THE SPECIES HISTORY, POPULATION GENETICS, AND BEHAVIORAL REPRODUCTIVE ISOLATING MECHANISMS OF TWO CHIHUAHUAN DESERT KATYDIDS (ORTHOPTERA: TETTIGONIIDAE)

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Abstract: Understanding the mechanisms and evolutionary processes that lead to reproductive isolation between populations is the major goal of speciation research. Here, I integrated approaches from phylogenetics, population genetics, and behavioral ecology to gain perspective on a behavioral isolating mechanism between two species of Chihuahuan desert katydids. Previously little was known about the genus *Obolopteryx* described over 100 years ago. In the first chapter I built the first molecular phylogeny from two mitochondrial DNA genes and compared my hypothesis to previous morphology-based hypotheses. In the second chapter I used Amplified Fragment Length Polymorphisms (AFLP) to compare total genetic similarity of allopatric and sympatric populations of the two focal species: *O. oreoeca* and *O. brevihastata*. I found substantial evidence that *O. oreoeca* was experiencing a gene-flow restriction between the allopatric population in the Chisos Mountains of the Big Bend National Park and the population in the Davis Mountains sympatric with *O. brevihastata*. I did not find equivalent support for differentiation between the two *O. brevihastata* populations. In the third chapter I explored the calling behavior of the males in both species, and the phonotactic responses of *O. oreoeca* females between allopatry and sympatry. I quantitatively described the calls of both species. I then tested whether various aspects of calls differed in allopatry and sympatry within each species. I tested for character displacement in call syllable durations of both species. I found that *O. oreoeca* populations showed no differences in most call features, but they did show character displacement in the syllable duration. Interestingly, while I failed to find character displacement in *O. brevihastata's* syllable duration, I found that other unexpected call features differ between their populations. Controlled experiments show that a high amplitude component of the male call is important for female *O. oreoeca* phonotaxis. Sympatric *O. oreoeca* females showed significantly decreased phonotactic responses to heterospecific calls compared to allopatric *O. oreoeca* females. This combined molecular and behavior data suggest a unique example of reinforcement in which females in a peripheral sky island population, sympatric with a non-sister species, have evolved strong heterospecific mating discrimination due to heterospecific competition for mates.

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CHAPTER I

INTRODUCTION

Speciation background

A prerequisite for studying reproductive isolation is an understanding the relationship of species. For instance, are the species closest relatives and still hybridizing, or are they distantly related and experiencing secondary contact? Species were traditionally clumped together by taxonomists based upon unique morphological traits that simply allowed one species to be clearly differentiated from another, but modern phylogeneticists are more interested in species histories based on common ancestry, using molecular data. The nodes in cladograms represent a moment in which the isolation of two species becomes complete. Mayr (1942) first defined the biological species concept as "species are groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups." This is still the most widely accepted species concept for eukaryotic organisms, and the most practical for understanding speciation. Coyne and Orr (2004) characterize species as having "substantial but not necessarily complete reproductive isolation", focusing on the process and mechanisms rather than the stringency of the biological species concept.

Understanding speciation, how species are formed, maintained, and delineated has been a fundamental goal in evolutionary biology, since the time of Darwin (1859). For eukaryotic

organisms which are the practical basis for the biological species definition (Mayr 1942), showing speciation means two things: pinpointing the first reproductive isolating barrier that delineates two populations, and secondly understanding the evolutionary forces driving that barrier (Coyne and Orr 2004). It is unlikely that we observe the emergence of reproductive isolating barriers *in situ*, so we generally explore other strategies. Two viable alternatives to *in situ* studies are forcing speciation in the laboratory through artificial selection experiments and alternatively modeling the genetics of speciation.

Two of the best examples of experimentally forcing speciation are Hurd and Eisenberg's (1975) *Musca domestica* experiment in which flies began to assortatively mate based on geotaxis preferences, and Thoday and Gibson's (1962) *Drosophila* experiments in which associative mating was experimentally achieved based upon artificial disruptive selection for bristle number (Thoday and Gibson 1970; Thoday 1972). Both experiments provide examples of the emergence of reproductive isolation. From these early experiments we learned that reproductive isolation happens fast, possibly within only a few generations, and that mate choice can play a role in reproductive isolation.

Genetic modeling has also contributed in a significant way to the field of speciation research. Dobzhansky (1937), Bateson (1909), and Mueller (1942) laid the foundation of nearly all modern genetic models of speciation based on a two-gene, two-allele model of genetic incompatibility (Orr 1996). Unfortunately, the exhaustive amount of theoretical work on speciation has mostly provided case-by-case scenarios under which reproductive isolation can arise from virtually all combinations of migration values (sympatric, parapatric, allopatric) and evolutionary mechanisms (drift, mutation, selection, assortative mating, etc.). There is little

consensus regarding how these individual results contribute to generalizations regarding speciation in nature. Gavrilets (2003) illustrates six of the most general contributions of modeling to speciation theory: 1) single adaptive peak shifts are unlikely, but should happen instantly so observing this in nature is highly unlikely, 2) drift and mutation alone can result in speciation, 3) the importance of drift and mutation depend on the adaptive landscape, 4) speciation resulting from drift and selection is slow, 5) speciation is generally triggered by environmental changes, and 6) sympatric speciation is possible with strong disruptive selection or assortative mating. These models provide specific predictions regarding mechanistic possibilities, but we are also interested in providing evidence that these mechanisms are driving speciation in nature. This type of evidence is possible only from organisms *in situ*.

In situ, reproductive isolation can be studied using recently diverged sister taxa. A sound approach is to first identify species pairs with one obvious reproductive isolating barrier, since other prezygotic barriers arise quickly after any initial barrier forms (Ramsey et al. 2003). Further, reproductive isolating mechanisms may be gained and lost through time, so once multiple mechanisms arise the initial mechanism may be lost. Second, we must use these species pairs to attempt to reject alternative hypotheses regarding the mechanisms involved in reproductive isolation leaving us with one good hypothesis. A variety of speciation mechanisms have been strongly supported in nature: sympatry (Schliewen et al. 1994; Gislason et al. 1999) versus allopatry (Knowlton et al. 1993; Xiang et al. 1998), selection (Nagel and Schluter 1998) versus drift (Gittenberger 1998), and ecological (Feder and Bush 1989) versus non-ecological (Wiernasz and Kingsolver 1992; Ryan and Rand 1993) isolation. To push forward our understanding of speciation, researchers must continue to broadly investigate reproductive

isolation between species pairs and increase the taxonomic breadth of our knowledge. Only then can we begin to infer generalities about how speciation occurs. I will restrict the rest of my discussion about reproductive isolation to animals, since plants are well known to become reproductively isolated by mechanisms related to ploidy level (Rieseberg and Willis 2007) as well as the aforementioned mechanisms.

Two problematic trends for identifying widespread patterns in speciation are the genetic focus on model organisms and the taxonomic deficit. The overexploitation of model organisms, sometimes chosen by convention rather than for biological appropriateness, has been a hindrance to many fields within biology (Bolker 2012). Increasing taxonomic breadth by exploring reproductive isolation between novel species pairs should be a major goal. There is also a large taxonomic gap and lack of job opportunities for those working in the field of taxonomy (Dubois 2010). Further, natural history museum funding faces steady decline (Kemp 2015). Taken together, these trends have slowed advances in speciation research. In spite of the recent decline in taxonomic expansion and natural history funding, the cost of genetic research (whole genome sequencing) is following a consistent decline (Wetterstrand). We are in the beginning of a new era in speciation research where researchers are increasingly recognize and have the resources to use non-model organisms to understand the traits, mechanisms, and genetics behind speciation (Wolf et al. 2010).

This dissertation will add to the body of speciation work by focusing on reproductive isolation between a species pair whose behavior was previously poorly understood. I begin by revising the current hypothesis about their phylogenetic relationship through a molecular phylogeny. Then I explore the finer-scale genetic architecture of both species inside and outside a

potential hybrid zone using AFLPs (Amplified Fragment Length Polymorphisms). Lastly I explore female behavior in the laboratory and variation in wild male calls to understand reproductive isolation between the species.

Species histories

Historically we only understood species histories by morphological, character-based clades, it is now widely accepted that these are often misleading (Scotland et al. 2003). One reason is that morphological characters are often based on many genes as well as their interaction with the environment. Morphological traits scored to make clades do not necessarily indicate stepwise changes, and these clades are based on stepwise changes in shared derived traits. Discreet morphological traits can result from an unknown number of changes in multiple genes, possibly with unequal effects. Insect genitalia, often used for morphology, has empirical support for pleiotropic effects (Arnqvist et al. 1997) Further, convergent evolution can produce nearly identical traits, which do not share the evolutionary history necessary to produce an informative clade. A final problem with morphological characters is that they are often under strong selective forces, which exaggerate the rate of change in more different traits when inferring species histories (Bachmann 1995). This allows us to incorrectly conclude species are more divergent than they actually are, or that they have diverged farther in the past than they actually have.

While morphological data can mislead us in understanding species relationships, phylogenies based on molecular data also have flaws. Phylogenies can misrepresent true species

histories (Degnan and Rosenberg 2015) because of issues such as long-branch attraction (Bergsten 2005), incomplete lineage sorting (Pamilo and Nei 1988), and mitochondrial capture (Toews and Brelsford 2012). With molecular phylogenies, however, we have the distinct advantage of knowing how sequences evolve. Sequencing technology is advancing and the cost is decreasing rapidly (Caulfield et al. 2013). In identifying closely related species, researcher most often begin with a molecular phylogeny for an initial perspective on the true evolutionary relationships of the species.

Population genetics

Reproductive isolation happens when gene flow ceases between populations. We now have a host of molecular tools that allow us to make inferences about gene flow between populations. Micro-satellites, mitochondrial haplotype mapping, various types of fragment length analyses, and now even whole genome population SNPs (single nucleotide polymorphims) are being implemented to document restrictions in gene flow, and observe reproductive isolation directly.

While microsatellites and whole genome sequencing are still relatively expensive, AFLPs (Amplified Fragment Length Polymorphisms) remains a powerful approach for understanding genetic structure for organisms whose genome is completely unknown (Vos et al. 1995). They can quantify restrictions in gene flow, similarities between populations, and detect hybrid individuals (Beismann et al. 1997; Mueller and Wolfenbarger 1999; Matsumoto et al. 2009). AFLPs are a product of using restriction enzymes, pre-selective PCR, and selective PCR to

amplify random medium-sized fragments of DNA through the entire genome. At the sacrifice of heterozygosity data, but the advantage of power, AFLPs are an excellent tool for exploring genome similarity between any two populations and are once again gaining popularity.

Behavioral reproductive isolating barriers

Reproductive isolating barriers can be any hindrance to groups of organisms producing viable offspring with one another. While these can be abiotic factors such as vicariance events, they are typically discussed in two broad biological categories: prezygotic and postzygotic. The former includes most behavioral reproductive isolating barriers whereas the latter includes many genetic incompatibilities that can arise between species. Reproductive isolating barriers occurring earlier in the mating process are greater in strength (Ramsey et al. 2003) since they eliminate future mating opportunities. Thus prezygotic mechanisms are stronger in absolute strength than postzygotic. For this reason, behavioral isolating mechanisms that prevent mating in the first place are thought to be among the strongest and most common in nature.

One of the great difficulties in studying speciation is that nearly all of the current species pairs on earth have multiple reproductive isolating barriers and it is impossible to determine which one arose first. Part of the problem is reinforcement. Reinforcement theory states that once there is a reduction in hybrid fitness, selection will now directly favor prezygotic isolating mechanisms that prevent mismatch matings (Dobzhansky 1937). Another problem is that in geographic isolation, over time, two species will gradually accumulate more and more barriers.

Regardless of the initial barrier, these prezygotic behavior barriers often arise quickly because of reinforcement and play a major role in the reproductive isolation of animals. By studying existing species pairs and their behavioral reproductive isolating barriers, we can learn about the mechanisms currently preventing species from hybridizing, and how selection works on these behavioral adaptations.

Katydids as a study system

Katydids, the family Tettigoniidae, are a diverse family within the orthopteran order. There are nearly 6400 named species in this family and at least 255 in North America. The reproductive biology of katydids is well understood (Gwynne 2001). Sexual selection is influential in the evolution of the spermatophylax (Bussiere et al. 2005, Vahed 2007), and nearly every measurable feature of acoustic advertisement calls (Gerhardt and Huber 2002), although male calls can also evolve in the absence of female preferences (Bush and Schul 2010). Character displacement of male signals has been demonstrated in closely related orthopterans (Jang and Gerhardt 2006).

Katydids have long a long history in studying acoustic communication (Gerhardt and Huber 2002). They make an excellent system for the study of mate-choice behavior for multiple reasons. The first is that acoustic calls are easily recorded and quantifiable. The second is that acoustic playback experiments are easy to perform. Playback experiments allow experimenters to isolate only the acoustic sound, and eliminate other evaluation criterion (visual, chemical, etc.), that individuals may incorporate when sampling live individuals (Otte 1977; Wells 1977; Loher

and Dambach 1989). In most species of katydids, individuals will walk directly to a speaker playing an acoustic call. In a laboratory setting, katydids will respond to synthetic acoustic calls created from "white noise". This allows experimenters to create calls of any frequency and temporal pattern they like and test female responses to different call features. Katydids are also an excellent system for studying acoustic behavior because of their simple neurobiology. They have one major auditory neuron and three interneurons responsible for transferring information about acoustic mating calls and researchers can directly measured the firing rate of all of these (Suga and Katsuki 1961; McKay 1969; Schul 1997). One final feature that makes katydids a good system is that many species exist with potential hybrid zones in with largely overlapping distributions, and this is true formyfocal genus *Obolopteryx* (Cohn et al. 2014). All of these features make them an ideal system for exploring reproductive isolating barriers with regards to mate choice behavior.

Summary

This dissertation adds to our understanding of reproductive isolation by identifying a reproductive isolating mechanism between a pair of understudied species. I first build the first molecular phylogeny for a newly revised genus of katydids: the *Obolopteryx*. Then, using population genetics tools I show a restriction in gene flow between two populations of *O. oreoeca,* but not *O. brevihastata.* I link this gene flow restriction between populations with a shift in the mating preferences of wild females to avoid heterospecific matings in sympatry. Additionally I quantify the male advertisement calls of both species highlighting character displacement in syllable

duration in *O. oreoeca*. This research provides a unique example of how selection from heterospecific competition can operate on discriminatory behavior in females. The genetic AFLP data combined with the results of behavior indicate that two populations of *O. oreoeca* located on sky islands are diverging from one another, probably due to reinforcement, in a peripheral population that has come into secondary contact with *O. brevihastata*.

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CHAPTER II

A MOLECULAR PHYLOGENY FOR A NORTH AMERICAN PHANEROPTERINE KATYDID GENUS *OBOLOPTERYX*

Abstract- *Obolopteryx* Cohn et al. 2014, formerly *Dichopetala* Rehn & Hebard 1914, is a newly erected genus of North American katydids that until recently fell under the 'dichopetaline' taxonomic group of phaneropterines. Here I produced the first molecular phylogeny for the genus *Obolopteryx* and compared my results to previous morphological studies. I used two mitochondrial genes (COI and Cytb) to resolve recent relationships in the genus. I failed to find congruence with any of the previous morphological hypotheses, suggesting that the genitalia traits used for morphological inferences are not necessarily phylogenetically informative. Further, I found some preliminary evidence that the recently erected genus, *Mactruchus*, is nested within the established *Obolopteryx*.

Introduction

Orthoptera is among the oldest orders of insects dating back more than 300 million years ago (MYA) by mitochondrial molecular clock estimates (Gaunt & Miles 2002), and deep relationships within the taxon are mostly resolved (Flook & Rowell 1998; Flook et al. 1999). The Tettigoniidae are of great interest in understanding both acoustic communication and sexual selection (Bailey & Rentz, 1990; Gerhardt & Huber 2002; Gwynne 2001). The Phaneropterinae (false katydid) subfamily alone, which includes the *Obolopteryx* genus, is currently estimated to include over 2300 species, most of which are subtropical (Grzywacz et al. 2014). The phyletic line formerly 'dichopetalines', is currently composed of 30 subtropical species with the exception of the temperate distribution of several *Obolopteryx* (Cohn et al. 2014).

The genus *Obolopteryx* is composed of eight morphologically similar species that overlap in various degrees in geographic distribution (Capinera et al. 2004). They range from central Texas west to Arizona and south through northeastern Mexico. Little is known about their ecology or reproductive biology, with the exception of two detailed taxonomic studies describing male and female genitalia (Cohn et al. 2014; Rehn & Hebard 1909). Phaneropterines display courtship behavior whereby females click in response to a specific "trigger pulse" of the male song, which in turn directs males to approach females. However, *Obolopteryx* are the sole North American genus within the subfamily that has reverted to the ancestral mate-choice behavior, consisting of females phonotaxing to singing males. The tegmina in *Obolopteryx* females are so diminished that they no longer touch each other, and are thus incapable of producing song. Worldwide, most phaneropterines have the diminished tegmina condition (Bey-Bienko 1965; Harz 1969), although the condition is rare in the New World.

The notable species diversity, overlapping distributions, and confounding morphology among *Obolopteryx* genitalia emphasize the need to understand their phylogenetic relationships.

Here, I provide the first molecular phylogeny for the genus *Obolopteryx*, and compare my results to inferences based on morphological traits.

Methods

Sampling and DNA isolation

Samples included in my analysis were compiled from field collections, museum vouchers and previous published material (Appendix A). I collected individuals of *Obolopteryx oreoeca*, *O. brevihastata*, and *O. castanea* from multiple locations in southwest Texas, and preserved them in 95% ethanol in the field. *Obolopteryx gladiator*, *O. catinata*, *O. emarginata*, *O. seeversii, Mactruchus serrifera,* and *Arethaea gracilipes* (a thread legged katydid) were loaned from the University of Michigan, Museum of Natural History. Within the *Obolopteryx* genus, I have omitted only the species *Obolopteryx poecilia*, as I was unable to obtain tissue from this Mexican species. I have included the species *Mactruchus serrifer*, since it was formerly a member of *Dichopetala*. I included *Arethaea gracilipes* as an outgroup. Also, I included two additional outgroup samples, an old world locust *Ruspolia dubia* and a cone-headed katydid *Locusta migratoria* (GENBANK accession numbers EF583824 and JN858153, respectively). For all tissues, I isolated DNA from preserved hind femurs using E.Z.N.A.® Insect D.N.A. Kits (Omega BioTek) according to the manufacturer's protocol.

