INVESTIGATING AN ADAPTIVE RADIATION IN TEMPERATE
NEOCONOCEPHALUS

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The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

INVESTIGATING AN ADAPTIVE RADIATION IN TEMPERATE
NEOCONOCEPHALUS

Presented by Katherine H. Frederick,

A candidate for the degree of Doctor of Philosophy

And hereby certify that, in their opinion, it is worthy of acceptance.

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TABLE OF CONTENTS

Chapters

iv

LIST OF ILLUSTRATIONS

LIST OF TABLES

Abstract

Evolution of communication system is seemingly complex requiring coevolution between sender and receiver mechanisms. My dissertation uses phylogenetic methods to investigate phenotypic diversification of *Neoconocephalus* acoustic communication, specifically how novel traits arise, and the evolutionary history of these traits.

Character state reconstructions found three lines of qualitative trait divergence in male calls, pulse rate, pulse pattern and call pattern. The combination of these call traits make up a species male call phenotype, each trait has an independent evolutionary history. The derived characters (slow pulse rate, double pulse pattern, and discontinuous call pattern) all have multiple independent origins. Double pulse pattern and slow pulse rate are only derived at the tips of the tree but discontinuous call pattern has two independent origins with subsequent reversals to the ancestral state.

The receiving side of the communication system appears to have even more diversity. Species that share a derived call trait do not necessarily have similar female recognition mechanisms or even females preferences for that trait. I found that female's preference for leaders was not due to a pre-existing sensory bias but instead has two independent origins one in each of the discontinuous calling clades.

The multiple independent origins of and subsequent state reversals found in the qualitative traits of acoustic communication make *Neoconocephalus* a great systems to study speciation. Specifically, seven species of temperate *Neoconocephalus* are found in a single clade. This clade has all the phenotypic diversity, or repertoire, of call traits found in the genus. I propose that the diversification of these temperate *Neoconocephalus*

is the result of an adaptive radiation triggered by post-glacial expansion of temperate grasslands and the adaptation of eggs diapause in order to overwinter.

Molecular-clock analysis reveled that the diversification in this clade occurred in a single glacial cycle. In fact, this adaptive radiation occurred 11 thousand years ago. The combination of pliable secondary sexual characters and frequent occurrences of drift along the colonization front across North America allowed this adaptive radiation to occur more in the timescale of African Laucustrine cichlids.

Previously the most rapid arthropod diversification event known occurred in Hawaiian *Laupala* crickets five million years ago. I found that temperate *Neoconocephalus* species were radiating into North America out of tropical grasslands at the same time that humans were entering North American by crossing the Bering Straight.

Chapter 1

Comparing phenotypic and genetic diversity in three sympatric species with acoustic communication.

Abstract

In acoustically communicating insects, male calls are among the most diverse traits between species and are directly correlated with fitness, because they serve as pre-zygotic barriers. Typically, species-specific call traits have little variation within species but are the most divergent traits between sister species. We studied three *Neoconocephalus* sympatric sister species, each with a unique derived call trait and species specific recognition mechanism. Males were recorded and sequenced using mtDNA and AFLP markers to investigate the relationship between phenotypic and genetic diversity. Mismatched genotypes, haplotypes and intermediate call traits indicate gene flow between species. Additionally, genetic analysis revealed incomplete lineage sorting between two of the focal species. Each species has distinct call traits, recognition and morphological phenotypes despite gene flow and low levels of interspecific genetic differentiation. The inheritance patterns and genetic analysis for double pulse pattern and

discontinuous calls indicate a genetic architecture of few genes of large effect. Genetic

architecture of the low frequency call trait is complicated because of its correlation with

body size. This study captures a snapshot of mid to late stage speciation, indicated by

quickly differentiated call phenotypes with otherwise little genomic differentiation

between species. Divergence in the communication system may serve as a reproductive

barrier while the fitness costs of hybridization accumulate and prevent fusion among

these species during secondary contact.

Introduction

In many insect and frog systems, acoustic communication brings males and

females together for mating (Gerhardt & Huber 2002). In most cases males produce

advertisement calls and receptive females respond, either by approaching the male or by

producing a response signal. Male signals convey information about species identity,

location, and potentially quality. Because communication directly affects reproduction, it

is a major determinant of fitness.

In groups with acoustic communication, male signals are among the most diverse

traits (Panhuis *et al.* 2001). Signals often differ substantially among closely related

species; in many cases, communication signals are the only detectable difference among

these species, at least to the human observer (cryptic species, *e.g.* Fitzpatrick & Gray

2001). Signals can vary in the value of one parameter (*e.g.* pulse rate) (Shaw & Herlihy

2000) or more qualitatively in the absence or presence of certain parameters (*e.g.* chirp

pattern) (Deily & Schul 2009). The evolutionary mechanisms generating and maintaining this signal diversity are not completely understood (Gerhardt & Huber 2002).

One potential scenario is that communication diversifies under selection for reproductive isolation (*e.g.* after secondary contact of geographically isolated populations) through reinforcement. Alternatively, diversity of the communication system that evolves in sympatry might result in reproductive isolation and ultimately drive speciation. Both scenarios predict significant phenotypic diversity of the communication system. However, since only a few genes might influence these traits, large differences in communication signals may not be paralleled with genotypic diversity in general (Carson & Templeton 1984).

Low genetic differentiation among populations of species may persist for several reasons. Relatively young populations may not have had enough time for genetic divergence, resulting in incomplete lineage sorting (Knowles 2000; Qu *et al.* 2012). Ongoing gene flow through hybridization and backcrosses may also result in little genetic divergence between species (Vedenina *et al.* 2007). Historic gene flow may confuse the analysis of genetic distances, if, for example, a captured mitochondrial haplotype persists in one or both populations (Roca *et al.* 2005).

To understand the relationship between genetic and phenotypic diversity we studied a young group of sympatric species that differ distinctly in the phenotypes of their acoustic communication system. The katydid genus *Neoconocephalus* (Orthoptera, Tettgigoniidae) has little morphological diversity, while male calls and female preferences are highly diverse among species. Call diversity occurs both in the spectral and in the temporal domains (Greenfield 1990; Schul & Patterson 2003). We chose three

sibling *Neoconocephalus* species (Snyder *et al*. 2009) for this study that each have one unique derived call trait. By comparing genotypic and phenotypic diversity between these species we find evidence for incomplete lineage sorting, mitochondrial capture, and recent hybridization.

Materials and Methods

Species collection

Neoconocephalus robustus (Scudder), *N. nebrascensis* (Bruner), and *N. bivocatus* Walker, Whitesell and Alexander are closely related species (Figure 1D) with widely overlapping ranges (Walker *et al.* 1973; Walker 2000; Snyder *et al.* 2009). These species have different habitat specification regarding soil moisture and vegetation, however, they are regularly found in close proximity (Walker 2000).

We collected males during July/August 2006 at three locations in Boone and Callaway County, Missouri (USA). One site ("Tucker Prairie" Latitude: 38.949 Longitude: -91.995) is a native prairie remnant, another site ("Three Creeks" Latitude: 38° 51' 3"Longitude: -92° 16' 33") a restored prairie. The third site ("McBaine" Latitude: 38° 53' 9" Longitude: -92° 26' 18") was among agricultural fields in the Missouri river bottoms. Specimens were located by ear and collected by hand.

Morphological Measurements

The species in this study are cryptic with limited morphological traits that can be used for species identification. However, in *N. nebrascencsis* the fastigium is long and slender with black coloration on the ventral side, while in the two other species it is broad, blunt

4

and without black coloration. The fastigium shape was categorized as *N. nebrascensis* type or *N. robustus/bivocatus* type respectively (Walker *et al.* 1973; Walker 2000) (Figure 1E). Classification by three researchers led to identical results.

Call recordings

The assumed ancestral call traits in *Neoconocephalus* are (1) a single pulse pattern in which pulses are repeated with a single period, (2) a continuous call, where pulses are repeated for minutes without a second order time structure, and (3) main spectral energy concentrated in a narrow band between 10 and 15 kHz (Greenfield 1994; Snyder *et al.* 2009). Bayes character state analyses confirmed these assumptions (Frederick & Schul, Chapter 2). Each of the three species has a derived character for one call trait, while being in the ancestral state for the two other traits. In *N. bivocatus* the pulse pattern contains two alternating pulse periods of different duration ('double pulse pattern', Figure 1B) (Greenfield 1994; Deily & Schul 2006). The calls of *N. nebrascensis* have a second order time structure, with verses of 1-2s duration repeated after silent intervals of about 1s duration (Fig. 1A) (Meixner & Shaw 1979; Deily & Schul 2009). In *N. robustus*, the spectral energy is shifted towards lower frequencies (6-9 kHz, Figure 1C) (Schul & Patterson 2003). We analyzed these three call traits and assigned each of them to the ancestral or derived state.

Male calls were recorded either immediately after collection or the following evening. Males were placed individually in recording cages (approximately 10x20x10 cm). Cages were spaced at least 3 m apart, with up to 15 males being recorded the same evening. Recordings took place outdoors between 2200 and 0400 at ambient temperature.

Males were most likely to call under these conditions; the evaluated call traits are not temperature dependent, thus the variable temperatures did not influence our results (Büttner 2002; Walker 1962).

Male calls were recorded individually with a Marantz PMD-671 solid-state recorder (48 kHz sampling rate, 16 bit) and an Audiotechnica ATR 55 microphone. The microphone was placed within 15 cm of the calling male, resulting in at least 30 dB signal to noise ratio. The temporal pattern of the calls was analyzed using custom made software at a resolution of 0.125 ms. Call spectra were calculated using (BatSound 1.0, Pettersson, Uppsala, Sweden).

Call data analysis

Discontinuous Calling: Males of all three species start each calling bout with discontinuous calling. In *N. bivocatus* and *N. robustus*, the calls become continuous within 1-2 minutes. In *N. nebrascensis* the calls remain discontinuous throughout the calling bout while settling in to a rhythmic verse pattern of 1-2 s verses repeated after slightly shorter silent intervals (Meixner & Shaw 1979; Deily & Schul 2009) (Figure 1A). We recorded at least 5 minutes of calling per male and used the duration of the longest uninterrupted stretch of calling as measure for continuous/discontinuous calling. As all males produce short call pieces at the beginning of the bout, this was a better measure than average verse duration.

Spectral Analysis: Amplitude spectra were calculated by fast Fourier transformation (FFT; Hamming window, frame length 256 samples) and averaged over a 1s section of

6

each call. The spectra of the calls of all species had a narrow-band low-frequency component (Fig. 1C). Amplitudes were calculated in relative dB. In female *N. robustus*, frequency components above 10 kHz have an inhibitory effect on phonotaxis (Deily & Schul 2006) and male calls of this species lack high amplitude components above 10 kHz, while in the other two species the low frequency component extends well above 10 kHz (Schul & Patterson 2003). Therefore, we measured the upper cut off frequency (at -10 dB) of the low frequency band of each male's spectrum.

Double Pulse Pattern: In the ancestral single pulse pattern, all pulse periods belong to the same statistical population. In the derived double pulse pattern, alternating pulse periods have different durations and the distribution of pulse periods clusters in two populations (Bush & Schul 2010). To quantify the pulse pattern, we calculated the 'CV ratio' as in (Bush & Schul 2010). In short, we compared the coefficients of variation (CV) of p1 and p2 to that of the pooled pulse periods (i.e. the mean of both p1 and p2), by calculating the CV ratio as $CV_{pooled}/((CV_{p1}+CV_{p2})/2)$. If p1 and p2 are from the same population (i.e. if the calls have a single pulse pattern), then CV_{pooled} should be similar to CV_{p1} and CV_{p2} and the CV-ratio should be close to 1. If p1 and p2 are from different populations (i.e. the calls have a double pulse pattern) the CV_{pooled} should be larger than CV_{p1} and CV_{p2} and the CV ratio >1.

To generate a baseline value for pure single pulsed calls, we calculated the CV ratio for two species that have no sympatric congener with the double pulse pattern making gene flow from double pulsed species extremely unlikely. In species with double pulse pattern, the CV ratios were typically larger than 2 (Bush & Schul 2010). We were

not able to determine a CV ratio for two individuals, as their recordings were too noisy to unequivocally measure the pulse periods of the calls; we assumed the ancestral state (i.e. single pulse pattern) for these individuals.

DNA collection

Extraction: After each male was recorded, hind legs were preserved in ethanol. DNA was extracted and isolated from hind femurs using a DNeasy Blood + Tissue Kit (Qiagen Inc., Valencia, CA, USA). The concentration and quality of each DNA sample was determined by spectrophotometry (NanoDrop 1000, Thermo Scientific, Wilmington, DE).

COI Amplifying: I used 825 base pairs of Cytochrome oxidase I as the mitochondrial locus for this study. MtDNA is inherited from the mother and can give us an idea of parental species identity. Details for mtDNA collection and sequencing are included in Snyder *et al.* (2009).

AFLP Amplifying: Amplified fragment length polymorphisms were acquired using the procedure described in Snyder *et al.* (2009). This procedure used the preselective PCR primers Eco+A (5'-GACTGCGTACCAATTCA-3') and Mse+A (5'-GATGAGTCCTGAGTAAA-3') (IDT, Coralville, IA, USA), as well as selective PCR, Eco primers Eco+AAC (6FAM), Eco+AGC (PET) (ABI, Foster City, CA, USA) and Mse primers Mse+ATA, Mse+AGA, Mse+AGC, Mse+ACA and Mse+AAC. Samples were genotyped at the DNA Core Facility, University of Missouri, on an ABI 3730 genetic analyzer, with Liz600 internal size standard (Applied Biosystems, Foster City,

CA, USA). AFLP genotypes were analyzed in GeneMarker v1.6 (Softgenetic Corp, State College, PA, USA) using the AFLP analysis setting.

Sequencing: Sequencing was performed either at the DNA Core Facility, University of Missouri, Columbia, MO or at Cornell Life Sciences Core Laboratories Center, Cornell University, Ithaca, NY, on ABI 3730 DNA Analyzers, using standard Big Dye Terminator cycle sequencing chemistry (Applied Biosystems, Foster City, CA, USA). Sequence data was edited and aligned in Sequencher v4.5 (Gene Codes Corp., Ann Arbor, MI, USA).

Phylogenetic and population genetic analysis
Alignment/File Building: During alignment and phylogenetic analysis, individuals were randomly assigned numbers so that species identity would not be biased. Alignments were manually generated in Sequencher with unambiguous gaps. Alignments were then formatted in Text Wrangler v3.1 (Bare Bones Software Inc., Bedford, MA, USA) for further analysis.

Haplotype Analysis: Mitochondrial gene COI (cytochrome oxidase I) alignments, with gaps removed, were analyzed in TCS v1.21. This program estimates non-bifurcating genealogical relationships between mitochondrial haplotypes using statistical parsimony (95%) (Clement *et al.* 2000). TCS uses the algorithms included in Templeton *et al.* (1992). Haplotype analysis included 54 of the 55 specimens, individual N27 was

excluded from the haplotype analysis because its short sequence could not cluster with either haplotype.

AFLP Network: Splits Tree4 was used to estimate phylogenetic information among individuals, using the neighbornet algorithm based on p-values between individual's AFLP banding patterns (Huson 1998). Network analysis of AFLPs was chosen over traditional phylogenetic analysis (cladogram) because relationships between individuals are not necessarily bifurcating. Individuals in this study may be siblings and/or hybrids these relationships can be more accurately interpreted in network form (Huson & Bryan 2006). AFLP analysis included 54 of the 55 individuals; N29 was excluded because of poor banding quality.

Measuring Gene Flow: Non-informative AFLP bands were removed and then all informative bands were input into AFLP-SURV v1.0 (Vekemans 2002). AFLP-SURV estimates the amount of gene flow between and within populations. In this study each species was treated as a population. We assigned individuals to populations based on their call phenotypes; individuals with intermediate or equivocal call phenotypes were excluded from the F_{ST} analyses. We applied 3 populations, method 4, 500 permutations, 100,000 bootstraps, 5,000 burnin, and assumed Hardy-Weinberg equilibrium.

Results

We collected and recorded the calls of 55 individuals. The fastigium of fourteen individuals had unequivocally the slender shape and black markings typically of *N. nebrascensis*. The remaining individuals had the broad fastigium shape of *N. bivocatus* and *N. robustus* without black markings.

Analysis of Call traits:

Continuous / discontinuous calls: The maximum chirp duration of fifteen individuals was between 1 and 2s which is the rhythmic verse pattern described for *N. nebrascensi*s. These individuals never produced continuous calls (5s or longer). The remaining 40 individuals we recorded had continuous calls of 5 plus seconds (Figure 2A); these individuals typically produced much longer calls (up to several minutes). In order to record many individuals in a single night we recorded for 5 seconds. We consider these 40 individuals as the ancestral 'continuous call type' and the 15 individuals with call durations shorter than 2s as the derived 'discontinuous call type,' typical for *N. nebrascensis*. We did not detect intermediate call types for this trait. All but one of the males with the discontinuous call type also had the fastigium shape typical for *N. nebrascensis*.

Call spectrum: The main energy of the call spectra was concentrated in a low frequency band between 6 and 12 kHz (Figure 1C). The upper cut-off frequency varied significantly among the 55 individuals. Twenty individuals had upper cutoffs below 8 kHz, while 32

11

individuals had values >10 kHz (Fig. 2B). Three individual had intermediate values (8.3, 8.8, 9.6 kHz). We consider cut-offs below 8 kHz as the derived 'low-frequency type,' (typical for *N. robustus*) and those above 10 kHz the ancestral spectrum. All of the individuals with cut-off frequencies below 10 kHz had the continuous call type (Figure 2B).

Single/double pulse pattern: To assess the pulse structure of the calls (Figure 1B) we calculated the CV ratio for each call. Two distinct groups appeared in the population: CV ratios >2.8 occurred in 15 individuals while 30 individuals had CV ratios between 0.99 and 1.2 (Figure 2C). Eight individuals had intermediate values ranging from 1.4 to 2.1. CV ratios of 2 individuals are missing due to poor recording quality. We consider the group with CV ratio values close to 1 (range 0.99-1.2) as the ancestral 'single pulse pattern' type and values >2.8 as the derived 'double pulse' type (typical for *N. bivocatus*). The remaining 8 individuals have an intermediate pulse pattern.

Intermediate call traits: The calls of 43 of the 55 individuals had a single derived call trait (13 discontinuous calls, 16 low frequency calls, 14 double pulse patterned calls), and were ancestral for the other two traits (Table 1). These individuals could be unequivocally assigned to one of the three species. Eight individuals had intermediate or derived phenotypes for two call traits, either double pulse pattern and low frequency (6x) or double pulse pattern and discontinuous calls (2x). This suggests that *N. bivocatus*, the species with the double pulse pattern, hybridizes with the two other species. The remaining four individuals were either ancestral for all three traits (2x) or ancestral for

12

two traits and intermediate for the third. These individuals provide evidence for backcrosses between hybrids and one of the parental species (see Discussion).

Genetic Analysis

Haplotype network: The network analysis of the mitochondrial CO1 sequences found two significantly different (95% confidence, haplotypes >12 step differences) haplotype groups among the 54 individuals. Haplotype group 1 (Fig. 3) contained 13 individuals. Haplotype group 2 contained 40 individuals. One individual did not sort with either haplotype group. Individuals in haplotype group 2 sorted into 2 sub-groups separated by at least 7 base pair differences. However, these sub-groups were not significantly different. Haplotype sub-group 2A included 27 individuals while 2B had 14 members. The three derived call traits largely align with the haplotype groupings (haplotype group 1 discontinuous calls; haplotype group 2 continuous calling: 2A low frequency and 2B double pulse pattern), however, each group also includes individuals of other call types (Fig. 3).

AFLP analysis of individual relationships: The amplified fragment length polymorphisms (AFLP) network analysis identified two clades among our study population. Clade 1 included all but one of the individuals of haplotype group 1 of the mitochondrial network. One individual of haplotype sub-group 2A also sorted in this clade (Fig. 4A). The second AFLP clade is comprised of the individuals from haplotype group 2 and one from haplotype group 1. No significant patterns of subtypes 2A and 2B were detected (Fig. 4A). All individuals of AFLP clade 1 had the discontinuous call trait,

13

while only one individual with this call trait was in clade 2. Two of the members of AFLP clade 1 were intermediate for the pulse pattern. Continuous calling individuals formed AFLP clade 2 but had no significant grouping of the derived call traits (low frequency, double pulse pattern).

F_{ST} analysis using AFLP data: We assigned all individuals that had a species specific call type, i.e. were derived for one of the three call traits and in the ancestral state for the two others, to their respective species. Individuals with 'mixed' or intermediate call traits were excluded from this analysis (leaving 41 individuals for analysis). We then calculated the F_{ST} within the total population and between the three species. The F_{ST} for the total experimental population (i.e. all three call types) was 0.1076 (global). There was moderate genetic differentiation (Rank 1992) between *N. nebrascensis* and both *N. robustus* ($F_{ST} = 0.1951$) and *N. bivocatus* ($F_{ST} = 0.1439$). However, we found no genetic distance between *N. robustus* and *N. bivocatus* ($F_{ST} < 0.0001$), indicating that these two species are not genetically distinct. The within-group variation was larger ($H_w = 0.1945$) than the global F_{ST}, indicating gene flow among the call types (Lynch & Milligan 1994).

Discussion

We studied the relationship of genetic and phenotypic diversity in the communication system of three sympatric *Neoconocephalus* species. We found little phenotypic variation within the derived call traits that define the three species specific calls. Individuals with intermediate phenotypes were detected indicating that

14

hybridization occurs between *Neoconocephalus* species. Although each species is clearly delineated by call phenotype, we found little genetic differentiation among the species.

