

PRE-MATING COMMUNICATION AND HYBRIDIZATION BETWEEN TWO
MEADOW KATYDIDS, *ORCHELIMUM NIGRIPES* AND *O. PULCHELLUM*
(ORTHOPTERA: TETTIGONIIDAE): MALE CALLING SONG
AND ASYMMETRIC FEMALE PREFERENCE

BY

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ABSTRACT

I investigated the role of male calling song and female preference on mate choice and pre-mating isolation in *Orchelimum nigripes* and *O. pulchellum*. In Chapter 1, I show that the calls of *O. nigripes* and *O. pulchellum* differ significantly in multiple temporal characteristics. Given that male signals are different, I next determined whether or not there was species discrimination by females in caged mating trials. In chapter 2, I show female *O. nigripes* mated preferentially with *O. nigripes* while *O. pulchellum* females mated with both *O. nigripes* and *O. pulchellum* males. To isolate the acoustic preference of females for male song from other signals and exchanges, I tested female song preference on a walking compensator (a “servosphere”) with playback of male song. In chapter 3, I demonstrate that female preference in both species is more complex than a simple preference for increased call energy, and that females of both species will orient to male songs of either species.

Katydid from within the hybrid zone were characterized in two ways, song (Chapter 1) and mitochondrial DNA (mtDNA, Chapter 2). Songs from hybrid populations collected within the zone differed in temporal patterning from songs of allopatric *Orchelimum nigripes* and *O. pulchellum*, and were less variable than songs of allopatrics. Individuals collected across the hybrid zone primarily carried *O. pulchellum* mtDNA. This is consistent with the hypothesis that the southeastern hybrid zone was formed by crosses between *O. pulchellum* females and *O. nigripes* males, and is also consistent with the results of the caged mating trials in Chapter 2.

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General Introduction

A fundamental question in evolution is how do new species form? To understand how evolution results in the rich biodiversity around us we must better understand the process by which new species are formed (reviewed by Coyne and Orr 2004; Wolf et al. 2010). If we use Mayr's (1963) criteria for species as groups of organisms that successfully interbreed, then speciation is the process by which groups become reproductively isolated. Understanding how this reproductive isolation occurs is difficult; reproductively isolated taxa are the end result of lineage splitting, i.e. speciation, but closely related taxa are studied more often than partially isolated taxa out of convenience. When studying fully isolated species, we cannot observe historical processes and must infer the route that led to reproductive isolation by looking at current differences between taxa. Differences between taxa that we observe today may be a combination of traits that initiated reproductive isolation and traits that have developed following divergence (reviewed in Coyne and Orr 2004).

Mechanisms that lead to speciation may be broadly divided into two categories: pre-mating and post-mating isolation. Pre-mating isolation is primarily the result of three mechanisms: temporal or habitat differences (species do not meet in nature), mechanical incompatibility (sperm transfer is not possible due to morphological differences between individuals) and behavioral isolation (individuals do not recognize one another as mates). Behavioral pre-mating barriers include courtship differences and intricate species specific signaler-receiver relationships (Greenfield 2002). When there is a fitness cost to hybridization, selection is expected to favor pre-mating barriers between species to avoid this cost (Dobzhansky 1940). Post-mating barriers may also reduce gene flow between

taxa through sterility or inviability. Hybridization between taxa may result in unviable eggs, production of sterile or less fecund hybrid offspring, or production of less fit individuals.

Hybrid zones are areas where individuals of two genetically distinct parental taxa interbreed, resulting in offspring of mixed ancestry (Harrison 1990). Hybridizing taxa and hybrid zones provide an opportunity to study the mechanisms that maintain species boundaries because reproductive barriers between these populations are incomplete (Barton and Hewitt 1985). Taxa that hybridize are thought to be the result of recent and incomplete speciation events, i.e., mating boundaries are not fully intact in at least one sex of each species (Barton and Hewitt 1985; Bridle et al. 2006; Gay et al. 2007; Matute and Coyne 2010).

Hybrid populations often have a range of genotypes and phenotypes greater than that found in either parental population and that are different from the parental types (Barton and Hewitt 1985; Rieseberg et al. 1999). We can observe the interactions among, and the effects of selection on, parental species and hybrid individuals to understand how the partial isolation between these parental taxa arose (Jiggins and Mallet 2000). My research focuses on pre-mating isolation of hybridizing acoustic Orthoptera.

Orthopteran hybrid zones

Many species of Orthoptera (grasshoppers, katydids and crickets) are known for their acoustic courtship signals (Otte 1970; Walker 1971; Walker 1974; Gwynne 2001). Their prominent courtship songs have been shown to be important in pre-mating reproductive isolation and species-recognition (e.g., Bailey and Robinson 1971; Bailey 1993; Allen 1995; Brown et al. 1996; Bailey and Field 2000; Faure and Hoy 2000; Gray

and Cade 2000; Olvido and Wagner 2004; Orci 2007; Bush et al. 2009). For example, males of the hybridizing grasshopper species, *Chorthippus brunneus* and *C. jacobsi* (Acrididae) have very different songs, and females use song to orient to males (Bridle et al. 2006); females of both species preferred conspecific male songs and discriminated against the songs of hybrid males. For these grasshoppers, song sharpens species boundaries. Songs of Orthoptera are normally species-specific, yet there are several cases in which taxa with distinct courtship songs hybridize. The field crickets *Gryllus firmus* and *G. pennsylvanicus* (Gryllidae) form a mosaic hybrid zone (a patchy hybrid zone resulting from environmental fragmentation); when individuals from allopatric populations were presented calls with conspecific and heterospecific pulse rates, they preferred the pulse rate of conspecific males (Doherty and Storz 1992). In other Orthoptera, differences in male song between species are not recognized by females. The crickets *Allonemobius fasciatus* and *A. socius* (Gryllidae: Nemobiinae) sing different songs and meet in a mosaic hybrid zone in the Eastern United States (Mousseau and Howard 1998). Females do not discriminate between the species by song differences between the species, and the song differences may be the result of genetic drift in these taxa (Doherty and Howard 1996; Olvido and Wagner 2004). The role of song, if any, in mate discrimination is unclear in these hybridizing Orthoptera.

Hybridization between Orchelimum nigripes and O. pulchellum

Here I examine species discrimination between two acoustic meadow katydids, *Orchelimum nigripes* and *O. pulchellum*, that are incompletely isolated (hybridizing) and for which no song differences have been previously documented (Walker 1971).

Orchelimum nigripes and *O. pulchellum* are allopatric throughout most of their native

ranges. *Orchelimum nigripes* is found throughout most of the Central United States, extending east to the Appalachian Mountain Range (Figure 1, Walker 1971; Morris and Walker 1976). *Orchelimum pulchellum* is found east of the Appalachian Mountain Range, extending to the East Coast of the United States (Figure 1, Walker 1971; Morris and Walker 1976). *Orchelimum nigripes* and *O. pulchellum* have been described as different species based upon their morphology (Scudder 1875; Davis 1909; Rehn and Hebard 1915; Blatchley 1920; Walker 1971). Across their distributions, males of these species have consistent morphological differences that include the shape and angle of the male's cercal tooth, the angle of the female's ovipositor, the shape of lateral pronotal lobes, and leg and eye color (Rehn and Hebard 1915; Shapiro 1996).

There is evidence of gene flow between *O. nigripes* and *O. pulchellum*. Shapiro (1998) identified two hybrid zones between these taxa based upon morphological and allozyme data. Shapiro (1998, 2000, 2001) focused on the northeastern hybrid zone located near the Potomac River Basin, Washington, DC, which likely formed in the past 75 years as a result of human introduction of *O. nigripes* into habitat occupied by *O. pulchellum*. My study focuses on the southeastern hybrid zone (Figure 1), which extends across much of Louisiana, Mississippi, and Alabama south of the Appalachian Mountain Range. This older zone likely formed naturally from secondary contact in the Pleistocene (Shapiro 1996).

Shapiro (2001) found asymmetrical mate choice between the species in the northern hybrid zone, such that *O. nigripes* discriminated against heterospecifics, while *O. pulchellum* did not. These preferences were maintained despite fitness costs for females who mated with the wrong species in terms of the number of offspring produced

and sterility of male offspring (Cabrero et al. 1999; Shapiro 2000). Given fitness costs of heterospecific matings between these species (in the northeastern hybrid zone), selection is expected to favor pre-mating discrimination against heterospecific individuals (Shapiro 2000), if associated fitness costs in the southeastern hybrid zone are similar to those found in the northeastern hybrid zone.

Inferences about hybrid zone structure and ecology

The southeastern hybrid zone between *O. nigripes* and *O. pulchellum* appears to be unimodal, because across the zone, only individuals with intermediate (hybrid) morphology are found. This hybrid zone is broad and long-lived (Shapiro 1998). The vegetation and predators (birds, anoles) are similar in the range occupied by parental taxa and across the hybrid zone. These katydids do not appear to be habitat specialists as both parentals and hybrids were found in a variety of habitats. For example, males of both parental species and the hybrids would sing from lower grassy vegetation as well as high in kudzu and trees. They were also observed feeding on seeds and insect prey (which were ubiquitous), so this hybrid zone is not likely an ecotonal zone.

Katydid were not found in many locations that appeared to be appropriate habitat, and the distribution of katydids appeared patchy. Across the zone, suitable habitats were fragmented by human development. Despite their ability to fly, I suspect (because of how patchy katydids are) they do not disperse much across the southern hybrid zone.

This hybrid zone has no obvious geographic boundaries between parental and hybrid populations. Both parental species are able to live in a variety of habitats as demonstrated by their north-south distributions. Because these habitats are much more variable than those found across the deep south, it appears that both parental species may

be able to live anywhere across the zone, but only intermediates were found across the zone. One of the parental species or the hybrids could potentially have a local advantage; however, I do not think it is likely that micro-habitat preferences separate these species in the field given that they are found in similar environments and feeding and calling from similar areas within those habitats (pers. obs.). This hybrid zone appears to be stable, and to have resulted from asymmetrical preference. This research demonstrates that stable hybrid zones are possible in spite of statistically significant courtship signals in the parental species.

Research summary

I investigated the role of male calling song and female preference on mate choice and pre-mating isolation in *Orchelimum nigripes* and *O. pulchellum*. Males of *O. nigripes* and *O. pulchellum* both sing broad frequency songs that consist of two repeated song elements. Both species' songs have a series of ticks followed by a brief silent interval, a buzz, then a longer silent interval. In Chapter 1, I show that the calls of *O. nigripes* and *O. pulchellum* differ significantly in multiple temporal characteristics. These differences in male signals may be used by females to correctly diagnose species of males.

Given that male signals are different, I next determined whether or not there was species discrimination by females in caged mating trials. In chapter 2, I show female *O. nigripes* mated preferentially with *O. nigripes* while *O. pulchellum* females mated with both *O. nigripes* and *O. pulchellum* males. The experiment was aimed at determining female mate choice, but it is unclear if the lack of matings between *O. nigripes* females and *O. pulchellum* males was due to male or female mate choice. In these crosses,

courtship proceeded until females became quiescent and males attempted to couple.

Male mate choice might be expected in this system as males transfer a large percentage of their body weight to females in the form of a spermatophore when mating, but males did not appear choosy. They readily courted con- and heterospecific females and males, and were seen depositing spermatophores into fine mesh covering the tops of their cages.

To isolate the acoustic preference of females for male song from other signals and exchanges, I tested female song preference on a walking compensator (a “servosphere”) with playback of male song. In chapter 3, I demonstrate that female preference in both species is more complex than a simple preference for increased call energy, and that females of both species will orient to male songs of either species.

Katydid from within the hybrid zone were characterized in two ways, song (Chapter 1) and mitochondrial DNA (mtDNA, Chapter 2). Songs from hybrid populations collected within the zone differed in temporal patterning from songs of allopatric *O. nigripes* and *O. pulchellum*, and were less variable than songs of allopatrics. The maternally inherited mtDNA marker was used to genotype katydids from outside and within the hybrid zone that Shapiro (1998) had previously identified on the basis of biparentally inherited morphological and allozyme markers. Individuals collected across the hybrid zone carried *O. pulchellum* mtDNA, and *O. pulchellum* mtDNA was found farther west than predicted on the basis of Shapiro's morphological and genetic work (Shapiro 1998). This is consistent with the hypothesis that the southeastern hybrid zone was formed by crosses between *O. pulchellum* females and *O. nigripes* males, and is also consistent with the results of the caged mating trials in Chapter 2. The low variation

among male songs within this zone suggests that it may have been initiated by a few individuals.

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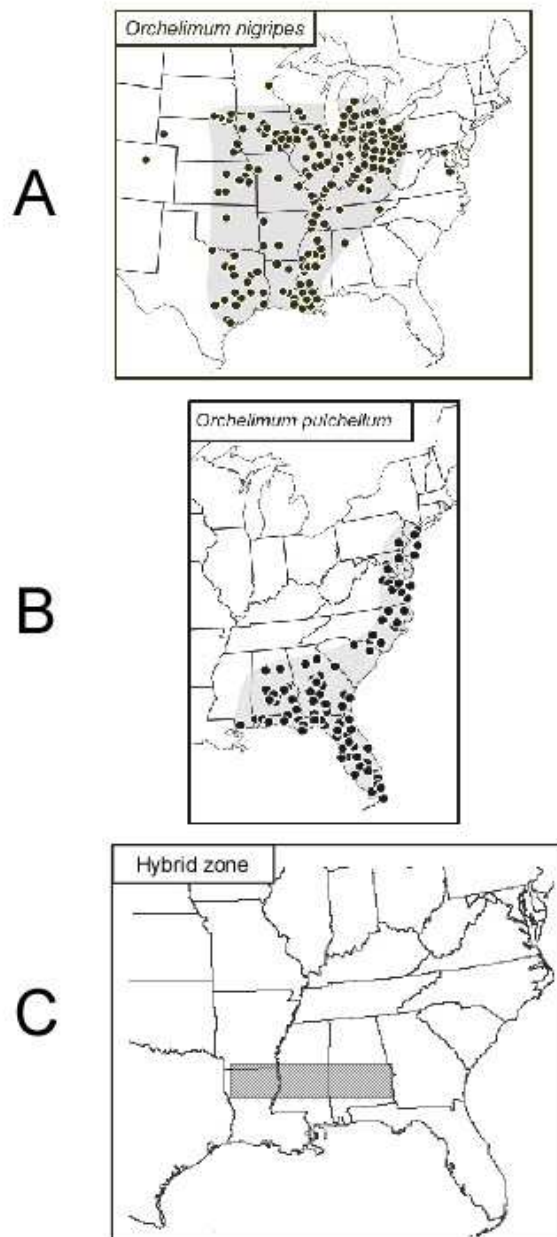


Figure 1. Distributions of parental taxa and location of hybrid zone between *Orchelimum nigripes* and *O. pulchellum*. (A) Range of *O. nigripes* (from SINA <http://www.entnemdept.ufl.edu/walker/buzz>, with permission). (B) Range of *O. pulchellum* (from SINA <http://www.entnemdept.ufl.edu/walker/buzz>, with permission). (C) Area where hybridization has been observed based upon the present work and Shapiro (1998).

Chapter 1: Species delineation by male calling song in two hybridizing meadow katydids (Orthoptera: Ensifera: Tettigoniidae)

INTRODUCTION

Speciation is the process by which populations diverge, resulting in genetically distinct groups. Various mechanisms, including genetic drift, natural selection, sexual selection, and founder effects, can all lead to isolation between populations (reviewed by Coyne and Orr 2004; Wolf et al. 2010). Understanding how reproductive isolation occurs is difficult as we cannot observe speciation directly. Studies often focus on differences between taxa where speciation is complete to infer the processes that led to reproductive isolation. However, existing species trait differences may not have initiated reproductive isolation and may have resulted from divergence following isolation. Therefore, we must be cautious in interpreting trait differences between fully isolated taxa and not assume that all observed differences between species were responsible for isolating populations.

An alternative to studying fully isolated taxa is to study partially-diverged taxa. In partially-diverged taxa, limited gene exchange still occurs and reproductive isolation can be studied as it is occurring. Observations of the interactions among partially-diverged taxa and their hybrid offspring can further our understanding of how the evolution of reproductive isolation occurs (Barton and Hewitt 1985; Bridle et al. 2006; Gay et al. 2007; Matute and Coyne 2010).

The areas where interbreeding occurs between taxa and individuals of mixed ancestry are present are called hybrid zones (Harrison 1990). Hybrid zones may be viewed as "natural laboratories" for the study of selection and fitness (Barton and Hewitt

1985; Rieseberg et al. 1999). They may contain hybrids that are first generation crosses between parental taxa, backcrosses to parental taxa, or be the result of many years of crosses by individuals of mixed ancestry. The genotypic and phenotypic variance found in hybrid populations is often greater than that found in either parental population. Traits of hybrids may be intermediate between parental taxa. For example, hybrids of the cholla cacti *Cylindropuntia spinosior* and *C. versicolor* are intermediate in height, color, spine density, and fruit shape (Grant and Grant 1971). Comparison of the fitness of these intermediate forms may help us understand how fruit shape or other intermediate characters affect the success of the cactus.

The width of a hybrid zone may provide insight into how far individuals are dispersing and the strength of selection (Barton and Hewitt 1985). High rates of dispersal increase hybrid zone width. In contrast, high fitness costs narrow the width of hybrid zones. If dispersal is low and/or few or no offspring are produced by hybrids, the hybrid zone will be narrow. If, however, dispersal is high and/or the fitness of hybrids is not significantly lower than the fitness of the parental taxa, a broad hybrid zone will result. Studies of hybrid zone width allow us to determine if selection is maintaining a hybrid zone or if both selection and dispersal together are maintaining a hybrid zone (Barton and Hewitt 1985).

I will use the term unimodal to refer to zones in which only hybrid taxa are found and bimodal to refer to zones containing parental and hybrid taxa (Jiggins and Mallet 2000). The composition of a hybrid zone may provide insights into the mechanisms by which it was formed and is maintained. For example, Jiggins and Mallet (2000) have suggested that taxa with bimodal hybrid zones often have strong assortative mating and

high fitness costs associated with hybrid matings. In broad hybrid zones, parental taxa may not encounter one another. Hybrids in broad, unimodal zones are often offspring from many generations of hybrid \times hybrid matings and may not suffer the fitness consequences often experienced by first generation hybrids.

The ecology of an area may influence hybrid zone size. For example, the intermediacy of hybrids may allow hybrids to thrive in areas where parental taxa are not found or out-compete parental taxa in an area. The size of the area that hybrids are more successful in will determine the size of these hybrid zones. Subspecies of *Macoma balthica* (Mollusca, Bivalvia) meet in a hybrid zone hundreds of kilometers long. Hybrids of these aquatic subspecies are found in areas of intermediate salinity. The environment appears to influence the size of this hybrid zone as populations show strong genetic divergence that corresponds to salinity changes (Nikula et al. 2008). Hybrids may also be less selective than parental taxa and exploit areas unsuitable to parental taxa because of the intermediacy of hybrid individuals. Hybrids between the Carrion Crow, *Corvus corone corone*, and Hooded Crow, *C. c. cornix* (Aves) forage in fields avoided by pure parental species along a long hybrid zone (Saino 1992).

The ecology of an area can also influence gene flow within a hybrid zone. A mountain range may prohibit individuals from crossing an area, therefore limiting migration of individuals between populations on either side of the mountain range. Environmental factors may also be more subtle. For example, if a suite of genes bestow a competitive advantage to organisms that live in wet regions while another suite of genes allow organisms to live in dry regions, there may be strong genetic shifts and shifts in traits between populations that correspond to habitat changes.

