MECHANISMS OF CALL RECOGNITION IN THREE SYMPATRIC SPECIES OF *NEOCONOCEPHALUS* (ORTHOPTERA: TETTIGONIIDAE): ASYMMETRICAL INTERACTIONS AND EVOLUTIONARY IMPLICATIONS.

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A Dissertation presented to the Faculty of the Graduate School

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In Partial Fulfillment of the Requirements for the Degree

Doctor of Philosophy

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by

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Dr. Johannes Schul, Dissertation Supervisor

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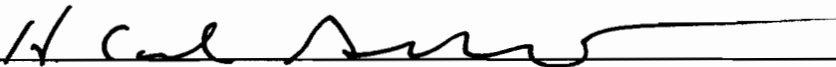The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled

MECHANISMS OF CALL RECOGNITION IN THREE SYMPATRIC SPECIES

OF *NEOCONOCEPHALUS* (ORTHOPTERA: TETTIGONIIDAE):

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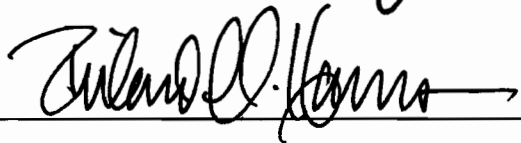
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a candidate for the degree of Doctor of Philosophy

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

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MECHANISMS OF CALL RECOGNITION IN THREE SYMPATRIC SPECIES OF *NEOCONOCEPHALUS* (ORTHOPTERA: TETTIGONIIDAE): ASYMMETRICAL INTERACTIONS AND EVOLUTIONARY IMPLICATIONS.

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Abstract

Scientists have debated the hypothesis of reinforcement. Nevertheless, recent empirical and theoretical work have dispelled doubts about its existence. Reinforcement is most often studied by establishing one of its predicted outcomes: reproductive character displacement. Empirical studies of reproductive character displacement often report that the interactions between species are asymmetrical, such that one species shows the expected pattern of displacement while the other does not. This may be a result of focusing on a limited set of isolation mechanisms.

Graphical presentation of character displacement often depicts a symmetrical displacement occurring along a single axis. Implied is the assumption that both species achieve reproductive isolation using similar mechanisms. However, this is not entirely accurate. Reproductive isolation may be achieved by any number of mechanisms, several of which may presumably act in any given species. Furthermore, the same mechanisms need not be responsible for isolation between interacting species.

To address the question of how reproductive isolation occurs among close relatives, I studied three sympatric species of the acoustically communicating genus *Neoconocephalus*. I considered all call parameters (temporal and spectral) that may be important for call recognition,

and identified and characterized the mechanisms of female call recognition using a walking compensator.

Female *N. robustus* responded to a continuous unmodulated 7 kHz sine wave. They also responded to pulsatile calls as long as the intervals were short or absent. Female responses were limited to a narrow frequency range around the center frequency of the conspecific call (7 kHz). The addition of higher frequencies resulted in strong inhibition of phonotaxis, which should help females avoid the calls of congeners using slightly higher (10 kHz) carrier frequencies.

Female *N. nebrascensis* also responded to sinusoids in which the intervals were short or absent. In addition, females of this species required a higher order temporal pattern (verse structure). Female responses were limited to a narrow frequency range around the center frequency of the conspecific call (10 kHz). The addition of higher frequencies resulted in a weak but significant inhibition.

Female *N. bivocatus* required a distinct pulsatile structure for call recognition, responding only when the pulse rate was approximately 87 Hz. Using the duration of the merged double-pulse corresponding to the conspecific pattern, pulse rates from 80-95 Hz elicited significant responses, while pulse rates of 74 and 105 Hz failed to elicit significant responses. Female responses were limited to a narrow frequency range around the center frequency of the conspecific call (10 kHz). The addition of higher frequencies resulted in little if any inhibition.

Strikingly different mechanisms are responsible for reproductive isolation of the three species. In *N. bivocatus* and *N. nebrascensis*, call recognition is based on derived temporal characteristics. In contrast, call recognition in *N. robustus* relies on several less effective cues, both temporal and spectral. This pattern indicates that *N. robustus*, through a reinforcement-like process, was pushed by the other two species to use a suboptimal carrier frequency and to sharpen its temporal recognition mechanisms. The evolutionary processes leading to the qualitatively new call traits and recognition mechanisms in *N. nebrascensis* and *N. bivocatus* are unknown.