The validity of the species classification is robust (Snyder *et al.* 2009). Male calls of the three species are each characterized by one derived call trait, which unequivocally distinguishes it from the two other species call phenotypes. Females can be identified unequivocally by morphological traits (Walker 2000). Female preferences for temporal and spectral call traits also differ among the three species. Each species evaluates a different acoustic parameter, resulting in strong preferences for the conspecific call phenotype (Deily & Schul 2004; 2006; 2009). Indeed, heterospecific calls evoke do not reliably evoke female responses, and acoustic communication apparently serves as the primary isolating mechanism for these species in sympatry (Deily 2006).

Interspecific hybrids in Orthopterans have been commonly observed in the field (e.g. Schul 1995; Shapiro 1998; Bailey *et al.* 2004; Miller 2010) and produced in the lab (Hoy 1974; Helversen & Helversen 1975a; Shaw & Herlihy 2000). Hybrid individuals typically have intermediate call traits with maternal effects (Helversen & Helversen 1975b; Shaw & Herlihy 2000; Gottsberger & Mayer 2007). Preferences of F1 hybrid females are commonly intermediate between the preferences of the parental species and in some cases they match the intermediate hybrid call (Helversen & Helversen 1975a; Shaw & Herlihy 2000). Intermediate call traits and preferences might not be compatible with the communication system of either parental species and thus render the hybrids behaviorally sterile (Shaw & Herlihy 2000; Gottsberger & Mayer 2007).

15

Mechanistic and genetic basis of derived call traits:

The ancestral call phenotype of *Neoconocephalus* consists of evenly spaced pulses repeated for extended periods of time. This simple rhythm with only one period is generated by a central pattern generator (CPG) located in the thoracic ganglia. The derived discontinuous call typical for *N. nebrascencis*, which has a second order rhythm at the second scale, requires a second rhythm to be generated in the nervous system, which switches the pulse CPG on and off (Gerhardt & Huber 2002). Since we did not find intermediates for this trait, this second pattern generator is either absent or present even in putative hybrids and thus represents a qualitative trait. Discontinuous call trait co-occurred largely with the *N. nebrascensis* fastigium shape, another qualitative trait without intermediates that identifies this species.

The second line of call diversification in *Neoconocephalus* is the evolution of a double pulse pattern with two alternating pulse periods. In Tettigoniids, this is a modification of the pulse pattern (*i.e.* the first order temporal pattern) rather than a chirp or second order time pattern (Pollack & Hoy 1979; Deily & Schul 2009). Accordingly, we find intermediate double pulses pattern within discontinuous calls (Table 1: N26, N35). The neuronal changes of the pulse pattern CPG underlying this derived call trait are not known. Despite introducing a new 'quality' (the presence of a second pulse period), the double pulse pattern shows quantitative inheritance. Seven of the eight males with intermediate CV values are likely hybrids, as indicated by the presence of another derived call trait (low frequency or discontinuous calls, Table 1). The one individual without another derived trait is likely a backcross.

The CV values of the males classified as the ancestral single pulse pattern ranged from 0.99 to 1.21. This variation is considerably larger than in other species with the similar call pattern that do not have a sibling or live in sympatry with double-pulsed pattern species. For example we found other katydid species *N. palustris* (ca. 230 Hz pulse rate) and *Orchelimum vulgare* (ca 70 Hz pulse rate) have CV values less than 1.01 (n= 19, 32) suggesting that even values close to 1.2 (i.e. classified as single pulse pattern) were likely influenced by gene flow from double pulsed populations.

The third line of call divergence is the shift of the dominant spectral component below 10 kHz in *N. robustus*. Female phonotaxis of this species is inhibited by the presence of frequencies above 10 kHz (Deily & Schul 2006), while females of the two other species respond equally well to the ancestral and derived spectra. *N. robustus* females show significant responses to the derived double pulse pattern (Deily 2006), so the low frequency spectrum is likely the outcome of reinforcement due to the sympatric occurrence of a double pulse species (likely *N. bivocatus*; Deily & Schul 2006). Thus, male *N. robustus* are under strong selection to maintain the low carrier frequency resulting in little variability for this trait (Fig. 2B). Females of the other two species are not selective for this trait, resulting in higher variability of males with the 'high' spectrum. *N. robustus* males are also significantly larger than males of the two other species and the only species with males being larger than females (Schul & Patterson 2003). The dominant frequency of the calls is strongly correlated to the size of the sound radiating structures on the forewings and thus to body size. Dominant frequency is thus a quantitative trait with intermediate trait values in hybrids and backcrosses.

Our sample populations included twelve individuals that did not match the call phenotype of any of the three species. These intermediate individuals had either more than one of the derived call traits (e.g. double pulses and low frequency), were intermediate for one or more of the derived traits, or were ancestral for all three call characters. This observation suggests recent gene flow among the three *Neoconocephalus* species. The diversity of 'mixed' calls also suggests that backcrosses occur, *i.e.* that at least some hybrids are not sterile. There is no evidence for interference among call traits and the absence/presence of one derived call trait does not constrain or influence the absence/presence of the other call traits.

Integrative and population genetic analysis:

The population genetic analyses revealed that call types were only weakly correlated to genotype. Discontinuous calls correlated to a single mitochondrial haplotype group. Individuals with this call trait also grouped together into AFLP clade 1 (Figs. 3, 4). The other two call traits were not significantly differentiated in the mtDNA (Fig. 3) and fell into a single AFLP clade. Stronger differentiation of the mitochondrial than of the nuclear genome is expected in taxa with incomplete lineage sorting (Knowles 2001; Qu *et al.* 2012). F_{ST} analyses indicate that only rudimentary sorting occurred between *N. robustus* and *N. bivocatus*, while *N. nebrascensis* diverged considerably further. The F_{ST} analysis also suggested substantial gene flow among the call types.

Intermediate call traits identified hybrid individuals between the three species (*e.g.* N26, N35, N38, N45 in Fig. 3 and 4). Male N06 is likely an example of mitochondrial capture (Roca *et al.* 2005) since its mitochondrial haplotype group 1,

18

which includes the discontinuous callers, neither matches its call phenotype (continuous, double pulsed) nor its AFLP clade (2, *N.robustus/bivocatus*). In contrast, the discontinuous call phenotype of N30 did not match either its position in the haplotype and AFLP networks, where it groups with the continuous callers (Figs. 3, 4) and could thus be interpreted as a case of "phenotypic capture." The common occurrence of hybrids (at least 7 out of 56 individuals) does not necessarily indicate high levels of gene flow, since hybrids may have low fitness and backcross only rarely. However, the examples of mitochondrial and phenotypic capture are evidence for at least some gene flow among the species.

Low genetic diversity establishes significant phenotypic differences:

The three *Neoconocephalus* species have distinct phenotypes in male calls, female recognition and morphology, despite their low genetic divergence. Below we propose a scenario which may explaining the maintenance of phenotypic differences of weakly diverged species in the presence of substantial gene flow.

Assume two populations diverged in their call and call recognition traits in allopatry while ecological and physiological divergence remained low. At the forefront of postglacial expansions, high levels of genetic drift (Hewitt 1996; Milá *et al.* 2007) could cause the divergence of the communication system, despite small differences in habitats. If these populations come into secondary contact, hybrids are likely reproductively viable and the fitness costs of hybridization would be limited to reproductive communication: Male hybrids have 'mixed' calls and are unlikely to attract females. Female hybrids with intermediate call recognition, however, will have a high chance of mating due to the male

biased sex ratio in *Neoconocephalus* populations. Gene flow, would thus occur through female hybrids backcrossing with either parental species. If type II genetic architecture (few genes with large effects) underlies the communication traits, such backcrosses would quickly restore the distinct call and recognition phenotypes of the parental species, as only individuals with matched alleles for calls and call recognition have high fitness (Carson & Templeton 1984). However, other nuclear loci will mix more freely, as the fitness consequences are weaker due to absence of genetic divergence.

The three *Neoconocephalus* species, and especially *N. bivocatus* and *N. robustus* fit into this scenario. They have widely overlapping ranges and occur often within few meters, indicating little ecological divergence. Two of the three diverged call traits (discontinuous, double pulse pattern) appear to have type 2 architecture as evidenced by the absence of intermediate calls (discontinuous) and the convergent evolution of the double pulse pattern in five *Neoconocephalus* species (Bush & Schul 2010). We could not detect AFLP bands associated with these call traits, this is expected since only a few loci likely encode these differences. Inheritance of male call frequency is probably more complex as it is strongly correlated with male body size (Schul & Patterson 2003). Therefore, the described scenario could explain our finding that the genes underlying communication traits diverged more rapidly than other portions of the genome and that this divergence is maintained in the presence of substantial gene flow.

Temperate *Neoconocephalus* are certainly young species. They require a habitat (temperate grasslands), which appeared only during the late Pleistocene, about 700,000 years ago (Hewitt 1996). The current ranges have only been habitable for *Neoconocephalus* since the end of the last glaciation about 20,000 years ago. The low

levels of genetic divergence agree with such short radiation times of this genus. The divergence of acoustic communication in *Neoconocephalus* is a model for the rapid diversification and species radiation into temperate grasslands during Pleistocene glacial cycles.

Table 1: Data set including information for each individual

| TAXA | Location | Genotype: | | Call Traits: | | | Morphology: |
		Haplotype	AFLP	Discontinuous (sec)	Call Spectrum (kHz)	Double Pulse (CV ratio)	Fastigium
N08	3Cr	2a	2	5.0	**8.0**	1.00	Broad
N37	McB	2a	2	5.0	**8.0**	1.00	Broad
N29	TP	1	x	**1.5**	11.4	1.00	Slender
N34	3Cr	1	1	**1.5**	11.8	1.01	Slender
N16	3Cr	2a	2	5.0	**7.2**	1.01	Broad
N14	TP	2a	2	5.0	**7.7**	1.01	Broad
N15	3Cr	2a	2	5.0	**7.6**	1.01	Broad
N52	McB	2a	2	5.0	**7.9**	1.01	Broad
N39	McB	2a	2	5.0	**7.4**	1.01	Broad
N22	3Cr	1	1	**1.8**	11.3	1.03	Slender
N31	3Cr	1	1	**1.4**	11.9	1.03	Slender
N32	3Cr	1	1	**1.2**	11.5	1.03	Slender
N55	McB	2a	2	5.0	**7.9**	1.04	Broad
N43	McB	2a	2	5.0	*8.8*	1.04	Broad
N24	3Cr	1	1	**1.8**	11.0	1.04	Slender
N30	3Cr	2a	2	**1.4**	11.4	1.04	Broad
N12	TP	2a	2	5.0	**7.9**	1.05	Broad
N36	3Cr	1	1	**1.5**	12.2	1.06	Slender
N21	McB	1	1	**1.4**	11.4	1.06	Slender
N42	McB	2a	2	5.0	**7.6**	1.06	x
N46	McB	2a	2	5.0	**7.7**	1.07	x
N23	3Cr	1	1	**1.6**	11.6	1.08	Slender
N56	McB	2a	2	5.0	**8.0**	1.09	Broad
N09	3Cr	2a	2	5.0	10.3	1.13	Broad
N25	3Cr	1	1	**1.6**	10.1	1.13	Slender
N11	3Cr	2a	2	5.0	**7.7**	1.15	Broad
N50	3Cr	2a	2	5.0	**7.7**	1.16	Broad
N54	McB	2b	2	5.0	**7.7**	1.19	Broad
N47	McB	2a	2	5.0	10.5	1.19	x
N33	3Cr	1	1	**1.3**	12.8	1.21	Slender
N41	McB	2a	2	5.0	**8.0**	*1.44*	Broad
N38	McB	2a	2	5.0	**8.0**	*1.47*	Broad
N45	McB	2b	2	5.0	*8.3*	*1.47*	x
N51	McB	2a	2	5.0	**7.3**	*1.57*	Broad
N40	McB	2b	x	5.0	11.7	*1.73*	Broad
N26	3Cr	2a	1	**1.5**	10.8	*1.77*	Slender
N13	3Cr	2a	2	5.0	**7.4**	*1.96*	Broad
N35	3Cr	1	1	**1.3**	10.7	*2.08*	Slender
N01	3Cr	2b	2	5.0	12.8	**3.00**	Broad
N02	3Cr	2b	2	5.0	13.1	**3.00**	Broad
N03	3Cr	2b	2	5.0	*9.7*	**3.00**	Broad
N04	3Cr	2b	2	5.0	14.3	**3.00**	Broad
N05	3Cr	2b	2	5.0	11.3	**3.00**	Broad
N06	3Cr	1	2	5.0	12.4	**3.00**	Broad
N07	3Cr	2b	2	5.0	11.4	**3.00**	Broad
N10	3Cr	2b	2	5.0	14.3	**3.00**	Broad
N17	3Cr	2b	2	5.0	12.6	**3.00**	Broad
N18	3Cr	2b	2	5.0	12.2	**3.00**	Broad
N19	3Cr	2b	2	5.0	11.6	**3.00**	Broad
N20	3Cr	2b	2	5.0	11.5	**3.00**	Broad
N44	McB	2a	2	5.0	11.7	**3.00**	Broad
N49	McB	2a	2	5.0	12.4	**3.00**	Broad
N53	McB	2a	2	5.0	11.1	**3.00**	Broad
N27	3Cr	x	1	**1.2**	14.3	x	Slender
N48	McB	2a	2	5.0	**7.4**	x	x

Locations are abbreviated (3Cr= Three Creeks, TP= Tucker Prairie, McB= McBaine). Haplotype groups indicated in Fig. 3. AFLP clades are indicated in Figure 4. Results from call trait analysis can be viewed in Figure 2. Morphological traits are depicted in Figure 1.

Figure 1
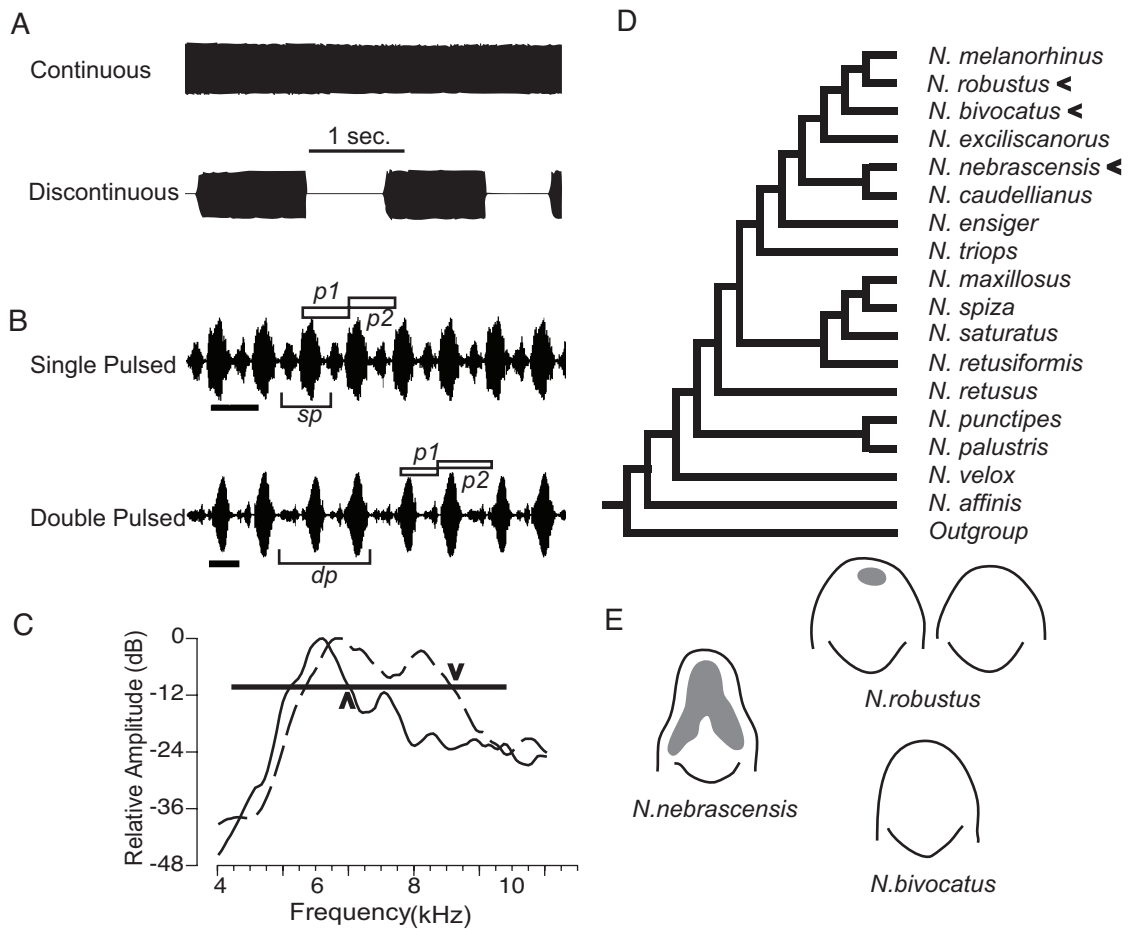
Figure 1: Description of study species.

A- Oscillograms of secondary time signatures depicting continuous and discontinuous calls. B- Oscillograms depicting pulse pattern (sp=single pulses, dp=double pulses) (p1 and p2 indicate the separate pulses), black bars indicate 5ms. C- Frequency spectra of a male *N. robustus* (solid line) call and a male *N. nebrascensis* (dashed line) call, arrows indicate highest frequency cutoff at -10dB. D- *Neoconocephalus* total evidence phylogeny, bold and arrows indicate study species. E- Species-specific morphology of the fastigium (ventral side of cone head) (Walker 2000).

Figure 2
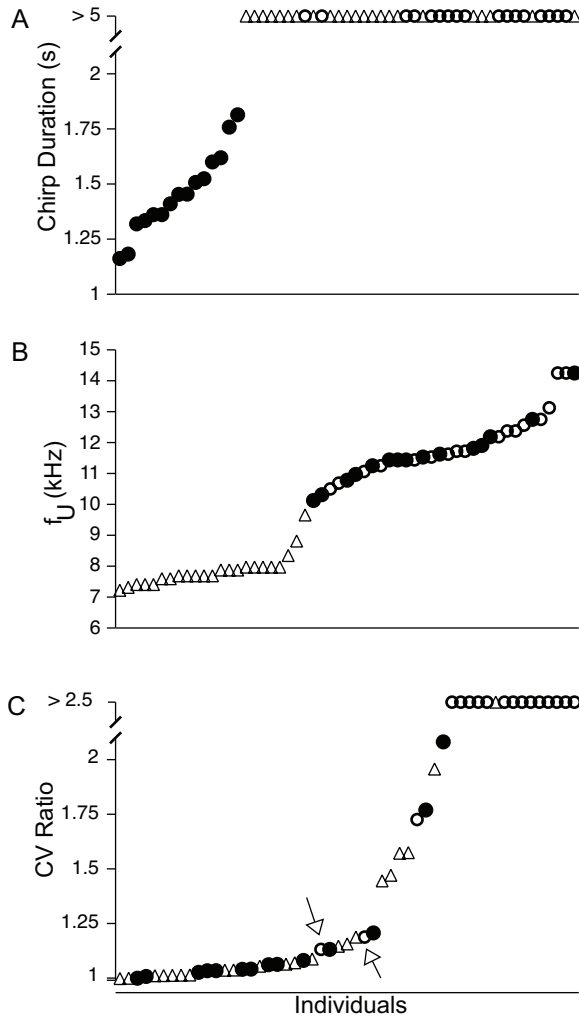
Figure 2: Call trait analysis- Each individual is a data point sorted by derived trait values in order to better visualize intermediate values. Solid individuals (of either shape) indicate discontinuous calls. Triangles indicate low frequency. A- Maximum calling length rhythmically produced without a silent gap (>2ms) by each individual calling male. Individuals are considered continuous if they produced a call longer than 2 seconds. Solid individuals are discontinuous. B- Highest frequency at -10dB bandwidth in calling male individuals. Those with <8 kHz are considered low frequency (represented with triangles). C- CV ratio indicating pulse pattern for each individual calling male. Those with CV ratios >2.5 are considered double pulsed. The

24

arrows indicate individuals that do not posses a single derived/species identifying call trait (*i.e.* their calls are continuous with single pulses presented at the high frequencies (-10dB > than 8khz).