In addition to ecology of an area, the paleogeographic history of a region can have an influence on hybrids. Organisms retreated into refugia during the last ice age. During this physical isolation, differences accumulated between populations in different areas of refuge. As temperatures warmed, the ranges of taxa expanded. Despite these accumulated differences, diverged taxa are often still morphologically similar and have comparable dispersal abilities. Eventually these taxa met along postglacial colonization routes (Hewitt 2000). Many hybrid zones are believed to be a result of post-glacial expansion of taxa following divergence during the last ice age. Swenson and Howard (2005) have found that contact points between closely related taxa can be predicted by post-glacial expansions following the Pleistocene. In other words, organisms recolonized areas following warming periods and populations that were once physically isolated meet at common "half-way" points between points of refugia. Hybrid zones are clustered in regions because many taxa shared common refugia during the last ice age (Hewitt 2000; Swenson and Howard 2004, 2005).

Orthopteran song and hybrid zones

The Ensifera (katydids and crickets) are known for their diverse calling songs (e.g., Otte 1970; Walker 1971; Walker 1974; Gwynne 2001). Song divergence between ensiferan taxa can occur rapidly and may precede morphological differentiation (Doherty and Hoy 1985). Songs of closely related orthopteran taxa are often similar in spectral properties (e.g., carrier frequency, harmonics), but differ in temporal features, such as duration of and spacing between pulses of sound (Otte 1992). Temporal patterns, such as pulse rate, have been suggested to evolve rapidly and result in reproductive isolation in the genus *Laupala* (Shaw 1996). Songs of closely related *Laupala* crickets (Orthoptera:

Ensifera: Gryllidae) are markedly similar in spectral characteristics, but differ in pulse rate among songs of different species (Shaw 1996). If the song structure of two closely related, morphologically similar species differs, this implies selection on song. Selection may result in increased differences in songs to facilitate conspecific mate choice and avoid hybridization through character displacement (Brown and Wilson 1956).

As song is often the first indication an animal receives of the presence of another individual, it may be an important species recognition feature that represents a behavioral pre-mating barrier (Otte 1992; Gwynne 2001). Songs of Orthoptera are normally species-specific, yet there are several cases in which taxa with distinct courtship songs hybridize. For example, males of the hybridizing grasshopper species, *Chorthippus brunneus* and *C. jacobsi* (Acrididae) have very different songs, and females use song to orient to males (Bridle et al. 2006); females of both species preferred conspecific male songs and discriminated against the songs of hybrid males. For these grasshoppers, song sharpens species boundaries. Song also strengthens species boundaries in the field crickets *Gryllus firmus* and *G. pennsylvanicus* (Gryllidae) that form a mosaic hybrid zone (a patchy hybrid zone resulting from environmental fragmentation); when individuals from allopatric populations were presented with calls with conspecific and heterospecific pulse rates, they preferred the pulse rate of conspecific males (Doherty and Storz 1992).

Alternately, song differences are not always recognized by females and variation in song may be the result of genetic drift or climatic differences experienced by populations, rather than sexual selection through female choice (Veech et al. 1996; Mousseau and Howard 1998). In other Orthoptera, differences in male song between species are not recognized by females. The crickets *Allonemobius fasciatus* and *A. socius*

(Gryllidae: Nemobiinae) sing different songs and meet in a mosaic hybrid zone in the Eastern United States (Mousseau and Howard 1998). Females do not discriminate between the species by song dissimilarity, and the song differences may be the result of genetic drift in these taxa (Doherty and Howard 1996; Olvido and Wagner 2004). The role of song, if any, in mate discrimination is unclear in these hybridizing Orthoptera.

*Hybridization between *Orchelimum nigripes* and *O. pulchellum**

My research focuses on pre-mating isolation of hybridizing acoustic Orthoptera. Here I examine species discrimination between two acoustic meadow katydids, *Orchelimum nigripes* and *O. pulchellum*, that are incompletely isolated (hybridizing) and for which no song differences have been previously documented (Walker 1971). *Orchelimum nigripes* and *O. pulchellum* are allopatric throughout most of their native ranges. *Orchelimum nigripes* is found throughout most of the Central United States, extending east to the Appalachian Mountain Range (Figure 1, Walker 1971; Morris and Walker 1976). *Orchelimum pulchellum* is found east of the Appalachian Mountain Range, extending to the East Coast of the United States (Figure 1, Walker 1971; Morris and Walker 1976). *Orchelimum nigripes* and *O. pulchellum* have been described as different species based upon their morphology (Scudder 1875; Davis 1909; Rehn and Hebard 1915; Blatchley 1920; Walker 1971). Across their distributions, males of these species have consistent morphological differences that include the shape and angle of the male's cercal tooth, the angle of the female's ovipositor, the shape of lateral pronotal lobes, and leg and eye color (Rehn and Hebard 1915; Shapiro 1996).

Orchelimum nigripes and *O. pulchellum* hybridize. Shapiro (1998) identified two hybrid zones between these taxa based upon morphological and allozyme data. Shapiro

(1998, 2000, 2001) focused on the northeastern hybrid zone located near the Potomac River Basin, Washington, DC, which likely formed in the past 75 years as a result of human introduction of *O. nigripes* into habitat occupied by *O. pulchellum*. This study focuses on the southeastern hybrid zone (Figure 1), which extends across much of Louisiana, Mississippi, and Alabama south of the Appalachian Mountain Range. This older zone likely formed naturally from secondary contact in the Pleistocene (Shapiro 1996).

Shapiro (2001) found asymmetric mate choice between the species in the northern hybrid zone, such that *O. nigripes* discriminated against heterospecifics, while *O. pulchellum* did not. These preferences were maintained despite fitness costs for females who mated with the wrong species in terms of the number of offspring produced and sterility of male offspring (Cabrero et al. 1999; Shapiro 2000). Given fitness costs of heterospecific matings between these species (in the northeastern hybrid zone), selection is expected to favor pre-mating discrimination against heterospecific individuals (Shapiro 2000), if associated fitness costs in the southeastern hybrid zone are similar to those found in the northeastern hybrid zone.

Acoustic communication in Orchelimum nigripes and O. pulchellum

Male *Orchelimum* produce a prominent calling song; females are silent and move toward males to begin courtship (Morris and Walker 1976). The songs of *O. nigripes* and *O. pulchellum* consist of two parts. The first is a series of loud ticks with silence between these sound pulses, followed by a silent interval (Figure 2). The second is a longer series of softer and more tightly spaced pulses referred to as a “buzz” (Morris and Walker 1976). These two parts are followed by a period of silence and then repeated. Otte

(1992) suggested the different parts of ensiferan song may serve different functions in communication—i.e., one portion may be used for male-male competition and the other female attraction. In *Orchelimum*, male songs are used both in male-male competition and in attracting females (Morris and Walker 1976; Feaver 1983; Feaver 1985).

In the present study, I compared the songs of male *Orchelimum nigripes* and *O. pulchellum* to determine whether there are differences in songs in these species. Songs of males within the southeastern hybrid zone were recorded and compared to those of pure species males at similar latitude. Prior to this study, no quantitative song differences between these species had been identified (Walker 1971; Shapiro 1996).

Songs of katydids were also recorded from individuals along a transect through the southeastern hybrid zone. Patterns of variation in signals through a hybrid zone can be used to infer how genes are moving through a zone, and whether or not selection is influencing signal characters. Additionally, the distributions of different traits may be compared to determine if selection was similar among traits. Segmental-linear regression was used to compare populations to determine whether the observed variation was best explained by a single regression equation (suggesting clinal variation) or separate regression equations obtained by applying a break point. The longitude at which song break points occur was compared to a maternally inherited mitochondrial DNA marker (Chapter 2).

METHODS

Male katydids were collected as adults and late-instar nymphs in the summer and fall of 2006–2009. Allopatric *Orchelimum nigripes* were collected from Missouri, Kansas, and Texas. Allopatric *O. pulchellum* were collected from two locations in Georgia and one location in Florida (Table 1, Figure 1). The remaining populations were collected within a previously identified hybrid zone (Chapter 2, Shapiro 1998). Animals were transported to the laboratory in insulated, cold-food storage containers to avoid exposing them to dramatic temperature shifts during transport. In the lab, males were kept at $25 \pm 2^\circ \text{C}$ on a 12:12 h light:dark cycle and reared on orthopteran food mix (Rentz 1996) with the addition of millet seed mix. Moisture was provided by hydrated Watersorb® crystals (Watersorb, Inc., Hot Springs, AR). Animals were housed in the laboratory a few days to several weeks before they were recorded, depending on whether they were collected as nymphs or adults and because of time constraints on the number of animals that could be recorded in a day.

The songs of five to fifteen individuals of each population were recorded. For recording, a male was placed in a cage (approximately $25 \times 25 \times 25 \text{ cm}^3$) covered by fine mesh netting in a semi-anechoic chamber maintained at $25 \pm 1^\circ \text{C}$. Temperature can affect calling song (Walker 1975; De Graaf et al. 2005; Beckers and Schul 2008); therefore, all animals were recorded in the laboratory within a narrowly bracketed temperature range. Ninety seconds of calling song were recorded for each male. Digital recordings were made with a Linear X microphone (Model M51, Tualatin, OR) sensitive to 75 kHz and captured at 151 kHz using a Pettersson interface with a National Instruments DAQ sound card and BatSound Pro (Pettersson Electronics AB, v. 3.30).

Before analyzing each recording, I applied, a 1-kHz high-pass filter to eliminate background noise. The pulse characteristics feature of BatSound Pro was used to mark the beginning and ending of volleys of sound from spectrograms. Pulse marks were edited manually. These marks were used to calculate the temporal patterns of songs.

From these laboratory recordings, I measured seven traits that frequently distinguish the calls of the 19 species of *Orchelimum* (Walker 1971). From four successive tick-buzz periods (exceptions noted below) of each male, I measured and calculated the mean of: (1) the number of ticks (before a buzz); (2) tick length; (3) tick period (length of time from the start of a tick to the start of the next successive tick); (4) buzz length; (5) buzz chirp period (length of time from the start of a chirp to the start of the next chirp within a buzz); (6) length of silent interval between end of ticks and start of a buzz; and (7) length of tick-buzz period (Figure 2). The buzz chirp period (5) represents an average of 10 chirps in the middle of each of the four successive buzzes. Central chirps were used because they are the most uniform. The last trait, (7) tick-buzz period (length of time from the start of a tick-buzz to the start of the next successive buzz), was averaged from the entire 90 sec recorded for each male (Figure 1).

Statistical analyses were performed with two computer programs. I used Minitab (version 13.31, Minitab Inc.) to run Nested ANOVAs (individuals within populations within species) and a MANOVA to determine if there were population- and/or species-level differences for each of the seven song parameters. Minitab was also used to calculate a discriminant analysis to determine how effectively song temporal patterning can be used to predict species identity. Longitudinal variation across the hybrid zone was compared with segmental linear regression calculated in SegReg (R. J. Oosterbaan, Water

Info) to determine if and where there was disjuncture in songs to compare to a mitochondrial DNA marker (Ch.2).

RESULTS

There are clear differences in temporal patterning between *Orchelimum nigripes* and *O. pulchellum*, but the structure of the song (multiple ticks followed by a buzz) was the same between species. Closely related katydids often have similar songs that differ in temporal patterning (Nityananda and Balakrishnan 2006). The songs of *O. nigripes* and *O. pulchellum* have an overlapping, broad frequency range with the majority of call energy (amplitude) between 10–45 kHz (data not shown). I found variation in temporal characters of calling songs for multiple song characters between species and significant differences among populations within a species for one song character.

Song-tick characters

There are no significant differences among populations of pure species for the three parameters associated with the tick portion of male calling song. However, significant variation exists between species in both the number of ticks a male produced (1; Figure 3) and the average tick length (2; Figure 4). Calls of male *Orchelimum pulchellum* have significantly more ticks preceding the buzz (1) than those of male *O. nigripes*; the length of the ticks of songs of *O. nigripes* is longer than that of songs of *O. pulchellum*. Tick period (3) is not independent of tick length (2) and is the single character measured that does not differ between these species (Figure 5). Thus, tick period cannot be used to distinguish between species.

Song-buzz characters

Buzzes begin softly and rapidly increase in amplitude across the first several chirps. The middle of the buzz has steady amplitude with end chirps decreasing in amplitude. For each species, the maximum amplitude of the buzz is less than that of ticks

(Figure 1). There are significant interspecific differences, but not inter-population variation of a species in both buzz length (4, Figure 6) and buzz-chirp period (5, Figure 7). The buzz length (4) of calls of male *Orchelimum pulchellum* is approximately twice as long as that of male *O. nigripes*. The chirps composing a buzz are tightly spaced, almost merging together; therefore, because it was difficult to identify the chirp length accurately, it was not measured. The period of buzz chirps in *O. pulchellum* is significantly longer than it is in *O. nigripes* (Figure 7).

Silence and overall call parameters

The silent interval (6) between the end of the ticks and the start of the buzz was significantly shorter in *O. nigripes* than in *O. pulchellum* (Figure 8). There was also a significant difference among populations within a species for the silent interval between the ticks and buzz. The silent interval (6) song characteristic is independent of all song parameters except the tick-buzz period. The tick-buzz period (7) of *O. nigripes* was significantly shorter than that of *O. pulchellum* (Figure 9) due to shorter buzzes, shorter silent intervals between ticks and buzzes, and shorter silent intervals between tick-buzz calls (data not shown) in *O. nigripes* song compared to *O. pulchellum* song.

Overall, compared to *Orchelimum nigripes*, males *O. pulchellum* spend about twice as long performing the tick portion of their call, approximately four times as long on their silent interval before the buzz, and twice as long on their buzz. The tick-buzz period (7) describes the overall timing of singing and is not independent of any characteristics measured, except number of ticks (1). Because tick period does not differ between the species, whereas tick length does, males are producing volleys of sound with different duty cycles: male *O. nigripes* are filling more of the tick period with sound

than are *O. pulchellum*. Therefore, the duty cycle of *O. nigripes* is shorter than that for *O. pulchellum*.

Note that the following population pairs are located less than a degree apart in longitude, but different at latitudes: KS & TX, MO & LA-1, and GA-2, FL. Despite differences in latitude, calls were similar in most temporal parameters. This suggests that differences in song do not merely reflect geographic variation as north-south shifts in habitat are much greater than those across the west-east zone (pers. obs.).

A MANOVA was performed to determine overall differences in songs based upon the above parameters. Tick-buzz period was not included as it is not independent of the other temporal features. Songs were significantly different between the species (MANOVA: $F_{4,56} = 46.145$; $P < 0.0001$). To determine how accurately song patterning predicted species identity, a discriminant function was calculated. The four independent song features used to calculate the discriminant analysis were: number of ticks (1), tick length (2), buzz length (4), and silent interval (6). The resulting function correctly identified songs ($n = 61$) 93.4% percent of the time, despite the variability within a species.

Hybrid zone song

Hybrid populations were different from parental populations in song. Analysis by segmental linear regression (Figure 10) identified break points for all song characters except tick period (3), which did not differ between these species (Figure 5). The residual values for number of ticks (1), tick length (2), buzz length (4), and buzz chirp period (5), were best fit to two individual horizontal lines with a break point corresponding to the eastern point where the *O. nigripes* COI mtDNA haplotype

terminates (Figure 10). There was covariance between longitude and both the silent interval (6) and tick-buzz period (7), resulting in two sloping lines of best fit for each of these song parameters (Figure 10). The disjuncture in both of these non-independent song parameters corresponds to the westernmost *O. pulchellum* mtDNA haplotype (Figure 10).

DISCUSSION

Species-specific song differences

Male calling songs between *Orchelimum nigripes* and *O. pulchellum* have significant differences in temporal patterning. These differences can be used to determine species identity of individuals by recording animals in the laboratory and analyzing songs with a discriminate analysis function. Overall, the calls of male *O. pulchellum* are temporally lengthened versions of the calls of male *O. nigripes*. Temporal characteristics of *O. pulchellum* males are longer in all but two parameters. The tick length of *O. pulchellum* is shorter than the tick length of *O. nigripes*. The tick period does not differ between *O. pulchellum* and *O. nigripes*. The most distinctive temporal features of male song are the number of ticks preceding a buzz (1) and the tick length (2).

The temporal differences in song between *Orchelimum nigripes* and *O. pulchellum* have been documented in other closely-related insects (Tregenza et al. 2000; Nityananda and Balakrishnan 2006; Sueur and Puissant 2007). Song evolution in closely-related taxa often occurs through differences in song parameter lengths (Tregenza et al. 2000; Nityananda and Balakrishnan 2006; Sueur and Puissant 2007). For example, in the katydid genus *Mecopoda* (Tettigoniidae) there are several sibling species that are morphologically cryptic but have distinctive calls that differ in temporal patterning (Nityananda and Balakrishnan 2006).

Despite having statistically significant temporal differences, songs are variable within a species. For many song features (Figures 5, 8–9), there is overlap in parameter length between the species. The overlap in songs between species decreases the likelihood of females distinguishing between hetero- and conspecific males during long-

range communication, and may result in recognition errors by females during phonotaxis. This could partially explain why isolation is incomplete between these species.

Acoustic calls used in species discrimination are normally distinctive (Gerhardt 1991). While songs provide the raw material for an individual to select a conspecific mate the majority of the time, the margin of error by females would be quite high if song alone were used for discrimination. Given four temporal traits, species can be correctly diagnosed from their calls by a discriminant function over 93% of the time. This is low for individuals using acoustic information for species discrimination. For example, a discriminant function analysis can be used to correctly identify the species of individuals 99% of the time between the sympatric burrowing seabirds (Aves), the Yelkouan shearwater *Puffinus yelkouan* and the Mediterranean Cory's shearwater *Calonectris diomedea diomedea* (Cure et al. 2009). Likewise, acoustic calls of the warblers (Aves), *Phylloscopus reguloides* and *Phylloscopus davisoni* were diagnostic between species (Packert et al. 2009). Songs of the lacewings the carnea group of *Chrysoperla* (Neuroptera: Chrysopidae) are also distinctive among the morphologically similar species, *C. agilis*, *C. carnea* and *C. pallida* (Henry et al. 2003). The overlap in temporal song traits between *Orchelimum nigripes* and *O. pulchellum* is larger than expected for song to be an effective pre-mating barrier.

There is asymmetric isolation between *Orchelimum nigripes* and *O. pulchellum* (Chapter 2). It is unclear if song contributes to this behavioral isolation. Females may, instead, distinguish between species later in courtship (Chapter 2). This study provides a framework for determining female preference for male song to determine what role, if any, song plays in isolating these katydids. An examination of female preference for

male song will clarify the importance of song characters in mate choice between *O. nigripes* and *O. pulchellum* (Chapter 3).

Songs across the hybrid zone

Although morphology is intermediate across this hybrid zone, male calling songs were not intermediate. Calling songs of pure *Orchelimum nigripes* and *O. pulchellum* did not differ significantly in tick period (3), and this trait did not vary across this zone. For the remaining six traits analyzed, songs were different between the species and segmental linear regression revealed disjuncture in temporal patterning across this zone.

