (Björklund 2013), it does not take away the importance of placing each zone into a larger framework. Currently, many studies discuss the "fitness" and "unfitness" of hybrids as if fitness was not context dependent (but see Abbott et al. 2013), both in terms of genetic background and environmental setting. Hybrids with severe genetic incompatibilities will be unfit in all contexts, but even then the severity of genetic incompatibilities may vary with the specific hybrid genotype (Taylor et al. 2009). Likewise, ecological and sexual selection often varies with environmental context (Seehausen et al. 1997, 2008; Grant and Grant 2002; Narraway et al. 2010). A stronger focus on the strength, direction, and specifically the geography of selection is needed to better understand how reproductive isolation evolves in these systems. For example, when multiple traits are involved in reproductive isolation (Labonne and

Hendry 2010), do they have similar directional effects (toward reduction or increase of isolation) or do they conflict? And how do these effects vary with the environment?

Laboratory experiments can be useful to determine specific mechanisms that may drive interactions between populations (Powell et al. 2012), but a laboratory setting lacks the multiple selective pressures of the natural environment. Experiments in a natural setting may better address mechanisms that stabilize hybrid zones, although it can be logistically difficult to move hybrids and parentals into and out of the appropriate habitats. Plants are easier to move and as such are better-studied, but animal examples are beginning to appear. For example, a range of hybrid fishes were used to test how the adaptive landscape drives adaptive radiations in fish (Martin and Wainwright 2013). Although Martin and Wainwright (2013) discussed how disruptive selection could drive adaptive radiation, their study also illustrated how hybrids could diversify into multiple lineages. Additional studies should be done on the potential fates of dispersing parental and hybrid individuals to understand better why zones expand or are prevented from doing so. For example, presenting both parentals and hybrids in mate choice tests, which is logistically difficult in many systems (like birds), allows some idea of what can prevent expansion of hybrid zones where hybrids have no intrinsic unfitness. Above I mentioned how hybrids, if viable, should disperse like parents, but changes in dispersal based on the genetic makeup of hybrids should also be studied in more detail (Edelaar and Bolnick 2012).

We currently use concordance of clines to see if traits and clines match (Barton and Hewitt 1985; Gay et al. 2008), but not all traits may match in the pattern and direction of selection that they experience. Selection may be in favor of one population's

traits and against the other; neutral for some traits but selected for others; or in favor of one population for some traits but vice versa for another set of traits. We estimate selection (Szymura and Barton 1986; Moore and Price 1993; Brelsford and Irwin 2009), but examining gene clines in the context of overall genomic introgression also provides additional, more detailed information on the type and direction of selection (Parsons et al. 1993; Parchman et al. 2013). Some work has been done with ecological speciation potential in guppies (Labonne and Hendry 2010) as a model, where sexual and natural selection pressures are in different directions. Different life stages can also have differing fitness (Moriarty Lemmon and Lemmon 2010). More empirical work in natural contact zones is crucial to understanding the relative influence of traits on overall isolation (Ramsey et al. 2003). Genetic work on the "genomic islands" (Ellegren et al. 2012) or genes (Feder 1998) underlying said traits is important to understand the patterns of selection that shape reproductive isolation between the hybridizing populations.

Language such as "incipient species" implies a particular outcome (Butlin et al. 2008) for populations in which data are limited. We do not have time machines that will allow us to see the fate of an individual hybrid zone thousands of generations in the future. However, explicit tests are important to validate our theory and models; these are possible with carefully designed experiments (Moriarty Lemmon and Lemmon 2010; Martin and Wainwright 2013) and study systems where comparisons over time are possible. Zones for which we have historical (Mettler and Spellman 2009; Carling and Zuckerberg 2011; Smith et al. 2013; Curry and Patten 2014) and long-term (Grant and Grant 2002) data are particularly useful to see how well they fit with predictions of

competing models. Such studies are becoming more numerous. We should be able to predict the fate of individual zones under the assumption that current conditions continue and once we know which selective pressures act, be they mate choice, intrinsic hybrid inviability, or environmental factors. Recently formed zones are especially useful as we can see if they change as predicted under our current estimates of selection. Being able to make these predictions will become increasingly important with invasive species (Rhymer and Simberloff 1996; Allendorf et al. 2001; Perry et al. 2002) and climate change bringing increasing numbers of formerly isolated populations into secondary contact (Barton and Hewitt 1985, 1989; Arnold 1997).

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Figures and tables

Table 1. Framework and assumptions of four current hybrid zone models (see Arnold 1997 and citations below).

Model	Evolutionary novelty	Mosaic	Bounded hybrid superiority	Tension zone
Selection on hybrids	For certain genotypes and/or in certain	Against or for	For, in zone	Against
Environmental dependence	Yes	Yes	Yes	No
Dispersal dependence	Not required	Colonization only	No	Yes
Citations	(Arnold 1997)	(Rand and Harrison,1989; Howard et al. 1993)	(Moore 1977)	(Barton and Hewitt 1985, 1989)

Figure 1. Mosaic zones are characterized by a patchwork of contacts in which hybridization is favored by environmental conditions. The zones (gray) of contact between two species (shaded black and white) vary by the environmental conditions in each patch, but within each patch hybridization will be governed by selection pressures as found in Figure 2. (A) shows a typical continuous hybrid zone. (B) shows a fragmented hybrid zone where environmental conditions result in similar gradients between the two species in both habitat patches. (C) shows a typical mosaic hybrid zone. The difference between the three is a matter of spatial scale and heterogeneity in contact conditions.

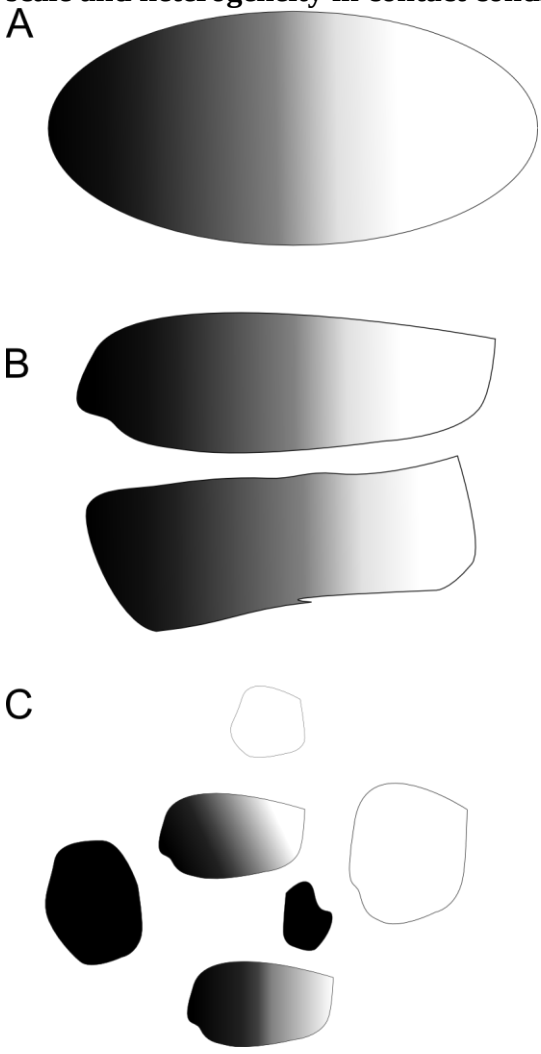
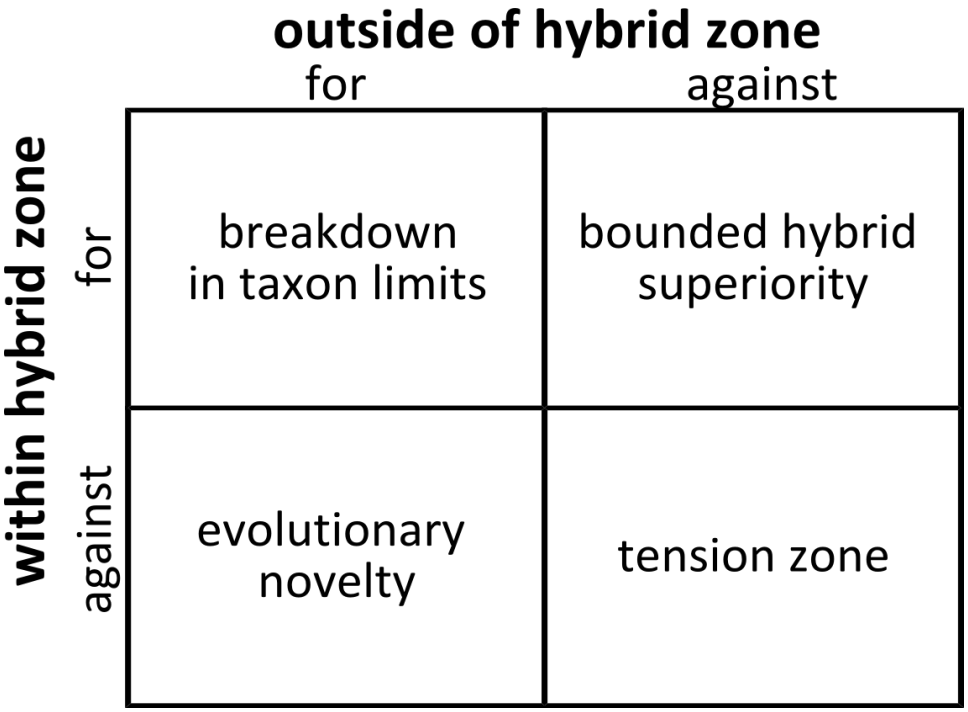


Figure 2. Categories of hybridization with names and spatially explicit definitions of current models. Selection on hybrids may be of any type (premating, postmating, extrinsic, or intrinsic). The important part for this categorization is simply whether selection favors or acts against hybrids in a particular geographic region. The mosaic zone is a special case defined by geography of scale rather than by direction of selection on hybrids.



Chapter 2: Current and historical extent of phenotypic variation in the Tufted and Black-crested Titmouse (Paridae) hybrid zone in the southern Great Plains

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Abstract

Hybrid zones, where phenotypically distinct populations interbreed, should expand or contract until reaching a balance between selection and dispersal. Few studies examine multiple contacts within one species complex to compare how their extent changes over time. Black-crested and Tufted Titmice (*Baeolophus atricristatus* and *B. bicolor*) hybridize extensively within a narrow zone in Texas and southwestern Oklahoma. In Texas, hybridization has been occurring for several thousands of years, while evidence suggests the southwestern Oklahoma contact is more recent, beginning within the past century. We quantify plumage and morphology of the two species across both the younger and older hybrid zones and compare the current and historical extent of phenotypic variation in the older Texas contact with that in the younger Oklahoma contact. Variation in plumage between species is similar in the younger and older contacts, while overlap in morphological characters is broader in the older contact. Recently and historically surveyed transects in the older zone have similar cline widths, indicating selection, at least on crest and forehead plumage, has reached equilibrium with dispersal over the time periods involved (comparing both the historically surveyed data from 1955 vs. the recently surveyed data from the 2000s in Texas). In the recently

surveyed younger Oklahoma contact, cline width is narrower, indicating potential for expansion if it follows the course of the older contact. This temporal complexity should make this species complex a productive system for future work, using plumage and additional traits such as song and genetics, on the relative influences of both natural and sexual selection on the evolution of reproductive isolation.

Introduction

Hybrid zones form where phenotypically distinct populations meet and interbreed at secondary contact (Moore 1977). These populations, which are partially reproductively isolated, provide important tests of the relative contributions of natural and sexual selection to speciation (Labonne and Hendry 2010; Moriarty Lemmon and Lemmon 2010). Hybrid zones are most commonly maintained by a balance of selection for or against, hybrids (Moore 1977; Barton and Hewitt 1985, 1989; Arnold 1997). These conditions often result in a zone that is narrow when compared to the parental ranges. Studying hybrid zones of differing ages is useful to understand what isolating barriers arise as speciation progresses. Zones with repeated sampling over time (Reudink et al. 2007; Brelsford and Irwin 2009; Mettler and Spellman 2009; Carling and Zuckerberg 2011; Roy et al. 2012; Smith et al. 2013) or containing contacts of different ages (Haavie et al. 2004; Rohwer and Martin 2007) are particularly enlightening because we can examine selective forces over time within the same species complex.

When sampling transects of differing ages and, for the moment ignoring the possibility of movement, four patterns of variation in hybrid zone width are possible (Table 2). First, dispersal and selection (Moore 1977; Barton and Hewitt 1985) have

reached equilibrium, resulting in stable clines of similar widths at each transect or time period (Moore and Buchanan 1985; Smith et al. 2013). Second, expansion in width of transects over time is expected when ongoing dispersal and introgression have not been balanced by selection either against hybrids within the zone or against hybrids dispersing out of suitable intermediate habitat. Mechanisms for such a pattern might include sexual selection on hybrids ((Rosenfield and Kodric-Brown 2003) and ecological adaptation (Donovan et al. 2010), both of which could allow the spread of hybrids. Neutral gene flow could also allow transects to continue widening over time (Table 2; scenario 2a). Third, narrowing of a zone over time should occur when the balance of dispersal and introgression has shifted to reduce the suitable area for hybridization. Hybrid unfitness can result from phenomena such as genetic incompatibilities resulting in inviability (Barton and Hewitt 1981) or reduced fertility (Alatalo et al. 1990), ecological disadvantages of hybrids (Tobler et al. 2009), preference for parental phenotypes (Merrill et al. 2011), or a combination of multiple factors (Patten et al. 2004). Hybrid disadvantages can then cause narrowing of the zone (Carling and Zuckerberg 2011) by shifts in population density (Barton and Hewitt 1989), reinforcement (Howard 1993), or shifts in environmental regimes (Swenson 2006). Forth, a lack of consistent temporal pattern is expected to occur when selection pressures have changed direction over time (Roy et al. 2012). Such a pattern could be caused by large-scale changes (anthropogenic or natural) within or between regions in habitat (Grant and Grant 2002; Arnold and Martin 2010) or in population density (Barton and Hewitt 1989). Additionally, a zone can move while remaining the same

width (Smith et al. 2013); this is expected to occur with shifts in population density (Barton and Hewitt 1989).

Many avian hybrid zones have demonstrated intrinsic selection against hybrids (Sætre et al. 1999; Bronson et al. 2003; Haavie et al. 2004), extrinsic selection for hybrids (Flockhart and Wiebe 2008), or a combination of both (Good et al. 2000; Gay et al. 2008); yet, few of these studies have examined more than one hybrid zone within the same species complex (Sætre et al. 1999; but see Haavie et al. 2004; Rohwer and Martin 2007; Vallin et al. 2012). Where multiple zones have been examined, such as for the Hermit/Townsend's Warbler (*Setophaga occidentalis* and *S. townsendi*) (Rohwer and Martin 2007) and Pied/Collared Flycatchers (*Ficedula hypoleuca* and *F. albicollis*) (Sætre et al. 1999; Haavie et al. 2004; Vallin et al. 2012) complexes, differences in gene flow (Sætre et al. 1999), phenotypic introgression (Rohwer and Martin 2007), and song characteristics (Haavie et al. 2004) have been found. One species pair with hybrid zones of different ages is the Black-crested Titmouse (*Baeolophus atricristatus*) and Tufted Titmouse (*B. bicolor*) complex. The older contact zone in central and north-central Texas (TX) (Dixon 1955, 1978, 1990) likely formed no later than 4000 years ago based on climatic data (Dixon 1978). An apparently younger contact zone in southwestern Oklahoma (OK) (Dixon 1955, 1978; Sutton 1967; Patten and Smith-Patten 2008) likely formed as a result of shrub, chiefly honey mesquite (*Prosopis glandulosa*) (Callahan, 2002), invasion in response to fire suppression and overgrazing within the past century (Sutton 1967; Rising 1983; Patten and Smith-Patten 2008)

The main margins of the TX hybrid zone appear to be phenotypically stable in previously studied areas of the TX coast, central TX, and north-central TX (Dixon

1990), and climatic evidence suggests that it has remained in the same region since the original putative secondary contact (Dixon 1955). However, the exact extent of the hybrid zone is poorly known outside of central TX (Dixon 1955). Additionally, some maps (i.e., Oberholser 1974) do not distinguish phenotypically intermediate individuals (Dixon 1990) and Pulich (1988) noted that many observers attempt to categorize all individuals as one of the parental species, even in areas of hybridization, making it difficult to map the hybrid zone using survey data not aimed explicitly at these species and their hybrids. Dixon's (1955, 1978, 1990) studies focusing on TX are the most recent works aimed specifically at the distribution of this hybrid zone. In the southwestern OK contact zone, the extent of interbreeding has not been studied. Thus, an update is warranted on the status of hybridization in this species complex.

The two titmouse forms diverged during the Pleistocene glaciations (Dixon 1978; Klicka and Zink 1997; Patten and Smith-Patten 2008) and currently are considered two species (Banks et al. 2002) on the basis of mitochondrial DNA sequences and DNA-DNA hybridization (Braun et al. 1984; Avise and Zink 1988; Sheldon et al. 1992) and vocalizations (Dixon 1955; Coldren 1992). The two species occupy different habitats, the Tufted wetter deciduous forest and the Black-crested a variety of arid, more open woodlands; interbreeding occurs across this ecological transition in TX (Dixon 1955). The species can be distinguished by crest and forehead color (Dixon 1955) and differ on average in song characteristics such as frequency, duration and spacing of phrases, spacing of notes and phrases, and number of phrases per song (Coldren 1992; Chapter 3). These phenotypic differences provide multiple

avenues to observe the relative effects of natural and sexual selection on introgression in the younger and older zones (Vallin et al. 2012).

Our study compares both plumage and morphology in north-central TX and southwestern OK, including the first quantitative analysis of plumage for these species. Further, our study compares the current extent of hybridization to previous records, yielding three time periods in which to compare cline width in two regions: historical (1955) and recent (2000s) for the older north-central TX and recent for the younger southwestern OK zone. We also evaluate the use of Dixon's (1955) hybrid index, developed for specimens, on live birds in the field.

Methods

Study area

This study was conducted in TX and OK, with banding at 20 public and private sites (Figure 3; Table 3). One banding site (Llano River Field Station) in central TX is excluded from the cline analyses because of its long distance from the main older transect. Banding sites are located in the following USGS Level III ecoregions in TX (Griffith et al. 2004) and OK (Woods et al. 2005): Cross Timbers (all TX hybrid sites), Central Great Plains, Edwards Plateau, Southwestern Tablelands, and East Central Texas Plains and occur across a gradient in precipitation. Additional sight records were gathered at locations throughout the two states near previously reported areas of hybridization (Dixon 1955; Patten and Smith-Patten 2008).

Sampling and measurements

We observed titmice in 2007, 2008, and March-June 2009 near the reported hybrid zone throughout TX and in southwestern OK. We observed and banded additional titmice from 2010-2012 in north TX and southwestern and central OK.

The original hybrid index for these species (Dixon 1955) classifies the range of crest and forehead plumage on a scale from 0 (pure Tufted) to 6 (pure Black-crested) by combining crest (0 to 3) and forehead (0 to 3) values. Outside of the hybrid zone birds are typically 0-1 (Tufted) or 5-6 (Black-crested), so hybrids show a large range of intermediate plumage within the narrow zone of contact (Dixon 1955). It was developed from and used on museum specimens, but we used the hybrid index to score live birds (Figure 3). Most observations of unbanded birds were made with 8×32 Leica Rangefinder binoculars. To compare hybrid index values to a quantitative measurement of plumage color, we scored the hybrid index and then used a Konica-Minolta CR-400 colorimeter to quantify forehead and crest colors on in-hand birds using the L*a*b* color scale (CIE 2004) with light-dark, red-green, and blue-yellow axes. The colorimeter was calibrated on a white standard before each use.

As female Black-crested Titmice can have paler crests (Dixon 1955), only males are included. We counted all singing birds for sight records as males. All singing banded birds for which blood samples have been analyzed were males (genetically sexed following Griffiths et al. 1998). Dixon (1955) included female titmice in the hybrid index site average, so those site averages are lowered (Figure 3).

To compare body plumage and morphology between the species and between TX and OK, we quantified color on the dorsum, flanks, breast, and side of head using

the colorimeter to compare with verbal descriptions given in Dixon (1955). Each banded bird was measured by one author (CMC) for wing chord, tail length, tarsus length, bill length, bill width, and bill depth following Pyle (1997) and crest length (Dixon, 1955).

These methods were approved by the University of Oklahoma Institutional Animal Care and Use Committee (R09-004, R12-009). Banding was conducted under U.S. Fish and Wildlife Service permit 23215-H, Oklahoma Department of Wildlife Conservation scientific collecting permits 4716, 4955, and 5210, and Texas Parks and Wildlife Department scientific collecting permit SPR-0310-019.

Data analyses

We estimated measurement error for all plumage and morphology data (Bailey and Byrnes 1990; Marantz and Patten 2010) using SAS 9.2 (SAS Institute Inc. 2002). Repeated measures were taken on randomly selected birds (n=14) in 2010 and 2011. Tarsus was excluded from analyses due to high measurement error (20.3%). Instead, we used wing chord for a size proxy. Forehead (measurement errors: light-dark 1.8%, red-green 3.8%, blue-yellow 11.8%) and crest (measurement errors: light-dark 10.5%, red-green 5.08%, 11.8%), likely due to their easily observed fixed locations, were just above or well below a 10% cut-off for measurement error, as were wing chord (3.0%) and tail length (3.7%). Because of measurement error >10% for bill measurements and many colorimeter measurements in 2010-2011, we began to average three replicates for the bill and all colorimeter measurements in 2012. Bailey and Byrnes (1990) suggested this as an acceptable approach for using variables with high measurement error, as

averaging multiple measurements and increasing the sample size allows for sound inference from such variables.

Birds are grouped in two ways in the following analyses: by using the hybrid index directly and by using the hybrid index to categorize birds with scores of ≥1 but ≤5 as hybrids; though occasional birds far from the hybrid zone (Figure 3) can also display specific hybrid-like variations. (These variations are always a paler crest [crest scored as 2] in Black-crested and a hint of brown forehead [forehead scored as 1] on a Tufted ; [C.M. Curry, pers. obs.]. On some species feather wear reveals colors [e.g., (Johnson and Johnson 1985)], and this may be the cause of hybrid-like variations on the titmouse crest and foreheads outside of the hybrid zone.) Although this is an arbitrary cut-off, it allows for convenient grouping of birds for illustrative purposes and for a few categorical tests.

To compare colorimeter values with the visually estimated hybrid index values, we performed canonical correlation analysis (CCA) in SAS 9.2 (SAS Institute Inc. 2002). The six crest and forehead colorimeter values (three color axes for each region) were one canonical axis; crest and forehead hybrid index values were the other canonical axis.

To test for spatial autocorrelation indicative of geographic variation within each parental species, we performed Mantel tests (Sokal 1979) using the R package 'ade4' (Dray and Dufour 2007). Tufted Titmouse is monotypic (Grubb and Pravasudov, 2008), whereas the Black-crested Titmouse has several weakly marked subspecies (Patten and Smith-Patten 2008). The Black-crested Titmice in this study include mostly *B. a. sennetti* with five individuals of *B. a. paloduro* from Palo Duro Canyon, all identified

by geographic range (Dixon 1955; Patten and Smith-Patten 2008). Hence, variation is not likely to be the result of subspecific identify, especially for Tufted Titmice. We tested for significance after using the Dunn-Šidák correction for family-wise error rates for morphology (six comparisons, $P<0.0085$) and plumage (18 comparisons, $P<0.0028$). For both the young and old zones combined, we described plumage and morphology using principal components analysis (PCA) with a correlation matrix in R 2.15.0 (R Development Core Team 2012), with axis loadings (Pearson correlations between components and raw variables) >0.33 interpreted; this corresponds to roughly 10% of variation explained (Comrey and Lee 1992). We illustrate these results for each species with the centroid of the first two principal components and 68% data concentration ellipses in the R package 'car' (Fox and Weisberg 2011); these ellipses are equivalent to approximately one standard deviation around the average assuming bivariate normality.

We used ArcGIS to create shape files of historical sightings (figures 9 and 12 in Dixon, (1955; figures 9 and 12). All additional mapping was done in Quantum GIS (QGIS) 1.8.0 (Quantum GIS Development Team 2012). Sightings from this study are identified as individuals. Dixon's sightings are identified by their hybrid index values as site averages (tables 14-16 in Dixon 1955).

To compare recent and historically surveyed cline widths and centers for hybrid index, we first created standardized transects through the main OK and TX banding sites (excluding one site far to the south of the main study areas) using standard (reduced) major axis regression in the R package lmodel2 (Legendre 2011) to determine the slope and intercept of each transect. Each transect starts at -102° W and ends at -95° W to allow for approximate comparison of the cline center from east to west, although

the transect slopes differ. We transformed coordinates into the Universal Transverse Mercator (UTM) projection in QGIS and calculated the distance of each individual's GPS points along the transect.

We then fit clines using the R package 'hzar' (Derryberry 2012) and CFit-8 (Gay et al. 2008). Both programs fit three-part cline equations to the data using maximum likelihood (Szymura and Barton 1986; Brumfield et al. 2001; Gay et al. 2008). However, the sigmoidal curves were a poor fit to the data. Instead, clines were described using loess smoothing, a type of local polynomial regression fitting, as implemented with the "loess" function in R 2.15 (R Development Core Team 2012). Loess smoothing is a type of local polynomial regression fitting with its shape dictated by a polynomial degree and a spanning parameter, which adjusts how many local points are used in the fit (Cleveland 1979). These values were adjusted for each cline but are comparable with 84% confidence intervals. As the historically surveyed data mainly covers hybrid values and lacks the parental asymptotic tails that are present in recently surveyed data, using span f = 0.5 (older) and f=0.6 (younger) for the recently surveyed transects (degree = 1 for both), and span f = 1 and degree = 2 for historically surveyed data produced appropriate fits. Recently surveyed data included plumage and morphology PCAs, so the first principal component for each was also plotted using this method.

Cline center was defined as the location where maximum slope was located. We calculated cline width in two ways: first as the absolute value of 1/(maximum slope) (Szymura and Barton 1986) and second as a range of minimum and maximum width. Because cline width, as estimated by the slope, does not generate a confidence interval,

we calculated the minimum and maximum range where birds with hybrid index values between one and five are found using the cline confidence intervals (see Figure 4 for an illustration of the calculations). Cline positions were compared by overlap of 84% confidence intervals generated by the "predict" function in R. Confidence intervals of this width overlap 95% of the time and thus are approximately equivalent to a significance test at $\alpha=0.05$ (Payton et al. 2003).

Dispersal estimates for our titmouse species were not available, so to estimate selection within each hybrid zone, we used dispersal distances from the sister group (Gill et al. 2005) to Tufted and Black-crested Titmice: the Juniper/Oak Titmouse complex (*B. ridgwayi* and *B. inornatus*; natal dispersal distances reported of 0.091-1.097 km, with an average of 0.343 km) (Cicero 2000a,b); and from the related Carolina Chickadee (*Poecile carolinensis*; Mostrom et al. 2002) (8 km) and Black-capped Chickadee (*P. atricapillus*; Weise and Meyer 1979) (11.2 km). We calculated selection strength with the equation $w=\sqrt{(8\ \sigma 2)}/s$ (Szymura and Barton 1986; Moore and Price 1993; Brelsford and Irwin 2009), where w=width of the cline, s=selection, and σ= dispersal. We also calculated the length of time that would result in the widths for each contact under neutral gene flow (Barton and Hewitt 1985 p. 130) using $1/T=2\pi(\sigma/w2)$, where T=number of generations, to determine the number of generations. We converted this time to years using this equation to calculate generation time: $G = \alpha + u/(\lambda - u)$ where u= survivorship, α=age at first breeding, and λ= population growth rate (Lande et al. 2002; Milá et al. 2007). We assumed $\alpha=1$ and $\lambda=1$ (one year of age at first breeding in a stable population) and survivorship was 0.54 (Grubb and Pravasudov 2008); this resulted in a generation time of 3.35 y.

Results

Hybrid index

Crest and forehead colorimeter values were correlated strongly with variance in crest and forehead hybrid index values (n=89, Wilks' Λ=0.091, P<0.0001; Figure 5).

Plumage and morphology

The Black-crested Titmouse showed no significant spatial autocorrelation in plumage or morphology. The Tufted Titmouse showed significant spatial autocorrelation for crest color on the red-green axis (Mantel's r=0.242, P<0.001). The crest is redder to the north (r=0.47, t=3.06, df=34, P=0.004) and west (r=-0.59, t=-4.25, df=34, P=0.0002). The difference in crest color is not obvious to the author in the field or from photos. The range in variation is also small (see Table 4-Table 7). The main variation in plumage for recently surveyed data (Table 8; Figure 6A; Figure 7A) is in the light-dark and red-green axes for crest and in the light-dark and blue-yellow axes for forehead colors, along with the blue-yellow axis on the side of the head. PC2, which captures mainly individual variation in both recently surveyed regions, shows additional variation in the light-dark and red-green axes on both the dorsum and side of head, which is usually a similar color to the dorsum (C. M. Curry pers. obs.). In general, Tufted Titmice have paler, greener crests, darker and bluer foreheads.

In both the younger and older zones for recently surveyed data, Black-crested Titmice are smaller with a longer crest and shorter, shallower bill (n=91; Table 9; Figure 6B; Figure 7B). Additional individual variation in these features, plus bill width and excepting crest length, occurs on PC2.

Distribution and clines

The current extent of hybridization between Tufted and Black-crested Titmice in TX generally matches previous records (Figure 3). New sightings of hybrids follow in similar areas north and south of past sightings for central TX. The cline centers are similar for the recently surveyed and historically surveyed older zone in north-central TX (Figure 8) but widths are 23 and 17 km, respectively. Parental phenotypes also come into closer contact in the younger zone (Figure 9). The 84% confidence intervals for the loess smooths do not overlap from approximately 410-470 km, indicating a slight shift in traits towards Black-crested in that region. However, the shift occurs within the same region (between 310 and 500 km), indicating the overall location and width of the hybrid zone is similar despite a shift in characters towards Black-crested within it and a potential widening. Compared to the recently surveyed older zone, the recently surveyed younger zone is located farther west (as one would expect from Figure 3) and is 16 km wide. When using the ranges for the hybrid index clines calculated using 84% confidence intervals, the recently surveyed younger zone is generally narrower at 18-23 km than the historically surveyed older zone (18-31 km) but overlaps with the recently surveyed older zone (10-29 km). The historically and recently surveyed older zone width ranges also overlap. Using hybrid index values of 2-4 as the range of the hybrid zone gives qualitatively similar ranges in width, with the recently surveyed younger transect being narrower than the historically surveyed older transect.

Using those width values and a range of potential dispersal distances to estimate selection strength, values range from almost zero in the recently surveyed older zone to over 10 in the recently surveyed older zone, depending on the parameters (Table 10).

Discussion

The recently and historically surveyed older zone phenotypic clines did not differ in width while the recently surveyed younger zone cline was narrower. Our current distributional data for north-central TX show a region of intermediate individuals (Figure 3) that is similar both in extent to previous reports (Dixon 1955) and to previous estimates of a zone 24-40 km (Lockwood and Freeman 2004) or 50-100 km wide (Dixon 1990) in north-central TX. Expansion of a zone over time is expected when ongoing dispersal and introgression has not been balanced by selection against hybrids (Barton and Hewitt 1985, 1989) balanced with dispersal of parental individuals into the zone (Barton and Hewitt 1989) or selection for hybrids within a narrow zone (Moore 1977). The narrower width for only the recently surveyed younger zone, which is the area with least time since presumed secondary contact, suggests that the recently surveyed younger zone is likely to become wider but then stabilize as a result of selection as appears to have happened with the recent and historical transects through the older zone (Table 2, scenario 2b). Additional evidence for selection is the amount of time estimated for the cline to reach its current width assuming neutral introgression. Either the younger zone is wider than it should be for its age (for titmouse values of dispersal) or narrower (for chickadee values of dispersal), indicating selection could, respectively, either be favoring introgression or preventing further expansion of the zone. The time to current width under neutrality for the older zone (both historical and

recent data) for the maximum titmouse and chickadee dispersal distances also suggest that selection is constraining expansion of the older contact zone. Additional work with genetic data in our system should allow better estimates of dispersal and selection; experimental work with male agonistic responses and female mate choice will clarify what processes may be exerting selection pressure.

The lack of expansion and small center shift (3 km) in the older north-central TX zone over the 60 y historical and recent resurveying periods matches results from other avian hybrid zones in the Great Plains and western North America that have been stable, such as Rose-breasted and Black-headed Grosbeaks (Mettler and Spellman 2009) and Yellow-rumped Warblers (Brelsford and Irwin 2009) or shifted only slightly, such as the "Red-shafted" and "Yellow-shafted" Northern Flickers (Moore and Buchanan 1985). A contact zone in Australia between two species of frogs, resurveyed after 60 y, also showed no change in width but a small change in cline center, possibly in response to changes in local population density (Smith et al. 2013). A similar process could be occurring in the titmouse zones; additional possible reasons for movement are discussed below. That the zone has not narrowed, as has occurred with Lazuli and Indigo Buntings (Carling and Zuckerberg 2011), indicates that selection, such as by reinforcement (Howard 1993) or by other natural or sexual selection (Barton and Hewitt 1981; Alatalo et al. 1990; Patten et al. 2004; Tobler et al. 2009; Merrill et al. 2011) is at least not overcoming dispersal into the zone. Another resampled zone, that of Townsend's and Hermit Warblers, has continued to move over recent resampling (Krosby and Rohwer 2010) consistent with past genetic evidence of its movement

(Krosby and Rohwer 2009). The related Carolina and Black-capped Chickadees have also shown obvious movement in their hybrid zone (Reudink et al. 2007).

Although there was no overall difference in width for the recently surveyed and historically surveyed data, a small local shift (as in Moore and Buchanan 1985) in the north-central TX zone has occurred since Dixon's (1989, 1990) work in the region; the cline center has moved by about 3 km to the east, matching with a shift towards Black-crested Titmouse on the hybrid index at any given hybrid locality for the older zone (Figure 8). Lockwood and Freeman (2004) described the Black-crested Titmouse as occurring east to Clay County, TX; we found hybrids occurring west to at least Wichita County, TX and a Tufted or near-Tufted hybrid in Clay County. Pulich (1988) noted the two forms interbreed freely west of Tarrant County, TX and north to the Red River. Dixon (1955) speculated the north-central TX zone may have expanded in response to changes in oak distributions; Dixon (1990) discussed slight distributional shifts on the basis of local weather events, such as drought and vegetation changes along coastal TX, but concluded the main area of the hybrid zone had not changed despite small details of movement. Slight movements in climatic gradients (Dixon 1955, 1989; Swenson 2006) or in population density (Barton and Hewitt 1989; Smith et al. 2013) could also be a causal factor. Genetic work should aid in determining any potential shifts in the extent (Sattler and Braun 2000) and rate of introgression of neutral markers (Krosby and Rohwer 2009; Mettler and Spellman 2009) and also determine if phenotypic and genetic clines coincide (Sattler and Braun 2000; Gay et al. 2008; Toews et al. 2011; Seneviratne et al. 2012).

We present herein the first detailed description of the younger southwestern OK contact zone, where the Black-crested Titmouse has expanded its range into southwestern OK likely in response to woody shrub encroachment (Van Auken 2000; Callahan 2002). Parids prefer not to cross large open areas (Desrochers and Bélisle 2007) so this is a reasonable cause for the range expansion. We posit east-west contact between the two forms; though, another possibility is that all titmice populations (parental and hybrids) have moved north- and westward together into southwestern OK. However, after a fairly smooth curve north along the Edwards Plateau, the contact orientation (Figure 3) jogs abruptly westward at the Red River, as evidenced by hybrids just south of the Red River and Tufted Titmice present north of it in south-central OK. This is consistent with a new east-west contact in southwestern OK. Dixon (1955) reported neither titmice nor appropriate habitat along the Red River in TX (Hardeman and Hall Counties); isolating a population of the Black-crested in the TX panhandle (*B. a. paloduro*) from those in north-central TX. A break is still possible between the TX and OK contact zones in the present day, but it is more likely to be continuous along the riparian woodlands of the Red River despite sparse titmouse habitat in southwestern OK and western north TX, as both the Black-crested and hybrids are now found in counties where formerly absent (see references in Patten and Smith-Patten 2008 and sight records in the current study). Ongoing genetic work may clarify the route of the range expansion (Chapter 4).

We present the first quantitative analysis of plumage within this species complex. Plumage variation across both contact zones (Figure 6A; Figure 7) is similar with minor differences between zones in coloration on the side of the head and breast.

Otherwise, excepting the obvious change in crest and forehead coloration between the species, most variation in plumage was individual scatter within species. Despite the individual scatter, however, the Black-crested Titmouse [which is comprised of four weakly marked subspecies (Patten and Smith-Patten 2008)], does not show any spatial autocorrelation in plumage, while the monotypic Tufted Titmouse (Grubb and Pravasudov 2008) shows only slight clinal variation in one plumage character. The larger sampling range in latitude for the Black-crested Titmouse (Figure 3) should result in easier detection of any potential clinal variation compared to the shorter expanse sampled for the Tufted Titmouse. The recent range expansion in the Black-crested Titmouse could result in the lack of clinal variation observed in the populations we sampled, which included approximately equal numbers of birds from the younger and older zones. For morphology, we found no significant spatial autocorrelation in size over the range of either species; but we sampled a much smaller range in latitude, particularly for the Tufted Titmouse, than did Dixon (1955). He noted a distinct size difference between populations of the Black-crested and Tufted Titmice at similar latitudes and clinal variation in size, with northern birds being larger. Tufted Titmice tend to have larger bills in both the TX and OK contacts; Dixon (1955) speculated the prevalence of "oaks and other mast-producing trees" in the Tufted range during winter might result in these slightly larger bills for handling these larger foods. In the recently surveyed younger zone, the species show less overlap along PC1 (Figure 6B). Perhaps continued introgression in the older zone has blunted body size differences (Figure 7B) and left only potentially ecologically important differences despite the widths of the plumage clines being similar in both regions for both recent and historical data.