Sequencing and molecular analyses

I analyzed partial sequences for the cytochrome c oxidase subunit 1 (COI, 704 bp.) and cytochrome b (Cytb; 751 bp.) mitochondrial genes for nine phaneropterines of the following species: *Arethaea gracilipes, Mactruchus serrifera, Obolopteryx catinata, O. castanea, O. emarginata, O. brevihastata, O. gladiator, O. seeversii,* and *O. oreoeca*. I amplified COI using published primers COI-F: 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3' and COI–R: 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3' (Snyder et al., 2009). Primers for Cytb, (Cytb-F: 5'-CAA ATA TCY TTY TGA GGR GC-3' and Cytb-R: 5'-GTT TTC AAA ACR TAY GCT T-3'), were designed for this study from an alignment of related orthopteran taxa from GenBank. I amplified both genes under the following PCR conditions: an initial denaturing of 94°C for three min, followed by 35 cycles of denaturation at 94°C for 30sec, 47°C for 1min and 72°C for 1min, with a final hold of 72°C for seven min. PCR products were gel-purified using the Wizard® SV Gel and PCR Clean-Up System (Promega). Sequencing was performed in both directions using the PCR primers above on an Applied Biosystems ABI3730 genetic analyzer at the Core Facility of Oklahoma State University. I initially aligned sequences using CLC Main Workbench 6.8.2, and then edited alignments manually in MEGA 5.2.2 (Tamura et al. 2011). I concatenated both mitochondrial genes in my alignment and treated them as one for all analyses. I acknowledge the fundamental problem of inflating bootstrap values when concatenated genes (Salichos & Rokas 2013), but mitochondrial DNA should be an exception to this problem since it is inherited as a single functional unit (Birky 2001; Wolstenholme 1985).

I created phylogenies using maximum likelihood (ML) and Bayesian methods. The software PAUP 4.0 (Swofford 2000) and MrBayes 3.2 (Huelsenbeck & Ronquist 2001) were used to estimate phylogenies for ML and Bayesian methods, respectively. Support for the ML topology was evaluated with 10,000 bootstrap replicates. For the Bayesian consensus tree I report

posterior probabilities based on 37,500 posterior likelihoods from the trace file, ignoring a 25% burnin period.

For the ML tree, I used Modeltest 3.7 to select the GTR+I+G evolutionary model with the following parameters: Lset Base=(0.3245 0.2175 0.1346), Nst=6, Rmat=(10.3206 49.7093 28.9093 6.2844 191.2924), Rates=gamma, Shape=1.2301, Pinvar=0.4995. I assigned the outgroup *Locusta migratoria*, and used the TBR branch swapping algorithm. For the ML tree, I enforced the constraint of monophyly of the *Obolopteryx* genus. For the Bayesian tree I used the following priors: Nst=6, rate=invgamma, and Nchains=6. I enforced a constraint on the monophyly of the Phaneropterinae subfamily, and assigned the outgroup *Locusta migratoria*. I set the MCMC for nreps=5,000,000 and sampled every 100 runs. The consensus tree was generated using the sumt command with a 25% burnin representing 37,500 samples.

In a separate analysis, I used Beast 1.7 (Drummond et al. 2012) to estimate divergence times within *Obolopteryx* using the same evolutionary model and similar parameters as used in my MrBayes analysis. I used the Birth Death tree model of speciation and fixed a strict, uniform clock rate of evolution to 2.3% divergence per myr between taxa (Brower 1994; Shapiro et al. 2006).

Results

I found that the two ancestral outgroups, *Ruspolia dubia* and *Locusta migratori*, shared an unresolved polytomy with the Phaneropterinae subfamily of katydids (Fig.1). However, the Phaneropterines outgroup *Arethaea gracilipes* was well supported in both phylogenetic analyses as sister to the remaining dichopetalines. Two pairs of sister taxa shared moderate support by both methods. The first was *O. castanea* as sister to *O. oreoeca*. The second was *O. brevihastata* as sister to the ancestor of *O. gladiator* and *O. seeversii*. Additionally, *O. gladiator* and *O. seeversii*

had moderate support as sister taxa in the Bayesian analysis. Deeper in the *Obolopteryx* phylogeny there was uncertainty about the placement of *O. emarginata* in relation to the other groups. *Obolopteryx* was paraphyletic with respect to *M. serrifera*, which was nested within the genus. *M. serrifera's* sister relationship to *O. catinata* was highly supported by both Bayesian and ML methods.

Using the evolutionary rate of 2.3% divergence per million years (MY), the median ages of the *Obolopteryx* nodes ranged from 5.17± CI [4.19,6.27] MYA to 14.06± CI [12.14,16.17] MYA. Six of the species in the *Obolopteryx* diverged from one another in the last 10.33± CI [8.92,11.90] years. *M. serrifera* and *O. catinata* appear to have diverged approximately 12.05± CI [9.94,14.30] MYA making them one of the oldest divergences in the clade.

Discussion

This phylogeny marks the first molecular hypothesis of the genus *Obolopteryx*, and my results provide an initial framework for understanding evolution in this group. Mitochondrial genes are established as good initial approximations of phylogenetic relationships in the Orthoptera (Flook & Rowell 1997), although I acknowledge the shortcomings of using mitochondrial DNA such as only reveling female lineages, nuclear mitochondrial gene transfers (NUMTS), and possible selective forces refuting the neutrality of mitochondrial markers (Toews & Brelsford 2012). Overall, most interspecific relationships were well supported. However, one exception involves the problematic position *O. emarginata* and its relationships with the remaining well-resolved *Obolopteryx*.

Interestingly, this phylogeny supports almost none of the original relationships proposed by Rehn and Hebard (1914) based on morphological characteristics of male cerci. Rehn and Hebard's phylogeny hypothesized sister relationships for three taxon pairs included in my study,

including *O. castanea* and *O. brevihastata*, *O. gladiator* and *O. emarginata*, and *O. oreoeca* and *O. catinata* although they refrain from further inferences among such pairs. My molecular analysis provides substantial evidence to the contrary for all three pairings. While my results are consistent with cerci shape unifying all *Obolopteryx*, such characters are not informative in elucidating sister relationships, and suggest that cerci shape is not apomorphic. Thus, my results caution against utility of cerci morphology to reveal close affinities, much like Cohn et al. cautioned for spinose ovipositors and the extent of tegmina reduction (Cohn 2014).

Cohn et al. (2014) also discussed the relationships within the *Obolopteryx*. They suggest that *O. gladiator, O. seeversii,* and *O. emarginata* share a common ancestor based on male cerci, subgenital plates, and epiprocts. Although I did not include *O. emarginata* in my analyses, my molecular results support the sister relationship of *O. gladiator* and *O. seeversii*, but additionally reveal *O. brevihastata* is a sister to the common ancestor of this pair.

Based on extant taxa in my analysis, it appears that the *Obolopteryx* species radiated from one another in a relatively slow and stepwise fashion between five and 14 MYA during the second half of the Miocene, marked by a long and gradual cooling period preceding the most recent ice age. Notably, *M. serrifera* and *O. catinata* shared a common ancestor approximately 12 MYA during the warmest point of the Miocene. The Miocene epoch was marked by the expansion of grasslands, however, it is unclear how modern distributions were influenced historically, since much of the North American fauna was subsequently affected by the repeated glacial cycles during the Pleistocene (Hewitt 1996). Some of the more recent branches preceding poorly supported nodes are a minimum of one million years apart and I suspect incomplete lineage sorting with respect to my genetic markers is unlikely the problem in resolving my tree. Rather, resolution will be improved by the addition of more slowly evolving nuclear markers and additional mitochondrial data.

Mactruchus has recently been moved to a separate genus based mostly on the lack of forked male cerci (Cohn et al. 2014). This genus rests on the epiphallus rather than on the shape of male cerci, which was used for most of the other dichopetalines, and the authors admit to finding the three species in this genus systematically perplexing. Within the genus there appears to be no epiphallus commonalities between the three existing species. The mitochondrial data presented here suggest that this new genus, *Mactruchus*, is nested within the *Obolopteryx*, and more molecular data may support either the merging of these two genera or possibly only moving this single species of the *Mactruchus* genus back into *Obolopteryx*. The close relationship between these taxa was among the best supported in my phylogeny, and further reinforces that rapid evolution of male genitalia can be a misleading character in determining species relationships in the dichopetalines.

When considering the spatial distributions of taxa included in my molecular analysis, two of the well-supported sister clades suggest contrasting models of speciation. Species distributions seldom provide sufficient evidence to infer speciation (Coyne and Orr 2004), and *O. castanea* and *O. oreoeca* occur in adjacent geographic proximity with only limited overlap, consistent with some type of allopatric divergence. However, *O. gladiator, and O. seeversii* fall entirely within (but near the margins) of the distribution *O. brevihastata*, and alternatively suggests a peripatric mode of speciation. This notion is consistent with my sampling over three years (2010-2013), and suggests that *Obolopteryx* species distributions are patchy in general, ephemeral from year to year, and potentially correlated with precipitation (BJK pers. comm.). *Obolopteryx* is typically abundant when adequate ground cover is available, but negligible when absent. Thus, it seems plausible that short periods of reproductive isolation are potentially generated by climatic influences in the already patchy deserts of the southwest U.S. and northeastern Mexico. Still, the above explanations are tentative, and do not alone provide a sufficient explanation for complete reproductive isolation between either of the above species pairs in *Obolopteryx*.

The contrasting molecular and morphological evidence suggests that in closely related species, male genitalia potentially evolves rapidly, and is decoupled from neutral molecular markers. Rapid evolution in katydids has been noted before, including the development of marked differences in genitalia as an effective means of reproductive isolation (Rentz 1972) and male genitalia in general (Eberhard 1985). While these traits are taxonomically informative for *Obolopteryx*, such patterns potentially result from convergent evolution, confounding species histories. I suggest caution using such traits for phylogenetic inference since hypotheses based primarily on male genitalia contrast markedly with molecular hypotheses.

My results provide an initial molecular perspective on relationships within and among the *Obolopteryx* occurring within the Unites States. Although logistical difficulties prevented me from including taxa from Mexico, recent work (Cohn et al. 2014) has since identified 20 additional species in closely related New World genera. For several species I was only able to extract DNA from a single representative specimen collected decades ago, and this left only a small amount of usable DNA for the species included in my study. My study provides a novel perspective on this expanding taxonomic group and provides a framework to compare the newly described taxa. One major consideration of future systematic work is that male genitalia in this group conflict withmyinitial molecular analysis. This is not surprising due to the overlapping distributions of many closely related species creating potential for rapid evolution of these traits.

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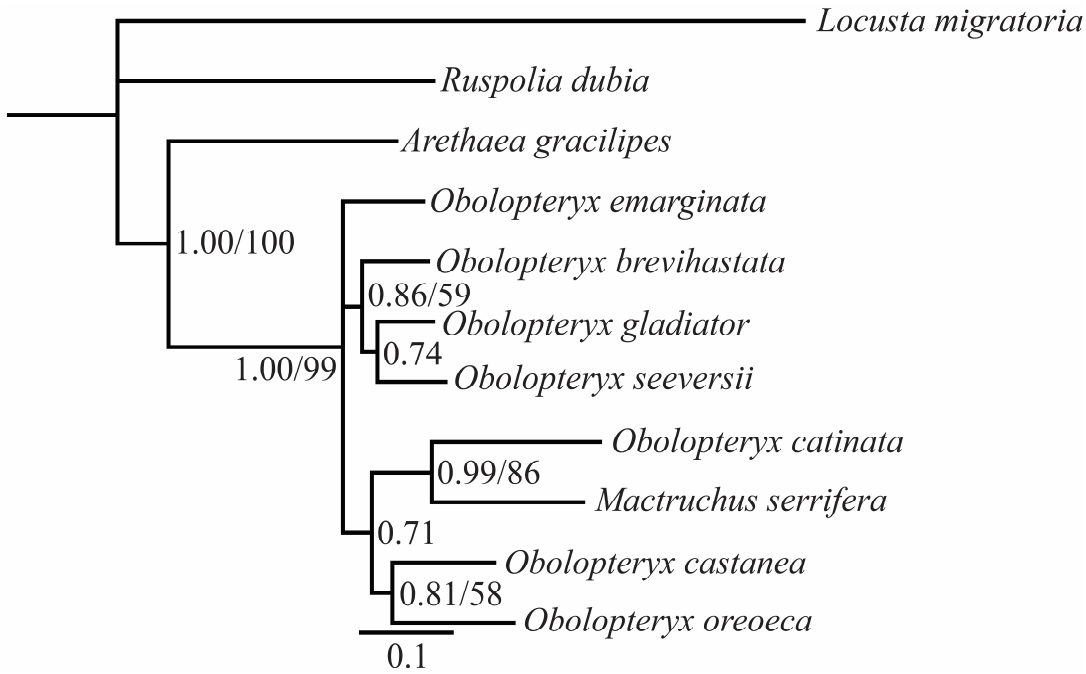
Figure Captions

Figure 2.1. Bayesian *Obolopteryx* phylogeny with posterior probabilities and ML bootstrap values. Scale bar indicates substitutions per site.

Fig 2.1


Locusta migratoria
Ruspolia dubia
Arethaea gracilipes
Obolopteryx emarginata
1.00/100
Obolopteryx brevihastata
0.86/59
Obolopteryx gladiator
0.74
1.00/99
Obolopteryx seeversii
Obolopteryx catinata
0.99/86
Mactruchus serrifera
0.71
Obolopteryx castanea
0.81/58
Obolopteryx oreoeca
0.1

CHAPTER III

POPULATION STRUCTURE BETWEEN SKY ISLANDS IN A NARROWLY DISTRIBUTED CHIHUAHUAN DESERT KATYDID

Abstract- I compared gene flow between multiple populations of two species of Chihuahuan desert katydids: *Obolopteryx oreoeca* and *O. brevihastata*. The higher elevation species, *O. oreoeca,* is restricted to sky islands while *O. brevihastata* occupies a wider range of elevations and is relatively continuous in its distribution. I used dominant AFLP markers to test for gene flow in both species. Global F_{ST} showed that *O. oreoeca* populations differ more from one another than *O. brevihastata* populations, (respectively 0.061 and 0.017). I found a significant difference between *O. oreoeca* populations in a pairwise F_{ST} permutation test ($p=0.019$), but found no difference between *O. brevihastata* populations. STRUCTURE analyses further supported the clustering the Chisos Mountain population of *O. oreoeca* in Big Bend National Park separately from the Davis Mountains populations. A low elevation, arid region acts as an apparent geographic barrier for these two management units.

Introduction

Obolopteryx brevihastata exists largely in allopatry from *O. oreoeca*. However, they are sympatric in the Davis Mountains where they overlap spatially and temporally (see Cohn et al. 2014). I have found syntopy more common than previously reported (BJK pers. comm.). Sky islands, or high elevation mountains surrounded by drastically different low elevation habitat, are typical of the Chihuahuan desert. While *O. brevihastata* are widely distributed at low elevations in the Chihuahuan desert, *O. oreoeca* exhibits a patchy distribution on sky islands throughout only the Davis Mountains and the Chisos Mountains of Big Bend National Park.

The newly revised *Obolopteryx* genus (Rehn and Hebard 1914; Cohn et al. 2014) consists of eight species and is unique in several ways. Mate-choice behavior typical of the subfamily Phaneropterinae (excluding *Obolopteryx*) consists of males chorusing, females returning a timed click to indicate choice, then the chosen male phonotaxing to the female (Bailey and Rentz 1990). The *Obolopteryx*, however have reverted to the ancestral katydid behavior in which males chorus, and females phonotax towards them. Also, *Obolopteryx* are presumably limited in dispersal ability due to their flightless, diminished wing and short body length. Together, patterns of geographic distribution, mate-choice behavior and limited dispersal ability make the *Obolopteryx* an ideal group to study reproductive isolation and address mechanisms of adaptive radiation.

Using Amplified Fragment Length Polymorphism (AFLP) markers I test both *O. oreoeca* and *O. brevihastata* populations for gene flow restrictions, and ask if the two species differ in the extent of how genetically distinct their populations are from one another. I hypothesize specifically that *O. oreoeca* suffers a dispersal restriction from the low elevation arid desert between sky islands, and thus predict they will have populations that are genetically distinct. In comparison, the range of *O. brevihastata* is more continuous (uniform habitats at lower elevations) and I predict they will be more genetically homogenous.

Methods

AFLP is an efficient tool for inferring genome-wide genetic similarity between groups where genetic markers are unavailable and information about the genome is lacking (Vos et al. 1995). As such, I screened *Obolopteryx* for AFLP to test for isolation by distance (IBD) and pairwise F_{ST} differences between multiple populations of both *O. oreoeca* and *O. brevihastata*. I also tested whether the Bayesian clustering program STRUCTURE would find greater differences between *O. oreoeca* populations compared to *O. brevihastata*.

Sampling

Katydids of both species were collected from three regions within the Chihuahuan desert that differ in respect to biogeographic patterns: allopatric *O. oreoeca* from Brewster Co., Texas (n=23; Fig. 3.1, F), allopatric *O. brevihastata* from Terrell Co., Texas (n=22; Fig. 3.1, E) and a sympatric zone in Brewster & Jeff Davis Co., Texas [*O. oreoeca* (n=29; Fig. 3.1, A-D), *O. brevihastata* (n=24; Fig. 3.1, A&D)]. I sampled multiple locations within the sympatric zone to attain adequate sample sizes for AFLP. All specimens are deposited at the K.C. Emerson Collection of Invertebrates curated by the Department of Entomology and Plant Pathology at the Oklahoma State University - Stillwater, Oklahoma (Appendix 4.1). Populations were identified as allopatric or sympatric based on historical records (Cohn et al. 2014) and substantiated by my surveys conducted over three years. Femurs were collected from each individual in the field over multiple years and placed directly in 95% ethanol.

Molecular techniques

DNA extractions were performed with E.Z.N.A.® Insect D.N.A. kits according to the manufacturer's protocol. I used one to two hind-femurs for each extraction depending on individual size. Males have smaller hind-femurs and typically required two femurs for adequate DNA concentrations. Digestion was performed with restriction enzymes EcoR I (Eco) and Mse I (Mse) (NEB, Ipswitch, MA, USA) using 50 ng/µl of DNA. Double-stranded adaptors were ligated to the fragment ends (IDT, Coralville, IA, USA). Preselective PCR used Eco+A (5-GACTGCGTACCAATTCA-3) and Mse+A (5-GATGAGTCCTGAGTAAA-3) primers under the following thermocycling conditions: 72°C for one min, 94°C for 50 s denature, 56°C for one min anneal, then 72°C for two min extension, repeated 20x with a 4°C hold at the end. For selective PCR I used the following primer pairs: labeled Eco+ACG (PET) with MSE+ACA, labeled Eco+AAG (VIC) with MSE+AGC, and labeled Eco+ACG (6FAM) with MSE+AAC. Labeled and unlabeled primer pairs were amplified in individual reactions under the following thermocycling conditions: 13 cycles with a 0.7°C annealing temp step down per cycle (94°C for 50 s, 65°C for one min, then 72°C for a two min), 23 cycles at (94°C for 50 s, 56°C for one min, then 72°C for two min, ending with a 4°C hold for two min). Selective PCR products for each of the three labeled primers were pooled and run in single lanes for sequencing at Oklahoma State University Recombinant DNA/Protein Core Facility using an ABI 3730 genetic analyzer, with GeneScan™ 600 LIZ® dye size standard v2.0 (Applied Biosystems, Foster City, CA, USA). GENEMARKER version 1.1.0 (SoftGenetics, LLC, CA, USA) was used for visualization and scoring.