Male songs patterns are bimodal for four of six song parameters that differed between the species. It is unclear why song traits are bimodal, but disruptive selection or other genetic mechanisms may contribute to this bimodal distribution. Few researchers have studied the genetics of orthopteran songs. A notable exception is the work of Shaw et al. (2007) that performed QTL mapping of male song in *Laupala* crickets (Gryllidae). The QTL's for male song individually did not explain much of the variation observed between species; therefore, Shaw et al. (2007) concluded additive genetic variance was likely responsible for the differences in song observed among *Laupala* crickets. The genetic architecture of *Orchelimum* songs has not been addressed.

For the songs of *Orchelimum nigripes* and *O. pulchellum*, the breakpoint of these four parameters (1-2, 4-5) was concordant with a change in a genetic marker that is maternally inherited and independent of male song, COI mtDNA (Chapter 2). This shift in male song occurred at the eastern point where the *Orchelimum nigripes* mtDNA marker terminated; only mtDNA of *O. pulchellum* was found east of this point.

The traits silent interval (6) and tick-buzz period (7) are not independent of one another, but they appear to be inherited independently of the others (1-2, 4-5), because the break points differ. The break point of (6) and (7) is shifted west relative to the break point of the other song parameters (1-2, 4-5). Traits (6 and 7) are not bimodal. They decrease and subsequently increase from West to East across the transect, thus, they covary with longitude (Figure 10) and have a point of disjuncture in Louisiana. This break point corresponds to the eastern most point at which *Orchelimum pulchellum* mtDNA was found (Chapter 2).

Multiple processes may result in displacement of characters including hybrid zone movement, genetic drift, and selection. Shapiro (Shapiro 1998) has suggested this hybrid zone is stable based upon morphological and genetic clines he identified, so hybrid zone movement is unlikely. Genetic drift is also improbable as it would result in a random pattern of character shifts rather than produce the two concordant shifts observed here, but it is difficult to effectively rule out drift from the available data (Barton and Hewitt 1985).

A third possibility that may explain the western shift of the silent interval (6) and tick-buzz period (7) relative to the other four characters is sexual selection. Asymmetric isolation in mating may be responsible for this shift. Mating asymmetry was observed between populations of pure species adjacent to the hybrid zone (Chapter 2). Tick-buzz period length appears to be a sexually selected trait in *Orchelimum nigripes* but not in *O. pulchellum* (Chapter 3). This difference in song preference between species (Chapter 3) along with mating asymmetry (Chapter 2) may be shifting silent interval (6) and tick-buzz period (7) west.

Hybrid zone ecology

Orchelimum nigripes and *O. pulchellum* are not the only species that meet in the southeastern United States. The distributions of many fish as well as crabs, birds, oysters, and terrapins abut in this region (reviewed in Avise 1992). Several species also hybridize in this region and disjunctions in mtDNA and other characters are often associated with drainages of major rivers. Numerous major waterways run roughly North-South through Alabama, including the Tombigee, Black Warrior, Cahaba, and Coose riverways. Chorus frogs of the *Pseudacris nigrita* complex form a narrow hybrid zone at the border of Louisiana and Mississippi along the Pearl River mixed hardwood bottomlands (Gartside 1980). In this ecologically distinct region, nearly all frogs are hybrids due to either frequent hybridization or significant backcrossing in this area (Gartside 1980).

An RFLP study of the Carolina Chickadee, *Parus carolinensis* (Aves) revealed a major West-East split at the Tombigee/Mobile bay drainage in Alabama, despite the ability of these birds to fly and readily cross waterways. A finer scale analysis of the habitat used by *Orchelimum nigripes* and *O. pulchellum* may clarify if ecology is also influencing the location of hybridization between these species.

Within the hybrid zone in the Southeastern United States between *Orchelimum nigripes* and *O. pulchellum* (Figure 1), only hybrid individuals are found (pers. obs.); this is a unimodal hybrid zone. The hybrid zone is broad, suggesting that either hybridization between *O. nigripes* and *O. pulchellum* is not costly or that dispersal is very high. Because parental taxa do not encounter one another, hybrids are later generation hybrids. The mountain hares *Lepus europaeus* and *L. timidus* also hybridize across a broad zone

(Thulin et al. 2006). Thulin et al. (2006) have suggested that one reason this zone is broad is that there is little genetic differential between these species. Similarly, dispersal prior to mating may not be costly between *O. nigripes* and *O. pulchellum* as the genetic differences between neighboring populations are likely small, but a future direction for this research includes assessing the relative fitness of individuals across this zone.

These species and hybrids are agile fliers, but were not found in many locations that appeared to be appropriate habitat, and the distribution of katydids appeared patchy. Across the zone, suitable habitats were fragmented by human development. Despite their ability to fly, I suspect (because of how patchy katydid populations are) they do not disperse much across the southern hybrid zone. This hybrid zone has no obvious geographic boundaries between parental and hybrid populations, but again, this was not closely examined as it was not a focus of this study. Both parental species are able to live in a variety of habitats as demonstrated by their north-south distributions. Because these habitats are much more variable than those found across the Deep South, it appears that both parental species may be able to live anywhere across the zone, yet only intermediates were found across the zone. One of the parental species, or the hybrids, could potentially have a local advantage. However, I do not think it is likely that micro-habitat preferences separate these species in the field given that they are found in similar environments and feeding and calling from similar areas within those habitats (pers. obs.).

These katydids do not appear to be habitat specialists as both parentals and hybrids were found in a variety of habitats. The vegetation and predators (birds, anoles) are similar in the range occupied by parental taxa and across the hybrid zone. For example,

males of both parental species and the hybrids would sing from lower grassy vegetation as well as high in kudzu and trees. They were also observed feeding on seeds and insect prey (which were ubiquitous), so this hybrid zone is not likely an ecotonal zone.

Hybrid zone implications

Regions where hybrid zones, species, and/or phylogeographic break points cluster are referred to as suture zones (Swenson and Howard 2004). The region of the southeastern United States where *Orchelimum nigripes* and *O. pulchellum* hybridize is posited to be a suture zone (Swenson and Howard 2005). There is some controversy regarding where these "hot spots" are located, but the hypothesized glacial refugia in eastern Texas and western Florida correspond to the ranges of *Orchelimum nigripes* and *O. pulchellum* respectively. The primary point of song disjuncture between these species is located in Alabama. There was no significant difference in size between these species (Chapter 2); they likely dispersed and extended their ranges in an equivalent manner. At this half-way point of postglacial expansion, populations of *O. nigripes* and *O. pulchellum* are likely to have encountered one another and initiated this hybrid zone.

Hybrid zones are not static; they may shift in location or disappear entirely over time (Buggs 2007). The fate of a hybrid zone is partially determined by the fitness of hybrids and how far individuals disperse each generation (Barton and Hewitt 1985, 1989; Harrison 1993). The hybrid zone between *Orchelimum nigripes* and *O. pulchellum* is a broad, stable zone (Shapiro 1998). Further study is needed to understand the fitness and dispersal of individuals in this zone and the role of male song in the stability of this hybrid zone.

Studying male song differences alone provides only half of the evolutionary picture of species recognition. Male song differences will have little impact unless females attend to the song differences. Future research should address mate preferences of hybrid females through behavioral choice tests, including preferences for the silent interval (6) and tick-buzz period (7) of male songs. Additionally, estimations of dispersal of hybrids using population genetics will further our understanding of this hybrid zone, a zone that appears to be stable despite significant differences in male calling song between parental species. This hybrid zone appears to be stable and to have resulted from asymmetric preference (Chapter 2). This research demonstrates that stable hybrid zones are possible in spite of statistically significant courtship signal differences between the parental species.

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| Site | Number of individuals recorded | Latitude | Longitude | Locality |
|------|--------------------------------|----------|-----------|-----------------|
| MO | 8 | 38.5268 | -93.5070 | Windsor, MO |
| KS | 9 | 38.9627 | -95.2553 | Lawrence, KS |
| TX | 15 | 32.4478 | -95.1699 | Tyler, TX |
| LA-1 | 9 | 32.5973 | -93.3257 | Minden, LA |
| LA-2 | 9 | 32.4781 | -92.1979 | West Monroe, LA |
| MS | 10 | 32.2953 | -90.3338 | Clinton, MS |
| AL-1 | 10 | 32.5149 | -87.8703 | Demopolis, AL |
| AL-2 | 9 | 32.2545 | -86.3620 | Montgomery, AL |
| AL-3 | 9 | 32.5568 | -85.4705 | Auburn, AL |
| GA-1 | 14 | 32.5425 | -83.5391 | Bonaire, GA |
| GA-2 | 10 | 33.6361 | -82.5930 | Thompson, GA |
| FL | 5 | 29.6338 | -82.3676 | Gainesville, FL |

Table 1. Collection sites for male *Orchelimum nigripes* and *O. pulchellum* recorded in this study. Locations are listed from west to east. Locality information provides the nearest city or town and the state in which specimens were collected. Latitude and longitude are given in decimal degrees; site abbreviations are the same as those in Figure 2.

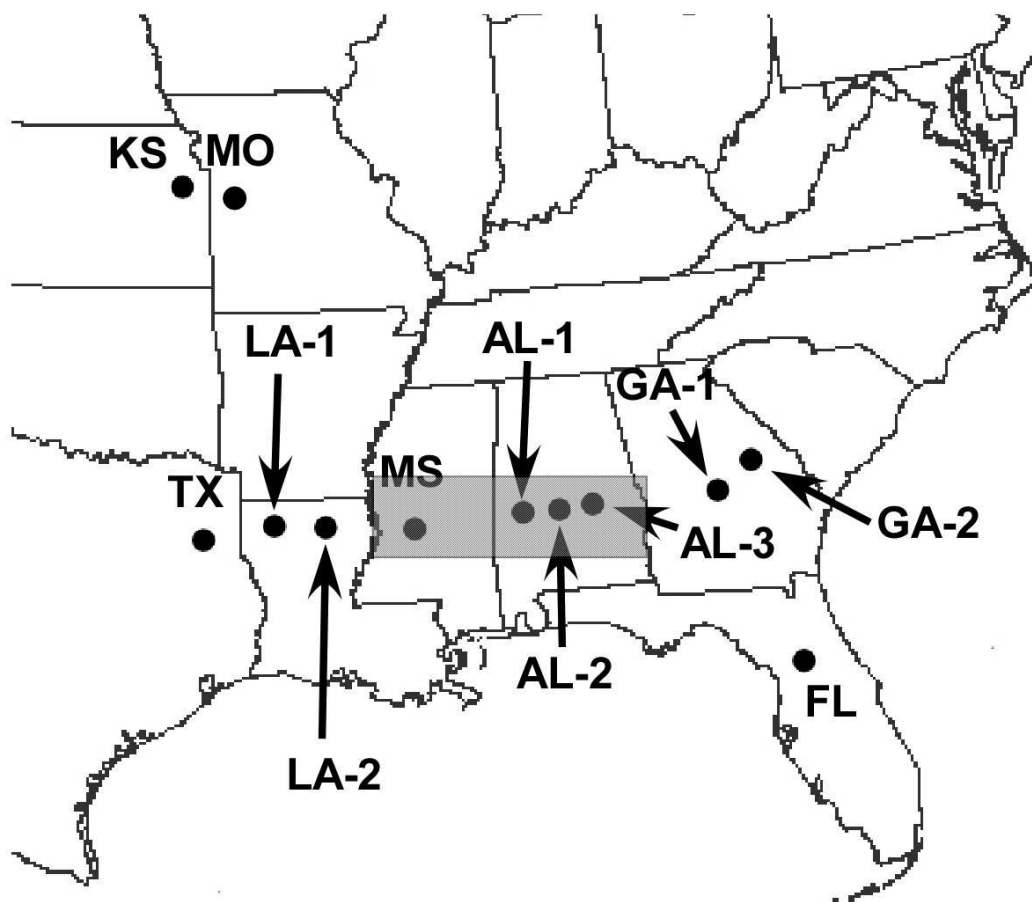


Figure 1. Collection localities of populations of male *Orchelimum nigripes* and *O. pulchellum* recorded in this study. Abbreviations as in Table 1. Grey bar indicates area where hybrid katydids are found based upon Shapiro's (1998) genetic and morphological work and my field work 2006–2009 (pers. obs.).

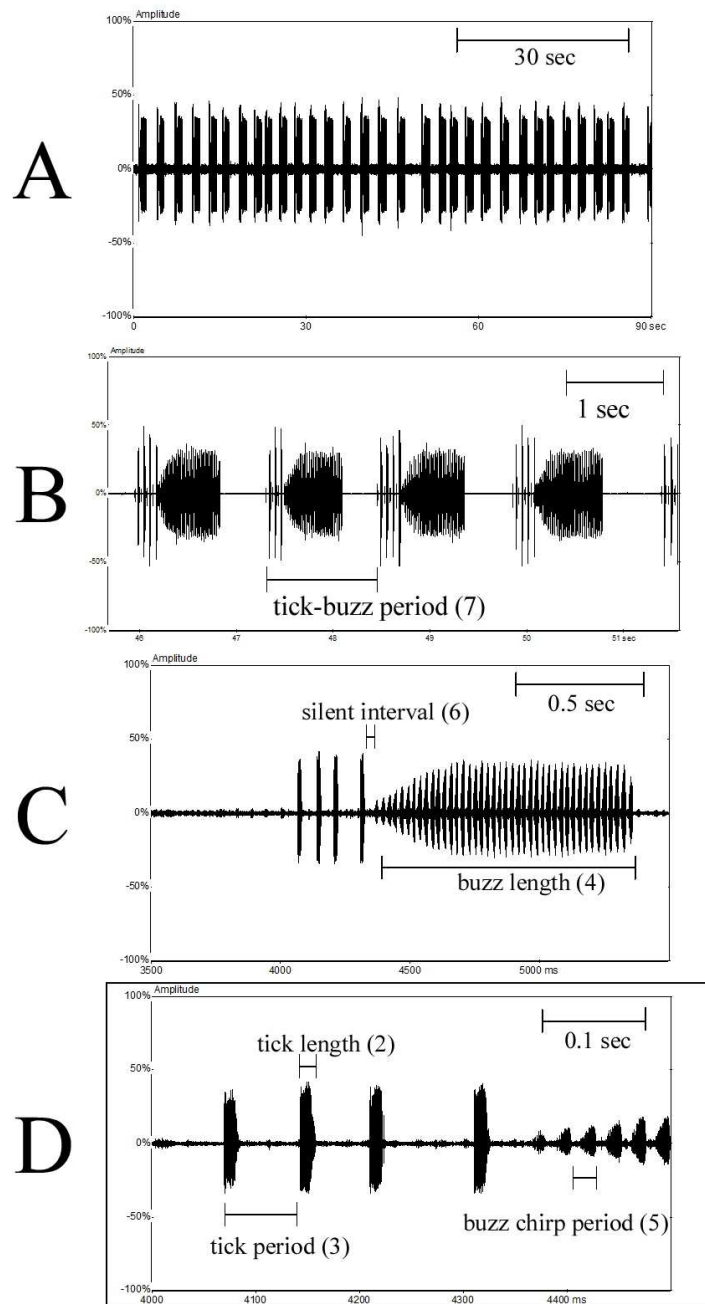


Figure 2. Oscillograms of *Orchelimum nigripes* male call to illustrate temporal measurements used in the study. Numbers in parentheses correspond to song parameters throughout text. (A) Ninety second male song showing multiple tick-buzz cycles. (B) Enlarged view of A showing measurement of tick-buzz period. (C) Enlarged view of B showing one tick-buzz period. The silent interval between the end of the ticks and the start of the buzz is shown along with the buzz length. (D) Enlarged view of C showing the ticks and beginning of a buzz. The tick length, tick period and buzz chirp period are shown.

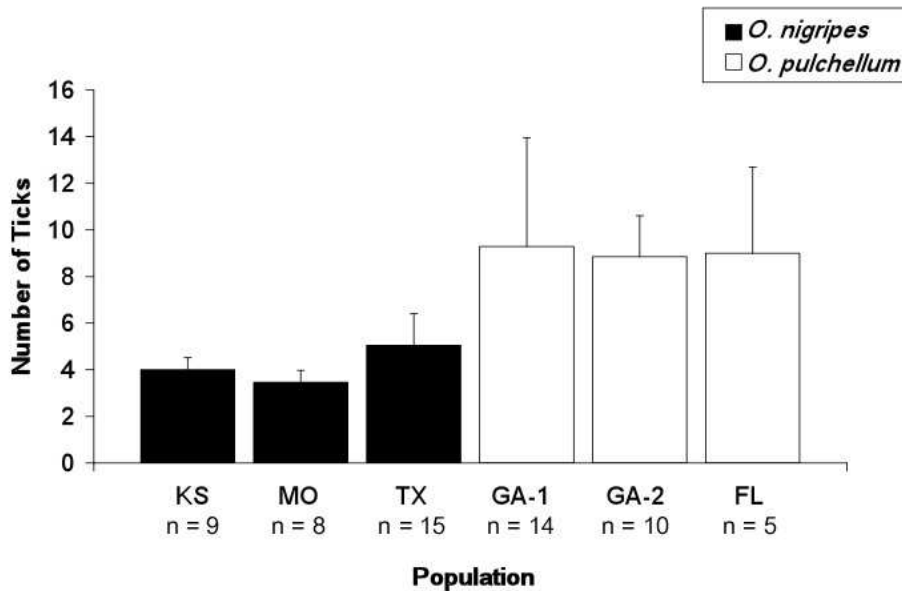


Figure 3. Number of ticks preceding a buzz of each species by population. Populations are arranged by the relative distance to the hybrid zone with the closest populations for each species flanking the center of the graph. Abbreviations are as in Table 1 and Figure 2. There is a significant difference in the number of ticks preceding the buzz between the species but not among populations (Nested ANOVA: $F_{1, 60}=75.70$, $p<0.0001$, species (population) $F_{5, 60}=0.55$, $p=0.699$). Error bars indicate 1 standard deviation.

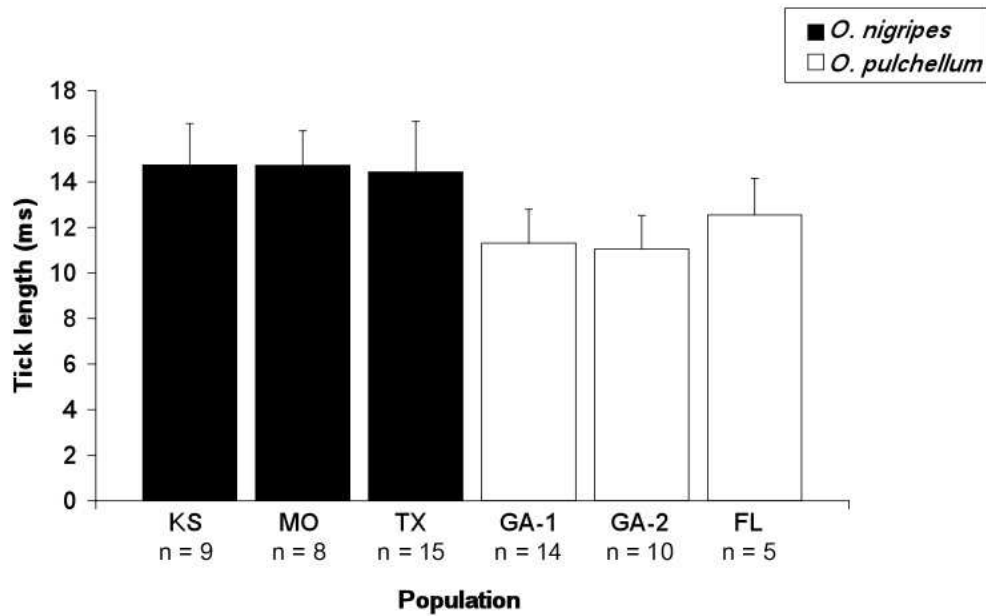


Figure 4. Tick length of each species by population. Populations are arranged by the relative distance to the hybrid zone with the closest populations for each species flanking the center of the graph. Abbreviations are as in Table 1 and Figure 2. There is a significant difference in the tick length between the species but not among populations (Nested ANOVA: $F_{1, 60}=54.39, p=0.001$, species (population) $F_{5, 60}=0.69, p=0.600$). Error bars indicate 1 standard deviation.