Chapter 1

Reinforcement, symmetrical versus asymmetrical interactions, and

Neoconocephalus **as a study system**

Reinforcement

In acoustically communicating insects and anurans, long-range advertisement calls serve to bring conspecific individuals together for mating. In many environments, however, multiple species' calls overlap in time and space, making the task of call recognition complex. Not only do species in such environments need to recognize conspecific signalers, but also there are frequently fitness costs associated with responding to heterospecific calls. These fitness costs may be associated with both post-mating and pre-mating consequences, and include the production of unfit or unviable hybrids, wasted gametes, or missed mating opportunities, as well as the energetic costs and/or predation risks associated with traveling to an inappropriate mate. The evolution of long-range acoustic isolation mechanisms to prevent these costly mistakes is one outcome of what is commonly referred to as reinforcement (sensu Blair, 1955), although this precise definition is not ubiquitous (reviews in Howard, 1993; Bradbury and Vehrencamp, 1998; Gerhardt and Huber, 2002; Greenfield, 2002; Coyne and Orr, 2004).

The theory of reinforcement is generally accredited to Dobzhansky (1937, 1940), and was enthusiastically accepted at the time (Mayr, 1942). Following this initial acceptance, however, reinforcement was called into question (Moore, 1957; Mayr, 1963; Walker, 1974) and largely abandoned as theoretical objections mounted in the 1980s

(Felsenstein, 1981; Barton and Hewitt, 1981; Patterson, 1978, 1982; Spencer et al., 1986). Reinforcement has since reemerged as a plausible evolutionary mechanism as empirical evidence has accumulated and theoretical models have been revisited (Coyne and Orr, 1989, 1997, 2004; Howard, 1993; Liou and Price 1994; Rundle and Schluter, 1998; Gerhardt and Huber, 2002; Marshall et al., 2002; Servedio and Noor, 2003; Höbel and Gerhardt, 2003). Turelli et al. (2001) perhaps captured the essence of this about-face in the following statement: "... [current] theoretical studies show that reinforcement is clearly plausible, which is reassuring given the growing evidence for its occurrence".

In light of the available evidence, the question is not whether reinforcement occurs in nature, but rather how important is this process in the generation of pre-mating isolation between species (e.g. long-range acoustic communication systems). Most empirical studies of reinforcement, however, consider only instances where it has left the signature of reproductive character displacement (review in Coyne and Orr, 2004), despite this theory's limiting prerequisites (Servedio and Noor, 2003).

Although some systems might have been shaped by reinforcement, they may not be good candidates in which to demonstrate reinforcement via reproductive character displacement. For example, many orthopteran species are quite good fliers and consequently have high rates of migration (Gwynne, 2001), meaning that differences between areas of sympatry and allopatry would likely be fleeting. However, this does not mean that reinforcement (or reproductive character displacement) has not acted to shape the communication systems in of this group of insects. It simply means that one might be unlikely to find the signature of reproductive character displacement.

Symmetrical versus asymmetrical interactions

Reproductive character displacement is only one potential outcome of reinforcement. Reinforcement may result in a variety of pre-zygotic isolation mechanisms, including geographic separation, ecological character displacement, behavioral isolation, temporal isolation, mechanical mating incompatibility, and the inability of gametes to fuse (Raven and Johnson, 2002). It is possible that several of these isolation mechanisms might be used in tandem by a single species, especially should one mechanism alone not be entirely effective. Of course which of these mechanisms becomes established in a given group depends upon the origins of the divergence, selective pressure and pre-existing variation.

Different mechanisms (or a different set of mechanisms) might act on each of a species pair. While it is of course possible, there is no reason why the response of each of a species pair should necessarily be symmetrical. For example, in a pair of acoustically communicating species, one species might achieve isolation solely through a more selective acoustic response toward pulse rate, while the other could achieve isolation through a combination of a more selective acoustic response toward pulse duration and an ecological displacement.