Figure 3
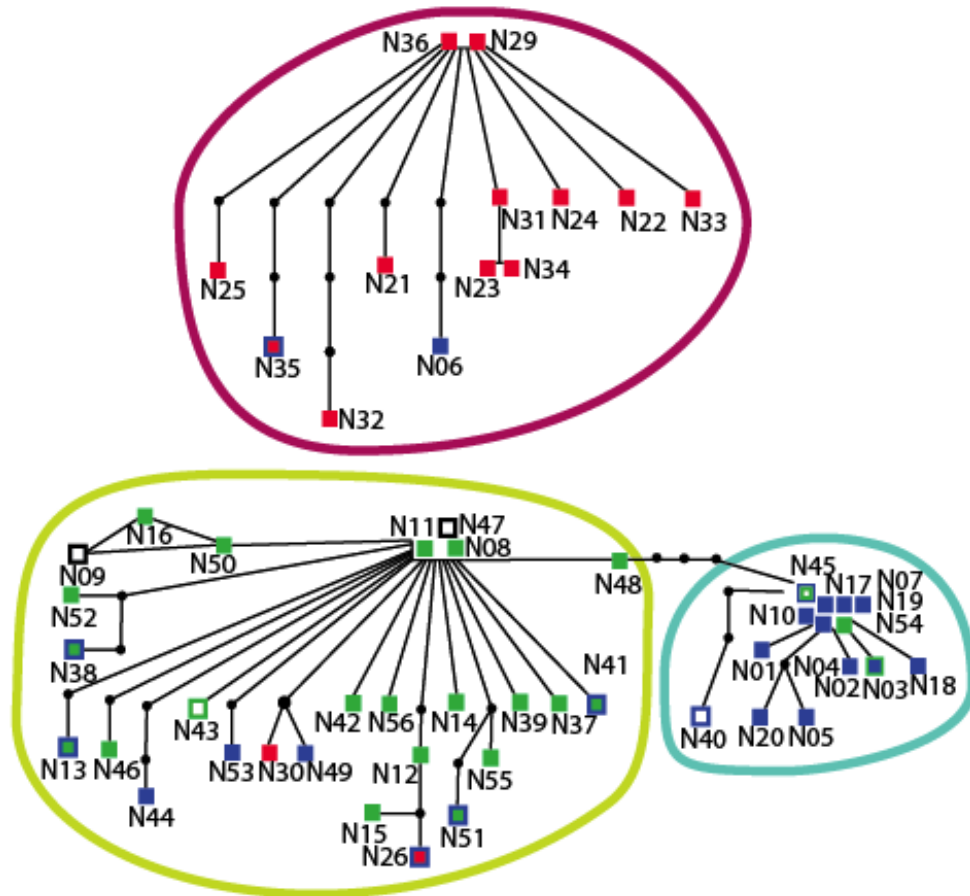
Figure 3: Mitochondrial haplotype network built from COI mtDNA using TCS. Each branch and node represents a single base pair change. The outlines indicate haplotype groupings. The chartreuse (2a) and aqua (2b) haplotype sub-groups are not significantly different from each other (sig. diff. 0.05>, +12bps differences). The raspberry (haplotype group 1) is significantly different than from haplotype group 2. Individuals are represented by squares which have been color-coded based on derived call traits: green indicates low frequency, blue indicates double pulses, red indicates discontinuous calls. Individuals with mixed traits have empty squares of the

corresponding to the color of the trait they are intermediate; black empty squares are individuals that completely lack all derived traits.

Figure 4

A

B

Clade 2

Clade 1

28
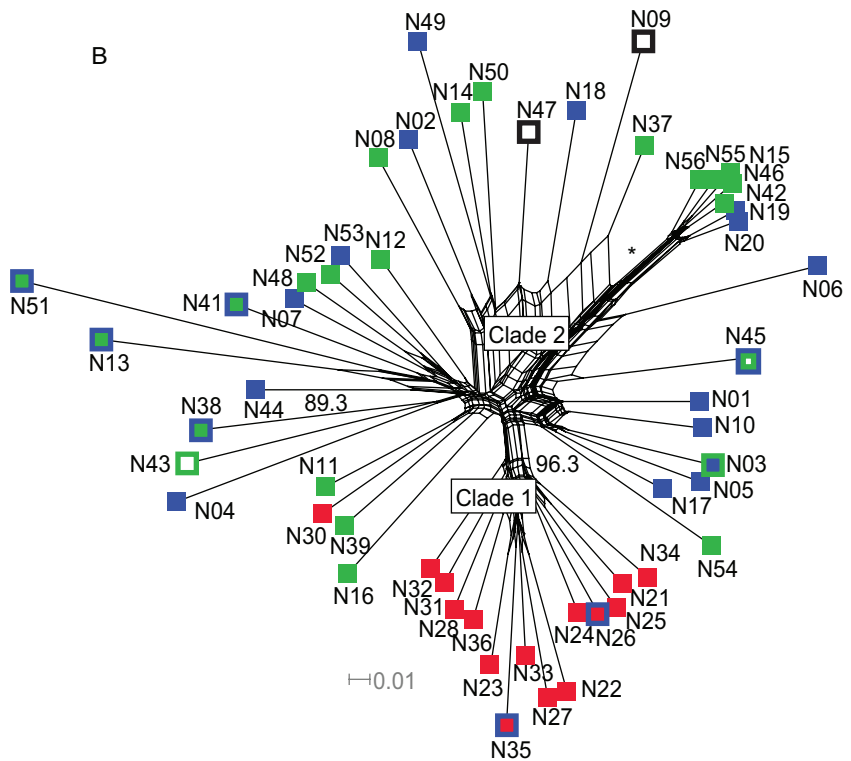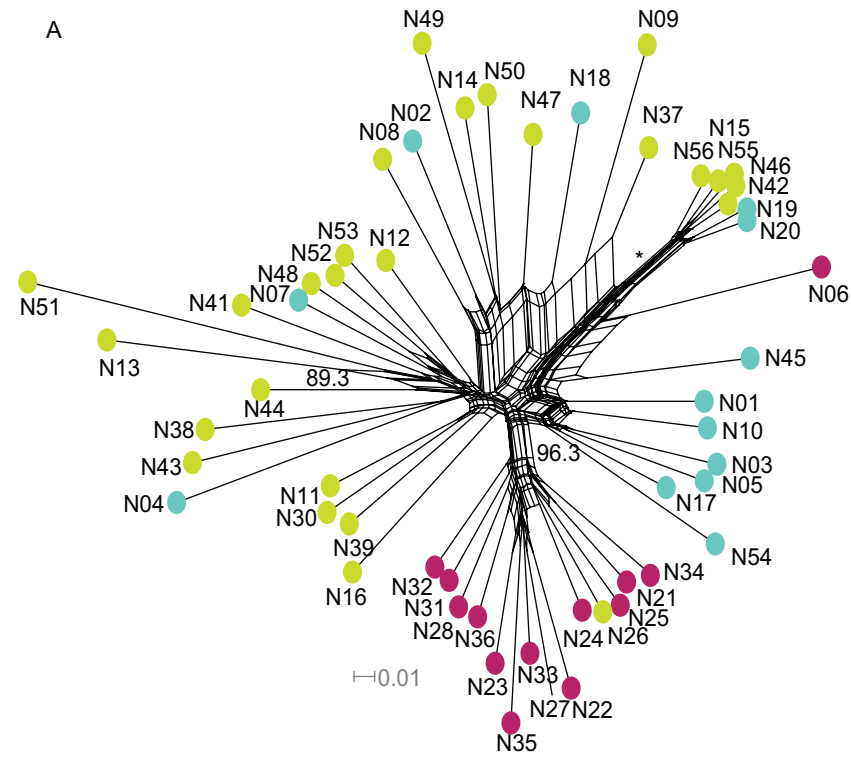
Figure 4: Minimum spanning network built from AFLP (amplified fragment length polymorphisms) A- Individuals are color coded by mitochondrial haplotype groups as outlines indicated in Figure 3. B- Individuals are colored by derived call trait: green indicates low frequency, blue indicates double pulses, red indicates discontinuous calls. Individuals with intermediate traits have empty squares of the corresponding color; black squares are individuals that completely lack derived traits.

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Chapter 2

Über-rapid divergence during post-glacial expansion

(This chapter will be submitted to Nature J. Schul an additional author)

Adaptive radiations are often triggered by major morphological or physiological innovations. Alternatively, significant changes in the environment can open new niches that allow radiations (Schluter 2000). Both triggers lead to rapid phenotypic divergence. Adaptive radiations are rapid at a geological time scale, however, the fossil record does not reveal divergence times at finer time scales. Glacial cycles during the Pleistocene provide known biogeographical time points and may add insight by which to scale radiation time scales.

North America experienced eight glacial cycles during the last 800 kyr, with the last glacial maximum (LGM) occurring about 18 Kya (Hewitt 1996). Each interglacial provided opportunity for radiations in temperate habitats, however subsequent glaciations likely reduced diversity through extinctions, leaving some species to persist in refugia

(Schluter 2000; Hewitt 1996). Thus, the diversity observed today could be the result of many radiation and extinction cycles accumulating in refugia. Alternatively, diversity could be largely the result of the radiation occurring in the current interglacial period.

Temperate grasslands in North America went through extreme geographic expansions and contractions. During the LGM, they were restricted to a narrow strip along the Gulf coast (south of the 31st parallel) and expanded within a few thousand years well beyond the 50th parallel (Adams & Faure 1997; Hewitt 1996). Grassland communities are species rich and many genera have high phenotypic diversity (Joern & Laws 2013; Hewitt 1999).

Species of the katydid genus *Neoconocephalus* are grassland obligates, relying on grasses for oviposition and food (Greenfield 1990). All species are strong fliers which can travel considerable distances; ranges of species can change significantly within few years (Hewitt 1999). This genus originated in tropical habitats and invaded temperate North America after the evolution of a novel life history (diapausing as eggs). Egg diapause evolution likely occured several times independently (Snyder *et al.* 2009).

The acoustic communication system entails the most diverse traits of *Neoconocephalus*. Temporal and spectral traits of male calls, as well as female pattern recognition mechanisms differ significantly among species and largely define species (Greenfield 1990; Schul & Patterson 2003). This phenotypic diversity together with the availability of a robust phylogenetic hypothesis (Snyder *et al.* 2009) (based on mtDNA, nDNA, and

AFLP data) makes *Neoconocephalus* a good study system for phenotypic diversification. Here we determine the timing of speciation and phenotypic diversification relative to glacial cycles and the evolutionary history of call diversity.

Seven North American *Neoconocephalus* species with temperate life history form a monophyletic clade (green in Figure 1). The sister of this clade is *N. triops* (Linnaeus 1758), a species with tropical life history, that ranges from South America into subtropical North America (Figure 1, Snyder *et al.* 2009). Ultrametric tree analysis of the mtDNA (CO1) revealed little genetic diversity within the temperate clade. Indeed, the genetic diversity among four geographically isolated populations of *N. triops* is greater than that among the seven species of the temperate clade.

Calibration of the ultrametric tree requires the application of estimated mutation rates. Standard mutation rate for invertebrate mtDNA is 2.3 percent base pair changes per million years (%/myr) (Brower 1994; Knowles 2000). Rates determined for specific Orthopteran groups were in a similar range (1.4 for Hawaiian *Laupala* crickets (Brower 1994; Mendelson & Shaw 2005), 3 to 4 for Hawaiian *Banza* katydids (Shapiro *et al.* 2006). Our mitogenomic study of the Copiphorini (the tribe including *Neoconocephalus* and *Banza*) resulted in a rate of 3.4 %/myr. The inclusion of data from all Orthopteran groups into this analysis resulted in 0.45 %/myr. The last value is likely too low due to an inherent sampling bias (*see methods*).

Using the standard rate of 2.3 %/myr, the estimate for the age of the temperate clade is 11.400 kyr (7.9-15.9 kyr; peak and 95% range of posterior density function). Mutation rates of 1.4 and 3.4 %/myr result in ages of 18.7 kyr (12.8-25.4 kyr) and 7.8 kyr (5.4-10.8 kyr). These estimates put the diversification of this clade close to or after the LGM (18 Kya). Even the slowest, likely too conservative estimate for mutation rates (0.45 %/myr) would put the age at 59 kyr (41-82 kyr) and thus within the most recent glacial cycle.

Thus, our analysis provides strong evidence that the species diversity of temperate *Neoconocephalus* evolved during postglacial expansion and was not the result of refugia divergence. The resulting speciation rate is several orders of magnitude faster than the fastest reported rates in arthropods (4.6/myr, (Mendelson & Shaw 2005) and 13.95/myr (Wessel *et al.* 2013)), and comparable to cichlid speciation rates within lakes of the African Rift Valley (Seehausen 2006).

Similarly fast divergence occurred within a clade of four tropical *Neoconocephalus* species ('tropical clade' purple in Figure 1) with present day ranges in the Caribbean and Central America. The end of glaciation significantly extended temperate ranges, but also changed tropical habitats due to increased precipitation (Hewitt 1996; Adams & Faure 1997). The dramatic changes in climate and habitat are likely the trigger for the 'über-rapid' speciation rates in this and the temperate *Neoconocephalus* clade.

It is worth noting that for several tropical species with wide geographic ranges (*N. triops, N. affinis, N. punctipes*) the genetic differentiation among geographically isolated populations is similar to that between species in the temperate and tropical clade (Figure 1). Population structure of these three species evolved thus during the same period as speciation occurred in the two clades. Within each of these three species, little phenotypic variability occurs in the communications system among geographically isolated populations (Beckers 2008; Greenfield 1990).

Acoustic communication in *Neoconocephalus* plays an important role in species diversity: males produce calls with species specific time pattern and females approach males for mating. Females are typically selective for the conspecific pattern, and prefer conspecific calls over the calls of other sympatric congeners.

The two young clades have considerable phenotypic diversity in male calls with an interesting pattern of synapomorphies, convergence and reversals. Bayesian character state analyses applied to the total evidence tree of *Neoconocephalus* (Snyder *et al.* 2009) revealed that the ancestral call had a single pulse pattern, a pulse rate above 100 Hz, and was continuous (Figure 2, *see methods* for detailed description of the temporal call traits). Three lines of call divergence occurred in the *Neoconocephalus*. First, the pulse pattern changed to a double pulse rhythm with two alternating pulse periods. Second, slow pulse rates below 50 Hz appeared. Double pulse pattern had five and slow pulse rate had three independent origins. Character state reconstruction places both derived traits only at tips of the tree (*i.e.* in extant species) and each occurrence of these traits represents

39

independent evolution of the derived trait. Discontinuous calls occur in two clades and have likely independent origins (Figure 2, red branches). Within each clade, reversals to the ancestral continuous state have also occurred.

All derived and ancestral call traits are present in both the temperate and the tropical clade (green and purple in Figures 1 & 2). The most recent common ancestor (MRCA) of the temperate clade likely had (posterior probability >0.98) discontinuous calls with fast pulse rate and single pulse pattern. Slow pulse rate and double pulse pattern each evolved in one species within each young clade (Figure 2). The discontinuous call pattern reverted to the ancestral state once, leading to three extant species with continuous calls. The MRCA of the tropical clade was ancestral for all three call traits (posterior probabilities > 0.97). Each of the derived call traits evolved once and the discontinuous pattern reverted once to continuous calls. Thus, rapid diversification of the communication traits occurred concomitantly with the über-rapid speciation in these two clades.

Extremely rapid diversification of communication signals was not expected, as signals have to co-evolve with female preferences to maintain the function of communication (Gerhardt & Huber 2002). Signal traits and female preferences are generally considered to be quantitative traits (Gerhardt & Huber 2002; Ritchie 2000), influenced by many loci with small effect. Selection on quantitative traits appears unlikely to be fast enough to generate the amount of diversity observed in the two young *Neoconocephalus* clades. There is, however, reason to argue that the derived call traits act more like qualitative traits controlled by few, possibly only one, loci with large effects (Chapter 1). Also,

40

derived call traits in *Neoconocephalus* may not affect the attractiveness to the females, as they do not change the call parameter that the ancestral female preference is based upon (Bush & Schul 2010; Beckers 2008). Thus, they may spread in a population to fixation, while female preferences remain in the ancestral state.

In groups that differ mostly in secondary sexual traits (*e.g.* calls) rather than in ecological or morphological traits, sexual selection is assumed to play a predominant role in driving divergence (Mendelson & Shaw 2005). In *Neoconocephalus*, sexual selection is unlikely to play a dominant role, as males introduce new call traits while females retain the ancestral preferences (Bush & Schul 2010; Beckers 2008). In addition, the extremely short divergence time in *Neoconocephalus*, which is 1 or 2 orders of magnitude faster than in the fastest diverging Arthropod group previously reported (Mendelson & Shaw 2005; Wessel *et al.* 2013), make sexual selection an unlikely candidate to drive divergence. What evolutionary force should then result in the über-rapid diversification reported here?

During rapid range expansions, as they occurred after the LGM, the leading populations experience extreme genetic drift, due to small population sizes and reduced migration rates (Milá *et al.* 2007). Bottlenecks and repeated extinction/re-colonization cycles (*e.g.* during the younger Dryas) increased the importance of genetic drift even further (Hewitt 1999; Knowles & Richards 2005). While selection by the rapidly changing environments probably had large impact on life history and ecological traits (Milá *et al.* 2000), genetic drift was likely the predominant evolutionary force acting on the communication

41

system(Barton & Charlesworth 1984). Assuming a type II genetic architecture of the calls (Carson & Templeton 1984; few loci with large impact), single mutations that result in novel phenotypes may drift quickly to fixation during postglacial expansion.

Female call recognition changes qualitatively between sibling species (*e.g.* Deily & Schul 2004; 2009; Bush *et al.* 2009) and few genetic changes with high impact may be responsible for these changes (Schul *et al.* 2014). In some species, a derived call trait has been established with females remaining in the ancestral state (Bush & Schul 2010) demonstrating that males may lead the divergence of the communication system and that female preferences are not necessarily the driving force (Barton & Charlesworth 1984). Other selective pressures appear unlikely to drive the call evolution in these species. Thus the analysis of the phenotypic pattern of calls and recognition also suggests drift as a potential evolutionary force resulting in the diversity of the communication system. The short divergence times after the LGM strongly support this view.

We describe here the fastest rates of speciation and signal diversification reported so far for insects. They are in the same range as the divergence rates reported in African cichlids and North American fishes (*e.g.* sticklebacks) (Seehausen 2006; Schluter 1996; Smith & Skulason 1996). However, in these fish systems, fast diversification occurs within lakes when dispersal is limited, *e.g.* due to changing water levels. In contrast, *Neoconocephalus* katydids hare highly mobile and each species occupies large geographic ranges which widely overlap with those of other congeners. Diversification took place at a much larger geographic scale than in cichlids or Sticklebacks. For mobile

organisms like *Neoconocephalus*, a whole continent may thus act like a single lake or island for species with more limited dispersal potential. The rapid quaternary climate changes in today's temperate zones might have been diversity hotspots, at least for organisms with high dispersal potential (Pulgarín-R & Burg 2012).

Our findings add to the increasing number of cases which document postglacial divergence (Milá *et al.* 2006; Lee & Lin 2012; Pulgarín-R & Burg 2012; Smith & Skulason 1996). In *Neoconocephalus*, postglacial divergence resulted not only in phenotypic diversity but also reproductive isolation that persists in extant sympatry. Highlighting how rapid speciation can occur during adaptive radiations, we find divergence rates several orders of magnitude faster that previously described in Orthopterans. This requires us to rethink the evolutionary mechanisms leading to the diversity in general and particularly in communication systems.

Methods

The divergence time analyses (Figure 1) and character state reconstruction (Figure 2) were based on the phylogenetic hypotheses of Snyder *et al.* (2009).

Phylogenetic analyses of Snyder et al. (2009)

Taxon Sampling: Neoconocephalus is a new world genus of acoustically communicating cone-headed katydids. This study includes seventeen *Neoconocephalus* species from North and Central Americas as well as the Caribbean islands. All but one species with temperate life history were included in the analyses; the missing *N. lyristes* is endemic to

few remnant habitats in the eastern USA. *Belocephalus davisi* Rhen and Hebard, 1916 and *Bucrates malivolans* (Scudder 1878), were used as outgroups in the phylogenetic analyses. Calling males were collected by hand, identified based on call and morphological traits (Walker & Moore 2000; Walker and Greenfield 1999; Froeschner 1954). Hind femurs and bodies were preserved in ethanol.

*Molecular Methods:*This study included 2,028 AFLP (Amplified Fragment Length Polymorphism) bands and 2,961 base pairs of DNA originally published in Snyder *et al.* (2009). Genomic DNA was isolated using a DNeasy Blood + Tissue Kit (Qiagen Inc., Valencia, CA, USA) and the concentration of each DNA sample was determined with spectrophotometry (NanoDrop 1000, Thermo Scientific, Wilmington, DE, USA). The presence or absence of AFLP banding patterns (sorted by fragment length) is informative for discerning taxa that are young or not very genetically distinct (Danley & Kocher 2001; Parsons & Shaw 2001). Bands were generated using a modified version of the protocol described in Vos *et al.* (1995). AFLP genotypes were analyzed in GeneMarker v1.6 (Soft- genetic Corp, State College, PA, USA) using the AFLP analysis setting.

Partial gene sequences were generated for two nuclear loci Internal Transcribed Spacer 1 and 2 (ITS1 & ITS2), the protein coding Histone 3 (H3) and one mitochondrial locus cytochrome oxidase I (COI). The data from ITS1 and ITS2 was combined into a single ITS analysis. The ITS loci are separated by the 5.8S rRNA gene, which is less than 1000 bp long. Given the close proximity of the two loci, we assume them to be tightly linked thus treat them as a single locus. ITS1 PCR primers were anchored in the flanking

44

18s and 5.8s genes and ITS2 PCR primers were anchored in the flanking 5.8s and 28s

genes. Primer sequences for both ITS genes correspond to the CAS18sF1 (ITS1 forward),

CAS5p8sB1d (ITS1 reverse), CAS5p8sFc (ITS2 forward) and CAS28sB1d (ITS2

reverse) of (Ji et al. 2003). H3 primers (H3 AF and H3 AR) sequences were from (Colgan

et al. 1998). COI primers (Ron and Calvin) were from (Lin & Wood 2002).

Sequencing was performed at the DNA Core Facility, University of Missouri,

Columbia, MO and at the Cornell Life Sciences Core Laboratories Center, Cornell

University, Ithaca, NY, on ABI 3730 DNA Analyzer. The resulting sequence data was

edited and aligned in Sequencher v4.5 (Gene Codes Corp., Ann Arbor, MI, USA). All

sequence and AFLP data are available at GenBank (Accession Numbers FJ913499-

FJ913766).

Phylogenetic Genetic Analysis: The AFLP data matrix includes binary data from 124

individuals. The restriction site model is binary (like F81 Felsenstein 1982), and to

analyze it, we used the 'noabsencesite' sub-model in MrBayes v. 3.1.2 (Huelsenbeck &

Ronquist 2001; Ronquist & Huelsenbeck 2003; Ronquist *et al.* 2012). The noabsencesite

sub-model best fits the nature of AFLP's, since the DNA fragments are anonymous and

analysis cannot distinguish between the absence of an allele and the absence of that locus

(Koopman *et al.* 2008). Sixty-four individuals were sequenced for the three to the DNA

markers in this matrix. Then jModelTest v0.1.1 (Posada 2011) was used to identify which

nucleotide substitution model that best fit each marker. The K80 model of nucleotide

substitution was selected for ITS1&2, this data set included 878 base pairs, 106 of which

were phylogenetically informative. H3 had 308 base-pairs and 24 informative characters and used the substitution model GTR+I+G. We also used the GTR+I+G substitution model for COI, which has 875 base-pairs with 287 informative characters.