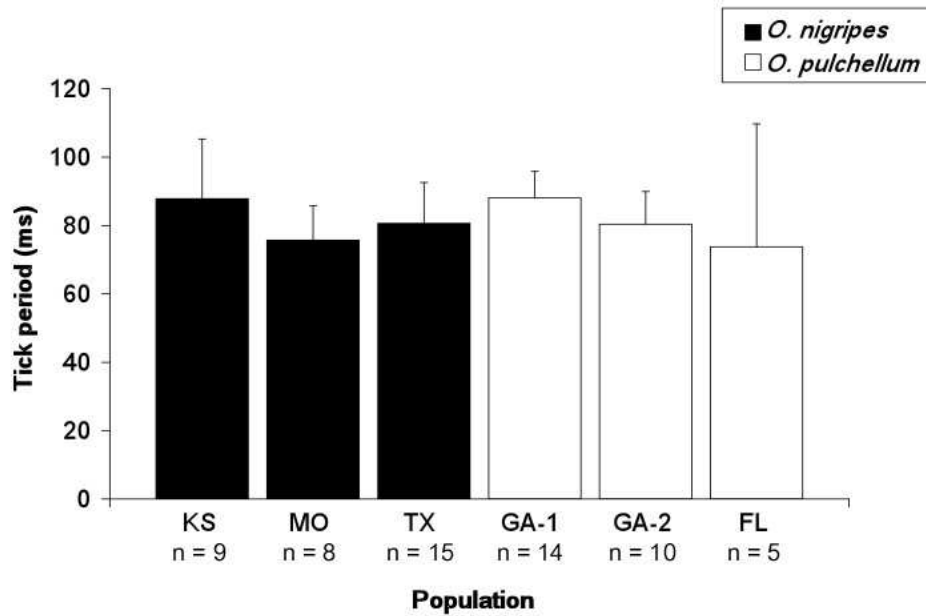


Figure 5. Tick period of each species by population. The length of time from the start of a tick until the start of the next tick is shown above by population. Populations are arranged by the relative distance to the hybrid zone with the closest populations for each species flanking the center of the graph. Abbreviations are as in Table 1 and Figure 2. There is a not a significant difference in the tick period between the species or among populations (Nested ANOVA: $F_{1, 60}=0.02$, $p=0.905$, species (population) $F_{5, 60}=0.69$, $p=0.159$). Error bars indicate 1 standard deviation.

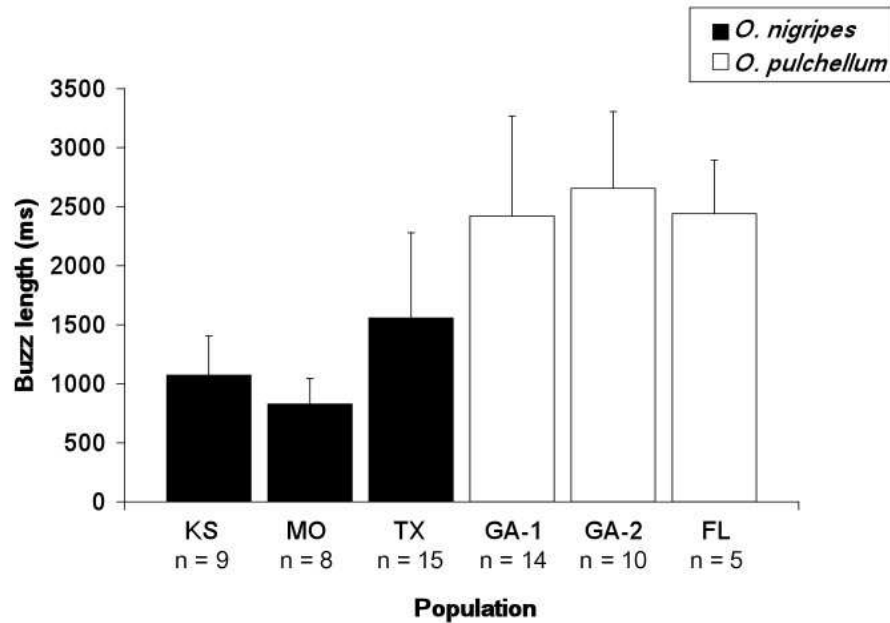


Figure 6. Buzz length of each species by population. Populations are arranged by the relative distance to the hybrid zone with the closest populations for each species flanking the center of the graph. Abbreviations are as in Table 1 and Figure 2. There is a significant difference in the buzz length between the species but not among populations (Nested ANOVA: $F_{1, 60}=29.30, p=0.005$, species (population) $F_{5, 60}=2.12, p=0.091$). Error bars indicate 1 standard deviation.

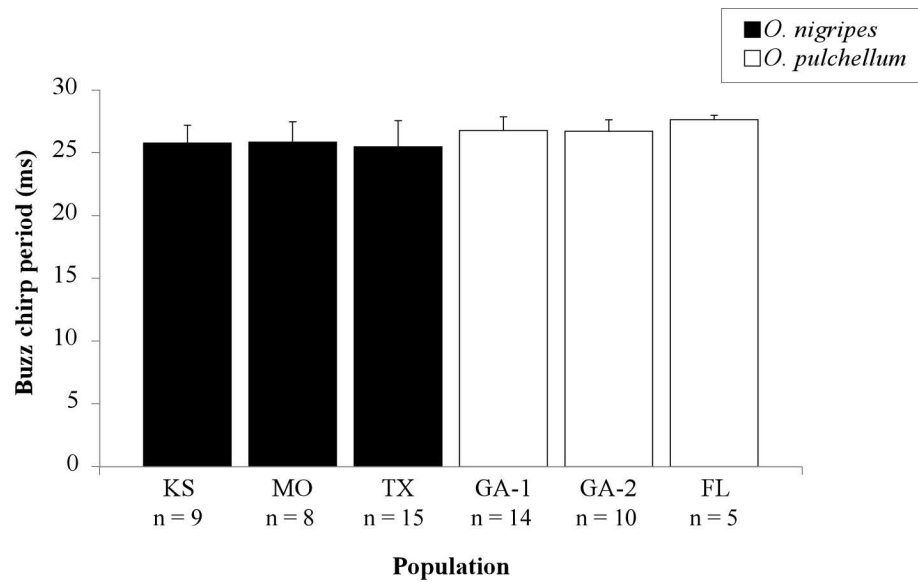


Figure 7. Buzz chirp period within the buzz of each species by population. Populations are arranged by the relative distance to the hybrid zone with the closest populations for each species flanking the center of the graph. Abbreviations are as in Table 1 and Figure 2. There is a significant difference in the chirp period between the species but not among populations (Nested ANOVA: $F_{1, 60}=21.43$, $p=0.005$, species (population) $F_{5, 60}=0.49$, $p=0.745$). Error bars indicate 1 standard deviation.

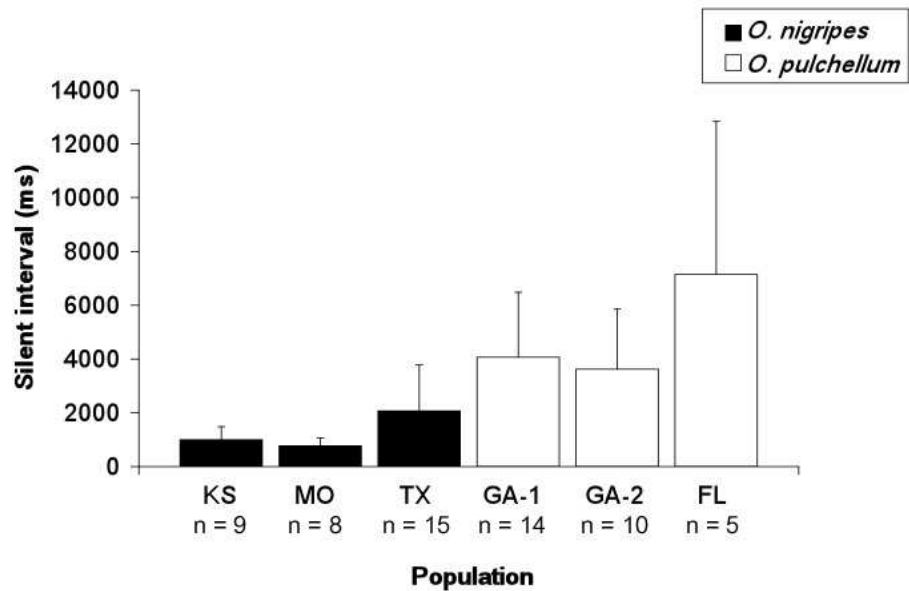


Figure 8. Silent interval between end of ticks and start of buzz of each species by population. Populations are arranged by the relative distance to the hybrid zone with the closest populations for each species flanking the center of the graph. Abbreviations are as in Table 1 and Figure 2. There is a significant difference in the length of the silent interval between ticks and buzzes between the species and among populations (Nested ANOVA: $F_{1, 60}=13.27$, $p=0.020$, species (population) $F_{5, 60}=2.65$, $p=0.043$). Error bars indicate 1 standard deviation.

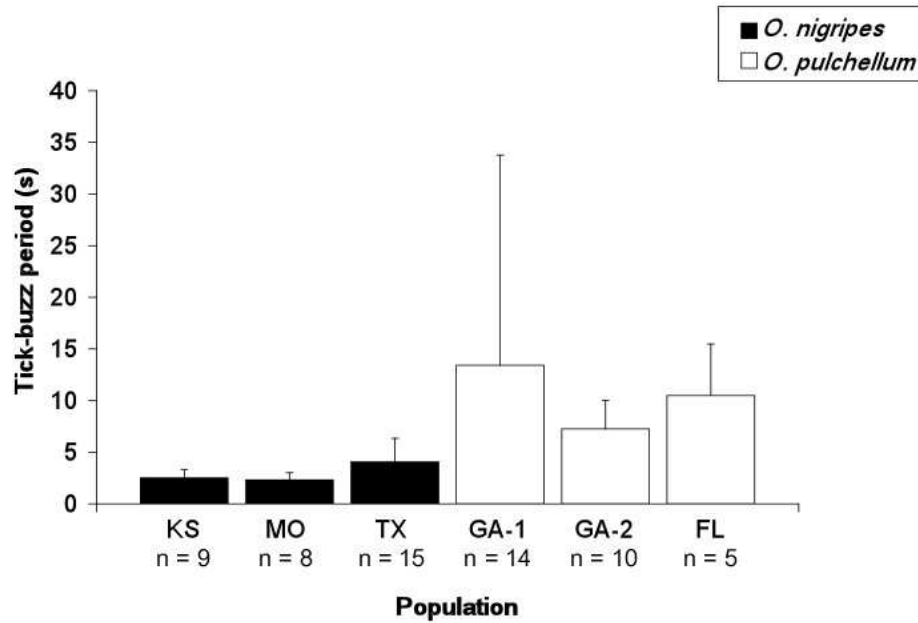


Figure 9. Tick-buzz period of *Orchelimum nigripes* and *O. pulchellum* by population. Populations are arranged by the relative distance to the hybrid zone with the closest populations for each species flanking the center of the graph. Abbreviations are as in Table 1 and Figure 2. Tick-buzz period differs significantly between species but not among conspecific populations (Nested ANOVA: $F_{1,60}=11.48$, $p=0.019$, species (population) $F_{5,60}=0.59$, $p=0.668$). Error bars indicate 1 standard deviation.

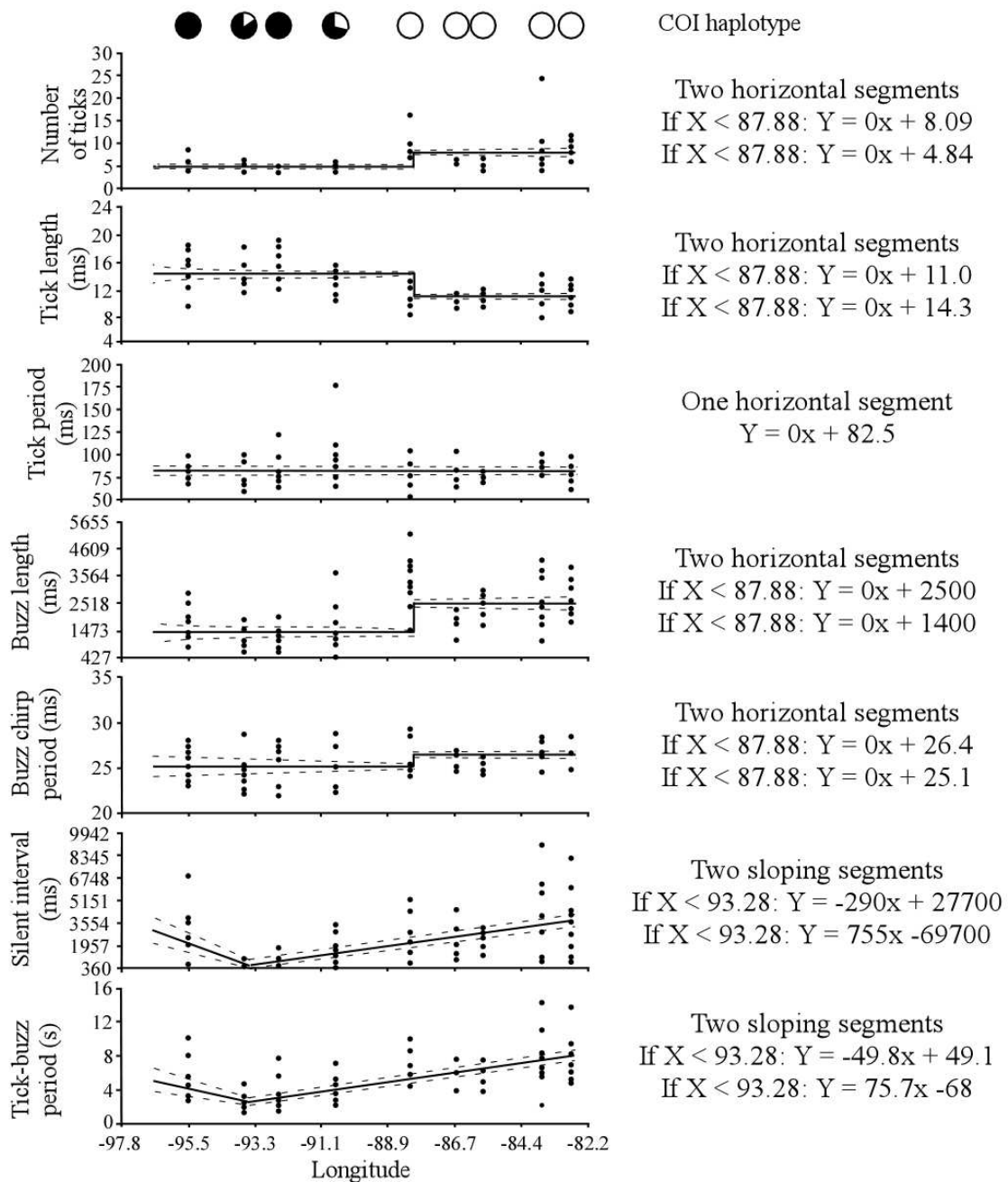


Figure 10. Segmental linear regression of temporal characters against longitude of katydids across the transect through the hybrid zone. Longitude is shown in decimal degrees. Lines of best fit are solid; dashed lines indicate 95% confidence intervals. Circles indicate frequencies of COI mitochondrial DNA haplotypes sampled from the same collection localities (black = *O. nigripes*, white = *O. pulchellum*; Ch. 2). Equations for the lines are given to the right of the graphs. The breakpoint (if present) in longitude is the value of X.

Chapter 2: Asymmetric mate choice and mtDNA introgression in two hybridizing meadow katydids, *Orchelimum nigripes* and *O. pulchellum*

INTRODUCTION

Mate recognition systems play a fundamental role in the origin and maintenance of species (Paterson 1985; 1994; Butlin and Ritchie 1994). Sexual selection may also play such a role in speciation (Lande 1981; Panhuis et al. 2001; Gage et al. 2002; Ritchie 2007). Although often regarded as mutually exclusive, specific mate recognition and sexual selection may instead be an extension of each other (Ryan and Rand 1993).

Individuals optimize their evolutionary fitness through mate choice, thus for closely related taxa, the relative roles of mate recognition and sexual selection are important for understanding species differences, or when those species differences break down through the formation of hybrid zones.

Mate choice can profoundly affect the dynamics of hybrid zones. Hybrid zone stability is affected by mate choice (reviewed in Buggs 2007) and species-specific characters may be introgressed asymmetrically by asymmetric mate choice (e.g., Harrison 1983; DeSalle et al. 1986; Parsons et al. 1993; Stein and Uy 2006). Mate choice may decrease hybridization through the process of reinforcement if assortative mating is under selection (reviewed in Servedio and Noor 2003). However, animals may hybridize because of insufficient mate recognition, scarcity of conspecifics (thus making the best of a bad situation by taking an opportunity to mate) and reaction to supernormal stimuli (reviewed in Randler 2002). Assessing mate preferences is essential to understanding the dynamics underlying a hybrid zone and the potential implications for speciation.

Orchelimum nigripes and *O. pulchellum* (Orthoptera: Tettigoniidae: Conocephalinae) are meadow katydids that hybridize in two zones in the USA. *Orchelimum nigripes* is found west of the Appalachian mountains whereas *O. pulchellum* is east (Walker 1971; Morris and Walker 1976). A northern hybrid zone is found in the Potomac River Basin (PRB, Shapiro 1998, 2000, 2001). By allozyme and morphological analysis, the southern hybrid zone extends from western Mississippi to eastern Alabama (Shapiro 1998). In this study, we examined both mate choice in populations adjacent to the southern hybrid zone and frequencies of mitochondrial DNA haplotypes that distinguish the two species in a transect through the hybrid zone to make inferences about the crosses involved in the formation of the hybrid zone.

Orchelimum nigripes and *O. pulchellum* are morphologically distinct based on the shape and angle of the male's cercal tooth, the angle of the female's ovipositor, the shape of lateral pronotal lobes, and leg and eye color (Scudder 1875; Davis 1909; Rehn and Hebard 1915; Blatchley 1920; Walker 1971; Shapiro 1998), but through the southern hybrid zone, populations have mixed morphologies (G.L. Miller pers. obs., Shapiro 1998). Given the variability in morphology and the size of the zone, and because hybrids appear to be healthy and well established, there may be an absence of a fitness cost to mating among hybrids, although this has not been directly measured. Matings with heterospecifics in the PRB have been shown to be costly because of reduced fertility (Shapiro 2000).

In the PRB hybrid zone, the two species demonstrate asymmetrical pre-mating isolation: female *Orchelimum nigripes* prefer conspecifics and female *O. pulchellum* do not discriminate (Shapiro 2001). Thus, hybrids in the PRB are expected to be formed from

one cross (female *O. pulchellum* with male *O. nigripes*). Mate choice in the southern hybrid zone has not been examined and here we ask if the same mate choice is observed through mating trials in which a female chooses among males of both species. In addition we examined traits involved in courtship (male size and singing) that may influence mate choice to determine if sexual selection potentially influences mate recognition in these species.