Analyses

I used the program GEOGRAPHIC DISTANCE MATRIX GENERATOR (Ersts) to estimate the geographical distances between the six collection sites (A-F in Fig. 3.1). I used the program ISOLATION BY DISTANCE (Bohonak 2002) to test for IBD in both species using a Mantel test. I calculated pairwise F_{STs}, Nei's genetic distances, and Reynold's genetic distances for both species in the program AFLP-SURV version 1.0, which also implements a permutation test for Pairwise F_{ST} differences between populations (Vekemans et al. 2002).

I evaluated population genetic structure using STRUCTURE (Pritchard et al. 2000), a model-based method for clustering individuals using multilocus data. It uses a Bayesian Markov Chain Monte Carlo method to quantitatively assign the proportion of each individual to a specified number of genetic groups (K). Ten individuals (five from each species) used in the Mantel test for IBD and pairwise F_{ST} permutation test were omitted from the STRUCTURE analysis due to high proportions of missing data, which interfered with clustering. I used this approach for two independent analyses: the first allowing clustering of all populations of both species and the second only used *O. oreoeca* in case shared alleles with *O. brevihastata* hindered STRUCTURE from finding clusters within this species.

For the first STRUCTURE analysis, I used the admixture model with sampling information as priors, and allowed the allele correlation (lambda) between populations to be estimated. I varied K from one to ten. I conducted an additional analysis only for *O. oreoeca* with the same parameters. For this analysis I varied K from one to seven. I implemented the Evanno method (Earl and vonHoldt 2012) in STRUCTURE HARVESTER and report the optimal K values, based on the largest increase in likelihood. The programs CLUMPP (Jakobsson and Rosenberg 2007) and DISTRUCT (Rosenberg 2004) summarize multiple runs of identical K and visualize the outputs.

Results

My survey of AFLP produced a total of 77 scoreable loci: 51 in *O. oreoeca* and 45 in *O. brevihastata*. Overall, the global F_{ST} between species was 0.097. Mantel tests for IBD revealed no correlation between raw genetic and geographic distance in *O. brevihastata* populations (n=3) using pairwise F_{ST} (Z=7861.59, R^2=0.29, p=0.16), Nei's genetic distance (Z=1455.10, R^2=0.31, p=0.32), or Reynold's genetic distance (Z=7924.67, R^2=0.28, p=0.16). Similarly, for *O. oreoeca* populations (n=5), I found no correlation between raw genetic and geographic distance using pairwise F_{ST} (Z=52429.05, R^2=0.00, p=0.54), Nei's genetic distance (Z=15682.79, R^2=0.03, p=0.62), and Reynold's genetic distance (Z=55657.78, R^2=0.00, p=0.58). Additionally, log transformations of both genetic or geographic distances did not affect significance. However, the power to detect significant associations was low due to my small number of sampling locations.

Global F_{ST} within species varied, and showed stronger differentiation among populations of *O. oreoeca* than among *O. brevihastata* with 0.061 and 0.017, respectively. Pairwise F_{ST} permutation tests, with 1000 permutations, verify this pattern. There was significant genetic divergence between *O. oreoeca* populations (p=0.019), but not between *O. brevihastata* populations (p=0.074).

In the first analysis with both species I used population assignment priors and the admixture ancestry analysis delineates the species well (Fig. 3.3). Generally, the majority of individuals from both *O. brevihastata* (orange) and *O. oreoeca* (yellow) are clearly assigned to their species. However, several individuals in each population cluster in several groups (blue, pink, and green) that were shared across species. Green is never the dominant ancestry block for any individuals. Blue is only the dominant ancestral block for members of the allopatric population of *O. oreoeca*, which is moderate support for the clustering of this group. The pink

cluster, which is dominant in many individuals across all populations, is uninformative in this regard and suggests a lack of power in my analyses.

In a second STRUCTURE analysis I evaluated evidence of genetic divergence between only the *O. oreoeca* populations. While the largest increase in natural log likelihoods is from K=1 to K=2, I see a peak in the natural log likelihoods for 10 replicate STRUCTURE runs at K=4 (Fig. 3.4). An Evanno analysis assigned K=2 as the optimal K based on the largest increase in likelihood, but I again find more biological meaning and support for the summary of runs from K=4, which is the likelihood peak and also has small error bars (Fig 3.4). While all five sampling sites share two of the ancestral blocks (orange & blue), the pink ancestry block is almost exclusively comprised of the allopatric population of *O. oreoeca*. Also, the yellow ancestral block is far more common in the other three populations in the Davis Mountains and is the dominant ancestral block in only one individual in the allopatric population.

Discussion

I found no effect of isolation by distance in either species. Finding IBD in *O. brevihastata* but not *O. oreoeca* would provide strong support for a low elevation, arid isolating barrier in *O. oreoeca*. If physical barriers exist for *O. oreoeca* they should produce a pattern of genetic structure resulting from geography rather than distance. However, it is not surprising that I did not find IBD in *O. brevihastata* due to the few sampling locations combined with the proximity of the populations sampled. It is possible that there is gene flow across the entire Chihuahuan desert in this species.

I reported a higher global F_{ST} for *O. oreoeca* than for *O. brevihastata,* and the pairwise F_{ST} permutation test revealed significant differences between *O. oreoeca* populations (p=0.019) but not for *O. brevihastata*. These results matched my expectation given the defined physical

boundary that separate *O. oreoeca,* but not *O. brevihastata* populations. The *O. oreoeca* in the Chisos Mountains (Fig. 3.1, F) are separated from the rest of the populations by an arid region at substantially low elevation, which rarely has patches of suitable habitat for any species of *Obolopteryx* in continuous years. I had little success collecting any katydid species in this gap over multiple years of sampling. *O. oreoeca* has also rarely been found at elevations below 915m (Cohn et al. 2014), so flightless dispersion seems unlikely. Contrastingly, *O. brevihastata* exhibits somewhat continuous habitat between the sampling locations that is more likely to foster ongoing gene flow between the populations sampled. Roadside drainages provide relatively stable corridors of dispersion for this species, since they are found at all but the highest elevations in the region and appear, according to their distribution and elevation, to be somewhat more heat tolerant than *O. oreoeca*.

My AFLP data showed distinct blocks of genetic similarity unifying each species (Fig. 3.3). With my limited number (77) of alleles, STRUCTURE was unable to completely separate the species or populations perfectly, but I did infer similarities by shared blocks of ancestry. Some blocks of ancestry were common to all populations and uninformative in my analysis. It has been well documented that STRUCTURE will not to find spurious patterns of genetic structure (Jakobsson and Rosenberg 2007), thus increasing the number of alleles will only strengthen the apparent divergence between *O. oreoeca* populations of the Davis and Chisos Mountains. The *O. oreoeca* in allopatry showed that a large portion of the population was from the blue block of ancestry, which almost none of the sympatric *O. oreoeca* individuals possess (Fig. 3.3). This pattern is consistent with results of the additional conspecific STRUCTURE analysis. Figure 3.5 summarizes the results of 10 runs of K=4, revealing the distinctiveness of *O. oreoeca* in allopatry compared to sympatry. The allopatric population of *O. oreoeca* in the Chisos Mountains almost exclusively shares the pink cluster in Figure 3.5. Further, the yellow block of ancestry, which dominates the sympatric individuals, is rare for the allopatric individuals (Fig. 3.5).

I initially ran the STRUCTURE analysis using all of the populations of both species. The largest increase in natural log likelihoods is from K=1 to K=2 and I see a general increase in the natural log likelihoods from K=1 to K=10 with the curve stabilizing around K=5 (Fig. 3.4). An Evanno analysis assigned K=2 as the most likely number of populations. But in using two different species it is not surprising that the STRUCTURE runs for K=2 produce such a large ΔK since the largest increase in likelihoods should result from separating species and not populations within the species. I present the results using K=5 because of increasing likelihood with reduced error bars compared to the Evanno selected K, and also better support of the AFLP-SURV results.

Ideally, I would also have tested for hybrids between species in the sympatric region of the Davis Mountains, but such an analysis requires a very powerful data set. With increased markers, this may be possible. While these species are not nearest sister relatives to one another (chapter 2), I cannot rule out the possibility of hybridization, although hybrids likely have reduced fitness since there are no obvious stable hybrid zones.

Taken together the Mantel test for IBD, pairwise F_{ST} permutation test, and STRUCTURE analyses all suggest that the allopatric *O. oreoeca* are more divergent from their neighboring conspecific population than the *O. brevihastata* populations are to one another. The lack of IBD also suggests that this difference is not due to distance alone, but a gene flow barrier. I suspect physical dispersal barriers are most likely due to the geology. On the U.S./Mexico border of the Chihuahuan desert, sky islands are known to produce gene flow restrictions in black bears (Atwood et al. 2011), which have presumably greater dispersal ability than these insects. Jumping spiders from the Chihuhuan desert have also shown patterns of gene-flow restrictions based on sky islands, although in complex and unpredictable ways (Masta 2000). The Davis and Chisos Mountains are well-established "stepping stones" in the Madrean Archipelago which connects the larger plateaus of both the Rocky Mountains to the North and the Sierra Madres of Mexico to the South (Warshall 1995). It is worth considering these results with regard to other invertebrates.

This pattern of restricted gene flow between the high elevation habitats of the Chisos and Davis Mountains may be ubiquitous for drought intolerant and flightless species.

The geography of sky islands likely play a role in diversity and endemism of katydids everywhere. Tettigoniidae show great diversity in mountainous regions (Çıplak 2003) and also high levels of endemism (Çıplak and Demirsoy 1995; Çıplak et al. 2002). The Chisos Mountains of the Big Bend National Park are one of only a few places in the U.S. where the narrowly distributed *O. oreoeca* can be found, and also the home of the rare, endemic Big Bend quonker katydid, *Paracyrtophyllus excelsus* (Hebard 1941). While range expansions post ice ages are often the historical null model for this great diversity (Hewitt 1996), models of habitat fragmentation incorporating these sky islands are increasing plausible models for the diversity of species in these unique ranges (Knowles 2001a; Knowles 2001b; DeChaine and Martin 2005; Saglam et al. 2014).

This study is the first attempt to quantify population structure in *O. oreoeca*, which is endemic only to the Davis and Chisos Mountains in the U.S. My results imply that there may be multiple populations that are isolated from one another genetically in varying degrees. The distribution of *O. oreoeca* throughout the Chihuahuan desert of Mexico is not well known, but there are at least five locations more than 100 miles south and west where this species has been reported in low abundance (Cohn et al. 2014). Small sky islands are distributed regularly throughout the region. The Sierra Madre plateau is geographically close and relatively large compared to either the Davis or Chisos Mountains and may be a large genetic reservoir for these populations if suitable habitat exists. Larger scale sampling on many islands, and using more markers will inevitably illuminate a clearer picture of the restrictions to the flightless invertebrates in the region. Given growing conservation concerns for sky island species, and particularly the sky islands of the Madrean Archipelago (Gottfried et al. 2013), these data supports the need to independently consider the populations on each island. With respect to

conservation, and given the limited data available, I recommend that the *O. oreoeca* population to the south should be considered a separate unit from the population to the north in the Davis Mountains. This gene flow restriction between the Chisos and Davis mountains provides an interesting case study for future research since selection might operate differentially on the Davis Mountain population, which exists sympatrically with other species in the genus.

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Figure Captions

Figure 3.1. Collection locations for AFLP samples in Texas, USA. A, B, C, and D are in the sympatric zone (Davis Mountains). Location E (low elevation plateau) and F (Chisos Mountains of Big Bend National park) are in the allopatric zones of *O. brevihastata* and *O. oreoeca*, respectively.

Figure 3.2. Mean natural log likelihood values ± SE of ten STRUCTURE runs for all populations of both *O. oreoeca* and *O. brevihastata*.

Figure 3.3. DISTRUCT average genetic assignment of individuals of *O. oreoeca and O. brevihastata* using structure with population information (K=5, 10 runs). Letters indicate collection locations from Figure 3.1.

Figure 3.4. Mean natural log likelihood values ± SE of ten STRUCTURE runs for five populations of *O. oreoeca*.

Figure 3.5. DISTRUCT average genetic assignment of individuals of O. *oreoeca* using STRUCTURE with population information (K=4, 10 runs). Letters indicate collection locations from Figure 3.1.

Fig. 3.1

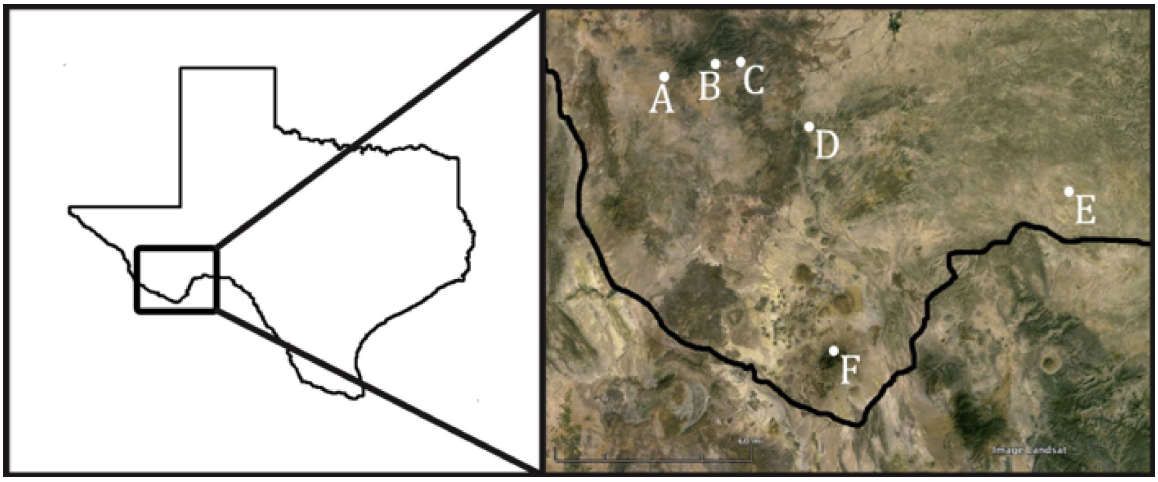
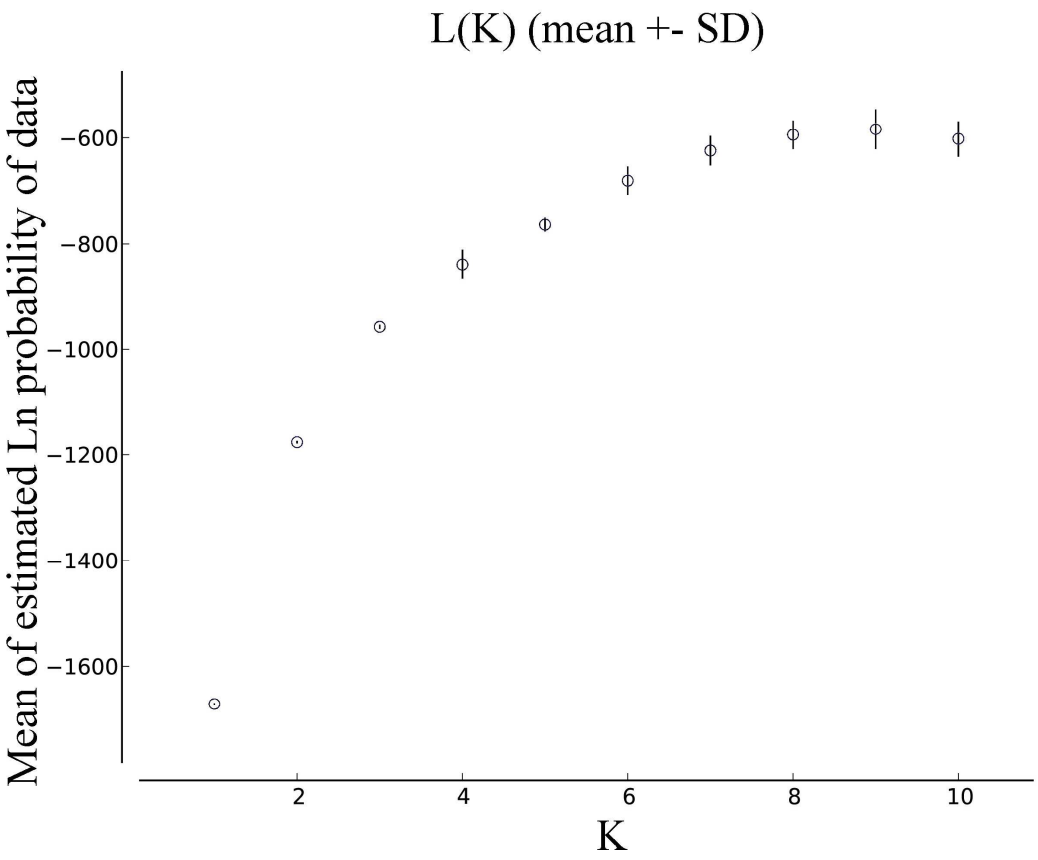


Fig. 3.2


L(K) (mean +- SD)
Mean of estimated Ln probability of data
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−800
−1000
−1200
−1400
−1600
2
4
6
8
10
K