A total evidence tree from Snyder *et al.* (2009) was generated by concatenating three gene alignments and AFLP data into a partitioned super matrix with 4089 characters and 128 individuals. Individual missing partition data were coded using a question mark or ambiguity character. For supermatrix analysis in MrBayes, models and priors were specific to the partition and unlinked between all partitions (genes, AFLP). This tree was produced from 2 runs, 10 chains each, and 2 million generations. The first 5000 trees of each Bayesian run were discarded as burnin, and the remaining trees in each analysis were used to calculate the posterior probabilities and a 50% majority rule consensus tree.

Divergence Time Estimates

Using the BEAST v1.7.4 software package (Drummond et al. 2012) we built an ultrametric tree (= chronogram) from the COI data with the same substitution model as above for 100 million generations. The starting tree was built in Mesquite v2.75 (W P Maddison & D R Maddison 2011) after randomly resolving polytomies and setting the root value to 1. This analysis resulted in a tree with the root node between *Neoconocephalus* and the outgroup that had the relative age of 1 and values at all other nodes reporting age relative to the root. The topology of this tree was used to infer the relative age of each clade. Runs were checked for convergence and normality using

Tracer v1.5. Tree files were combined in LOGCOMBINER 1.7.4 (Drummond *et al.* 2007), excluding the first 25 million generations burn-in.

The ultrametric tree was calibrated using literature collected mutation rates (Table 1) as well as rates calculated from an Orthopteran mitogenomic study (description below), which produced taxon specific mutation rates for the Copiphorini and the Orthoptera. For each mutation rate, we ran a lognormal relaxed molecular clock analysis with a speciation birth-death model (uniform prior distribution for ucld.mean 0.5 initial value =IV, range 0-10K; BirthDeath.relativeDeathRate IV= 0.5 range 0-1; BirthDeath.meanGrowthRate Prior Distribution Prior Distribution IV= 1.0 range 0-10000). Divergence times were derived from the pooled post-burnin results and TreeAnnotator v1.7.4 was used to compute a maximum clade credibility tree. Divergence times were calculated as mean node heights of the 95% highest posterior density intervals. Divergence times and the posterior probabilities of inferred clades were visualized on a chronogram in FigTree v1.3.1 (Rambaut 2009).

Sequencing the Neoconocephalus mitogenome: DNA extraction was performed as described above and the mitogenomes for two individuals *N. robustus* and *N. nebrascensis*. The massively parallel sequencing techniques were performed in the Pires lab by Roxi Steele in the as described in (Steele & Pires 2011; Steele *et al.* 2012). DNA was sheared and ligated to barcoding adaptors from the NEB Prep kit E600L (New England Biolabs, Ipswich Massachusetts, USA). PCR was used to enrich the fragments using universal primers (forward 5′-AAT GAT ACG GCG ACC ACC GAG ATC TAC

ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC* T-3′; reverse 5′-CAA GCA

GAA GAC GGC ATA CGA GAT CGG TCT CGG CAT TCC TGC TGA ACC GCT

CTT CCG ATC*-3′; both HPLC purified). PCR product as run on a 2% low-melt agrose

gel, samples were cut out and purified using a Quiagen Gel Extraction Kit. Product was

sent for the Missouri DNA Core for quantitation, fragment size verification and

sequencing on the Illumina GAIIx Genome Analyzer using the Illumina Cluster

Generation Kit v2-GA II, Cycle Sequencing Kit v3, and the image analysis using

Illumina RTA 1.4.15.0. These samples were ran on one sixth of an Illumina lane with

single-end 80-120 bp reads.

Assembling Mitogenome Libraries: Setting *Ruspolia dubia* (Zhou *et al.* 2007) as our

reference genome, we used YASRA (Yet Another Short Read Assembler

http://www.bx.psu.edu/miller_lab/) to assemble the short reads from the sonified and

sequenced DNA and put together the mitogenome. Short sequences were combined into

contigs using Geneious Pro v5.0.4 (Drummond *et al.* 2011) aligning overlapping regions.

Contigs annotations were checked using DOGMA comparing annotations to the reference

sequence (Wyman *et al.* 2004).

Building a Datasets: This study used all complete Orthopteran mitogenomes available on

GenBank. The mitogenomic data set included 35 Orthopterans downloaded from

GenBank (Appendix) and two *Neoconocephalus* mitogenomes sequenced and assembled

in the Pires lab. To get more complete taxonomic sampling of Orthopterans we also built

a dataset using the mitochondrial locus cytochrome oxidase I (COI). The CO1 sequences of 107 Orthopterans were downloaded from GenBank. This COI dataset also included seven *Neoconocephalus*.

Sequence Alignment: For the newly assembled genomes (*N. nebrascensis* and *N. robustus*) we used a reverse BLASTX search to map mitogenomic features, including tRNAs, genes, sRNA, and the control region onto our sequence. These were then spot checked by eye to correct gapped and overhanging calls. We used MUSCLE v3.8.3 (Edgar 2004) to align each of the coding sequences by amino acid for all 38 mitogenomes. Aligned amino acid sequences were then transformed to codon informed alignments using the nucleotide sequences. Non-coding regions (*i.e.*, tRNA and sRNA) were aligned by nucleotide, using MUSCLE. The control region aligned poorly and was excluded from this analysis. For the 122 COI sequences, we implemented the same codon informed nucleotide alignment method that we used for the coding sequences in the mitogenomes.

Phylogenetic Analysis: Nucleotide substitution model testes were performed using jModeltest v0.1.1 (Posada 2011) for the COI tree and individual partitions of the Mitogenome tree (*GTR+G*: ATP6, ATP8, CO1, CO11, CO111, CYTB, ND1, ND2, ND4L, ND4, ND5, tRNA; *TN93*: ND6; *HKY*: 12SrRNA, 16SrRNA). We obtained preliminary phylogenetic estimates of the COI gene tree and the concatenated Mitogenome Orthopteran tree using MrBayes v3.1.2 on the Cipres Portal (Miller *et al.*

2010) with the following settings: Chain length of 10,000,000, sampling every 1,000

trees with a burnin of 5,000 trees. Tracer v1.5.0 (Rambaut & Drummond 2003) was used

to check convergence of the Markov chains and to ensure sufficient sampling. In all cases

the chains were run long enough to achieve high effective sample sizes (ESS≥1000) for

all parameters. Polytomies were resolved and ultrametric trees were generated in the

same methods as described above (see *Ultrametric Tree*).

Bayesian settings for all mitogenome alignments had unlinked substitution models

for each partition, but linked clock and tree models across partitions. For the COI and

Mitogenome tree we specified the ancestral nodes to be calibrated with 18 fossils and

biogeographical time points, in order to estimate the age of internal nodes (Table 2). The

Speciation Birth-Death Process tree prior and the Relaxed Clock Uncorrelated Lognormal

Clock model (Drummond *et al.* 2006) were selected to account for rate variation among

lineages. Two independent runs were analyzed using LogCombiner v1.7.4 to test for

chain convergence with MCMC chain lengths of 10 million generations, sampling every

1,000 iterations with a 5,000 tree burnin. We then used TreeAnnotator 1.7.4 to generate a

Max-clade credibility consensus tree and calculate significant mutation rate changes

across the tree.

Character State Reconstruction for discrete Call Traits

Three lines of call divergence of the temporal call pattern occurred among the species

included in our analyses. Each trait can be classified as two discrete states, as described

below. We classified each species for each of the three call traits based on previous call

descriptions. Our own recordings and call analyses agreed with the descriptions in the literature.

Continuous/Discontinuous call pattern: Most *Neoconocephalus* species produce continuous call bouts with the basic pulse pattern repeated with a rhythmic second order time structure. Discontinuous calls typically last several minutes. During such continuous calls no silent intervals longer than 10 ms appear. Several other species produce discontinuous calls in that they group their pulses into a distinct second order time structure ('chirps'). These rhythmically repeated chirps have durations from about 30 ms to 1-2s. We follow here the classification of Greenfield (1990) except for *N. ensiger*. This species produces a slow pulse rate (about 10/s) continuously. Between pulses a silent interval of about 30-40 ms occurs which lead to Greenfield's classification as discontinuous call. We interpret the call here as continuous, as only a single rhythm occurs in the call (the pulse rate).

Pulse Pattern: In *Neoconocephalus* males, each wing closing produces a distinct sound pulses. In most species, the pulses are repeated at a single rate (=single pulse pattern). In five species, pulses are repeated with alternating pulse rates, resulting in pulses pairs, which are separated from the following pulse pair by a longer silent interval (= double pulse pattern) (Figure 2C). Five species have double pulse pattern: *N. bivocatus, N. affinis, N. retusus, N. maxillosus, N. triops* (Bush & Schul 2010; Walker and Greenfield 1999; Walker *et al.* 1973).

Pulse Rate: Calls of most *Neoconocephalus* species have unusually fast pulse rates for Tettigoniids (100-250/s)(Greenfield 1990). Several species have dramatically slower pulse rates at about 50/s (*N. retusiformis*) or 10/s (*N. ensiger, N. affinis*). We classify these three species as 'slow pulse rate' and all other species as 'fast'.

For these three lines of call divergence we used 10,000 post-burnin trees from the Bayesian total evidence phylogeny to reconstruct the ancestral character states with the program Discrete, implemented in BayesTraits v1.0 (Pagel *et al.* 2004; Pagel & Meade). This method reconstructs the most probable character states at each tested node, maximizing the likelihood of the character states observed in terminal taxa. Call state reconstructions were completed in batch mode using the concatenated data matrix by running MCMC, 2 million generations 100k burnin, at a 0.2 acceptance rate.

Mutation Rate %/myr	Temperate clade age (Kya)	Taxa	Source
Literature			
1.40	18.70	*Laupala*	Shaw (pers comm) Brower 1994
2.30	11.40	Melanoplinea	Knowles and Otte 2000, Brower 1994
Mitogenomic estimated rates			
0.46	59.00	Orthopterans	Average Orthopteran Rate
3.45	7.80	Orthopterans	Copiphorini rate

Table 1 **Mutation rates and clade age estimates**
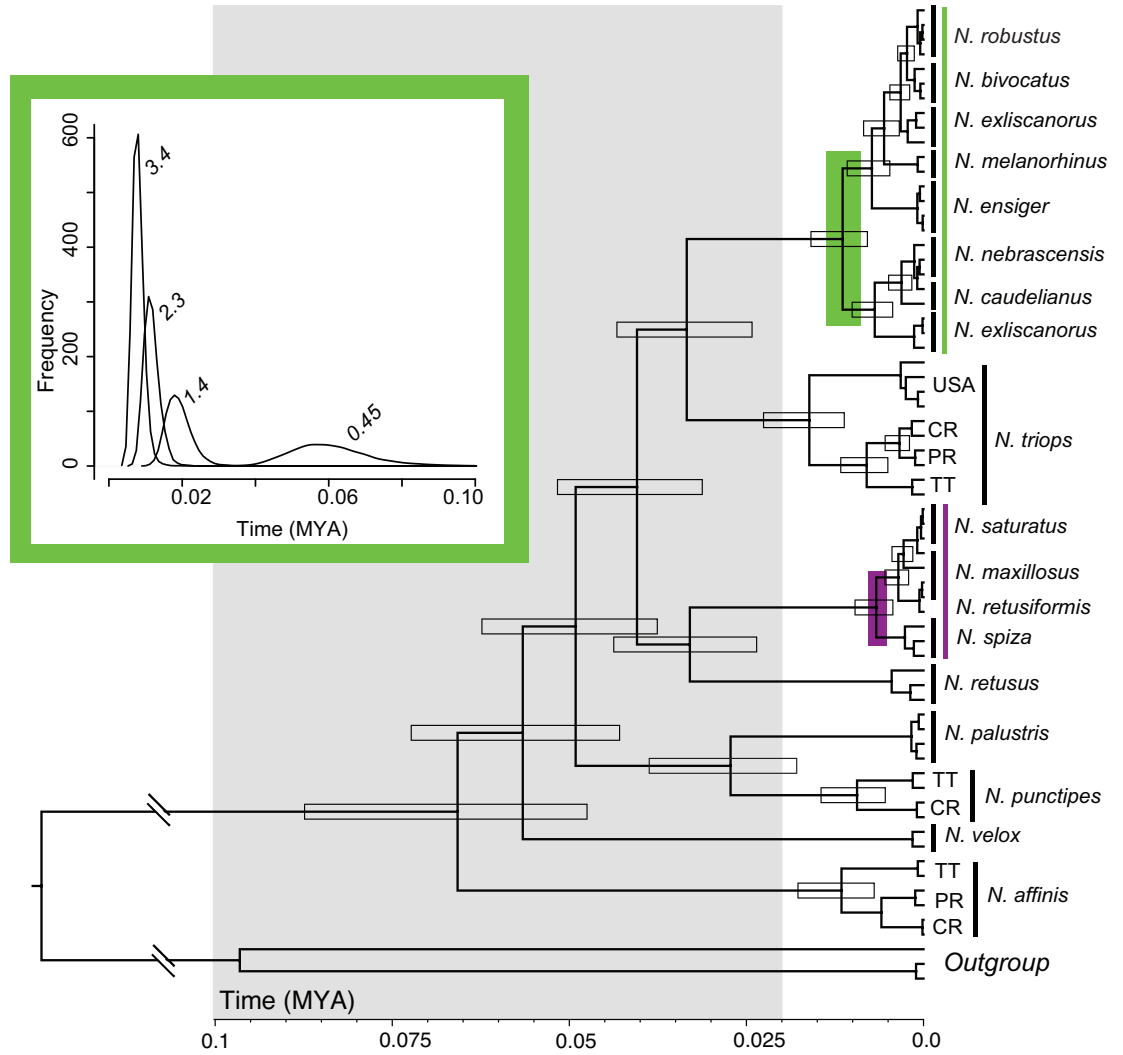
Figure 1

Figure 1: *Neoconocephalus* time calibrated phylogeny based on *mtDNA* and calibrated with 2.3%/myr mutation rate (base pair changes per million years). Bars at nodes are 95% HPD (highest probability distribution) of divergence time estimates. Grey area indicates the LGM (last glacial maxima). This tree is scaled to the geological time scale with absolute time given in millions of years. Green inset shows the age of the temperate node (indicated by green bar) calculated with varying mutation rates. Purple bar indicates the young and phenotypically diverse tropical clade.
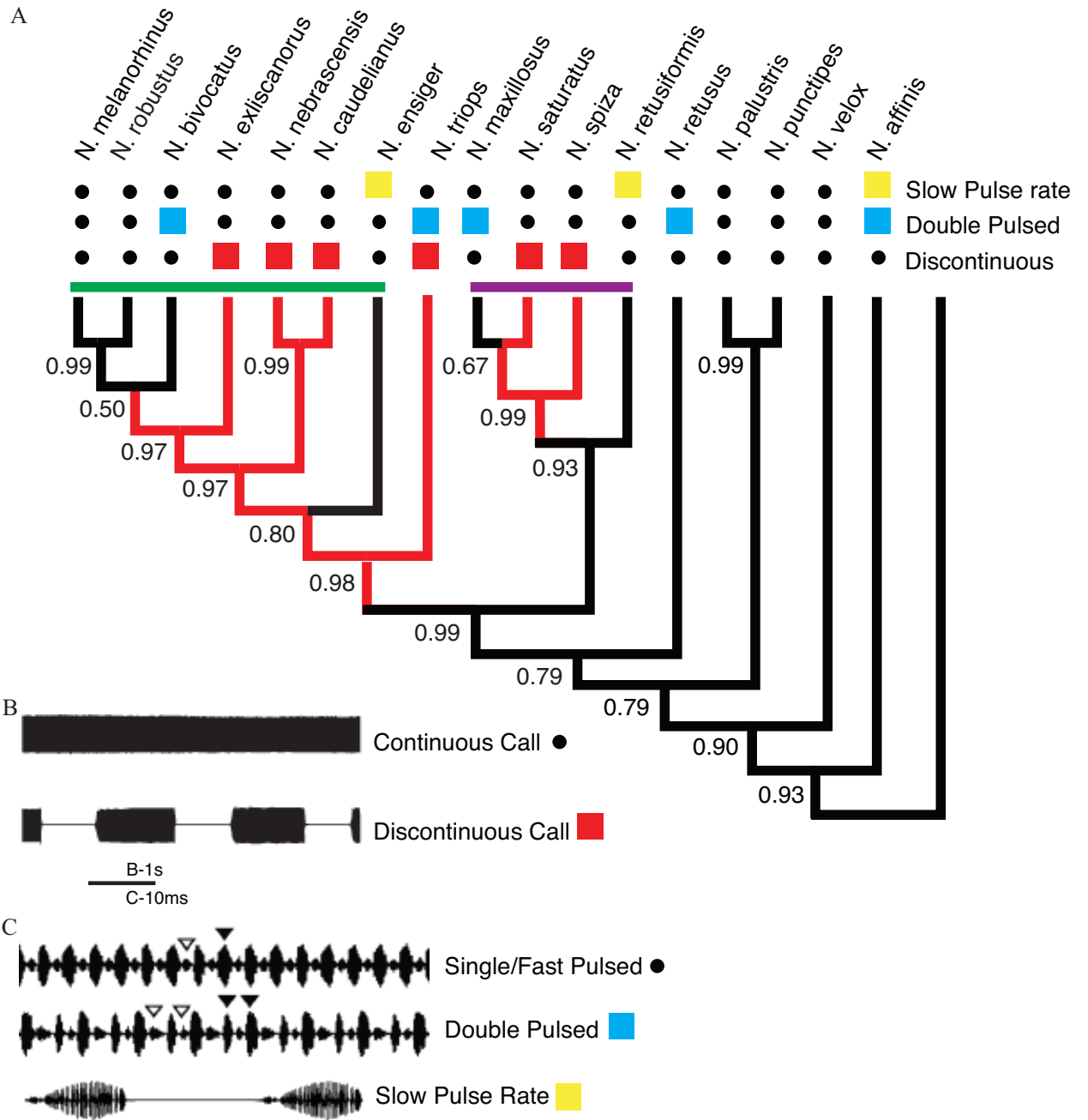
Figure 2: Call trait evolution in *Neoconocephalus*

A- Character state reconstruction for Continuous/Discontinuous calls traced onto the

Neoconocephalus total evidence tree. Red branches indicate discontinuous calling.

Values at nodes indicate probability of the character state at that node. Boxes at tips

indicate call phenotype by showing presence of derived traits at species tips: Black dots = Ancestral state, Red = Discontinuous call, Blue = Double pulse pattern, Yellow = Slow pulse rate. The two young species rich clades are indicated purple bar = tropical clade and green bar = temperate clade. B,C- Oscillogram traces depicting call traits at two time scales: B- Second order time scale: Ancestral continuous calling, derived discontinuous calling C- Millisecond time scale: Ancestral single pulsed, fast pulse rate call. Derived double pulse pattern (blue box), males call with alternating pulse periods (empty triangles indicate opening pulses, solid triangle indicate closing pulses). Additionally, derived slow pulse rate (yellow box), males call with pulse rates less than 50 Hz.

Table 2 **Fossil calibration for Orthopteran COI and Mitogenome trees**			
Node	Mean (myr)	Standard Deviation	Citation
A	35.5	1.5	Scudder, SH, 1890
C	31.25	2.45	Mantell, GA, 1844
D	30.6	23.5	Zeuner, FE, 1937
E	226.95	25.65	Brongniart, C, 1893
F	15.45	8.35	Heyden,CHG von, 1862
G*	39.3	15.5	Germar, EF & Berendt, GC, 1856
H	44.25	10.55	Gorochov, A.V., 1989
J	183	89.5	Gorochov, AV, 1989
K	116.5	4.5	Zeuner, F.E., 1989
M	120	21.55	Sharov, A.G.. 1968
N*	44.25	10.55	Zhang,JF, Liu,D & Shangguan Y, 1989
O*	150.7	8.7	Hagen, H, 1862
P*	15.45	8.35	Zhang,JF, Liu,D & Shangguan Y, 1989
Q	1.3	0.01	Shapiro et al 2006
R	0.5	0.01	Shapiro et al 2006

Figure 3: Cytochrome oxidase 1 Orthopteran phylogeny. This phylogeny is based on COI, contains 122 individuals (Appendix) and 15 fossil calibration points, which are indicated by boxed letters (Table 2. Branch widths indicate Bayesian posterior

probabilities (wider branches have greater support), values at nodes indicate estimated

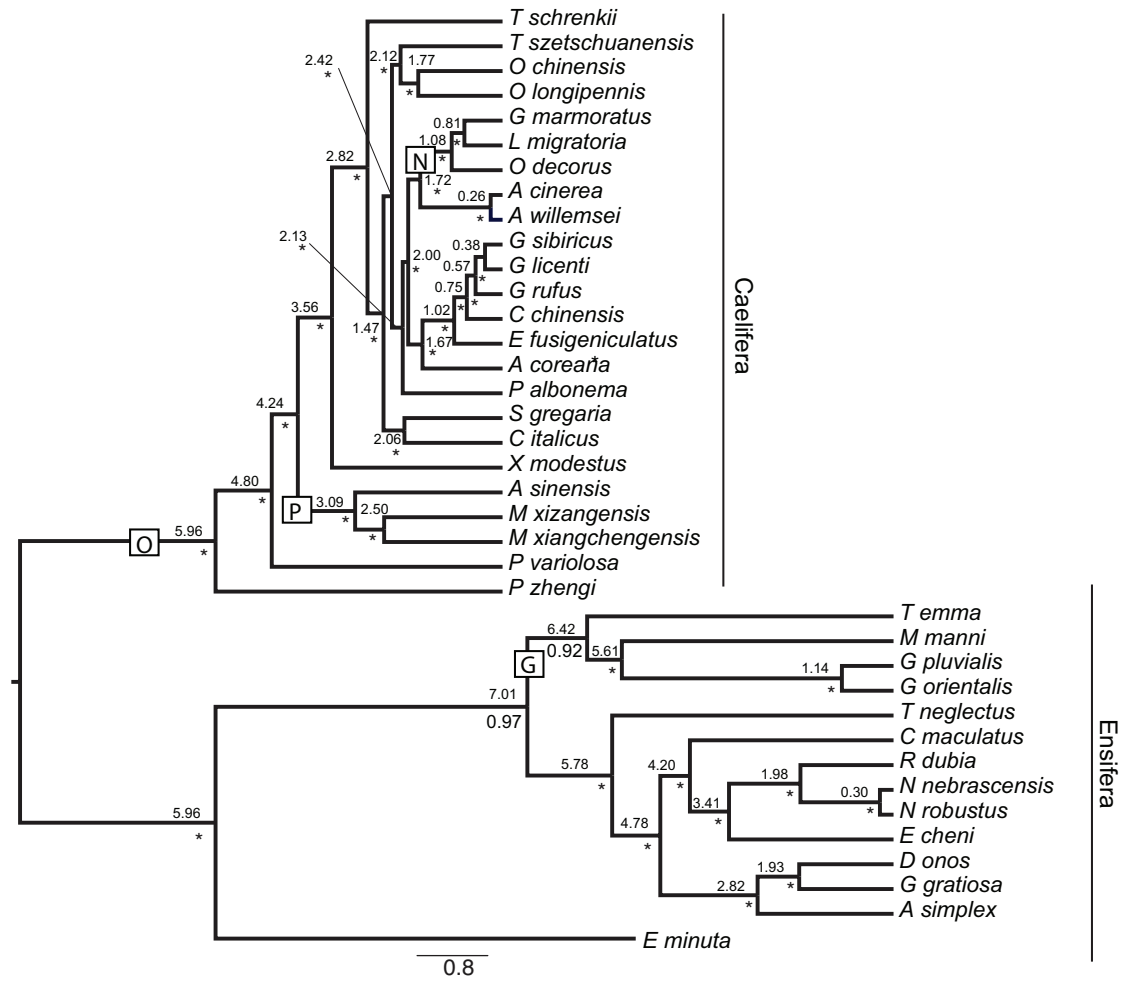mutation rates (%bp change/myr).