In these two katydid species, males call to attract females, females are silent and females move toward calling males. In many species of Orthoptera, females identify species by calling song and choose among members of their own species by calling song traits (Tuckerman et al. 1993; Hedrick and Weber 1998; Champagnon and del Castillo 2008). Prior to mating, female *Orchelimum* often approach multiple males (Feaver 1983). Once in close range, a male and female antennate, thereby exchanging olfactory and gustatory information, and may visually inspect one another (Shapiro 2001). Males cannot force copulations in these taxa; a female must stand still to allow a male to orient and couple, thus females may actively choose mates (G.L. Miller, pers. obs., Shapiro 2001). Females in many Orthoptera choose among potential mates by their size and their singing ability (Tuckerman et al. 1993; Hedrick and Weber 1998; Bateman et al. 2001; Champagnon and del Castillo 2008; Lehmann and Lehmann 2008). Size may affect spermatophore production: in many species, large males have the largest spermatophores. Nutrient rich spermatophores may represent male parental investment in katydids and confer direct benefits to the female via nutrition (Gwynne 1988; Wedell and Ritchie 2004). Male *Orchelimum*, like other katydid males, produce a spermatophore that is transferred to the female with a successful mating. Therefore, a female may

choose a large male over small males for a greater benefit to her offspring, but similarly, males may exhibit mate choice through differential investment in females through the spermatophore (Simmons et al. 1999).

Female preference for calling song ability may reflect choice for indirect (genetic) benefits if males that call more frequently are higher quality than males that call less frequently (e.g., Tuckerman et al. 1993). Production of song makes a male more visible to parasites and predators so that time spent calling increases risk of parasitism and predation (e.g., Shapiro 1995; Allen et al. 1999; Kolluru and Zuk 2001; Muller and Robert 2001; Lehmann and Lehmann 2006). If singing is an honest indicator of male quality (Zahavi 1974, 1977), a female may have superior offspring by choosing a male that sings more compared to other males.

Females may also reflect their preferences not only through actual choice of mate, but mating latency and copulation duration as well. Mating latency, the time from mate meeting to the time copulation begins, reflects female preference as it is equivalent to the propensity to mate (Jennions and Petrie 1997). Other studies have used copulation latency to demonstrate that females take longer to mate with less preferred males (Ritchie et al. 1999; Acebes et al. 2003; Taylor et al. 2008). Copulation duration, the time spent *in copula*, can also be influenced by female choice (Eberhard 1994) with females spending more time copulating with preferred over non-preferred males. Males can also influence duration of copulation (Bonduriansky 2001) so this particular trait is not under the influence of only one sex.

Given that hybrid formation is directional in the PRB hybrid zone, in addition to characterizing mate choice in the southern zone, we also measured the frequency of

haplotypes of a mitochondrial (mt) gene across the southern hybrid zone. In the formation of the zone, if matings were between males and females of both species, haplotypes belonging to both species should be found through the hybrid zone. If matings were primarily unidirectional, the haplotype of the “mother species” will predominate across the hybrid zone (Wirtz 1999). We compare to the mtDNA results to the mate choice trials to infer how the hybrid zone formed.

METHODS

Natural history

Katydidids were collected across a transect that stretched from Georgia through Texas at 32-34 degrees north latitude. Across the transect (Table 1) katydidids were found in the same type of vegetation and in the presence of the same predators, thus there were not obvious differences in microhabitat for *Orchelimum nigripes* and *O. pulchellum* (G.L. Miller, pers. obs., Shapiro 2001). The katydidids were generalists within their habitat and were found in a variety of areas within collection sites (from emergent aquatic vegetation to grassy vegetation and tall trees) and were seen feeding on seeds and insects. Both species live across their north-south distributions, east and west of the Appalachians, in habitats that are more variable than habitats across the Deep South where the hybrid zone is located. Thus, there are no obvious ecological boundaries between these taxa. Across the transect, appropriate habitat was fragmented by human development and not all areas with appropriate habitat had katydidids (G.L. Miller, pers. obs.); therefore *Orchelimum* distributions were patchy.

Across the hybrid zone (Table 1), all individuals in populations designated as hybrid by Shapiro (1998), were morphologically hybrids, i.e., there were no individuals that morphologically matched the parental species. Within a population, individuals shared intermediate traits. For example, some populations had legs intermediate in color between the black legs of *Orchelimum nigripes* and the red legs of *O. pulchellum*. Other populations had violet colored eyes, intermediate to the red eyes of *O. nigripes* and the blue eyes of *O. pulchellum*. Thus, members of hybrid populations tended to resemble each other more than they did the parental species or other hybrid populations.

Specimen collection

Katydid were collected from outside and within the hybrid zone in the summers of 2006-2009 (Table 1) from three populations of *Orchelimum nigripes* (KS, TX, LA-1), three populations of *O. pulchellum* (GA-1, GA-2 and FL) and five populations from within the hybrid zone (LA-2, MS, AL-1, AL-2, AL-3). Animals were transported in portable, insulated, food storage coolers to the University of Kansas and housed at $25^{\circ}\text{C} \pm 5^{\circ}\text{C}$ on a 12:12 light cycle. Animals were reared on an orthopteran food mix (Rentz 1996), and moisture was provided by hydrated Watersorb® crystals (Watersorb, Inc., Hot Springs, AR).

Behavioral tests

Late instar male and female nymphs were collected from areas adjacent to the southern hybrid zone during the summers of 2007 and 2008. *Orchelimum nigripes* were collected from Texas and Louisiana (TX, LA-1). *Orchelimum pulchellum* were collected from two locations in Georgia (GA-1, GA-2) and Florida (FL). Collections were determined to be outside of the hybrid zone by reference to previous work based on morphology and allozymes (Shapiro 1998). Nymphs were separated and group housed by sex in separate constant temperature chambers to acoustically isolate females from male song. Once adult, females were placed in individual containers. Females used in behavioral experiments were 10 to 30 days old. Male age was not controlled, but only males old enough to sing were used in behavioral tests. Katydid were housed in the lab for at least two days before being used in trials.

In choice tests, a single female was given the choice of four males, two each from each species. Males were placed through a cloth sleeve into a cage that was 30.5 cm in all

three dimensions; the other sides had wire mesh and the bottom was metal. Observations were made from the side opposite the sleeve. Males were given a minimum of 20 minutes to acclimate before a single, virgin female was introduced to the cage. Trials were concluded when either the female finished mating with a male or 90 minutes had passed, if no mating occurred. One or two trials were conducted simultaneously. The species of male with which the female mated was recorded as well as latency to mating (time from introduction of female until successful copulation) and copulation duration (time spent *in copula*) to the nearest minute. Males that successfully mated were not used for trials for a minimum of three days to ensure that males were able to remate and successfully produce a spermatophore (Shapiro 2001). No females were used twice.

During the 2008 trials, the calling status (calling or non-calling) of each male was recorded for a 15 second period every two minutes. To determine if female choice was correlated with the amount of time a male spent singing, the males in each trial were ranked by the number of observations in which they sang. Males were grouped into two categories for statistical analysis: the two males singing the most and the two males singing the least.

Also for the 2008 trials, males were weighed prior to the start of the trial to the nearest 0.1 gram. To determine if female choice was correlated with male size, males were grouped into two categories for statistical analysis: the two largest males and the two smallest males. Males successful in copulating were weighed after mating: the difference in male weights before and after the trial was used as a proxy for spermatophore size.

Molecular genotyping

A total of 175 individuals across the hybrid zone were genotyped for a mtDNA marker (Table 1). DNA was extracted from the hind femur muscle of freshly killed or frozen (-80°C) katydids using the DNEasy Tissue extraction kit (Qiagen, Valencia, CA). To first identify a diagnostic difference between the two species, the mitochondrial gene Cytochrome Oxidase I (COI) was amplified by polymerase chain reactions (PCR), cloned and sequenced in an *Orchelimum nigripes* individual from Texas and an *O. pulchellum* individual from the GA-1 population. Each 25 µl PCR reaction contained 1X buffer (Bio-Rad, Hercules, CA), 0.32 mM each dNTP, 0.3 pmol/µl of each primer (LCO1490 and 2672e; Table 2), 1.5 mM MgCl₂, 0.5 units of *iTaq* DNA Polymerase (Bio-Rad, Hercules, CA) and approximately 10 ng of template DNA. The resulting approximately 1200 bp PCR fragment from each species was cloned using the TOPO® TA® Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA). Plasmid DNA was isolated and purified using the Wizard® Plus SV Minipreps (Promega, Madison, WI). Inserts were sequenced in both directions using T3 and T7 plasmid primers by the DNA Sequencing Laboratory at the University of Kansas. Sequences were trimmed of vector and poor quality ends using Sequencher (Gene Codes Corporation, Ann Arbor, Michigan) and the sequences of the two species were aligned in MegAlign using the Clustal W algorithm (DNASTAR, Inc., Madison, WI). SeqBuilder (DNASTAR, Inc., Madison, WI) was used to identify a restriction site difference between the two species in COI. The *O. nigripes* sequenced had a restriction site for PstI while *O. pulchellum* did not.

To determine if the mitochondrial haplotypes identified were species-specific, new primers specific to the two *Orchelimum* species were designed using Primer 3 (Rozen

and Skaletsky 2000) by first aligning the sequences in Sequencher and specifying that the primer be placed in areas of 100% identity between the two species that flanked the restriction site (Table 2). A 390 bp fragment of COI was amplified using the same PCR mix as above with the new primers, 0.5 units BIOLASE DNA Polymerase and 1 X BIOLASE buffer (Bioline USA Inc., Boston, MA). Cycling conditions are shown in Table 2. The resulting PCR products were digested with the restriction enzyme PstI (Promega, Madison, WI), which cut approximately 40 bp from the end of the *O. nigripes* PCR fragment. Twenty μ l reactions containing 10 μ l PCR product, 1 X Buffer H (Promega, Madison, WI), 0.1 μ g/ μ l BSA, and 10 units PstI were incubated at 37°C overnight prior to electrophoresis on a 2% agarose gel and visualized with ethidium bromide. To control for the efficiency of the reaction, 1 μ g of lambda DNA (USB Corp., Cleveland, OH) was digested in addition to the PCR products and checked for the expected restriction fragment length pattern. Katydid with a 390 bp band were denoted as having the *O. pulchellum* haplotype and those with a fragment of 350 bp were denoted as having the *O. nigripes* haplotype. Populations outside the hybrid zone (KS, TX, GA-1, GA-2, and FL; Table 1) were first examined to make sure the restriction difference was diagnostic between species. This same procedure was then performed for the remaining populations to determine the haplotypes of individuals.

RESULTS

Mate choice

Female *Orchelimum nigripes* were more discriminating and failed to mate more often than female *O. pulchellum*. A higher proportion of the trials conducted resulted in no mating for *O. nigripes* than for *O. pulchellum* (Fisher Exact Probability Test, two tailed, $P = 0.028$, Figure 1). Of the females making a choice, mate choice for these two species is asymmetrical: female *O. nigripes* mated more with conspecifics than heterospecifics (cumulative binomial probability, two tailed, $p=q=0.5$, $P < 0.0001$; Figure 1) whereas female *O. pulchellum* mated with both species of males (cumulative binomial probability, two tailed, $p=q=0.5$, $P = 0.2295$; Figure 1).

Two male characters that may influence female choice are male size and male calling song. In 2008, males that successfully mated did not differ significantly in weight between the two species (ANOVA: $F_{1,79}=1.58$ $P = 0.212$). *Orchelimum pulchellum* mated with one of the two largest males significantly more often than with one of the two smallest males (cumulative binomial probability, two tailed, $p=q=0.5$, $P = 0.0215$; Figure 2), while female *O. nigripes* mated with males of both size ranks (cumulative binomial probability, two tailed, $p=q=0.5$, $P = 0.7539$; Figure 2). Thus, these two species differ in their mate choice by size but because males of the two species do not differ in size, this does not lead to assortative mating by species.

A different choice pattern was seen with amount of time spent calling. For the males that successfully mated in 2008, there was a significant interaction between the amount males sang by species and the species of female present (ANOVA: $F_{1,79}=0.05$, $P = 0.028$). This result is confounded by interactions between the female and male in close-range

courtship: when a female approached a male that was not calling, the male would usually begin calling (G.L. Miller, pers. obs.). While interacting with a female at close range, males called continuously until they coupled with the female. Calling had a large effect on female *Orchelimum nigripes*, which only mated with one of the two males singing the most (cumulative binomial probability, two tailed, $p=q=0.5$, $P = 0.002$; Figure 3).

Female *O. pulchellum* mated both with the two most and two least singing males (cumulative binomial probability, two tailed, $p=q=0.5$, $P = 0.1094$; Figure 3) with one female *O. pulchellum* mated with a male that did not sing at all during the trial.

Mating parameters

Latency to mating did not differ significantly by type of mating (combination of male and female; ANOVA: $F_{3,44}=0.18$, $P = 0.908$; Figure 4). There was no significant difference in duration of copulation by type of mating (ANOVA: $F_{2,40}=1.84$, $P = 0.172$; Figure 4).

Not included in the analysis is the one mating between a female *Orchelimum nigripes* and a male *O. pulchellum* because the two stayed coupled until they died two days later.

For a mating to result in successful insemination, the male must produce and transfer a spermatophore to a female. Spermatophores were produced in all matings, even the single mating of a female *Orchelimum nigripes* with a male *O. pulchellum* in which the pair could not uncouple. There was no significant correlation between male size and spermatophore size for male *O. nigripes* (Pearson correlation = 0.298, $P = 0.322$; data not shown) or male *O. pulchellum* (Pearson correlation = 0.255, $P = 0.626$; data not shown).

Although males did not differ in size, and male weight and spermatophore weight were not correlated, we used the percentage weight change of the male before and after the trial as a proxy for spermatophore proportion to control for differences in male size.

Male *O. nigripes* lost an average of 10.1% of their body weight. Male *O. pulchellum* lost an average of 14.5% of their body weight, but the proportion of body weight transferred was highly variable for both species and was not significantly different between species (ANOVA: $F_{1,18}=1.34$, $P = 0.263$; Figure 5). For male *O. pulchellum*, spermatophore size did not differ significantly with species of female with which they mated (ANOVA: $F_{1,12}=0.69$, $P = 0.423$; Figure 5).

Hybrid zone genetic analysis

For the COI fragment amplified, the restriction enzyme PstI cut PCR products from *Orchelimum nigripes* populations (KS, TX) and not from *O. pulchellum* populations (FL, GA-1, GA2; Table 1). Based on the previous work in which two allozyme markers were only of the *O. nigripes* genotype (Shapiro 1998) and on morphological identification (G.L. Miller, pers. obs), LA-1 was designated as *O. nigripes*. This population has haplotypes of both *O. pulchellum* and *O. nigripes*, whereas the populations of *O. nigripes* further from the hybrid zone do not. Through Mississippi and Alabama, allozyme markers are mixed, though of predominantly *O. pulchellum* origin, in all but the western most population of Mississippi (Shapiro 1998). Samples here through the hybrid zone are either mixed (MS) or entirely of the *O. pulchellum* mitochondrial haplotype (all 3 Alabama populations).

DISCUSSION

Mate recognition and sexual selection appear to be causing sexual isolation for *Orchelimum nigripes*. Because there is a difference in the amount males sing between the species (at least when interacting with female *O. nigripes*) and because females prefer the males that sing the most, this leads to female *O. nigripes* predominantly mating with conspecifics. For *O. pulchellum*, a preference for large males, without a difference in size between the two species, does not lead to species discrimination. The asymmetry in sexual isolation thus has the potential to affect gene flow asymmetrically through the southern hybrid zone.

This mating asymmetry is the same as that found in the PRB hybrid zone (Shapiro 2001). This implies that throughout the species range, female *Orchelimum nigripes* are more discriminating with respect to species recognition. However, in this experimental design because females were presented with a choice among males, we cannot determine if female *O. nigripes* also recognize *O. pulchellum* as potential mates because of the overwhelming preference for male *O. nigripes* when given a choice. However, based on the crossability of these two species when originating in the PRB, female *O. nigripes* can recognize male *O. pulchellum* males as mates (Shapiro 2000).

The signals and signal modalities females use when picking mates can differ between closely related species (e.g., Hebets and Maddison 2005), as appears to be the case here with the predominant effects being singing for *Orchelimum nigripes* and size for *O. pulchellum*. However, many other characteristics of these katydids may play a role, such as cuticular hydrocarbons, but these were not assessed. Nonetheless, choice seems to be governed by female *O. nigripes*. An alternative explanation is that male *O. pulchellum*

may not court female *O. nigripes*. However, during the trials, male *O. pulchellum* were observed to attempt to mount female *O. nigripes*. Males of both species tried to mount other males. Thus, males do not discriminate among mating partners and female choice is driving the mating asymmetry observed.

Latency to mating was the same for all mating combinations, though there was only one heterospecific choice for a female *Orchelimum nigripes*, and female *O. nigripes* did not mate more than they chose to mate. Female *O. pulchellum* did not spend more time choosing a conspecific than a heterospecific male, so males of both species met their courtship requirement. If females mated more rapidly with heterospecifics than with conspecifics, that would imply that *O. nigripes* courtship is a supernormal stimulus. If female *O. pulchellum* had greater latency with heterospecifics, the extended latency period might indicate that close-range courtship of *O. nigripes* is unattractive. The equivalent length of time choosing males, regardless of species chosen, suggests female *O. pulchellum* are able to orient and couple readily with males of both species.

Copulation duration can be affected by female mate choice and male mate choice (Eberhard 1994; Bonduriansky 2001). At least for female *Orchelimum pulchellum*, copulation times did not differ by species of male. Genitalic differences may prevent successful mating between female *O. nigripes* and male *O. pulchellum* because the one pair that mated was not able to uncouple. However this requires further investigation as Shapiro (2000) obtained successful crosses (although with reduced fecundity compared to conspecific crosses) between female *O. nigripes* and male *O. pulchellum* from the PRB. In many species, males and females can influence mate choice through copulation duration. For example, hangingfly (*Bittacus apicalis*) females terminate copulation

before sperm transfer is completed if given a small nuptial gift (Thornhill 1976). For *Orchelimum pulchellum* females, copulation duration did not differ by male species. The same is true for male *O. nigripes*. Thus neither species is controlling mate choice through copulation duration, though there are not enough data to make inferences about female *O. nigripes* or male *O. pulchellum*. Furthermore, most female orthopterans control post-copulatory choice through the timing of spermatophore removal (Mautz and Sakaluk 2008), though this was not measured here.

As with copulation duration, success in spermatophore transfer may be influenced by male mate choice (Simmons et al. 1999). In all successful matings, spermatophores were transferred, implying no significant morphological incompatibilities between female *O. pulchellum* and male *O. nigripes*. For male *O. nigripes*, spermatophores of equivalent portion of body size were transferred to both conspecific and heterospecific females, implying no differential male reproductive allocation between the species.