Future work on this temporally complex hybrid zone should focus on forces acting to hold the older clines in equilibrium and monitoring the younger cline for potential expansion and movement. Plumage may be under sexual selection at least in the Black-crested Titmouse (Dixon 1955), preventing the clines from spreading in the older zones. In the future, we will compare mitochondrial and nuclear genetic markers for concordance of clines (Barton and Hewitt 1985), as they are expected to be coincident where selection against hybrids is balanced against dispersal into the zone (Barton and Hewitt 1985). Additionally, if the younger hybrid zone is a new secondary contact as we postulate and not a northwestward range expansion of both parental and hybrid titmouse populations, neutral genetic clines should also be narrower in the younger hybrid zone, reflecting a shorter time for introgression. Finally, differences in song between the two species (Coldren 1992; Chapter 3) provide an additional avenue for exploration. If hybrids have reduced fitness, whether it is intrinsic or extrinsic, conspecific song preferences may have not yet evolved (Labonne and Hendry 2010) in response to selection pressures in the younger zone, allowing it to potentially spread to a width comparable to those in the older zone (Labonne and Hendry 2010) Our study describing the size of and phenotypic variation across this temporally complex hybrid zone provides a baseline for such further studies of behavioral, ecological, and genetic processes shaping its maintenance (Harrison 1990).

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Figures and tables

Table 2. Predicted changes in relative cline width over time under varying scenarios. Bars represent relative cline width.

	Most time<------>Least time since contact		
	Modern old (TX)	Historical old (1950s)	Young (SW OK)
Scenario			
1. Equilibrium.	|---|	|---|	|---|
2a. Expansion over time.	|------|	|---|	|-|
2b. Expansion over time, with no change between modern and historical sampling.	|------|	|------|	|-|
3a. Narrowing over time.	|-|	|---|	|------|
3b. Narrowing over time, with no change between modern and historical sampling.	|-|	|-|	|------|
3c. Narrowing over time, with no change between historical and young sampling.	|-|	|------|	|------|
4a. Temporal variation, one possibility.	|------|	|-|	|------|
4b. Temporal variation, potential alternative scenario.	|-|	|------|	|-|

Table 3. Main study sites with transect (older or younger contact zone), sample size (n), average hybrid index scores ± standard deviation (range), and GPS coordinates in decimal degrees

Site	Transect	n	Forehead hybrid index	Crest hybrid index	Total hybrid index
Texas Tech University Llano River Field Station, TX (30.474 N, -99.783 W)	Older (but *see* Methods)	2	3.0±0 (3)	3.0±0 (3)	6.0±0 (6)
Abilene State Park, TX (32.235 N, -99.886 W)	Older	7	3.0±0 (3)	3.0±0 (3)	6.0±0 (6)
Fort Griffin State Historic Site, TX (32.924 N, -99.216 W)	Older	5	3.0±0 (3)	2.4±0.55 (2-3)	5.4±0.55 (5-6)
City of Graham sewage treatment plant, TX (33.098 N, -98.599 W)	Older	4	2.5±0.58 (2-3)	2.8±0.5 (2-3)	5.3±0.96 (4-6)
City of Graham Fireman's Park, TX (33.115 N, -98.595 W)	Older	1	2.0	3.0	5.0
Fort Richardson State Park, TX (33.225 N, -98.152 W)	Older	4	2.0±0 (2)	2.0±0.81 (1-3)	4.0±0.82 (3-5_
Lyndon B. Johnson National Grasslands, TX (33.379 N, -97.579 W)	Older	5	0.8±0.45 (0-1)	0.2±0.45 (0-1)	1.0±0.71 (0-2)

Site	Transect	n	Forehead hybrid index	Crest hybrid index	Total hybrid index
Private land near Greenwood, TX (33.388 N, -97.488 W)	Older	1	0.0	0.0	0.0
Ray Roberts State Park, TX (33.328 N, -97.034 W)	Older	10	0.0±0 (0)	0.0±0 (0)	0.0±0 (0)
Caddo National Grasslands, TX (33.745 N, -95.991 W)	Older	7	0.0±0 (0)	0.0±0 (0)	0.0±0 (0)
Palo Duro Canyon State Park, TX (34.960 N, -101.669 W)	Younger	5	3.0±0 (3)	2.8±0.45 (2-3)	5.8±0.45 (5-6)
Matador Wildlife Management Area, TX (34.137 N, -100.387 W)	Younger	4	3.0±0 (3)	3.0±0 (3)	6.0±0 (6)
Private land near Eldorado, OK (34.389 N, -99.652 W)	Younger	3	3.0±0 (3)	3.0±0 (3)	6.0±0 (6)
Private land near Wellington, TX (34.951 N, -100.191 W)	Younger	5	2.6±0.55 (2-3)	2.8±0.45 (2-3)	5.4±0.89 (4-6)
Sandy Sanders Wildlife Management Area, OK (35.013 N, -99.818 W)	Younger	4	2.3±0.58 (2-3)	2.3±0.58 (2-3)	4.7±1.15 (4-6)

Site	Transect	n	Forehead hybrid index	Crest hybrid index	Total hybrid index
Quartz Mountain Nature Park, OK (34.911 N, -99.300 W)	Younger	9	1.7±0.71 (0-2)	1.1±0.60 (0-2)	2.8±1.09 (0-4)
Mountain Park Wildlife Management Area, OK (34.797 N, -98.998 W)	Younger	2	1.5±0.71 (1-2)	1.0±0 (1)	2.5±0.71 (2-3)
Wichita Mountains Wildlife Refuge, OK (34.763 N, -98.763 W)	Younger	8	0.0±0 (0)	0.0±0 (0)	0.0±0 (0)
Fort Cobb State Park, OK (35.190 N, -98.455 W)	Younger	5	0.0±0 (0)	0.0±0 (0)	0.0±0 (0)
Oliver's Woods Preserve, OK (35.180 N, -97.446 W)	Younger	3	0.0±0 (0)	0.0±0 (0)	0.0±0 (0)

Table 4. Old zone morphology measurements. Table 4 through Table 7 show average±1 sd, (range), and sample size for birds at each site. Tarsus is included here for comparison with Dixon (1955), despite >>10% measurement error in our study (see Methods).

Bill depth	Bill length	Site
5.0±0.02, (5.0-5.03), n=2	8.3±0.5, (8.0-8.7), n=2	Texas Tech University Llano River Field Station, TX "LLANO"
5.2±0.2, (4.9-5.5), n=7	9.1±0.4, (8.4-9.5), n=7	Abilene State Park, TX "ABILENE"
5.3±0.1, (5.2-5.4), n=5	9.1±0.6, (8.3-9.6), n=5	Fort Griffin State Historic Site, TX "FTGRIFFIN"
5.2±0.2, (5.0-5.4), n=4	9.1±0.3, (8.6-9.3), n=4	City of Graham sewage treatment plant, TX "GRAHAM"
5.2, (5.2), n=1	9.1, (9.1), n=1	City of Graham Fireman's Park, TX "FIREMAN"
5.2±0.2, (5.0-5.3), n=4	9.2±0.2, (9.0-9.3), n=4	Fort Richardson State Park, TX "FTRICHARDSON"
5.3±0.3, (4.9-5.5), n=5	8.9±0.5, (8.1-9.3), n=5	Lyndon B. Johnson National Grasslands, TX "LBJNG"
5.4, (5.4), n=1	9.5, (9.5), n=1	Private land near Greenwood, TX "CURRY"
5.4±0.3, (5.0-5.9), n=10	9.1±0.8, (8.0-10.7), n=10	Ray Roberts State Park, TX "GREENBELT"
5.3±0.3, (5.0-5.7), n=7	9.1±0.5, (8.4-9.7), n=7	Caddo National Grasslands, TX "CADDO"

Tail	Wing chord (right)	Tarsus (left)	Bill width
63.5±0.7, (63-64), n=2	73.8±0.2, (73.7-74), n=2	21.2±0.3, (21.0-21.4), n=2	4.9±0, (4.9), n=2
70.4±2.3, (68-73), n=7	78.5±1.5, (77-81), n=7	21.4±0.5, (20.5-21.8), n=7	5.0±0.3, (4.6-5.6), n=7
66.1±1.9, (64-68), n=5	73.9±3.0, (69.3-77), n=5	20.4±0.6, (19.9-21.4), n=5	5.0±0.3, (4.5-5.3), n=5
69.3±3.4, (65.3-73), n=4	77.0±2.0, (76-80), n=4	21.0±0.8, (20.1-21.9), n=4	5.1±0.3, (4.7-5.3), n=4
67, (67), n=1	79, (79), n=1	20.9, (20.9), n=1	4.9, (4.9), n=1
70.0±3.6, (67-74), n=4	78.2±3.3, (75.6-82), n=4	20.2±0.7, (19.2-20.8), n=4	5.0±0.3, (4.6-5.2), n=4
73.4±4.2, (69-79), n=5	81.9±2.9, (78-86), n=5	21.5±0.8, (20.2-22.5), n=5	5.0±0.3, (4.6-5.4), n=5
71, (71), n=1	75, (75), n=1	22.0, (22.0), n=1	4.9, (4.9), n=1
72.2±2.9, (67.7-76), n=10	79.3±2.1, (76-83), n=10	21.3±0.9, (19.8-22.8), n=10	5.0±0.2, (4.6-5.3), n=10
71.1±2.4, (68-74), n=7	80.7±2.8, (76-85), n=7	21.1±0.7, (19.9-21.8), n=7	5.0±0.3, (4.5-5.4), n=7

Crest length
18.3±0.9, (17.7-19), n=2
21.3±0.6, (20.3-22), n=7
19.9±0.9, (19-21), n=5
20.0±1.4, (18-21), n=4
19, (19), n=1
18.9±0.8, (18-19.7), n=4
18.4±2.2, (17-22), n=5
19, (19), n=1
19.1±1.5, (17-21), n=10
17.5±0.8, (17-19), n=7

Table 5. Young zone morphology measurements.

Bill length	Site
9.1±0.4, (8.7-9.6), n=5	Palo Duro Canyon State Park, TX "PALODURO"
9.0±0.4, (8.4-9.4), n=4	Matador Wildlife Management Area, TX "MATADORWMA"
8.2±0.4, (7.8-8.5), n=3	Private land near Eldorado, OK "OSBORNE"
8.9±0.5, (8.3-9.4), n=5	Private land near Wellington, TX "HENARD"
8.5±0.4, (8.1-8.9), n=3	Sandy Sanders Wildlife Management Area, OK "SSWMA"
9.1±0.4, (8.3-9.6), n=9	Quartz Mountain Nature Park, OK "QUARTZMTN"
8.7±0.1, (8.6-8.7), n=2	Mountain Park Wildlife Management Area, OK "MTNPARKWMA"
9.2±0.7, (8.0-10.1), n=8	Wichita Mountains Wildlife Refuge, OK "WITCHITAMTNS"
9.7±0.4, (9.2-10.5), n=5	Fort Cobb State Park, OK "FTCOBB"
9.0±0.6, (8.5-9.7), n=3	Oliver's Woods Preserve, OK "OWP"

Wing chord (right)	Tarsus (left)	Bill width	Bill depth
74.5±1.1, (73-76), n=	21.1±0.5, (20.3-21.7), n=5	4.9±0.2, (4.6-5.2), n=5	5.3±0.1, (5.2-5.4), n=5
75.8±1.7, (74-78), n=4	21.2±0.2, (20.9-21.3), n=4	5.2±0.3, (4.8-5.4), n=4	5.3±0.1, (5.2-5.4), n=4
77.7±0.6, (77-7), n=3	20.7±0.8, (19.8-21.2), n=3	5.2±0.6, (4.8-5.9), n=	5.2±0.2, (5.1-5.4), n=3
75.0±2.4, (72-78), n=5	20.7±1.2, (19.0-22.1), n=5	5.0±0.6, (4,4-5.8), n=5	5.3±0.1, (5.2-5.4), n=5
74.4±4.7, (69-77.3), n=3	19.7±0.8, (18.7-20.3), n=3	5.1±0.7, (4.3-5.5), n=3	5.0±0.1, (4.9-5.1), n=3
77.3±1.6, (74.3-79), n=9	21.4±1.6, (18.8-23.4), n=9	5.0±0.3, (4.6-5.2), n=9	5.2±0.2, (4.9-5.4), n=9
77.0±0, (77), n=2	20.3±0.3, (20.1-20.5), n=2	4.9±0.3, (4.7-5.1), n=2	5.0±0.1, (4.9-5.0), n=2
81.0±1.5, (79-83), n=8	22.0±0.6, (21.0-22.8), n=8	5.3±0.3, (4.8-5.9), n=8	5.4±0.3, (4.9-6.1), n=8
82.1, ±1.3, (81-84), n=5	22.1±0.8, (20.8-22.7), n=5	5.1±0.3, (4.8-5.4), n=5	5.5±0.1, (5.4-5.7), n=5
80.5±3.0, (77-82.5), n=3	21.7±0.1, (21.7-21.8), n=3	5.1±0.5, (4.5-5.5), n=3	5.4±0.2, (4.8-5.8), n=3

Crest length	Tail
20.5±0.8, (19.3-21), n=5	65.4±1.1, (64-67), n=5
20.5±1.3, (19-22), n=4	66.5±1.0, (65-67), n=4
20.3±0.6, (20-21), n=3	71.6±2.5, (68.7-73), n=3
19.2±1.4, (17-20.8), n=5	66.7±3.3, (63.7-72), n=5
20.1±1.0, (19-21), n=3	69.7±2.3, (67-71), n=3
19.5±1.5, (17.3-22), n=9	70.5±1.5, (68-72), n=9
19.5±0.7, (19-20), n=2	67.5±2.1, (66-69), n=2
18.2±1.4, (16-20), n=8	71.5±2.1, (68-74), n=8
17.7±1.1, (16-19), n=5	73.6±3.0, (72-79), n=5
17.7±1.3, (16.5-19), n=3	71±2.6, (68-73), n=3

Table 6. Old zone plumage measurements.

Crest light-dark	Sites
14.9±4.7, (11.6-18.2), n=2	Texas Tech University Llano River Field Station, TX "LLANO"
13.7±1.7, (12.3-16.8), n=7	Abilene State Park, TX "ABILENE"
17.3±3.1, (13.4-21.4), n=5	Fort Griffin State Historic Site, TX "FTGRIFFIN"
19.1±2.7, (16.6-22.9), n=4	City of Graham sewage treatment plant, TX "GRAHAM"
19.0, (19.0), n=1	City of Graham Fireman's Park, TX "FIREMAN"
17.7±3.0, (15.1-21.1), n=4	Fort Richardson State Park, TX "FTRICHARDSON"
30.9±3.7, (26.1-35.9), n=5	Lyndon B. Johnson National Grasslands, TX "LBJNG"
33.1, (33.1), n=1	Private land near Greenwood, TX "CURRY"
32.0±1.7, (29.9-34.7), n=10	Ray Roberts State Park, TX "GREENBELT"
30.7±1.5, (29.2-33.2), n=7	Caddo National Grasslands, TX "CADDO"

Forehead red-green	Forehead light-dark	Crest blue-yellow	Crest red-green
1.8±0.1, (1.75-1.9), n=2	39.3±3.0, (37.1-41.4), n=2	2.8±0.1, (2.7-2.9), n=2	1.7±0.4, (1.5-2.0), n=2
2.9±0.8, (2.3-4.5), n=7	37.3±3.8, (31.7-41.1), n=7	2.7±0.5, (1.9-3.4), n=7	1.8±0.2, (1.7-2.2), n=7
2.1±0.4, (1.6-2.5), n=5	37.5±4.5, (31.7-42.8), n=5	3.7±1.3, (2.8-6.0), n=5	2.0±0.5, (1.5-2.8), n=5
2.5±0.3, (2.2-2.8), n=4	33.6±3.3, (28.9-36.3), n=4	2.9±0.5, (2.6-3.6), n=4	1.7±0.4, (1.3-2.2), n=4
4.5, (4.5), n=1	28.7, (28.7), n=1	4.5, (4.5), n=1	1.4, (1.4), n=1
4.2±1.0, (3.1-5.2), n=4	29.7±3.2, (26.6-32.9), n=4	3.4±0.4, (3.1-3.6), n=4	1.8±0.3, (1.5-2.0), n=4
2.7±0.8, (2.0-3.9), n=5	22.9±6.3, (13.7-30.7), n=5	3.0±0.8, (2.3-4.3), n=5	0.9±0.3, (0.6-1.3), n=5
2.2, (2.2), n=1	18.7, (18.7), n=1	2.2, (2.2), n=1	0.7, (0.7), n=1
2.1±0.3, (1.3-2.4), n=10	16.6±4.0, (11.2-23.7), n=10	2.8±0.6, (1.8-3.5), n=10	0.6±0.2, (0.4-0.9), n=10
1.9±0.7, (1.0-3.0), n=7	14.8±6.0, (6.8-24.4), n=7	2.5±0.3, (2.2-3.0), n=7	0.7±0.2, (0.3-0.8), n=7

Dorsum blue-yellow	Dorsum red-green	Dorsum light-dark	Forehead blue-yellow
3.5±0.3, (3.3-3.7), n=2	0.7±0.1, (0.6-0.7), n=2	37.2±0.8, (36.7-37.7), n=2	11.5±0.5, (7.4-10.4), n=2
3.8±1.9, (0.6-5.3), n=7	0.5±0.4, (0.2-1.1), n=7	35.4±2.2, (32.2-38.1), n=7	12.5±2.4, (10.0-16..8), n=7
4.2±0.8, (3.3-5.4), n=5	0.9±0.2, (0.6-1.2), n=5	34.9±1.3, (32.8-36.0), n=5	11.1±1.1, (10.0-12.6), n=5
3.9±0.8, (2.8-4.7), n=4	0.6±0.1, (0.57-0.7), n=4	37.6±1.4, (36.4-39.4), n=4	10.8±1.5, (9.3-12.4), n=4
5.4, (5.4), n=1	0.8, (0.8), n=1	35.0, (35.0), n=1	14.1, (14.1), n=1
5.5±0.9, (4.5-6.1), n=4	0.5±0.5, (-0.1-0.8), n=4	38.7±0.2, (38.6-38.9), n=4	12.4±2.0, (10.9-14.7), n=4
3.3±0.6, (2.3-3.8), n=5	0.7±0.3, (0.2-1.0), n=5	34.9±2.5, (33.0-38.8), n=5	8.2±1.2, (7.4-10.4), n=5
4.0, (4.0), n=1	0.4, (0.4), n=1	41.3, (41.3), n=1	4.0, (4.0), n=1
3.7±1.2, (1.6-5.3), n=10	0.5±0.2, (0.2-0.9), n=10	35.2±2.2, (29.5-37.4), n=10	6.5±0.6, (5.6-7.5), n=10
3.4±0.6, (2.6-4.1), n=7	0.6±0.3, (-0.01-1.0), n=7	34.7±1.9, (32.6-38.0), n=7	5.0±1.3, (3.1-6.7), n=7

Side of head blue-yellow	Side of head red-green	Side of head light-dark
3.0±0.03, (2.99-3.03), n=2	0.6±0.1, (0.5-0.7), n=2	42.3±1.7, (41.2-43.5), n=2
3.6±0.7, (2.9-4.8), n=7	0.7±0.2, (0.5-1.0), n=7	38.5±2.4, (35.3-41.6), n=7
4.6±1.3, (3.2-6.6), n=5	1.0±0.4, (0.7-1.6), n=5	39.0±3.8, (34.2-44.6), n=5
3.0±0.2, (2.7-3.3), n=4	0.6±0.1, (0.4-0.8), n=4	38.9±3.6, (35.4-42.6), n=4
4.3, (4.3), n=1	0.6, (0.6), n=1	41.2, (41.2), n=1
3.0±0.8, (2.3-3.8), n=4	0.7±-/2, (0.5-0.9), n=4	37.2±6.0, (30.4-40.8), n=4
2.7±0.6, (2.0-3.3), n=5	0.7±0.1, (0.6-0.9), n=5	38.1±3.5, (34.3-42.0), n=5
2.4, (2.4), n=1	0.6, (0.6), n=1	38.1, (38.1), n=1
2.0±0.5, (1.3-3.0), n=10	0.5±0.2, (0.3-0.9), n=10	36.7±2.3, (32.1-39.2), n=10
2.1±0.2, (1.9-2.3), n=7	0.6±0.2, (0.2-0.7), n=7	34.7±4.0, (29.0-40.5), n=7

Flank blue-yellow	Flank red-green	Flank light-dark
19.6±8.3, (13.8-25.5), n=2	4.5±4.0, (1.6-7.3), n=2	61.8±6.4, (57.3-66.4), n=2
25.3±1.6, (23.6-27.3), n=7	7.4±1.6, (5.4-10.1), n=7	54.5±2.9, (49.1-57.8), n=7
20.4±3.2, (17.6-24.2), n=5	4.3±1.4, (3.1-6.6), n=5	60.0±2.9, (57.4-64.9), n=5
24.0±3.7, (19.7-28.1), n=4	7.1±1.8, (5.2-8.9), n=4	62.9±18.2, (47.5-63.7), n=4
24.8, (24.8), n=1	6.5, (6.5), n=1	56.2, (56.2), n=1
23.2±7.1, (15.0-27.8), n=4	5.8±3.0, (2.4-7.8), n=4	59.1±3.3, (56.5-62.8), n=4
23.1±2.4, (19.7-25.9), n=5	7.4±1.9, (5.6-10.3), n=5	53.7±3.0, (48.9-56.1), n=5
24.0, (24.0), n=1	10.1, (10.1), n=1	46.4, (46.4), n=1
20.9±4.5, (11.9-27.2), n=10	6.3±3.1, (0.9-11.8), n=10	54.0±7.0, (43.9-67.1), n=10
24.0±2.6, (19.6-27.4), n=7	8.3±1.6, (6.6-10.6), n=7	51.5±4.9, (44.0-56.9), n=7

Breast blue-yellow	Breast red-green	Breast light-dark
8.2±1.8, (7.0-9.5), n=2	-0.3±0.5, (-0.7-0.03), n=2	68.9±3.4, (66.6-71.3), n=2
6.7±1.6, (5.4-9.3), n=7	-0.2±0.4, (-0.9-0.3), n=7	67.2±4.4, (63.0-74.2), n=7
7.0±1.3, (5.0-8.3), n=5	-0.3±0.2, (-0.5- -0.1), n=5	67.3±3.7, (61.7-70.9), n=5
7.5±1.3, (5.6-8.6), n=4	-0.3±0.2, (-0.5- -0.1), n=4	72.1±3.4, (67.3-74.6), n=4
12.2, (12.2), n=1	0.7, (0.7), n=1	68.0, (68.0), n=1
6.2±0.8, (5.3-6.7), n=4	-0.4±0.04, (-0.5- -0.4), n=4	70.1±4.6, (65.5-74.8), n=4
7.0±2.8, (3.6-9.9), n=5	0.04±0.1, (-0.2-0.1), n=5	68.1±6.1, (61.2-76.3), n=5
8.1, (8.1), n=1	-0.2, (-0.2), n=1	74.3, (74.3), n=1
8.1±1.8, (5.3-10.4), n=10	-0.2±0.2, (-0.6-0.2), n=10	71.3±4.3, (66.8-78.2), n=10
5.8±1.2, (4.0-7.4), n=7	-0.1±0.3, (-0.9-0.1), n=7	67.8±7.5, (58.3-76.7), n=7

Table 7. Young zone plumage measurements.

Crest light-dark	
16.6±3.0, (13.1-20.9), n=5	Palo Duro Canyon State Park, TX "PALODURO"
15.7±3.0, (13.4-20.0), n=4	Matador Wildlife Management Area, TX "MATADORWMA"
14.5±1.1, (13.7-15.8), n=3	Private land near Eldorado, OK "OSBORNE"
17.0±5.0, (13.0-25.3), n=5	Private land near Wellington, TX "HENARD"
14.7±0.9, (13.6-15.2), n=3	Sandy Sanders Wildlife Management Area, OK "SSWMA"
26.7±7.2, (14.4-34.1), n=9	Quartz Mountain Nature Park, OK "QUARTZMTN"
23.6±3.2, (21.3-25.8), n=2	Mountain Park Wildlife Management Area, OK "MTNPARKWMA"
29.7±1.8, (26.7-32.3), n=8	Wichita Mountains Wildlife Refuge, OK "WITCHITAMTNS"
32.1±0.9, (31.2-33.5), n=5	Fort Cobb State Park, OK "FTCOBB"
31.2±2.7, (28.2-33.6), n=3	Oliver's Woods Preserve, OK "OWP"

Forehead red-green	Forehead light-dark	Crest blue-yellow	Crest red-green
2.9±0.5, (2.2-3.7), n=5	40.5±5.2, (31.9-46.2), n=5	3.9±0.8, (3.0-5.0), n=5	2.1±0.3, (1.8-2.7), n=5
1.9±0.2, (1.7-2.0), n=4	35.2±5.2, (29.9-42.1), n=4	3.3±0.6, (2.6-3.9), n=4	2.0±0.4, (1.5-2.3), n=4
2.8±0.1, (2.7-2.9), n=3	38.2±2.9, (35.0-40.8), n=3	3.1±0.6, (2.6-3.7), n=3	1.9±0.2, (1.7-2.1), n=3
2.7±0.8, (1.6-3.5), n=5	33.3±4.7, (26.9-39.2), n=5	2.9±0.6, (2.1-3.7), n=5	1.8±0.3, (1.4-2.1), n=5
3.2±1.1, (2.5-4.5), n=3	36.5±4.5, (33.3-41.7), n=3	3.4±0.3, (3.2-3.7), n=3	2.0±0.2, (1.9-2.2), n=3
4.7±1.4, (1.7-6.7), n=9	26.5±5.5, (15.5-32.1), n=9	3.6±0.7, (2.7-4.8), n=9	1.4±0.6, (0.7-2.5), n=9
3.9±0.7, (3.4-4.3), n=2	24.3±1.5, (23.3-25.3), n=2	4.4±0.4, (4.1-4.7), n=2	1.4±0.2, (1.3-1.6), n=2
2.2±0.5, (1.5-3.2), n=8	19.0±7.5, (10.8-30.9), n=8	3.1±0.5, (2.5-3.9), n=8	1.0±0.2, (0.7-1.4), n=8
2.1±0.4, (1.7-2.5), n=5	18.5±8.5, (8.8-31.2), n=5	3.2±0.7, (2.3-4.0), n=5	0.9±0.1, (0.7-1.0), n=5
2.0±0.7, (1.4-2.8), n=3	13.7±1.9, (11.6-15.4), n=3	3.1±0.3, (2.8-3.4), n=3	0.6±0.2, (0.4-0.7), n=3

Dorsum blue-yellow	Dorsum red-green	Dorsum light-dark	Forehead blue-yellow
4.3±0.7, (3.4-5.1), n=5	1.0±0.3, (0.7-1.3), n=5	36.5±3.7, (31.6-40.1), n=5	13.1±2.5, (9.6-15.8), n=5
4.6±0.3, (4.2-4.9), n=4	0.9±0.2, (0.8-1.3), n=4	34.4±2.4, (31.7-37.1), n=4	9.5±2.5, (5.8-10.8), n=4
2.5±2.1, (0.1-4.1), n=3	0.7±0.4, (0.3-1.1), n=3	36.3±1.6, (34.9-38.0), n=3	12.7±2.1, (11.3-15.1), n=3
3.7±1.1, (2.3-5.3), n=5	0.8±0.4, (0.4-1.4), n=5	35.8±2.7, (32.9-38.6), n=5	11.9±3.2, (7.4-16.4), n=5
3.9±0.4, (3.4-4.1), n=3	0.9±0.2, (0.6-1.0), n=3	37.1±1.7, (35.2-38.5), n=3	14.8±1.7, (12.9-16.0), n=3
4.7±1.1, (3.5-6.7), n=9	0.6±0.2, (0.3-0.9), n=9	36.2±2.6, (31.8-41.1), n=9	12.0±3.6, (5.5-18.1), n=9
3.1±0.6, (2.6-3.5), n=2	0.8±0.03, (0.79-0.84), n=2	34.2±0.3, (34.0-34.4), n=2	10.7±1.4, (9.8-11.7), n=2
2.8±0.8, (1.1-3.6), n=8	0.8±0.3, (0.4-1.2), n=8	35.2±2.6, (30.8-37.7), n=8	6.0±0.9, (4.5-7.0), n=8
4.1±1.0, (2.6-5.5), n=5	0.8±0.2, (0.5-1.1), n=5	34.0±1.6, (31.9-35.6), n=5	6.2±2.3, (3.7-10.0), n=5
4.1±1.3, (2.9-5.5), n=3	0.7±0.3, (0.5-1.0), n=3	33.8±1.1, (32.7-34.7), n=3	3.8±1.1, (2.8-5.0), n=3

Flank light-dark	Side of head blue-yellow	Side of head red-green	Side of head light-dark
58.0±4.4, (51.8-63.3), n=5	3.9±0.7, (3.4-5.0), n=5	1.0±0.2, (0.8-1.3), n=5	37.7±2.3, (34.5-40.0), n=5
57.9±1.0, (56.9-59.3), n=4	3.7±0.9, (2.9-4.9), n=4	1.2±0.5, (0.6-1.9), n=4	34.6±4.5, (30.7-40.5), n=4
54.8±0.7, (54.0-55.3), n=3	3.8±1.8, (2.2-5.8), n=3	0.9±0.5, (0.5-1.5), n=3	39.9±3.5, (36.5-43.6), n=3
58.8±3.9, (54.3-64.2), n=5	3.0±0.6, (2.4-3.8), n=5	0.8±0.1, (0.6-0.9), n=5	37.9±3.8, (31.3-40.6), n=5
58.7±2.0, (57.4-61.0), n=3	2.6±0.9, (1.6-3.3), n=3	0.7±0.2, (0.6-0.9), n=3	38.9±0.8, (38.1-39.6), n=3
55.3±5.4, (48.1-66.1), n=9	2.9±0.4, (2.3-3.6), n=9	0.6±0.1, (0.4-0.8), n=9	38.5±2.5, (35.3-41.9), n=9
56.2±0.9, (55.5-56.8), n=2	2.8±0.5, (2.5-3.2), n=2	0.7±0.2, (0.6-0.8), n=2	38.2±0.8, (37.7-38.8), n=4
52.9±5.2, (46.5-62.6), n=8	2.5±0.6, (1.7-3.4), n=8	0.8±0.2, (0.6-1.1), n=8	34.9±3.7, (30.3-41.2), n=8
50.0±6.7, (42.9-56.7), n=5	2.5±0.6, (1.7-3.4), n=5	0.7±0.2, (0.3-1.0), n=5	34.6±1.9, (32.1-37.4), n=5
49.2±7.2, (42.7-57.0), n=3	1.6±0.6, (1.1-2.2), n=3	0.5±0.1, (0.4-0.6), n=3	34.5±2.3, (32.0-36.6), n=3

Breast red-green	Breast light-dark	Flank blue-yellow	Flank red-green
-0.1±0.7, (-1.0-0.7), n=5	68.3±6.6, (58.1-73.7), n=5	20.9±5.6, (12.5-26.7), n=5	4.8±2.5, (0.8-6.7), n=5
-0.5±0.5, (-1.0-0.1), n=4	64.9±3.1, (60.5-67.6), n=4	24.0±1.9, (22.2-26.6), n=4	6.1±0.7, (5.3-6.8), n=4
0.2±0.5, (-0.2-0.7), n=3	74.3±1.7, (72.4-75.5), n=3	24.6±0.5, (24.3-25.1), n=3	7.1±0.3, (6.8-7.4), n=3
-0.4±0.3, (-0.7-0), n=5	69.3±3.1, (65.4-73.7), n=5	17.8±2.9, (14.1-21.6), n=5	2.9±1.2, (1.8-4.2), n=5
-0.1±0.03, (-0.2- -0.1), n=3	68.2±4.5, (63.9-73.5), n=3	21.7±4.4, (17.3-26.2), n=3	4.8±1.7, (3.0-6.4), n=3
0.1±0.3, (-0.2-0.7), n=9	69.1±4.5, (60.0-74.2), n=9	19.8±5.8, (10.1-25.0), n=9	5.2±2.8, (1.5-8.8), n=9
-0.03±0.1, (-0.1-0.1), n=2	68.5±0.6, (68.1-68.9), n=2	19.9±3.9, (17.2-22.6), n=2	4.3±2.9, (2.3-6.3), n=2
0.1±0.2, (-0.3-0.5), n=8	66.7±7.1, (50.4-72.6), n=8	19.7±6.4, (5.3-26.7), n=8	6.0±2.6, (0.9-9.4), n=8
0.04±04, (-0.3-0.8), n=5	70.2±3.0, (67.8-75.4), n=5	21.2±1.5, (19.5-22.7), n=5	7.4±1.3, (5.4-8.5), n=5
0.5±0.6, (0.2-1.2), n=3	72.7±4.4, (67.9-76.6), n=3	24.8±0.9, (24.0-25.7), n=3	9.4±1.6, (7.6-10.7), n=3

Breast blue-yellow
7.7±2.1, (5.2-10.4), n=5
5.8±1.0, (4.4-6.7), n=4
8.9±0.4, (8.4-9.1), n=3
6.4±1.4, (4.6-8.5), n=5
5.6±1.9, (3.4-7.0), n=3
6.8±2.6, (3.3-10.6), n=9
7.6±0.1, (7.5-7.6), n=2
5.5±1.3, (3.2-7.3), n=8
6.9±2.7, (3.5-10.9), n=5
7.1±1.2, (6.4-8.7), n=3

Table 8. PCA loadings for plumage. Loadings r>0.33 are in bold.

	Both zones combined (n=89)	
Plumage character	PC1	PC2
Crest light-dark	**0.370**	0.113
Crest red-green	**-0.395**	0
Crest blue-yellow	-0.215	0.289
Forehead light-dark	**-0.360**	-0.162
Forehead red-green	-0.183	0
Forehead blue-yellow	**-0.369**	-0.176
Dorsum light-dark	0	**-0.383**
Dorsum red-green	-0.158	**0.399**
Dorsum blue-yellow	-0.106	0
Side of head light-dark	-0.116	**-0.368**
Side of head red-green	-0.250	**0.359**
Side of head blue-yellow	**-0.332**	0.160
Flank light-dark	-0.237	-0.205
Flank red-green	0.222	0
Flank blue-yellow	0	-0.148
Breast light-dark	0.101	-0.306
Breast red-green	0.113	0.274
Breast blue-yellow	0	-0.102

Table 9. PCA loadings for morphology. Loadings >0.33 are in bold.

	Both zones combined (n=91)	
Morphological character	PC1	PC2
Bill length	**-0.418**	**0.525**
Bill depth	**-0.455**	**0.402**
Bill width	-0.187	**0.359**
Wing chord	**-0.500**	**-0.459**
Tail	**-0.469**	**-0.469**
Crest length	**0.336**	0

Table 10. Selection strength and neutral generation time estimates for 15 ranges of dispersal and width. Estimates are shown as selection range, neutral time for this width in generations (years with generation time as 3.35 y; generations and years are rounded after calculations).

Recently surveyed younger (18-23 km)	Recently surveyed older (10-29 km)	Historically surveyed older (18-31 km)	
0.0000013-0.0000020; 5667-9252 generations; 18,983-30,994 y	0.00000079-0.0000066; 1749-14,709 generations; 5859-49,274 y	0.00000069-0.0000020; 5667-16,807 generations; 18,983-56,305 y	Low titmouse (0.0091 km)
0.0018-0.0029; 150-245 generations; 504-822y	0.0011-0.0094; 45-390 generations; 155-1307 y	0.00098-0.0029; 150-446 generations; 503-1494 y	Average titmouse; (0.343 km)
0.018-0.030; 47-77 generations; 157-257 y	0.011-0.096; 15-122 generations; 49-56 y	0.010-0.030; 47-139 generations; 157-467 y	Max. titmouse; (1.097 km)
0.97-1.58; 6-11 generations; 22-35 y	0.61-1.77; 2-17 generations; 7-56 y	0.53-1.58; 6-19 generations; 22-64 y	Carolina Chickadee; (8 km)
1.90-3.10; 5-8 generations; 15-25 y	1.19-10.04; 1-12 generations; 5-40 y	1.04-3.10; 5-14 generations; 15-46 y	Black-capped Chickadee; (11.2 km)

Figure 3. Recent (2007-2012) and historical (Dixon 1955) distribution of hybrid index values across the hybrid zones in TX and OK. Tick marks on transects represent 100 km intervals for comparison with Figure 7. Annual precipitation gradient is a 30 arc-second grid from WorldClim (Hijmans et al. 2005). Contour lines for the hybrid zone were created using empirical Bayesian kriging in ArcGIS 10.1 via the Geostatistical Analyst extension (subset size 100, overlap factor 1, 100 simulations, no transformation, standard circular neighborhood, 10-15 neighbors, 4 sectors, 0 angle, and radius 5) (ESRI 1999). Inset: Color interpretation of Dixon's hybrid index, based on black-and-white illustrations in Dixon (1955). Crest values: 0=Gray, similar color as neck and back; 1=Dark gray, obviously different from neck and back although color may blend at edges; 2=Dull black or very dark gray, blackish color can blend at edges; 3=Shiny black or black crest with crisply defined border, may extend down back of crest into back of neck. Forehead values: 0=Black or only small amounts of brown at the edge of the forehead; 1=Dark chestnut or dark brown; 2=Light brown or chestnut color that is distinct from facial color; 3=White or pale color that blends into facial area, not a distinct patch of color.