Figure 4: Mitogenome Orthopteran tree. This Orthopteran only phylogeny was formed from concatenation of 38 individual's whole mitochondrial genomes (Appendix) and four calibration points, indicated by boxed letters (Table 2). Top values at the nodes indicate estimated mutation rate (%bp change/myr), bottom values indicate Bayesian posterior probabilities (BPP) (* BPP ≅.99).

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Chapter 3

Evolution of a novel female preference- Leader preference

Abstract

Using a comparative approach, we studied the evolutionary history of a novel preference, leader preference. In *Neoconocephalus,* males call to attract mates. Some of these males have discontinuous calls, which they produce in imperfect synchrony with neighboring males. Imperfect synchrony causes one male to lead relative to neighboring males. Leader preference is common in acoustically communicating organisms (Gerhardt & Huber 2002). We know that *Neoconocephalus* species have two evolutionary trajectories associated with discontinuous calls. *N. spiza* females have leader preference and males competitively synchronize their calls. Conversely, *N. nebrascensis* females lack leader preference and males cooperatively synchronize (Greenfield & Roizen 1993 and Greenfield & Schul 2008). To understand the evolutionary explanation (*i.e.* preexisting sensory bias, runaway selection and direct or indirect benefits) for this preference and the

two evolutionary trajectories, we conducted behavioral female preference tests in six *Neoconocephalus* species using a walking compensator. We found leader preference in only *N. ensiger,* showing that leader preference lacks a phylogenetic signal and is not the result of a preexisting sensory bias. Leader preference is an example of phenotypic convergence with two evolution origins. Additionally, female preference for leaders has driven the evolution of competitive synchrony among males. Females of *N. ensiger* and *N. spiza* may prefer leaders because fast chirp rates may signal a fitness benefit for the female. Preliminary evidence shows that leader preference in *N. ensiger* is likely associated with an emergent property of the sensory system, by including an additional interneuron for mate recognition. Further studies are needed to investigate the benefits associated with leader preference as well as the adaptive advantage *N. ensiger* gains from the newly found emergent property. This study illustrates the phenotypic diversity in *Neoconocephalus* acoustic communication systems and their divergent evolutionary explanations.

Keywords: communication, leader preference, exaggerated male trait

Introduction

Female preferences for exaggerated male traits are common in reproductive communication systems. Visual preferences, for example, are for larger feathered crests, longer tails, or elaborate displays (Andersson & Simmons 2006). In acoustic

67

communication systems female preferences are commonly for longer, faster, or louder signals (Kirkpatrick & Ryan 1991; Helversen & Helversen 1994; Gerhardt & Huber 2002). Numerous studies have addressed how sexual selection acts on behavior and existing traits (Ryan & Rand 1999; Brown 1982). The evolutionary origins of mate choice have been more elusive, however, and our understanding of the underlying evolutionary mechanisms remains under debate (Andersson & Simmons 2006).

Several explanations for female preferences of exaggerated traits have been put forward (Andersson & Simmons 2006; Endler & Basolo 1998). First, the exaggerated male trait may indicate direct benefits for the female, such as resources or increased fecundity (Moller & Jennions 2013). Second, the male trait under selection advertises the male's genetic quality or compatibility (indirect benefits). Third, a genetic correlation between male trait and female preference may lead to self-reinforcing co-evolution (*runaway selection*; Kirkpatrick 1982; Pomiankowski & Iwasa 1998). Fourth, female choice may result from preexisting sensory biases, which evolved in a different context (*e.g.,* foraging, Proctor 1992), or as an epiphenomenon of sensory mechanisms (Enquist & Arak 1993). There is no doubt today that the described models of preference evolution are internally valid, *i.e*., each explanation could work (Kirkpatrick & Ryan 1991). However, the evolutionary origin of preferences (as opposed to the existence of preferences) has been thoroughly studied in only a few systems, and even in the best-studied systems (*e.g*., Túngara frogs) well-founded conclusions (Ryan & Rand 1993a; 1993b; 2003) leave at least some potential for debate (Ron 2008).

In acoustically communicating organisms, female choice may not only act on call traits (such as pulse duration or chirp rate) but also on the relative timing of the calls

produced by neighboring males (Greenfield 2005). In discontinuous calling species, neighboring males typically synchronize their calls. This synchrony is imperfect with the calls overlapping and one call leading the other. At least in some species, males compete for the leading position, as females have a strong preference for leading calls (= leader preference, LP). In the absence of a LP, call synchrony can be explained by several cooperative hypotheses (*e.g.,* preserving the pattern, beacon effect, or enemy confusion; Greenfield 2005).

Leader preferences occur in several frog and insect species (Römer *et al.* 2002; Snedden & Greenfield 1998; Gerhardt & Huber 2002); in some cases, females prefer lagging calls (Grafe 1999). When the leading position is an outcome of male competition, it may be an indicator of male quality. Thus direct or indirect benefits are likely explanations for this preference (Greenfield *et al.* 1997). A preexisting sensory bias has been proposed as an alternative explanation for LP in Tettigoniids (Römer *et al.* 2002). Contralateral inhibition in the ascending auditory pathway, which functions in directional processing, may lead to a suppression of responses on the side of the following call, thus rendering the female 'deaf' for the following call. According to this hypothesis, the LP should occur inevitably after discontinuous calls evolve. The preexisting sensory bias hypothesis would thus predict that species with continuous calls would also have LP, if they are stimulated with a leader/follower duet. Here, we test the distribution of leader preference in a group of Tettigoniids, where discontinuous calls evolved twice independently.

In the Tettigoniid genus *Neoconocephalus,* a species-rich group with diverse acoustic communication (Greenfield 1990; Bush & Schul 2010; Schul *et al.* 2014),

discontinuous calls have evolved twice independently from the ancestral continuous calls with several occurrences of reversals to continuous calling (see Chapter 2, Figure 1). Females of at least one species with discontinuous calls (*N. spiza*) have a significant LP (Greenfield & Roizen 1993), while at least one other species does not have LP (*N. nebrascensis*, Greenfield & Schul 2008). Accordingly, males of *N. spiza* compete for the leading position, while males of *N. nebrascensis* have cooperative synchrony. Thus, at least two evolutionary trajectories exist in this genus after the evolution of discontinuous calls, one with LP and competitive call synchrony, and the other lacking LP with cooperative call synchrony. The evolutionary forces leading to these trajectories are not understood. A well supported phylogeny (Snyder *et al.* 2009) together with the behavioral diversity in *Neoconocephalus* acoustic communication provide a unique opportunity to study evolutionary origin of this female preference.

Methods

Study Organisms

Females of five *Neoconocephalus* species (*N. bivocatus* Walker, Whitesell & Alexander 1973, *N. ensiger* (Harris 1841), *N. exciliscanorus* (Davis 1887), *N. nebrascensis* (Brunner 1891) and *N. retusus* (Scudder 1878)) were collected as nymphs from Adair, Boone, and Stoddard Co. Missouri (Table 1). *N. triops* (Linnaeus 1758) were lab reared from Puerto Rican stock populations collected in 2009. Specimens were identified to species using morphological taxonomic keys (Walker & Moore 2000). Katydids were maintained on a lab diet of wheat grass seedlings, cut grass, apples and cricket food with a 14:10 hour

light:dark cycle at 20-25ºC. Females were tested two to six weeks after their adult molt.

Phonotaxis Experiments

Two choice behavioral tests were conducted in an anechoic chamber at 25±1°C. Females were placed on top of a spherical walking compensator (*Kramer treadmill*; Weber *et al.* 1981). The females were free to walk, compensatory rotations of the sphere kept the female in place as signals were presented with stimuli (synthetic male calls) from two loudspeakers (Motorola KSN1218C). Loudspeakers were mounted at a distance of 150 cm in the horizontal plane of the female and separated by 105º. Tests were conducted in the dark with an overhead infrared light source used to monitor the movement of the insect. This light did not provide directional cues to the female. As the female walked her intended direction, walking speed and distance were recorded from control circuitry.

Experimental Protocol: Females were placed on the walking compensator and allowed to acclimate without stimulus for 1-2 minutes. Next, I presented a call model (as described below) from one loudspeaker only. After 90-120s the signal was switched to the second speaker. If the female responded reliably and her orientation followed the change of the loudspeaker, I proceeded to testing leader/follower situations.

Two signals were played simultaneously (except for the experimental delay) and with equal amplitudes (except for the calibration experiments, see below) from the two loudspeakers. After 120s, the signals were switched between the loudspeakers, so that the leading signal now came from the direction of the initially following signal. After the 4 min presentation of one experimental delay, one minute of silence was kept. Then

the next delay was tested. Every 2 or 3 delays, I tested a call model from one speaker

alone, to control for the responsiveness of the female. Delays were tested in different

sequences among the individuals, to avoid sequence effects. I could not detect any

indication of the preceding stimulation affecting the orientation during a test. Individual

females were tested for 30-50 minutes.

Data Acquisition: As described in Schul *et al.* 1998, data is reported from the walking

compensator at intervals of 1 cm of compensated walking, the angle of orientation and

elapsed time were calculated and recorded by a computer to reconstruct the female's path

and velocity. The walking angles of the two presentations of the same stimulation but

switched speakers were pooled by mirroring the walking angles along the midline

between the two loudspeakers. I used the vector angle of the resulting response vector as

measure for female preference, as it ultimately determines which loudspeaker the female

would reach. The vector length was used to control for the responsiveness of the female

and the attractiveness of the stimuli (data not shown).

For each individual, I calculated the vector angle (=average walking

direction) for each stimulus. The midline between the loudspeaker is at 0º and positive

values indicate orientation towards the louder or leading loudspeaker. I present the

individual angles of orientation as well as the population median for each species.

Stimulus Generation

Stimuli were generated with a custom-developed DA-converter/amplifier system (16 bit

resolution, 250 kHz sampling rate). The stimuli were produced from frequency filtered

frozen noise with energy either from 9 to 15 kHz (low frequency) or 10 to 20 kHz (high frequency). I used for each frequency band one noise signal of 20 s duration and reverted it to generate two spectrally equivalent carrier signals for simultaneously presented signals. I then multiplied the noise files with envelope files which mimicked the species specific temporal pattern (described below). The envelope files incorporated the delays between leading and following calls. The low frequency noise was used for *N. bivocatus, N. exciliscanorus, N. nebrascensis,* and *N.triops* females and stimuli for *N. retusus* and *N. ensiger* used the high frequency noise. I chose the spectra based on the spectrum represented in the male calls (Schul & Patterson 2003).

I calibrated the signal amplitude to 80±1 dB peak SPL (re 20x10^{-5} Pa) using a Bruel and Kjaer sound level meter (B&K 2231) and a 1/4" condenser microphone (G.R.A.S. 40BF) that was positioned 1 cm above the apex of the sphere. Signal amplitudes were adjusted using a computer-controlled attenuator.

Temporal pattern of the amplitude modulation: Female call recognition mechanisms of the species used here have been described in detail (Deily 2006; Deily & Schul 2004; 2009; Beckers & Schul 2008; Bush & Schul 2010; Frederick KH, Schul J unpublished; Kong XL, Schul J unpublished). I used temporal parameters which were likely to be attractive for females; they are given below.

N. bivocatus: Stimuli had pulse rate of 87/s with pulse duration of 7.5 ms and silent intervals of 4 ms. Each pulse represented a double pulse of the male call (Deily & Schul 2004). Calls of *N. bivocatus* are continuous, but females responded in preliminary experiments well to chirp durations of 1000 ms repeated after silent intervals of 400 ms. I

used delays of 0, 23, 46, 103, 195, and 299 ms to test for leader preference. The delays were integer multiples of the pulse period, so that all pulses were synchronous between leading and following calls. Additionally, I tested 250 ms chirps presented after silent intervals of 100 ms in this species. Delays used for this stimulus were 0, 11.5, 34.5, and 46 ms.

N. nebrascensis: Females of this species were tested with chirps of 1000 ms duration repeated after silent intervals of 800 ms (Greenfield & Schul 2008). Each chirp consisted of many pulses (3 ms duration including 0.5 ms rise and fall time) repeated after a 2 ms silent interval (*i.e.,* pulse rate of 200/s) (Deily & Schul 2009). Stimuli were presented at delays of 0, 50, 150, and 300 ms, integer multiples of the pulse period, so that all pulses were synchronous between leading and following calls.

N. retusus: Calls of *N. retusus* are continuous, but females responded in preliminary experiments well to chirp durations of 1000 ms repeated after silent intervals of 98 ms. Each chirp consisted of pulses (3.5 ms duration including 0.5 ms rise and fall time) repeated after a 3.5 ms silent interval (*i.e.* pulse rate of 140/s) (Bush & Schul 2010). I tested delays of 0, 28, 49, 70, 98, and 130 ms.

N.triops: I tested *N. triops* with chirp durations of 950 ms repeated after 150 ms interval durations. *N. triops* males produce calls with distinct double pulse structure, females respond to temporal patterns which merge the two pulses into one long pulse (Beckers & Schul 2008; Bush & Schul 2010). Each chirp contained 6.5 ms pulses (including 0.5 ms rise and fall time) and a 2.5 ms interval duration between pulses. I used delays of 0, 27, 54, 81, 135, and 325 ms to test for leader preference. Pulses were synchronized between leading/following stimuli.

N. ensiger: The stimuli used in this species consisted of continuous noise bursts of 36 ms duration with 16 ms rise time and 5 ms fall time. These chirps were repeated after silent intervals of 44 ms. In previous experiments (Kong XL & Schul J, unpublished) this temporal pattern was highly attractive. I used delays of 0, 6, 12, 18, 24 and 40 ms.

N. exiliscanorus: Here I used chirps consisting of continuous noise bursts with 76 ms duration, repeated after silent interval of 314 ms duration. Although male calls have a distinct pulse structure within each chirp, females respond equally well to continuous chirps without pulse pattern (Frederick & Schul unpublished). I tested delays of 0, 15, 30, 50, 76, and 195 ms. A delay of 195 ms resulted in alternating chirps without a leader or follower. The 76 ms delay resulted in the following chirp abutting the leading chirp without overlap. Neither the abutting nor alternating chirps occur in male choruses (Schul J, Frederick KH, Guerra P, unpublished).

Calibrating Leader Preference: To determine whether the shift of orientation during leader/follower stimulation would translate into a significant preference in a true choice situation (*i.e.* where the female would actually reach the loudspeaker or male), I compared it to the orientation towards a louder signal. If everything else is equal, a 2 dB amplitude advantage results in a significant female preference (Römer et al. 1998). We presented identical signals from the two loudspeakers at 80 dB SPL and then attenuated one loudspeaker by 3, 6, and 12 dB (*N. ensiger* only). The different relative amplitudes were tested using the same protocol as above. I used the resulting deviation of the angle of orientation towards the louder speaker to quantify the female leader preference.

I analyzed the relationship between angle of orientation and relative amplitude differences between speakers using linear mixed model regression (model <- lmer (data = file, formula = DependentVariable ~ FixedEffect + (1|RandomEffect)) with a Gaussian distribution calculated in R (RDC Team) with the lme4 library (Bates *et al.* 2013). This analysis reported the interaction value of shift of orientation and amplitude differences (unit: degree/dB). I report these values for each species as well as for all individuals pooled. The significance of these interactions were reported in Student's *t*-values and converted to *p*-values using a Student's *t*-test data table (Soper 2006).

Results

Female preference for louder signals

In the first set of experiments, I tested female orientation toward two synchronous stimuli that differ in relative amplitude. When both signals had the same amplitude, females oriented along the midline between the two loudspeakers. When the amplitude of one signal was reduced by 3 or 6 dB, orientation turned towards the louder speaker (Figure 2 for *N. nebrascensis*). The mean shift of orientation was dB-linear with deviations changing by 3-5° per dB amplitude difference across the six species tested (Tab. 2). Linear mixed model regression reported the interaction between relative amplitude and angle of orientation for all 6 species (41 total females) as 3.4°/dB (p= 0.02).

In choice experiments, amplitude differences of 2 dB result in significant preferences for the louder signal. I evaluate the shift of orientation during stimulation with leading and following call using this finding: shifts of orientation equivalent to those caused by 2 dB or more amplitude difference should be biologically significant. I used the across species value of 3.4°/dB as significance threshold for all species.

Responses to leader follower duets

Species with discontinuous calls- I tested four species with discontinuous calls for the presence of LP. Three of these species (*N. ensiger, N. nebrascensis, N. exciliscanorus*) have male call synchrony with leader follower relationships among neighboring males. In *N. triops* males do not synchronize their chirps and leader/follower relationships do not occur. During stimulation with call models in perfect synchrony (*i.e.,* a delay of 0 ms) female orientation was close to 0º, *i.e.* along the midline between the speakers (Figure 3 & 4).

Females of *N. ensiger* showed strong leader preference at delays between 6 and 24 ms. The mean shift of orientation towards the leading loudspeaker was up to 24º at 24 ms, equivalent to an advantage of the leading call of more than 6 dB (Figure 3). A delay of 40 ms resulted in alternating chirps (Table 1) with no side being the leader. Accordingly, mean orientation was not shifted from the midline.

Chirps of the *N. exciliscanorus* stimulus were longer (76 ms) and repeated after longer intervals (324 ms) than the *N. ensiger* stimuli. Female *N. exciliscanorus* did not

show leader preference for delays up to 50 ms, with median deviation less than 3.5º (Figure 4a). For the 76ms delay, i.e. when the following calls abuts the leading call, females showed a significant LP (median deviation of 16.2º equivalent to 3 dB amplitude advantage). This situation, however, has not been observed in male choruses (see Discussion), so that this LP is unlikely to influence mate choice. For the alternating situation (200 ms delay), again no significant deviation of the orientation occurred.

In *N. nebrascensis*, females did not show any leader preference. The median deflection was for all delays up to 300 ms below the significance threshold of 6.8º (Figure 4b). At 300 ms delay, the variability increased due to decreasing quality of orientation, as the shortened silent intervals reduced the attractiveness of the stimulation (Deily & Schul 2009).

Male calls in *N. triops* are discontinuous, but neighboring males do not synchronize their chirps; accordingly no leader/follower relationships occur. Females of this species show no leader preference for any of the delays tested (Figure 4c)

Species with continuous calls: We tested two species with continuous calls to test the predictions of the preexisting sensory bias hypothesis. Females of *N. retusus* and *N. bivocatus* species showed in preliminary experiments consistent phonotaxis to discontinuous calls, if durations of chirp and inter-chirp intervals were adequately chosen. This allowed me to test these species for 'hidden' leader preferences. Chirp durations of 1000 ms repeated after silent intervals of 98 ms were attractive for female *N. retusus*. I could not detect any LP for delays between 0 ms and 130 ms (Figure 4d) in this species. Female *N. bivocatus* had low selectivity for chirp pattern and responded well to

chirp/interval durations of 1000/400 ms and 250/100 ms. For both chirp patterns, female had no significant deviation from the midline for any delay tested (Figure 4e,f) *i.e.,* they did not have leader preference.

Discussion

I determined the phylogenetic pattern of occurrences of leader preference in the genus *Neoconcephalus*. I found strong a leader preference in one species (*N. ensiger)* in addition to the previously described leader preference in *N. spiza* (Greenfield & Roizen 1993). The other species with discontinuous calls did not have significant leader preferences. I also found no leader preference in species with continuous calls.