Together, all of the data suggest that the female controls mate choice in these two species, at least under lab conditions: female *Orchelimum nigripes* discriminate against male *O. pulchellum* whereas female *O. pulchellum* will mate with male *O. nigripes*. The mtDNA results are consistent with this asymmetry: *O. pulchellum* mitochondrial haplotypes extend west through the hybrid zone to an area previously demonstrated to be dominated by *O. nigripes* nuclear markers (two allozymes, Shapiro 1998). Although this sampling was not in exactly the same locations, the samples were from the same latitude as Shapiro's northern transect through the southern hybrid zone (1998). Thus, *O. pulchellum* mtDNA has introgressed further west than nuclear markers, though at the same time, *O. nigripes* nuclear alleles extend further east (although at very low

frequency) than *O. nigripes* mtDNA haplotypes. The pattern of mtDNA frequencies indicates that females carrying *O. pulchellum* haplotypes are indiscriminately mating across the hybrid zone.

The western Louisiana population (LA-1, Table 1) was used as a source for *Orchelimum nigripes* individuals for the mating choice trials. The mtDNA results indicate that this was probably not the best choice of populations as there was a low frequency of *O. pulchellum* mtDNA haplotypes (this study). No *O. pulchellum* allozyme alleles were found in Louisiana (Shapiro 1998) and individuals collected in LA-1 were identified morphologically as *O. nigripes* (G.L. Miller, pers. obs). These females (13 of the 20 that mated in these trials) still chose male *O. nigripes*, despite potentially having a small percentage of *O. pulchellum* DNA. If these females had biased the results by not being pure *O. nigripes*, carrying DNA of *O. pulchellum* would increase the probability of these females to choose male *O. pulchellum*, but this was not observed.

Based on the mitochondrial DNA data alone, we cannot definitively determine that the predominant cross is between a female *Orchelimum pulchellum* and a male *O. nigripes*, because if there are significant differences between the reciprocal crosses in post-mating success (i.e., all progeny of a female *O. nigripes* mated with a male *O. pulchellum* die) then the same pattern would be expected (reviewed in Wirtz 1999). However, combined with the pattern of mate preferences observed in the mating trials, the cross between a female *O. pulchellum* and a male *O. nigripes* was probably instrumental in the formation of the hybrid zone.

The implications of these results are that, in the absence of any post-mating isolation barrier, gene flow through the hybrid zone should continue to be asymmetric. Post-

mating isolation barriers have been examined for the PRB hybrid zone with demonstrated fecundity decreases for heterospecific crosses compared to conspecific crosses (Shapiro 2000). However, the individuals involved in the PRB hybrid zone have different characteristics than the individuals here. The PRB was probably formed by human introduction of *Orchelimum nigripes* into the area about 75 years ago (Shapiro 1998). In that area, *O. nigripes* are smaller than *O. pulchellum* and have a faster development time, which implies that *O. nigripes* can outcompete *O. pulchellum* (Shapiro 2000). Together with asymmetric mate choice, pure *O. nigripes* individuals can be identified in PRB populations. This contrasts with the southern hybrid zone in which males do not differ in size and for which distinct hybrid populations are found: the zone is not a mosaic of pure species and hybrid individuals but is dominated by hybrids only. This implies that post-mating barriers may not be as strong in the southern hybrid zone, but this remains to be tested.

In addition, because calling song influences female *Orchelimum nigripes* in their mate choice, we have undertaken a study of male calling song variation and differentiation between these species and through the hybrid zone (G.L. Miller, unpublished). The species do differ in some diagnostic traits, but future work will test whether these calling song traits may be contributing to species identification and/or mate choice.

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Table 1. Collection localities.

| Site | Locality ¹ | Latitude ² | Longitude ² | Species ³ | mtDNA study | |
|------|-----------------------|-----------------------|------------------------|-----------------------------------|-------------|--|
| | | | | | Sample size | Number with <i>O. nigripes</i> haplotype |
| KS | Lawrence, KS | 38.96278 | -95.25528 | <i>O. nigripes</i> | 25 | 25 |
| TX | Tyler, TX | 32.44785 | -95.16989 | <i>O. nigripes</i> ⁴ | 10 | 10 |
| LA-1 | Minden, LA | 32.59731 | -93.32567 | <i>O. nigripes</i> ⁴ | 13 | 11 |
| LA-2 | West Monroe, LA | 32.47816 | -92.19786 | hybrid | 6 | 6 |
| MS | Clinton, MS | 32.29539 | -90.33382 | hybrid | 14 | 10 |
| AL-1 | Demopolis, AL | 32.51498 | -87.87031 | hybrid | 32 | 0 |
| AL-2 | Montgomery, AL | 32.25450 | -86.36199 | hybrid | 30 | 0 |
| AL-3 | Auburn, AL | 32.55682 | -85.47054 | hybrid | 9 | 0 |
| GA-1 | Bonaire, GA | 32.54251 | -83.53914 | <i>O. pulchellum</i> ⁴ | 15 | 0 |
| GA-2 | Thompson, GA | 33.63611 | -82.59299 | <i>O. pulchellum</i> ⁴ | 11 | 0 |
| FL | Gainesville, FL | 29.63383 | -82.36755 | <i>O. pulchellum</i> ⁴ | 10 | 0 |

¹Locality information, listed from west to east, includes the nearest city or town to the collection site.

²Latitude (degrees North) and longitude are in decimal degrees.

³Species were designated as determined by morphology and previous description of the hybrid zone (Shapiro 1998).

⁴Individuals from these populations were used in the mate choice study.

Table 2. PCR primers used to amplify COI gene.

| Primer name | Sequence (5' to 3') | Cycling conditions | Source |
|--------------------------|---------------------------|---|--------|
| Sequencing primers | | | |
| LCO1490 | GGTCAACAAATCATAAAGATATTGG | 95° 3 min, 35 cycles (92° 10 sec, 48° 15 sec, 72° 1 min), 72° 5 min | 1 |
| JCOI2630 | GCAAATACAGCTCCTATTGATAAAA | 72° 5 min | 2 |
| Population assay primers | | | |
| COIORCFOR | TGACCCTGCTCCTTGCGAG | 95° 3 min, 35 cycles (92° 1 min, 48° 1 min, 72° for 1 min), 72° 5 min | 3 |
| COIORCREV | TAAGTGGTTGATAGAGAAT | | 3 |

1 Folmer (1994)

2 Designed from *Drosophila* sequences

3 Custom primers designed from *Orchelimum nigripes* and *O. pulchellum* sequences

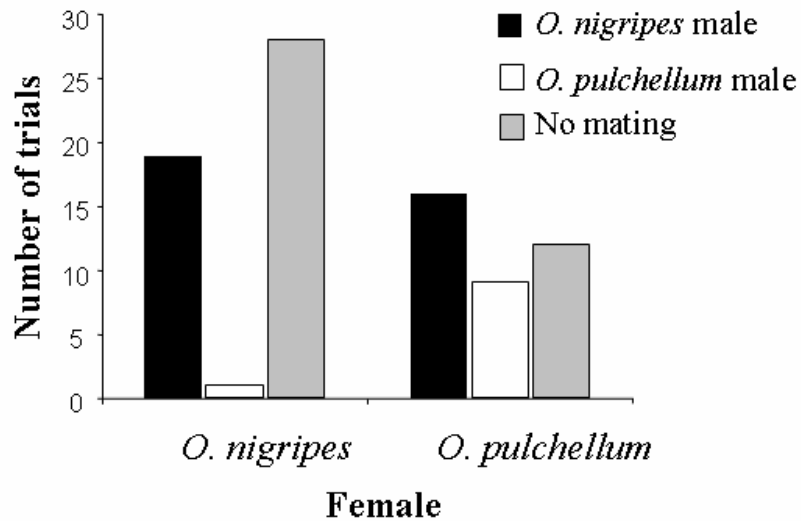


Figure 1. Mate choice of female *Orchelimum nigripes* and *O. pulchellum*. The number of matings between each species of female and male is shown for trials with mating to *O. nigripes*, *O. pulchellum* and no mating. Female *O. nigripes* mated with male *O. nigripes* significantly more often than they mated with male *O. pulchellum* (cumulative binomial probability, two tailed, $p=q=0.5$, $P < 0.0001$). Female *O. pulchellum* mated with both species (cumulative binomial probability, two tailed, $p=q=0.5$, $P = 0.2295$).

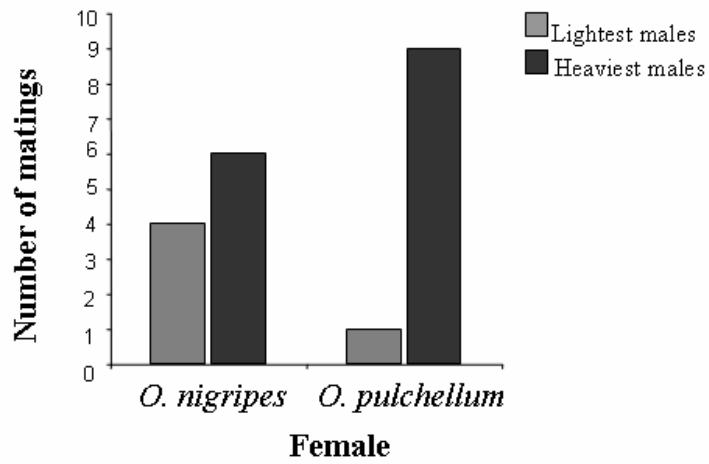


Figure 2. Female mate choice by male size. Female *Orchelimum nigripes* mated with males of all sizes (binomial test, 2-tailed, $P = 0.7539$) while *O. pulchellum* mated with one of two heaviest males significantly more (binomial test, 2-tailed, $P = 0.0215$).

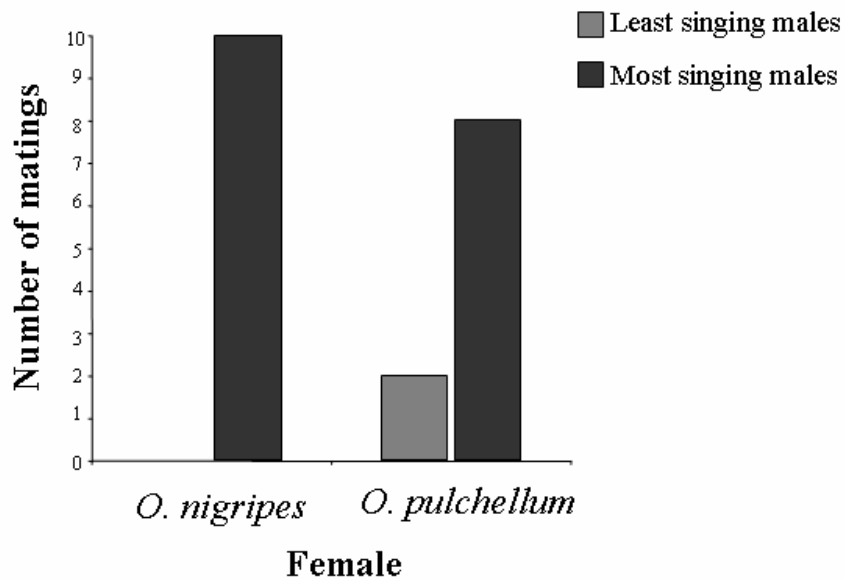


Figure 3. Female mate choice by relative time male spent singing. Female *Orchelimum nigripes* mated only with one of the two males that sang the most (cumulative binomial probability, two tailed, $p=q=0.5$, $P = 0.002$). Female *O. pulchellum* did not mate with one of the two males that sang the most significantly more often (cumulative binomial probability, two tailed, $p=q=0.5$, $P = 0.1094$).

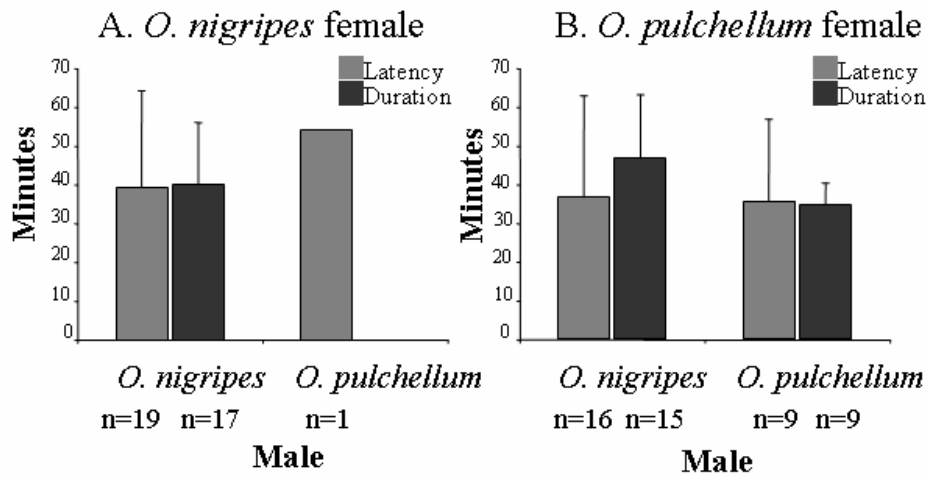


Figure 4. Latency to mating and duration of copulation. Females are shown separately: A. *O. nigripes* and B. *O. pulchellum*. Error bars represent one standard deviation. There was no significant difference in either latency to mating by type of mating (ANOVA: $F_{3,44} = 0.18, P = 0.908$) or in the duration of copulation by type of mating (ANOVA: $F_{2,40} = 1.84, P = 0.172$). Sample sizes are given under each bar. Uncoupling was not observed to the nearest minute in 3 matings, so the duration of these was not included.

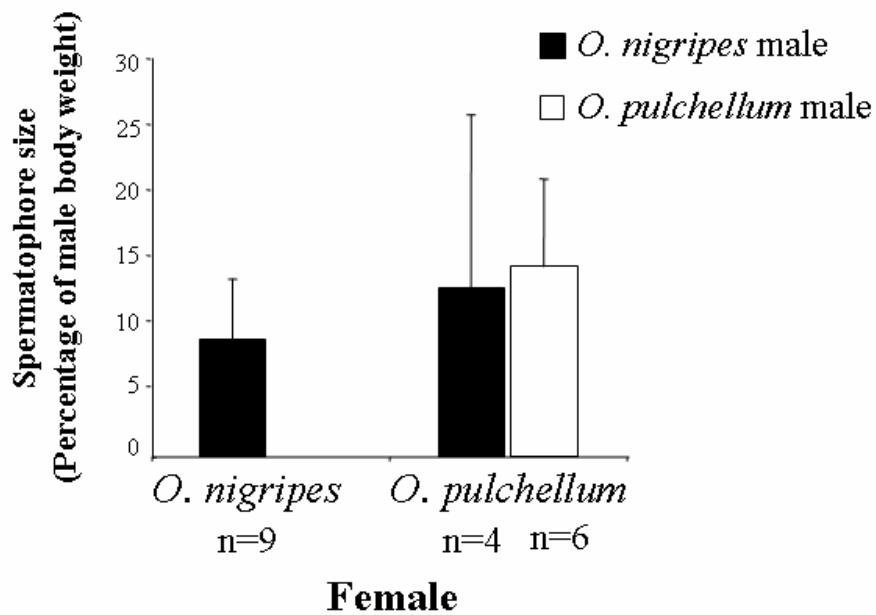


Figure 5. Spermatophore as percentage of male body weight by cross. Error bars represent one standard deviation. The spermatophore proportion of male *O. nigripes* did not differ by species of females with which they mated (ANOVA: $F_{2,12} = 0.69$, $P = 0.423$). The single cross between the female *O. nigripes* and male *O. pulchellum* is not included because they stayed *in copula* until dying two days later. Sample sizes are given under each bar.

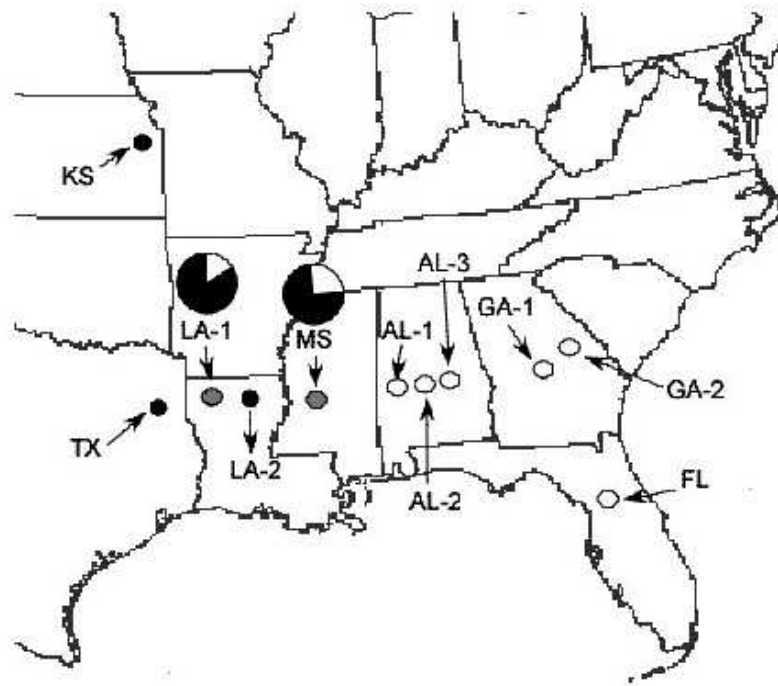


Figure 6. Mitochondrial DNA haplotypes by location. The proportion of the population with an *O. nigripes* haplotype is shown in black and the proportion of *O. pulchellum* haplotypes is in white in each pie chart. The gray area is the approximate region of the hybrid zone as determined by Shapiro (1998). Sample sizes are given in Table 1.

Chapter 3: Female preference for male calling song in two hybridizing meadow katydids (Orthoptera: Tettigoniidae)

INTRODUCTION

Behavioral isolation plays an important role species recognition and sexual selection for many taxa. A signal may operate in one or both of these roles. Ryan and Rand (1993) have demonstrated that species recognition and sexual selection may form a continuum rather than operating on separate signals. Mate choice based upon a signal may operate at a coarse level as choice for a conspecific mate (with heterospecific signals being much less attractive) and at a finer level in selecting the most attractive conspecific available (Ryan and Rand 1993).

Identifying how signals are encoded between signalers and receivers is fundamental to our understanding of mate choice. The acoustic channel is particularly suited to studies of mate choice. In many acoustic taxa, songs may be readily recorded and played back to females, making them particularly tractable systems for studies of mate choice. Songs are also easily altered to allow one to explore the effects of song modification on female preference to determine the range over which females find songs attractive. The relative attractiveness of songs may also be assessed.

The Orthoptera figure prominently into studies of speciation and sexual selection (i.e., Ritchie 1996; Shaw 2000; Shaw and Herlihy 2000; Bridle and Butlin 2002; Ferreira and Ferguson 2002; Robinson and Hall 2002; Izzo and Gray 2004; Bridle et al. 2006; Forrest et al. 2006; Jang and Gerhardt 2006; Safi et al. 2006; Bailey et al. 2007). Within the Orthoptera, the Caelifera use a mix of sensory modalities (primarily acoustic and visual) in mate choice, but ensiferan (katydids and crickets) pair formation is primarily

acoustic, at least during the initial stages of courtship. The Ensifera are known for their diverse species-specific acoustic courtship signals that are used in mate recognition and in sexual selection in many taxa (e.g., Bailey and Robinson 1971; Bailey 1993; Allen 1995; Brown et al. 1996; Bailey and Field 2000; Faure and Hoy 2000; Gray and Cade 2000; Olvido and Wagner 2004; Orci 2007; Bush et al. 2009). There are taxa in which song has been secondarily lost. A mutation in the wing of the cricket *Teleogryllus oceanicus* (Gryllidae) has silenced males in an area of high predation (Bailey et al. 2008; Logue et al. 2010). In another, *Hapithus agitator* (Gryllidae) calling is rare and females silence males through wing chewing (Alexander and Otte 1967), but silent males are uncommon in Ensifera.