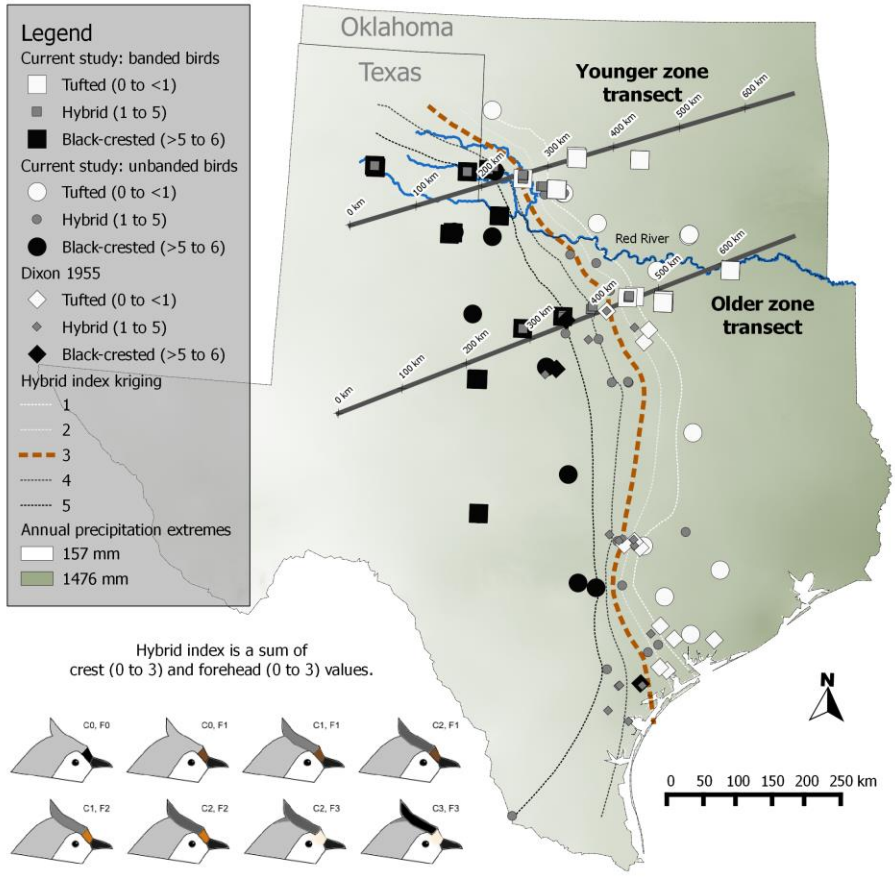


Figure 4. Cline width figure showing how cline width ranges were calculated using upper and lower 84% confidence intervals. The distance between each cut-off line (1 and 5) to the loess smoothing prediction was minimized to find the point closest to the intersection of the cut-off line and the loess smoothing predicted values. The point does not always match the visual of the smoothing line for the smoothing because the graph interpolates between the predicted loess smoothing values, but the estimate is repeatable. For each of the three transects, the two closest values were subtracted to find the minimum width, while the two most distance values were subtracted to find the maximum width

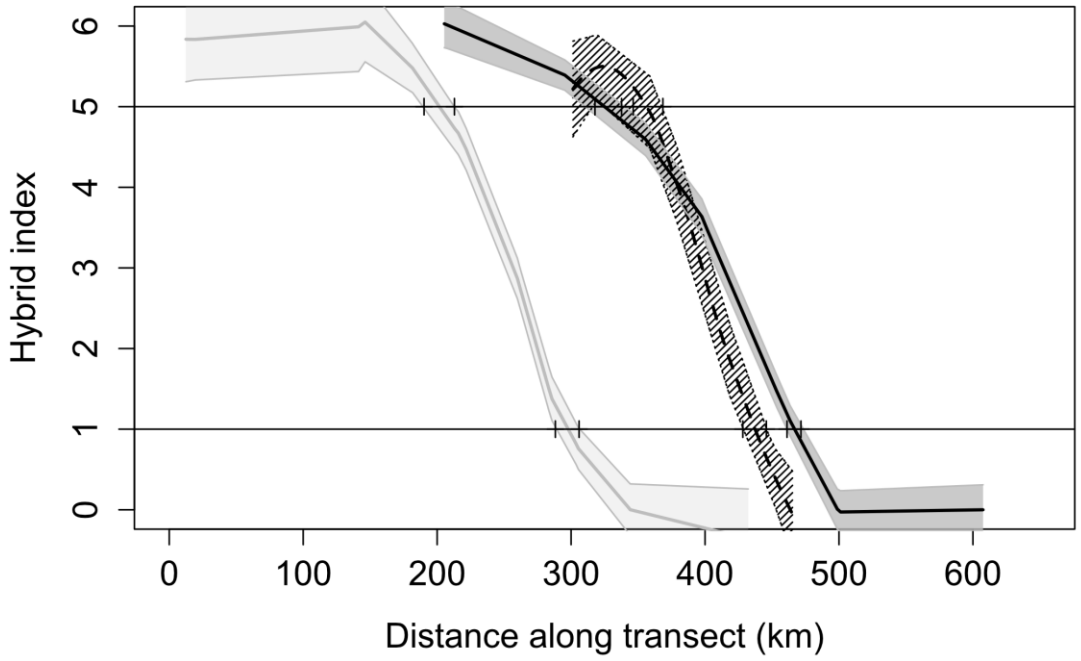


Figure 5. Canonical correlation between hybrid index and colorimeter values for CC1 ($F_{12,162}$ =31.4, p<0.0001). The hybrid index CC1 variate accounts for 97.4% of variance in the hybrid index values; the colorimeter CC1 variate accounts for 55.9% of variance in colorimeter values. Variance in colorimeter values for crest and forehead explain 86.9% of variance in the hybrid index. While CC2 is significant ($F_{5,82}$=3.1, p=0.04), very little (0.4%) variance in the hybrid index values are explained by variance in colorimeter values on the CC2 variates and so is not illustrated further. Actual hybrid index values (crest and forehead values summed) are shown for each individual.

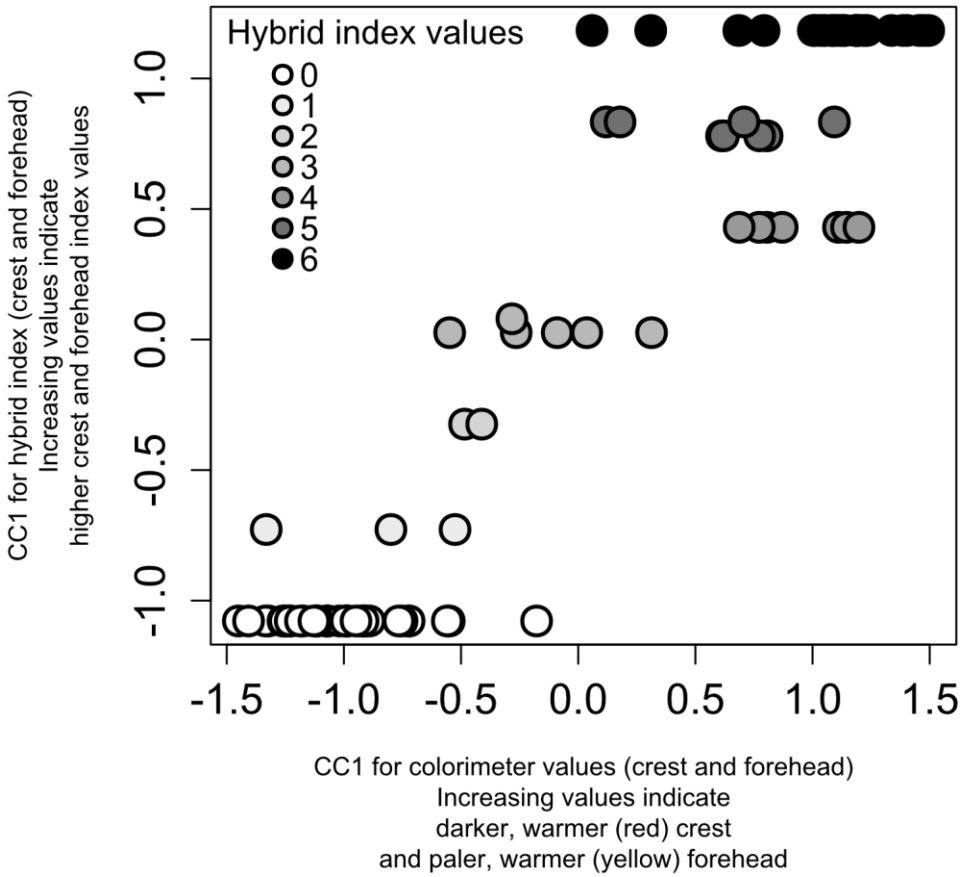
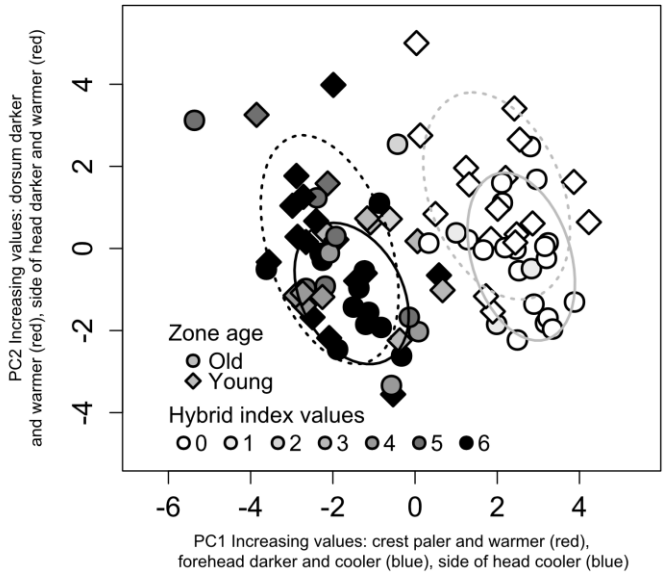


Figure 6. PCA averages and 68% data concentration ellipses for (A) plumage and (B) morphology. Ellipses represent birds with a hybrid index of <1 as Tufted, 1-5 as hybrids, and >5 as Black-crested, although slight variation within parental species is possible (see Methods). Symbols: black = Black-crested Titmouse, gray = hybrid, white = Tufted.

A



B

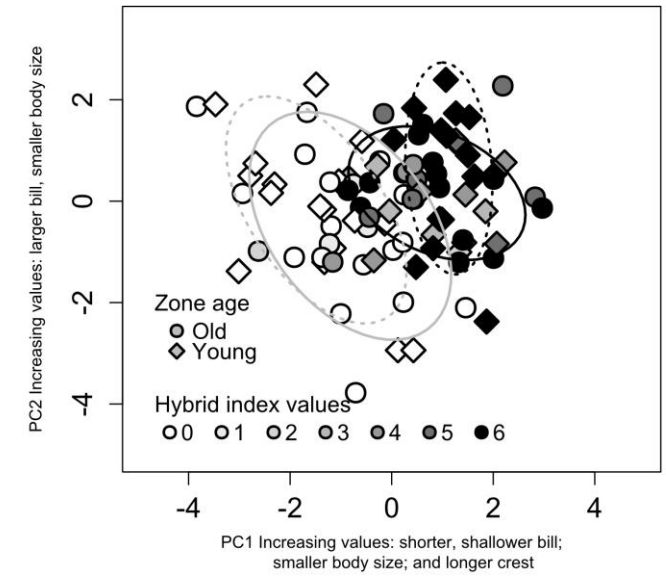
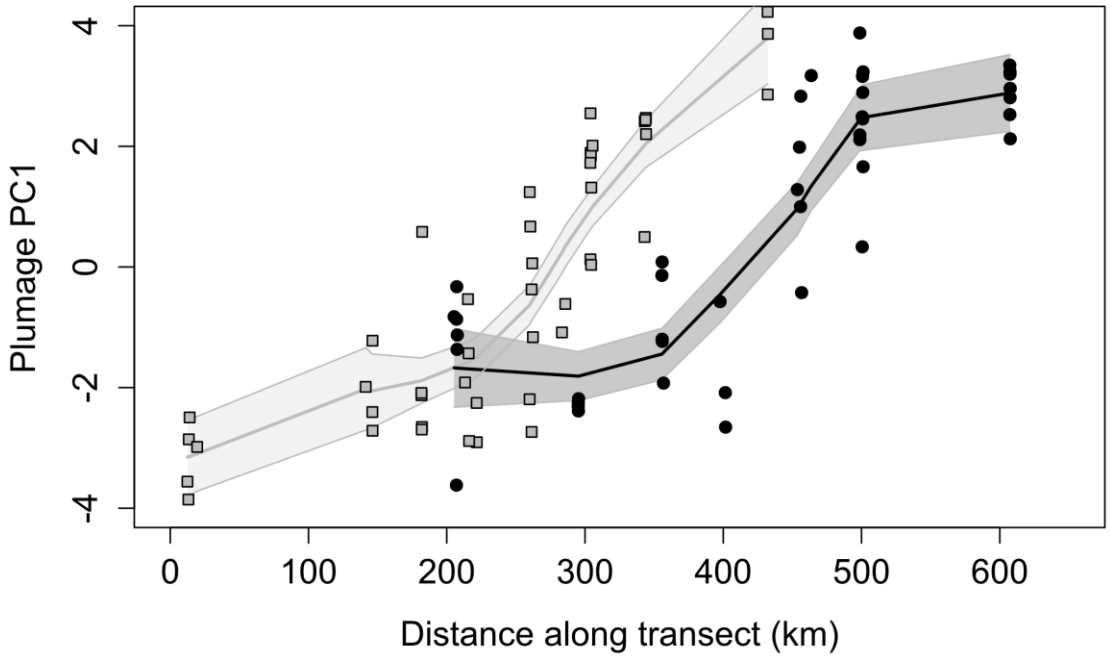


Figure 7. Phenotypic clines fit with loess smoothing. (A) plumage PC1 and (B) morphology PC1. These data are the same as in Figure 6, but instead PC1 is shown across the hybrid zone. The leftmost cline is the younger hybrid zone (gray lines, symbols, and fill). The recently surveyed older zone (black line and circles, dark gray fill) is to the right. Fill areas represent 84% confidence intervals, while each symbol is an individual bird. Clines were fit as described in Methods.
A



B

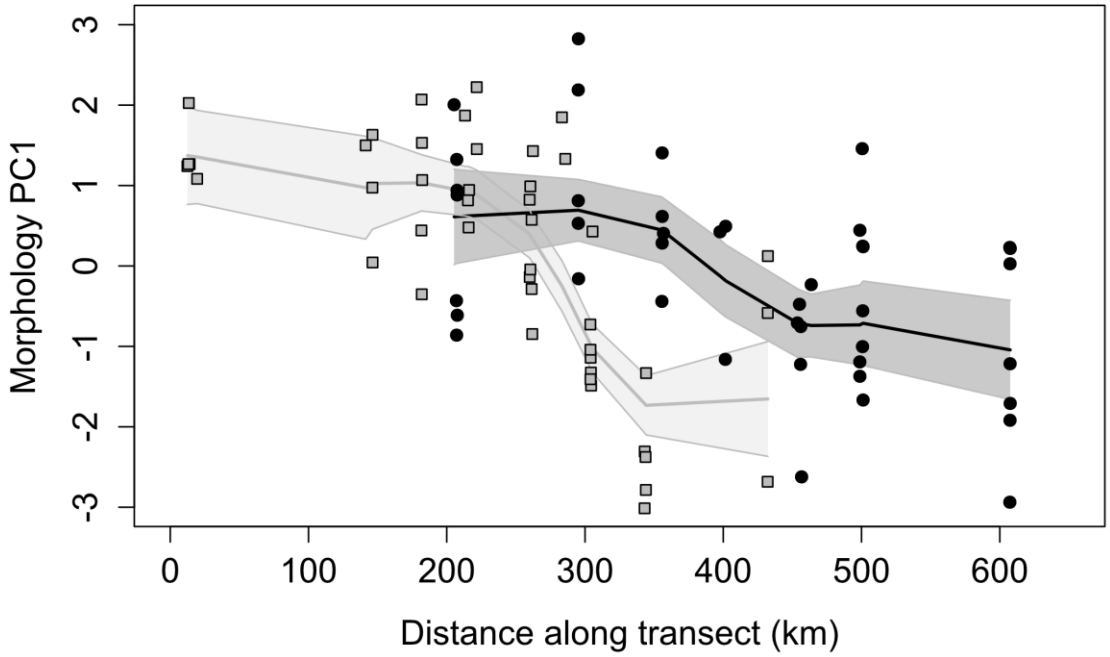

Morphology PC1
3
2
1
0
-1
-2
-3
0
100
200
300
400
500
600
Distance along transect (km)

Figure 8. Historical and modern older (north-central TX) and younger (southwestern OK) hybrid zone clines. The leftmost cline is the younger hybrid zone (gray lines, symbols, and fill). The remaining clines are the modern older zone (black line and circles, dark gray fill) and the historical older zone (white squares, dashed line, and hatched fill). Fill areas represent 84% confidence intervals, while each symbol is an individual bird (recently surveyed data) or a population (historically surveyed data). Vertical lines are placed at the cline center (location of maximum slope). Clines were fit as described in Methods.

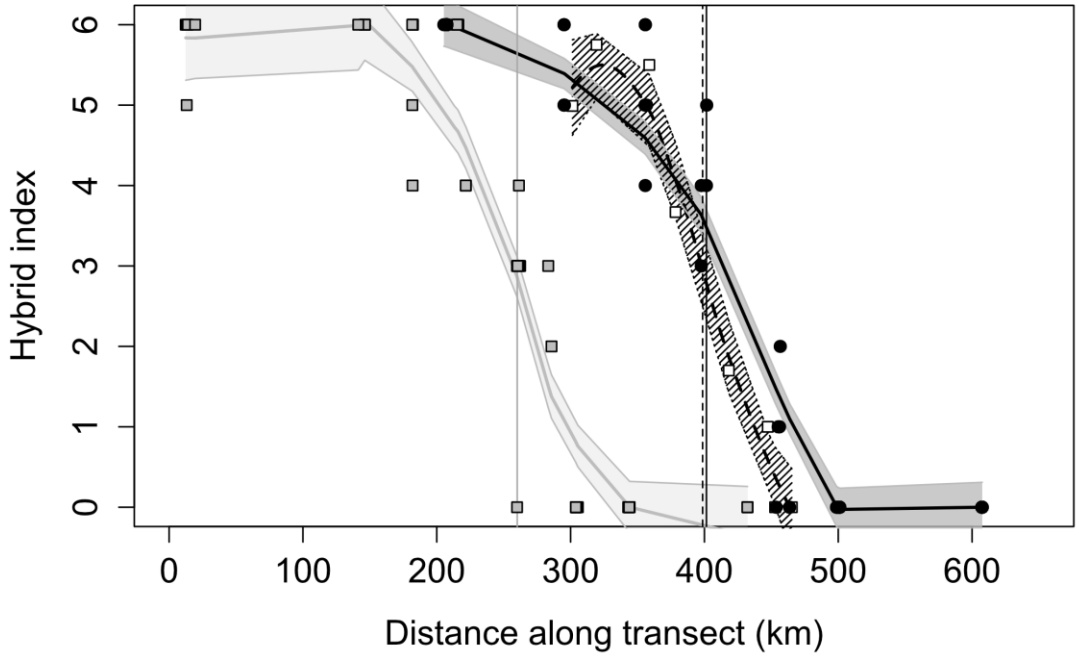
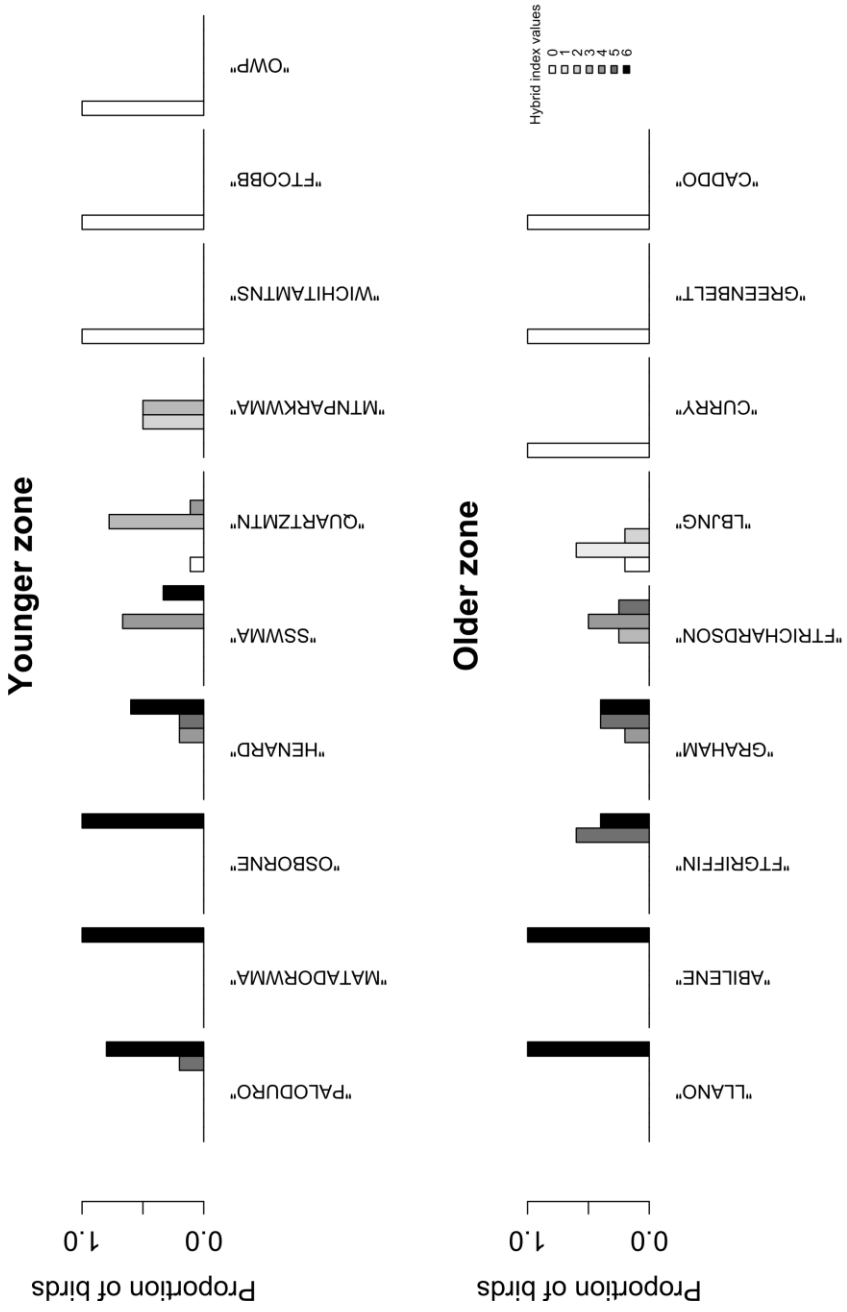


Figure 9. Histogram showing proportion of birds with a given hybrid index value at each site. [For this figure, Graham and Fireman's Park have been merged into one site (Graham) for ease of visualization. The site codes are shown in Table 4-Table 7.] Despite potential selection pressure as shown in Table 10, no parental forms are found at the center of the older contact zone; more potential mixing occurs in the younger contact zone.



Chapter 3: Environmental and morphological constraints on song variation across a temporally complex passerine hybrid zone

Abstract

Hybrid zones are an excellent place to examine mechanisms of signal divergence, particularly because these areas where distinguishable populations meet often occur across environmental gradients. Tufted (*Baeolophus bicolor*) and Black-crested (*B. atricristatus*) titmice are passerine sister taxa with structurally similar songs that differ slightly in features such as song duration, number of notes per phrase, number of phrases per song, and center frequency; their hybrid zone occurs across an environmental gradient. A difference in time since contact for the two zones allows investigation of whether divergent song features are maintained with ongoing interactions between these taxa. I used the older and younger contact zones to see how the environmental and morphological differences between the two species have affected patterns of song differentiation with continued contact. Morphological features that are potentially biomechanically limiting vary across the hybrid zone, but no biomechanical constraints were found. In the young zone, noise and vegetation structure were correlated with several song characteristics such as song duration, spacing within the song, and center frequency. In the older zone, these features did not correlate with song despite similar differences in song features across that zone. These data suggest that despite overall similarities in song characteristics, birds singing in the older zone may be more constrained by sexual selection, whereas songs in the young zone are constrained by the environment.

Introduction

Bird songs are sexually selected vocal signals used for male defense of territory and female choice of mates (Catchpole and Slater 2008). Because these signals are so important in reproductive activities, they can reproductively isolate populations whose signals have diverged (Patten et al. 2004). Acoustic signal divergence can be shaped by many factors (Podos and Warren 2007) such as natural selection via biomechanical constraints (Podos 2001; Podos et al. 2004; Huber and Podos 2006; Herrel et al. 2009), background noise (Doutrelant and Lambrechts 2001; Slabbekoorn and Smith 2002a), the physics of signaling through different environments (Morton 1975; Wiley and Richards 1978; Tobias et al. 2010), and, for learned songs, by cultural divergence via drift or sexual selection (Olofsson and Servedio 2008; Byers et al. 2010). Learned birdsong sometimes even can diverge faster (Lachlan and Servedio 2004). Thus, studying the causes and patterns of song divergence between species is useful for understanding both signal evolution and the generation and maintenance of biodiversity.

Hybrid zones, where populations with distinguishable differences interbreed (Harrison 1993; Arnold 1997), are an excellent place to examine mechanisms of signal divergence, particularly because hybrid zones often occur across environmental gradients (Moore 1977). Thus in such a zone one can use the extrinsic and intrinsic selection on individuals (Moore 1977; Barton and Hewitt 1989) and the phenotypic variability that can be associated with hybridization (Grant and Grant 1994) across a gradient to gain insight into specific causes and maintenance of signal divergence.

Tufted (*Baeolophus bicolor*) and Black-crested (*B. atricristatus*) titmice are passerine sister taxa with songs that differ subtly, though the overall structure is similar

(Coldren 1992). The hybrid zone occurs across an environmental gradient (Dixon 1955), with Black-crested occuring in more arid, open environments (Dixon 1955, 1978; Grubb and Pravasudov 2008; Patten and Smith-Patten 2008). Black-crested Titmice are slightly smaller in body size (Dixon 1955; Curry and Patten 2014). A difference in time since contact for the two zones (Rising 1983; Patten and Smith-Patten 2008; Curry and Patten 2014) allows investigation of whether divergent song features are maintained with ongoing interactions between these taxa. These morphological and environmental differences, combined with the temporally complexity of the hybrid zone, make this species complex ideal for investigating causes and changes in signal divergence with continued contact.

In this paper I will use the older and younger contact zones to see how the environmental and morphological differences between the two species have affected patterns of song differentiation with continued contact. Specifically, I will describe (1) whether song and morphological features potentially involved in biomechanical constraints, vegetation structure, and environmental noise vary between the species; (2) whether potential biomechanically limiting morphological characters (body size and bill morphology) are correlated with song characteristics; (2) whether two aspects of the environment (environmental noise and vegetation structure) are correlated with song characteristics; and (3) whether these correlations differ in the younger and older zones and the relative importance of each possible constraint.

First, the bird's bill size and shape and body size can be affected by its diet and ecology, but these features also constrain what the bird can physically sing (Herrel et al. 2009). Birds with stouter bills cannot sing as rapidly (Podos 2001; Huber and Podos

2006) and the difference in body size between the titmouse species (Dixon 1955) has been suggested previously as a potential cause of frequency differences between the two (Coldren 1992). Thus I predict that titmice with wider, deeper, and shorter bills will sing at a slower rate (more spacing between notes or phrases) and that larger titmice will sing at a lower frequency (Ryan and Kime 2003). Secondly, the environment can directly filter the transmission of signals (Doutrelant and Lambrechts 2001; Slabbekoorn and Smith 2002a; Catchpole and Slater 2008), affecting both receipt of the signal and learning of signals (Peters et al. 2012). Different vegetation structures should distort signals in particular ways (Morton 1975; Wiley and Richards 1978), with birds in open habitats predicted to have longer notes, longer spacing between notes, higher mean frequencies, and more complex songs (Coldren 1992). Additionally, background noise, be it from other birds (Weir et al. 2012), insect noise (Kirschel et al. 2009), or anthropogenic noise (Slabbekoorn and Peet 2003; Verzijden et al. 2010), can also constrain the frequency and other characteristics of vocal signals. Finally, I look for differences between the younger and older zones in these constraints for three reasons. First, environmental features of vegetation structure and background noise might be different across the two zones. Second, morphology (including bill depth and length and body size) appears to transition more sharply between the species in the younger zone than the older zone (Curry and Patten 2014), although the statistical significance of that transition was not tested; such variation might be reflected in biomechanical constraints. Finally, this paper addresses natural selection such as vegetation structure and environmental noise, but how the species respond to heterospecifics versus conspecifics might differ with continued contact because of evolution via sexual selection. If sexual

selection is shaping songs (which it appears to be at least in the older zone; Chapter 5), then one would expect a stronger correlation of songs with environmental and biomechanical constraints in the younger zone than in the older zone where sexual selection has had a longer time to act.

Methods

Study species and sites

Most songs were recorded with a Marantz Professional PMD661 Solid State Recorder using a Telinga 570mm parabola and ME-62 Sennheiser omnidirectional microphone. Additional songs were recorded with a Sharp MD-MT15 portable digital MiniDisc recorder and 18" foam-backed metal parabola; the microphone was suspended at the focus point and covered with foam. Dr. Michael A. Patten provided additional vocalizations recorded with a Nagra ARES-BB+ digital recorder with Telinga Pro parabolic reflector. Recordings were made at sites throughout Texas and Oklahoma spanning the hybrid zone (Figure 10; banded sites usually have vegetation and morphology data; noise data are from both banded and unbanded sightings). I used a plumage-based hybrid index (Dixon 1955; see Curry and Patten 2014 for a color representation) to classify individuals by species for these analyses. [Chapter 4 provides genetic evidence supporting this classification; Curry and Patten (2014) show that the hybrid index correlates strongly with quantitative measurements of plumage.] These methods were approved by the University of Oklahoma Institutional Animal Care and Use Committee (R09-004, R12-009). Banding was conducted under U.S. Fish and Wildlife Service permit 23215-H, Oklahoma Department of Wildlife Conservation

scientific collecting permits 4716, 4955, and 5210, and Texas Parks and Wildlife Department scientific collecting permit SPR-0310-019.

Acoustic measurements

Song characteristics center frequency and duration (Charif et al. 2009) were measured for phrases, notes, and elements (Figure 11; elements are portions within notes shown in Coldren 1992) in Raven Pro 1.4 (Cornell Laboratory of Ornithology 2003-2009). I averaged songs from each individual and used that individual average for analysis to prevent pseudoreplication (Searcy et al. 1997) that would result from using the multiple songs from the same bird as independent data points. I measured environmental noise using center and peak frequency for blocks above and below the songs in the recordings used to get the songs (Figure 11). Each banded bird was identified as an individual by its color bands (see Morphology). Unbanded birds were assumed to be different individuals because of territory locations; i.e., the same sites and territories were not re-visited for recordings.

Morphology

Banded birds were measured for bill length, bill depth, bill width, and wing chord (used as a proxy for body size) following Pyle (1997). Individuals were marked with USFWS aluminum bands and ten colored plastic bands for individual identification. Although female titmice do sing on rare occasions (Coldren 1992; C.M. Curry pers. obs.), all banded birds found singing were genetically sexed (Griffiths et al. 1998) as males, so I assumed all unbanded singing birds were males as well.

Vegetation

Vegetation structure variables are adapted from Patten et al. (2004). Plots (10×10 m) were centered where the singing titmouse was first observed. I conducted a point-quarter estimate on each plot, measuring the distance from plot center, height, and radius of the nearest shrub or tree in each of four quadrats (northwest, northeast, southeast, southwest), from which mean and standard deviation were calculated.

Statistical analyses

All analyses were conducted in R (R Development Core Team 2013). Species-specific characteristics used in the following analyses were described by performing a canonical correlation analysis with song characters versus a plumage-based hybrid index; the dataset used for this included unbanded birds. (Internote interval was not used in this analysis as it eliminates one-note songs; it was only retained for the morphology analysis.) I used canonical correlation analyses to correlate song (a set of species-specific characters or a set of characters predicted to change for each hypothesis, where appropriate ln-transformed for normality) and predictor variables (Patten et al. 2004) for each zone (older and younger) separately and the zones combined. Canonical R^2 values give the percent overlapping variance, showing the proportion of variation in the two variates associated with each other (Rotenberry et al. 1996; Tabachnick and Fidell 2007). Loadings are used to aid interpretation of variates by showing the correlation of each untransformed variable on the canonical variate (Tabachnick and Fidell 2007). Redundancy analysis (Rotenberry et al. 1996; Tabachnick and Fidell 2007) also shows the proportion of variance in each variate associated with its own variables and with the opposite variables.

To check if the predictor variables changed across the hybrid zone, I correlated each predictor set with the hybrid index. This allowed me to see if any of the variable sets might be covarying with species. Even then, one would not necessarily expect the song and predictor variables to change together in the same way, but these additional correlations help rule out the possibility of covariation as a cause.

Predictor variable sets were: (1) morphology variables to test for biomechanical constraints (bill width, depth, and length , all regressed to wing chord to standardize for body size; and wing chord as a proxy for body size); (2) vegetation structure (mean and standard deviation of distance to nearest tree or shrub, height of nearest tree or shrub, and radius of nearest tree or shrub); and (3) environmental noise above and below the songs [frequency at which the maximum power (loudness) occurs above and below the songs]; and (4) all variables from previous significant analyses involving song versus all song variables (since all were significant in at least some analyses with hybrid index). Figure 12 illustrates hypothesized relationships for predictor sets 1-3.

Results

Species-specific song variables

All song characteristics were significantly correlated with hybrid index in at least one of the hybrid zones (Table 11; Figure 13A-C). Four variables (song duration, phrases per song, notes per phrase, and center frequency) that had loadings ≥ 0.33 in all three analyses (young, old, both zones), so these were used as the species-specific song variables in later analyses. In the younger zone (Wilks' $\Lambda=0.20$, $N=32$, $p=0.01$), Black-crested Titmice had fewer notes per phrase, longer song duration, more phrases per song, longer note duration, shorter phrase durations, and higher center frequency

(Canonical R^2 =0.52; Figure 13A). In the older zone (Wilks' Λ=0.52, N=46, p=0.014), Black-crested Titmice loaded with similar variables excepting note and phrase duration and adding interphrase interval (Canonical R^2 =0.49; Figure 13B). In both zones combined (Wilks' Λ=0.55, N=78, p<0.001; Canonical R^2 =0.44; Figure 13C) loadings were all characteristics except phrase duration. Variation in hybrid index explained 13%, 12%, and 11% of variation in song in the younger, old, and both zones combined, respectively. Each song variate extracts 25%, 25%, and 26% of variance from the song variables for the young, old, and both zones, respectively.

Constraints

Species-specific song variates were not correlated with any of the constraints (Table 12). Only morphology varied with hybrid index (wing chord and relative bill width decrease towards Black-crested Titmouse; Table 11), but it did not vary significantly with any of the song variables predicted for morphology.

Vegetation structure correlated with songs in the younger zone (and in the dataset of both zones combined, presumably due to the younger's influence in the overall dataset), with shorter note durations occuring in vegetation that is shorter and less evenly spaced (Figure 14; Table 11). Variation in vegetation structure explained 8.6% of variation in song in the younger zone (Canonical R^2=0.91) (6.6% in both zones combined, canonical R^2=0.55). The young zone vegetation variate only extracted 9.4% of vegetation variables, while the song variate explained even less (6.8%) of song variables. Although the vegetation for Tufted and Black-crested appeared to cluster somewhat for the younger zone (Figure 14), there was no correlation for hybrid index and vegetation structure, nor was there a significant difference (Type III MANOVA:

Wilks'$_{4,18}$=0.77, p=0.30) between parental species alone (hybrid index 0 as Tufted and 6 as Black-crested).

Environmental noise above and below songs both correlated with song frequency in the older zone (Canonical R^2=0.14), and in the dataset of both combined, again presumably due to the older's influence in the overall dataset (Canonical R^2=0.09), such that songs are higher frequency where noise below has decreased and noise above has increased frequency (i.e., there is a wider "gap" in noise available) (Figure 15; Table 11). Variation in noise explained 14% variation in song in the older zone and 9.4% in both zones combined. The noise variate extracts 51% and 54% of variance from noise variables in the older and both zones combined, respectively. (The song variable is 100% of variance, as it contains only one variable: center frequency.)

All noise and vegetation variables combined (an "environmental constraint" variate) was significant when correlated with all song variables in the younger zone (Wilks' Λ=0.000016, N=16, P=0.001; Canonical R^2=0.998), marginally so in both zones combined (Wilks' Λ=0.14, N=37, P=0.08; Canonical R^2=0.62), and not significant in the older zone (Wilks' Λ=0.03, N=21, P=0.44). Loadings (Table 11) showed that, in the younger zone, songs had shorter interphrase intervals, more phrases per song, longer song durations, and higher center frequencies in locations with short, unevenly-spaced trees with lower-pitched peak noise below the song (Figure 16). Together these environmental constraints explained 12% of the song variation. The environmental constraint variate explains 10% of the variance in its variables, while the song variate explains 12% of its own variables. In the marginally significant analysis of both zones' data combined, the environmental constraints together explained 7.2%.