Orientation and choice on the walking compensator

During choice situations on the walking compensator, female *Neoconocephalus* did not walk directly to one loudspeaker, but the vector angle pointed somewhere between the two loudspeakers. If the two signals were equally attractive, the vector angle was close to the midline between the loudspeakers. As the relative attractiveness of one signal increased, the vector turned gradually to this speaker (Figure 2). Other Tettigoniids showed similar behavior in comparable situations (Schul *et al.* 1998, Helversen *et al.* 2001). This orientation behavior is not an artifact of compensated walking on the treadmill: *Poecillimon* katydids showed similar orientation during a choice experiments performed in their habitat as well as on a treadmill (Helversen *et al.* 2001). Thus the

79

deflection away from the midline is a measure for the preference strength of females during choice situations on the walking compensator.

The main advantage of the walking compensator, that females remain during the trials in a fixed position and stimulus situation, prevents them from reaching a loudspeaker (or male) and thus provides a direct measure for their 'choice' (*i.e.* the probability to reach a specific signal or male). To evaluate the preference strength during leader/follower trials, I used the female preferences for louder signals. The relationship between vector angle and amplitude difference was linear and in the same order of magnitude for all species tested (3 to 5 °/dB). Similar experiments with katydids that respond to very different signals (short clicks <1 ms duration) yielded a similar relationship of orientation/amplitude difference (Helversen *et al.* 2001).

Females show significant and consistent preferences to louder signals, if the difference is

≅2 dB (*e.g.* Römer *et al.* 1998). Such amplitude differences lead to approximately 6.8º shift of the vector angle (Table 2). Given the consistent shift of orientation across species/subfamilies, I am confident that a similar shift caused by leader/follower relationship also would result in a significantly higher probability to choose the leading male. I therefore consider median shifts of orientation >6.8º as significant preferences for leading calls.

Absence and Presence of leader preference in Neoconocephalus

I found strong leader preference in *N. ensiger* females for delays between 6 and 24 ms. Delays among pairs of calling males typically fall between 0 and 25 ms, thus the

LP occurs in a behaviorally relevant range of delays. Female orientation was shifted between 10 and 24º towards the leading calls, equivalent to amplitude advantages of 3 to 7 dB. This was as strong, or stronger than the preference for leading calls reported in the katydids *Mecapoda elongata* (Römer *et al.* 2002) and *N. spiza* (Snedden & Greenfield 1998) which could be eliminated by the follower being 6 dB louder. A preference strength of >6 dB means that leading males are more attractive even if they are twice as far away from the female as the following male. This illustrates the selective pressure put on males by the preference for leading calls of *N. ensiger* females.

Male *N. ensiger* compete for the leading position by adjusting the pulse timing on a pulse to pulse basis. Males either advance or delay their next pulse to put it in a leading position, depending on the relative timing of their neighbor's pulse. If the males are evenly matched they will frequently switch or alternate leader/follower roles. If one male is not competitive, he commonly skips pulses to gain the leader position or drops out of the duet, if he cannot match his competitors chirp rate (Thompson N, Murphy M, Schul J, in prep.). The competitive synchrony in *N. ensiger* largely resembles that of *N. spiza* (Greenfield 2005, Greenfield & Schul 2008). It is likely to be the outcome of the female preference for leading calls (Greenfield & Roizen 1993).

I also found a preference for leading calls in *N. exciliscanorus*, which was, however, weaker (16º or 4 dB) and occurred at a single delay only (76 ms). At this delay, the following chirp starts at the end of the leading chirp, *i.e.,* no overlap or silent intervals occur. For all other delays, females tracked the midline between the speakers and had no leader preference. In choruses and duets of *N. exciliscanorus*, leader/follower relationships are stable with delays between 10 and 35 ms. We have never observed

timing relationships representing the abutting calls that resulted in a leader preference

(Schul J, Frederick KH, Guerra P, unpublished). Thus, the observed LP in *N.*

exciliscanorus is biologically irrelevant and will not affect female mate choice. It may,

however, provide an interesting opportunity to understand the neuronal basis of the leader

preference.

The two other species with discontinuous calls (*N. triops, N. nebrascensis*) did not

show any evidence of leader preference. Accordingly, males of *N. nebrascensis, N.*

exciliscanorus, and *N. triops* are not competing for the leading position. Male *N. triops*

are not interacting at all regarding their chirp timing and neighboring males call with

random timing relationships. Neighboring males of *N. nebrascensis* and *N.*

exciliscanorus synchronize their chirps by settling into stable leader/follower

relationships, with the individual starting the calling bout staying the leader (Greenfield

& Schul 2008; Schul H, Frederick KH, Guerra P, unpublished). Potential reasons for this

'cooperative synchrony' include preserving the species specific chirp pattern, or the

beacon effect (Deily & Schul 2009).

Finally, neither of the two species with continuous calls had a "hidden"

preference for leading calls, when tested with stimuli that might reveal it. Note that *N.*

retusus represents the ancestral state of this call pattern, while *N. bivocatus* is most likely

a reversal back to the continuous calls from derived, discontinuous calls (Figure 1).

Phylogenetic pattern of discontinuous calls, LP, and call synchrony

Discontinuous calling has two independent evolutionary origins in *Neoconocephalus*. Within each of the clades with discontinuous calls, subsequent reversals to the continuous pattern occurred (Figure 1). Similarly, the female leader preference evolved at least twice independently, once in each of the 'discontinuous' clades. The species rich temperate clade plus *N. triops* allows me to develop an evolutionary scenario leading to the absence and presence of LP as described here.

Discontinuous calls evolved from the ancestral continuous pattern basally in this clade (arrow in Figure 1). The extant call pattern of *N. triops* could be interpreted as an early or transitional stage: here, only short interruptions of 50-150 ms duration separate relatively long buzzes (0.8-2 s duration). Under some developmental conditions, temperate populations even express continuous calls (Whitesell & Walker 1978; Beckers & Schul 2008). At least for these populations, the 'chirp pattern' is not required for call recognition, as females respond well to continuous calls (Beckers 2008). A proposed function of the call interruptions is to provide a quiet window for listing to other calling males (Michelsen & Larsen 1993).

Within the sister clade to *N. triops*, all species with discontinuous calls have a distinct chirp pattern with silent intervals as long or longer than the chirps. Females of these species require the chirp pattern for recognition (Deily & Schul 2009, Kong *et al.* in prep, KH Frederick, unpublished). Among these species, two evolutionary trajectories exist (Greenfield & Schul 2008), one leading to a LP (in *N. ensiger*), the other to the absence of a LP (*N. exciliscanorus, N. nebrascensis*). Male chirp synchronization matches these differences and evolved likely as consequence of female preferences: In

the absence of a LP, males cooperatively synchronize, likely to preserve the chirp pattern for female recognition (Deily & Schul 2009). If, however, a LP evolved, males compete for the leading position and the resulting competitive synchrony differs significantly from the cooperative synchrony (Greenfield & Schul 2008).

The loss of the chirp structure and reversal to the ancestral continuous call pattern occurred within the clade without LP and with cooperative synchrony. It was likely preceded by the loss of the female preference for chirped calls, as such a female preference would stabilize the male call trait. It is not clear whether both evolutionary trajectories also exist in the second clade with discontinuous calls, as *N. saturatus* has not been tested for LP yet. However, anecdotal observations of male calling behavior suggest male *N. saturatus* cooperatively synchronize their chirps (J. Schul, pers. comm.). This would suggest that no LP exist in this species.

Origin of the leader preference

The phylogenetic pattern of leader preferences suggest that they evolved after the evolution of discontinuous calls. This, and the absence of leader preferences in continuously calling species, argues against a preexisting sensory bias hypothesis, which predicts that a hidden preference for leading calls should exist before call discontinuous calls evolve. Thus, directional processing in the thoracic ganglion resulting in a LP, as it was hypothesized for *Mecapoda* (Hartbauer *et al.* 2012; Römer *et al.* 2002) is most likely not the case in *Neoconocephalus*.

A likely explanation for the leader preferences in this group is that females receive indirect (=genetic) or direct (=non-genetic) benefits (Anderson & Simmons 2006)

from choosing a leading male. As the leading position is the outcome of competitive interactions, it is a costly trait and potentially an honest indicator of male quality. The benefits hypothesis makes testable predictions about fitness gains of mating with the leader, which could be tested in breeding experiments (*e.g.* Welch *et al.* 1998).

An alternative explanation for the LP is that during the evolution of chirp recognition neuronal circuitry was incorporated in the call recognition mechanisms which, in the ancestral state, was not involved in call processing. Candidates would be, for example, neurons encoding predator signals. These circuits may introduce a bias to leading calls into call processing that may result in a leader preference. Thus, the leader preference would evolve as an emergent property of evolutionary change of call recognition without inherent adaptive value. A recent neurophysiological study (Hartmann 2013) suggests that one auditory interneuron (TN1), which likely functions in bat detection, shows a strong leader bias when stimulated with the *N. ensiger* temporal pattern. As it encodes the chirp rate of *N. ensiger* calls, it appears a likely candidate for incorporation into the *N. ensiger* recognition mechanisms. Comparative studies of the role of this neuron for female phonotaxis could test this hypothesis.

Table 1: Species specific stimuli generated from known female preferences.

Species	Year Tested	Collecting Locality	Chirp Duration (ms)	Internal Duration (ms)	Stimuli Pulse Structure	Delays Tested (ms)	Carrier Freqency (kHz)	Female Preference Citation
N. bivocatus	2008	Boone Co. Mo	1000	400	7.5ms pulses	0, 23, 46, 103, 195, 299	9 to 15	Deily and Schul 2004
			250	100	7.5ms pulses	0, 11.5, 34.5, 46	9 to 15	
N. ensiger	2010-11	Adair Co. Mo	35	39	Noise Burst	0, 6, 12, 18, 24, 40	10 to 20	Kong and Schul unpublished
N. exciliscanorus	2010-12	Stoddard Co. Mo	76	316	Noise Burst	0, 15, 30, 50, 76	9 to 15	Frederick and Schul unpublished
N. nebrascensis	2007	Boone Co. Mo	1000	800	3ms pulses	0, 50, 150, 300	9 to 15	Deily and Schul 2009
			250	200	3ms pulses	0, 12.5, 37.5, 75	9 to 15	
N. retusus	2008	Boone Co. Mo	1000	98	3.5ms pulses	0, 28, 49, 70, 98, 130	10 to 20	Bush et al. 2010
N. triops	2010	Lab Reared	1000	150	6.5ms pulses	0, 27, 54, 81, 135, 325	9 to 15	Beckers and Schul 2010

Table 2: Angular deviation response to amplitude differences (dB SPL)

Species	Angle*dB	t-value	p-value	Sample Size
N. bivocatus	3.15	11.43	0.02	9
N. ensiger	3.67	13.64	0.02	10
N. exciliscanorus	3.63	4.03	0.07	5
N. nebrascensis	4.28	9.78	0.03	8
N. retusus	5.08	9.41	0.03	4
N. triops	3.74	4.63	0.06	5
POOLED	**3.40**	**12.98**	**0.02**	**41**

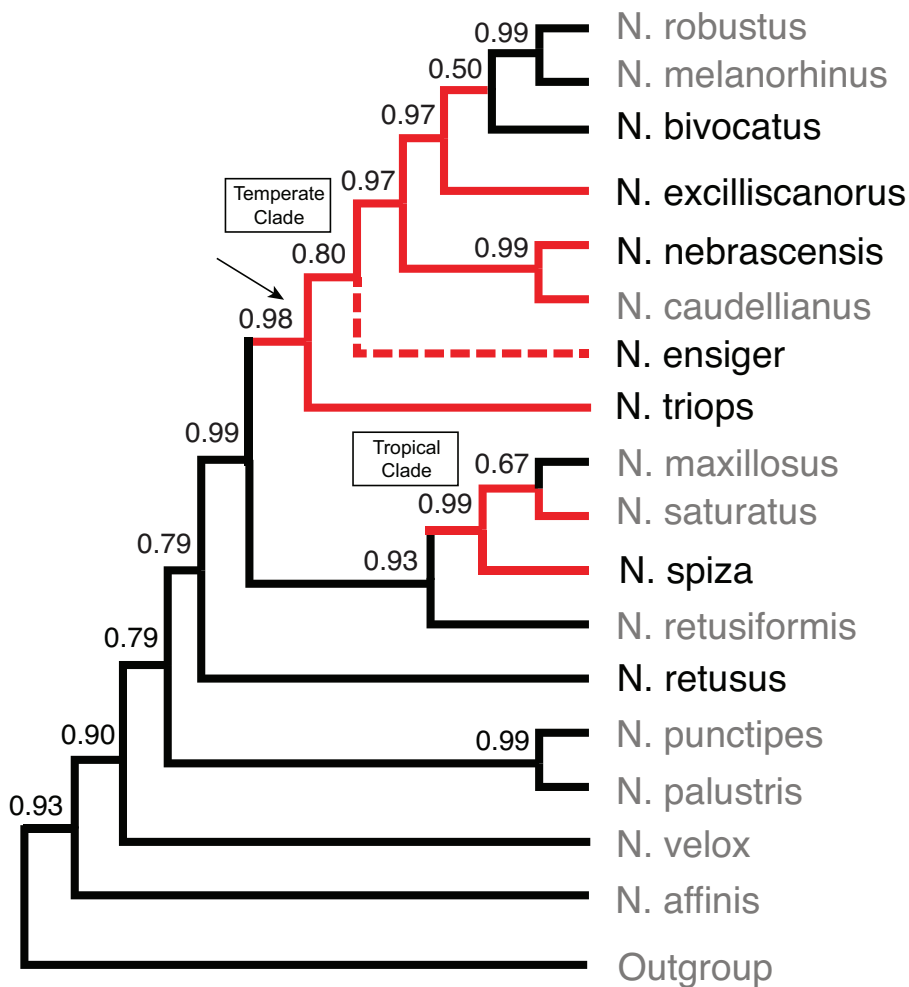p-values are one-tailed calculated from Student's t-test values.

Figure 1: *Neoconocephalus* character state reconstruction of discontinuous calls. Bayesian total evidence *Neoconocephalus* phylogeny from Snyder *et al.* (2009). Red branches indicate discontinuous calling, values at nodes indicate the probability of the state at that node. There are two independent origins of discontinuous calling. Black species names indicate study species. *N. ensiger* is treated as discontinuous because it is perceived as a chirp- single noise burst followed by silent interval.
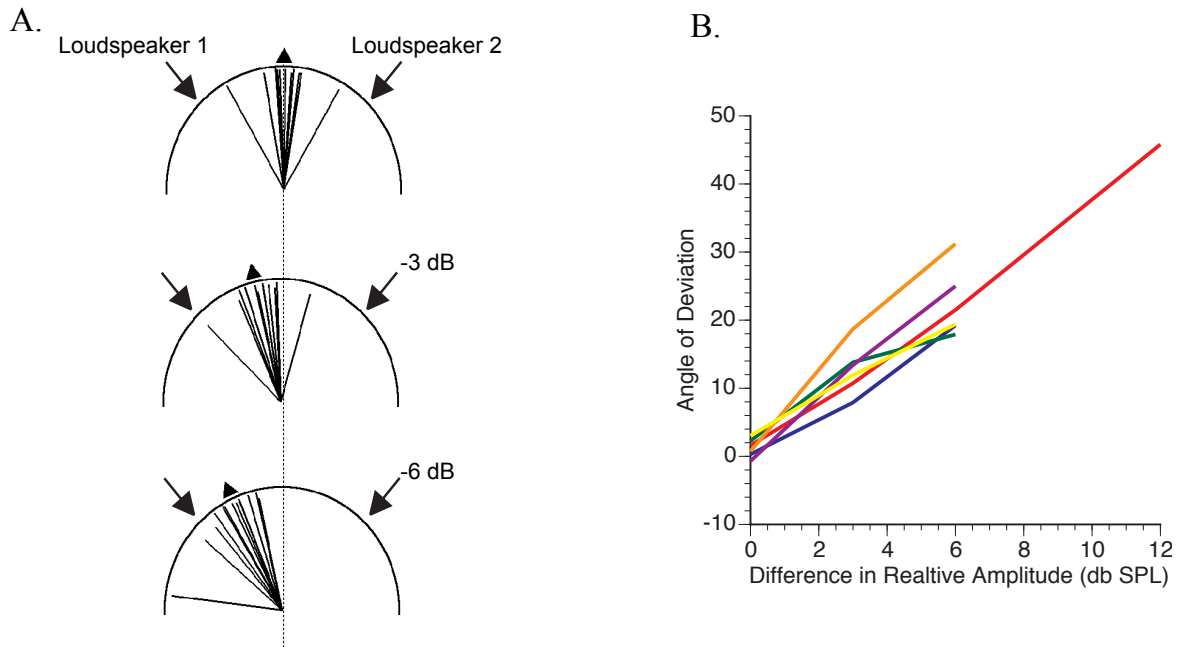
Figure 2

A.

B.

Figure 2: Linear relationship of amplitude differences A- Shows the traces of a *N. nebrascensis* female walking on the 'kugel' spherical walking compensator during behavioral experiments where loudspeakers differ in amplitude. The black triangle indicates the vector angle. Dashed lines indicate the centerline. The female steers towards loudspeaker 1 as the amplitude difference increases between speakers. B- Median angle of deviation per relative amplitude difference at 0, 3 and 6 dB SPL for each study species; *N. bivocatus* - blue, *N. ensiger*- red, *N. exciliscanorus*- green, *N. nebrascensis*- purple, *N. retusus*,- orange, *N. triops*- yellow.
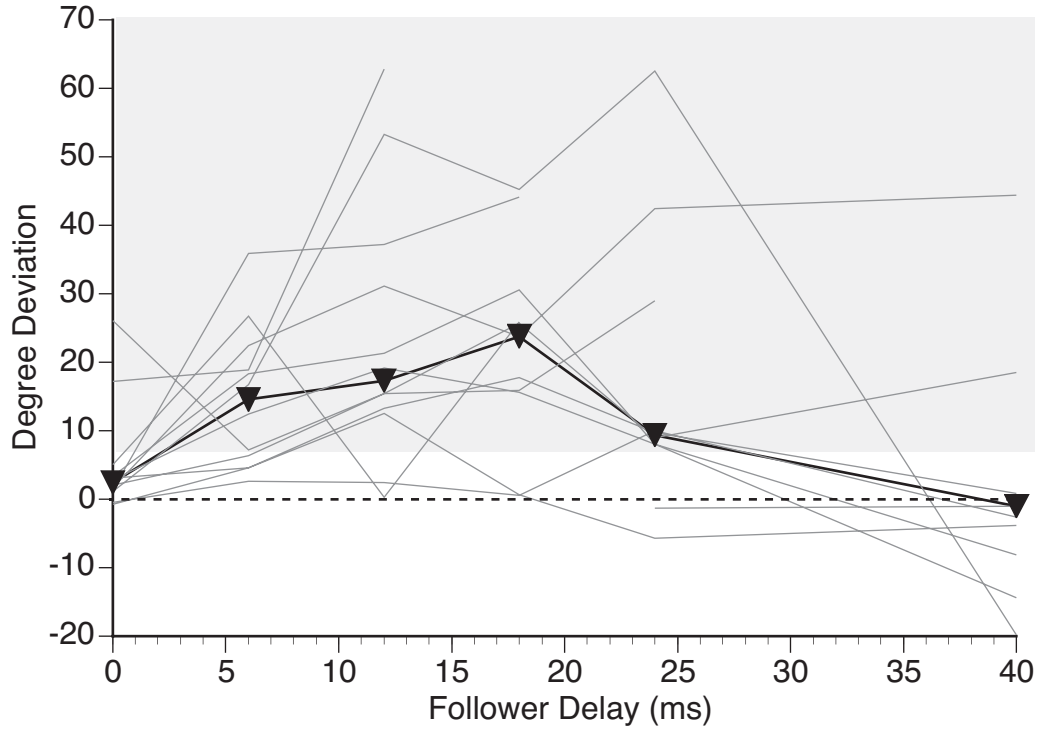
Figure 3: *N. ensiger* female responses to leader/follower duets. Twelve individual female responses are shown with thin grey lines and the median response is reported by a thick black line tested at delayed onsets of 0, 6, 12, 18, 24 and 40 ms. Significant leader preference is indicated by an angle of orientation greater than 6.8° (grey box).