Fig. 3.3

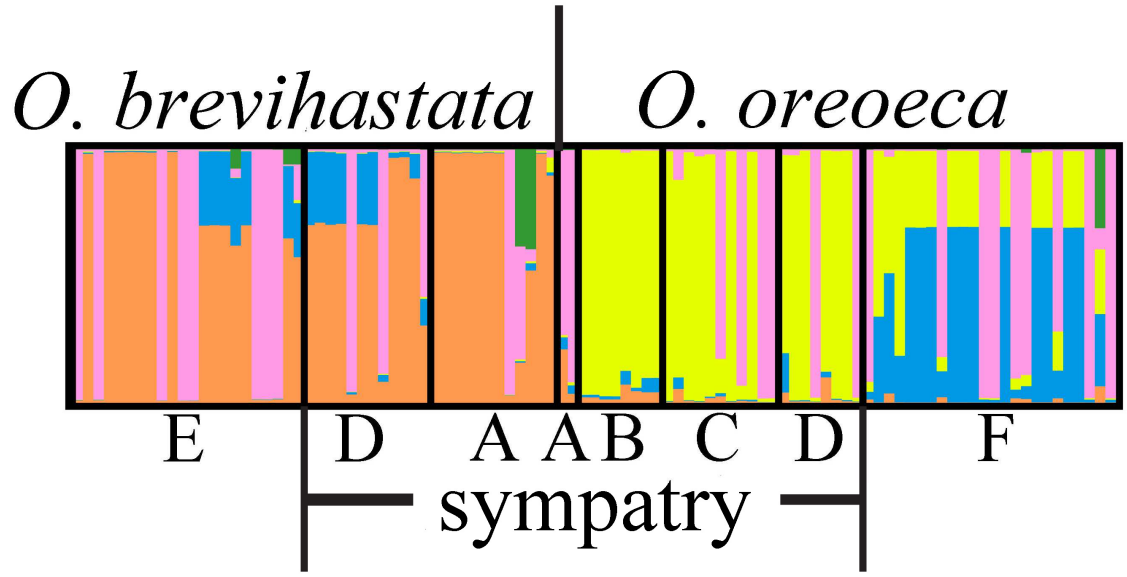
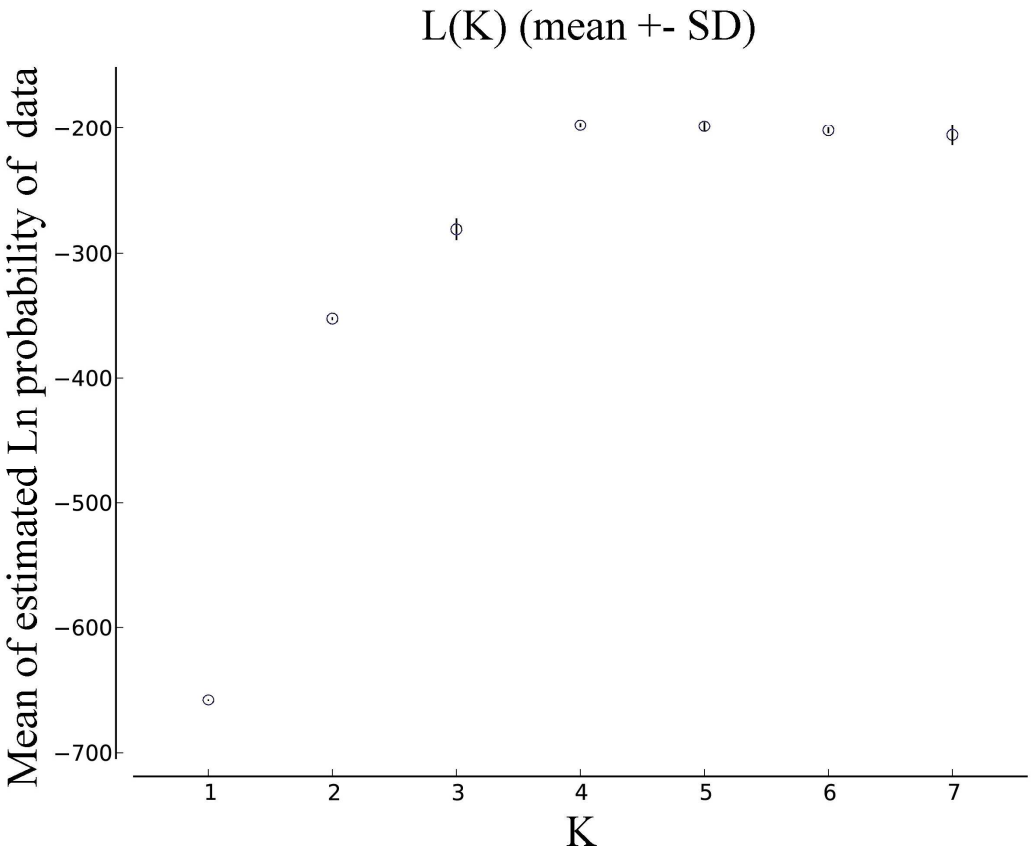


Fig. 3.4


L(K) (mean +- SD)
Mean of estimated Ln probability of data
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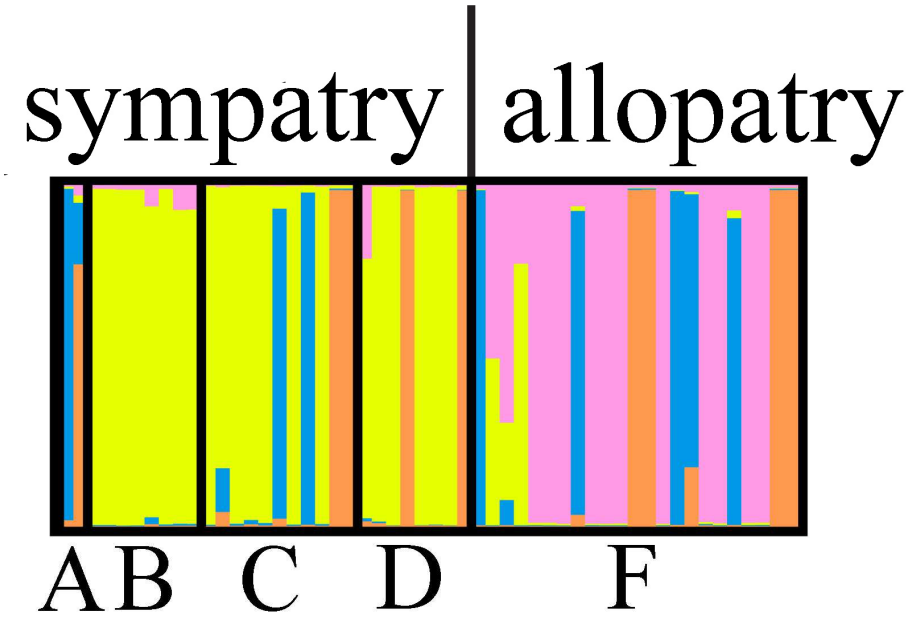
−300
−400
−500
−600
−700
1
2
3
4
5
6
7
K

Fig. 3.5



CHAPTER IV

INCREASED FEMALE MATING DISCRIMINATION AND CHARACTER DISPLACEMENT IN MALE CALLS IN SYMPATRIC CHIHUAHUAN DESERT KATYDIDS

Abstract- Female katydids use acoustic advertisement calls to locate and choose appropriate mates. In this type of mating system, selection can act on both the calls of one sex and on call preferences of the other. Here, I provide a quantitative male advertisement call description of two closely related species of Chihuahuan Desert katydids: *Obolopteryx oreoeca* and *O. brevihastata*. I analyzed the calls of both species, highlighting population differences in calls when in sympatry and allopatry. I found character displacement in syllable duration of my focal species *O. oreoeca*. With a no-choice playback experiment I demonstrated that a high amplitude pulse element is important for *O. oreoeca* female responses. With a second no-choice playback experiment I showed that *O. oreoeca* females from a population sympatric with *O. brevihastata* discriminated strongly against heterospecific calls, but that allopatric females responded well to the heterospecific call. While these population differences in *O. oreoeca* females' heterospecific call discrimination were stark, differences in male advertisement calls were subtler. I found strong support for a decrease in male *O. oreoeca's* syllable duration in the sympatric population, and no support for differences in any of the other temporal properties of *O. oreoeca's* calls. My data

moderately supported population differences in multiple features of *O. brevihastata's* calls, but the main effect was relatively longer interval durations between syllables in the sympatric population. These data for *O. oreoeca* combined with previous molecular data provide an example from nature in which the choosiness of females and the calls of males have evolved in combination with a gene flow restriction between two sky islands.

Introduction

A large body of empirical and theoretical work on sexual selection suggests that acoustic advertisement calls are a costly signal (Andersson 1994; Stoddard and Salazar 2011). Advertisement calls aid receivers in species recognition (Wells 1977a; Wells 1977b) and as indicators of individual quality (Ryan and Rand 1993; Ptacek 2000). Female choice is well documented as an evolutionary driver of male calls (Ryan 1985). In mating systems where males call and females use calls for species recognition, male calls (Andersson 1994; Gerhardt and Huber 2002; Groening and Hochkirch 2008) and female preferences (Kirkpatric 1982, Liou and Price 1994) are under strong selection. When there is a cost to heterospecific mating, a common situation in sympatric regions of closely related species (Shapiro 2000), reinforcement theory specifically predicts that species will evolve stronger heterospecific discrimination (Dobzhansky 1940; Noor 1999). Indeed, females may even compromise fitness in these situations to avoid heterospecific matings (Pfennig 2000)**.** There is empirical support for this prediction, with sympatric populations having stronger heterospecific mating discrimination than allopatric populations (Howard 1993; Butlin 1995).

When males call to attract potential mates and females use calls to find and select males, the selection can act on the sexes in different ways (Svensson et al. 2007). Females are generally thought to be the more discriminatory sex (Bateman 1948, Trivers 1972), and in some species they show stronger heterospecific mating discrimination than males (Saetre et al. 1997; Wirtz 1999). Although females are generally assumed to shift their call preference up or down to discriminate against heterospecific matings, they can also narrow the variation in call preferences, or switch cues entirely and rely on other non-acoustic signals (Martin and Pfennig 2011). Male mate choice has received less attention but males are also known to discriminate against heterospecific matings (Baxter et al. 2015). More commonly, male advertisement calls shift in sympatric regions due to character displacement (Brown and Wilson 1956). Male calls can

diverge in multiple ways including call rate (Jang and Gerhardt 2006), frequency (Hobel and Gerhardt 2003; Kirschel et al. 2009), and qualitative features (Ryan 1985). Interestingly, character displacement in male calls has been documented even in populations that do not hybridize (Etges et al. 1999), so reinforcement is not the only explanation for patterns of divergent traits in sympatry. While both female discriminatory behavior and male character displacement due to reinforcement have some clear empirical support, more examples from nature are needed to improve improve our understanding of these phenomena.

Identifying and understanding the mechanisms behind reproductive isolation in recently diverged taxa is a major goal of speciation research (Coyne and Orr 2004). While model organisms for speciation such as *Drosophila* have provided valuable insights into the evolutionary processes behind reproductive isolation, the overexploitation of model organisms has been a major hindrance to nearly all fields within biology (Bolker 2012). Many times these are chosen by convention rather than biological appropriateness. This study is an attempt to increase our taxonomic breadth in understanding reproductive isolation, which some have suggested is the next shift in speciation research (Wolf et al. 2010).

Most tettigonids (Orthoptera: Tettigoniidae) have a unidirectional courtship system in which females walk toward a singing male (Gwynne 2001). The Phaneropterinae subfamily has a bidirectional courtship system in which the sexes duet before mating. A calling male typically walks toward a stationary, calling female (Hartley et al. 1974; Robinson 1990). This system is favored by natural selection as a way to avoid eavesdropping predators (Belwood and Morris 1987). Within the European genus *Poecilimon* three lineages have reverted back from a bidirectional to a unidirectional courtship system (Heller 1984; Stumpner and Heller 1992; Chobanov and Heller 2010). *Obolopteryx* cannot duet since females' wings are too small to touch (Rehn and Hebard 1914). They represent a unique new world genus, which has probably lost the ancestral duetting courtship system common to all phaneropterines.

Many *Obolopteryx* species' distributions overlap to varying degrees, creating the potential for hybrid zones. *O. oreoeca* is sparsely distributed only in the sky island region of the Chihuahuan desert. Conversely, *O. brevihastata* is the most widely distributed species in this genus and can be found from Arizona to Texas and south through eastern Mexico. Only in the Davis Mountains can these two species be found sympatrically (Cohn et al. 2014). Because the adults mate in the same place at the same time, hybridization is possible, but undocumented. This is an excellent opportunity to test the hypothesis that male advertisement calls and female choice may evolve to prevent heterospecific matings. My study focuses on two populations of *O. oreoeca* that show signs of genetic differentiation, and two *O. brevihastata* populations that do not (chapter 3).

I first describe both species calls qualitatively and quantitatively. I compare the call features of both species in allopatry and sympatry across a temperature gradient and search for differences in call features between sympatric and allopatric populations of each species. Lastly, I test for character displacement in syllable duration of calls between species. Syllable duration is the only qualitatively comparable feature of the calls in these two species. Based on previous genetic data, I predict that differences in calls between sympatry and allopatry will be greater for *O. oreoeca* than *O. brevihastata*. This prediction is consistent with the hypothesis that character displacement occurred (Brown and Wilson 1956; Lemmon 2009).

I also use two playback experiments for the female *O. oreoeca.* I first test which elements of synthetic calls affect the probability of phonotactic responses in females. I then test if sympatric females have a lower probability of phonotactic response to heterospecific calls compared to allopatric females. I predict that the sympatric population has evolved to avoid heterospecific calls, but the allopatric population has not.

Methods

Test subject collection

I collected 14 male *O. oreoeca,* 18 male *O. brevihastata* males, and 14 female *O. oreoeca* from four locations (Fig. 4.1, A-D). Locations A and B are within a sympatric region of the two species. I collect the two species from separate sympatric locations where I was able to attain adequate sample sizes for behavior and previous genetic analyses for each species. Location C and D are within allopatric regions of *O. brevihastata* and *O. oreoeca*, respectively. Location D is central to the Chisos Mountain Range of Big Bend National Park and I collected animals with permission of the United States Department of the Interior National Park Service, (Permit #BIBE-2012-SCI-0032). I collected all katydids by hand and at night. I housed male and female katydids in the field separately in wood framed cages (30 x 30 x 60 cm) with well-ventilated steel mesh walls and a Plexiglas sliding door. I provided water (sprayed on interior Plexiglas), apple slices, celery, and lettuce ad libitum in the field and lab.

I acclimated females for three days before testing on a 14/10-hour light/dark cycle. Once transferred to the lab, they were individually stored in plastic containers (34.9 x 20.3 x 12.7 cm) with holes drilled in the longer walls for ventilation. The lab temperature was approximately 27.0 ± 1.0 °C. I provided females with densely packed cotton for oviposition to retain receptivity to calls. Phaneropterines are capable of mating many times over the breeding season (Simmons and Gwynne 1991; Gwynne 2001) and virginity and previous acoustic experiences are both known to have effects on female Orthopteran mating preferences (Bateman et al. 2001; Bailey and Zuk 2008). I collected wild *Oreoeca* females as adults and was unable to determine virginity.

I recorded the calls of 32 males after transporting them to Oklahoma State University: nine allopatric *O. brevihastata* (Fig. 4.1, C), nine sympatric *O. brevihastata* (Fig. 4.1, A), five allopatric *O. oreoeca* (Fig. 4.1, D), and nine sympatric *O. oreoeca* (Fig. 4.1, B). Sympatric *O.*

brevihastata were recorded in 2011, allopatric *O. oreoeca* were recorded in 2012 and allopatric *O. brevihastata* and sympatric *O. oreoeca* were recorded in 2013. I recorded all calls in a temperature-regulated chamber with humidity held constant at 55±10%. All male advertisement calls were recorded using a Marantz® Professional solid-state recorder PMD-671, and a G.R.A.S. ® SPL Transducer Type 21SB 1/2" condenser microphone. I recorded each individual at a minimum of two temperatures (between 18.0° and 35.3°C) because pulse-rate (the inverse of syllable duration) in insect calls varies in a linear manner with temperature (Walker 1957; Walker 1962; Walker 1975; Gray and Cade 2000). I eliminated echoes with circular sound foam wrapped around cylindrical calling cages and lowering the microphone inside the foam.

All specimens, male and female, were preserved in 95% ethanol, and deposited in the K.C. Emerson Collection of Invertebrates at the Oklahoma State University (Appendix C).

Statistical Analyses

All statistics were done in R. The lme4 and MuMIn packages were used for all generalized liner models. To analyze data for males and females I used Akaike Information Criterion (AICc) and model selection. For all AICc analyses I eliminated pretending variables by eliminating models that had ΔAICc greater than simpler versions of the model. I also eliminated models with ΔAICc greater than seven (Richards 2005; Anderson 2007).

Male advertisement calls

I chose four individuals per species from the allopatric populations and recorded them between the temperature range of 23.3 and 24.3°C. I annotate typical calls of both species (Fig. 4.2). For the general call description of the gross call features I took a ten second sample of a continuous

trill and measured the average pulse durations, interval durations, syllable duration, and ratio of peak amplitudes (Fig. 4.2). I also measured pronotum length (mm) by hand as a metric for size. For description of the fine-scale features of male calls I averaged the impulse periods (duration of time from the beginning of one tooth impact to the next) over ten syllables for each pulse in the call. I quantified all calls using custom software (SONGX), designed by Johannes Schul.

For *O. oreoeca*, which has variability in number of pulses per syllable (Fig. 4.2, A&B), I used model comparison based upon AICc to find what factors affected the probability of five-pulse versus four-pulse syllables in each 30-second clip. I used the probability of five-pulse versus four-pulse calls as a measure of the number of pulses per syllable. I ignored the less common types of syllables with greater or fewer pulses per syllable for this analysis. Higher probability values represent more pulses per syllable. I used a generalized linear mixed model (GLMM) with individual treated as a random effect and syllable duration, temperature, sympatry, and pronotum length treated as fixed effects with a logit link function.

To ask if male calls within species differed between allopatric and sympatric population. I used a model comparison approach to specifically ask what call features best predicted the population type a call came from. Only continuous syllable bouts of at least ten seconds were analyzed to estimate each individual's call. I averaged the call features described in Tables 4.1 and 4.3 for each individual at each temperature. I analyzed *O. oreoeca's* four-pulse calls since it was by far the most common call type I recorded. Individual size (pronotum length in mm) was excluded from my population analysis after an initial linear regression showed a weak relationship between pronotum length and syllable duration/temperature within each species: *O. oreoeca* ($F=4.36$, $df=39$, $p=0.04$, $R^2=0.08$), *O. brevihastata* ($F=3.46$, $df=60$, $p=0.07$, $R^2=0.04$). This suggests that within each species, when I control for temperature, individual body size has only a relatively small effect on calling rate.

For *O. oreoeca*, I controlled for the effect of temperature by dividing each temporal component of the call by the syllable duration, thus I was using the relative proportions of pulse durations and interval durations, or the proportion of the syllable duration filled by each temporal component. All of the resulting proportions of syllable durations were uncorrelated with temperature with the highest of the eight R^2 values being 0.06. Relative amplitudes (RA) were also not significantly affected by temperature, but they were significantly correlated with each other: RA1-RA2 ($R^{2=}$0.81, F=170.50, df=39, p=0.00), RA1-RA3 ($R^{2=}$0.62, F=66.09, df=39, p=0.00), and RA2-RA3 ($R^{2=}$0.68, F=87.29, df=38, p=0.00). Thus, I only included the relative amplitude of the last two pulses (RA3). I used a GLMM to predict the population a call was from with individual treated as a random effect and P1/S, P2/S, P3/S, P4/S, I1/S, I2/S, I3/S, I4/S, and RA3 treated as fixed effects with a logit link function.

For *O. brevihastata*, I again controlled for the effect of temperature by dividing each temporal component of the call by syllable duration. All of the resulting proportions of syllable durations were uncorrelated with temperature with the highest of the six R^2 values being 0.08. The two relative amplitudes RA1 and RA2 were uncorrelated (RA1-RA2, $R^{2=}$0.00, F=0.76, df=60, p=0.38), so I included both in the full model. I used a GLMM to predict the population a call was from with individual treated as a random effect and P1/S, P2/S, P3/S, I1/S, I2/S, I3/S, RA1, and RA2 treated as fixed effects with a logit link function.

I tested for character displacement in syllable duration between the species. Syllable duration is the only qualitatively comparable feature between species. I log transformed syllable duration to normalize this variable. I used a GLMM to predict the log of syllable duration with individual treated as a random effect and species, sympatry, and temperature treated as fixed effects. I included the interactive effects of species and sympatry as well as temperature and sympatry with an identity link function.

I additionally tested for character displacement using a parametric test. I controlled for temperature and eliminated repeated measures as follows. I used the best model for each species, predicting log of syllable duration treating temperature as a fixed effect and individual as a random effect with an identity link function. Using the species-specific coefficients for temperature, I then standardized each recorded call duration to 25°C. I calculated the average of standardized log of syllable durations for each individual from these calls. I performed an analysis of variance (ANOVA), testing if the four populations (allopatric *O. oreoeca*, sympatric *O. oreoeca*, sympatric *O. brevihastata*, and allopatric *O. brevihastata*) differed in their standardized log of syllable durations, and a Tukey HSD post hoc when significance was found.