Discussion

Species-specific differences and constraints on song

My results for species-specific differences are generally consistent with previous results from central TX (Coldren 1992), where Black-crested Titmice were found to have higher frequency songs with more phrases per song and shorter phrase durations (this latter feature I found in the younger zone only). Coldren's interphrase intervals differed by site but not species; in my study it differed by species in the old zone (which is where his study sites were located) but not in the younger zone. In the young zone interphrase interval differs with vegetation and noise (see below), so this song feature seems to be variably influenced by the environment.

Morphology failed to correlate with any song features, despite having a significant difference between species; Black-crested are smaller with relatively narrower bills. This is not surprising considering the how slight the differences are in overall size (Dixon 1955; Curry and Patten 2014), for frequency, despite previous speculations that frequency differences might be due to the size difference (Coldren 1992). Additionally, the previous work done on biomechanical bill constraints is also for birds that trill at rapid rates (Podos 1997). Titmouse songs are comparatively slow repeated whistles without trills. Additionally, while between species body size is correlated with frequency, within species a relationship has not always been found (Ryan and Kime 2003).

When songs were analyzed separately for the old (here including both north-central and central TX) and younger (OK) hybrid zones, the distinguishing song characteristics were similar excepting interphrase interval and note and phrase duration

(both of which are predicted to vary with vegetation structure), which were significant in the old zone (interphrase interval) and young zone (durations). Note duration, which is loaded at 0.44 in the younger zone for songs vs. hybrid index, is found to decrease in shorter, less evenly-spaced trees; however, this change is opposite of predictions (Figure 14). It might be that another distorting feature of the environment, such as potentially increased wind in an open environment ("nonstationary heterogeneities" of Wiley and Richards 1978), is present in that habitat structure. With disturbances causing the amplitude of a signal to vary wildly to the receiver (Wiley and Richards 1978), shorter notes might be helpful for conveying information without distortion in between lower amplitude moments. More repeats of each phrase and a longer song (as are found in Black-crested Titmice) could also help in getting the information to the receiver between hypothetical gusts of wind. Additionally, the loadings on the variate (unevenly spaced, shorter trees) could be characteristic of either an understory (which would be more "dense") or a scattered savannah-like environment. The radius of the trees would be required to distinguish such, but radius has a very small loading (0.068) in this analysis. Future work should record weather data along with habitat variables to better address these potential distortions of the signaling environment.

When noise and vegetation environmental variables are combined, results are as predicted for the acoustic adaptation and noise hypotheses (Figure 12) except for distance (Figure 16). However, phrases per song and song duration are not expected to correlate with noise or vegetation structure. Decreasing song duration and fewer phrases per song might be simpler to detect in a noisy environment as the peak frequency of noise below the song increases (i.e., gets closer to the song itself). Songs might instead

be repeated more often for detection (Brumm and Slabbekoorn 2005), although the little work done on this has found that individual signals are lengthened, not shortened as here, to increase detectability. On the other hand, repeated songs should be shorter to save on energetic costs (Ryan and Kime 2003). I do not have data on rates of song repeats themselves (only the phrases and notes within the songs), so more research into this aspect of noise and song would be worth investigation. Songs from locales with tall, widely spaced trees have longer interphrase intervals as predicted (though predicted, intervals were not found significant in the meta-analysis of Boncoraglio and Saino 2007). However, these birds in the "open" environment are Tufted and Tufted-like (Figure 16), contrary to statements in the literature ("widely spaced trees" of Oberholser 1974; Dixon 1978) about Black-crested Titmice inhabiting more arid and open environments. This apparent contradiction may be a problem of the human perspective as to what constitutes an open environment. Scattered but dense clumps of trees and a savanna-like woodland are somewhat similar for human purposes in that one walks through open spaces to get to a tree containing the bird. In a continuously forested area (such as that said to be characteristic of Tufted Titmice), the habitat is more continuous, and hence denser to our perspective. Nonetheless, at the actual spot where the titmouse is, the vegetation may be dense (such as in a brush-tangled small clump of trees surrounded by open areas for a Black-crested Titmouse, or in a forest with a dense understory for Tufted) or open (riparian zones with large trees and little understory in either species). Corroborating this, the measured structures in my study show no significant differences between species in MANOVA nor do they show a correlation across the hybrid index (i.e., across the hybrid zones) in canonical correlation analysis.

A close examination of habitat descriptions in Dixon (1955) supports this, because although the habitats for a Black-crested Titmouse sound more open from the "human" perspective, they do include dense tangles of brush in its more broadly open environment.

Overall patterns

From my analyses, titmouse songs in these populations do correlate well with vegetation and noise, particularly in the younger zone, just not necessarily with regards to species boundaries and with low explanatory power for the variance (vegetation explaining less than 10% of song variation). The four variables that load on the song variate in the combined constraints analysis all do correlate with species (Table 11) in at least one or both zone. Noise and vegetation do not appear to be additive, as the percent of song explained does not increase much in the combined analysis when compared to the individual analyses. As noise could include anything from other birds to insects at frequencies above or below the focal songs, to low-frequency wind-induced vegetation movement (Brumm and Slabbekoorn 2005), some interaction is to be expected. Acoustic adaptation is less common than predicted (Ey and Fischer 2009), tends to have low explanatory power as I have found here (Boncoraglio and Saino 2007), and is difficult to disentangle from other factors (Nottebohm 1975). Noise, while important, also does not always vary with song characteristics in other species (Ripmeester et al. 2009). Thus, the main message here is that the constraints on song are complex. Recent studies are now attempting to examine multiple factors simultaneously (Slabbekoorn and Smith 2002b; Derryberry 2009; Kirschel et al. 2009), so my study adds important data with the additional twist of having two gradients, one where the birds have been in

contact for a longer period of time. My data suggest that the younger zone is more influenced by vegetation structure and noise. This is as expected if sexual selection has a longer time period to act in the older zone (within environmental constraints), which appears to be the case in this system (Chapters 4 and 5). Features such as number of notes per phrase, song duration, and number of phrases per song are all different between the two species while only the latter two are correlated with vegetation (in the young zone only). This leaves additional space for such characteristics to be sexually selected, within environmental constraints (Wilkins et al. 2013) as interactions continue between the two species. This suggests that the influence of environmental and social factors will change with continued contact.

Acknowledgments

J. Curry and P. Curry aided with parabola and recorder issues. A. Ainsworth measured environmental noise in numerous song recording files.

Figures and tables

Table 11. Loadings for canonical correlation analyses. Boldface values are interpreted as they are significant (Table 12) and >0.33.

	young	old	both
Song vs. hybrid index (one variable, no loadings shown)			
Song duration	**0.542**	**0.789**	**0.681**
Phrases per song	**0.457**	**0.488**	**0.44**
Notes per phrase	**-0.886**	**-0.54**	**-0.746**
Center frequency	**0.325**	**0.544**	**0.492**
Interphrase interval	0.251	**0.425**	**0.415**
Note duration	**0.44**	0.262	**0.424**
Phrase duration	**-0.361**	-0.102	-0.134
Veg. vs. song			
Song variate			
Notes per phrase	0.072	0.533	-0.282
Note duration	**-0.895**	0.025	**-0.859**
Phrase duration	-0.053	-0.73	0.113
Center frequency	0.09	-0.018	**-0.33**
Interphrase interval	0.083	-0.396	0.183

	young	old	both
Vegetation variate			
Distance (mean)	0.219	-0.352	-0.194
Distance (sd)	**0.634**	0.411	**0.541**
Height (mean)	**-0.718**	-0.706	**-0.426**
Radius (mean)	0.068	-0.433	**0.655**
Song (one variable, no loadings shown) vs. noise			
Peak frequency above	0.888	**0.329**	**0.586**
Peak frequency below	-0.574	**-0.956**	**-0.86**
Morphology vs. hybrid index (one variable, no loadings shown)			
Bill length residual	-0.115	0.062	-0.055
Bill depth residual	-0.231	-0.326	-0.32
Bill width residual	-0.466	-0.23	**-0.346**
Wing chord	-0.817	-0.828	**-0.839**
All environmental variables vs. all song variables			
Environmental variate			
Distance (mean)	**0.334**	-0.227	0.127
Distance (sd)	**-0.51**	-0.555	-0.743
Height (mean)	**0.47**	0.054	0.373

	young	old	both
Radius (mean)	0.255	0.105	0.232
Peak frequency above	0.11	-0.231	0.426
Peak frequency below	**0.548**	0.743	-0.013
Song variate			
Song duration	**-0.361**	-0.412	-0.631
Phrases per song	**-0.527**	0.073	-0.261
Notes per phrase	-0.024	-0.755	0.274
Center frequency	**-0.343**	0.318	0.188
Interphrase interval	**0.556**	-0.12	0.206
Note duration	-0.252	0.249	0.108
Phrase duration	-0.03	0.231	-0.393

Table 12. Canonical correlation results for three predictor variable sets.

		Environmental	Vegetation	Morphology
Species-specific song variables	Young	Wilks' Λ=0.7, N=31, P=0.299	Wilks' Λ=0.37, N=17, P=0.801	Wilks' Λ=0.18, N=12, P=0.831
	Old	Wilks' Λ=0.73, N=43, P=0.145	Wilks' Λ=0.58, N=24, P=0.86	Wilks' Λ=0.32, N=18, P=0.581
	Both	Wilks' Λ=0.83, N=74, P=0.112	Wilks' Λ=0.66, N=41, P=0.555	Wilks' Λ=0.58, N=30, P=0.664
Predicted for hypothesis	Young	Wilks' Λ=0.9, N=31, P=0.235	**Wilks' Λ=0.05, N=17, P=0.042**	Wilks' Λ=0.32, N=12, P=0.801
	Old	**Wilks' Λ=0.86, N=43, P=0.046**	Wilks' Λ=0.36, N=24, P=0.56	Wilks' Λ=0.35, N=18, P=0.322
	Both	**Wilks' Λ=0.91, N=74, P=0.03**	**Wilks' Λ=0.35, N=41, P=0.013**	Wilks' Λ=0.53, N=30, P=0.206
Predictor variables vs. hybrid index	Young	Wilks' Λ=0.94, N=31, P=0.425	Wilks' Λ=0.74, N=17, P=0.418	Wilks' Λ=0.34, N=12, P=0.079
	Old	Wilks' Λ=0.95, N=43, P=0.354	Wilks' Λ=0.88, N=24, P=0.643	Wilks' Λ=0.54, N=18, P=0.072
	Both	Wilks' Λ=0.96, N=74, P=0.233	Wilks' Λ=0.86, N=41, P=0.223	**Wilks' Λ=0.49, N=30, P=0.001**

Figure 10. Sites for song recordings across the hybrid zone (shown with contour lines; see Chapter 5 for details of calculation).

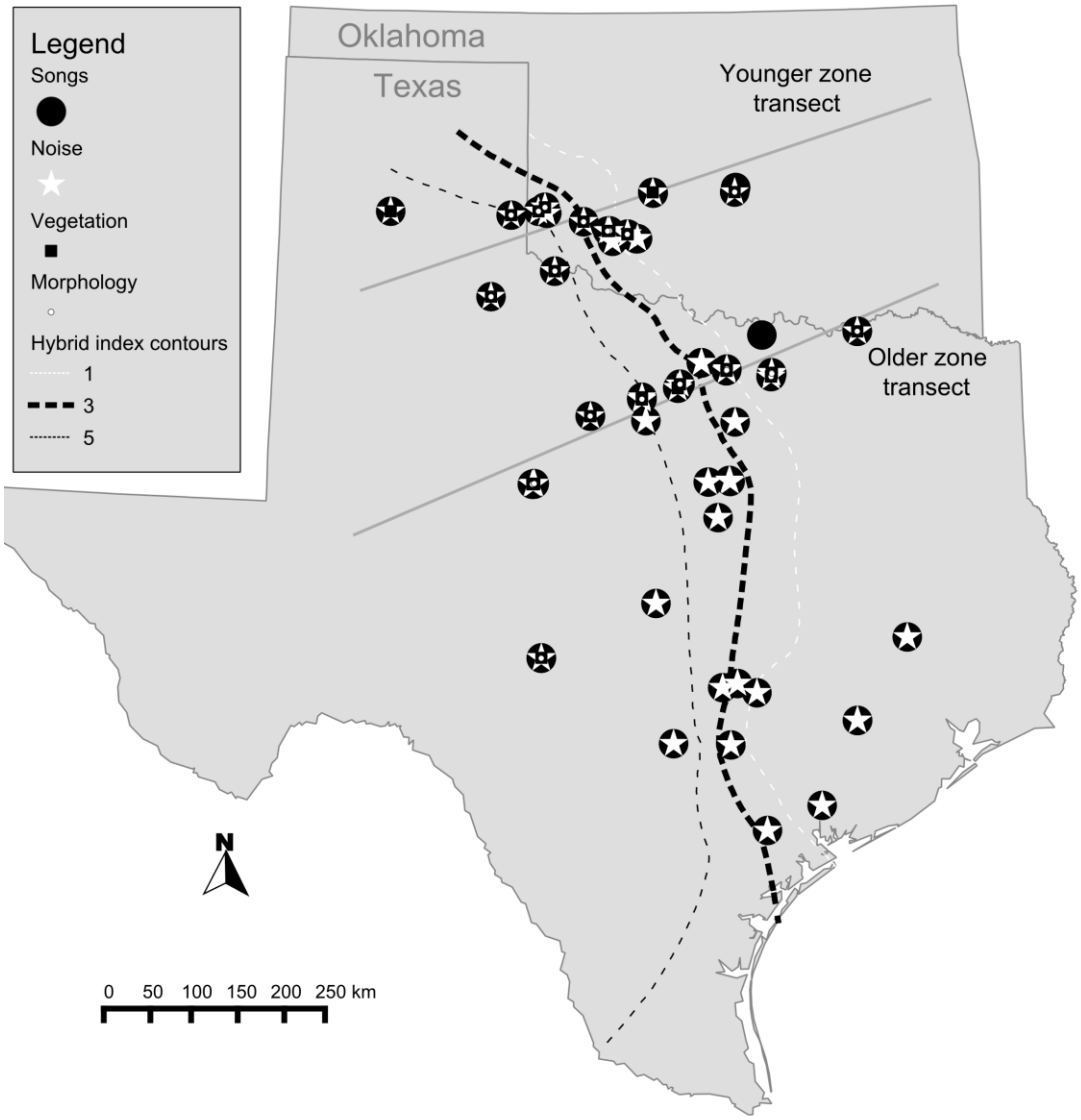


Figure 11. Acoustic measurements for an idealized titmouse song (shown with two notes; birds with one-note songs obviously lack the internote interval)

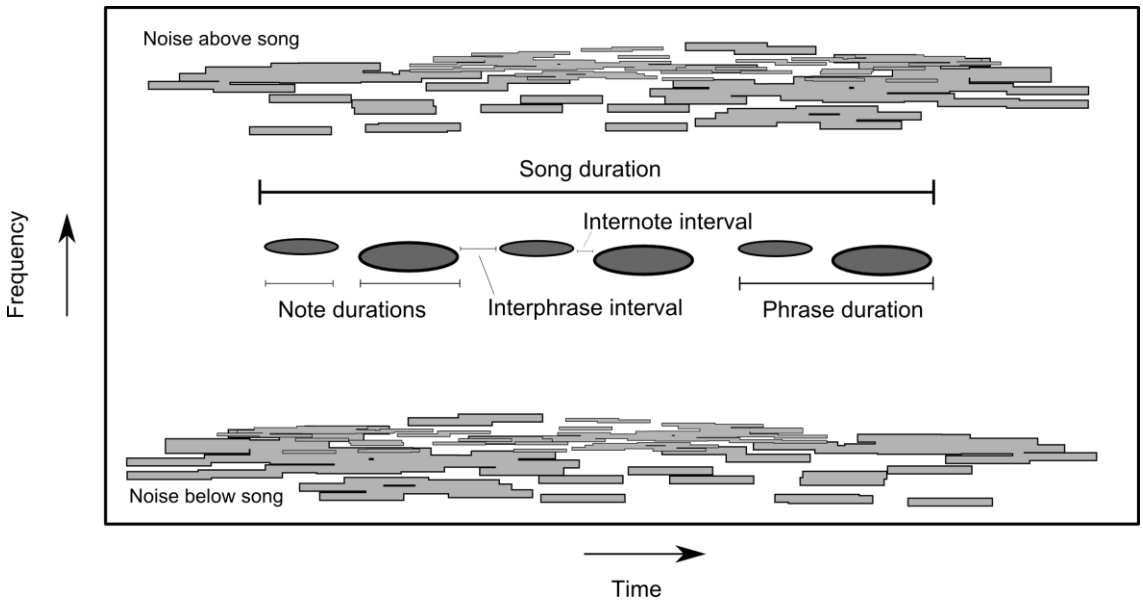


Figure 12. Predictions for morphology, noise, and vegetation structure.

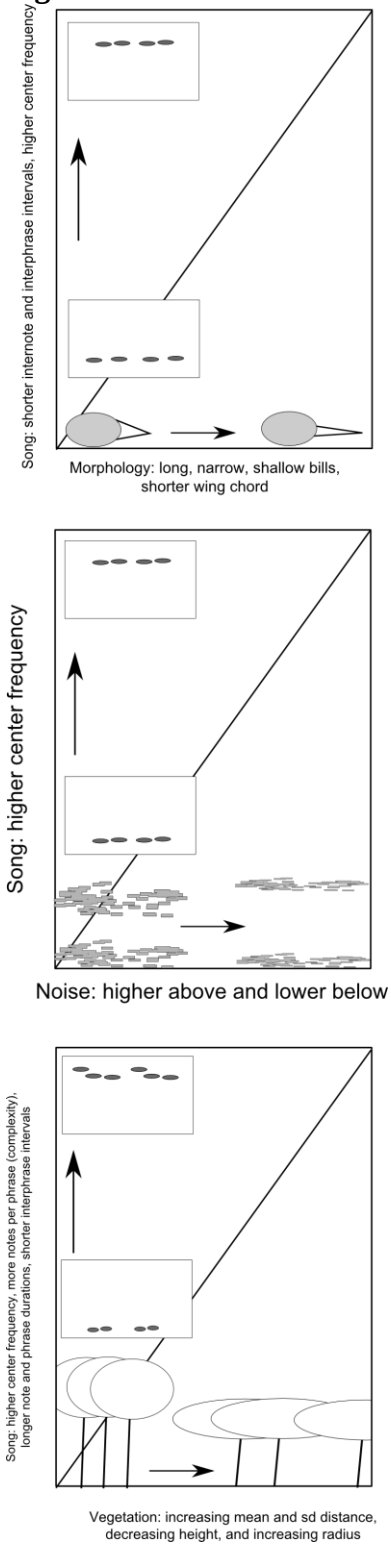
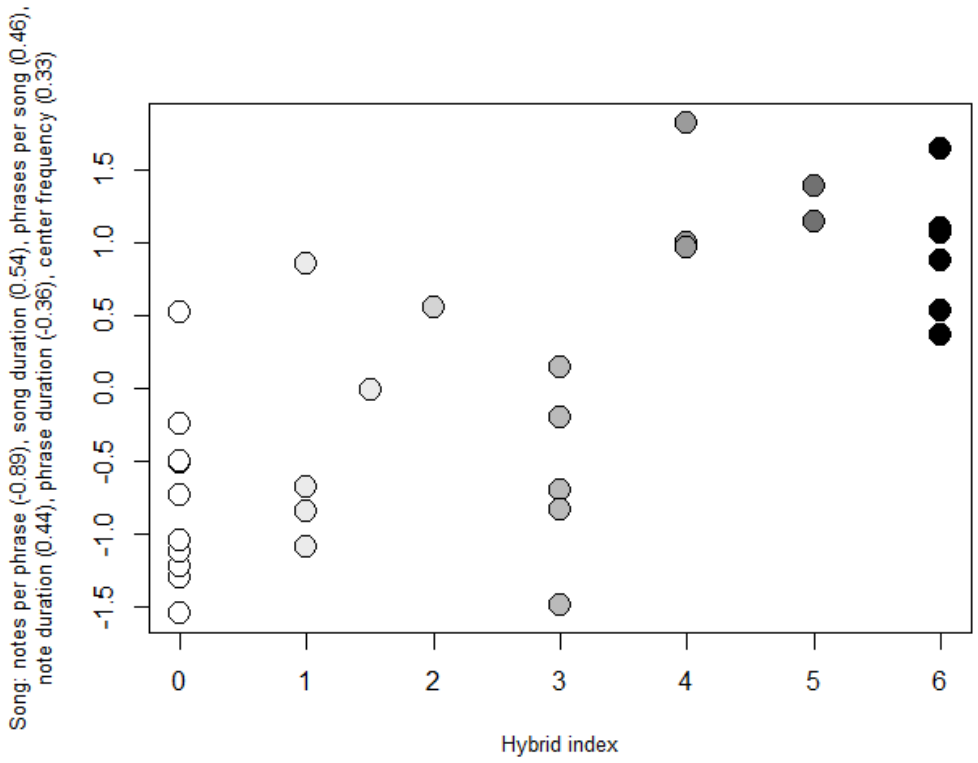
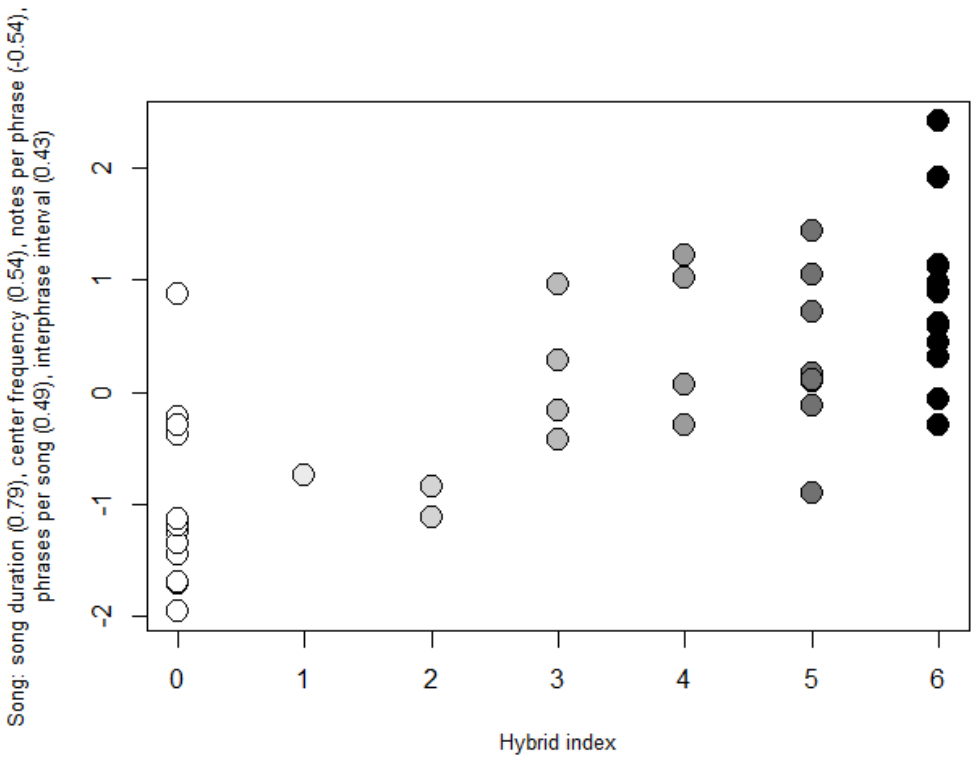


Figure 13. Canonical correlation analysis for hybrid index versus song structure in (A) the young zone; (B) the old zone; and (C) both zones combined. The song variate is shown with variable loadings >0.33 in order of absolute weight. Variables with positive loadings increase along the y-axis; variables with negative loadings decrease along the y-axis.

A



B


Song: song duration (0.79), center frequency (0.54), notes per phrase (-0.54),
phrases per song (0.49), interphrase interval (0.43)
-2
-1
0
1
2
0
1
2
3
4
5
6
Hybrid index

C

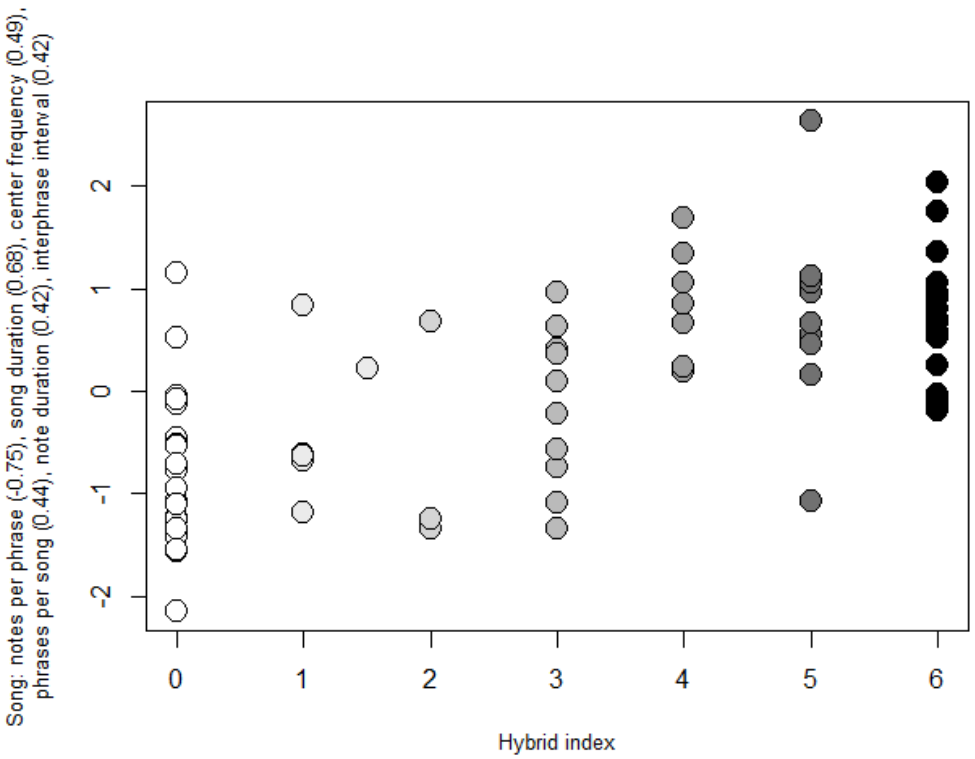

Song: notes per phrase (-0.75), song duration (0.68), center frequency (0.49),
phrases per song (0.44), note duration (0.42), interphrase interval (0.42)
-2
-1
0
1
2
0
1
2
3
4
5
6
Hybrid index

Figure 14. Young zone vegetation versus song variables predicted by the acoustic adaptation hypothesis. The song and vegetation variates are shown with variable loadings >0.33 in order of absolute weight. Variables with positive loadings increase along each axis; variables with negative loadings decrease along each axis.

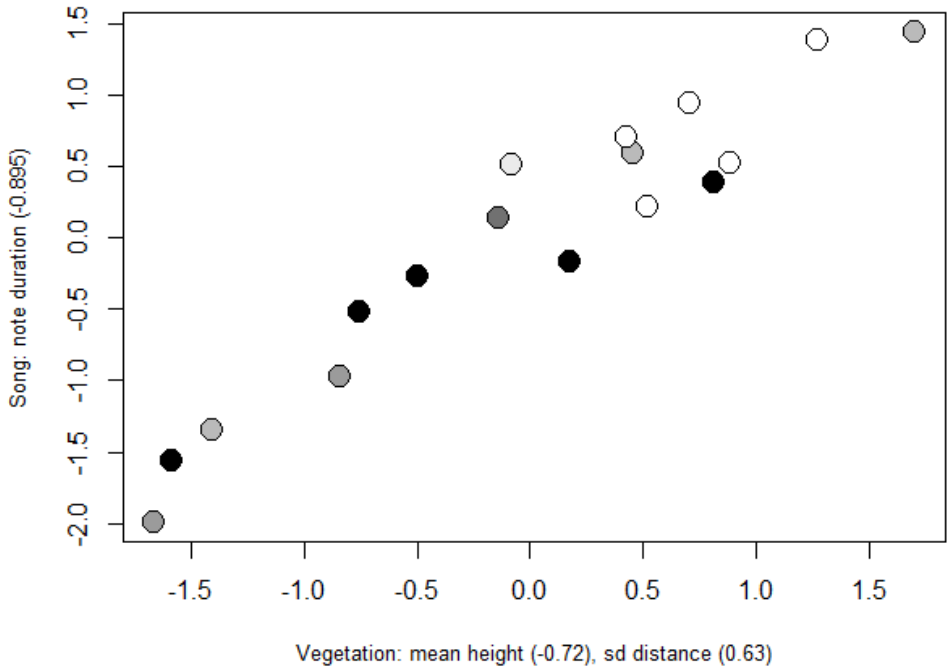


Figure 15. Noise vs. song (center frequency) for the old zone. The two outliers have reasonable values for noise and song, but simply are placed farther along the noise variate. The song and noise variates are shown with variable loadings >0.33 in order of absolute weight. Variables with positive loadings increase along each axis; variables with negative loadings decrease along each axis.

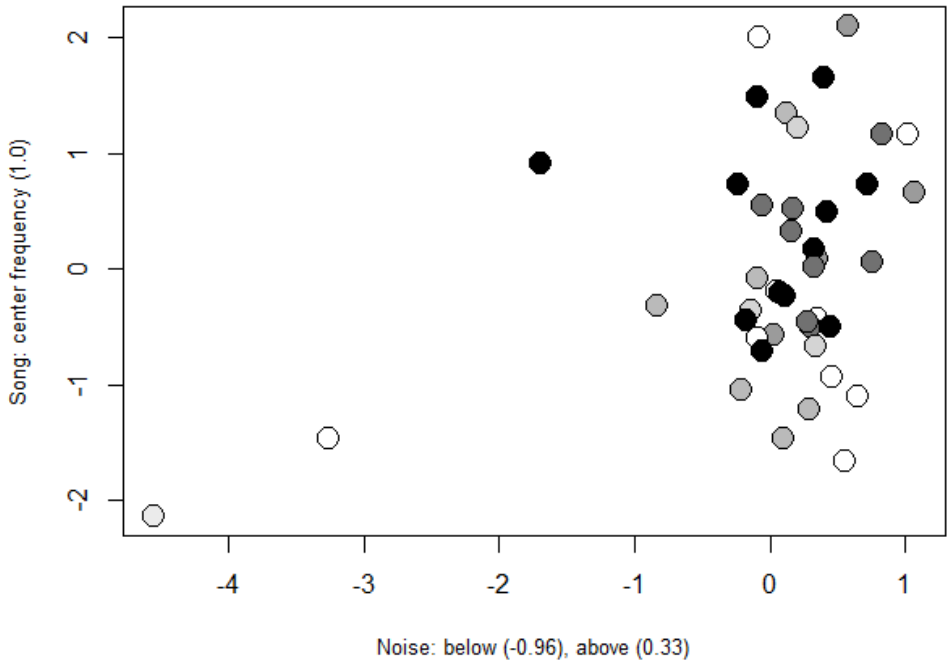
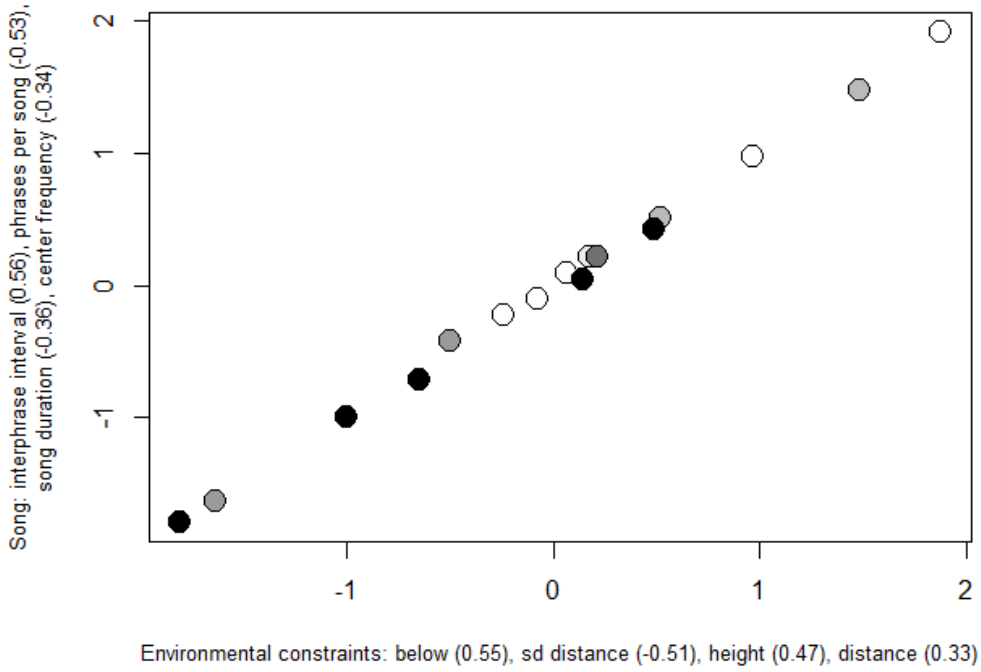


Figure 16. Environmental constraints on all song variables in the young zone. The song and environmental constraint variates are shown with variable loadings >0.33 in order of absolute weight. Variables with positive loadings increase along each axis; variables with negative loadings decrease along each axis.



Chapter 4: Introgression and range expansion in the temporally complex Tufted and Black-crested Titmouse hybrid zone

Abstract

Analyzing patterns of gene flow between species with partial reproductive isolation is important to our understanding of how speciation progresses, especially if I examine zones over time or multiple zones within the same species complex to see how introgression changes with continued contact. Black-crested and Tufted Titmice hybridize in two regions: an older zone in Texas and a younger zone in southwestern Oklahoma. I examined patterns of genetic introgression and diversity in the two species and these zones using mtDNA cytochrome b and 44 nuclear single nucleotide polymorphisms (SNPs) with fixed differences between the species. I found that hybrids possess mtDNA haplotypes usually of the species to which they are phenotypically more similar, and mixtures of SNP genotypes also related to the phenotypic hybrid index. SNP cline widths, where significantly different, are narrower in the younger zone (10/44 SNPs), as is predicted for a younger cline either under neutral introgression or if selection has not yet balanced dispersal for loci under selection. Cytochrome b nucleotide diversity and haplotype networks add to the evidence (previously based on occurrence records and habitat changes) for a recent range expansion of Black-crested Titmouse to form the younger hybrid zone. These data give evidence for the postulated hybrid zone ages in this system and provide a backdrop for behavioral studies (Chapters 3 and 5) by confirming that phenotypic and genotypic classifications of individuals are similar.

Introduction

Hybridization, interbreeding between populations with distinguishable heritable differences (Harrison 1993; Arnold 1997), is useful in understanding how speciation occurs because it provides insights into intermediate stages of speciation where reproductive isolation is not yet complete. Genes exchanged with successful interbreeding then can introgress varying distances depending on the dispersal of the organism and selection against the locus (Barton and Hewitt 1985, 1989). Identifying genetic cline widths for different genes and for different contact zones allows us to begin to determine what selective pressures maintain species boundaries.

Studies with re-sampling of genes allow us to see how zones have changed over time with regards to the extent of gene flow and introgression (Mettler and Spellman 2009; Carling and Zuckerberg 2011; Smith et al. 2013), and with sufficiently detailed analyses, if the genes that introgress are the same in repeated contacts (Nolte et al. 2009). Another way to approach this is to look at hybridizing pairs that have contact zones of different ages, allowing us to see how characteristics change with continued contact (Haavie et al. 2004; Curry and Patten 2014). To my knowledge, however, there has been more focus on behavioral differences in zones of different ages (Haavie et al. 2004) than on genetic differences between such zones.

Black-crested (*Baeolophus atricristatus*) and Tufted (*B. bicolor*) titmice diverged ca. 200,000-250,000 years ago or more (Gill and Slikas 1992; Klicka and Zink 1997). Climatic data indicate a break in continuous habitat around 10,500 BP (Dixon 1978). The two species group separately with good support in recent phyologenetic work (Johansson et al. 2013). Allozyme divergence (Nei's genetic distance=0.063;

Braun et al. 1984) is larger than mtDNA divergence (0.4-0.6%; Avise and Zink 1988; Gill and Slikas 1992), perhaps due to mtDNA introgression (Gill and Slikas 1992).

Until the early 20th century (Sutton 1967; Rising 1983; Patten and Smith-Patten 2008), Black-crested Titmice were not recorded in southwestern Oklahoma. This postulated range expansion, which is also supported by habitat changes (Van Auken 2000, 2009; Callahan 2002) should leave a unique genetic signature (Savolainen et al. 2002; Crandall et al. 2008; Puritz et al. 2012) with reduced diversity and star-like patterns of haplotypes (few differences between haplotypes in the recently colonized areas and ancestral range) in the younger zone Black-crested Titmice compared to the younger zone Tufted Titmice.

The goal of this chapter is to describe patterns of genetic introgression and diversity in the two species to corroborate inferences made about phenotypic (Curry and Patten 2014) introgression, on which other work in this dissertation is based. To those ends, I answer three questions:

1. What mtDNA haplotypes and SNP genotypes do hybrids possess?
2. Does introgression (i.e., cline widths for genes) differ between the young and old zones?
3. Do patterns of haplotype change and nucleotide diversity match the hypothesized range expansion of Black-crested Titmice?