Figure 4
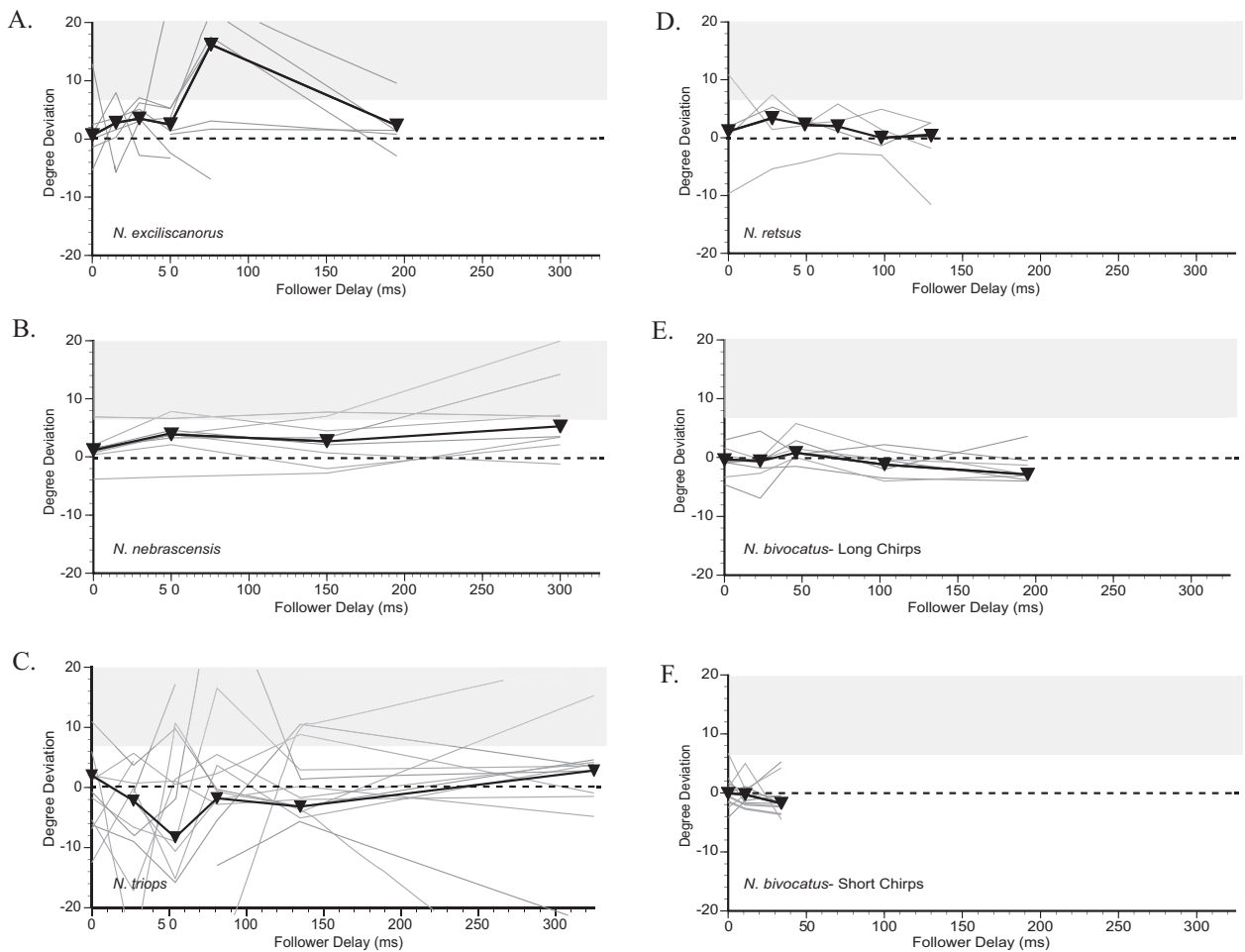
Figure 4: Female responses to leader/follower duets. Two choice tests where females were presented with simultaneous signals with differing onsets (Table 1). Individual responses for each female are shown in thin grey lines, the median response is a thick black line. Significant leader preference is indicated by an angle of orientation greater than 6.8° (grey box). A-C represents discontinuous calling species: A. *N. exciliscanorus*- Eight females were tested finding leader preference only at 76 ms delay. B. *N. nebrascensis* (N=8) no LP. C. *N. triops* (N=14) non-synchronous species, no LP. D-F represent continuous calling species, D. *N. retusus* (N=4) no LP and E. *N. bivocatus* (N=8) tested with 1000 ms chirps, no LP. F. *N. bivocatus* (N=9) tested with shorter duration 250 ms chirps no LP.

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Chapter 4

The interaction between genetic drift and genetic architecture of the *Neoconocephalus* acoustic communication resulted in rapid phenotypic diversification.

Overview

In *Neoconocephalus* most species' defining traits are associated with acoustic communication. Little genetic divergence is needed to generate these species-defining call phenotypes (Chapter 1), (Carson and Templeton 1984; Templeton *et al.* 1992). The derived call traits (double pulse pattern, slow pulse rate, and discontinuous call pattern) all have multiple independent origins (Chapter 2), and derived female preferences may also involve many convergent evolutionary events (Chapter 3). Additionally, drift may drive diversification in male call traits not driven by sexual selection alone. Diversification of male call traits can occur without female selective pressure. The pliability of the acoustic communication system has the "potential" for rapid diversification but calls traits repeatedly converge on similar phenotypes instead of

96

forming new phenotypes. The ways in which call traits can change are likely constrained, thus limiting the possibility of novel traits (see Chapter 1) The diversification of the full call repertoire in *Neoconocephalus* communication has occurred in two clades since the Last Glacial Maxima (LGM) (see Chapter 2).

Below I describe the phenotypic patterns of species radiations in biogeographical hotspots. Specifically, I compare the African Laucasutrine Cichlid (Perciformes: Cichildae) radiation, which occurred after the Last Glacial Maxima, with the *Laupala* cricket (Orthoptera: Gryllidae) radiation event that occurred on the Hawaiian Islands. Then I describe phenotypic diversity of secondary sexual characters (acoustic communication) in *Neoconocephalus*. I evaluate the rapid radiation of *Neoconocephalus* diversity and the possibility of a temperate biogeographical hotspot. Finally, I suggest future directions for research in *Neoconocephalus* focusing on phenotypic diversification and investigating the genetic architecture and evolutionary mechanisms in place that allowed rapid evolution in this genus.

Evolution of a key innovation, dispersal into a new habitat or extinction of antagonists are Simpson's suggested triggers for an adaptive radiation (Simpson 1940, 1953). Adaptive radiations or radiation events result in a group of organisms diversified into many new forms all sharing a single common ancestor. The number of new forms is only limited by the available niche space (Brooks and McLennan 2002). Although we commonly use the term adaptive to imply new forms generated through radiation as a result of natural selection, many different evolutionary mechanisms have been proposed to generate the evolutionary novelties observed as a result of a radiation.

97

Additionally, these supposed rapid diversification event or radiations occur at timescales that differ by orders of magnitude and results in various amounts of phenotypic diversity and species richness. Comparing radiation events and resulting phenotypic variation, I propose a mechanism for *Neoconocephalus* radiation and a possible correlation between young radiation events and great amounts of phenotypic diversity, as is seen in cichlids and temperate *Neoconocephalus* species.

Diversification at differing timescales

African Lacaustrine Cichlids- More than 3,000 species of cichlids occur in the lakes of the African Rift Valley making this area known as a hotspot of biodiversity (Seehausen 2006). Lakes in this area function as islands along a region of unstable tectonic plates. Cichlid radiation events are tied to the drastic ecological changes that have occurred in these lakes over the last 10 myrs. Most lakes in the African Rift Valley have had independent radiation events (Wagner *et al.* 2012), Some of these radiation events have occurred as recently at 10Kya, this recent radiation was likely linked to the desiccation of many lakes during the LGM (Seehausen 2006).

In addition to many ecological opportunities, the evolution of pharyngeal jaw apparatus was a key morphological innovation. Evolution of this jaw allows cichlids to exploit many new niches through variation in muscle and ligament attachment altering their prey capturing abilities (Danley and Kocher 2001). The resulting diversification in cichlids has occurred at a rate of one new species every 46 years (230 species in the last 10kyr; Seehausen 2006). Scale coloration and patterns are extremely diverse in African cichlids, usually most diverse between closely related species. Scale coloration and

patterns are critical for mate detection, recognition and choice. There are many cases of convergent evolution of male coloration and female preferences observed between lakes. Coloration phenotypes are diverse even with little genetic divergence between the species (incomplete lineage sorting in young species) the differences in these phenotypes is primarily qualitative throughout their evolutionary history (Kocher 2004). In this cichlid system convergent evolution of color pattern is common and can even be predicted given community dynamics and an understanding of the evolutionary pathways that lead this phenotypic diversity (Gillispie 2013).

Hawaiian Laupala crickets- The Hawaiian Islands are recognized as a hotspot of biodiversity, and the formation of new islands through volcanic activity provides a unique opportunity to understand the natural history of the islands. Many different species complexes have experienced radiation events across these islands. Most of these event show similar patterns of speciation and colonization occurring over the last 5 myr as species travel from the oldest to youngest islands (Fleischer *et al.* 1998). Older island communities are often more closely related and less phenotypically diverse (process of specialization). Here I will use the 'explosive radiation' of *Laupala* crickets (Mendelson and Shaw 2005) as a comparison of diversity to the recent cichlid radiations. *Oliaris* (Hawaiian cave dwelling plant hoppers) have a speciation rate an order of magnitude faster than *Laupala,* however, little is known about the phenotypic diversity in this system (Wessel *et al.* 2013).

Laupala crickets have species specific male advertisement calls that vary in pulse rate (Shaw 1996). These calls are a key component of courtship, used to attract females,

99

and are the primary distinguishing trait between these sympatric ecologically similar species (Brooks and McLennan 2002). AFLP and QTL analyses show that the genetic information associated with differences in pulse rate (quantitative trait in *Laupala*) between species are associated with 8 loci (Shaw and Lesnick 2009). The 'rapid' evolution of divergence in *Laupala* communication results of many small synchronous genetic changes between female preferences and male calls through genetically coupling (Lande 1981; Oh *et al.* 2012; Shaw and Parsons 2002; Templeton 1981).

It appears that the quantitative vs. qualitative phenotypic differences between *Laupala* calls and cichlid coloration are due to the timescale at which we are observing each system. Both examples have similarities in the phenotypic diversification of secondary sexual traits, and in both systems the rapid diversification of these traits has been attributed to the interaction between ecological and sexual selection (Oh *et al.* 2012; Seehausen 2006).

Laupala speciation events occurred over millions of years, resulting in extant species that are ecologically similar and vary primarily by quantitative acoustic communication phenotypes (Brooks and McLennan 2002; Mendelson and Shaw 2005). A similar pattern is found in the *Tetragnatha* spiders that also radiated across the Hawaiian islands (Gillespie 2004), where similarity in spider communities varied by island age. On young islands species were randomly assorted into guilds by prey-capturing phenotypes. As the islands aged, communities became more closely related and phenotypic diversity between species became more quantitative and specialized through local extinctions and replacement during habitat succession (Gillespie 2004). This pattern of specialization in older communities has also been observed in cichlids that inhabit Lake Tanganyika (the

oldest lake) of the African Rift Valley (Brooks and McLennan 2002).

Neoconocephalus diversification

Qualitative traits of both female preference and male calls make up much of the diversity in *Neoconocephalus* acoustic communication. Comparing young sympatric species we have found that derived male call traits are inherited independently. In this study we also found distinct phenotypes that define species even when the species are otherwise not genetically distinct (incomplete lineage sorting) providing evidence for Type II genetic architecture. Type II architectures (few loci with big impact) occur when a small population size is exposed to drift and traits change rapidly, as seen in *Neoconocephalus.* Additionally, the derived traits evolve repeatedly and rapidly and may be convergent due to parallel evolution (shared a genetic pathway) common when similar traits evolve in adaptive radiations (Gilliespie 2013) (Figure 1A. & 2A.). Finally, female preferences do not drive male call trait diversity, in fact there are multiple occurrences of species with derived male calls traits and females with ancestral call preferences e.g. *N. retusus* and *N. maxillosus* in Figure 1 (Bush and Schul 2010) and *N. triops* in Figure 2 (Beckers 2008). Mapping male call trait diversity and known female preferences on to the phylogeny we have little if any evidence for genetic linkage between sender and receiver as seen in the *Laupala* system (Oh *et al.* 2012).

Five species of *Neoconocephalus* have male calls with the derived double pulse pattern, but only three of those species have female preference for double pulses. Additionally, the three species with female preference for double pulse pattern have three different recognition mechanisms to interpret the pattern. These are cases of derived

101

males traits paired with ancestral female preference in both pulse pattern and call pattern phenotypes, providing little evidence for genetic coupling between signal and receiver and indicating that male call traits are not driven by female preference. Alternatively, the genetic architecture of acoustic communication traits in combination with vicariance events likely generated the call variation found in extant *Neoconocephalus*.

Receding glaciers and succession of grasslands into North America and subsequent climate fluctuation during the Younger Dryas, caused temperate North America to be a hotspot of biodiversity, provided grassland species with many ecological opportunities for niche expansion. Capable of flying great distances, *Neoconocephalus* species could easily colonize the tropical grasslands. Adaptation of egg diapause (key innovation) allowed invasion of the temperate habitats. Founder effect and small population sizes greatly influence traits with Type II genetic architecture (few loci with large effect) like male call traits in *Neoconocephalus* (Carson and Templeton 1984; Roderick and Gillespie 1998; Templeton 1981). Founding *Neoconocephalus* were likely small and isolated populations with randomly divergent calls spread across the expansive open habitat. Over time, as call traits established and small populations grew, species with divergent calls could share habitats. Grassland communities may host many species of *Neoconocephalus* seemingly only limited by availability of an acoustic niche, constrained by the ways in which call traits can change.

As species colonize a new area, genetic drift and small population sizes can generate large amounts of variation, both allelic and phenotypic. The grassland communities in this study are still quite young, which likely explains why most diversity in acoustic communication is from qualitative traits. The evolutionary history of

102

phenotypic diversity in *Neoconocephalus* acoustic communication is similar to the diversity in coloration and patterns in cichlid fish and also has diversified in a similar timescale. In both clades the phenotypic patterns are likely explained by the influence of drift, as high amounts of convergence are often found in clades diversifying through drift (Stayton 2008). Dominey (1984) suggested that the variation seen in these secondary sexual traits of cichlids is also the result of Type II qualitative traits and vicariance events (Seehausen 2006). As niches are filled the speciation rate will slow down limiting further colonization. As is often observed in older communities of cichlids, *Neoconocephalus* communities may also become more specialized and their quantitative traits may influence the shape of sender or receiver phenotypes.

Future Directions

Expanding the *Neoconocephalus* phylogeny further to include *Ruspoila* (old world coneheaded katydids) would allow for the investigation of phenotypic diversification through time in communities that have experienced radiations. I would like to expand the *Neoconocephalus* phylogeny to include endemic species from North America, South America and Old World *Ruspolia.* Then look for similar patterns of diversification during the South American colonization of cone-headed katydids from Africa. Acoustic niche availability in *Neoconocephalus* determines the community structure (Schul unpublished). Grassland communities in North and Central America (*e.g.* New Jersey, Missouri, Costa Rica, Panama) commonly contain five species with four call phenotypes the ancestral call phenotype and each of the three derived call traits. Additionally, there is often a species that has found a new acoustic niche by calling later in the season or later

at night (allochronic). I would like to investigate patterns of phenotypic diversity in older communities and whether these communities have lost phenotypic diversity, or if they have gained species by further partitioning the acoustic niches through quantitative traits diversity like that observed between species from older radiation events like those that occurred on the Hawaiian Islands.

Convergent evolution is common in populations that experience drift, founding populations that colonize of a new continent definitely experience drift. *Neoconocephalus* makes a great study system because of the ease in which they can be collected and kept in culture, the phenotypic diversity of their acoustic communication system and the simplicity of their neurological system as a model for sensory ecology. Transcriptomics allow for studies of gene expression even in non-model systems and is the logical next step in this system, allowing for gene expression and search of candidate genes. I would like to look at the prevalence of convergent evolution in temperate species of *Neoconocephalus* starting with the key innovation that allowed for colonization of North America, egg diapause. I would like to compare the differences in gene expression between species with and without egg diapause. Sampling four species (two tropical and two temperate) and comparing gene expression among ground embryos at two developmental stages for each species (pre-diapausing and diapausing). A similar Orthopteran study, *Allonemobius socius* (Reynolds and Hand 2009), found that pre-diapausing eggs and eggs that will never diapause (tropical life history) show similar gene expression. Four species were chosen because of their phylogenetic relationship and life history. Tropical *N. punctipes* is sister to temperate *N. palustris* (has egg diapause) these two species will make a good comparison because of their close relationship and

differing developmental stages. Additionally, two more species with egg diapause will be selected, *N. retusus* and *N. robustus* a species from the temperate radiation. Comparing the gene expression of the three temperate species will determine if independent origins of egg diapause and colonization of North America result from the activation of the same genetic pathway or simply share a phenotype through convergence. Differences in expression of 8 genes were found to promote or regulate diapause in the cricket.

Bias in the production of phenotypes even under selective pressure, is due to constraint. Neurophysiological constraint likely limits variation of call phenotypes in this system. Tests of species-specific female preferences have been completed for many species in the *Neoconocephalus* phylogeny. Characterizing these preferences in a comparative manner (*e.g.* how pulse rate preferences differ between species) would determine the amount of variation in this system. Preliminary evidence suggests that there may be more ways to determine a preference for a pattern than there are to generate a pattern. Muscle movements that generate a call pattern are synchronized by a central pattern generator. We do not yet know the effect of mutations to the CPG or its influence on female preference and recognition mechanisms. Studies of neurophysiological experiments to investigate temporal filters in the brain will help us understand pattern recognition in female *Neoconocephalus*. These experiments are more effective when informed by a well-supported phylogeny; understanding species relationships and pleisiomorphic traits help target the most interesting species and traits to be tested. Ultimately, I would like to use comparative methods to categorize female preferences and show over all diversity in acoustic communication in this genus highlighting the disparity between female preferences and male calls and the evolutionary mechanisms that led to

such variation.

Ultimately, I would like to study genomic patterns of phenotypic diversity. Behavioral systems, like those that have acoustic communication, provide an excellent opportunity to compare patterns of diversity across many species as well as within a single species. This is a seemingly untapped field, where the phenotypic diversity has been thoroughly studied with little correlation to genetic relationships between individuals. Studies in comparative transcriptomics or other genomic techniques for non-models systems are definitely the next step in behavioral ecology. I hope to develop techniques and toolkits to carry my lab in this direction.
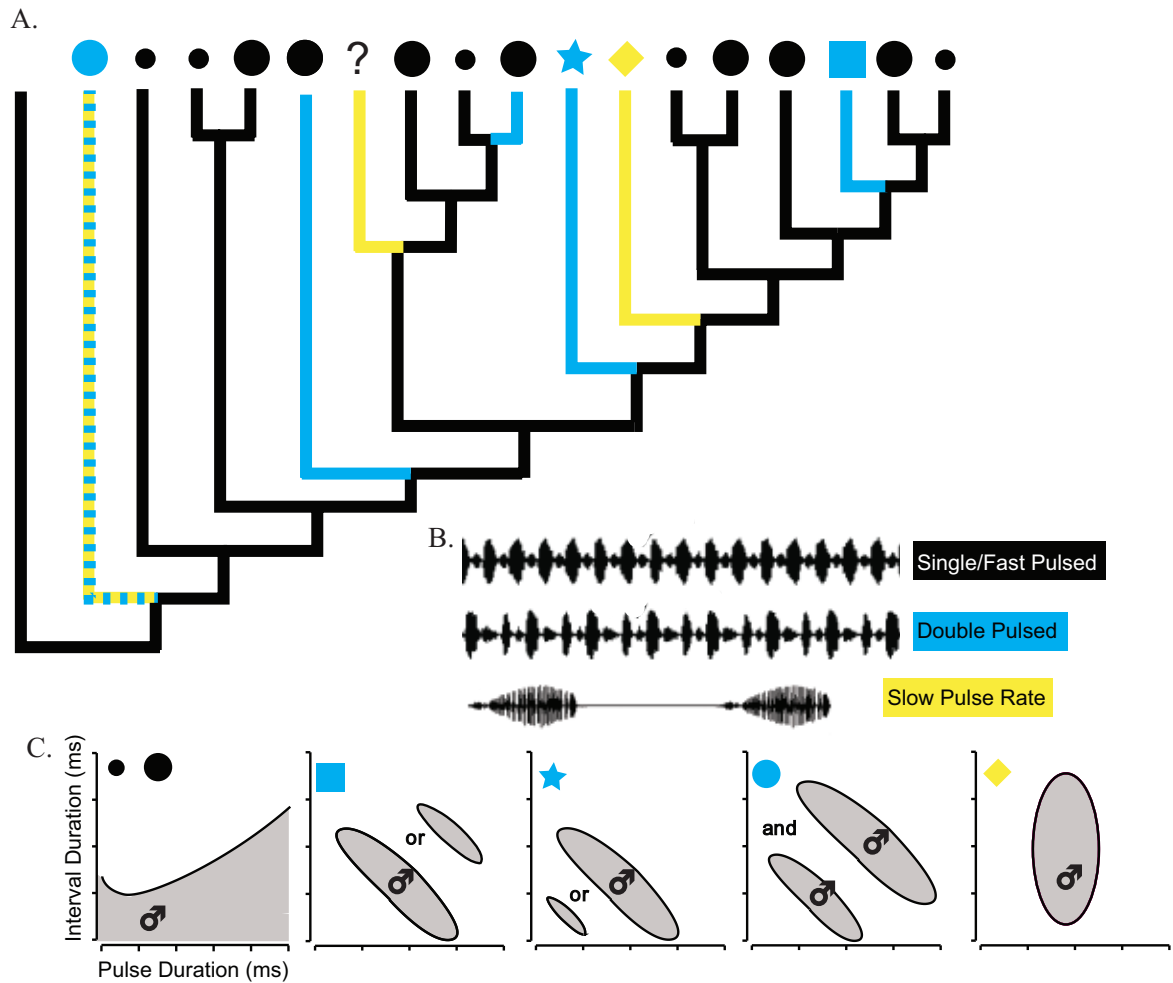
Figure 1

Figure 1: Two call traits (Pulse Pattern and Pulse Rate) and female preferences mapped onto the *Neoconocephalus* phylogeny. A. Branch colors correspond with male call phenotypes shown in (B). Symbols at tips correspond with the female preference fields show in (C). B. Traces of temporal call phenotypes at the millisecond time scale. C. Schematics of positive female phonotaxis fields (grey area) based on interval and pulse duration. These fields are intended to show the diversity of female preferences and do not represent actual values. Male symbols shows where average male calls for that species.