Female phonotactic response protocol

I played females synthetic calls from a pair of Motorola KSN1218C loudspeakers mounted on a horizontal plane at opposite ends of a temperature regulated (25.5±1.0° C) chamber (182.9 x 121.9 x 61.0 cm). The chamber was illuminated by red light. A custom designed amplifier/attenuator ran LABVIEW7 EXPRESS® software to play calls. I adjusted signal amplitude to 86 dB peak SPL and used a G.R.A.S. ® SPL Transducer Type 21SB with 1/2" condenser microphone before each round of tests to orient the speakers in the horizontal plane and directly toward the center of the arena. I wrapped the chamber in black fabric, which hid contours and speakers. Speakers were centered on the short wall of the chamber five cm above the double-layered foam. White tape was also used to create a 20 cm radius around each speaker at ground level and on the wall around the speaker. A 6.4 cm diameter by 7.6 cm tall cylinder with a mechanical string overhead pulley housed the animals on the center "X" during the acclimation period. I performed no-choice experiments, but to insure females were not simply attracted to speaker noise, one speaker on the opposite side of the chamber from the experimental treatment

speaker was simultaneously playing the call with negligible amplitude. I randomized the speaker in which the experimental versus control call was played between trials.

For playbacks, females were housed in the center of the chamber in the cylindrical wire-mesh cage. I used a two-minute acclimation period before manually releasing the female from the cage with a mechanical pulley. A phonotactic response was scored if females entered a 20cm radius of the speaker on either the floor or the wall. Trials were ended after a ten-minute period, so the females had eight minutes to respond after the initial acclimation period. Preliminary trials showed that most females responded within this time frame or buried in the sound foam for an extended period of time. To increase the power of my small sample of females, each female went through two trials of each stimulus. I randomized the order of stimuli in each trial since previous acoustic experience is known to influence female mate-choice preferences in Orthopterans (Bailey and Zuk 2008). I treated individual and trial as random effects in my analyses.

Female responses to call elements

To test which signal elements were important for female *O. oreoeca* responses in the laboratory I measured the binomial responses of females to two artificial calls composed of different signal elements in randomized no-choice tests. I used allopatric *O. oreoeca* females (n=6). I generated synthetic calls (Fig. 4.4, A-C), from custom designed SONGX software (16-bit resolution, 250 kHz sampling rate) (Deily and Schul 2004). I used frequency filtered (17kHz-40kHz) noise produced in ADOBE COOL EDIT. Pulse envelopes were trapezoid shaped with long rises and shorter falls mimicking the natural call. I first produced a full synthetic call that mimicked the natural call of *O. oreoeca* (Fig. 4.4, A). To make this call five *O. oreoeca* males from the allopatric region were recorded at 25.5 ± 1 °C. I used the mean duration of each pulse, the duration of intervals between, and the relative amplitudes of the pulses to create a synthetic call with the same temporal

properties and relative amplitudes as an average natural call syllable at experimental lab temperature (P1=0.083s, P2=0.089s, P3=0.102, P4=0.181, P5=0.033, I1=0.031, I2=0.034, I3=0.038, I4=0.076, I5=0.188, RA1=0.18, RA2=0.20, RA3=0.25, RA4=0.30, syllable duration=0.855). The second call contained only the low amplitude element (LAE) of this call and the third call contained only the high amplitude element (HAE) (Fig. 4.4, B&C). I concatenated a string of syllables into a continuous ten-minute long trill for all three calls.

I used a GLMM to predict the responses of females with individual and trial number treated as random effects and LAE, HAE, the interaction of LAE with HAE, and temperature as fixed effects with a logit link function.

Female responses to heterospecific calls

I tested how a female's population (sympatric or allopatric) affected the probability that she responded to heterospecific calls. I measured the binary responses of both populations of female *O. oreoeca* to both heterospecific and conspecific synthetic calls in randomized no-choice trials (Fig. 4.5). I used *O. oreoeca* females from two locations: allopatric females (n=5) and sympatric females (n=8). I used the normal, male *O. oreoeca* call from the previous experiment. To create a synthetic heterospecific male call, I recorded five *O. brevihastata* from the sympatric region and averaged their syllable parameters again in SONGX (P1=0.302s, P2=0.008s, P3=0.019s, I1=0.140s, I2=0.052s, I3=0.261s, RA1=0.14, RA2=0.78, syllable duration=0.782s).

I used a GLMM to predict females' responses with individual and trial treated as random effects and call, sympatry, the interaction between call and sympatry, and temperature as fixed effects with a logit link function.

Results

Male advertisement calls

Each wing closing movement produces a pulse, and within each pulse are impulses, that are the impacts of a sharp edged scraper on the right wing against the teeth of a file on the left (Dumortier 1963; Bailey 1970). I annotate two distinct types of pulses: low amplitude and high amplitude (Fig. 4.2). Depending on the species, specific combinations of these two pulse types are combined into syllables, which repeat as a continuous trill. For both species, the pulses have broadband frequencies (16-45 kHz) with peak energy at 27 kHz.

O. oreoeca syllables vary from two to six, but typical syllables contain four or five total pulses (Fig. 4.2, A&B). The final pulse in a syllable is always a stereotyped high amplitude pulse. The impulse periods and amplitude of impulses gradually increases from beginning to end of each pulse. *O. brevihastata* always produces a three-pulse call: one pulse similar to *O. oreoeca's* numerically variable pulses, and two consecutive high amplitude pulses (Fig. 4.2, C). Like *O. oreoeca*, *O. brevihastata's* impulse periods and amplitude of impulses gradually increases from beginning to end of each pulse. While I occasionally observed both species producing only the high amplitude pulse portion of the call, I always observed full calls preceding mating events in the laboratory.

The average syllable duration for *O. oreoeca* was 734 ms when making a four-pulse syllable and 789 ms when making a five-pulse syllable. For both call types, each low amplitude pulse increased in duration from the previous. Average pulse durations were 82, 91, 170 and 22 ms for the four-pulse call, and 69, 77, 94, 154 and 24 ms for the five-pulse call. The low amplitude pulses ranged in average amplitude between 12 and 25 percent the intensity of the high amplitude pulse. The low amplitude pulses' impulse periods averaged between 2.17 ms and 3.40

ms. The high amplitude pulse's impulse period fell within the range of low amplitude pulse interval values for both four-pulse (Fig. 4.3) and five-pulse syllables.

The average syllable duration for *O. brevihastata* was 792 ms. The average pulse durations are 335, 11 and 24 ms. The impulse period in the low amplitude pulse averaged 8.03 ms while the high amplitude pulse impulse periods averaged 4.69 and 4.20 ms. These impulse periods were long compared to the observed maximum average impulse period for any pulse in *O. oreoeca* being 3.40 ms. Overall *O. brevihastata* has longer duration pulses in high amplitude pulses compared to *O. oreoeca* (Fig. 4.3). The first and second pulse amplitudes averaged 23 percent and 53 percent the intensity of the final pulse. The average low amplitude pulse fell within the intensity range of *O. oreoeca's* low amplitude pulses, but the first high amplitude pulse averaged double the relative amplitude of *O. oreoeca's* low amplitude pulses.

I tested whether mean impulse periods were proportional to mean pulse duration, which would indicate the number of teeth impacts per pulse were fixed. Mean impulse periods and mean pulse duration were not significantly correlated in either *O. oreoeca* (P1, R^2=0.23, F=0.43, df=2, p=0.58), (P2, R^2=0.02, F=0.93, df=2, p=0.44), (P3, R^2=0.31, F=2.33, df=2, p=0.27), (P4, R^2=0.25, F=1.98, df=2, p=0.29), or *O. brevihastata* (P1, R^2=0.44, F=0.07, df=2, p=0.81), (P2, R^2=0.32, F=0.28, df=2, p=0.64), (P3, R^2=0.49, F=0.02, df=2, p=0.90). This suggests that the number of tooth impacts in pulses varies.

The best model predicting the probability of *O. oreoeca* producing syllables with five-pulse versus four-pulse calls was simply syllable duration (Table 4.4). Temperature, sympatry, and size (pronotum length in mm) have negligible effects by comparison, although temperature is strongly negatively correlated with syllable duration ($R^{2=}$0.48, F=37.83, df=39, $p < 0.01$). *O. oreoeca* produce more pulses per syllable when syllable durations increase (Fig. 4.6), and as temperature decreases.

For *O. oreoeca* the best model predicting whether a call came from a sympatric or allopatric population was the null model with an Akaike weight of 1.00 (Table 4.5). Thus, there was no evidence for any of the factors improving how well I could predict what population a call came from. These results strongly support that there has been no differentiation in relative temporal or amplitude features between allopatric and sympatric populations of *O. oreoeca*.

For *O. brevihastata* the best model predicting whether a call came from a sympatric or allopatric population included I3/S and RA1 (Table 4.6). I3/S, which represents the relative duration of the interval between the final high amplitude pulse and the first low amplitude pulse between any two syllables, appeared in nearly every supported model, and the sum of Akaike weights for I3/S was 0.96. This can be interpreted as the probablility of the best model including I3/S as 0.96. Sympatric populations have larger gaps between syllables. RA1 and P3/S were also moderately supported as factors that aid in predicting what population a *O. brevihastata* came from (Table 4.6). Sympatric populations had lower RA1 and lower P3/S values.

I found evidence for character displacement in syllable duration for the two species. The best model for predicting syllable duration included interactive effects of species with population (sympatric versus allopatric), and the interactive effect of and species with temperature. It also included the factor temperature. No other models received a ΔAICc < 7. Evidence for character displacement is given by the inclusion of the species term interacting with population term in the one and only supported model. Both *O. oreoeca* and *O. brevihastata* have lower syllable durations in sympatry, but *O. oreoeca's* decrease is orders of magnitude greater. Temperature decreases syllable duration. *O. brevihastata's* syllable duration is similar in sympatry and allopatry. *O. oreoeca* in allopatry has the highest syllable duration, and in sympatry has the lowest of all four populations (Fig 4.6).

I also found evidence that character displacement has occurred between the species with the main effect being the sympatric *O. oreoeca* population decreasing its syllable duration compared to the other three populations. Differences in temperature controlled log syllable durations between the four populations were highly significant ($F=27.57$, $df=3$, $p<0.001$). A Tukey HSD post hoc test reveals a significant difference between sympatric *O. oreoeca* with all three of the other populations at significance levels of $p<0.001$ (Fig. 4.9).

Female responses to call elements

Female *O. oreoeca* responded nearly as often to high amplitude pulse element (HAE) as they do to a full call in the laboratory (Fig. 4.9), and they never responded to only the low amplitude pulse element (LAE) of the male call. The best model included only the variable HAE (Table 4.8) and had an Akaike weight of 1.00. This suggests that the HAE is fully supported as the most crucial factor eliciting a phonotactic response. However, the probability of response increased from 0.5 with only the HAE to 0.75 with the full call, strongly suggesting that the full call is more attractive.

Female responses to heterospecific calls

Females from the sympatric population were less likely to respond to the heterospecific calls than the allopatric females. The best model included call, sympatry, and the interaction of the two, and was supported by an Akaike weight of 0.48 (Table 4.9). The large negative effect of the interactive factor shows that sympatric individuals responded less to heterospecific calls than did allopatric individuals (Fig. 4.10). Females from the sympatric population had a 0.69 probability

of response to the conspecific call and a 0.13 probability of response to the heterospecific call. By comparison, allopatric females had a 0.60 probability of response to the heterospecific call.

Discussion

I found no differences in the relative proportions of call features or relative amplitudes of call features between allopatry and sympatry in *O. oreoeca* male calls (Table 4.5). However, the sympatric population does have shorter syllable durations compared to the allopatric population and either of the *O. brevihastata* populations (Fig. 4.7, 4.8). While the rate of calling differs between *O. oreoeca* populations, there was no indication that the relative proportions of each feature within syllables were shifted. This result is consistent with the hypothesis character displacement in syllable duration has occurred, and *O. oreoeca* shows a decrease between allopatric populations and sympatric populations than do *O. brevihastata*. One study reports that in multiple contact zones that the rarer species consistently shifted their call (Lemmon 2009), and in my experience *O. oreoeca's* relative abundance in the sympatric region is consistent with this result; I have collected over 30 *O. brevihastata* individuals from multiple sympatric locations in a single season, but have never collected this many *O. oreoeca* from a single location. Unlike *O. oreoeca* the sympatric *O. brevihastata* populations differ in several temporal call features, with the most support for differences in IS/3 (Table 4.6). The sympatric *O. brevihastata* have higher I3/S, but lower RA1 and P3/S than the allopatric population.

The impulse periods within all pulses for *O. oreoeca* and *O. brevihastata* are generally very short in duration. The exception is that *O. brevihastata* has an observably longer impulse period in the P1 pulse. While my results suggest that females use high amplitude pulses to find mates (Fig. 4.9), there may be biological significance in this slow impulse period as females evaluate males more proximately. The average impulse period for P1 in *O. brevihastata* was

8.30ms whereas all other pulses were approximately 5ms. It is possible that *Obolopteryx* are physiologically capable of discrimination between the individual impulses for *O. brevihastata's* P1 at either the sensory neuron or interneuron processing level (Schul 1994; Schul 1997). Alternatively they may process all pulses at the maximum firing rate of their neurons.

The high amplitude pulse element (HAE) of *O. oreoeca's* calls is important factor for eliciting a phonotactic response from females in this species (Table 4.7), and I was unable to elicit a phonotactic response from the low amplitude element (LAE) alone. I was also unable to detect a difference in females' responses to the call with only the HAE compared to the full call with both elements using model selection, however there is an obvious increase in response to the full call from the HAE alone (Fig. 4.9). In nature, I observe that males always call with the HAE, but sometimes drop the LAE. However in long calling bouts with multiple males I rarely observe the HAE alone. It is possible that males are energetically constrained and cannot produce the full call over an entire evening, and are conserving energy regarding its use. Estimates of katydid energetic calling efficiency is higher than many insects, but cost estimates vary greatly between species (Bailey et al. 1993). In other duetting phaneropterines, males wait for information that females are proximate, then increase their chirp rate significantly after a female responds to a trigger pulse (Hartley et al. 1974).

Females from the sympatric population of *O. oreoeca* responded substantially less to the heterospecific call than the conspecific call (Fig. 4.10). While call and population alone both affected responses, the top model (ΔAICc weight of 0.48) included the interaction between call and population. This supported my hypothesis that females in the sympatric *O. oreoeca* population have evolved to be choosier, and my prediction that they would decrease their responsiveness to heterospecific calls compared to the allopatric population. Increased female discrimination in sympatry has been found in other wild populations (Coyne and Orr 1989; Rundle and Schluter 1998), and my results broaden the list of taxa in which this phenomenon is

observed. Interestingly in *Drosophila* a single locus mutation can result in this type of instant female discriminatory behavior against heterospecific male calls (Doi et al. 2001). Other *Drosophila* species also have populations that have a gradient of reproductive discrimination against heterospecific populations (Bewick and Dyer 2014). Since the probability of sympatric *O. oreoeca* females is greater than zero, I suspect that reproductive discrimination in *Obolopteryx* is more of a gradient than, than the type of absolute discriminatory behavior found in *Drosophila*. Sampling additional populations at various depths into contact zones could provide additional insight into this pattern.

Although sympatric female *O. oreoeca* show distinct differences in phonotactic responses to heterospecific calls, the sympatric males only differ by character displacement in the form of shorter syllable durations. Further behavioral experiments could determine whether females from the two populations have call duration preferences, but my data allow no inferences to this question. Other studies have found that male calls sometimes evolve in the absence of female preferences (Bush and Schul 2010). Sympatric female *O. oreoeca* discriminate against heterospecific calls but it seems that call duration is not the most likely features females use to discriminate, partially because of overlapping variation in both species (Fig. 4.7), but also since many other qualitative features differ between the species calls (Fig 4.2, A-C).

Amplitude is very important to mate-choice preferences (Gerhardt and Doherty 1988; Forrest and Raspet 1994; Schul and Fritsch 1999). My data suggest that *O. oreoeca* females rely heavily on the HAE for initially locating males, at least at the acclimation amplitude (96 dB SPL) for my setup. Amplitude was held constant from the speaker, but from the females' perspective it changed exponentially as a function of distance. They probably reached a critical amplitude threshold as they walked toward calls at which the LAE became important, and the female discrimination experiments suggest that females are indeed using this part of the call to make choices. While the HAE is important for locating males I am limited in the conclusions I can

make beyond that. Experiments performed with a walking compensator, which holds the female at a fixed distance from a speaker regardless of her rate of speed, would likely exaggerate the difference in response between LAE and the full call (probability of .50 versus .75) (Fig. 4.9). Increasing the samples size or elimination of the random variables would also likely result in statistical significance between the HAE and the full call. Females may also rely on other cues to evaluate males once they are located such as visual or olfactory cues (Otte 1977; Loher and Dambach 1989), or some combination of acoustic calls and other signals (Hebets and Papaj 2005).

This population difference in female discrimination provides circumstantial evidence that heterospecific competition may be influencing the evolution of female discrimination in this population. Amplified fragment length polymorphism (AFLP) data suggest population differentiation between the sympatric and allopatric *O. oreoeca* populations (chapter 3). Taken together, these data suggest that the two populations are in the early stages of divergence from one another. The allopatric population of the Chisos Mountains of Big Bend National Park is separated from the sympatric population of the Davis Mountains by at least 45 miles of low elevation, arid terrain. To my knowledge, no one has collected this species between my two sampling sites, and this space probably acts as a gene flow barrier. Female choice is possibly under different selection pressure on the two islands. The ancestral female discriminatory behavior is unknown, but it is possible that either the Chisos Mountains population has been released from selection pressure and they have evolved to be indiscriminant, or that the Davis Mountains population has evolved to be more discriminant. The Davis Mountains population is at the periphery of the species distribution, so I find it more plausible that this population has become isolated and discriminatory behavior has increased due to heterospecific competition for mates. While it is interesting that females in sympatry discriminate strongly it is potentially just as interesting that females in allopatry do not discriminate. The calls of both species are

qualitatively different and differ in the number, duration, and relative amplitudes of of pulses. The two female phonotaxis experiments, taken together, suggest that the HAE is important for locating mates, but the LAE increases attractiveness of calls and the LAE is the obvious part of the call that females are using to discriminate between the two species' calls. I pose heterospecific competition as a strong selective force on the sympatric females' discriminatory behavior.