Methods

This chapter includes four main datasets: data collected by the author, data from the San Antonio TX region provided by Dr. Troy Murphy (Trinity University, San Antonio), museum tissue samples (Table 13) for the SNP data, and GenBank (Benson et

al. 2012) sequences: AF347957 as a Tufted Titmouse reference and AF347956 [Bridled Titmouse (*B. wollwebberi*)] and AY607659-AY607688 [Juniper (*B. ridgwayi*) and Oak titmice (*B. inornatus*)] to use as outgroups. Figure 17 shows sampling localities for Tufted and Black-crested Titmice and their hybrids. Birds are categorized via Dixon's hybrid index (1955); see Curry and Patten (2014) for a color representation. I categorize birds with a hybrid index score of 0 as Tufted and 6 as Black-crested; all others are labeled as hybrids (Curry and Patten 2014). These hybrid index values are only available for my dataset and are assumed to be 0 (Tufted) and 6 (Black-crested) for birds far from the hybrid zone (as in the museum tissue samples).

DNA was extracted from my and San Antonio blood samples in stored in Queen's lysis buffer (Seutin et al. 1991) using Qiagen DNeasy Blood & Tissue kits (Qiagen, cat. no. 69504) following a modified protocol for nucleated blood (50 µL amount of blood, and two final elutions of 50-100 µL with TE 10:0.1 buffer). For museum tissue samples (Table 13), I used Qiagen DNeasy kits following the tissue protocol with the modification using two 50 µL elutions with TE 10:0.1 buffer.

mtDNA

To amplify ca. 1000 base pairs of cytochrome b, I used primers L14990 (Gill et al. 2005) and H16064 (Sorenson et al. 1999) (Table 14) using the TopTaq DNA Polymerase kit (Qiagen, cat. no. 200203) in a 30 µL reaction: 23.85 µL water, 3 µL 10X TopTaq PCR Buffer, 0.6 µL 10 µM dNTP, 0.6 µL each 10 µM primer, 0.15 µL TopTaq DNA Polymerase, and 1.2 µL whole-extract DNA) with thermal conditions of initial denaturing 93°C (5 minutes); 30 cycles of 92°C denature (1 min), anneal 50°C (1 min), and polymerize 72°C (1.5 min); and final polymerization at 72°C (3 min). An Applied

Biosystems Inc 3130xl Genetic Analyzer was used to sequence the gene using the same primers. To prevent amplifications of numts (nuclear copies of mtDNA genes: Collura and Stewart 1995), sequence traces were checked for coamplified peaks and mismatches in overlapping sequences from the same individual (Sorenson and Quinn 1998). No numts were found.

Sequences were automatically trimmed in Geneious 6.1.7 (Biomatters, Ltd. 2005-2013) using default settings during de novo assembly (0.05 error and 2 maximum ambiguities on both the 3' and 5' ends, assembled with medium sensitivity) for the L14900 and H16064 strands. Low quality sequences beyond these trimmed ends were deleted; the resulting consensus sequences ranged in length from 331-1009 bp (my sequences and San Antonio). GenBank sequences ranged in length from 900-1059 bp. Sequences were mapped to a reference Tufted Titmouse sequence from GenBank (AF347957), then aligned using ClustalW (IUB matrix, 6.66 gap extend cost, and 15 gap open cost) in Geneious 6.1.7. After alignment, the sequences were trimmed to 650 bp region. The resulting sequences (46 from my samples; 17 from San Antonio) were used for all additional mtDNA analyses.

SNPs

Genotyping-by-sequencing (GBS) following Elshire et al. (2011) was performed on extracted DNA from blood or tissue, resulting in 119 individuals with single nucleotide polymorphisms (SNPs). Using the GBS method, DNA was digested with the PSTI restriction enzyme to generate tens of thousands of DNA fragments. These fragments were Solexa/Illumina sequenced for ca. 100 base pairs and run through the UNEAK pipeline (using the default parameter settings) in the program TASSLE 4.0

(Bradbury et al. 2007), which generated 16,382 SNP loci located across the genome for the samples of each species pair. This complete set of SNPs was used in the Structure analysis. A subset (7242 SNPs where the SNP was present in 80% of the individuals) was used in the remaining analyses. This procedure was conducted by Dr. Jason Weir and his lab (University of Toronto). SNPs were then searched for loci that had fixed differences using the samples highlighted in Figure 17 as "pure".

Hybrid genotypes

I created haplotype networks (Templeton et al. 1992) in the R package 'pegas' (Paradis 2010) using my sequences to examine which mtDNA cytochrome b haplotypes occurred in hybrids. The haplotype network shows the number of mutational steps from one haplotype to the next. I created a network for both species and their hybrids, with one version color-coded to show distribution of haplotypes by species and zone and a second version color-coded by hybrid index.

I used my sequences, the San Antonio sequences, and GenBank sequences to estimate phylogenetic relationships and determine where hybrids are grouped using mtDNA cytochrome b. To choose the best model, I used jModelTest (Guindon and Gascuel 2003; Darriba et al. 2012) and chose the best model available in each program as estimated using AICc. For a Bayesian tree, I used MrBayes 3.2.1 (Huelsenbeck and Ronquist 2001; Ronquist et al. 2012) (via a Geneious plugin) using HKY+Γ substitution model, Γ rate variation (1 tree), 4 Γ categories, 1,100,000 chain length, 4 heated chains, 0.2 heated chain temp, 200 subsampling frequency, 100,000 burn-in length, and unconstrained branch lengths exponential 10. I created a maximum likelihood tree using RaxML 7.4.2 (Stamatakis 2006) in raxmlGUI (Silvestro and Michalak 2012) with

thorough bootstrapping and 10 runs of 500 replicates with the model GTR+Γ. Bridled Titmouse was set as the outgroup for both trees.

Principal components analysis (PCA) was used to summarize the SNPs (Parchman et al. 2013). I plotted the principal components with hybrid index values and zone age. I then used STRUCTURE (Pritchard et al. 2000) to estimate population ancestry using 16,382 SNP loci. I used 5,000 iterations for burn-in (during which alpha and Fst stabilized) and 1,000 iterations with runs for K=1-20. K (number of populations) was selected based on ln likelihood values (visualized using Earl and vonHoldt 2012) and biological interest of populations. Data from the two zones were run together to see if populations were shared between zones. Museum samples from distant sites were used in both the young and old zone graphs but were only included once in the STRUCTURE run.

Geographic clines

To describe the geographic clines, I used three-part cline equations fit using maximum likelihood (Szymura and Barton 1986; Brumfield et al. 2001; Gay et al. 2008) to test if the width of genetic clines differs between the younger and older transects. I used a fixed difference at base 31 in my 650 base pair region of cytochrome b and 44 fixed SNP loci (determined using samples far from the hybrid zone; see Figure 17) to create a total of 45 clines for each zone. I used the R package 'hzar' (Derryberry 2012) to implement these cline methods.

Details on transect placement and calculation are given in Curry and Patten (2014). I averaged individual birds' GPS coordinates by site to use as distances for each location along the transect. Museum samples from distant sites were used in both the

young and old clines, with their distance recalculated for each transect. To fit the model parameters, I used Markov chain Monte Carlo chain lengths of 20,000 (burn-in 500; 10 iterations of the chain). I used Akaike Information Criterion corrected for small sample size (AICc; Derryberry 2012) to compare which models best fit each locus with the fewest parameters (Sokal and Rohlf 2012). Two-parameter models fit center and width only; four-parameter models fit center, width, tau (a cline tail slope coefficient), and delta (distance from the center at which to begin the cline tail); six-parameter models fit center, width, and tau and delta for both tails of the cline separately.

To determine if cline widths were significantly different between the younger and older zones, I used a likelihood ratio test (Hilborn and Mangel 1997, Mettler and Spellman 2009) to compare likelihoods for cline fits. For each comparison, I set the null width as the mean of the young and old widths (Mettler and Spellman 2009) and generated a constrained cline with that width for both the old and young zones. I then summed the likelihoods for the two unconstrained values (young and old) and the two constrained values (young and old). The test statistic R is two times the absolute value difference between ln likelihood values of constrained and unconstrained sums. R is compared to a χ^2 table (degrees of freedom equals the number of zones compared minus one, i.e., df=1 for all comparisons) (Brumfield et al. 2001, Mettler and Spellman 2009). Values for center and width are reported along with two log-likelihood unit support limits.

Range expansion

I used haplotype networks (described above) and calculated nucleotide diversity (π) for cytochrome b in Black-crested Titmouse and Tufted Titmouse using the R

package 'pegas' (Paradis 2010) to check for the reduced diversity that is expected of a recently expanded population. I also used ‘pegas’ to compare mismatch distributions to look for the “wave” in pairwise genetic differences that is characteristic of suddenly expanding populations (Rogers and Harpending 1992).

Results

Hybrid genotypes

Haplotype networks (Figure 18) showed all Tufted and Black-crested Titmouse clustering with conspecifics; hybrids were scattered in both groups. Birds with similar phenotypic hybrid indices (Figure 18A) generally clustered together. Older zone hybrids have haplotypes throughout the network, whereas younger zone hybrids cluster nearer to the younger zone parentals (Figure 18B).

The Bayesian tree (Figure 19A) showed a similar pattern with good support, with all Black-crested Titmice clustered together and hybrids scattered throughout the tree. The ML tree (Figure 19B) had lower support values but a similar topology. One bird had a hybrid index of 5 but is closer to Tufted Titmice in both the trees and the haplotype networks; this individual was found at the western (Black-crested) edge of the older hybrid zone at site “Graham”, where the other birds had Black-crested haplotypes. For a fixed difference between the two species (as used in the geographic clines), it was categorized as a Tufted haplotype. PCA showed that hybrid phenotypes are ordered approximately with SNP genotypes (Figure 20).

Structure results (Figure 21) showed a distinct separation between Black-crested and Tufted Titmice, with hybrid individuals sharing ancestry. K=2 shows a simple view of the transition between the two species (Figure 21A). K=10 (Figure 21B) had the

highest mean ln likelihood (-1282965.9). With K=10, more of the Black-crested genotype occurs east into phenotypically Tufted individuals in the older zone than in the younger zone, such as a few individuals birds at site "Greenbelt". Additionally, Tufted birds show more population structure than Black-crested in both zones with K=10. The hybrid individual at site "Graham" with a Tufted haplotype (the rightmost bird in "Graham") also had four more-characteristically Tufted populations in its SNP genotype (Figure 21B), compared to 2-3 blocks of Tufted-like inferred ancestry for other birds at that site.

Geographic clines

Most loci were best fit with two-parameter clines; however, older zone clines for SNPs 1352 and 2965 were best fit with four parameters (Table 15). Where there was a significant difference between the younger and older zone cline widths (10 of 44 SNPs), the younger zone is always narrower (Table 16; Figure 22). Five loci (4 of 44 SNPs, plus cytochrome b) had wider younger zone clines, but these widths were not significantly different.

Range expansion

All haplotypes found within Black-crested populations were only one mutational step away from the most common haplotype found in Tufted populations, or two steps away from another Black-crested, regardless of whether it was from the young or old zone (Figure 18B). Tufted haplotypes were found up to four mutational steps away from each other, including birds in the young zone. Nucleotide diversity for Black-crested Titmouse (π =0.0016) was lower than for Tufted Titmouse (π =0.0048). When San Antonio samples were included from the main portion of the Black-crested range, this

value did not increase substantially (π =0.0018). Mismatch distributions (Figure 23) also showed a strong wave in pairwise genetic distance frequencies for Black-crested (this included San Antonio samples) and a weaker wave for Tufted.

Discussion

Hybrid genotypes

The Bayesian tree (Figure 19A) supported Black-crested Titmice as a monophyletic clade; this is consistent with recent work (Johansson et al. 2013). Hybrids are scattered throughout the trees and haplotype network as would be expected with extensive backcrossing or symmetric introgression. Structure plots indicate a more sudden transition between the species in younger zone than in the older zone, which matches the known patterns of transition in plumage and morphology (Curry and Patten 2014). Consistent with the greater nucleotide diversity and more complex haplotype networks for mtDNA in Tufted, Tufted Titmice showed much more population structure in both the younger and older hybrid zones. In both K=2 and K=10, Black-cresteds are mostly assigned to only one population, also consistent with a recent range expansion (see below).

Geographic clines

Ten of 45 clines (22%) were significantly narrower in the younger zone than in the older zone, which is what one would expect with neutral loci that have had a shorter time for introgression in that region. Although the cytochrome b cline had no significant difference in width between the zones, it appears to be placed farther west in the older zone (Figure 22). With a Tufted haplotype present at at site farther west (site "Graham"), Tufted haplotypes seem to have introgressed farther into the Black-crested-

like birds. With SNPs, three populations in Figure 21 (shown in green, orange, and yellow) also move from Tufted into Black-crested individuals with a longer "tail" going west than do Black-crested genes in either the younger or older zones. Nonetheless, none of the clines (SNPs or cytochrome b) were best fit with a six-parameter model, which would have allowed for asymmetry in cline shape via differing parameters for each tail.

The younger zone cline is significantly narrower than the older zone cline in 10 SNPs; 29 SNPs that lack a significant difference in width; and a remaining 5 (including cytochrome b) have the younger zone cline wider than the older zone cline. The latter 34 loci that are statistically similar in width between the two zones may have reached equilibrium quickly in both zones. It could also be that there is difference between the zones that is simply not large enough to reach statistical significance. If the differences in width are real for some or all of these 34 loci, then the 29 narrower young zone clines are to be expected with less time for introgression, but the five with wider young zone clines are of particular interest. Cline width is a balance between selection and dispersal (Barton and Hewitt 1985, 1989), so such a pattern would indicate differences in dispersal or selection between the zones. A difference in dispersal is a possibility, as some taxa are known to disperse farther with lower population densities (Trewhella et al. 1988; McGuire et al. 1993) and in more fragmented habitats (Matthysen et al. 1995). Southwestern Oklahoma, where the younger zone is located, does have a lower population density than other parts of the titmouse range (Price et al. 1995) and contains only patchy titmouse habitat compared to more central localities in the titmouse range. Hence the locus could be under similar selection regimes in both zones, but might be

carried farther by dispersing individuals in the younger zone. (Other loci with stronger selection would then still have the expected narrower young zone in this case, as sufficiently strong selection would slow expansion to the equilibrium width.) A difference in selection based on differing conditions (such as climate or habitat) in each zone could also cause a difference in widths between the younger and older zones that is opposite of the expected narrower young zone.

The preceding discussion does not imply that the 10 loci that do have significantly differing widths are either neutral or that they are under selection (directly or linked). Instead, if those loci with differing widths are under selection, the two zones have not yet reached equilibrium (as would be predicted for a balance of selection and dispersal) or that the selection pressure on each locus differs between the two zones. It seems unlikely that differing selection pressures between the two zones would result in all significant differences being in the same direction (narrower younger zone clines) unless the pressures are the same but stronger in the younger zone. Additional investigation using genomic clines to detect which of these loci are under selection and which are selectively neutral, and the type of selection they are experiencing (Gompert and Buerkle 2009; Gompert et al. 2012) would be useful. Mapping the SNPs to a known genome (Cai et al. 2013; Qu et al. 2013) could also allow for detection of what specific selective pressures may have shaped the species during divergence (Qu et al. 2013) and if divergence is occuring in genomic islands (Ellegren et al. 2012) or is scattered across the genome (Parchman et al. 2013).

Range expansion

All Black-crested haplotypes cluster and have few mutational steps from the most common haplotype, as would be expected from a rapid range expansion, whereas the Tufteds in both zones have more steps between one another (Figure 18). All Black-cresteds also seem to share one main inferred ancestry in SNPs, even in the older zone (Figure 21), as might be expected for a species that is recently derived from an ancestral Tufted-like population. Additionally, the lower Black-crested nucelotide diversity, even when including the San Antonio birds from central TX, would be expected if they have diverged in allopatry only recently from a subset of the Tufted Titmouse, as has been hypothesized based on climatic data (Dixon 1978). The mismatch distribution for Black-crested is also consistent with a "wave" that is expected for a recent and sudden range expansion (Rogers and Harpending 1992). The Tufted Titmouse mismatch distribution matches this to a lesser extent. Explanations for this could be that movement of Tufted haplotypes into Black-crested birds is equivalent to a range expansion by pure birds and that the expanding habitat for titmice in SW OK might also be reflected in Tufted Titmice potentially moving westward. These data combined with expanding habitat for titmice in SW OK and western TX (Van Auken 2000, 2009; Callahan 2002) and known historical distributions (Sutton 1967; Dixon 1978; Patten and Smith-Patten 2008) of the Black-crested Titmouse in OK support the hypothesis of recent range expansion for Black-crested Titmouse both into SW OK creating the younger hybrid zone and of the species itself moving into central TX from diverged titmouse populations in Mexico (Dixon 1978).

Overall patterns

Hybrids showed a range of genotypes and were clustered with each parental species mostly based on phenotype. In both zones, birds closer to the hybrid zone sometimes contained genes from the other species, despite being phenotypically pure using Dixon's hybrid index, but this was more common in the older zone, as would be expected with continued introgression. The cline widths, particularly for SNPs, support a longer time since secondary contact for the older zone. Phenotypic clines (Curry and Patten 2014), habitat changes (Van Auken 2000, 2009; Callahan 2002), and mtDNA haplotype networks and nucleotide diversity from this study also all support this hypothesis of longer secondary contact in the Texas zone. Structure data suggests a possibility for slight directional introgression of Tufted genes into otherwise Black-crested birds. Female mate choice experiments (Chapter 5) indicate a slight preference for Tufted plumage and song, which could be a cause of this potentially directional introgression. However, fixed SNP loci do not show any asymmetry in cline shapes. Additional analyses examining selection pressures on SNP loci, both the fixed ones in this study and remaining loci that are polymorphic in one species but not the other, would be useful to gain a deeper understanding of the genetic introgression occuring between the two species and how this affects their reproductive isolation.

Acknowledgments

The following museums kindly provided tissue samples: LSU Museum of Natural Science Collection of Genetic Resources (D.L. Dittman), University of Kansas Biodiversity Institute (M.B. Robbins), and University of Washington Burke Museum (S. Birks). J. Weir helped with several discussions of SNP analysis methods; he and his

lab provided the SNP sequencing. A. Barrera provided help with using STRUCTURE. A. Harris with the OU Biology Core Molecular Lab sequenced mtDNA and provided general advice on lab work. S.H. Stuart and A. Ainsworth assisted with lab work. R. Broughton provided advice on methods and analysis. F. Zhang provided help with phylogenetic trees. A.J. Contina helped with using STRUCTURE and read a draft of the chapter.

Figures and tables

Table 13. Sample numbers, species, and museum for 51 museum tissue samples.

Sample number	Species	Museum
KU6270	Tufted	University of Kansas Biodiversity Institute
KU7061	Tufted	University of Kansas Biodiversity Institute
614	Tufted	LSU Museum of Natural Science
615	Tufted	LSU Museum of Natural Science
616	Tufted	LSU Museum of Natural Science
617	Tufted	LSU Museum of Natural Science
618	Tufted	LSU Museum of Natural Science
876	Tufted	LSU Museum of Natural Science
877	Tufted	LSU Museum of Natural Science
878	Tufted	LSU Museum of Natural Science
3899	Black-crested	LSU Museum of Natural Science
5306	Tufted	LSU Museum of Natural Science
5332	Black-crested	LSU Museum of Natural Science
5341	Black-crested	LSU Museum of Natural Science
5371	Black-crested	LSU Museum of Natural Science
5373	Black-crested	LSU Museum of Natural Science
5636	Black-crested	LSU Museum of Natural Science
5667	Tufted	LSU Museum of Natural Science
5796	Tufted	LSU Museum of Natural Science
8433	Black-crested	LSU Museum of Natural Science

8434	Black-crested	LSU Museum of Natural Science
8693	Tufted	LSU Museum of Natural Science
21795	Black-crested	LSU Museum of Natural Science
23784	Tufted	LSU Museum of Natural Science
23785	Tufted	LSU Museum of Natural Science
36023	Tufted	LSU Museum of Natural Science
37038	Black-crested	LSU Museum of Natural Science
43224	Black-crested	LSU Museum of Natural Science
45503	Tufted	LSU Museum of Natural Science
45588	Black-crested	LSU Museum of Natural Science
46888	Black-crested	LSU Museum of Natural Science
47290	Tufted	LSU Museum of Natural Science
49266	Tufted	LSU Museum of Natural Science
49267	Tufted	LSU Museum of Natural Science
49268	Tufted	LSU Museum of Natural Science
49269	Tufted	LSU Museum of Natural Science
49270	Tufted	LSU Museum of Natural Science
49271	Tufted	LSU Museum of Natural Science
49272	Tufted	LSU Museum of Natural Science
49273	Tufted	LSU Museum of Natural Science
52745	Black-crested	LSU Museum of Natural Science
55064	Tufted	LSU Museum of Natural Science

55097	Tufted	LSU Museum of Natural Science
59057	Tufted	LSU Museum of Natural Science
59498	Tufted	LSU Museum of Natural Science
59616	Tufted	LSU Museum of Natural Science
59620	Tufted	LSU Museum of Natural Science
62385	Tufted	LSU Museum of Natural Science
69156	Tufted	LSU Museum of Natural Science
69562	Tufted	LSU Museum of Natural Science
81916	Tufted	LSU Museum of Natural Science
UWBM 67870	Tufted	University of Washington Burke Museum
UWBM 78020	Tufted	University of Washington Burke Museum
UWBM 78021	Tufted	University of Washington Burke Museum
UWBM 85545	Tufted	University of Washington Burke Museum
UWBM 85573	Tufted	University of Washington Burke Museum
UWBM 85587	Tufted	University of Washington Burke Museum
UWBM 86804	Tufted	University of Washington Burke Museum
UWBM 86806	Tufted	University of Washington Burke Museum
UWBM 86967	Tufted	University of Washington Burke Museum
UWBM 86968	Tufted	University of Washington Burke Museum
UWBM 90142	Black-crested	University of Washington Burke Museum
UWBM 90180	Black-crested	University of Washington Burke Museum
UWBM 90181	Black-crested	University of Washington Burke Museum

Table 14. Primers for mtDNA amplification of cytochrome b (Sorenson et al. 1999; Gill et al. 2005).

Primer name	Sequence (5' to 3')
L14990	CCATCCAACATCTCAGCATGATGAAA
H16064	CTTCAGTTTTTGGTTTACAAGACC

Table 15. Parameter selection for 44 SNPs and 1 base of cytochrome b. Values are AIC_c. Boldface text highlights the two loci that were best fit with a four-parameter model.

	Younger zone				Older zone			
	Two	Four	Six	Best model	Two	Four	Six	Best model
SNP 7	6.0	10.6	15.6	2	11.7	12.8	17.9	2
SNP 205	9.3	13.9	18.9	2	16.6	16.9	20.9	2
SNP 268	7.4	11.2	16.4	2	13.8	14.7	18.2	2
SNP 601	11.8	16.1	18.3	2	13.0	15.7	20.6	2
SNP 706	11.9	15.3	18.5	2	11.5	16.0	19.1	2
SNP 753	7.6	12.0	15.8	2	7.1	11.6	16.1	2
SNP 916	7.2	11.8	16.1	2	14.6	18.1	21.0	2
SNP 968	6.0	10.6	15.6	2	7.4	11.9	16.7	2
SNP 1352	8.7	13.2	17.2	2	**14.5**	**13.2**	**18.7**	**4**
SNP 1354	14.2	18.3	23.6	2	8.3	12.8	17.1	2
SNP 1700	4.6	9.2	14.3	2	8.0	12.5	17.0	2
SNP 1939	6.1	10.6	14.9	2	5.8	10.2	15.0	2
SNP 1946	10.5	15.1	18.6	2	8.8	13.0	17.1	2
SNP 1980	5.7	10.3	15.0	2	13.7	14.5	19.3	2
SNP 1984	9.0	12.4	17.7	2	11.4	15.9	20.0	2

	Younger zone				Older zone			
	Two	Four	Six	Best model	Two	Four	Six	Best model
SNP 2092	5.2	9.8	14.8	2	9.1	11.9	16.8	2
SNP 2221	6.4	10.6	15.5	2	12.9	14.2	18.7	2
SNP 2332	11.3	13.2	20.3	2	6.2	10.7	15.4	2
SNP 2799	7.2	11.8	16.8	2	12.3	13.1	18.0	2
SNP 2929	11.1	15.3	17.2	2	9.9	12.6	16.2	2
SNP 2965	8.7	13.2	16.9	2	**20.0**	**17.2**	**21.6**	**4**
SNP 3265	6.9	11.5	16.1	2	8.9	12.9	16.1	2
SNP 3292	6.2	10.8	15.9	2	27.2	28.3	29.2	2
SNP 3486	8.0	12.6	17.2	2	11.8	15.1	19.5	2
SNP 3577	6.4	11.0	15.8	2	11.7	12.7	17.5	2
SNP 3593	6.0	10.6	15.6	2	6.4	10.8	15.6	2
SNP 3636	8.8	13.3	17.6	2	8.5	11.6	16.0	2
SNP 3753	9.1	13.7	18.7	2	13.9	18.3	22.6	2
SNP 4020	5.7	10.3	15.4	2	10.0	12.4	16.8	2
SNP 4037	7.5	12.1	16.8	2	6.0	10.5	15.3	2
SNP 4226	8.7	13.3	18.3	2	10.8	14.3	18.5	2

	Younger zone				Older zone			
	Two	Four	Six	Best model	Two	Four	Six	Best model
SNP 4331	6.0	10.6	15.6	2	13.0	14.1	18.7	2
SNP 4554	14.3	18.9	21.8	2	6.1	10.5	15.1	2
SNP 4962	6.9	11.5	16.3	2	9.7	14.0	17.4	2
SNP 5151	9.0	13.1	18.4	2	12.4	12.5	17.1	2
SNP 5208	4.7	9.3	14.3	2	14.1	16.5	22.9	2
SNP 5491	8.1	12.6	17.5	2	6.1	10.5	15.3	2
SNP 5991	5.0	9.6	14.6	2	12.1	14.2	18.3	2
SNP 6211	7.6	12.1	16.2	2	12.1	13.0	19.3	2
SNP 6235	6.8	11.4	16.3	2	10.7	14.6	17.6	2
SNP 6345	6.0	10.6	15.6	2	14.5	15.3	19.7	2
SNP 6404	7.6	11.9	16.3	2	8.6	12.9	17.1	2
SNP 6528	8.6	13.2	16.9	2	8.5	12.2	16.8	2
SNP 6533	7.3	11.7	16.9	2	15.6	18.2	21.5	2
Cytochrome b	10.2	18.0	31.0	2	4.9	11.3	20.4	2

Table 16. Test for cline widths in the younger and older zones. Width and center are given with a two-log-likelihood range in parentheses. Boldface rows have a significant difference in width with the likelihood ratio test at P=0.05 (test statistic R). Rows in italic have the older zone wider than the younger zone.

	Younger zone			Older zone			Unconstrained		Constrained				
Locus name	Width	Center	ln L_y	Width	Center	ln L_o	Σ ln L_u	Average	ln L_y	ln L_o	Σ ln L_c	$\Delta = \Sigma$ ln L_c	R
SNP 7	64 (30-145)	254 (227-273)	-0.87	149 (85-282)	424 (384-462)	-3.77	-4.64	106	-1.70	-4.44	-6.13	1.49	2.98
SNP 205	129 (71-270)	251 (214-281)	-2.52	209 (125-369)	403 (355-449)	-6.23	-8.75	169	-2.84	-6.51	-9.35	0.61	1.21
SNP 268	129 (72-270)	247 (209-278)	-1.57	211 (126-375)	406 (358-452)	-4.82	-6.39	170	-1.88	-5.13	-7.01	0.62	1.24
SNP 601	134 (78-283)	243 (201-275)	-3.77	247 (151-440)	401 (349-454)	-4.4	-8.17	190	-4.26	-4.93	-9.19	1.02	2.04
SNP 706	*166 (92-347)*	*266 (225-304)*	*-3.83*	*133 (73-258)*	*434 (396-469)*	*-3.66*	*-7.5*	*150*	*-3.88*	*-3.73*	*-7.6*	*0.11*	*0.22*
SNP 753	72 (34-160)	259 (232-280)	-1.68	153 (87-293)	436 (395-475)	-1.48	-3.15	112	-2.34	-2.05	-4.39	1.23	2.47

	Younger zone			Older zone			Unconstrained		Constrained				
Locus name	Width	Center	$\ln L_y$	Width	Center	$\ln L_o$	$\Sigma \ln L_u$	Average	$\ln L_y$	$\ln L_o$	$\Sigma \ln L_c$	$\Delta = \Sigma \ln L_c$	R
SNP 916	**113 (62-241)**	**262 (229-291)**	**-1.49**	**335 (205-611)**	**428 (368-500)**	**-5.22**	**-6.71**	**224**	**-3.15**	**-6.48**	**-9.62**	**2.92**	**5.83**
SNP 968	65 (30-145)	254 (227-274)	-0.87	88 (44-187)	437 (404-463)	-1.61	-2.48	76	-0.96	-1.69	-2.66	0.17	0.35
SNP 1352	*75 (36-164)*	*265 (238-287)*	*-2.21*	*69 (17-129)*	*411 (384-426)*	*-2.28*	*-4.49*	*72*	*-2.21*	*-2.39*	*-4.60*	*0.12*	*0.23*
SNP 1354	103 (55-222)	279 (250-308)	-4.96	166 (97-300)	400 (356-439)	-2.06	-7.02	135	-5.22	-2.31	-7.53	0.51	1.02
SNP 1700	33 (14-92)	269 (254-285)	-0.2	88 (44-185)	433 (400-460)	-1.91	-2.11	61	-0.89	-2.50	-3.39	1.28	2.57
SNP 1939	71 (37-162)	277 (254-299)	-0.94	78 (39-161)	398 (368-428)	-0.79	-1.73	75	-0.95	-0.80	-1.75	0.01	0.03
SNP 1946	99 (54-206)	246 (214-272)	-3.15	200 (121-354)	389 (340-433)	-2.3	-5.45	149	-3.86	-2.94	-6.8	1.35	2.7

	Younger zone			Older zone			Unconstrained		Constrained				
Locus name	Width	Center	$\ln L_y$	Width	Center	$\ln L_o$	$\Sigma \ln L_u$	Average	$\ln L_y$	$\ln L_o$	$\Sigma \ln L_c$	$\Delta = \Sigma \ln L_c$	R
SNP 1980	99 (51-204)	253 (221-279)	-0.71	144 (83-273)	425 (384-461)	-4.77	-5.48	121	-0.91	-4.94	-5.86	0.37	0.75
SNP 1984	**102 (53-223)**	**294 (267-325)**	**-2.38**	**293 (183-516)**	**390 (331-448)**	**-3.6**	**-5.98**	**197**	**-3.84**	**-4.89**	**-8.73**	**2.75**	**5.5**
SNP 2092	77 (37-166)	255 (227-277)	-0.47	117 (63-229)	428 (393-461)	-2.46	-2.93	97	-0.66	-2.62	-3.28	0.35	0.7
SNP 2221	**82 (42-178)**	**263 (234-285)**	**-1.06**	**216 (132-389)**	**418 (369-465)**	**-4.33**	**-5.39**	**149**	**-2.30**	**-5.32**	**-7.62**	**2.23**	**4.46**
SNP 2332	75 (38-167)	279 (256-302)	-3.5	188 (114-342)	413 (367-457)	-1	-4.5	132	-4.51	-1.88	-6.39	1.89	3.78
SNP 2799	72 (34-158)	256 (229-277)	-1.48	143 (84-272)	420 (380-457)	-4.05	-5.53	107	-2.05	-4.56	-6.62	1.08	2.17
SNP 2929	127 (72-263)	243 (205-272)	-3.45	151 (89-276)	385 (343-424)	-2.85	-6.3	139	-3.48	-2.89	-6.37	0.07	0.15

	Younger zone			Older zone			Unconstrained		Constrained				
Locus name	Width	Center	$\ln L_y$	Width	Center	$\ln L_o$	$\Sigma \ln L_u$	Average	$\ln L_y$	$\ln L_o$	$\Sigma \ln L_c$	$\Delta = \Sigma \ln L_c$	R
SNP 2965	*117 (69-248)*	*260 (225-289)*	*-2.21*	*92 (44-184)*	*442 (415-460)*	*-4.28*	*-6.50*	*105*	*-2.29*	*-4.46*	*-6.74*	*0.25*	*0.50*
SNP 3265	73 (36-158)	250 (220-271)	-1.33	128 (69-243)	429 (393-464)	-2.36	-3.69	101	-1.69	-2.61	-4.3	0.61	1.22
SNP 3292	**50 (21-124)**	**264 (242-281)**	**-0.99**	**448 (278-844)**	**369 (287-446)**	**-11.52**	**-12.51**	**249**	**-7.07**	**-14.63**	**-21.7**	**9.19**	**18.38**
SNP 3486	79 (40-170)	252 (223-274)	-1.87	177 (107-322)	408 (363-449)	-3.81	-5.69	128	-2.75	-4.53	-7.28	1.59	3.18
SNP 3577	62 (28-144)	259 (233-278)	-1.07	149 (85-280)	424 (383-462)	-3.77	-4.84	105	-1.90	-4.48	-6.38	1.53	3.07
SNP 3593	64 (29-145)	254 (227-274)	-0.87	92 (47-189)	432 (400-460)	-1.08	-1.96	78	-0.99	-1.18	-2.17	0.21	0.43
SNP 3636	121 (68-256)	261 (227-291)	-2.26	185 (109-340)	423 (378-466)	-2.16	-4.41	153	-2.47	-2.4	-4.86	0.45	0.9

	Younger zone			Older zone			Unconstrained		Constrained				
Locus name	Width	Center	$\ln L_y$	Width	Center	$\ln L_o$	$\Sigma \ln L_u$	Average	$\ln L_y$	$\ln L_o$	$\Sigma \ln L_c$	$\Delta = \Sigma \ln L_c$	R
SNP 3753	198 (112-463)	191 (108-234)	-2.42	373 (238-686)	388 (318-458)	-4.87	-7.29	285	-3.01	-5.49	-8.5	1.21	2.42
SNP 4020	**46 (15-114)**	**250 (225-267)**	**-0.74**	**167 (96-305)**	**418 (377-459)**	**-2.88**	**-3.62**	**106**	**-2.59**	**-4.13**	**-6.73**	**3.1**	**6.21**
SNP 4037	99 (51-211)	270 (240-296)	-1.64	140 (78-262)	416 (375-451)	-0.92	-2.55	119	-1.79	-1.04	-2.82	0.27	0.54
SNP 4226	160 (91-334)	237 (189-272)	-2.24	198 (117-351)	408 (361-452)	-3.3	-5.54	179	-2.31	-3.36	-5.67	0.13	0.26
SNP 4331	65 (29-146)	254 (227-273)	-0.87	151 (87-281)	429 (388-467)	-4.42	-5.3	108	-1.75	-5.01	-6.76	1.47	2.94
SNP 4554	*172 (99-375)*	*234 (182-271)*	*-5.04*	*94 (50-188)*	*409 (374-438)*	*-0.96*	*-5.99*	*133*	*-5.44*	*-1.48*	*-6.92*	*0.92*	*1.85*
SNP 4962	**74 (37-157)**	**245 (216-268)**	**-1.32**	**182 (107-329)**	**413 (368-455)**	**-2.77**	**-4.09**	**128**	**-2.49**	**-3.56**	**-6.05**	**1.96**	**3.92**

	Younger zone			Older zone			Unconstrained		Constrained				
Locus name	Width	Center	$\ln L_y$	Width	Center	$\ln L_o$	$\Sigma \ln L_u$	Average	$\ln L_y$	$\ln L_o$	$\Sigma \ln L_c$	$\Delta = \Sigma \ln L_c$	R
SNP 5151	101 (53-211)	257 (225-282)	-2.36	136 (76-261)	425 (385-460)	-4.08	-6.45	119	-2.49	-4.19	-6.67	0.22	0.45
SNP 5208	**36 (14-101)**	**267 (250-282)**	**-0.24**	**163 (93-300)**	**419 (379-458)**	**-4.94**	**-5.18**	**100**	**-2.25**	**-6.45**	**-8.69**	**3.51**	**7.03**
SNP 5491	92 (47-196)	266 (237-291)	-1.95	132 (78-247)	406 (367-441)	-0.97	-2.92	112	-2.11	-1.13	-3.24	0.32	0.64
SNP 5991	70 (37-161)	274 (250-296)	-0.36	172 (105-310)	390 (345-430)	-3.93	-4.29	121	-1.25	-4.78	-6.03	1.74	3.49
SNP 6211	159 (88-332)	256 (214-292)	-1.66	213 (130-375)	378 (327-423)	-3.97	-5.63	186	-1.79	-4.12	-5.9	0.28	0.55
SNP 6235	**39 (20-96)**	**278 (262-293)**	**-1.27**	**256 (158-445)**	**387 (333-437)**	**-3.26**	**-4.53**	**148**	**-5.25**	**-5.73**	**-10.98**	**6.46**	**12.91**
SNP 6345	**64 (29-145)**	**254 (226-274)**	**-0.87**	**220 (135-399)**	**432 (384-483)**	**-5.15**	**-6.02**	**142**	**-2.86**	**-6.53**	**-9.4**	**3.38**	**6.76**

	Younger zone			Older zone			Unconstrained		Constrained				
Locus name	Width	Center	ln L_y	Width	Center	ln L_o	Σ ln L_u	Average	ln L_y	ln L_o	Σ ln L_c	$\Delta = \Sigma$ ln L_c	R
SNP 6404	**72 (35-159)**	**259 (232-280)**	**-1.67**	**201 (124-353)**	**378 (329-423)**	**-2.22**	**-3.9**	**136**	**-3.03**	**-3.31**	**-6.34**	**2.44**	**4.88**
SNP 6528	168 (95-359)	248 (201-285)	-2.18	194 (116-353)	413 (366-458)	-2.14	-4.33	181	-2.20	-2.18	-4.38	0.06	0.11
SNP 6533	135 (74-278)	258 (220-289)	-1.54	177 (105-321)	406 (361-447)	-5.68	-7.22	156	-1.65	-5.78	-7.43	0.2	0.41
Cyt b	*79 (27-258)*	*265 (216-312)*	*-2.51*	*27 (3-254)*	*356 (297-409)*	*0*	*-2.51*	*53*	*-2.66*	*-0.03*	*-2.68*	*0.17*	*0.33*

Figure 17. Sampling localities with the young and old hybrid zones. The main transect regions are shown as black lines. Pure samples (large circles) were used to find fixed SNPs. When these large circles are overlaid by a smaller symbol, this indicates the type of collection (triangles are museum tissues; squares are blood samples collected by the author). Small circles are San Antonio area Black-crested Titmice used in the nucleotide diversity analysis.