Pair formation in Ensifera is generally initiated by males singing and females walking towards male songs that they find attractive (phonotaxis). Studying male song differences alone provides only half of the evolutionary picture of species recognition and sexual selection. Song differences are irrelevant unless females attend to these differences; therefore, female preferences must be measured to determine if song is operating effectively in pre-mating isolation and how attractiveness is encoded in male song.

We normally think of song as an important part of pre-mating isolation, but how does song operate when isolation between taxa is incomplete? For song to be effective in isolating species, females must preferentially respond to conspecific song over heterospecific song. The role of song and preference in mate recognition varies among hybridizing Orthoptera.

The male songs of the hybridizing grasshoppers *Chorthippus brunneus* and *C. jacobsi* (Acrididae) differ greatly. Females of these species use the male songs to orient to conspecific males and discriminate against heterospecifics and hybrids; thus, in these species, song is an effective pre-mating barrier (Bridle et al. 2006). A similar pattern is observed in the hybridizing field crickets *Gryllus firmus* and *G. pennsylvanicus* (Gryllidae); females respond to differences in male song and choose among mates based on pulse rate (Doherty and Storz 1992).

Song preferences may sharpen species boundaries when taxa are found in sympatry. For example, female katydids of *Conocephalus nigropleurum* (Tettigoniidae) approach signals of *C. attenuatus* (a species with pulse trains twice as fast as conspecific song), but do not approach speakers playing songs of *C. brevipennis* (a species with more similar songs). The differential response to song is proposed to result from "selective heterospecific recognition" as *C. nigropleurum* is found sympatrically with *C. brevipennis*, but does not co-occur with *C. attenuatus* (Gwynne and Morris 1986). Thus, pre-mating isolation is reinforced for sympatrically occurring taxa.

Song differences do not influence mate recognition in some taxa. The crickets *Allonemobius fasciatus* and *A. socius* (Gryllidae: Nemobiinae) have species-specific songs and meet in a mosaic hybrid zone in the Eastern United States (Mousseau and Howard 1998). Females do not respond differently to con- and heterospecific song, and it has been suggested that the observed song differences may be the result of genetic drift in these taxa (Doherty and Howard 1996; Olvido and Wagner 2004). No known pre-mating barriers isolate these species. Isolation results from a post-mating fertilization barrier (Gregory and Howard 1994).

Song also plays a role in sexual selection for most taxa (e.g., Weber et al. 1981; Verburgt et al. 2008). For example, calls may be used to assess overall male quality by female choice for call energy (amount of sound per unit of time). In the field cricket, *Gryllus lineaticeps* (Gryllidae), females choose males with high energy calls (Hoback and Wagner 1997). Female preference for call energy may allow females to choose males in good condition (i.e., males healthy enough to produce a great deal of calling song).

In taxa with multi-component calls, phonotaxis studies can be used to determine which song elements are necessary to evoke female orientation and in what context (i.e., mate choice, sexual selection) individual song parts are used (Stumpner and von Helversen 1992; Schul 1998; Bush et al. 2002; Bush and Schul 2006; Bush et al. 2009). In the sympatric cricket pair *Teleogryllus commodus* and *T. oceanicus* (Gryllidae): female *T. commodus* attend to both chirps and trills of the male's call, but only male chirps of *T. oceanicus* are necessary to elicit phonotaxis from conspecific females (Hennig and Weber 1997). The short-horned grasshopper *Chorthippus dorsatus* (Acrididae) also produces a two element call, both parts of which are necessary to attract mates (Stumpner and von Helversen 1992). Testing female preference is necessary in determining the function of complex calls (i.e., if multiple song elements are necessary to elicit phonotaxis or if females respond to a subset of song elements).

In hybrid zones, there is at least a partial breakdown in mate recognition. If the breakdown is incomplete, asymmetrical matings may result. Songs may play a role in such asymmetry. I measured phonotaxis in asymmetrically isolated meadow katydids (Chapter 2), *Orchelimum nigripes* and *O. pulchellum* (Tettigoniidae) to determine if female preference for male song plays a role in mate recognition.

Orchelimum nigripes and *O. pulchellum* hybridize in two regions of the United States (Shapiro 1998). I examined preference of populations adjacent to a hybrid zone in the southeastern United States. Male *Orchelimum* call; females are silent. There are species-specific temporal differences in male calls (Chapter 1). Pair formation is initiated by female phonotaxis to male calling song (Feaver 1983).

Mating trials revealed that female *Orchelimum nigripes* mated with conspecifics while female *O. pulchellum* mated with both hetero- and conspecific males (Chapter 2). The asymmetrical mating pattern observed between these two species suggests that female *O. nigripes* are selective in mate choice and female *O. pulchellum* are not selective. Therefore, I predicted female *O. nigripes* would respond to conspecific song only, whereas female *O. pulchellum* would respond to both con- and heterospecific songs. Female *O. nigripes* mated with males that sang the most in caged mating trials, which may reflect a preference for call energy. Thus, I predicted that *O. nigripes* would prefer calls with increased call energy, such as short tick-buzz periods (as increased singing was associated with an increased probability of mating). In contrast, I predicted female *O. pulchellum* would approach a broad range of songs as female preference may be based on visual cues, rather than song, as they chose large males. Finally, I determined if females needed multi-component calls for orientation (i.e., both the ticks and the buzz elements of the male call).

METHODS

Care of experimental animals

I collected late instar female nymphs from areas near the western and eastern ends of the southeastern hybrid zone (Figure 2). Collecting females as nymphs ensured their virginity, because animals that have previously mated may respond differently to male song (Morris et al. 1975; Prosser et al. 1997). *Orchelimum nigripes* were collected from Texas and Louisiana (TX, LA1) in the summer of 2007. *Orchelimum pulchellum* were collected from Georgia (GA-1, GA-2) in the summer of 2008. Animals were transported to the laboratory in insulated cold food storage containers to avoid exposing animals to dramatic temperature shifts during transport. Nymphs were group housed at $25^{\circ}\text{C} \pm 5^{\circ}\text{C}$ on a 12:12 light:dark cycle. Animals were reared on an Orthopteran Food Mix with the addition of millet seed (Rentz 1996), and moisture was provided by hydrated Watersorb® crystals (Watersorb, Inc., Hot Springs, AR). At eclosion, females were placed in individual containers. Female age was not controlled, but females were not used in behavioral experiments until they were a minimum of 10 days post-adult eclosion to ensure they were old enough to respond to male song (Shapiro 1996; Shapiro 2001).

Protocol for no-choice testing

Phonotaxis experiments were performed in a temperature controlled anechoic chamber at $25 \pm 2^{\circ}\text{C}$ in the dark to eliminate visual signals that could influence phonotaxis behavior. Females were observed from a separate room with an infrared camera during testing. Preference tests were performed on a Kramer-Kugel walking compensator(servosphere) (Weber et al. 1981; Huber et al. 1984) according to the methods outlined by Schul (1998). The walking compensator adjusted mechanically with

the movement of the female to keep reflective tape on the female's pronotum centered beneath the camera (and thus the female directly on top of a spherical walking surface). The movements of the walking compensator were recorded as a series of x, y coordinates (to calculate the direction the female walked) and the elapsed time following every one cm of movement of the servosphere.

Females were randomly chosen from the captive pool of females for testing. Prior to testing, a small circle of reflective tape was secured to the pronotum of the female. The female was then allowed to rest at least 30 minutes before being placed on the servosphere. Females were free to walk in any direction on the servosphere. Females were given five minutes to acclimate before beginning stimulus presentation.

A song was presented to a female through one of two speakers located 90° apart and 1 cm above the top of the walking compensator. Speaker volumes were adjusted to 85 dB at the location of the female. Each song stimulus (test or control) was played from one speaker for 1.5 minutes and then from the other speaker for 1.5 minutes. The female was allowed to rest with silence for one minute before the next stimulus was played. A control song (conspecific male song) flanked each group of test stimuli. Only trials in which the female responded consistently to the flanking controls (before and after) were included in the analysis to exclude females that became tired, habituated to male song, or were non-receptive (Schul et al. 1998). Every other female was presented with stimuli in the reverse order to counteract order effects. For each trial, females were presented with each set of test stimuli once. Females were used in multiple trials but with at least one day between trials.

A custom computer program written by Johannes Schul (Division of Biological Sciences, University of Missouri-Columbia) calculated three parameters from each trial: the distance the female walked, the walking speed, and the direction the female walked with a test stimulus relative to the direction she walked when the control stimulus was presented. Walking direction was calculated relative to the control stimulus rather than the speaker position because phonotaxis to calls is never perfect; slight asymmetries in females can lead to imperfect directionality (Schul 1998). Preference was scored from a ratio of the phonotaxis score (PS) of the test stimulus to the PS of the control stimulus (Bush and Schul 2006). The PS is the product of the distance a female walked multiplied by the cosine of deviation of mean direction in which the female walked between the control and test stimulus (Bush and Schul 2006). A PS of + 1 reflects perfect orientation towards a stimulus and a walking speed equal to that of the control. A PS of – 1 reflects perfect orientation away from a stimulus and a walking speed equal to that of the control (Bush and Schul 2006).

Preference testing stimuli

The songs of male *Orchelimum nigripes* and *O. pulchellum* have two elements (Figure 1). The first call element produced by males is a series of ticks. Ticks are loud pulses of sound with silent intervals between them. The second element is the buzz, a long, low amplitude, continuous series of pulses (Walker 1971; Morris and Walker 1976). Collectively, the tick-buzz call with the silent interval (until the next tick-buzz call is produced) is the tick-buzz period. Male *O. nigripes* call more rapidly, have fewer but longer ticks, shorter buzzes, and shorter silent intervals between tick-buzz cycles than male *O. pulchellum* (Chapter 1).

Male calls were recorded at 151 kHz and were temporally similar to the average of that species. For *Orchelimum nigripes* I used the song of a male from LA, and for *O. pulchellum* I used that of a male from GA-1 (Table 1). Later studies indicated that LA had a mix of mtDNA haplotypes (Chapter 2), but acoustically and morphologically it was identified as *O. nigripes*. The unmodified, single tick-buzz cycle from a natural male call served as a control stimulus and was repeated in a continuous loop in a custom playback program written by Johannes Schul. To construct control stimuli, I cropped a single tick-buzz period from a recording of a male that was representative of each species using Cool Edit (v. 1.53, Syntrillium Software, 1996). To test the effect of conspecific versus heterospecific song on female phonotaxis, the natural male calls (control stimuli) of both species were used without modification, with conspecific call again serving as a control. This resulted in the stimuli having a species-specific tick-buzz period. The response to these stimuli approximated how likely females were to orient to males of each species if they encountered them in nature.

Two different song modifications were constructed. To construct songs to test the effect of differing tick-buzz periods on female phonotaxis, the length of the silent interval between tick-buzz cycles was altered. This was done by reducing the interval by approximately half and increasing the interval by approximately two and four times the length of the normal interval. No other modifications of the song were made. The long buzz of *Orchelimum pulchellum* song limited the range over which songs could be tested, i.e., complete removal of the silent interval of *O. pulchellum* songs still resulted in their tick-buzz period being longer than that of *O. nigripes* songs.

To construct songs to test the effect of ticks and buzzes separately on female phonotaxis, either the ticks or buzz of a call were digitally replaced with silence. This resulted in an increased silent interval and preserved the tick-buzz period instead of altering the tick-buzz period by subtraction of the call element from the song. These three stimuli were tested with natural conspecific song as a control.

Statistical analysis

Statistical analysis was performed with Minitab (version 13.31, Minitab Inc.). A Friedman test was used to compare phonotaxis scores of test stimuli. This non-parametric test was chosen as female response data may not be normally distributed, and, thus, not meet the assumptions required of a repeated measures ANOVA. Where there were significant differences among stimuli, a Mann-Whitney test was used to compare between each pair of stimuli. Significance was determined after application of a Holm's Sequential Bonferroni correction factor.

RESULTS

Females of both species respond equally to calls of both species of males (Figure 3), despite the asymmetrical mate choice that caged mating trials demonstrate (Ch. 2, Shapiro 2001). Female *Orchelimum pulchellum* were expected to orient to male calls of both species as they accepted both species as mates. Female *Orchelimum nigripes* were not expected to respond to calls of male *O. pulchellum* as they were not chosen as mates.

For female *Orchelimum nigripes*, tick-buzz period may be a directional preference because they respond to calls with a shortened silent interval, but not at a greater rate than to natural song (Figure 4). Their phonotaxis drops significantly when the tick-buzz period is quadrupled in length relative to that of the natural song (Figure 4). This suggests that decreased call energy reduces phonotaxis. There is a significant impact on response by *O. pulchellum* to stimuli with different tick-buzz periods, but unlike in *O. nigripes*, this preference appears to be stabilizing, with the non-significant trend of response decreasing when the tick-buzz period is increased or decreased relative to the natural male song (Figure 5). Thus, female *O. pulchellum* do not show a preference for increased call energy.

Female *Orchelimum nigripes* respond to both parts of the song, tick and buzz. There is a significant reduction in phonotaxis when either "ticks only" or "buzz only" are presented (Figure 6). In contrast, female *O. pulchellum* do not decrease phonotaxis significantly when the "ticks only" or "buzz only" stimulus is presented (Figure 7). Because the "ticks only" stimulus silences a larger portion of the song than the "buzz only" stimulus, a female receiving "ticks only" experiences much less call energy than when that female experiences the other two songs. Thus, female *O. nigripes* may either

require both song elements or be responding to increased call energy, and female *O. pulchellum* do not appear to attend to call energy.

DISCUSSION

Because female *Orchelimum pulchellum* readily mated with males of both species (Chapter 2), an equivalent phonotaxis response to hetero- and conspecific song was expected and observed. This suggests that female *O. pulchellum* will readily approach the calls of male *O. nigripes* and *O. pulchellum* in the wild. Because female *O. nigripes* preferentially mated with conspecifics, I predicted that they would preferentially orient to song of conspecific males, if song were a pre-mating barrier. The equivalent response of female *O. nigripes* to hetero- and conspecific song demonstrates that song is not an effective pre-mating barrier in long-range courtship. Mating trials may involve complex interactions between males and females during close-range courtship that influences mate choice. Thus, short-range aspects of courtship must be further examined to determine where courtship breaks down between female *O. nigripes* and male *O. pulchellum* to result in the asymmetrical mating pattern observed (Chapter 2). While song is important in initiating phonotaxis to bring the sexes into contact, calling song, despite species specific differences, is not a barrier to hybridization.

If song differences between taxa are not used in species discrimination, song differences may be evolving through drift, as in the hybridizing field crickets *Allonemobious fasciatus* and *A. socius* (Olvido and Wagner 2004). The song differences between *Orchelimum nigripes* and *O. pulchellum* were not sufficient to isolate these species. The observed song differences may primarily be a result of drift, as they are not used by females in species discrimination.

The observed lack of species-specific song preference does not imply that song is irrelevant to females. The tick-buzz period may be used in intraspecific mate choice.

Female *Orchelimum nigripes* attend to differences in the tick-buzz period and prefer short period lengths; thus, tick-buzz period length may be evolving through sexual selection. The response of female *Orchelimum pulchellum* to tick-buzz period length did not drop significantly until it was increased to twice (32,000 ms) the maximum value observed in surveyed populations (range: 4,000–16,000 ms, Chapter 1), so their response was equivalent across the natural range of calls these females encounter. This flat response implies that tick-buzz period is not under sexual selection in *O. pulchellum*; differential response would be expected for a sexually selected trait.

Preferences for song can influence the rate and direction of gene flow between hybridizing taxa. In *Orchelimum nigripes* and *O. pulchellum*, behavioral and genetic data suggest mtDNA genes of *O. pulchellum* are introgressing into the hybrid zone from the east, but mtDNA genes of *O. nigripes* are not introgressing into the hybrid zone from the west (Chapter 2). This may be caused by mating asymmetry (Chapter 2). Multiple factors may lead to mating asymmetry; these include differences in male vigor and/or female receptivity. For these species, female receptivity may be higher in *O. pulchellum* than in *O. nigripes* (Chapter 2). Males of the two species may court with different intensities during caged mating trials or preferentially court one species of female. Possible differences in male vigor and courtship may also be responsible for the mating success of male *O. nigripes* and need to be tested.

Mating asymmetries may also result from sexual selection operating differently in these species. Wirtz (1999) posited that asymmetry in mating may result when females exert choice and are found at low densities. When a species is rare, mating with any male of a closely related species is more likely to result in offspring than not mating.

Therefore, if species densities differ, asymmetrical female choice may result from relaxed discrimination by low density females. During all three field seasons, population density of *O. pulchellum* was lower than that of *O. nigripes* (pers. obs.). The difference in density between these species may contribute to the observed asymmetry in mate choice.

Although song may not function effectively in species recognition in these taxa, song may function in sexual selection, particularly in *Orchelimum nigripes*. Phonotaxis scores of female *O. nigripes* decreased in response to lengthened tick-buzz periods compared to natural song when female *O. nigripes* were presented with calls with lengthened tick-buzz periods. The lengthened tick-buzz period was achieved by lengthening the silent interval between successive calls. Thus, call energy decreased as the silent interval increased (Figure 4). Calls with lengthened silent intervals may not have adequate call energy to cause strong phonotaxis.

In *Orchelimum nigripes*, the presence of both parts of male song (ticks and buzz) significantly increases phonotaxis compared to songs of only ticks or only the buzz element of male song (Figure 6), suggesting a multi-component call type is necessary for phonotaxis. Multi-component calls are also necessary for attracting females, as has been found in the grasshopper *Chorthippus dorsatus* (Acrididae) (Stumpner and von Helversen 1992). The preference of *O. nigripes* for two song elements is confounded by preference for call energy (songs with both elements contain more call energy than songs with one element silenced), but this potential preference for call energy is consistent with the preference for short tick-buzz periods and the preference for males that called the most during caged mating trials (Chapter 2). Females may be using call energy to choose males of high quality as calling can be energetically costly, increase risk of parasitism

from acoustically orienting flies (Shapiro 1995), and attract birds (pers. obs.) or other predators.

Sexual selection may be operating on different male traits between these species. Female *Orchelimum pulchellum* responded equally well to a broad range of male calls and were less selective than female *O. nigripes* with respect to songs. Phonotaxis response was independent of call composition for female *O. pulchellum*, so both song elements are not required for female *O. pulchellum* to approach calls.

The pattern of response between these closely-related species to the two song elements is similar to that found in female *Teleogryllus*, where one species requires both parts of song, while the other will respond to either, implying songs are filtered and processed differently in the nervous system (Hennig and Weber 1997). Encoding and filtering of male songs is a potential area of future research for *Orchelimum* to understand why the species respond differently to song. Female *O. nigripes* respond equally to song at or above the call energy of a natural male call, but shorter than natural intervals do not increase phonotaxis. Thus, a minimum call energy may be necessary for phonotaxis. The broad song preference of *O. pulchellum* suggests that call energy is unimportant in mate choice in these females. Selection may instead be for male size in *O. pulchellum*, as females selected larger males in mating trials (Chapter 2), while female *O. nigripes* mated with males of any size.