O. oreoeca and *O. brevihastata* are closely related, but they are not sister species. Morphological data (Cohn et al. 2014) and molecular data (chapter 2) provide conflicting accounts of the phylogenetic relatedness. Many species in the genus are closely related enough that it is difficult to tell them apart from morphology. The traits most consistently diagnostic of species are in male genitalia (Rehn and Hebard 1914). My phylogeny based on two mitochondrial genes suggest that the common ancestor of these two species diverged at least long enough ago that the separate species experienced range changes due to Pleistocene glacial recessions (Hewitt 1996). These glacial periods combined with the other known complications of sky island biogeography (Masta 2000; Knowles and Alvarado-Serrano 2010) make it difficult to infer historical distributions of any of the species in the *Obolopteryx* genus. The current discriminatory behavior of the sympatric *O. oreoeca* females most likely represents an example of reinforcement. They have probably come into secondary contact, and have evolved this discriminatory behavior due to the associated costs of heterospecific mating. Females in the allopatric population are free from these costs and this selection pressure. It has been suggested that peripheral isolation or neighboring populations combined with heterospecific competition can cause reproductive isolation between the populations (Pfennig and Rice 2014), and these populations appear to be on the cusp of reproductive isolation.

While the allopatric *O. brevihastata* population interestingly has differences in several call features. I found no genetic differences between these populations from AFLPs (chapter 3). It's not clear how these differences relate to *O. oreoeca*, but the *Obolopteryx* genus is a mosaic of

overlapping species distributions. The allopatric population of *O. brevihastata* lies at the edge of the range of a third species, *O. castanea*. It is possible that the allopatric *O. brevihastata* are being influenced by heterospecific competition from this third species.

Taken together, the genetic data (chapter 3) and the behavior data presented here provide an interesting case study in which two sky island populations have diverged genetically and behaviorally from one another. Although I only compared these two populations in the only known sympatric population of these two species, evidence suggests that heterospecific competition has caused a peripheral sky island population's females to evolve strong discrimination against heterospecific matings. This is a unique example of a reproductive isolating mechanism in females, occurring in a peripheral population, suggestive of reinforcement. I also found strong support for character displacement in syllable duration of male calls between these two populations, but further experimentation is needed to understand how females in each population respond to different syllable durations.

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Figure Captions

Figure 4.1. The satellite image on the right illustrates the collection locations of all males and females (Texas, USA). Locations A and B both lie within the sympatric region, whereas C and D are located within the allopatric regions for *O. brevihastata* and *O. oreoeca*, respectively.

Figure 4.2. Continuous two-second trills of *O. oreoeca* and *O. brevihastata*. Panel A and B are *O. oreoeca's* four and five-pulse calls, respectively, recorded from the same individual at 24.3°C. While individuals produce variable numbers of low amplitude, they always produce one high amplitude pulse in each syllable. Panel C is *O. brevihastata's* call recorded at 23.5°C. Their call is composed of one low amplitude pulse followed by two high amplitude pulses. Individual pulses are produced from a single wing closure and within pulses the vertical lines represent impulses (individual tooth impacts), many of which have such a small impulse period that they are difficult to distinguish from one another in this waveform. I use the following nomenclature to describe calls: the first low amplitude pulse in each syllable is named P1 and I1 follows P1, etc (e.g. Panel B). Peak amplitudes (PA) for each pulse are annotated and I used the ratio of these in my analyses.

Figure 4.3. Panel A and B are the expanded highest amplitude pulses of *O. oreoeca's* four-pulse call (Fig. 4.2, A) and *O. brevihastata's* call (Fig. 4.2, B) respectively. Shown are impulses (individual tooth impacts), at a magnified time scale. Impulse periods are shown as dark bars.

Figure 4.4. Waveform of a single syllable of each element used for the female phonotactic response to call elements experiment. Panel A is the full call, panel B is the high amplitude element (HAE), and panel C is the low amplitude element (LAE). Each of these syllables was repeated to fill a ten-minute loop of continuous calling.

Figure 4.5. Waveform of a single syllable of each call type used for the female heterospecific call discrimination experiment. Panel A is the normal *O. oreoeca* call (averaged from allopatric calls), panel B is the normal *O. brevihastata* call (averaged from sympatric calls. Each of these syllables was repeated to fill a ten-minute loop of continuous calling.

Figure 4.6. Probability of *O. oreoeca* producing a five-pulse versus a four-pulse syllable. The best model predicting probability of a five-pulse call contained only the variable syllable duration.

Figure 4.7. Character displacement in log syllable duration between *O. oreoeca* and *O. brevihastata* populations in sympatry. Syllable duration was log transformed for normality. Both populations of both species are shown to illustrate the interaction between sympatry and species. The best model applied to each of the four populations represents central tendencies.

Figure 4.8. Mean ± SE for temperature controlled values of log syllable duration standardized to 25°C for all four populations. Letters indicate significant differences from the Tukey HSD.

Figure 4.9. Mean binary responses ± SE of *O. oreoeca* females (n=6) to synthetic full calls, low amplitude pulses (LAE), and high amplitude pulses (HAE).

Figure 4.10. Mean binary responses ± SE of sympatric (n=8) and allopatric (n=5) *O. oreoeca* females to synthetic conspecific and heterospecific calls.

Table 4.1. Quantitative description of an *O. oreoeca* syllable for a four-pulse call. These calls were recorded between 24.1 and 24.3 °C. P=pulse, I=interval preceding pulse, RA=relative peak amplitude ratio.

Four-pulse call

	Mean ± SE	n
P1 duration (ms)	82.83 ± 4.03	4
P2 duration (ms)	91.25 ± 4.08	4
P3 duration (ms)	170.11 ± 5.04	4
P4 duration (ms)	22.25 ± 1.26	4
I1 duration (ms)	191.57 ± 10.79	4
I2 duration (ms)	35.67 ± 2.37	4
I3 duration (ms)	36.84 ± 1.85	4
I4 duration (ms)	99.69 ± 19.38	4
Syllable duration (ms)	734.66 ± 31.51	4
P1 impulse period (ms)	2.28 ± 0.44	4
P2 impulse period (ms)	2.40 ± 0.41	4
P3 impulse period (ms)	3.40 ± 0.26	4
P4 impulse period (ms)	2.72 ± 0.27	4
RA1 (P1 amplitude/P4 amplitude)	0.16 ± 0.01	4
RA2 (P2 amplitude/P4 amplitude)	0.18 ± 0.02	4
RA3 (P3 amplitude/P4 amplitude)	0.25 ± 0.02	4

Table 4.2. Quantitative description of an *O. oreoeca* syllable for a five-pulse call. These calls were recorded between 24.1 and 24.3 °C. P=pulse, I=interval preceding pulse, RA=relative peak amplitude ratio.

Five-pulse call

	Mean ± SE	n
P1 duration (ms)	69.16 ± 6.17	4
P2 duration (ms)	77.46 ± 1.11	4
P3 duration (ms)	94.13 ± 5.33	4
P4 duration (ms)	154.73 ± 11.09	4
P5 duration (ms)	24.22 ± 2.62	4
I1 duration (ms)	172.67 ± 6.43	4
I2 duration (ms)	35.41 ± 3.21	4
I3 duration (ms)	34.98 ± 2.25	4
I4 duration (ms)	38.42 ± 1.82	4
I5 duration (ms)	88.36 ± 10.29	4
Syllable duration (ms)	789.45 ± 19.40	4
P1 impulse period (ms)	2.17 ± 0.38	4
P2 impulse period (ms)	2.24 ± 0.33	4
P3 impulse period (ms)	2.47 ± 0.36	4
P4 impulse period (ms)	3.00 ± 0.34	4
P5 impulse period (ms)	2.66 ± 0.13	4
RA1 (P1 amplitude/P5 amplitude)	0.12 ± 0.01	4
RA2 (P2 amplitude/P5 amplitude)	0.13 ± 0.02	4
RA3 (P3 amplitude/P5 amplitude)	0.15 ± 0.03	4
RA4 (P4 amplitude/P5 amplitude)	0.21 ± 0.03	4

Table 4.3. Quantitative description of the stereotyped syllable of *O. brevihastata*. These calls were recorded between 23.3 and 23.5 °C. P=pulse, I=interval preceding pulse, RA=relative peak amplitude ratio.

Stereotyped three-pulse call

	Mean ± SE	n
P1 duration (ms)	335.18 ± 25.97	4
P2 duration (ms)	11.30 ± 1.33	4
P3 duration (ms)	24.35 ± 2.64	4
I1 duration (ms)	263.00 ± 18.39	4
I2 duration (ms)	112.69 ± 26.05	4
I3 duration (ms)	44.33 ± 1.53	4
Syllable duration (ms)	791.75 ± 9.86	4
P1 impulse period (ms)	8.03 ± 1.03	4
P2 impulse period (ms)	4.69 ± 0.19	4
P3 impulse period (ms)	4.20 ± 0.20	4
RA1 (P1 amplitude/P3 amplitude)	0.23 ± 0.05	4
RA2 (P2 amplitude/P3 amplitude)	0.53 ± 0.03	4

Table 4.4. AICc results for the probablility of *O. oreoeca*'s producing a five-pulse versus a four-pulse call. SD=syllable duration. The full model was probability of five-pulse syllable ~ temperature + pronotum length + population + syllable duration + (1|individual). The best model was probability of a five-pulse syllable ~ -12.28 + 0.01*syllable.

model	ΔAICc	df	AICc weight
SD	0.0	0	1.00

Table 4.5 AICc results for the probability an *O. oreoeca* call was from a sympatric population. The full model was population ~ P1/S + P2/S + P3/S + P4/S + I1/S + I2/S + I3/S + I4/S + RA3 + (1|individual).

model	ΔAICc	df	AICc weight
null model	0.0	2	1.00

Table 4.6. AICc results for the probability an *O. brevihastata* call was from a sympatric population. The full model was population ~ P1/S + P2/S + P3/S + I1/S + I2/S + I3/S + RA1 + RA2 + (1|individual). The best model was population ~ - 476.30 + 8192.00*I3/S - 160.70*RA1.

model	ΔAICc	df	AICc weight
I3S + RA1	0.0	4	0.26
I3S + P3S + I1S	0.1	5	0.19
I3S + P3S + P1S	0.8	5	0.16
I3S + P3S	0.9	4	0.12
I3S + I1S + I2S	1.0	5	0.07
I3S + I1S + P1S	1.2	5	0.07
I3S + P1S + I2S	2.8	5	0.06
null model	3.6	2	0.05

Table 4.7. AICc results for predicting the log of syllable duration. Character displacement is evidenced by interaction between species and population. SYM=sympatry, T=temperature, OREO=*O. oreoeca*, BREV=*O. brevihastata*. The full model was log (syllable duration) ~ species * SYM + species * T + (1|individual). The best model is log (syllable duration) ~ 8.19 - 0.07*T - 0.51*OREO + 0.02*T:OREO -0.01 * SYM:BREV - 0.19*SYM:OREO.

model	ΔAICc	df	AICc weight
SYM*Species + T*Species + T	0.0	5	1.00

Table 4.8. AICc results for *O. oreoeca* females' responses to calls elements (LAP=low amplitude pulse, HAP=high amplitude pulse). The full model was response ~ HAE * LAE + T + (1|individual) + (1|trial). The best model by AICc was response ~ -44.36 + 44.87*HAE (Table 4.8) and had an AICc weight of 1.00.

model	ΔAICc	df	AICc weight
HAE	0.0	4	1.00

Table 4.9. AICc results for two populations (sympatric/allopatric) *O. oreoeca* females' responses to conspecific and heterospecific calls. SYM=sympatry, T=temperature, HETERO=heterospecific call, CON=conspecific call. The full model was response ~ CALL * SYM + T + (1|individual) + (1|trial). The best model was response ~ 0.99 - 2.64*SYM:HETERO - 0.07*SYM - 0.52 * HETERO.

model	ΔAICc AICc	df	AICc weight
CALL * SYM	0.0	6	0.48
CALL + SYM	0.9	5	0.30
CALL	1.9	4	0.18
T	6.1	4	0.02
SYM	7.6	4	0.01
null model	8.4	3	0.00

Fig. 4.1

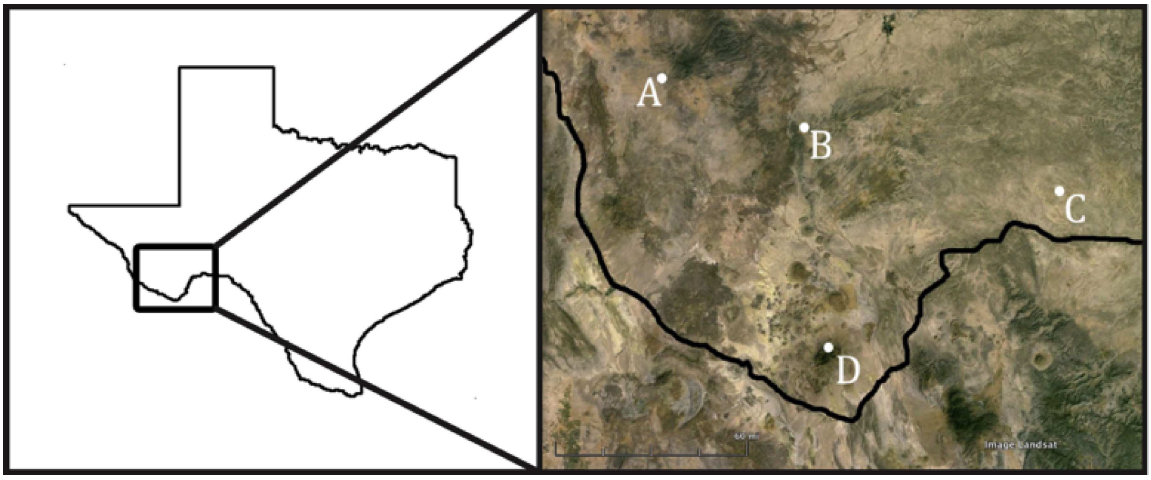


Fig. 4.2

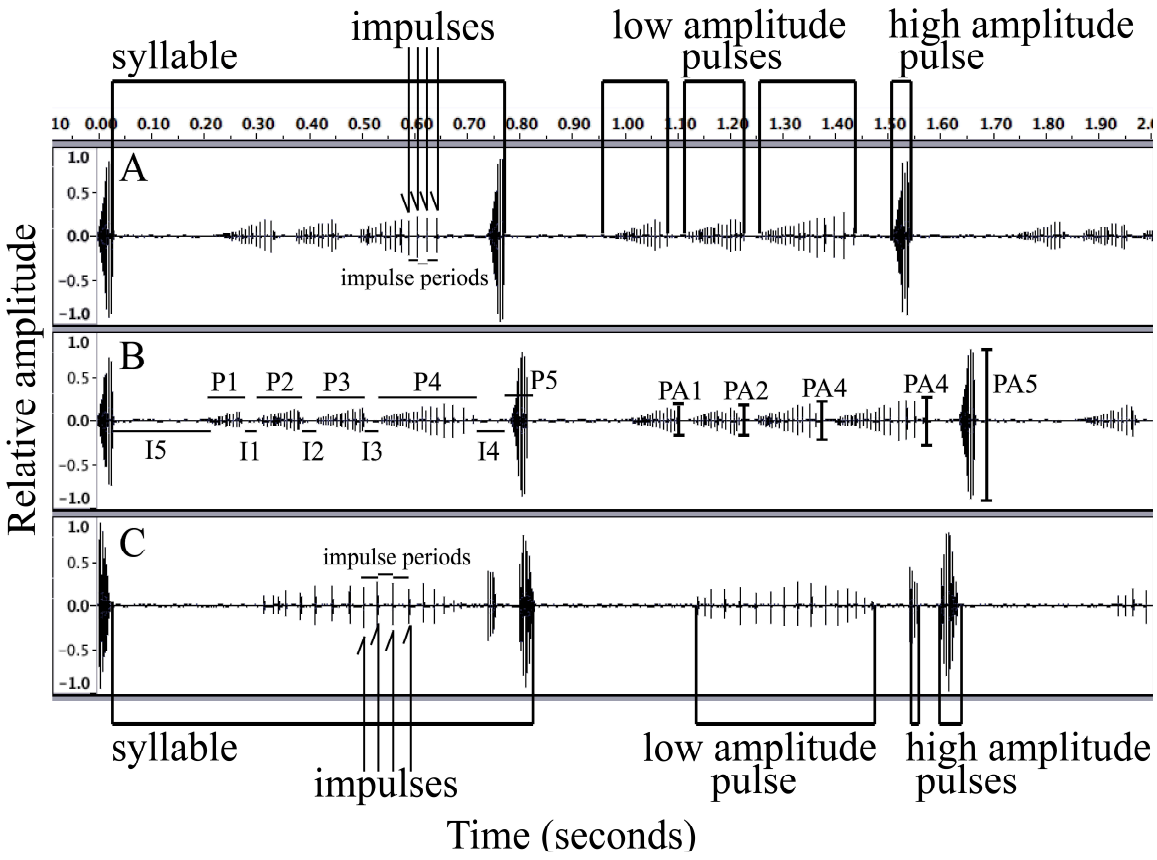

syllable
impulses
low amplitude pulses
high amplitude pulse
A
impulse periods
B
P1
P2
P3
P4
P5
PA1
PA2
PA4
PA4
PA5
I5
I1
I2
I3
I4
C
impulse periods
syllable
impulses
low amplitude pulse
high amplitude pulses
Relative amplitude
Time (seconds)

Fig. 4.3

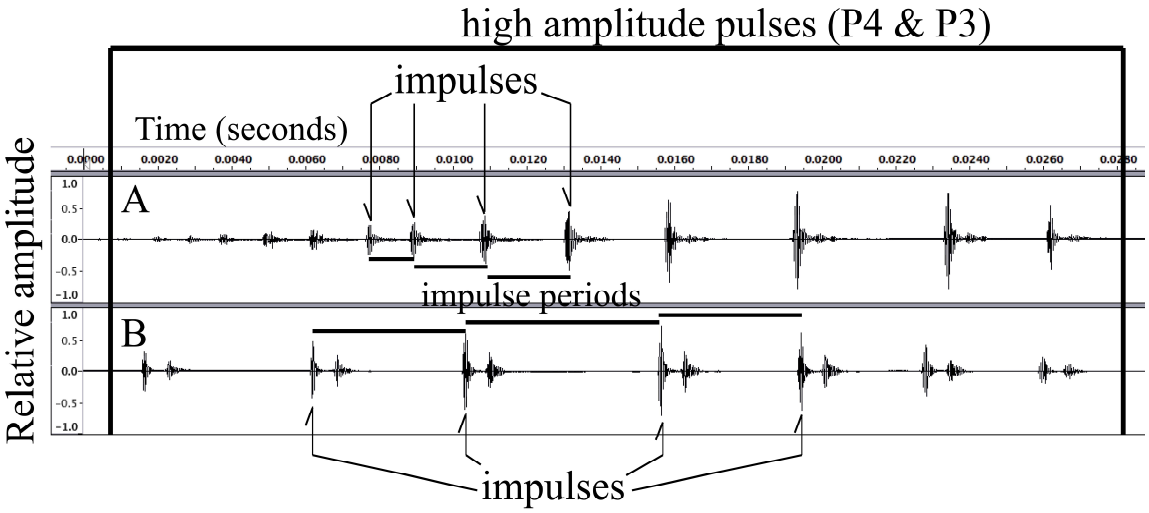

high amplitude pulses (P4 & P3)
impulses
Time (seconds)
0.0000
0.0020
0.0040
0.0060
0.0080
0.0100
0.0120
0.0140
0.0160
0.0180
0.0200
0.0220
0.0240
0.0260
0.0280
Relative amplitude
A
B
1.0
0.5
0.0
-0.5
-1.0
impulse periods
impulses