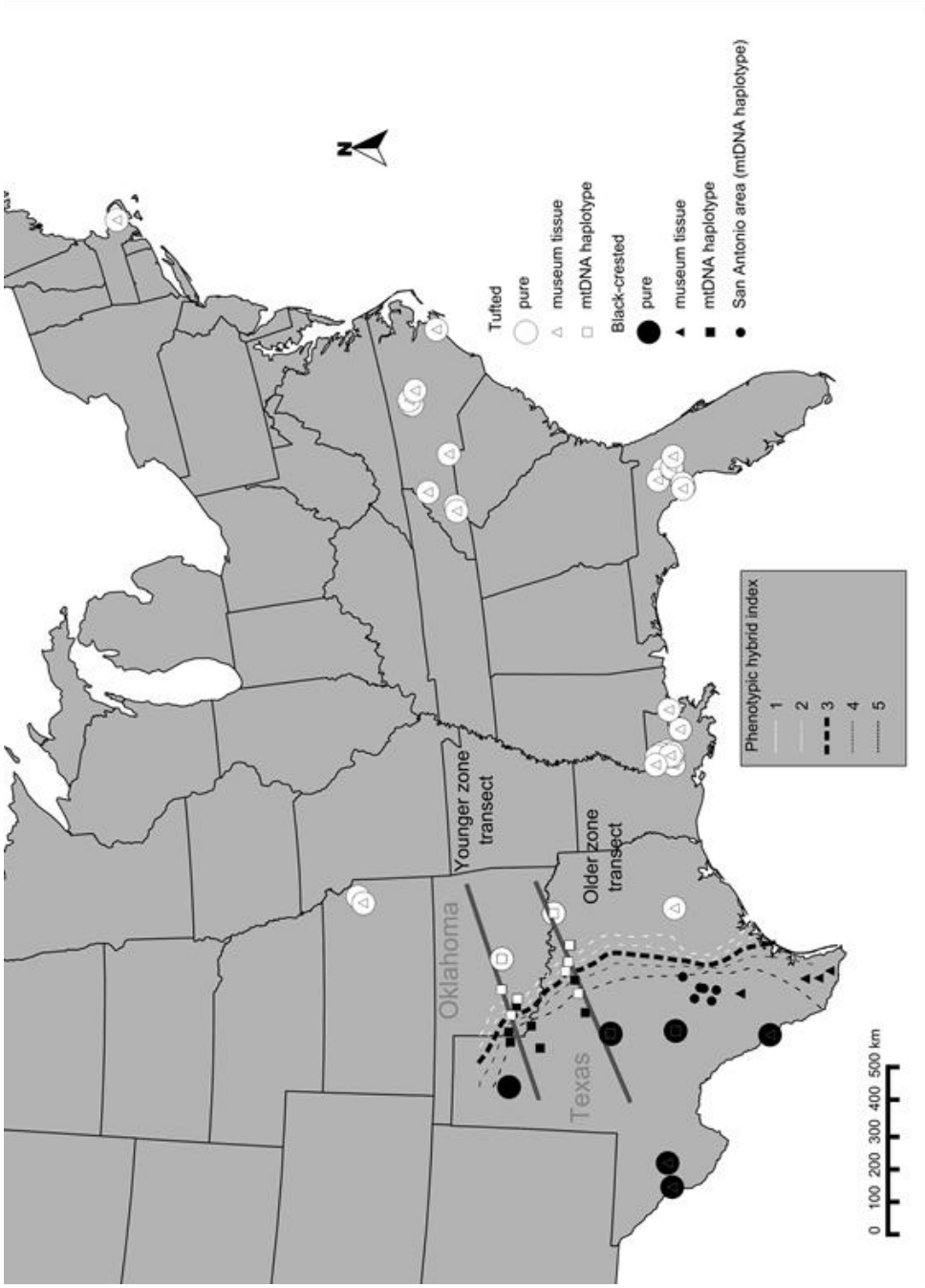
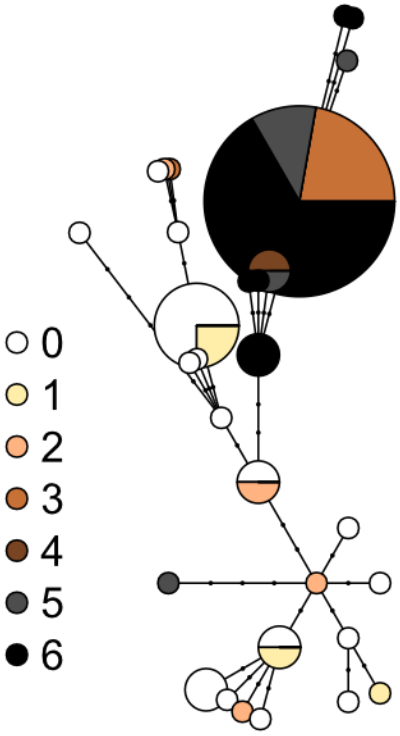


Figure 18. The haplotype network for my samples of Tufted, Black-crested, and hybrid titmice shown with respect to (A) hybrid index (Dixon 1955) and (B) species and zone. Each dot represents one base pair change; haplotype "pie" size is proportional to the number of individuals sampled.

A



B

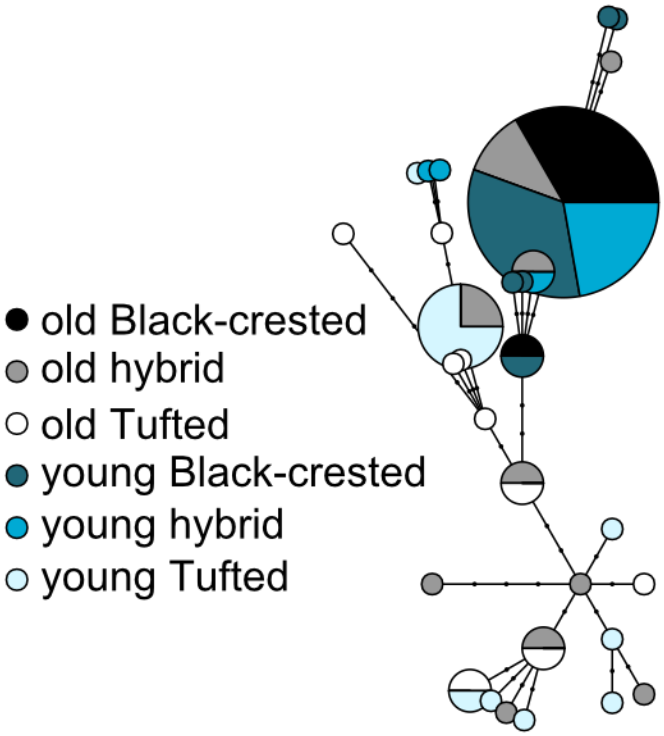
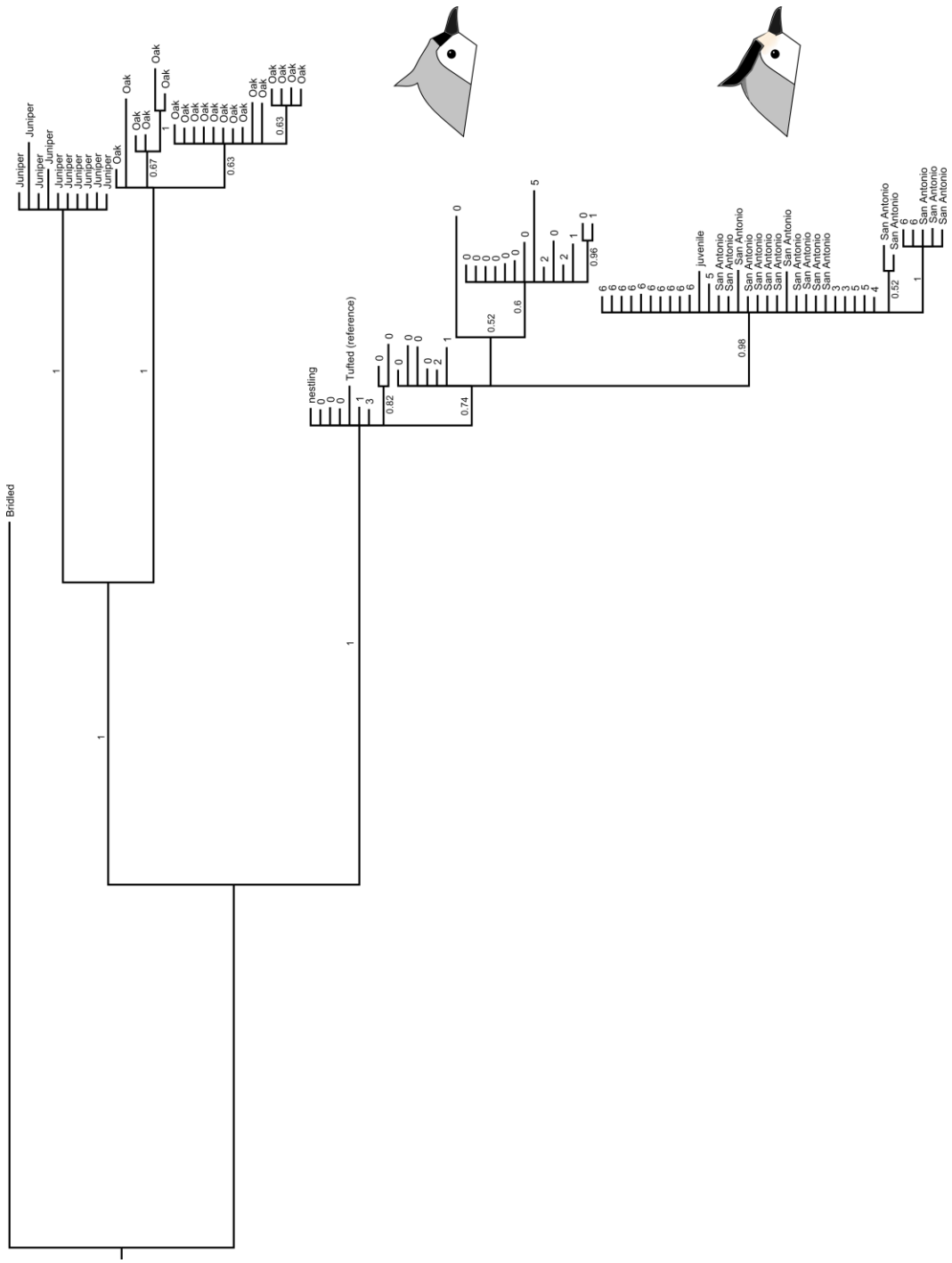

old Black-crested
old hybrid
old Tufted
young Black-crested
young hybrid
young Tufted

Figure 19. (A) Bayesian and (B) maximum likelihood trees. Support values are posterior probability (>0.50 shown) for the Bayesian tree and bootstrap (>50 shown) support values for the maximum likelihood tree. For Tufted, Black-crested, and hybrid titmice, I show hybrid index values at the tip where available. (These values are not available for San Antonio birds or young birds.)
A



B

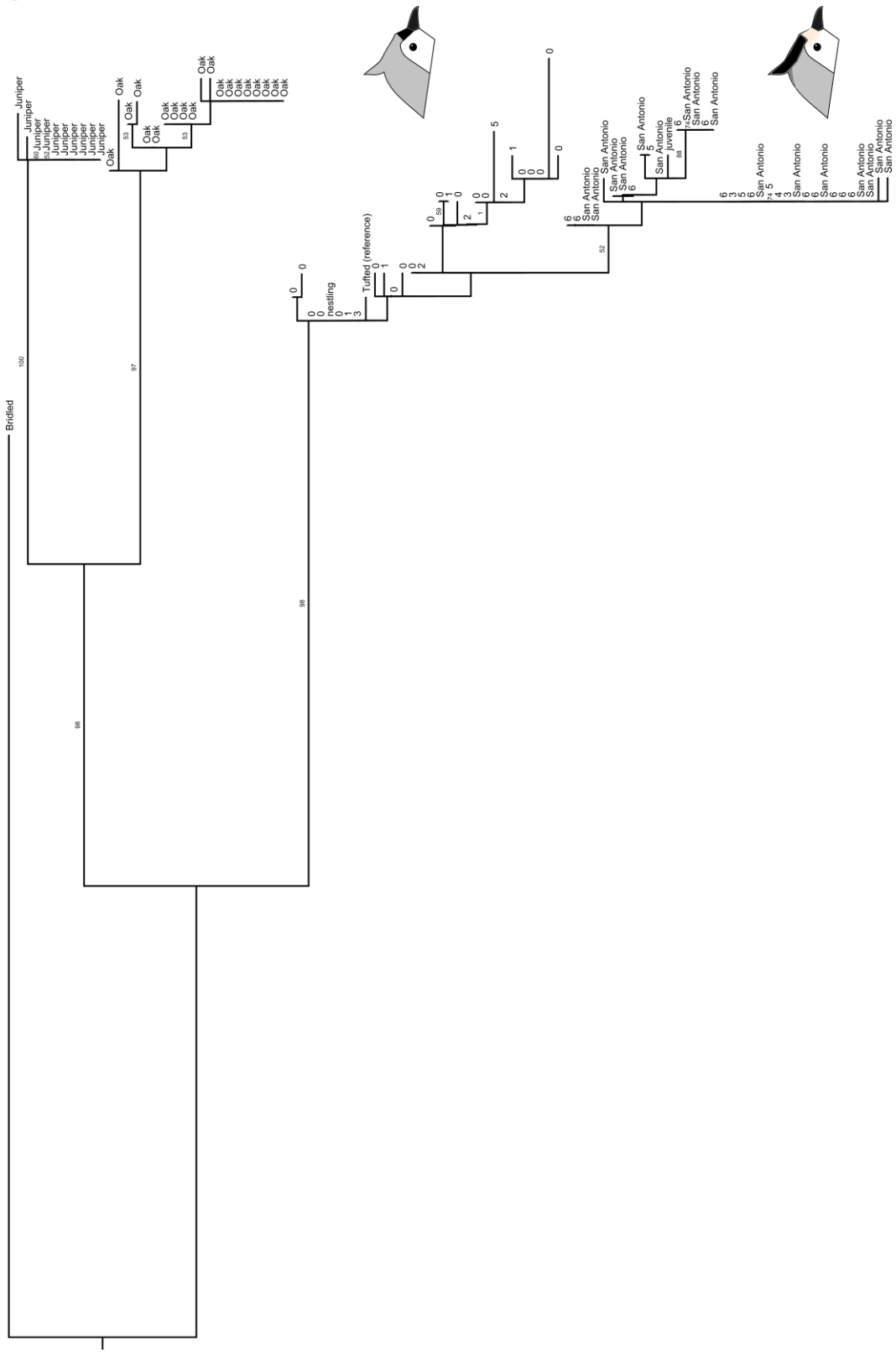

Bridled
Juniper
Oak
nestling
Tufted (reference)
San Antonio
San Antonio juvenile

Figure 20. First two principle components for 7242 SNP loci, color-coded by hybrid index and hybrid zone (shape).

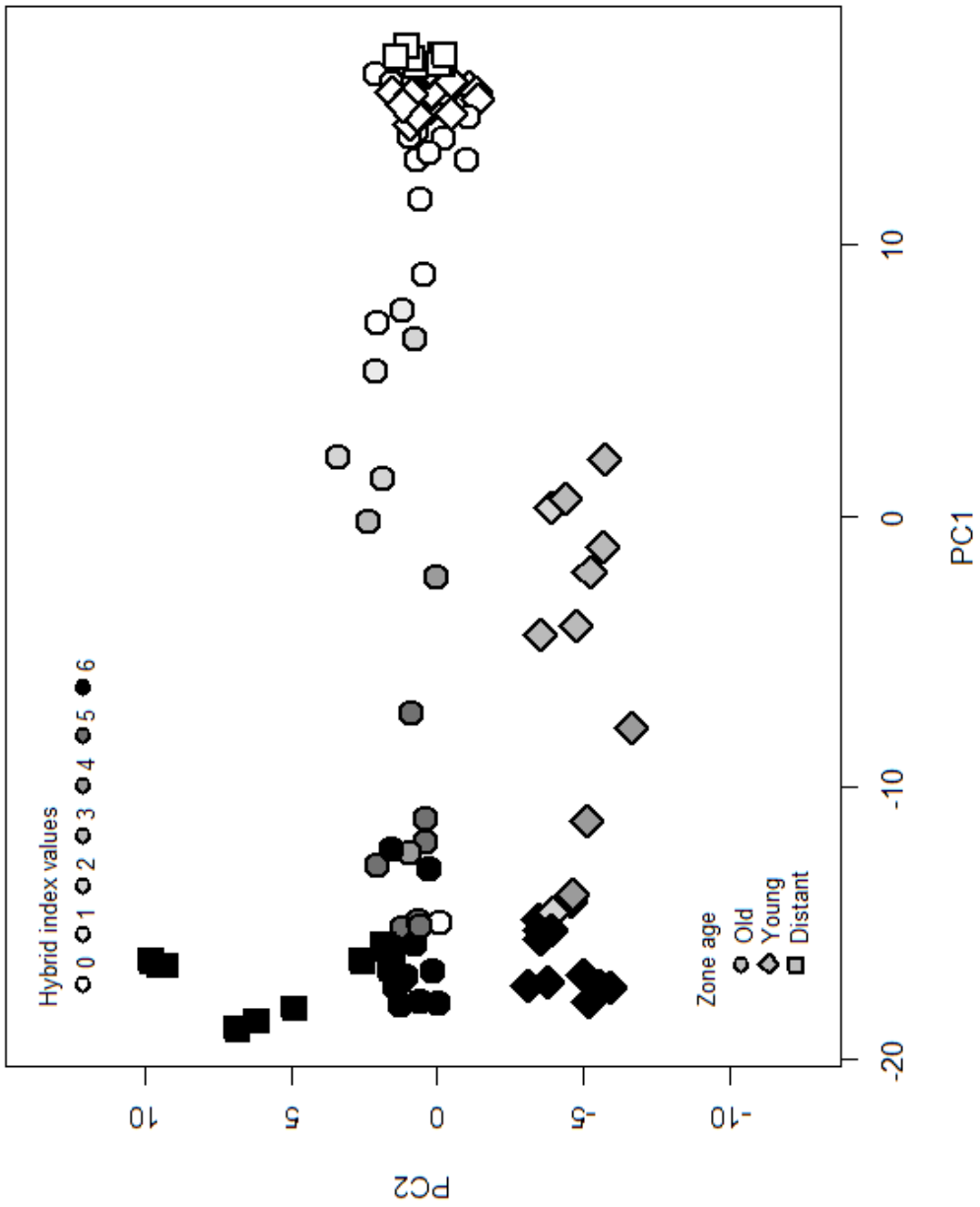
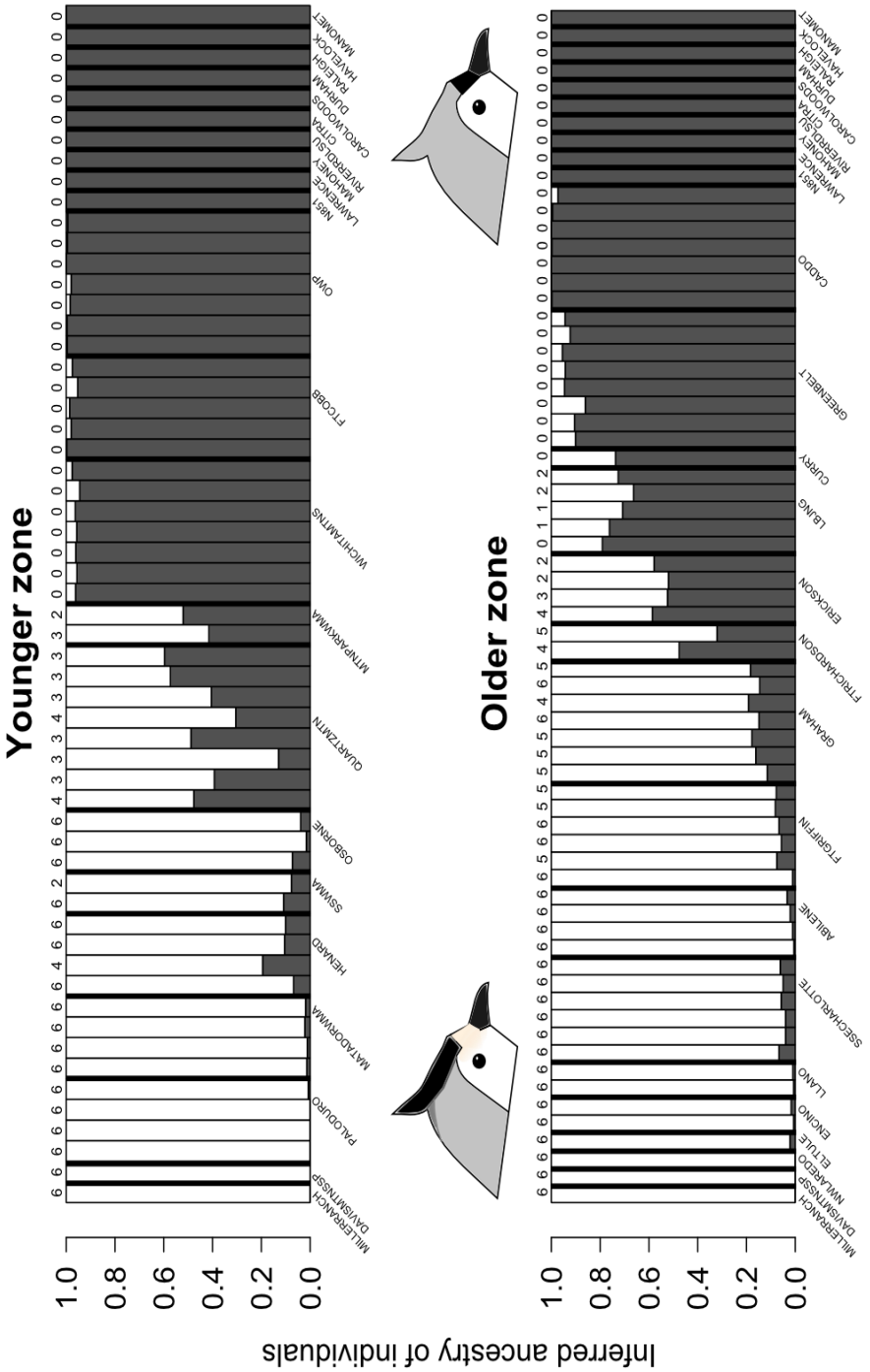


Figure 21. Structure plots with (A) K=2 and (B) K=10. Each bar represents one individual. Numbers above each plot show the phenotypic hybrid index (Dixon 1955; Curry and Patten 2014); phenotypic Black-cresteds are 6; phenotypic Tufteds are 0. Darker lines separate sampling populations, which are labeled below each population.

A



B

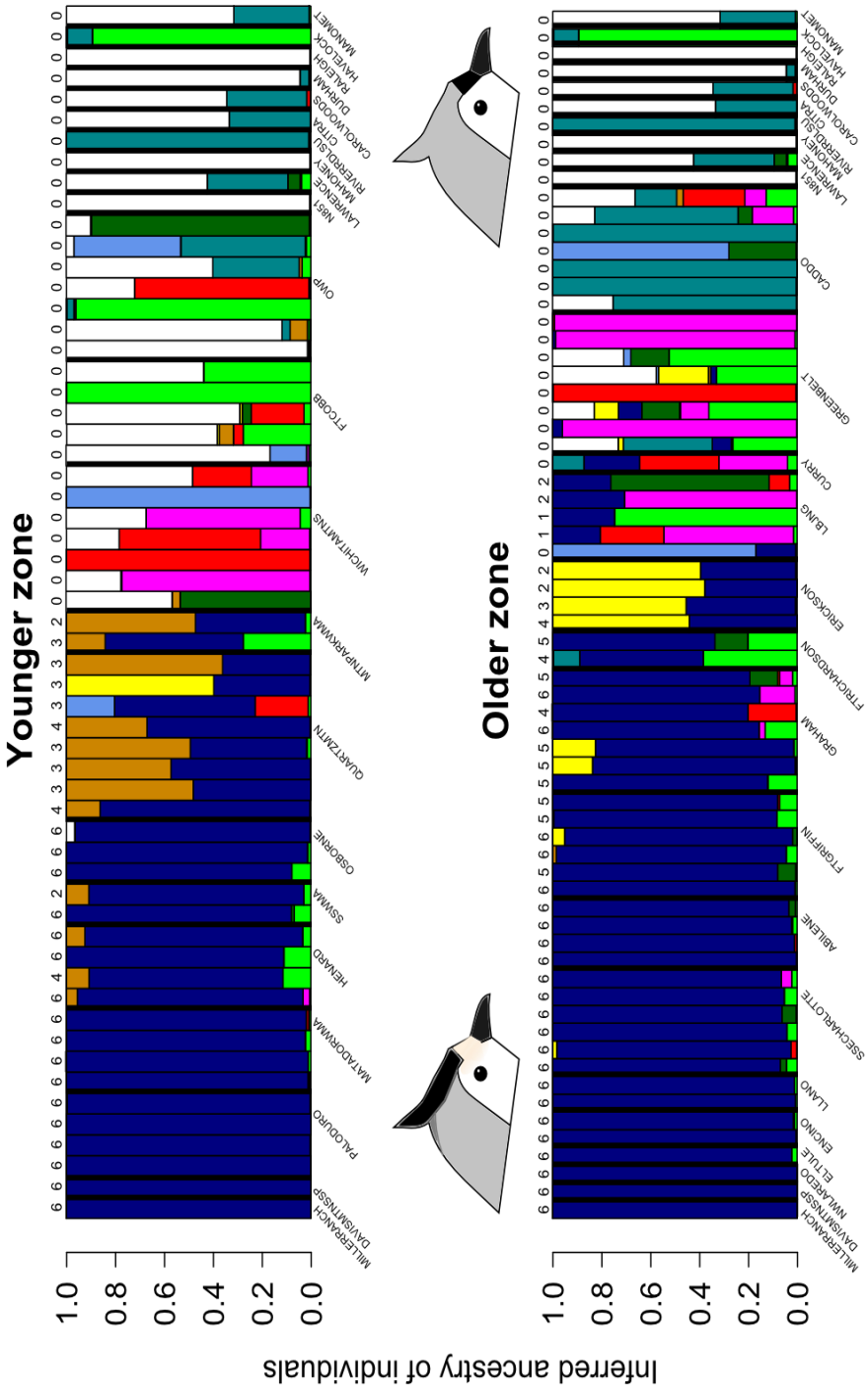

Younger zone
Older zone
Inferred ancestry of individuals
1.0
0.8
0.6
0.4
0.2
0.0
MILLERRANCH
DAVISMTNSP
PALODURO
MATADORWMA
HEMARD
SSWMA
OSBORNE
QUARTZMTN
MTNPARKWMA
WICHITAMTNS
FTCOBB
OWP
N9ST
LAWRENCE
MAHONEY
RIVERSIDE_SU
CTRA
CAROLWOODS
DURHAM
PALEGGY
HAVELOCK
MANOMET
CADDO
GREENBELT
CURRY
L&LNG
ERICKSON
FTRICHARDSON
GRAHAM
FTGRIFFIN
ABILENE
SSECHARLOTTE
LLANO
ENCINO
ELTULE
NWLAREDO

Figure 22. Geographic clines for 44 SNPs and cytochrome b. + show mtDNA frequencies at older zone sites; × shows mtDNa frequencies at younger zone sites. Gray lines represent SNPs with two-parameter models; of these, dashed lines are younger zone clines and solid lines are older zone clines. The dashed and solid black lines are the younger and older zone mtDNA clines, respectively. Dotted black lines are the two older zone SNP clines fit with four-parameter models (Table 15).

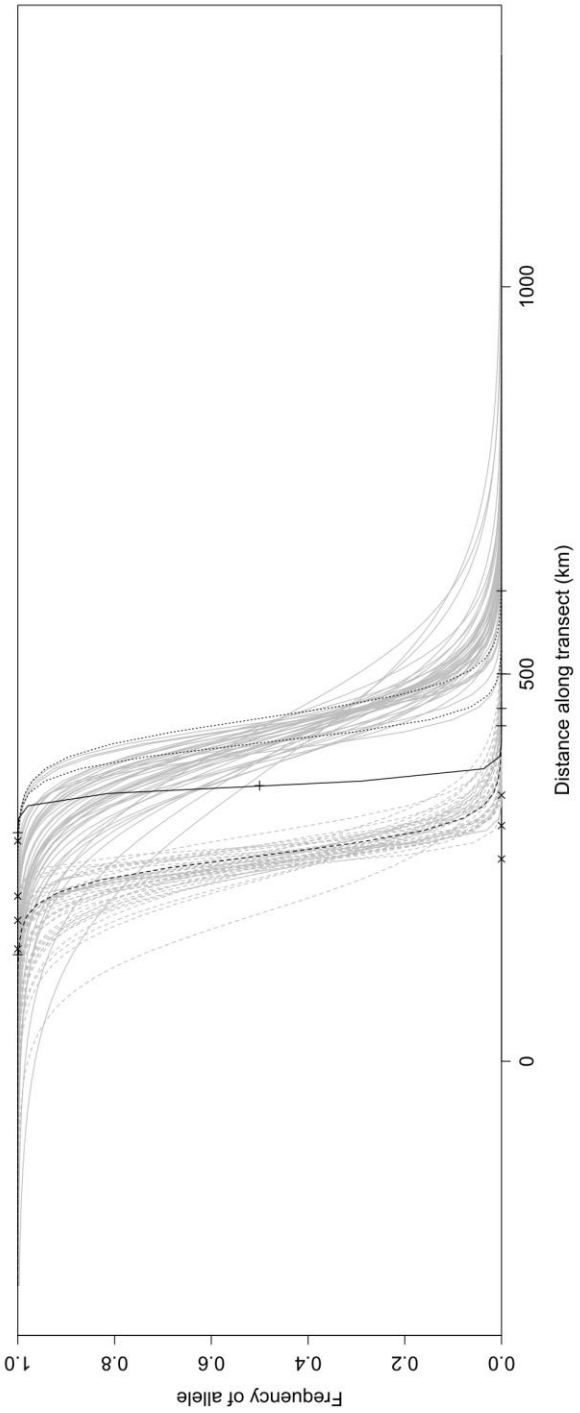
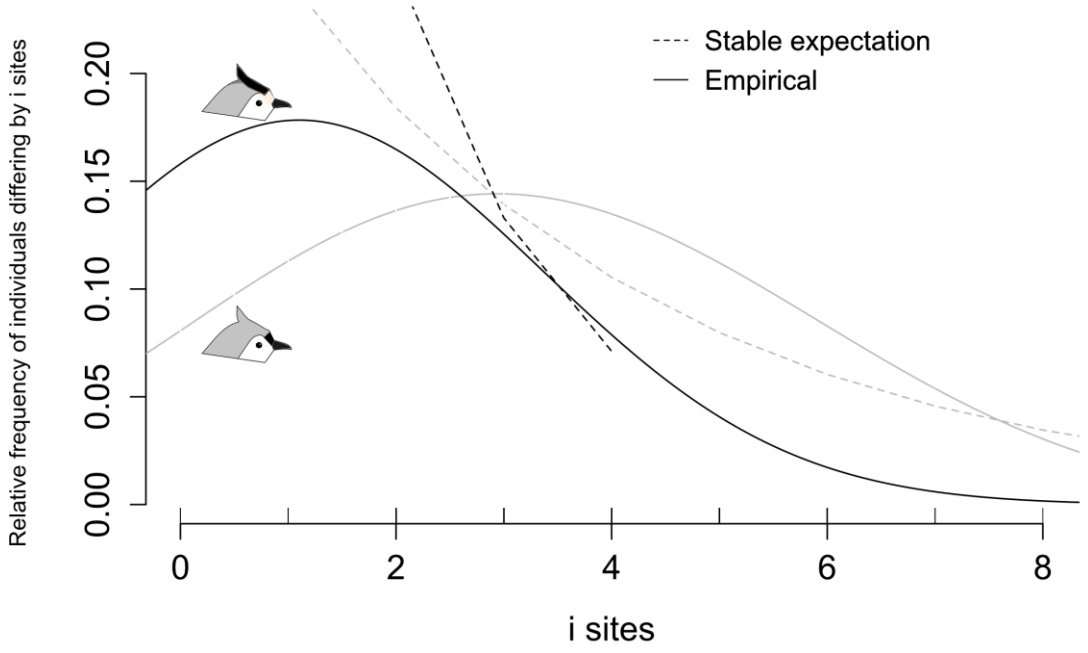


Figure 23. Mismatch distributions for Black-crested (black lines) and Tufted (gray lines). Black-crested includes San Antonio samples.



Chapter 5: Shadow of a doubt: premating and postmating isolating barriers in a temporally complex songbird hybrid zone

Abstract

Understanding the relative importance of reproductive isolating barriers between populations allows us learn what processes are most prevalent in causing speciation. Hybrid zones, where distinguishable populations interbreed, are particularly good systems in which to study how isolating barriers evolve because of the interaction between populations with incomplete reproductive isolation. Examining a hybrid zone over time or with contacts of different ages allows us to sort out which comes first—selection against hybrids, innate preferences for hybrid or parental types, or if one barrier type additionally evolves as a result of the other. One such temporally complex hybrid zone is that of two oscine songbirds, the Black-crested (*Baeolophus atricristatus*) and Tufted (*B. bicolor*) Titmice (family Paridae) in the southern Great Plains of North America; they differ in song, plumage, and genetics. In Texas, the two populations have been interbreeding for several thousands of years across a natural ecotone, while in Oklahoma the two species have contacted within the past century. I specifically tested 1) if males treat songs from other populations as potential competition as measured by agonistic responses and if this differs by zone age; 2) if females show a preference for song or plumage of parentals or hybrids; and 3) for postmating isolation by assessing if there are reproductive consequences for hybridizing individuals by estimating nest success. Males in the older zone respond most strongly to conspecifics, while in the younger zone preferences are occasional or absent. Females may prefer Tufted song and plumage although data are few. Our study provides

evidence that for the titmouse system, there is not a strong initial preference for either species, albeit there may be some sensory bias. These data best match a tension zone model for the hybrid zone, but intrinsic postmating isolation appears to be absent even in the older part of this system. Future studies should focus on potential ecological or behavioral post-mating isolation barriers that prevent the region of hybridization from spreading.

Introduction

Under the biological species concept, speciation results when populations become reproductively isolated (Coyne and Orr 2004). Reproductive isolation can result from premating or postmating barriers. Premating barriers include behavioral isolation (Patten et al. 2004; Guerra and Ron 2008; Ward and McLennan 2009; Dingle et al. 2010; Dopman et al. 2010), temporal isolation (Quinn et al. 2000; Dopman et al. 2010), and habitat differences (Feder 1998; Powell et al. 2012). Postmating barriers can be pre-zygotic, such as mechanical incompatibilities upon mating (Sánchez-Guillén et al. 2012), failure of sperm to fertilize eggs (Palumbi and Metz 1991), or reduced survival as a result of interspecific matings (Matute and Coyne 2010). Barriers can also be post-zygotic, with a range of offspring inviability and infertility possible (Sasa et al. 1998; Price and Bouvier 2002). Reductions in viability can be either intrinsic, because of genetic incompatibilities (Bono and Markow 2009; Matute and Coyne 2010), or extrinsic as a result of poor adaptation to their environment (Hatfield and Schluter 1999; Rundle 2002).

The initial divergences that begin to isolate populations may not always be the same ones that isolate at the later stages of the speciation process (Kim et al. 2013), but

it is difficult if not impossible to follow a speciation event in progress over the typically long periods of time necessary to reach complete reproductive isolation. Understanding the relative importance of these barriers allows us to learn which processes are most prevalent as isolating barriers in speciation. Many studies examine just a few barriers, because of logistical constraints, but increasingly studies examine the relative strengths of more than one barrier (Matsubayashi and Katakura 2009; Dopman et al. 2010; Moriarty Lemmon and Lemmon 2010; Egan et al. 2011; Sánchez-Guillén et al. 2012). Combining these approaches with studying populations at varying stages of divergence (Stelkens et al. 2010; Bracewell et al. 2011; Merrill et al. 2011) allows us to start teasing apart the relative influences of the various barriers and the evolution of them.

Hybrid zones, areas where populations with distinguishable heritable differences meet and interbreed (Harrison 1993; Arnold 1997), are particularly good systems in which to study how isolating barriers evolve because of the interaction between populations with incomplete reproductive isolation. By studying what prevents the two populations from merging completely, we can better understand how, when, and why barriers to reproduction evolve. Several models posit different selection regimes to maintain hybrid zones and each assumes differing hybrid fitness and selection pressures that influence hybrid fitness (Figure 24).