Within the southeastern hybrid zone between *Orchelimum nigripes* and *O. pulchellum*, individuals bearing the characteristics of the pure parental species were not found (pers. obs., Shapiro 1998). Thus, *O. nigripes* and *O. pulchellum* are not sympatric in this region. Neighboring populations to pure species, most likely encountered during

dispersal, may not be very different from one another across the southeastern US (Shapiro 1998). Thus, selection against hybridization (with neighboring populations) may be relatively weak. The cost of mating with a neighboring population in terms of hybrid fitness needs to be measured. Adjacent populations may not have as high of a reduction in fitness as matings between parental species. Therefore, female choice may be relaxed between these populations as compared to between parental species.

Female choice may have operated differently during the initial formation of this hybrid zone. Selection also may have been relaxed when this hybrid zone was formed. The pattern of the leading edge of range expansions between *Orchelimum nigripes* and *O. pulchellum* is believed to have occurred while they were isolated in glacial refugia (Shapiro 1998). As ranges of the taxa expanded, they likely initially encountered one another following divergence in Alabama. Some offspring were produced from crosses between these taxa, most likely from matings of an *Orchelimum nigripes* male and an *O. pulchellum* female (Chapter 2). It is unclear how often these matings occurred and produced offspring, but male songs of hybrid populations were less variable than those of pure species adjacent to the hybrid zone (Chapter 1), which suggests that this zone was likely formed by few individuals initially. It is probable that hybrids readily mated with one another successfully, establishing populations across this zone. Continued introgression may have occurred from matings between hybrid females and *O. nigripes* males.

All evidence suggests that *Orchelimum nigripes* are more selective than *O. pulchellum* and, therefore, female *O. nigripes* at the edge of the hybrid zone are less likely to mate with hybrids than female *O. pulchellum* are to mate with hybrids.

Continued introgression to the west is expected from dispersal and mate choice, but the patchiness of populations may result in low dispersal thus stabilizing the position of this hybrid zone.

Further genetic studies of individuals across this hybrid zone may help elucidate how hybridization is proceeding between these taxa. Additionally, to better understand the influence of song on gene flow between these species, song preferences of populations across the southeastern hybrid zone must be examined. The preference of hybrid females for songs of male *Orchelimum nigripes*, *O. pulchellum*, and hybrid individuals may provide insight into the dynamics of mate choice and their potential influence on gene flow between these species.

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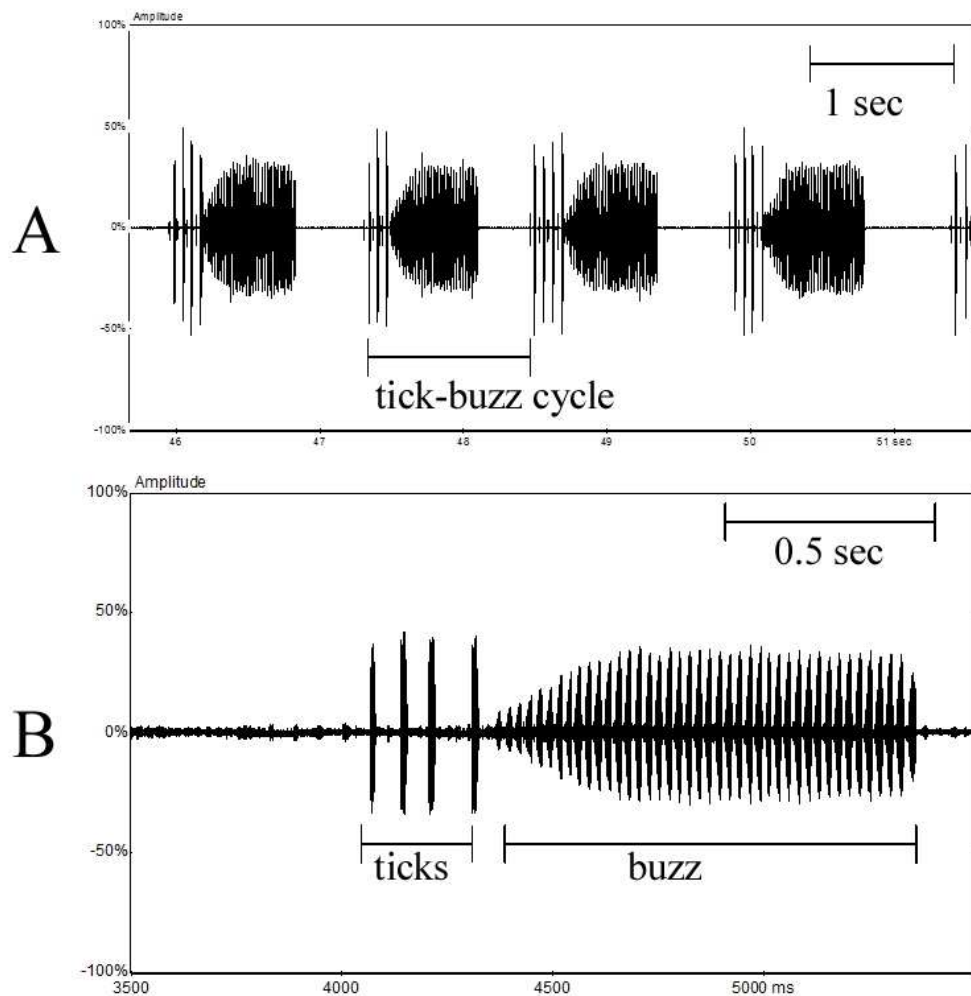


Figure 1. Oscillograms of *Orchelimum nigripes* male calling song to illustrate temporal measurements used in study. (A) Multiple tick-buzz cycles are shown. The average length of this cycle is the tick-buzz period. Silence was altered between calls to make test stimuli that varied in tick-buzz period. (B) Close-up of a tick-buzz call. Pulses were inserted and deleted from the middle of the buzz to make test stimuli that varied in buzz length. To test the effects of song elements on female preference, the ticks or buzz were silenced. Additionally, the natural (unaltered) *O. nigripes* and *O. pulchellum* songs were tested. *Orchelimum pulchellum* calling song does not differ from *O. nigripes* calling song in structure but differs in timing (Chapter 1).

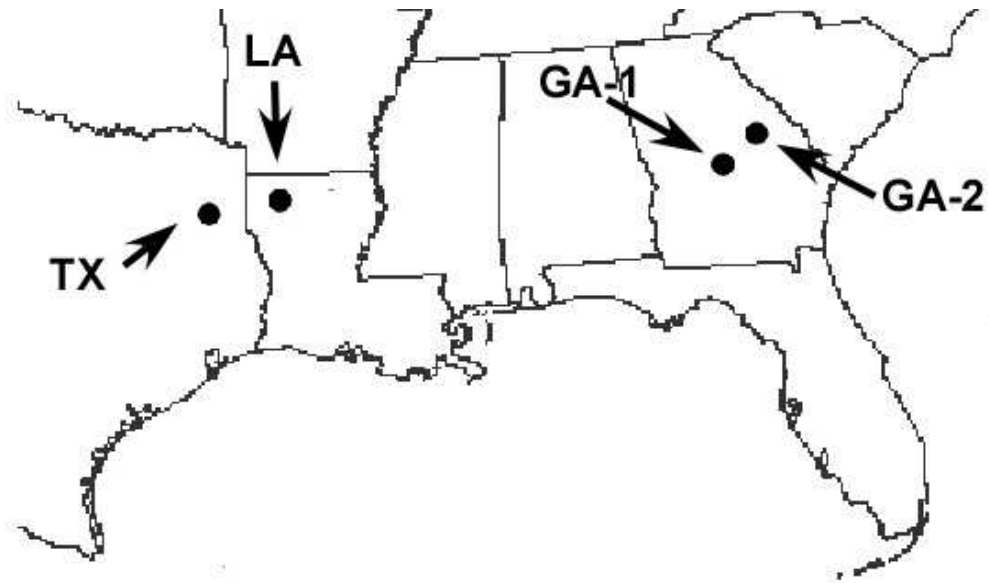


Figure 2. Collection localities of females used in phonotaxis studies.

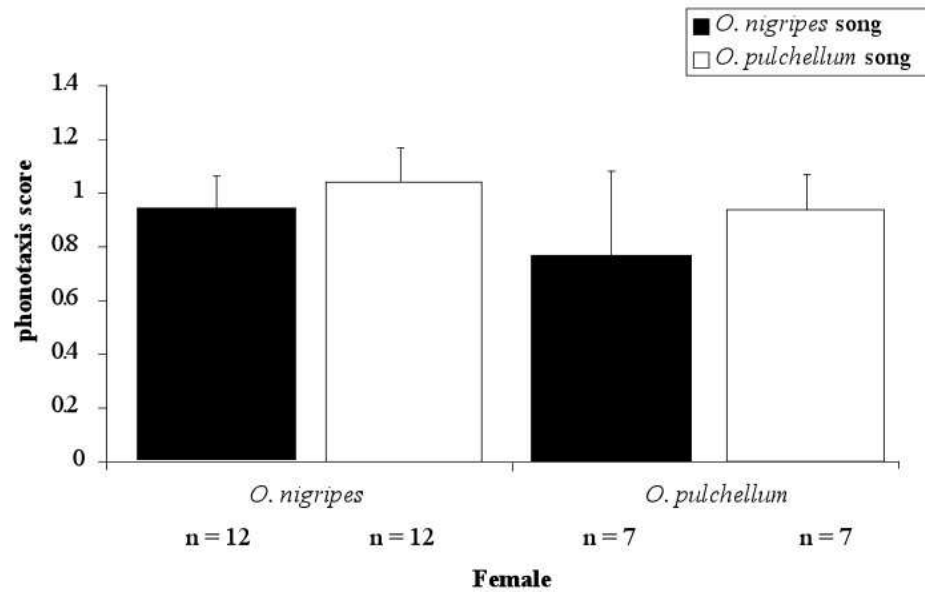


Figure 3. Phonotaxis by females to male calls of *Orchelimum nigripes* and *O. pulchellum*. There was not a significant difference in phonotaxis between calls of both species of males in female *O. nigripes* (Friedman test: $S_1 = 3$, $P = 0.083$) or *O. pulchellum* females (Friedman test: $S_1 = 1.28$, $P = 0.257$).

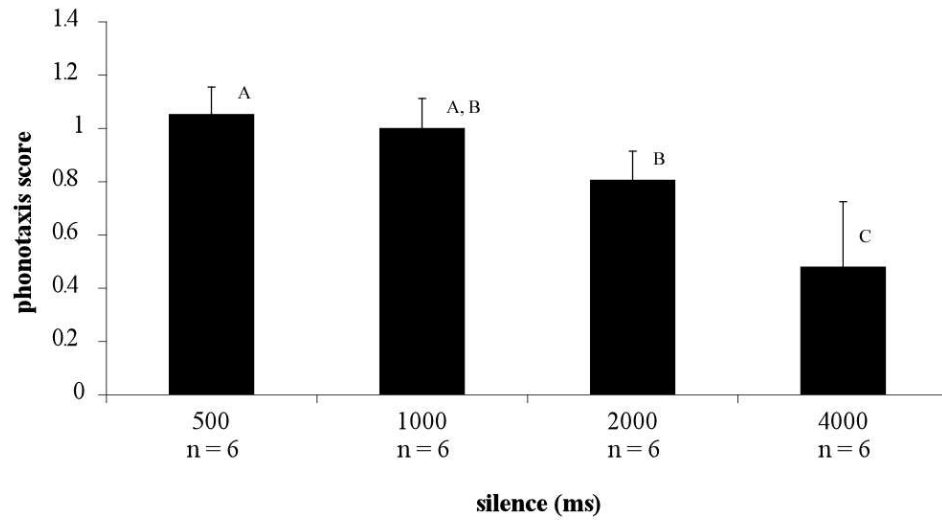


Figure 4. Phonotaxis scores of *Orchelimum nigripes* in response to different tick-buzz periods. The tick-buzz periods tested differ in the length of the silent intervals between successive tick-buzz calls. A tick-buzz period of 1000 ms corresponds to the *O. nigripes* natural male call. There was a significant difference in phonotaxis by tick-buzz length (Friedman test: $S_3 = 15.80$, $P = 0.001$). Pairwise comparisons using a Mann-Whitney test corrected with a Holm's sequential Bonferroni factor were performed to determine which pairs differed significantly from one another. Bars with the same letter indicate phonotaxis was not significantly different between stimuli.

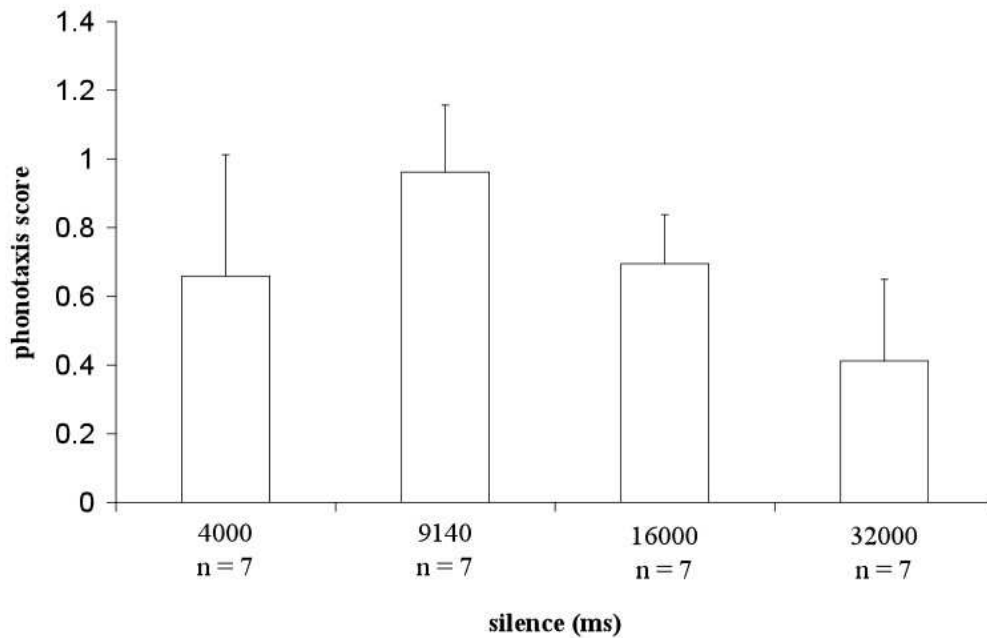


Figure 5. Phonotaxis scores of *Orchelimum pulchellum* in response to different tick-buzz periods. A silent interval of 9140 ms corresponds to the natural *O. pulchellum* call. There was a significant difference in phonotaxis based on the length of time between repeating tick buzz calls (Friedman test: $S_3 = 12.77$, $P = 0.005$). Pairwise comparisons using a Mann-Whitney test corrected with a Holm's sequential Bonferroni factor indicate the 9140 and 32000 ms silent intervals were the only pair of stimuli significantly different from one another.

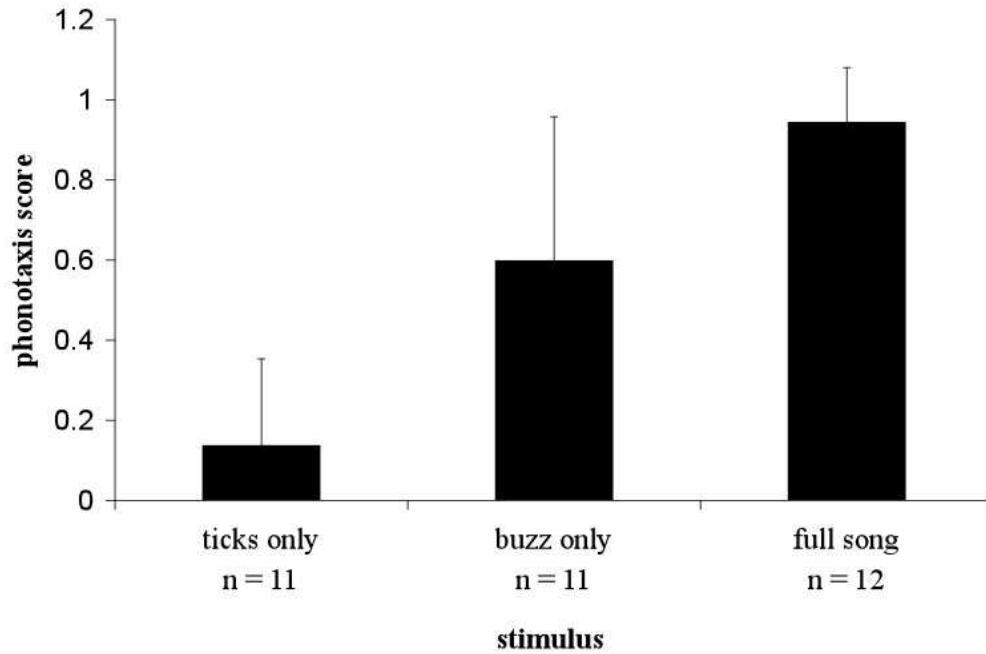


Figure 6. Phonotaxis scores of *Orchelimum nigripes* in response to portions of conspecific song. There was a significant difference in phonotaxis based on the presence of both parts of the tick buzz male song (Friedman test: $S_2 = 16.55$, $P < 0.0001$). Pairwise comparisons using a Mann-Whitney test corrected with a Holm's sequential Bonferroni factor indicate that all three categories are significantly different from one another.

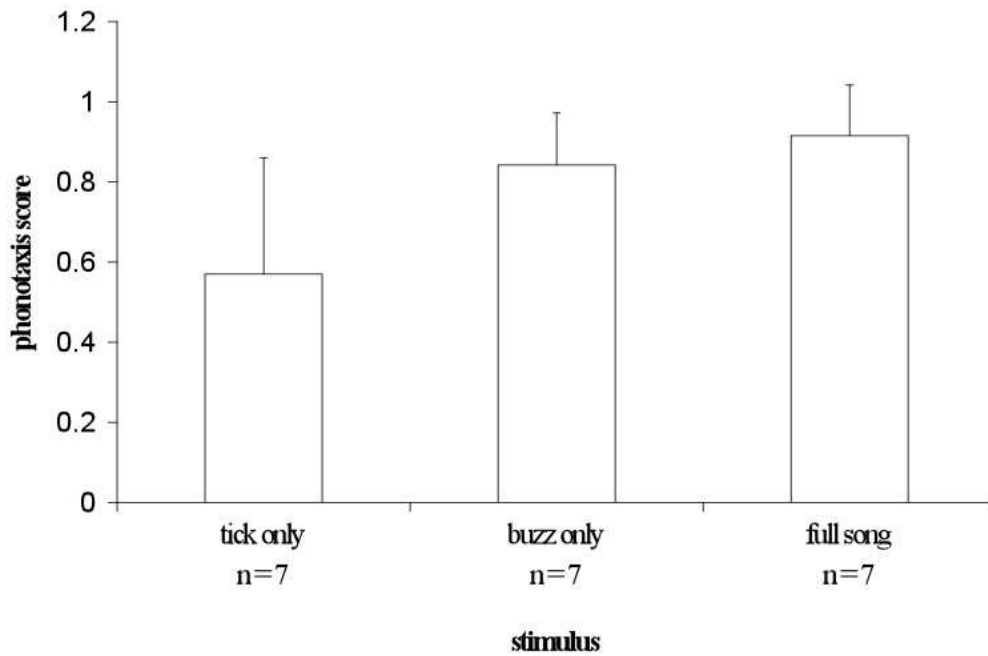


Figure 7. Phonotaxis scores of *Orchelimum pulchellum* in response to portions of conspecific song. There was not a significant difference in phonotaxis based on the presence of both parts of the tick buzz male song (Friedman test: $S_2 = 5.43$, $P = 0.066$).