Fig. 4.4

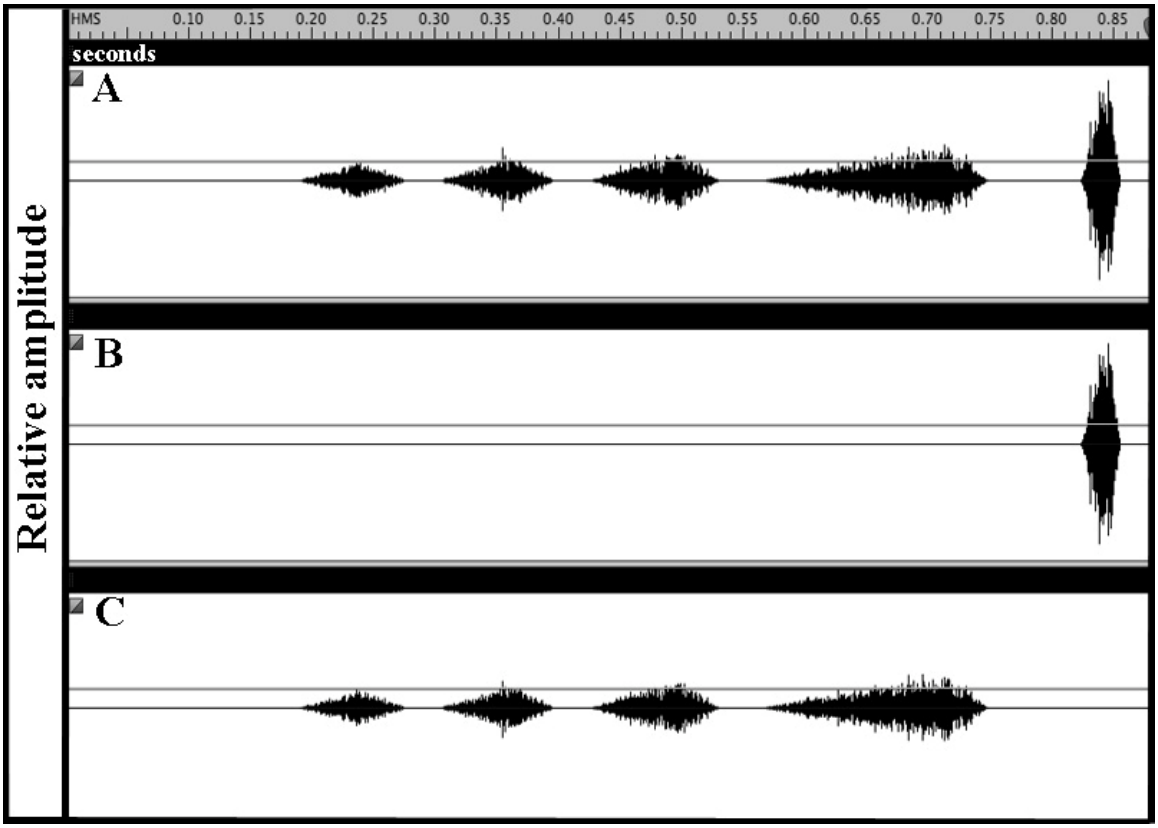

HMS
0.10
0.15
0.20
0.25
0.30
0.35
0.40
0.45
0.50
0.55
0.60
0.65
0.70
0.75
0.80
0.85
seconds
A
B
C
Relative amplitude

Fig. 4.5

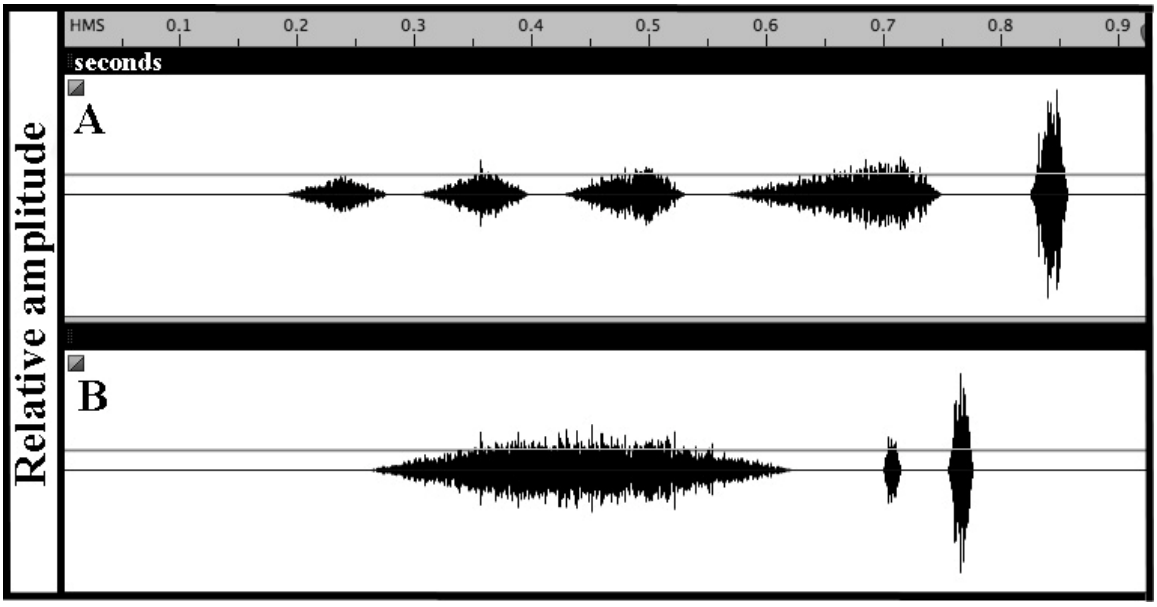


Fig. 4.6

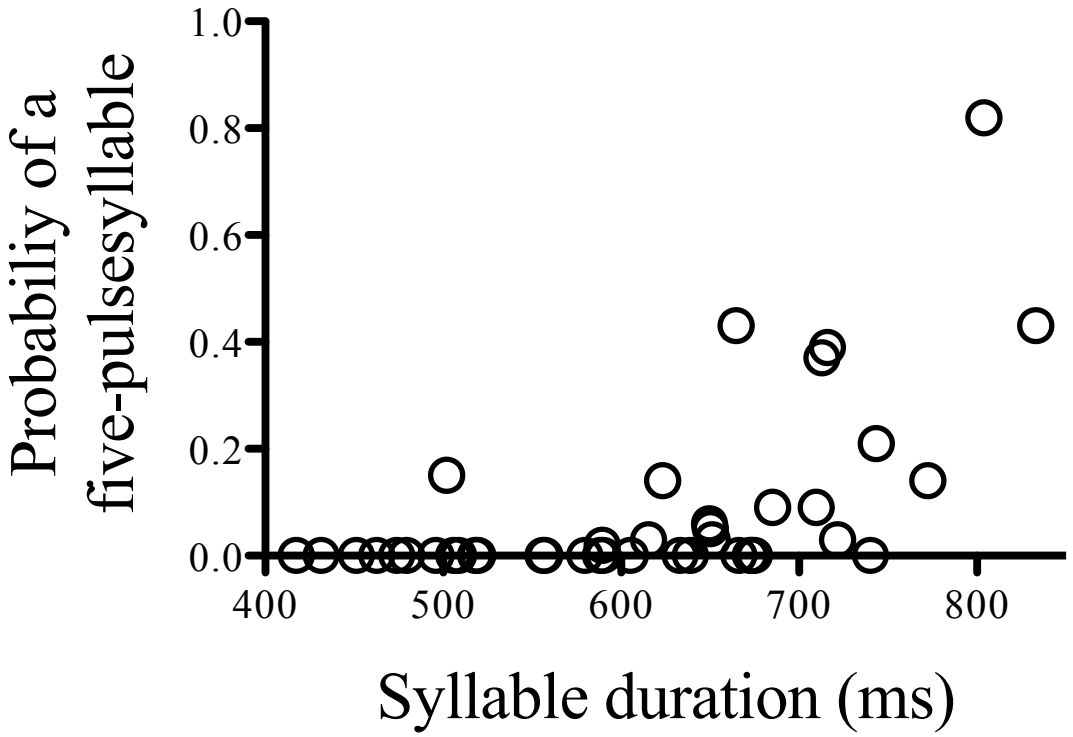

Probabiliy of a five-pulsesyllable
1.0
0.8
0.6
0.4
0.2
0.0
400
500
600
700
800
Syllable duration (ms)

Fig. 4.7

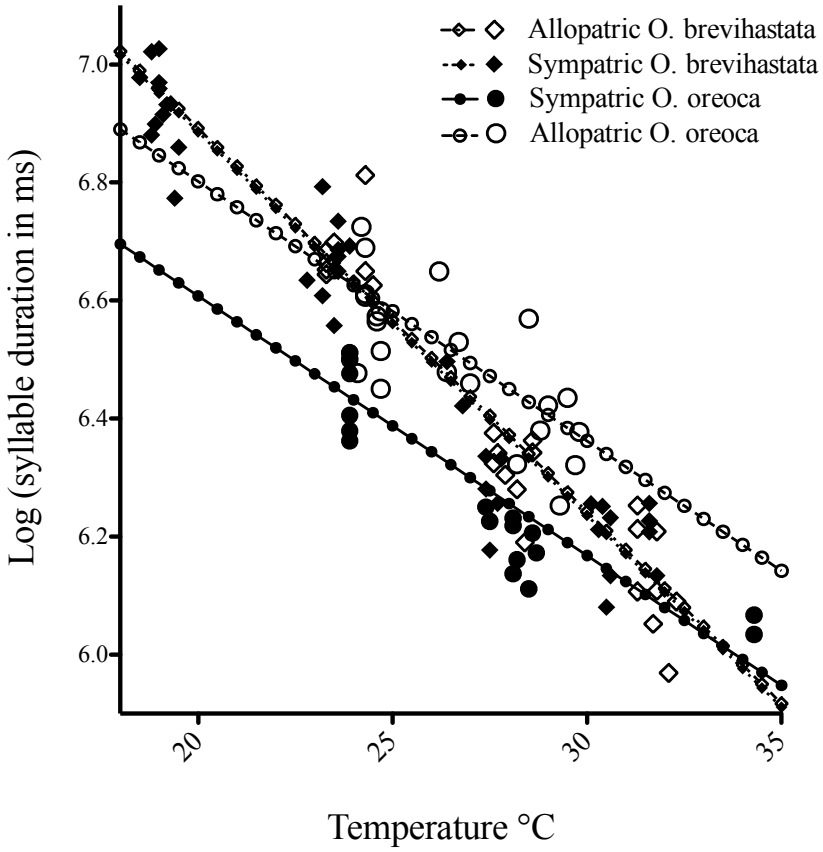

Allopatric O. brevihastata
Sympatric O. brevihastata
Sympatric O. oreoca
Allopatric O. oreoca
Log (syllable duration in ms)
7.0
6.8
6.6
6.4
6.2
6.0
20
25
30
35
Temperature °C

Fig. 4.8

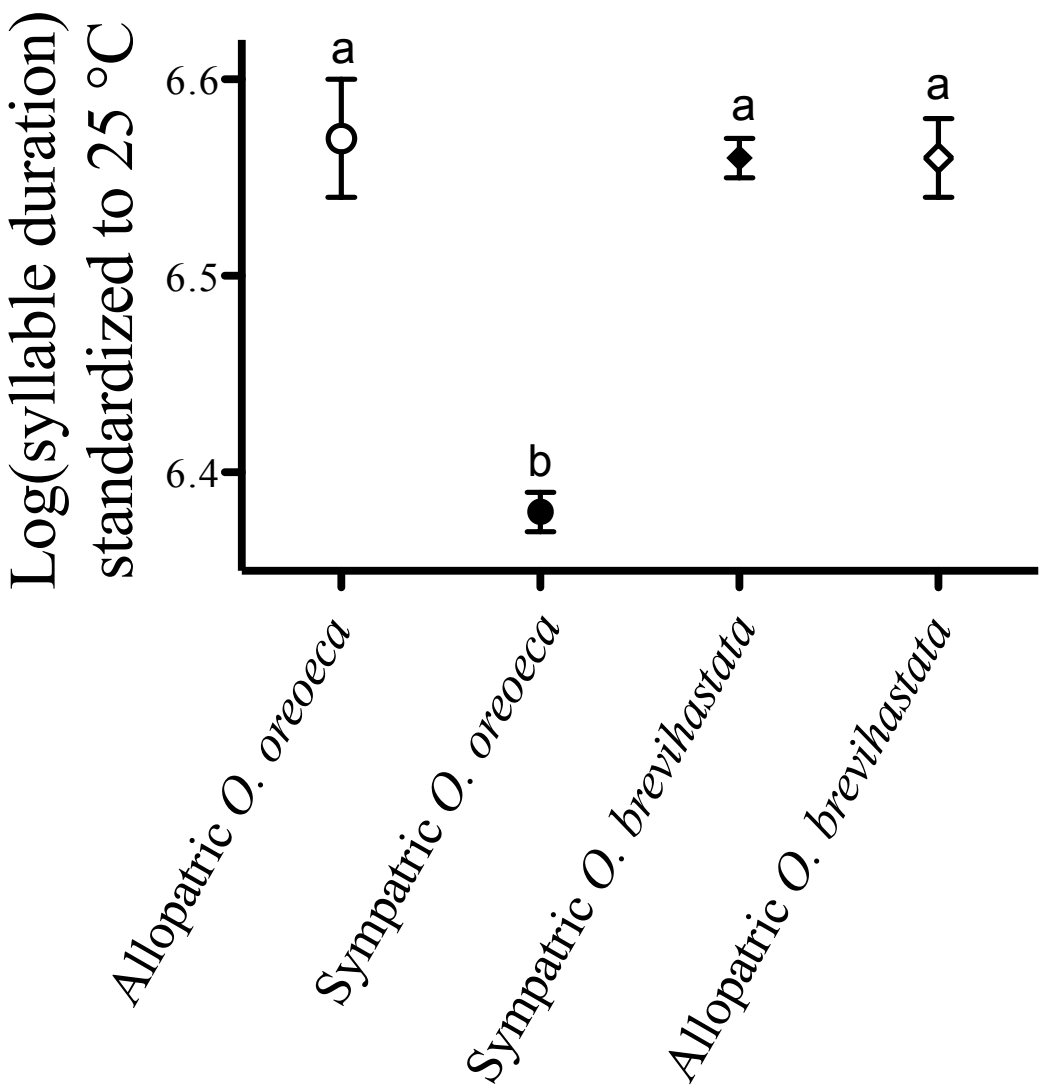

Log(syllable duration) standardized to 25 °C
6.6
6.5
6.4
a
b
a
a
Allopatric O. oreoeca
Sympatric O. oreoeca
Sympatric O. brevihastata
Allopatric O. brevihastata

Fig. 4.9

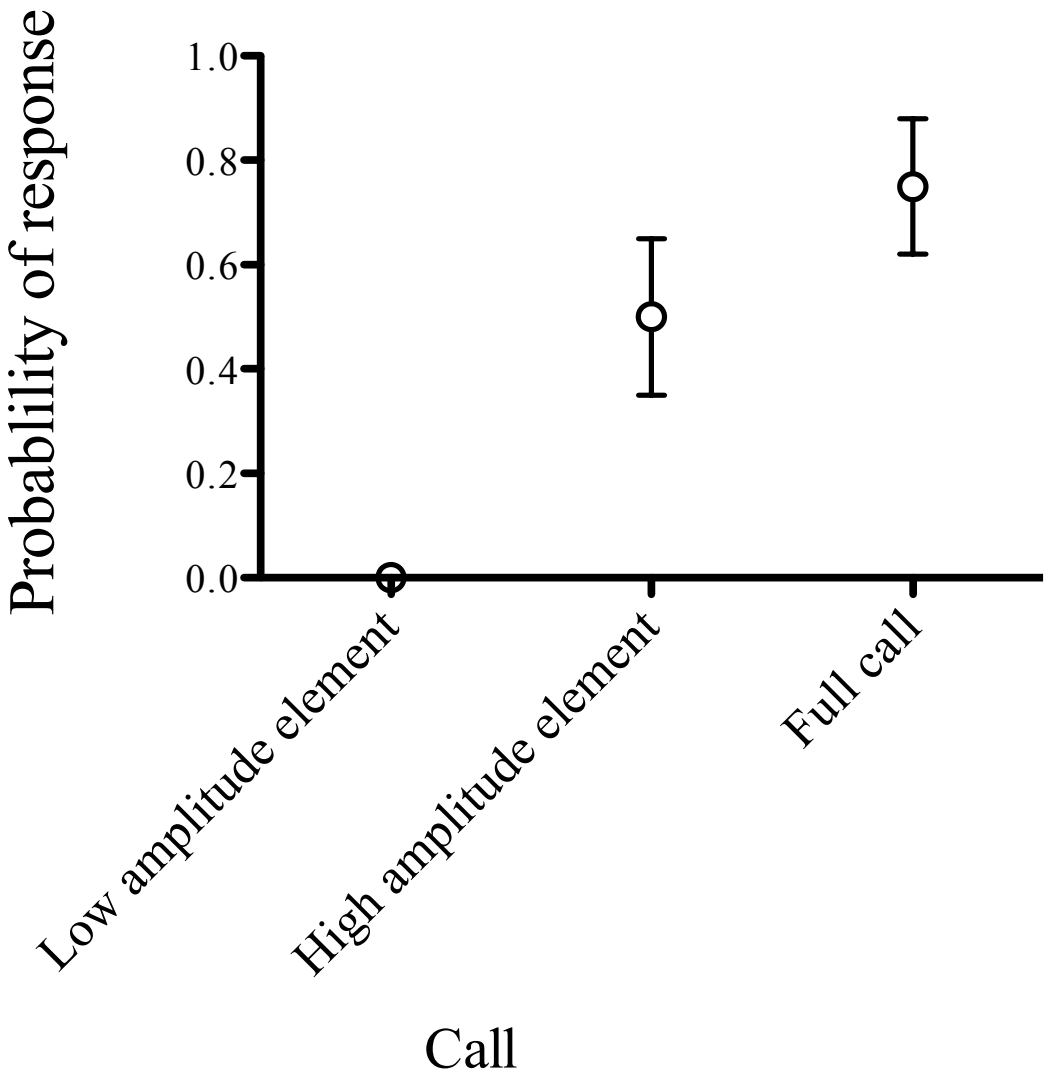
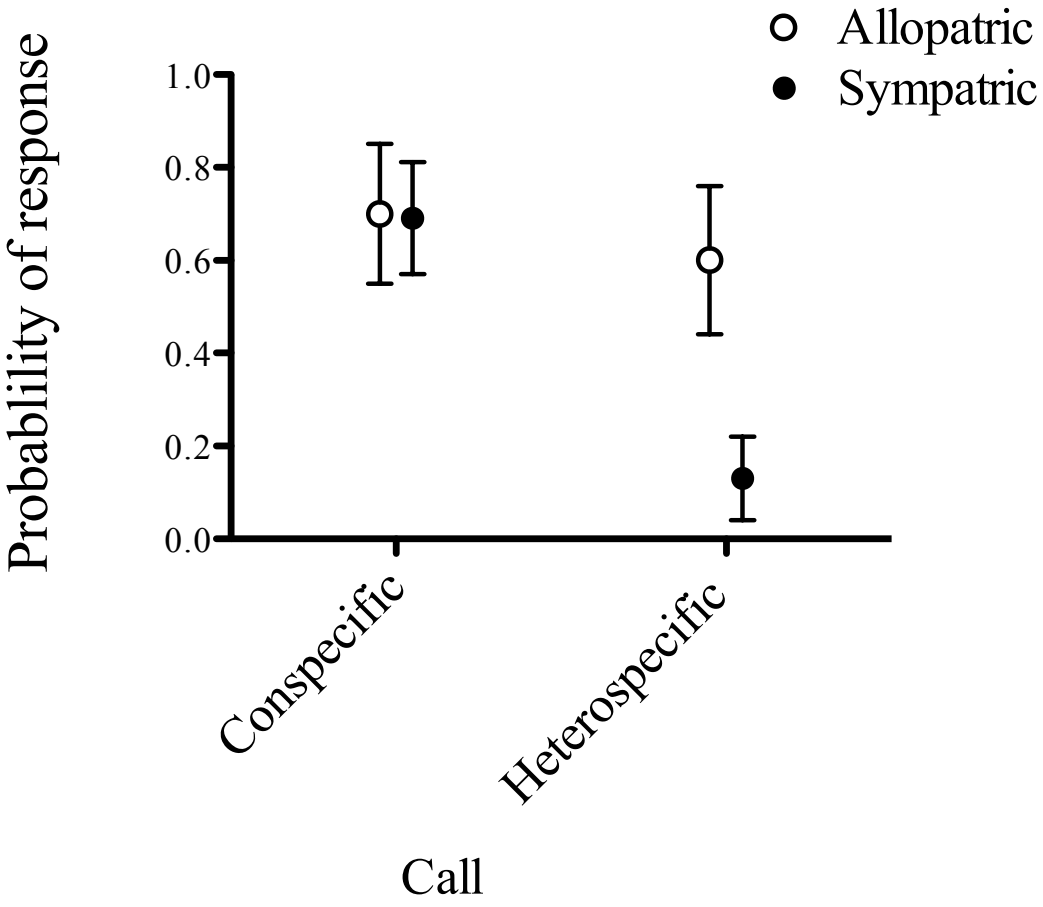