Despite the potential for premating isolation to develop quickly even with few or no genetic incompatibilities (Dopman et al. 2010; Furin et al. 2012), upon secondary contact, hybridization does not always result in premating isolation either being found or developing (Brelsford and Irwin 2009; Hughes et al. 2011); in such cases one looks to postmating factors (not necessarily genetic) that prevent breakdown in taxon limits

(Kim et al. 2013). Postzygotic selection can also result in enhanced premating isolation, a process known as reinforcement (Howard 1993), although other processes such as direct selection on preferences or ecological selection can result in similar patterns (Noor 1999; Servedio 2001; Coyne and Orr 2004). Either premating or postmating barriers can change the direction of evolution of reproductive isolation in hybridizing taxa, or if the genes involved are linked the two processes can enhance one another (Servedio and Saetre 2003). Examining a hybrid zone over time or with contacts of different ages allows us to sort out which comes first—selection against hybrids, innate preferences for hybrid or parental types, or if one barrier type additionally evolves as a result of the other.. Temporally complex hybrid zones (Haavie et al. 2004; Vallin et al. 2012; Curry and Patten 2014) or those with historical sampling (Mettler and Spellman 2009; Carling and Zuckerberg 2011; Smith et al. 2013; Curry and Patten 2014), in particular, allow us to see if and how the relative influences of premating and postmating isolation in reproductive isolation changes at different stages of divergence within the same species and same biogeographic region. The hybrid zone models provide a framework for specific predictions about behavior and reproductive success of hybrids in zones with continued contact (Figure 24). In other words, is reproductive isolation a consequence of post-zygotic isolation resulting in a divergence in preferences and species recognition at secondary contact (Sætre and Sæther 2010), or does drift or selection on preferences in these differentiated populations result in premating isolation upon secondary contact? Combined with data on how widths of hybrid zones change or remain stable, such data allow us to determine which isolating barriers evolved first (Palumbi 1994; Wilkins et al. 2013) and assess the fit of our

hybrid zone to the competing hybrid zone models, which all ascribe different importance to intrinsic and extrinsic isolating barriers (Arnold 1997).

One such temporally complex hybrid zone, ideal for studying the evolution of reproductive isolation, is that of two oscine songbirds, the Black-crested (*Baeolophus atricristatu*s) and Tufted (*B. bicolor*) Titmice (family Paridae) in the southern Great Plains of North America. In Texas, the two populations have been interbreeding for several thousands of years (Dixon 1978) across a natural ecotone (Dixon 1955), while in Oklahoma the two species have contacted within the past century (Dixon 1955, 1990; Rising 1983) as a result of shrub invasion (Callahan 2002; Patten and Smith-Patten 2008). The two species, which diverged ca. 0.2 Mya during the Pleistocene (Klicka and Zink 1997), differ in plumage (Dixon 1955; Curry and Patten 2014), song (Dixon 1955; Coldren 1992), mtDNA (Avise and Zink 1988; Gill and Slikas 1992), and allozymes (Braun et al. 1984). Comparing the historical distribution of hybrids to present-day distribution in the older and younger regions suggests that the older zone is stable but that the younger zone is comparatively narrow, perhaps indicating more expansion will occur (Curry and Patten 2014). Thus, some isolating barrier in the older zone prevents expansion, while this selective pressure has not yet balanced with dispersal in the younger zone. This situation provides an excellent opportunity to examine what premating and postmating isolating barriers occur in the younger and older zones and if such barriers differ with continued contact, as one would expect with the stability of the older zone and the potential for the younger zone to expand.

To determine what selection regimes (i.e., the hybrid zone models in Figure 24) the data best match, I need to examine several aspects of premating and postmating

isolation. We specifically tested 1) if males treat songs from other populations as potential competition as measured by agonistic responses (Curé et al. 2010; Dingle et al. 2010). 2) Do females show a preference for song or plumage of parentals or hybrids? Female preference for potential mates should be even more indicative of the level of reproductive isolation between taxa because of their energy investment in reproduction compared to males (Trivers 1972) but is less frequently tested in birds (but see Patten et al. 2004; Danner et al. 2011) than in other taxa (Mayr 1946; Carmody et al. 1962; Guerra and Ron 2008; Kozak and Boughman 2009; Ward and McLennan 2009) because of logistical difficulties (Searcy 1992). 3) postmating isolation by assessing if there are reproductive consequences for hybridizing individuals by estimating nest success. I discuss our findings in the context of how isolation has changed with continued contact in the younger and older zones, known patterns of hybrid zone stability in this system (Curry and Patten 2014), and what these mean for the evolution of premating and postmating isolation. Several studies with repeated sampling or temporally complex hybrid zones examined patterns, not directly the isolating barriers, although to our knowledge few examined premating (Haavie et al. 2004) isolating barriers in both regions, so our studies provides new insights into the evolution of reproductive isolation over time.

Methods

These studies were conducted under Federal Bird Banding Permit 23215H, Federal Fish and Wildlife Permit MB148195-2, Oklahoma Department of Wildlife Conservation Scientific Collecting Permits 4716, 4955, 5210, and 5507; Texas Parks and Wildlife Department Scientific Collecting Permit SPR-0310-019; and University of

Oklahoma IACUC protocols R09-004 and R12-009. Birds with a plumage hybrid index ≥1 but ≤5 are classified as hybrids, although a few individuals far from the hybrid zone are classified as hybrids in this way (Curry and Patten 2014)

Experimental design

Male playback

To test agonistic responses of males to different song types, I conducted playback experiments at 16 sites in both hybrid zones (Figure 25) between sunrise and noon in April-June 2011-2013. Each color-banded male received one playback per day with a randomly selected exemplar of Tufted, Black-crested, or hybrid song. Each playback lasted 15 minutes with standardized song spacing and volume (67±3 dB at 1 m) on a mini-amplifier/speaker (Radioshack cat. no. 277-1008C) from a Sandisk Sansa MP3 player. Due to file damage on some 2011 playback recordings, I transcribed and analyzed only the first 7 minutes of each playback for all years. The four exemplars for each species (12 total) were from across Texas and Oklahoma and chosen for their average song characteristics for their type (C.M. Curry, unpublished data). Each exemplar track contained 2-3 songs from individual males, with 6 seconds between each song, repeated for 15 minutes.

Female mate choice

Birds were captured with feeder traps (Bacon 1987) and mist nets at 5 sites in both hybrid zones (Figure 25). Captive birds were kept at 60°F and received *ad libitum* sunflower seeds and peanuts, at least 6 mealworms a day, and water with a liquid multi-vitamin supplement (EcoTrition Vita-Sol, item no. D312). Males were released as soon as feasible after genetic sexing (Griffiths et al. 1998). Until then, males and females

were kept visually but not audibly isolated. Females were kept on a spring-like light cycle of 06:40 to 20:15 central daylight time light for at least 10 days before dosing with 0.5 mg β-estradiol (Sigma-Aldrich cat. no. E8875) (Searcy 1992; Searcy et al. 1997; Patten et al. 2004) suspended in 25 μL corn oil, injected under the skin. The mate choice assay began after a minimum of 48 hours after hormone application.

Each assay lasted 20 minutes. The first 15 minutes were an adjustment period with no stimuli. At 15-16 minutes, the female's behavior before stimuli was recorded. From 16-19 minutes, females were presented with a 3-minute video of a male with a background track of a song. Videos were saved as display size 854 X 480 pixels, bit rate 5.69 Mbps, and were presented in full screen mode, video size 200%, using Windows Media Player v 9.00.00.4507 (Microsoft Corporation, 1992-2002) on an IBM ThinkVision monitor at 1024 X 768 resolution from a Windows XP Dell Latitude D600; sounds were presented at using the same speaker as for the male playback experiment, at a standardized volume. At 19-20 minutes, the female's behavior after stimuli was recorded. All assays were transcribed from videos of the female's behavior.

Videos of exemplar males were recorded with a Logitech Webcam Pro 9000 (Logitech, Silicon Valley, CA) with NTSC anti-flicker 60 Hz, "Right Light" automatic settings, and focused on the perch in Windows Live Movie Maker (2010, Microsoft Corporation, Redmond, WA) against a standardized pale canvas background. Males were from both hybrid zones. Male plumage is similar within titmouse type across both hybrid zones (Curry and Patten 2014), so video from each transect were selected for use based on video quality. Footage for each male was looped to reach 3 minutes if needed. The songs were the same as used in the male playback experiments.

Song and video stimuli were presented in a factorial design with all combinations of Black-crested, hybrid, and Tufted Titmouse song and male plumage plus a control (Carolina Chickadee song and video) to ensure that birds were responding to the presentation of titmice and not merely to the movement and sound (Danner et al. 2011). At least four hours were left between each test for a given female, so each bird received 1-3 tests per day. Video order and selection from the 4 videos and 4 songs were randomized so no given individual received repeats of a given stimulus in any combination.

Videos are rarely used for mate choice experiments in birds (but see Moravec et al. 2010; O'Loghlen and Rothstein 2010); this application of video is most common in fish studies (Schlupp 2000). Other options are live birds (Baker et al. 1986) or specimens posed in life-like positions (Patten et al. 2004). Live birds should provide the most realistic stimulus, but each male may behave differently or respond to the female's presence differently (C.M. Curry, personal observation). Glass to block the male's view of the female may filter wavelengths of light from her vision of his plumage. Mounts retain the realistic color and can be posed singing but obviously do not move. Video allows the female to see a live bird under standardized conditions. Video screens do not emit UV wavelengths (Baldauf et al. 2008), so UV photographs were taken of two specimens each of the two species (B. McDonald and K. Carter, unpubl. data). No UV reflectance was present on the crest or forehead areas that differ between the two species. The dark areas on the crest (Black-crested) and forehead (Tufted) absorb UV, but in such as pattern to emphasis the pattern already visible to human eyes. As this

slight alteration is present in all videos of both species, it should not have elicited any differential responses by females.

Reproductive fitness

I used data was provided by the Cornell Laboratory of Ornithology's Project NestWatch. In this dataset, nest species identities were provided as either Black-crested or Tufted Titmice. As titmice are rarely identified as hybrids even in known areas of hybridization (Pulich 1988; Curry and Patten 2014), I created a contour map of estimated hybrid index values using known hybrid localities (Curry and Patten 2014). I created a map of values using empirical Bayesian kriging in ArcGIS 10.1 via the Geostatistical Analyst extension (parameters: subset size 100, overlap factor 1, 100 simulations, no transformation, standard circular neighborhood, 10-15 neighbors, four sectors, 0 angle, and radius 5). Each nest then was given an estimated hybrid index based on the map and its location (given in latitude and longitude). As the older hybrid zone has been stable during the past 60 years (Dixon 1990; Curry and Patten 2014), this provides a good estimate of the average hybrid index of individuals nesting in the hybrid zone. Figure 25 shows three contours based on the kriged surfaces for hybrid index values of 1 (the phenotypic boundary for Tufted Titmouse), 3 (the most intermediate hybrid), and 5 (the phenotypic boundary for Black-crested Titmouse) (Curry and Patten 2014). The NestWatch dataset does not cover the younger zone in southwestern Oklahoma but provides information on hybrid fitness in Texas. Attempts at nest monitoring in southwestern Oklahoma were defeated by the birds' refusal to use provided nestboxes; nonetheless, data from the older zone should still provide insight into any potential intrinsic fitness problems with hybridization between the two species.

Statistical analysis

Except where noted, all analyses were conducted in R 3.0.1 (R Development Core Team 2013).

Male playback

Trials where the focal male did not respond at any distance were excluded. I used three response variables: count of 10-second intervals spent vocalizing < 16 m from the playback speaker, count of 10-s intervals present <16 m from the playback speaker, and minimum approach distance to the playback speaker in categories of 0-1 m, 1-2 m, 2-4 m, 4-8 m, 8-16 m, and >16 m. Time spent <16 m includes both vocal and silent birds.

To test the average response strength for each population using time spent present and time spent vocalizing (both <16 m from the playback speaker), I used mixed model general linear modeling in lme4 (Bates et al. 2011) with a Poisson distribution. I conducted a mixed model ANOVA (Fox and Weisberg 2011; Fox et al. 2012) for each focal species and transect with Tukey's Honestly Significant Difference (HSD) posthoc (Hothorn et al. 2012) to determine which test song (fixed effect) generated the strongest response for a given species and transect. Banded individuals were random effects. To quantify the strength of response, I calculated effect sizes (Del Re 2012) as small, medium, or large (Cohen 1992). Distance from the exemplar recording location to the focal bird's playback site and playback order number (first, second, or third playback to an individual) were included as a covariate and an independent variable, respectively, but neither were significant and so they were excluded from subsequent analyses.

To test the average response strength for each population using minimum approach distance, I used a Wilcoxon matched-pairs signed-ranks test implemented in the R package coin (Hothorn et al. 2008) for each species in the young and old zones separately. Playback songs coded as "same" (conspecific song for a parental focal bird and a hybrid song for a hybrid focal bird) or "different" (heterospecific or hybrid song for a parental focal bird and parental song for a hybrid focal bird).

To test which song types to which individual focal birds responded mostly strongly via time spent present and vocalizing, we used repeated measures multivariate ANOVA (profile analysis) in SAS 9.2 (SAS Institute Inc. 2002). A profile analysis is a multivariate repeated measures ANOVA with tests for levels (here, the focal species), and groups (here, the test stimulus), and parallelism (here, the interaction between focal species and test stimulus) (Tabachnick and Fidell 2007). This takes into account individuals in the repeated measures instead of as a random effect in the mixed model analysis, allowing this test to see how individuals respond to different stimuli.

Female mate choice

Females rarely gave a copulation solicitation display (Searcy 1992; Patten et al. 2004), so I used time spent on the side of the cage closest to the video and sound stimuli (similar to Ward and McLennan 2009). The response variable was count of 10-s intervals in the 3-min stimuli assay spent in the quarter of the cage closest to the stimuli. Time spent in the quarter of the cage in the minute before the stimuli assay was used as a covariate, as some individuals stayed on one side of the cage more and this was correlated with time spent on that side during the assay. A quarter was used in place of half as it is more conservative in detecting any preference for one side of the cage.

To test population preferences for song and plumage stimuli, I conducted a mixed model ANOVA (individual as random effect; all other variables as fixed effects) to determine if captive females of each focal species preferred conspecific, heterospecific, or combinations of cues and if mixed or matching cues (song and plumage from the same species or different species) were preferred. This test looks at population (Black-crested, Tufted, and hybrid or parental type vs. hybrid) responses to different classes of stimuli (Black-crested, Tufted, and hybrid). I used *a priori* contrasts (Venables and Ripley 2002) to see if there was a preference for parental vs. hybrid, control vs. treatment (any titmouse stimulus), and Black-crested vs. Tufted and if there were differences in focal species responses (parental vs. hybrid and Black-crested vs. Tufted).

I also analyzed the responses of each species to "same" (conspecific for parentals and hybrids for hybrids), "different" (heterospecific or hybrids for parentals and parentals for hybrids), and "mixed" (the video and song type do not match; for example a hybrid song with a Tufted video). Both "same" and "different" stimuli are matching in that the song and video species match. For the mixed model ANOVA, I used *a priori* contrasts for effect to focal bird: control vs. treatment (any titmouse stimulus), non-mixed same vs. different stimuli, mixed vs. matching stimuli, and for focal species responses (parental vs. hybrid and Black-crested vs. Tufted).

I also analyzed the song, video, and preference for mixed versus matching stimuli using profile analysis (as described for male playback). Levels were focal species and groups were the test stimuli (species for video and song; whether song and video matched for the mixed vs matching test).

Finally, to quantify the strength of response (regardless of statistical significance), I calculated effect sizes (Del Re 2012) as small (>0.2), medium (>0.5), or large (>0.8) (Cohen 1992) for the response to song and video stimuli of each focal species in each zone. I compared these effect sizes to male playback effect sizes.

Reproductive fitness

The NestWatch dataset (120 nests at 102 locations) response variable was success or failure at each nest check interval. I estimated nesting success using the logistic exposure method (Shaffer 2004) with estimated hybrid index and nest stage (building, egg, and young) as predictor variables. To test if nest success depended on those variables, I used AIC model selection as described in Shaffer (2004). An additional 80 locations had summary data available for nests. This combined total of 195 nests was used to calculate average clutch size, average brood size, average number of young fledged, the ratio of nestlings to clutch size (as a measure of hatching success), and the ratio of fledged young to nestlings for each species (as a measure of survival from the nestling stage to fledging). This should provide a reasonable examination of whether any stages are more susceptible to failure in hybrids (though causes could range from differences in intrinsic survival, ability of parents to care for young, or ability of parents to select nest sites that reduce vulnerability to predation) in addition to the logistic exposure analysis.

Results

Male playback

Population preference

As measured by time spent vocalizing, both Black-crested and Tufted Titmice preferred their own species in the older zone but not in the younger zone (Table 17 and Table 18, Figure 26A). Focal Tufted males had a medium effect size for conspecifics, but Black-cresteds preferred conspecifics over both hybrid and Tufted but did not distinguish between the two. Hybrids showed no preference. In the younger hybrid zone, there was a marginally significant medium preference by hybrids of fellow hybrids over Black-crested (Tukey's post hoc $p=0.07$).

The results for time present <16 m (Figure 26B) were similar (Table 17 and Table 18). In the younger zone, hybrids preferred Tufted and hybrid over Black-crested song. In the older zone Tufteds showed no preference for any song type.

Our final measure of focal males' interest in test species was distance to closest approach (Figure 26C). I used 23 individuals for which I have data on both same and different song (some individuals may have only a conspecific and heterospecific song, for example, but not hybrid song presented, due to logistical constraints). Tufted Titmice in the younger zone approached same songs more closely, but no other comparisons were significant.

Individual preference

For both zones combined (Figure 27), there were no significant interactions between the test species and focal species (vocalizing: Wilks' $\Lambda_{4,16}=0.74$, $p=0.64$, partial $\eta^2=0.14$; present: Wilks' $\Lambda_{4,16}=0.89$, $p=0.92$, partial $\eta^2=0.05$). There was a significant

difference between responses to the test species for time present, but not vocalizing (vocalizing: Wilks' $\Lambda_{2,8}$ =0.62, p=0.15, η^2=0.38; present: Wilks' $\Lambda_{2,8}$ =0.43, p=0.03, η^2=0.57). There were no significant differences between response by the focal species (vocalizing: $F_{2,9}$=0.93, p=0.43, η^2=0.17; present: $F_{2,9}$=0.68, p=0.53, η^2=0.13).

Female mate choice

I conducted all the following analyses with the young and old zones combined due to the low sample size (n=8). Female choice effect sizes (Table 18) did not differ consistently from male effect sizes for both video and song nor did they suggest generally stronger responses by females in the younger versus older zones (Figure 28).

Population preference

At the population level (mixed model ANOVA), the song used (Figure 29A; Table 19*)* was significant, with significant *a priori* contrast where parental songs were preferred over hybrid songs (p<0.001). Contrasts also interacted in complex ways: parental birds generally preferred treatment over control while hybrids were less responsive to titmouse songs (p=0.03). (This seems to be based on one old-zone hybrid individual that responded much more strongly to the chickadee stimuli than the titmice stimuli (Figure 28A), so presumably this was not generally a dislike of hybrids for titmouse songs, but an individual outlier.) Parental and hybrid focal birds responded differently to Tufted and Black-crested song (p<0.01): hybrids preferred Tufted song whereas parental species each had their own response pattern. Black-crested and Tufted focal birds responded differently to hybrid and parental songs (p<0.01); this seems to be a reflection of the Black-crested avoidance of Tufted in the final contrast. Black-crested and Tufted focal birds responded differently to Black-crested and Tufted song, with

Black-cresteds preferring their own song type over Tufted and Tufted responding to all equally ($p=0.04$).

The video used (Figure 29B; Table 19) was significant, with Tufted videos receiving higher interest from all species than Black-crested videos ($p<0.01$). An interaction of control vs. treatment stimuli and focal parental vs. hybrid ($p=0.01$) suggests that hybrids were less responsive overall to titmouse videos. The time spent on the source cage side before the stimuli was significant in both song and video (Table 19). When comparing how each species responds to mixed or matching stimuli, there was an interaction between focal species and the effect to the focal bird (Table 19); this showed up in contrasts focal hybrids responded poorly to mixed stimuli and preferred non-mixed (pure same or different) stimuli while focal parentals preferred mixed stimuli over either non-mixed (different and same) ($p<0.01$; Figure 30).

Individual preference

There was no detectable significant difference for individual females (Figure 31A-B) using profile analyses. There was no significant interaction between the test species and focal species (song: Wilks' $\Lambda=0.19$, df=6,6, $p=0.38$, partial $\eta^2=0.57$; video: Wilks' $\Lambda=0.24$, df=6,6, $p=0.48$, partial $\eta^2=0.51$) (i.e., profiles are parallel). There was no difference among responses to the test species (song: Wilks' $\Lambda=0.57$, df=3,3, $p=0.59$, $\eta^2=0.37$; video: Wilks' $\Lambda=0.15$, df=3,3, $p=0.10$, $\eta^2=0.85$), regardless of focal species (for both song and video: $F_{2,5}=0.21$, $p=0.82$, $\eta^2=0.08$).

When analyzing the data for response to same vs. different and mixed versus matching (both song and video matched in species or not) stimuli (Figure 31C), there was no significant interaction between the test species and focal stimulus combination

(Wilks' Λ=0.21, df=6,6, p=0.43, partial η^2=0.54) (i.e., profiles are parallel). There was no difference among responses to the test stimulus combination (Wilks' Λ=0.68, df=3,3, p=0.73, η^2=0.32), or by focal species ($F_{2,5}$=0.0.08, p=0.92, η^2=0.03).

Reproductive fitness

The best fitting models for estimated daily survival rate at nests in the older hybrid zone were nest stage (building, incubation, and young) and constant survival (Table 20). Particularly, stage has the highest Akaike weight. Mean numbers for clutch size, brood size, and fledged young, plus ratios of brood/clutch size and fledged/brood size also support a lack of post-mating reproductive fitness disadvantage to hybridization (Table 21).

Discussion

Male playback

Titmice showed fewer preferences in the younger zone compared to the older zone, where both parental species prefer conspecifics and Black-cresteds also respond less to hybrids. Effect sizes for both male metrics ranged from small to large, with effect sizes generally being stronger in the older zone (although the hybrid preference for hybrids over Black-crested in the young zone is strong and significant). The weaker preferences by males in the younger hybrid zone (Table 18) may be because titmouse songs are structurally similar, with differences only in detail (Coldren 1992 and Chapter 3). In the older zone, hybrids are the only different titmouse that each parental individual is likely to encounter given that pure parental forms of the two species do not occur together at the present (Dixon 1990); i.e., parental individuals will only encounter other conspecifics or hybrids. In the younger zone, morphologically parental individuals

are much closer to occurring together at the same site (Curry and Patten 2014) suggesting that instead of thousands of years having passed since they saw each other, it has occurred in the recent past or they may still occasionally encounter one another. These data suggest selection against hybrids after prolonged contact, best matching a tension zone model.

Female mate choice

Direct studies of female choice experiments are less common in studies of avian hybrid zones (but see Patten et al. 2004) in birds because they are logistically complex (Searcy 1992). Other tests of postmating isolation can include checking for extrapair preferences (Reudink et al. 2006; Hughes et al. 2011), or checking in the wild pairing rates (Dixon 1955), both tests that restricts birds to a range of currently available mates instead of all possibilities as they might have encountered at initial secondary contact. However, direct studies of female choice allow us to see if preference for conspecifics, heterospecifics, or hybrids drives or selects against hybridization.

Hybrids themselves prefer Tufted song and plumage, and Tufteds seem to not have a strong preference either way. This result hints at sensory bias (Fuller et al. 2005) and suggests a mechanism for Tufted genes spreading into Black-crested. Nevertheless, as hybridization has ceased to spread, in the older zone at least (Curry and Patten 2014), some factor must select against hybrids. In general, hybrids preferred either pure parental plumage or song over signals where song and plumage did not match, while both Tufted and Black-crested prefer heterospecific signals (the other species or hybrids) and mixed signals even more (song and plumage do not match). That both species preferred mixed combinations of plumage and song (despite the overall

preference for either Tufted signal on its own) explains why the hybridization has not expanded in one direction only (compare Shriver et al. 2005). The strong dislike of hybrid signals by hybrids themselves may partially explain why the zone does not continue to expand. At an individual level, no comparisons were significant, but considering the mixed model analyses (and profile analysis plots, Figure 31), this is likely due to the small sample size for a multivariate analysis.

Effect sizes are larger in the older zone than in the younger zone, as predicted by the tension zone model (Figure 24) and consistent with male data (Table 18). Some female choice effect sizes are also larger than male effect sizes in the old zone, which would be predicted by reinforcement. Yet reinforcement is such a controversial topic (Howard 1993) that stronger evidence is needed before declaring that has operated. Also, I found no starting preference for either species in the younger zone (Figure 28), so the fitness disadvantage for hybrids, stopping the spread of the hybrid zone, likely lies elsewhere than in selection against hybridization.

Reproductive fitness

Intrinsic postmating isolation appears to be absent in this system. Postmating isolation generally develops much later than premating isolation and with increased genetic divergence in birds (Price 1984, 2008) and other taxa (Sasa et al. 1998; Stelkens et al. 2010). As the titmice are recently diverged (Braun et al. 1984; Avise and Zink 1988; Gill and Slikas 1992; Klicka and Zink 1997), a lack of intrinsic genetic incompatibility is unsurprising.

Implications for evolution of isolating barriers

The data in our study provide evidence that for the titmouse system, male titmice (and probably females) do not have strong initial preferences for either species, albeit there may be some sensory bias. Postmating isolation appears to be absent even in the older part of this system. What, then, is the selection pressure maintaining this stable hybrid zone (Dixon 1990; Curry and Patten 2014)? One possibility is that ongoing hybridization in the older zone has resulted in only fitter hybrids reproducing (Arnold 1997), leaving no disadvantages obvious there. In the younger zone patterns of reproductive fitness could differ, although logistical constraints prevented us from investigating this. During the field work for this study, the author observed similarly sized groups of fledglings with adult titmice in both zones, suggesting that reproductive fitness may not hold the answers in this system.

Another possibility is a non-genetic postmating barrier, such as ecological disadvantages to hybrids. The two species forage in similar habitat and microhabitats (Dixon 1955), so it seems unlikely that direct incompatibilities between feeding behaviors and the environment are the cause. However, in a morphological analysis of the younger and older zones, the younger zone cline in morphology is sharper and overlap in morphology is greater in the older zone (Curry and Patten 2014). Subtle differences could exist that were not evident from Dixon's work on feeding niches. Another ecological barrier could be territory size. Population densities are lower in western Oklahoma and Texas (Price et al. 1995), and low population densities allow for larger territory sizes (Knapton and Krebs 1974), as does habitat fragmentation (Patten et al. 2011). Because songbird territories often contain both nesting and foraging areas

(Nice 1941), inappropriate territory sizes could result in too much time or energy spent on defense or too few resources available for survival and reproduction. Territory size has been shown to affect fitness in some but not all parids via nestling growth rate and the probability of a pair nesting (Both and Visser 2000). Young titmice sometimes stay with their parents through the non-breeding season (Van Tyne 1948; Pielou 1957; Brackbill 1970), so territory size could affect subsequent survival of offspring. Finally, territory size can be heritable (Price 1984). Birds in Black-crested habitat with Tufted genes might face selective pressure to prefer a mate that will defend an appropriately sized territory with sufficient resources (Sherman and Eason 1998). More studies are needed on these potential ecological postmating barriers, particularly since preliminary data suggests that Black-cresteds may have larger home ranges. All else being equal, such would prevent unidirectional gene flow from Tufted to Black-crested and a westward shift of the hybrid zone, neither of which is supported by current evidence.

Plumage might still also be a behavioral isolating mechanism, but not via female sexual selection (Tarvin and Murphy 2012). Both males and females having a contrasting black crest, often raised and lowered in interactions (Dixon 1955), so crest coloration is a good place to begin investigations. Black-crested Titmice are thought to have diverged from ancestral populations of gray-crested birds due to different visual signaling conditions in their new, more arid habitats (Dixon 1978). It might be that the gray-crested Tufted plumage is more attractive as closer to the ancestral plumage, while Tufteds do not mind Black-crested (which would also make it easier for the original ancestral Black-crested population to diverge in allopatry if Black-crested-like birds had no inherent disadvantage from mate choice). The presumed purpose of the black crest is

signaling (Dixon 1978), so both male and female hybrids might be at a disadvantage in interactions with pure Black-crested. If the ornament (the crest) is important in their social hierarchy (Murphy et al. 2009a,b), hybrids could be at a disadvantage as the range of crest darkness may not be correlated with sex (females sometimes have paler crests in Black-crested; Dixon 1955) or social status as it might be in pure Black-crested. It is unclear how or if Tufted Titmice use their crests in intraspecifc interactions. Such a disadvantage to hybrid plumage could prevent continued asymmetric spread of the favored Tufted phenotypes.

Finally, it is possible that environmental conditions in both zones may result in different dynamics, perhaps via asymmetry in population abundance (Lepais et al. 2009) instead of the younger zone reflecting what the older zone has looked like in the past. Some indications of this might be each zone having different genes under selection (Nolte et al. 2009), which could be detected with separate analyses of the older and younger zones with genomic cline admixture analyses (Gompert and Buerkle 2009).

Most resampling studies look at width of morphological and genetic clines (Mettler and Spellman 2009; Carling and Zuckerberg 2011; Roy et al. 2012; Curry and Patten 2014). While we can deduce interactions from these patterns, tests of mechanisms maintaining the zones provide deeper insight into the processes producing these patterns. Additionally, having two contact zones of different age provides an additional insight. If I had sampled only the older zone, I would have concluded that sexual selection on both male and females in plumage and song has maintained species limits. If I had sampled only the younger zone, I would have concluded that sexual selection based on these phenotypes plays a far less important role in maintaining the

zone. Instead, it appears that other selection pressures are changing the patterns of sexual selection over time until reaching a stable width at an older age (Curry and Patten 2014), after which preferences for conspecifics may maintain it as shown by the data from the older zone.

Acknowledgements

I thank the volunteers who collected the nesting data for this study through The Cornell Laboratory of Ornithology's Nest Box Network, The Birdhouse Network, NestWatch, the Smithsonian Migratory Bird Research Center's Neighborhood NestWatch, and various state nest monitoring projects that have contributed their historic data. T. Murphy, B. Sandercock, and J.M. Martin discussed several aspects of this chapter with me; J.M. Martin also facilitated access to the NestWatch dataset.

Figures and tables

Table 17. Significance tests for each species in the young and old hybrid zones for three responses to song playback. Mixed model ANOVA (type III Wald's χ^2 tests) for counts of time intervals spent vocalizing and present; Wilcoxon's signed-ranks test for minimum distance.

Zone	Response metric	Focal Black-crested	Focal hybrid	Focal Tufted
Young	Vocalizing	ald's $\chi^{22}_{1,2}$ =4.69, p=0.10	Wald's $\chi^2_{1,2}$ =7.03, p=0.03	Wald's $\chi^2_{1,2}$ =1.76, p=0.41
	Present	Wald's $\chi^2_{1,2}$ =1.52, p=0.47	Wald's $\chi^2_{1,2}$ =17.11, p<0.001	Wald's $\chi^2_{1,2}$ =2.70, p=0.26
	Minimum distance	Z=-0.45, p=0.65	Z=-1.34, p=0.18	Z=-2.02, p=0.04
Old	Vocalizing	Wald's $\chi^2_{1,2}$ =30.33, p<0.001	Wald's χ^2=3.83, p=0.15	Wald's $\chi^2_{1,2}$ =15.76, p<0.001
	Present	Wald's $\chi^2_{1,2}$ =33.48, p<0.001	Wald's $\chi^2_{1,2}$ =4.58, p=0.10	Wald's $\chi^2_{1,2}$ =3.46, p=0.18
	Minimum distance	Z=-0.37, p=0.72	Z=0.18, p=0.18	Z=0, p=1.00

Table 18. Effect sizes (Cohen's d) are shown for each pairwise comparison of focal and test species in the young and old hybrid zones. Values are marked *small, **medium, and ***large (Cohen 1992). Boldface values are statistically significant at p<0.05 in Tukey's post hoc test for males. The two male columns are for two different but related metrics of interest: time spent vocalizing <16 m from the speaker and time spent present <16 m from the speaker. Female profile analyses of song and video were not significant; female mixed models were compared using a priori analyses, so no post hoc analyses were conducted.

Test comparisons								
Tufted vs. Black-		Black-crested vs. hybrid		Tufted vs. hybrid				
Old	Young	Old	Young	Old	Young	Zone		
0.44*	0.49*	**0.78****	0.10	0.52**	0.45*	Vocalizing	Male	Focal Black-crested
0.46*	0.36*	**0.54****	0.27	0.17	0.04	Present		
1.36***	NA	0.04	NA	2.17***	NA	Song	Female	
0.83***	NA	0.27*	NA	0.48**	NA	Video		
0.67**	0.18	0.36*	0.26*	0.15	0	Vocalizing	Male	Focal hybrid
1.62***	0.24*	0.49*	**0.84*****	0.42*	**0.24***	Present		
0.58**	0.17	0.15	0.20*	0.19	0.37*	Song	Female	
0.90***	0.20*	0.03	0.20*	0.64**	0.32*	Video		
0.52**	0.56**	0.36*	0.01	0.98***	0.47*	Vocalizing	Male	Focal Tufted
0.28*	0.15	0.36*	0.19	0.66**	0.37*	Present		
0.82***	0.08	0.82***	0.19	NA	0.11	Song	Female	
0.82***	0.04	-	0.17	0.82***	0.30*	Video		

Table 19. Analysis of Deviance Table (Type III Wald's χ^2 tests) for tests of effects of stimuli: song, video, and "mixed and matched" song/video. Boldface values are statistically significant at p=0.05

	Wald's χ^2	df	P
Song species	11.58	3	**0.009**
Focal species	0.45	2	0.80
Time spent in quarter before treatment	35.22		**<0.001**
Song species: focal species	29.45	6	**<0.001**
Video species	10.29	3	**0.016**
Focal species	0.49	2	0.78
Time spent in quarter before treatment	29.58		**<0.001**
Video: focal species	9.51	6	0.15
Effect to focal bird	2.73	3	0.44
Focal species	0.25	2	0.88
Effect to focal bird: focal species	21.02	6	**0.002**

Table 20. Model selection for estimated daily survival showing the effective number of observations, K (the number of parameters), the corrected AIC value, the change in corrected AIC between models, and the Akaike weights.

Model	Effective N_{obs}	K	AIC_c	ΔAIC_c	w_i
Stage	3558	3	288.42	0	0.72
Constant survival	3558	1	292.06	3.64	0.12
Hybrid index	3558	2	292.70	4.28	0.08
Species	3558	3	294.10	5.68	0.04
Species*stage	3558	9	294.35	5.93	0.04

Table 21. Summary of nest descriptors by species and outcome: mean±standard deviation (range).

Species	Outcome	N	Clutch size	Brood size	Fledglings	Hatching success	Nestling success
Black-crested	Fail	16	4.5±1.5 (2-6)	1.0±1.8 (0-5)	0	0.20±0.36 (0-1)	0
	Success	99	5.6±1.2 (2-10)	5.2±1.3 (2-10)	5.1±1.4 (2-10)	0.92±0.13 (0.4-1)	0.91±0.10 (0.4-1)
	Overall	115	5.5±1.3 (2-10)	4.6±2.0 (0-10)	4.4±2.2 (0-10)	0.82±0.31 (0-1)	0.94±0.21 (0-1)
Hybrid	Fail	3	2.7±2.1 (1-5)	0	0	0	NA
	Success	29	5.6±1.1 (2-7)	5.3±1.2 (2-7)	5.3±1.3 (2-7)	0.96±0.11 (0.5-1)	0.99±0.05 (0.8-1)
	Overall	32	5.3±1.5 (1-7)	4.8±2.0 (0-7)	4.8±2.0 (0-7)	0.87±0.30 (0-1)	0.99±0.05 (0-1)
Tufted	Fail	4	4.8±2.2 (2-7)	1±2.0 (0-4)	0	0.14±0.29 (0-0.57)	0
	Success	44	5.4±1.0 (3-8)	5.1±1.3 (2-8)	5.0±1.3 (2-8)	0.94±0.13 (0.5-1)	0.98±0.08 (0.67-1)
	Overall	48	5.4±1.1 (2-8)	4.8±1.7 (0-8)	4.6±1.9 (0-8)	0.87±0.26 (0-1)	0.96±0.30 (0-1)

Figure 24. Hybrid zone model predictions for fitness and sexual selection.
The bounded hybrid superiority model (left column) assumes the environment stabilizes the hybrid zone, whereby hybrid individuals are best adapted to the intermediate habitat of the hybrid zone (Moore 1977). Extrinsic selection is acting. The evolutionary novelty model (not shown, but similar to bounded hybrid superiority) assumes a range of extrinsic and intrinsic selection is possible but that at least some hybrid genotypes will be positively selected and thus able to move on their own evolutionary trajectory (Arnold 1997). The tension zone model (middle column) assumes that hybrids are intrinsically unfit and that the hybrid zone is maintained stably by a balance between selection against hybrids and dispersal of parental individuals into the zone (Barton and Hewitt 1985, 1989). Females may show a slight increase in preference from males due to their larger investment in reproduction (Trivers 1972), but it should be similar across the range. Finally, reinforcement (right column), selection against hybridization resulting in increased premating isolation (Howard 1993), can be difficult to distinguish from other types of selection against hybrids (selection not specifically against interbreeding), such as selection based on ecological differences that might also result in increased premating isolation (Howard 1993; Noor 1999). Selection not specifically against hybridization need not result in comparatively stronger female isolation, so in those other ecological cases disadvantages should be shared evenly between males and females (Coyne and Orr 2004) and neither sex would be predicted to be more strongly isolated.