The ancestral state (black dots) females prefer a minimal interval duration. Small black dots indicate hypothesized preferences based on male call trait, behavioral tests of female preference have not been completed for those species.
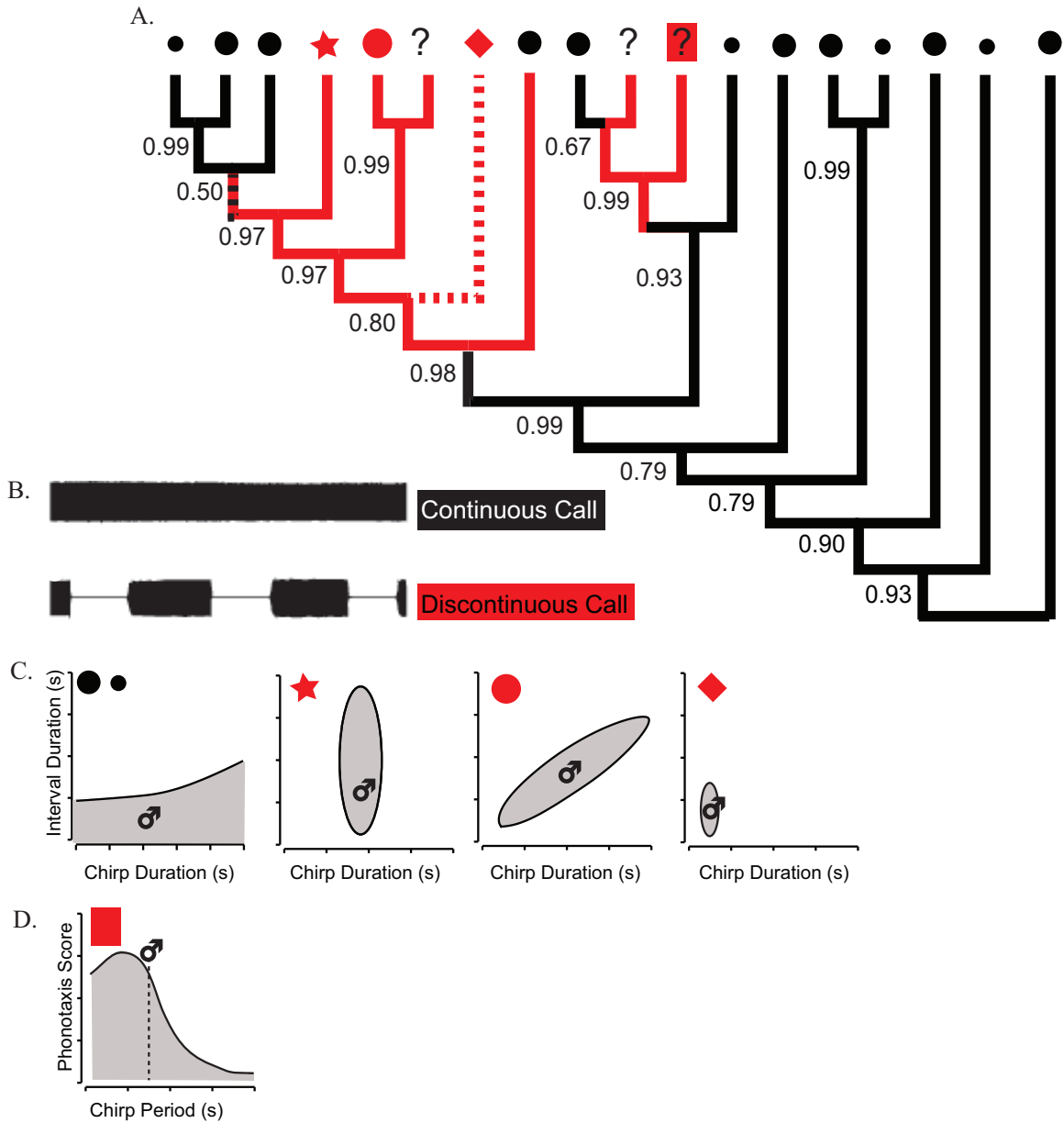
Figure 2

Figure 2: Male chirp pattern and female chirp preference mapped onto *Neoconocephalus* phylogeny. A- Red branches indicate discontinuous calls (call with chirps), hatched branch is N. ensiger the female percieve the call as discontinuous and have a preference for chirps (C: diamond shape) Symbols at tips correspond with the female preference

fields show in (C). B- Traces of continuous and discontinuous male calls on a second time scale. C- Schematics of positive female phonotaxis fields (grey area) based on interval and chirp duration. These fields are intended to show the diversity of female preferences and do not represent actual values. Male symbols shows where average male calls for that species. The ancestral state (black dots) females prefer a minimal interval duration. Small black dots indicate hypothesized preferences based on male call trait, behavioral tests of female preference have not been completed for those species. Question marks indicate unknown chirp preference. D- Chirp and interval duration prefernce in *N. spiza* has not yet been fully studied. Instead we show that females of *N. spiza* have directional selection for fast chirps (faster chirps than the males produce, male chirp period is indicated by the dashed line).

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Appendix: Taxa list for COI and Mitogenome datasets

Genus	Species	Order	Suborder	Family	Subfamily	Mitogenome	COI
Acrida	cinerea	Orthoptera	Caelifera	Acrididae	Acridinae	GU344100.1	GU344100.1
Acrida	willemsei	Orthoptera	Caelifera	Acrididae	Acridinae	EU938372.1	EU938372.1
Phlaeoba	albonema	Orthoptera	Caelifera	Acrididae	Acridinae	EU370925.1	EU370925.1
Calliptamus	italicus	Orthoptera	Caelifera	Acrididae	Calliptaminae	EU938373.1	GQ355945.1
Traulia	szetschuanensis	Orthoptera	Caelifera	Acrididae	Catantopinae	EU914849.1	EU914849.1
Schistocera	gregaria	Orthoptera	Caelifera	Acrididae	Cyrtacanthacridinae	GQ491031.1	
Arcyptera	coreana	Orthoptera	Caelifera	Acrididae	Gomphocerinae	GU324311.1	GU324311.1
Chorthippus	chinensis	Orthoptera	Caelifera	Acrididae	Gomphocerinae	EU029161.1	EU029161.1
Euchorthippus	fusigeniculatus	Orthoptera	Caelifera	Acrididae	Gomphocerinae	HM583652.1	JQ301458.1
Gomphocerippus	rufus	Orthoptera	Caelifera	Acrididae	Gomphocerinae	GU294759.1	GU294759.1
Gomphocerus	licenti	Orthoptera	Caelifera	Acrididae	Gomphocerinae	GQ180102.1	GQ180102.1
Gomphocerus	sibiricus	Orthoptera	Caelifera	Acrididae	Gomphocerinae	HM131804.1	HM131804.1
Hypochlora	alba	Orthoptera	Caelifera	Acrididae	Melanoplinae	EU914848.1	EU914848.1
Melanoplus	infantilis	Orthoptera	Caelifera	Acrididae	Melanoplinae	AF260536.1	AF260536.1
Melanoplus	infantilis	Orthoptera	Caelifera	Acrididae	Melanoplinae		AY083412.1
Ognevia	longipennis	Orthoptera	Caelifera	Acrididae	Melanoplinae	AF260548.1	AF260548.1
Gastrimargus	marmoratus	Orthoptera	Caelifera	Acrididae	Oedipodinae	EU513373.1	EU513373.1
Locusta	migratoria	Orthoptera	Caelifera	Acrididae	Oedipodinae	X80245.1	
Oedaleus	decorus	Orthoptera	Caelifera	Acrididae	Oedipodinae	EU513374.1	EU513374.1
Oxya	chinensis	Orthoptera	Caelifera	Acrididae	Oxyinae	EF437157.1	EF437157.1
Pielomastax	zhengi	Orthoptera	Caelifera	Episactidae	Episactinae	JF411955.1	JF411955.1
Vandiemenella	viatica	Orthoptera	Caelifera	Morabidae	Morabinae		EU121441.1
Vandiemenella	viatica	Orthoptera	Caelifera	Morabidae	Morabinae		EU121467.1
Vandiemenella	viatica	Orthoptera	Caelifera	Morabidae	Morabinae		FJ605707.1
Warramaba	virgo	Orthoptera	Caelifera	Morabidae	Morabinae		DQ308528.1
Warramaba	virgo	Orthoptera	Caelifera	Morabidae	Morabinae		DQ308516.1
Warramaba	virgo	Orthoptera	Caelifera	Morabidae	Morabinae		DQ308527.1
Warramaba	virgo	Orthoptera	Caelifera	Morabidae	Morabinae		
Prionotropis	hystrix	Orthoptera	Caelifera	Pamphagidae	Thrinchinae		GU122505.1
Thrinchus	schrenkii	Orthoptera	Caelifera	Pamphagidae	Thrinchinae	GU181288.1	GU181288.1
Thrinchus	schrenkii	Orthoptera	Caelifera	Pamphagidae	Thrinchinae		GU181288.1
Physemacris	variolosa	Orthoptera	Caelifera	Pneumoridae		GU945504.1	GU945504.1
Atractomorpha	sinensis	Orthoptera	Caelifera	Pyrgomorphidae	Pyrgomorphinae	EU263919.1	EU263919.1

Mekongiana	*xiangchengensis*	Orthoptera	Caelifera	Pyrgomorphidae	Pyrgomorphinae	HM583653.1	JQ301461.1
Mekongiella	*xizangensis*	Orthoptera	Caelifera	Pyrgomorphidae	Pyrgomorphinae	HM583654.1	JQ301462.1
Xyleus	*modestus*	Orthoptera	Caelifera	Romaleidae	Romaleinae	GU945503.1	GU945503.1
Saussurella	*borneensis*	Orthoptera	Caelifera	Tetrigidae	Batrachideinae	EU414813.1	EU414813.1
Bolivaritettix	*guibeiensis*	Orthoptera	Caelifera	Tetrigidae	Metrodorinae	FJ545408.1	FJ545408.1
Ellipes	*minuta*	Orthoptera	Caelifera	Tridactylidae	Tridactylinae	GU945502.1	
Tristira	*magellanica*	Orthoptera	Caelifera	Tristiridae	Tristirinae		JX913765.1
Penalva	*flavocalceatus*	Orthoptera	Ensifera	Anostostomatidae	Anostostomatinae		EU676759.1
Gryllus	*assimilis*	Orthoptera	Ensifera	Gryllidae	Gryllinae		EF050744.1
Gryllus	*multipulsator*	Orthoptera	Ensifera	Gryllidae	Gryllinae		EF050741.1
Gryllus	*ovisopis*	Orthoptera	Ensifera	Gryllidae	Gryllinae		GOU88333
Gryllus	*pennsylvanicus*	Orthoptera	Ensifera	Gryllidae	Gryllinae		GOU88333
Gryllus	*rubens*	Orthoptera	Ensifera	Gryllidae	Gryllinae		U88332.1
Gryllus	*rubens*	Orthoptera	Ensifera	Gryllidae	Gryllinae		AY234796.1
Gryllus	*rubens*	Orthoptera	Ensifera	Gryllidae	Gryllinae		AY234793.1
Gryllus	*texensis*	Orthoptera	Ensifera	Gryllidae	Gryllinae		AY234789.1
Gryllus	*texensis*	Orthoptera	Ensifera	Gryllidae	Gryllinae		AY234804.1
Gryllus	*texensis*	Orthoptera	Ensifera	Gryllidae	Gryllinae		AY234805.1
Gryllus	*veletis*	Orthoptera	Ensifera	Gryllidae	Gryllinae		AY234802.1
Itaropsis	*parviceps*	Orthoptera	Ensifera	Gryllidae	Gryllinae		U88334.1
Itaropsis	*parviceps*	Orthoptera	Ensifera	Gryllidae	Gryllinae		JN411870.1
Itaropsis	*parviceps*	Orthoptera	Ensifera	Gryllidae	Gryllinae		JN411868.1
Teleogryllus	*commodus*	Orthoptera	Ensifera	Gryllidae	Gryllinae		JN411857.1
Teleogryllus	*emma*	Orthoptera	Ensifera	Gryllidae	Gryllinae		GU592222.1
Teleogryllus	*infernalis*	Orthoptera	Ensifera	Gryllidae	Gryllinae	EU557269.1	EU557269.1
Gryllotalpa	*orientalis*	Orthoptera	Ensifera	Gryllotalpidae	Gryllotalpinae	AY660929.1	AY660929.1
Gryllotalpa	*pluvialis*	Orthoptera	Ensifera	Gryllotalpidae	Gryllotalpinae		JQ301446.1
Myrmecophilus	*manni*	Orthoptera	Ensifera	Myrmecophilidae	Myrmecophilinae	EU938370.1	EU938370.1
Troglophilus	*cavicola*	Orthoptera	Ensifera	Rhaphidophoridae	Troglophilinae	EU938371.1	EU938371.1
Troglophilus	*neglectus*	Orthoptera	Ensifera	Rhaphidophoridae	Troglophilinae	EU938374.1	AY793624.1
Ceuthophilus	*gracilipes*	Orthoptera	Ensifera	Rhaphidophoridae	Ceuthiphilinae		AY793593.1
Dolichopoda	*steriotisi*	Orthoptera	Ensifera	Rhaphidophoridae	Dolichopodainae		EF217016.1
Dolichopoda	*steriotisi*	Orthoptera	Ensifera	Rhaphidophoridae	Dolichopodainae		EF217017.1
Stenopelmatus	*mahogani*	Orthoptera	Ensifera	Stenopelmatidae	Stenopelmatinae		EF030182.1
Stenopelmatus	*mahogani*	Orthoptera	Ensifera	Stenopelmatidae	Stenopelmatinae		EF030184.1

Stenopelmatus	mahogani	Orthoptera	Ensifera	Stenopelmatidae	Stenopelmatinae		EF030162.1
Stenopelmatus	mahogani	Orthoptera	Ensifera	Stenopelmatidae	Stenopelmatinae		EF030116.1
Stenopelmatus	mahogani	Orthoptera	Ensifera	Stenopelmatidae	Stenopelmatinae		EF030178.1
Stenopelmatus	mahogani	Orthoptera	Ensifera	Stenopelmatidae	Stenopelmatinae		EU833612.1
Stenopelmatus	mahogani	Orthoptera	Ensifera	Stenopelmatidae	Stenopelmatinae		EF030186.1
Stenopelmatus	mahogani	Orthoptera	Ensifera	Stenopelmatidae	Stenopelmatinae		EF030189.1
Deracantha	onos	Orthoptera	Ensifera	Tettigoniidae	Bradyporinae	EU137664.1	EF540817.1
Deracanthina	deracanthoides	Orthoptera	Ensifera	Tettigoniidae	Bradyporinae		EF540818.1
Zichya	baranovi	Orthoptera	Ensifera	Tettigoniidae	Bradyporinae		DQ649479.1
Banza	brunnea	Orthoptera	Ensifera	Tettigoniidae	Phaneropterinae		DQ649480.1
Banza	brunnea	Orthoptera	Ensifera	Tettigoniidae	Phaneropterinae		DQ649495.1
Banza	nitida	Orthoptera	Ensifera	Tettigoniidae	Phaneropterinae		DQ649494.1
Banza	nitida	Orthoptera	Ensifera	Tettigoniidae	Phaneropterinae		DQ649493.1
Banza	nitida	Orthoptera	Ensifera	Tettigoniidae	Phaneropterinae		FJ913701.1
Banza	nitida	Orthoptera	Ensifera	Tettigoniidae	Phaneropterinae		FJ913702.1
Banza	nitida	Orthoptera	Ensifera	Tettigoniidae	Phaneropterinae		JQ301445.1
Bucrates	malivolans	Orthoptera	Ensifera	Tettigoniidae	Conocephalinae		FJ913731.1
Bucrates	malivolans	Orthoptera	Ensifera	Tettigoniidae	Conocephalinae		FJ913747.1
Conocephalus	maculatus	Orthoptera	Ensifera	Tettigoniidae	Conocephalinae		FJ913759.1
Neoconocephalus	nebrascensis	Orthoptera	Ensifera	Tettigoniidae	Conocephalinae		FJ913757.1
Neoconocephalus	robustus	Orthoptera	Ensifera	Tettigoniidae	Conocephalinae		FJ913755.1
Neoconocephalus	triops	Orthoptera	Ensifera	Tettigoniidae	Conocephalinae	HQ711931.1	FJ913757.1
Neoconocephalus	triops	Orthoptera	Ensifera	Tettigoniidae	Conocephalinae	X	AY165738.1
Neoconocephalus	triops	Orthoptera	Ensifera	Tettigoniidae	Conocephalinae	X	DQ649490.1
Neoconocephalus	triops	Orthoptera	Ensifera	Tettigoniidae	Conocephalinae	EF583824.1	EF583824.1
Orchelimum	nigripes	Orthoptera	Ensifera	Tettigoniidae	Conocephalinae		EU309760.1
Orchelimum	gladiator	Orthoptera	Ensifera	Tettigoniidae	Conocephalinae		EU309761.1
Ruspolia	dubia	Orthoptera	Ensifera	Tettigoniidae	Conocephalinae		EU309759.1
Elimaea	cheni	Orthoptera	Ensifera	Tettigoniidae	Phaneropterinae	GU323362.1	GU975807.1
Horatosphaga	regularis	Orthoptera	Ensifera	Tettigoniidae	Phaneropterinae		GU975804.1
Horatosphaga	regularis	Orthoptera	Ensifera	Tettigoniidae	Phaneropterinae		GU975806.1
Horatosphaga	regularis	Orthoptera	Ensifera	Tettigoniidae	Phaneropterinae		
Horatosphaga	regularis	Orthoptera	Ensifera	Tettigoniidae	Phaneropterinae		
Odontura	aspericauda	Orthoptera	Ensifera	Tettigoniidae	Phaneropterinae		
Odontura	glabricauda	Orthoptera	Ensifera	Tettigoniidae	Phaneropterinae		
Odontura	macphersoni	Orthoptera	Ensifera	Tettigoniidae	Phaneropterinae		
Anabrus	simplex	Orthoptera	Ensifera	Tettigoniidae	Tettidoniinae	EF373911.1	EF373911.1
Atlanticus	sinensis	Orthoptera	Ensifera	Tettigoniidae	Tettigoniinae		JQ301444.1
Gampsocleis	gratiosa	Orthoptera	Ensifera	Tettigoniidae	Tettigoniinae	EU527333.1	EU527333.1

Genus	Species	Order	Suborder	Family	Subfamily	GenBank accession
Platycleis	*intermedia*	Orthoptera	Ensifera	Tettigoniidae	Tettigoniinae	EU203971.1
Tettigonia	*viridissima*	Orthoptera	Ensifera	Tettigoniidae	Tettigoniinae	JN609409.1
Tettigonia	*viridissima*	Orthoptera	Ensifera	Tettigoniidae	Tettigoniinae	JN609410.1
Tettigonia	*viridissima*	Orthoptera	Ensifera	Tettigoniidae	Tettigoniinae	JN609416.1
Tettigonia	*viridissima*	Orthoptera	Ensifera	Tettigoniidae	Tettigoniinae	JN609408.1
Tettigonia	*viridissima*	Orthoptera	Ensifera	Tettigoniidae	Tettigoniinae	EF515120.1
Tettigonia	*viridissima*	Orthoptera	Ensifera	Tettigoniidae	Tettigoniinae	EF540827.1
Tettigonia	*viridissima*	Orthoptera	Ensifera	Tettigoniidae	Tettigoniinae	JN609416.1
Tettigonia	*viridissima*	Orthoptera	Ensifera	Tettigoniidae	Tettigoniinae	JN609420.1
Tettigonia	*viridissima*	Orthoptera	Ensifera	Tettigoniidae	Tettigoniinae	JN609408.1
Tettigonia	*viridissima*	Orthoptera	Ensifera	Tettigoniidae	Tettigoniinae	JN609408.1
Agathemera	*mesoauriculae*	Phasmatodea		Agathemeridae	Agathemerinae	JN228873.1
Micadina	*phluctainoides*	Phasmatodea		Diapheromeridae	Necrosciinae	AB477466.1
Entoria	*okinawaensis*	Phasmatodea		Phasmatidae	Cliturnianae	AB477459.1
Ramulus	*hainanense*	Phasmatodea		Phasmatidae	Cliturnianae	FJ156750.1
Extatosoma	*tiaratum*	Phasmatodea		Phasmatidae	Extatosomatinae	AB642680.1
Phraortes	*illepidus*	Phasmatodea		Phasmatidae	Lonchodinae	AB477460.1
Phobaeticus	*serratipes*	Phasmatodea		Phasmatidae	Phasmatinae	AB477467.1
Megacrania	*alpheus*	Phasmatodea		Phasmatidae	Platycraninae	AB477471.1
Timema	*cristinae*	Phasmatodea		Timematidae		AB477463.1
Ramulus	*irregulariterdentatus*	Phasmatodea		Phasmatidae		JQ339236.1
Blattella	*germanica*	Blattodea		Blattellidae		EU854321.1
Eupolyphaga	*sinensis*	Blattodea		Corydiidae		FJ830540.1
Cryptocercus	*relictus*	Blattodea		Cryptocercidae		JX144941.1
Periplaneta	*americana*	Blattoidea		Blattidae		GU947663.1
Periplaneta	*fuliginosa*	Blattoidea		Blattidae		AB126004.1

Vita

Katherine (Katy) Harrington Frederick was born on April 11, 1982 in Springfield, Missouri (USA). Katy graduated from Ozark High School in Ozark, Missouri in May 2000. In August 2000, Katy started her undergraduate education at Truman State University in Kirksville, Missouri. In May 2004, Katy earned her Bachelors of Science degree in Biology, focusing on Ecology and Evolution. During her undergraduate education Katy was advised by Dr. Jon Gering and published a paper concerning an exotic pest, the Asiatic Oak Weevil. In August 2004, Katy started her Master's of Science research at Truman State University still advised by Jon Gering and Michael Kelrick. Katy completed her Master's of Science in June 2006, her thesis was titled *Phylogenetic structure of katydid communities in Northeast Missouri Grasslands*. In August of 2006, Katy enrolled at the University of Missouri and earned her PhD in Biological Sciences in July of 2013. Katy accepted a teaching position at the University of Missouri and will continue to study novel trait evolution in *Neoconocephalus* with Johannes Schul throughout duration of this position.