Probablility of response
1.0
0.8
0.6
0.4
0.2
0.0
Low amplitude element
High amplitude element
Full call
Call

Fig. 4.10


Allopatric
Sympatric
Probablility of response
1.0
0.8
0.6
0.4
0.2
0.0
Conspecific
Heterospecific
Call

CHAPTER V

CONCLUSIONS

This dissertation explored a reproductive isolating barrier between two relatively unstudied Chihuhuan desert katydids: *O. oreoeca* and *O. brevihastata*. I presented the first molecular phylogeny for Obolopteryx. I used population genetics tools to search for gene flow restrictions between two species in this genus that share a potential hybrid zone. I quantified the calls of both species and tested for population differences in their calls. I tested for character displacement in the only comparable call feature between species, syllable duration. Lastly I used playback experiments for *O. oreoeca* females to test what feature of the advertisement call is important for phonotaxis, and if females from the sympatric population respond less to heterospecific calls.

The molecular phylogeny presented in chapter two, based on two mitochondrial genes (COI and Cytb) provides the first hypothesis for the species history of the *Obolopteryx* genus. My main findings were that *O. oreoeca* and *O. brevihastata* are not supported as sister taxa, but are also not the most distantly related. Of the well-supported nodes in my tree, I found complete discordance with the previous hypotheses. Specifically, my phylogeny refuted the following pairs: *O. castanea* and *O. brevihastata*, *O. gladiator* and *O. emarginata*, and *O. oreoeca* and *O.*

catinata. This result is not surprising, since morphology and molecular characters often produce different trees. Also, previous researchers used only male genitalia as their primary characteristic, which is problematic because of potentially strong selection on that trait.

The AFLP results in chapter three showed that there is a restriction in gene flow restriction between the sympatric and allopatric populations of *O. oreoeca*. I found a significantly higher global F_{ST} for *O. oreoeca* than for *O. brevihastata,* and the pairwise F_{ST} permutation test revealed significant differences between *O. oreoeca* populations (p=0.019) but not between *O. brevihastata* populations. This combined with the structure results indicate that there is restriction of gene flow restriction between *O. oreoeca* populations, but not between sampled *O. brevihastata* populations. This result is most interesting because it implies that *O. oreoeca* are experiencing a gene flow restriction because of their biogeography on a sky island system.

In chapter four I explore male and female mate choice behavior. I analyzed male calls for both species in sympatry and allopatry. The relative duration of the interval between syllables in the calls of *O. brevihastata* have shifted between populations. I can't explain this pattern based on heterospecific competition with *O. oreoeca*, however the allopatric population of *O. brevihastata* are at the periphery of another potential hybrid zone with a third species, *O. castanea*. This third species may be a source of heterospecific competition for allopatric *O. brevihastata*. For *O. oreoeca*, I found no significant differences between the relative pulse durations, pulse interval durations, or relative amplitudes between allopatry and sympatry populations. However, I did find character displacement in syllable duration with the most dramatic effect being that sympatric *O. oreoeca* decreased its syllable duration compared to all other populations. It is unclear how females use syllable duration when choosing mates, but this is one way that assortative mating could emerge between the sky islands; females could develop preferences for their local call rate. Future experiments could shed light into the population preferences for syllable duration.

Female *O. oreoeca* respond to synthetic calls in the laboratory only when played the high amplitude element (HAE) in the male call. Adding the low amplitude element (LAE) had no statistical effect on responses, but it had the observable of effect of increasing the probability of response by 25%. I also found compelling evidence that sympatric *O. oreoeca* drastically reduced response to heterospecific calls compared to allopatric females. It is rare that learning affects female preferences in insects, so this difference in behavior is most likely genetic. It appears that females in sympatry with *O. brevihastata* have evolved stronger heterospecific discrimination. The current discriminatory behavior of the sympatric *O. oreoeca* females most likely represents an example of reinforcement. Since this is the most peripheral population of *O. oreoeca*, the two species have probably come into secondary contact, and this discriminatory behavior is due to the associated costs of heterospecific mating. Females in the allopatric population are free from these cost and this selection pressure.

Overall, the female discriminatory behavior documented taxonomically broadens our understanding of behavioral reproductive isolating mechanisms, and is one of few demonstrations of female insects evolving discriminatory behavior in a sympatric population. Demonstrating a cost of heterospecific mating would solidify this as an example of reinforcement. Interestingly, my data suggest that competition from non-sister taxa can still drive the evolution of female preferences. My AFLP data suggest that *O. oreoeca* females are genetically different between populations overall, although there is no direct linkage to the genetic basis of the female choice behavior. The sympatric and allopatric *O. oreoeca* populations used in my experiments are partially divergent genetically and the most peripheral sympatric population has evolved strong heterospecific call avoidance probably due to reinforcement. Geography and competition apparently both play a role in this apparent divergence.

APPENDICES

Appendix 2.A. List of material examined for phylogenetic analyses.

Species	N	Locality	Deposit
Arethaea gracilipes	1	Doña Ana Co., New Mexico	UM[1]
Obolopteryx catinata	1	Cameron Co., Texas	UM[1]
Mactruchus serrifera	1	Municipio de Huimilpan, Queretaro/ Mexico	UM[1]
Obolopteryx castanea	1	Webb Co., Texas	OSU[2]
Obolopteryx emarginata	1	McMullen Co., Texas	UM[1]
Obolopteryx brevihastata	1	Brewster Co., Texas	OSU[2]
Obolopteryx gladiator	1	Kenedy Co., Texas	UM[1]
Obolopteryx seeversii	1	Bandera Co., Texas	UM[1]
Obolopteryx oreoeca	1	Jeff Davis Co., Texas	OSU[2]

[1]University of Michigan Museum of Zoology, 1109 Geddes Ave
Ann Arbor, MI 48109

[2]Oklahoma State University K.C. Emerson Entomology Museum, 127 Noble Research Center
Stillwater, OK 74078

Appendix 3.A. Specimen information for all individuals used for AFLP analyses. IDs are personal collection numbers attached to each specimen in KCEM collection of invertebrates at OSU. Species: O=*O. oreoeca*, B=*O. brevihastata*. UTM GPS coordinates are reported.

ID	date	species	sex	map	zone	N	W
1050	09/02/10	OREO	f	C	13R	608829	3388734
1051	09/02/10	OREO	f	C	13R	608829	3388734
1052	09/02/10	OREO	f	C	13R	608829	3388734
1053	09/02/10	OREO	f	C	13R	608829	3388734
1054	09/02/10	OREO	f	C	13R	608829	3388734
1094	09/02/10	OREO	f	B	13R	597547	3386403
1095	09/02/10	OREO	f	B	13R	597547	3386403
1096	09/02/10	OREO	f	B	13R	597547	3386403
1144	09/29/11	BREV	m	A	13R	568280	3383007
1146	09/29/11	BREV	m	A	13R	568280	3383007
1148	09/29/11	BREV	m	A	13R	568280	3383007
1149	09/29/11	OREO	m	A	13R	568280	3383007
1150	09/29/11	BREV	m	A	13R	568280	3383007
1151	09/29/11	BREV	f	A	13R	568280	3383007
1153	09/29/11	BREV	f	A	13R	568280	3383007
1154	09/29/11	BREV	f	A	13R	568280	3383007
1155	09/29/11	BREV	f	A	13R	568280	3383007
1157	09/29/11	BREV	f	A	13R	568280	3383007
1159	09/29/11	BREV	f	A	13R	569126	3389097
1162	09/29/11	BREV	f	A	13R	568280	3383007
1163	09/29/11	BREV	f	A	13R	568280	3383007
1181	09/29/11	OREO	f	A	13R	568280	3383007
1528	09/10/12	OREO	m	F	13R	667010	3240742
1529	09/10/12	OREO	m	F	13R	667010	3240742
1530	09/10/12	OREO	m	F	13R	667010	3240742
1531	09/10/12	OREO	m	F	13R	667010	3240742
1532	09/10/12	OREO	m	F	13R	667010	3240742
1533	09/10/12	OREO	m	F	13R	667010	3240742
1399	08/10/12	BREV	f	D	13R	651987	3349444
1400	08/10/12	OREO	f	D	13R	651987	3349444
1401	08/10/12	BREV	f	D	13R	651987	3349444
1402	08/10/12	BREV	m	D	13R	651987	3349444
1404	08/10/12	BREV	f	D	13R	651987	3349444
1405	08/10/12	BREV	f	D	13R	651987	3349444
1406	08/10/12	BREV	m	D	13R	651987	3349444
1407	08/10/12	BREV	f	D	13R	651987	3349444
1409	08/10/12	BREV	f	D	13R	651987	3349444
1410	08/10/12	BREV	f	D	13R	651987	3349444
1411	08/10/12	OREO	f	D	13R	651987	3349444
1412	08/10/12	OREO	f	D	13R	651987	3349444
1413	08/10/12	BREV	f	D	13R	651987	3349444

1415	08/10/12	OREO	f	D	13R	651987	3349444
1416	08/10/12	BREV	f	D	13R	651987	3349444
1417	08/10/12	OREO	m	D	13R	651987	3349444
1419	08/10/12	OREO	m	D	13R	651987	3349444
1420	08/10/12	OREO	m	D	13R	651987	3349444
1422	08/10/12	OREO	f	D	13R	651987	3349444
1423	08/10/12	BREV	f	D	13R	651987	3349444
1442	08/14/12	BREV	m	E	13R	784820	3323174
1443	08/14/12	BREV	m	E	13R	784820	3323174
1444	08/14/12	BREV	m	E	13R	784820	3323174
1446	08/14/12	BREV	m	E	13R	784820	3323174
1448	08/14/12	BREV	m	E	13R	784820	3323174
1452	08/14/12	BREV	m	E	13R	784820	3323174
1454	08/14/12	BREV	m	E	13R	784820	3323174
1455	08/14/12	BREV	m	E	13R	784820	3323174
1456	08/14/12	BREV	m	E	13R	784820	3323174
1457	08/14/12	BREV	m	E	13R	784820	3323174
1458	08/14/12	BREV	m	E	13R	784820	3323174
1459	08/14/12	BREV	m	E	13R	784820	3323174
1461	08/14/12	BREV	m	E	13R	784820	3323174
1462	08/14/12	BREV	m	E	13R	784820	3323174
1464	08/14/12	BREV	m	E	13R	784820	3323174
1465	08/14/12	BREV	f	E	13R	784820	3323174
1469	08/14/12	BREV	m	E	13R	784820	3323174
1470	08/14/12	BREV	f	E	13R	784820	3323174
1501	08/14/12	BREV	f	E	13R	784820	3323174
1502	08/14/12	BREV	f	E	13R	784820	3323174
1504	08/14/12	BREV	f	E	13R	784820	3323174
1505	08/14/12	BREV	f	E	13R	784820	3323174
1342	08/06/12	OREO	m	F	13R	667010	3240742
1343	08/06/12	OREO	m	F	13R	667010	3240742
1344	08/06/12	OREO	m	F	13R	667010	3240742
1345	08/06/12	OREO	m	F	13R	667010	3240742
1346	08/06/12	OREO	m	F	13R	667010	3240742
1348	08/06/12	OREO	m	F	13R	667010	3240742
1349	08/06/12	OREO	m	F	13R	667010	3240742
1350	08/06/12	OREO	m	F	13R	667010	3240742
1355	08/06/12	OREO	f	F	13R	667010	3240742
1356	08/06/12	OREO	f	F	13R	667010	3240742
1357	08/06/12	OREO	f	F	13R	667010	3240742
1359	08/06/12	OREO	f	F	13R	667010	3240742
1360	08/06/12	OREO	f	F	13R	667010	3240742
1361	08/06/12	OREO	f	F	13R	667010	3240742
1362	08/06/12	OREO	f	F	13R	667010	3240742
1363	08/06/12	OREO	f	F	13R	667010	3240742
1364	08/06/12	OREO	f	F	13R	667010	3240742

1365	08/06/12	OREO	f	F	13R	667010	3240742
1003	09/05/10	OREO	f	C	13R	608829	3388734
1084	09/05/10	OREO	m	C	13R	608829	3388734
1086	09/05/10	OREO	m	B	13R	597547	3386403
1087	09/05/10	OREO	m	B	13R	597547	3386403
1089	09/05/10	OREO	f	B	13R	597547	3386403
1031	09/05/10	OREO	f	B	13R	597547	3386403
1032	09/05/10	OREO	f	B	13R	597547	3386403
1045	09/05/10	OREO	f	C	13R	608829	3388734
1046	09/05/10	OREO	f	C	13R	608829	3388734
1047	09/05/10	OREO	f	C	13R	608829	3388734
1048	09/05/10	OREO	f	C	13R	608829	3388734

Appendix 4.A. Specimen information for all individuals used for behavioral analyses. IDs are personal collection numbers attached to each specimen in KCEM collection of invertebrates at OSU. Species: O=*O. oreoeca*, B=*O. brevihastata*. Experiments: D=call description, CA=call analysis HD=heterospecific call discrimination ER=call element response. UTM GPS coordinates are reported.

ID	date	species	sex	map	zone	N	W	experiments
1640	08/29/13	OREO	f	B	13R	614062	3391361	HD
1641	08/29/13	OREO	f	B	13R	614062	3391361	HD
1642	08/29/13	OREO	f	B	13R	614062	3391361	HD
1643	08/29/13	OREO	f	B	13R	614062	3391361	HD
1644	08/29/13	OREO	f	B	13R	614062	3391361	HD
1645	08/29/13	OREO	f	B	13R	614062	3391361	HD
1646	08/29/13	OREO	f	B	13R	614062	3391361	HD
1647	08/29/13	OREO	f	B	13R	614062	3391361	HD
1497	08/13/12	OREO	f	D	13R	667010	3240742	ER
1487	08/13/12	OREO	f	D	13R	667010	3240742	HD, ER
1488	08/13/12	OREO	f	D	13R	667010	3240742	HD, ER
1491	08/13/12	OREO	f	D	13R	667010	3240742	HD, ER
1492	08/13/12	OREO	f	D	13R	667010	3240742	HD, ER
1493	08/13/12	OREO	f	D	13R	667010	3240742	HD, ER
1496	08/13/12	OREO	f	D	13R	667010	3240742	HD, ER
1171	09/05/11	BREV	m	A	13R	569539	3388206	CA
1172	09/05/11	BREV	m	A	13R	568280	3383007	CA
1173	09/05/11	BREV	m	A	13R	569539	3388206	CA
1174	09/05/11	BREV	m	A	13R	568280	3383007	CA
1175	09/05/11	BREV	m	A	13R	568280	3383007	CA
1176	09/05/11	BREV	m	A	13R	568280	3383007	CA
1177	09/05/11	BREV	m	A	13R	568280	3383007	CA
1178	09/05/11	BREV	m	A	13R	568280	3383007	CA
1167	09/05/11	BREV	m	A	13R	569126	3389097	CA
1614	08/27/13	BREV	m	C	13R	787113	3320810	CA
1615	08/27/13	BREV	m	C	13R	787113	3320810	CA
1619	08/27/13	BREV	m	C	13R	787113	3320810	CA
1631	08/27/13	BREV	m	C	13R	787113	3320810	CA
1636	08/27/13	BREV	m	C	13R	787113	3320810	CA
1620	08/27/13	BREV	m	C	13R	787113	3320810	CA, D
1622	08/27/13	BREV	m	C	13R	787113	3320810	CA, D
1623	08/27/13	BREV	m	C	13R	787113	3320810	CA, D
1630	08/27/13	BREV	m	C	13R	787113	3320810	CA, D
1611	08/29/13	OREO	m	B	13R	614062	3391361	CA
1612	08/29/13	OREO	m	B	13R	614062	3391361	CA
1600	08/29/13	OREO	m	B	13R	614062	3391361	CA
1601	08/29/13	OREO	m	B	13R	614062	3391361	CA
1602	08/29/13	OREO	m	B	13R	614062	3391361	CA

1604	08/29/13	OREO	m	B	13R	614062	3391361	CA
1605	08/29/13	OREO	m	B	13R	614062	3391361	CA
1607	08/29/13	OREO	m	B	13R	614062	3391361	CA
1609	08/29/13	OREO	m	B	13R	614062	3391361	CA
1531	09/10/12	OREO	m	D	13R	667010	3240742	CA
1529	09/10/12	OREO	m	D	13R	667010	3240742	CA, D
1530	09/10/12	OREO	m	D	13R	667010	3240742	CA, D
1532	09/10/12	OREO	m	D	13R	667010	3240742	CA, D
1533	09/10/12	OREO	m	D	13R	667010	3240742	CA, D

VITA

Bart James Kensinger

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