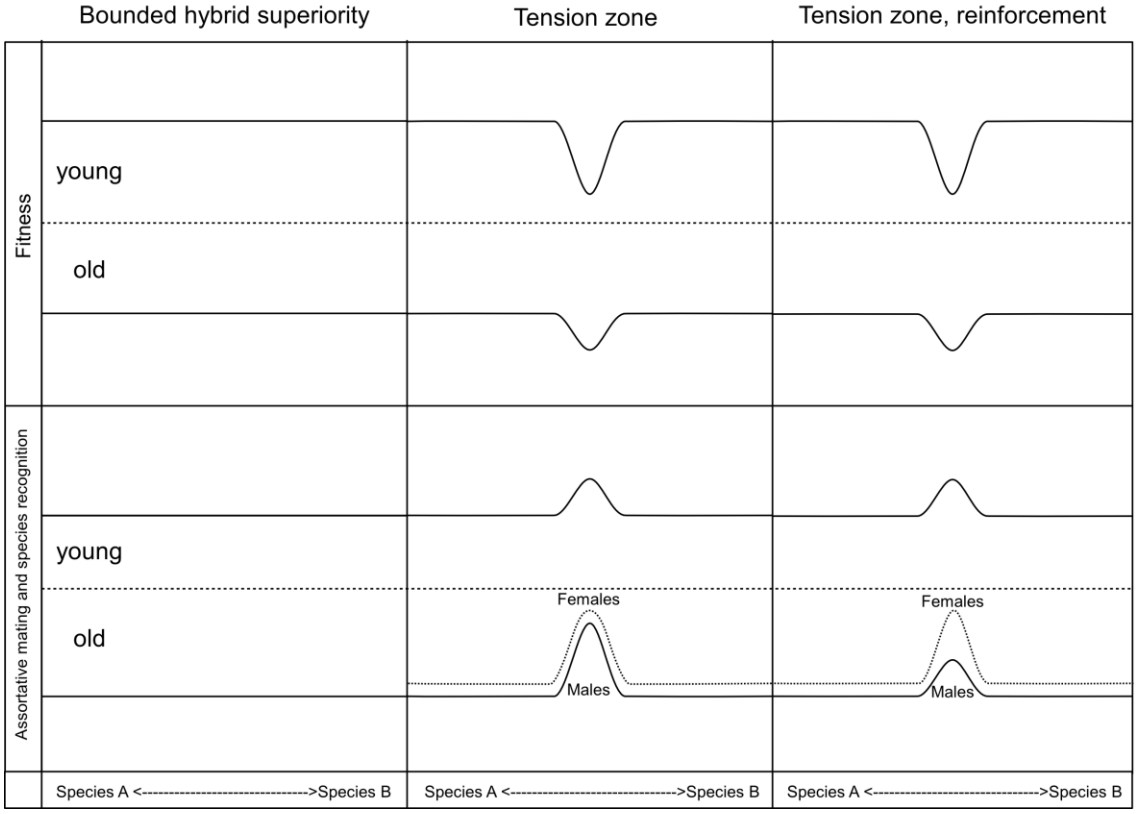


Figure 25. Map of the study sites showing NestWatch data points and locations for playback experiments and where females for the mate choice experiments were collected. Males are shown by "M", females by "F", and sites where both experiments were conducted are "B".

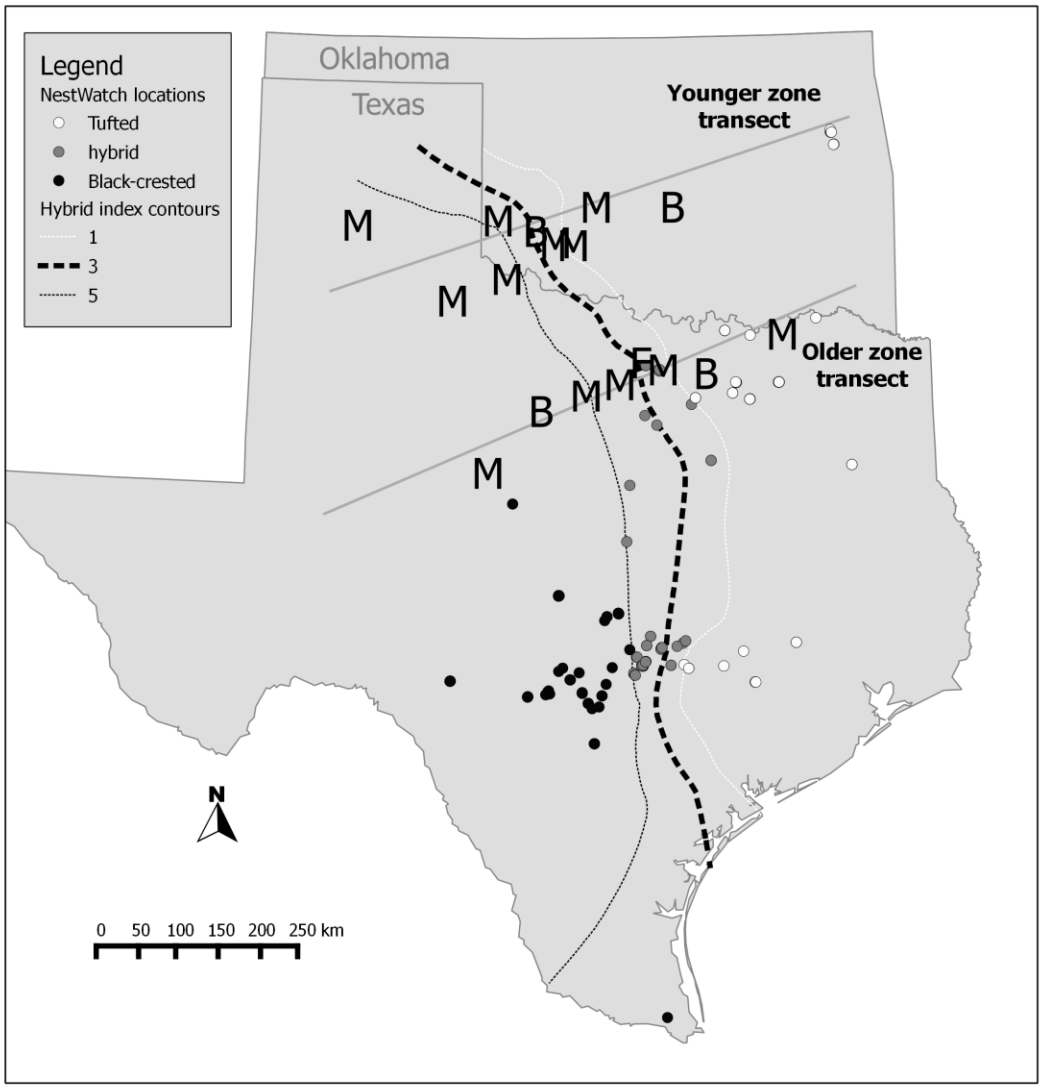
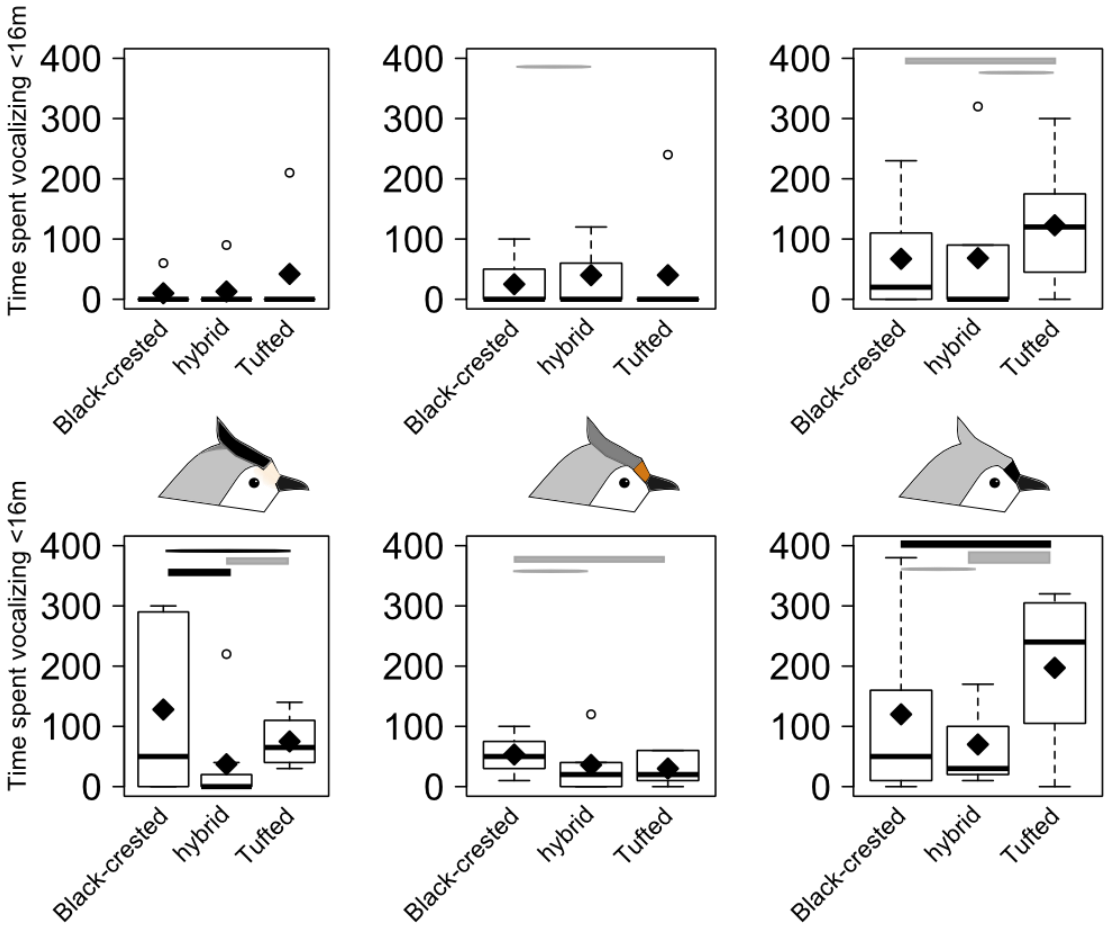
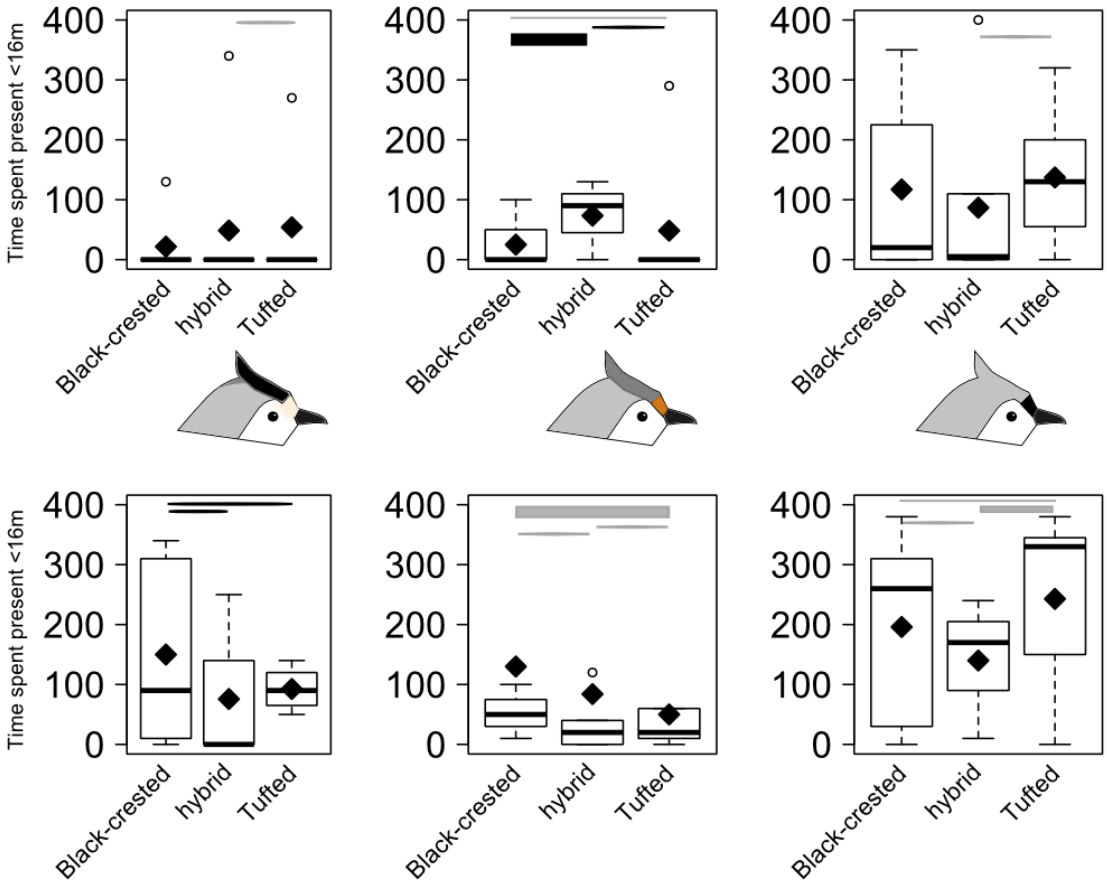


Figure 26. Response to song playback in the younger (top row) and older (bottom row) zone to Black-crested (left column), hybrid (middle column), and Tufted (right column) focal males. Horizontal bars indicate effect sizes (Cohen 1992) in small, medium, and large (actual values in Table 2). Black bars are significant after Tukey's post hoc; gray bars are not significant. Diamonds indicate means, whereas lines in box-and-whisker plots indicate median, outer boxes and error bars are quartiles, and dots are outliers. (A) Time spent vocalizing (singing or calling) <16 m from the playback speaker. (B). Time spent <16 m from the playback speaker in any activity (vocal or silent). (C) Minimum approach distance by each species in each zone with the test song categorized as "same" or "different" (see Methods).

A



B


Time spent present <16m
400
300
200
100
0
Black-crested
hybrid
Tufted

C

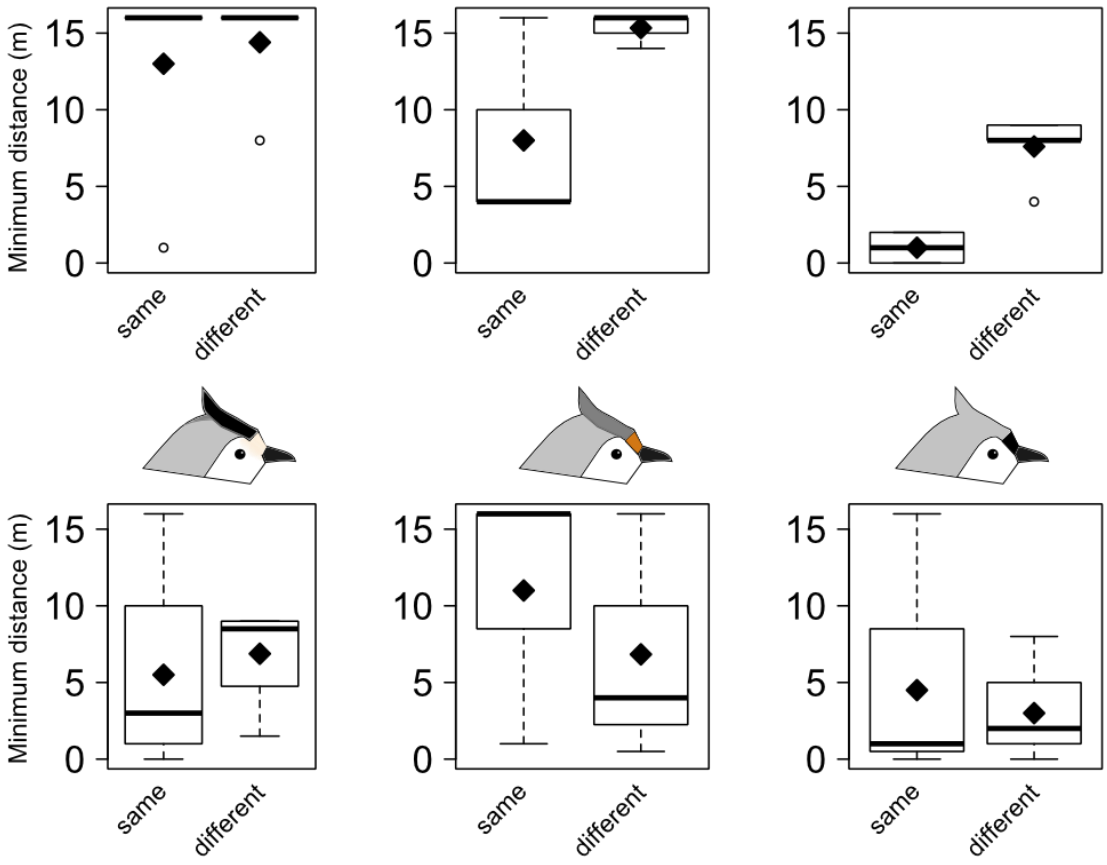
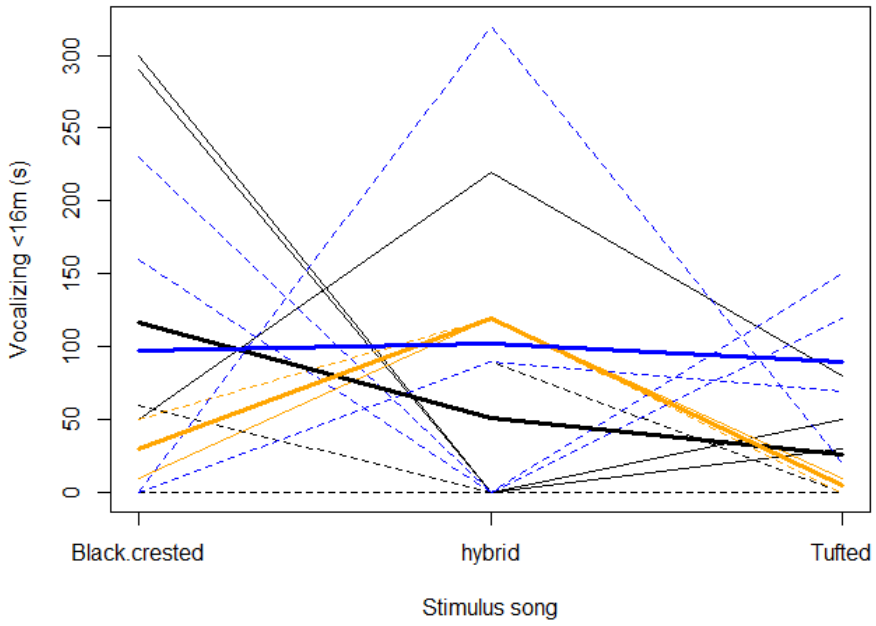


Figure 27. Profile analysis for male reactions of (A) vocalizing <16m and (B) present <16m. Lines connect individuals from the young (dashed) and old (solid) hybrid zones. Thicker lines show averages for each focal species: Black-crested (black), hybrid (orange), and Tufted (blue).

A



B

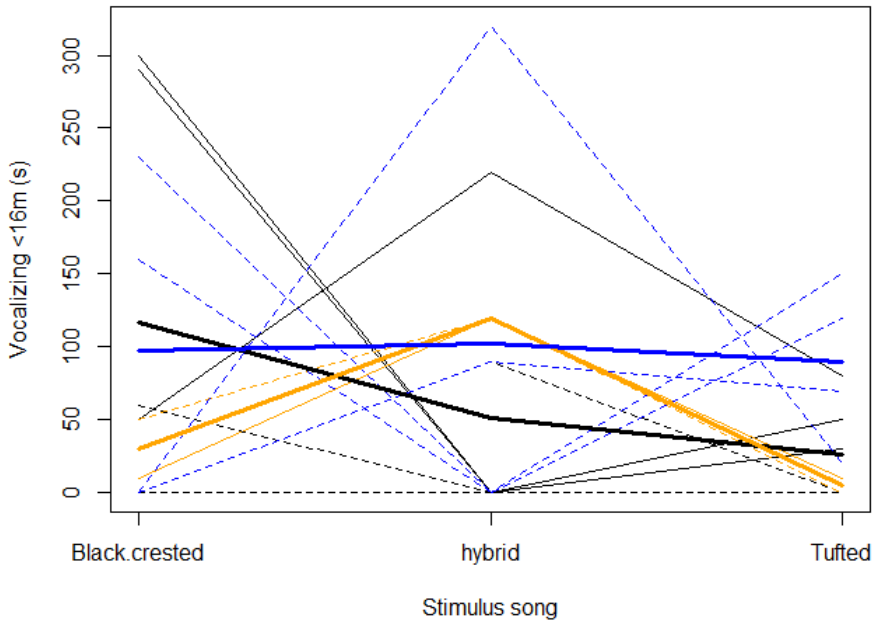
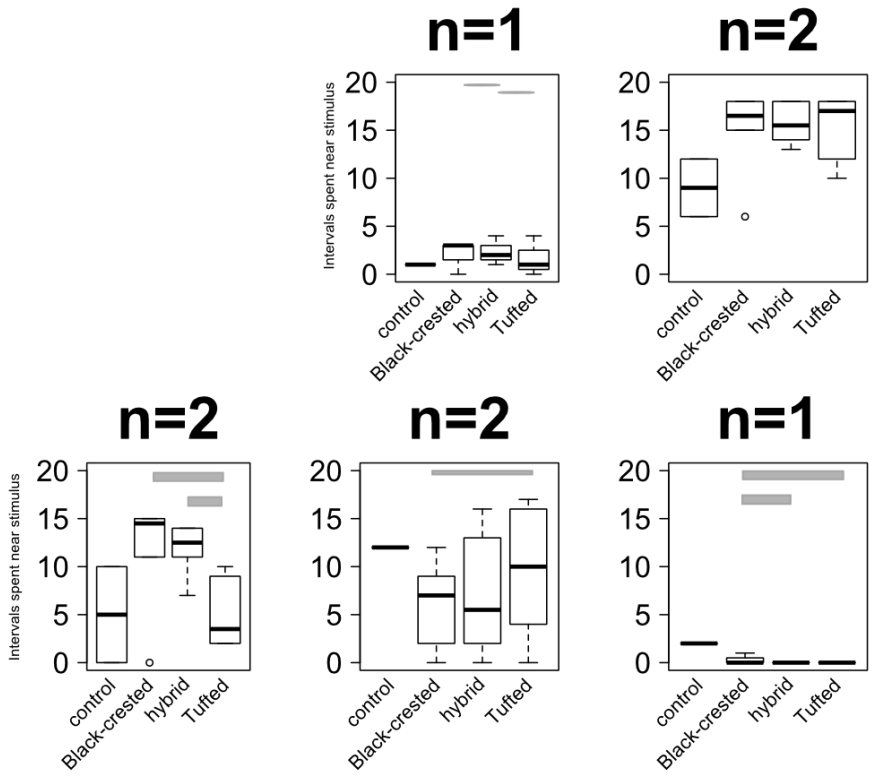


Figure 28. Female (A) song and (B) video responses measured by time spent in the quarter of the cage closest to the song and video stimuli. Younger zone birds are in the top row; older zone birds are in the bottom row. Focal female species are by column left to right: Black-crested, hybrid, and Tufted. Bars indicate effect sizes in small, medium, and large (Cohen 1992). No post hoc tests were conducted; see Table 19 for ANOVAs and text for a priori contrasts.

A



B

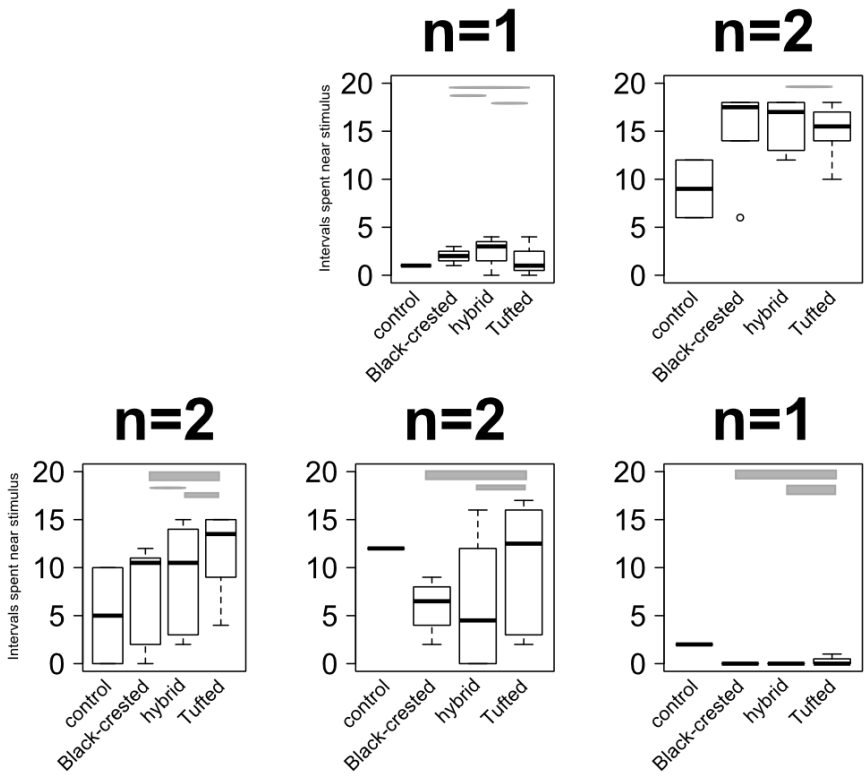
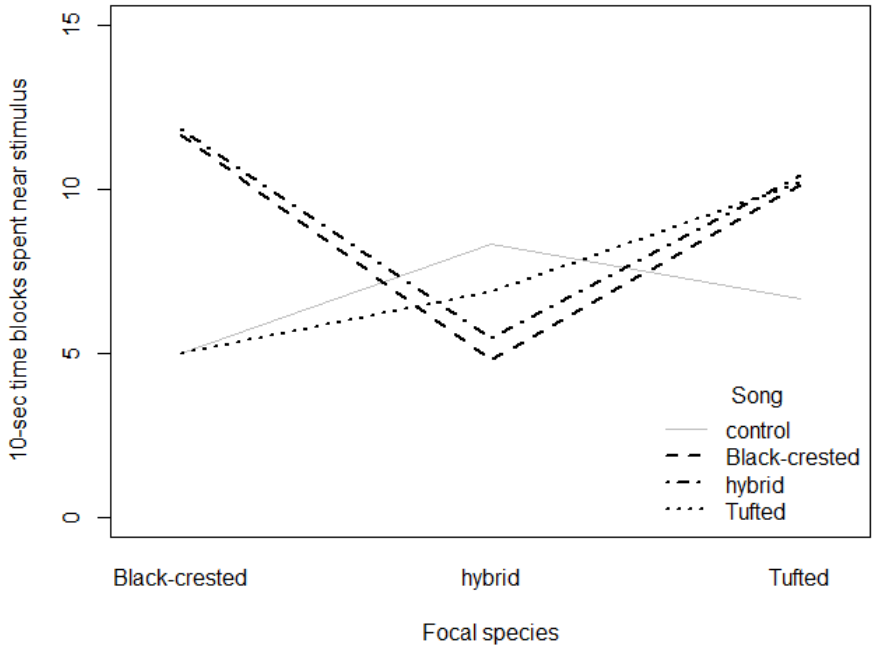

n=1
n=2
n=2
n=2
n=1
Intervals spent near stimulus
0
5
10
15
20
control
Black-crested
hybrid
Tufted

Figure 29. Female mate choice results for (A) song and (B) video. Lines show averages for each test stimulus. Boxplots and effect sizes for all comparisons in both the younger and older hybrid zones are in Figure 29 and Table 18.

A



B

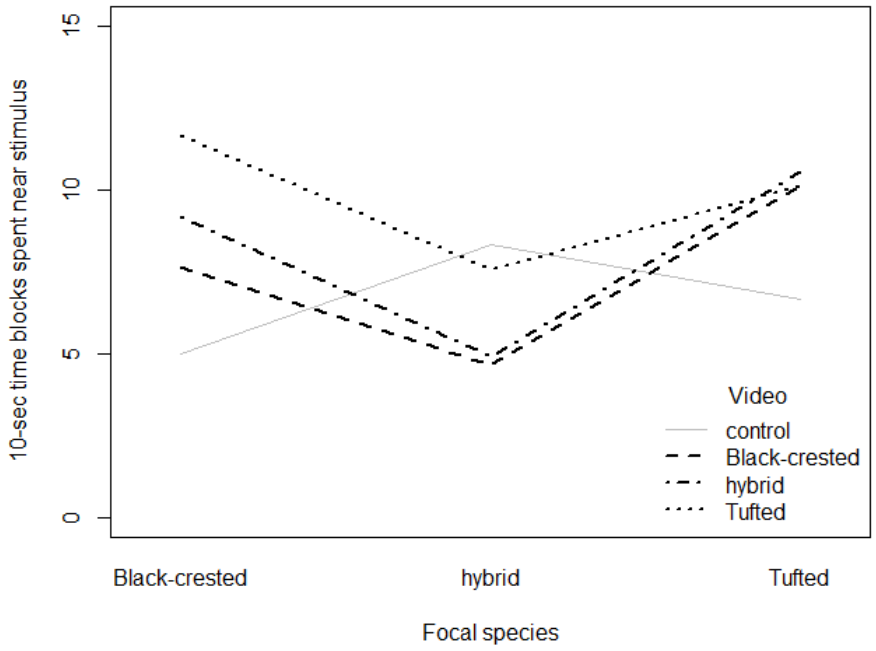


Figure 30. Female choice data organized by type of stimulus. Lines show averages for each test stimulus. "Same" stimuli shows a bird of the same type as the focal bird in both video and song; "different" stimuli shows a bird of a different type (either the heterospecific or hybrid for a parental, or a parental for a hybrid bird), and "mixed" stimuli shows mismatched types for song and video (for example, a parental song with a hybrid plumage).

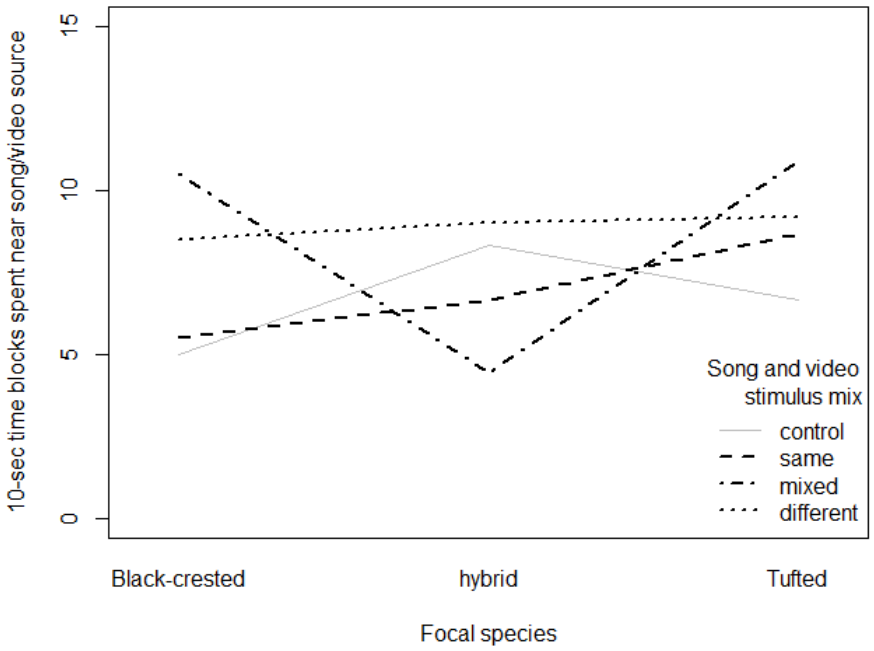
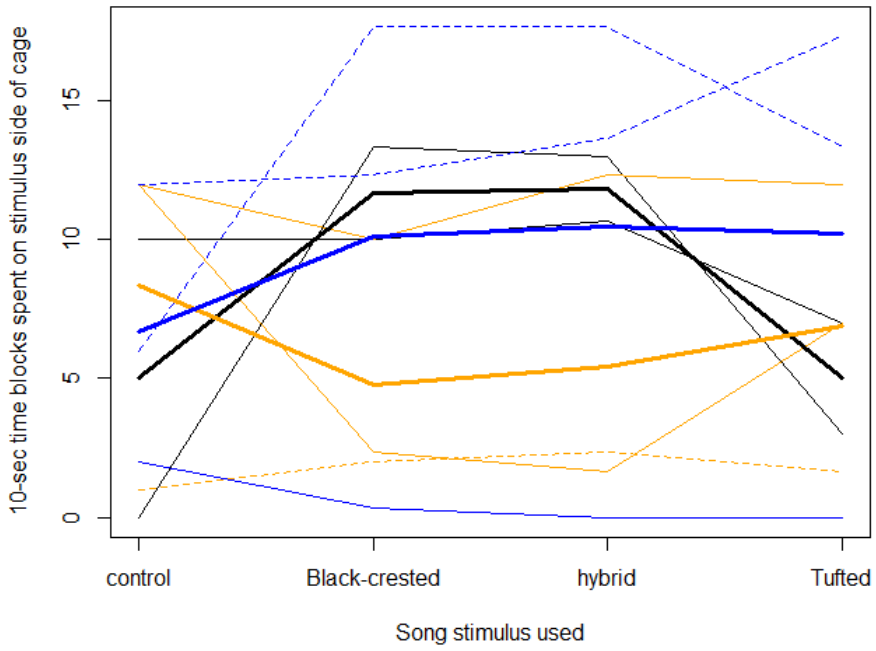
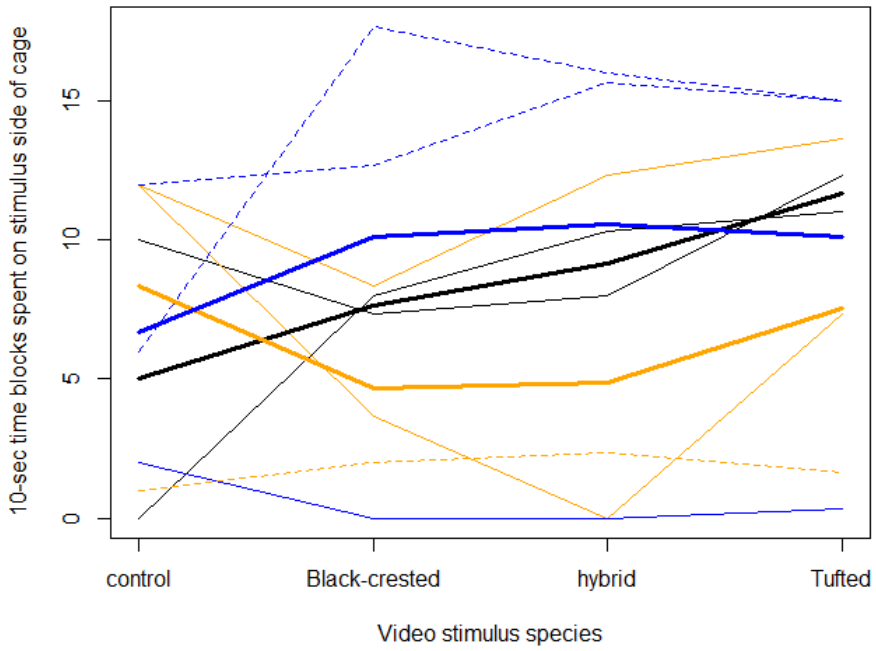


Figure 31. Profile analysis for (A) song, (B) video, and (C) combinations of song and video stimuli. Lines connect individuals from the young (dashed) and old (solid) hybrid zones. Thicker lines show averages for each focal species: Black-crested (black), hybrid (orange), and Tufted (blue).

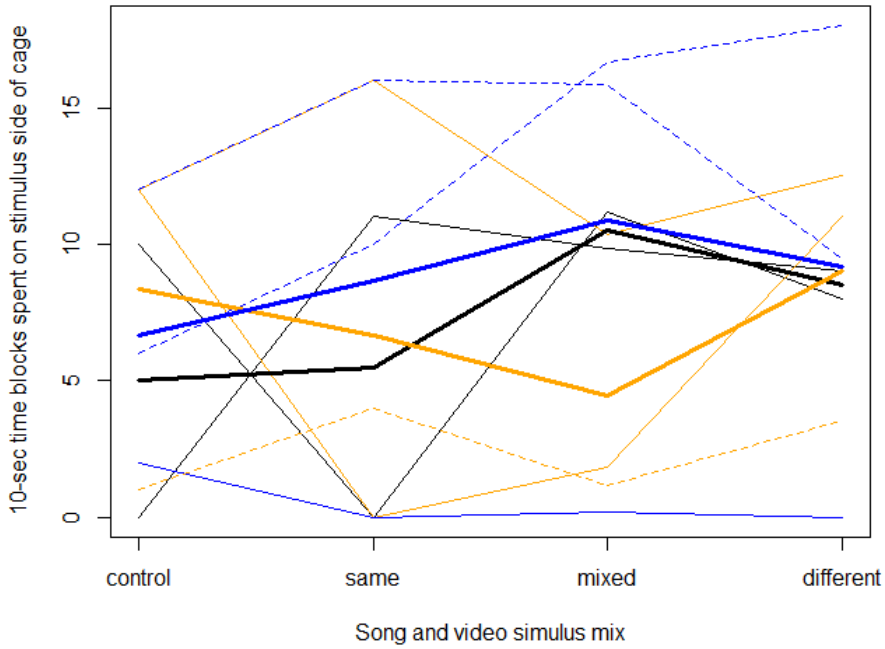
A



B


10-sec time blocks spent on stimulus side of cage
0
5
10
15
control
Black-crested
hybrid
Tufted
Video stimulus species

C


10-sec time blocks spent on stimulus side of cage
0
5
10
15
control
same
mixed
different
Song and video simulus mix

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