However, graphical or narrative descriptions of reinforcement (e.g. reproductive *or* ecological character displacement) typically represent it as occurring along a single trait value or preference dimension, where both species respond in opposite (symmetrical) directions (e.g., Losos, 2000; Pfennig and Murphy, 2003; Servedio, 2004;

review in Coyne and Orr, 2004). Again, while this may occur, reinforcement is also likely to occur along several dimensions.

Interactions between closely related species may also be asymmetric if only one of a pair of interacting taxa diverged significantly from their common ancestor prior to the interaction. This should be relatively common as small peripheral populations are isolated from and then rejoin a larger parent population. In this case, the isolating mechanism used by the peripheral population would need to be highly effective to prevent reabsorption or displacement by the larger parental population. In this sense, the derived isolating mechanism used by the divergent population evolved to discriminate between the two groups. However, the parental population likely would not have any such effective mechanism, having evolved in the absence of the derived communication system. The parental population might therefore sharpen any existing differences in order to achieve reproductive isolation from the divergent population. The parental population thus responds to the divergent population by modifying an existing communication system.

The asymmetry predicted by this scenario is for the divergent species to utilize one highly effective isolation mechanism, and for the parental species to use a variety of perhaps only partially effective isolation mechanisms.

The preceding paragraphs discuss the fact that reinforcement may result in a variety of isolating mechanisms, that several of these mechanisms may function in a given species, that different mechanisms may function in each of a species pair, and that

these mechanisms might be influenced by the order of divergence. These ideas may account for the numerous examples of asymmetrical character divergence reported in widely separate clades, where only one of a species pair exhibits reproductive isolation using the parameter under investigation (e.g., Kaneshiro, 1980; Wasserman and Koepfer, 1980; Vigneault and Zouros, 1986; Sperling and Spence, 1991; Emms et al., 1996; Schul, 1998; Shapiro, 2001; Coyne et al., 2002; Gerhardt and Huber, 2002; Smadja et al., 2004). Similarly, this scenario could explain why character displacement is sometimes expected but not found (Walker, 1974; reviews in Howard, 1993; Coyne and Orr, 2004).

Essentially, the expected result of reinforcement is potentially asymmetric or absent only when considered from the perspective of a single isolating mechanism (or a limited set of mechanisms).

Expected form of reproductive isolation in *Neoconocephalus*

Although reinforcement has many potential outcomes (see above), some outcomes may result in lower overall costs than others. For example, ecological isolation, temporal isolation, and/or isolation via long-range acoustic signals would avoid the energy expenditures, missed mating opportunities, and/or predation risks associated with traveling to an inappropriate mate, where other isolation mechanisms may then function.

The katydid species *N. robustus*, *N. nebrascensis* and *N. bivocatus* utilize similar habitats and breed synchronously, suggesting that ecological or temporal isolation are unlikely. Hybrids, although rare, have been found for these species (Büttner, 2002), indicating that mechanical or gametic incompatibilities are absent. However, the calls of

these species do contain species-specific cues, suggesting that the communication systems of these species could function in reproductive isolation.

Neoconocephalus

Neoconocephalus is a speciose clade of katydids, with approximately 17 species in North America, 7 of which occur in Missouri (Whitesell, 1969); additional species occur in South and Central America (Greenfield, 1990). Among Missouri species, *N. robustus*, *N. nebrascensis* and *N. bivocatus* are close relatives and, in preliminary results from a molecular phylogeny, are monophyletic (Snyder and Schul, unpublished data). *N. robustus* and *N. bivocatus* are sibling species and until relatively recently were also cryptic species (Walker et al., 1973).

N. robustus, *N. nebrascensis* and *N. bivocatus* occur throughout Missouri and large areas of North America. The geographical ranges of *N. nebrascensis* and *N. bivocatus* are smaller than and occur within the range of *N. robustus*. *N. robustus* occurs throughout the US east of the Rocky Mountains and in some areas of California, although the California populations might be recent introductions from the east (Fig. 1; Walker et al., 1973; GrylTett database, compiled by Thomas J. Walker).

These katydids species live in grasslands, and while their habitat requirements are similar, there are subtle differences. *N. robustus* prefers relatively moister grasslands than *N. bivocatus*, and *N. nebrascensis* most often occurs along wooded edges and fence-rows (Walker et al., 1973; Meixner and Shaw, 1979; personal observations). Despite these modest habitat differences, their respective habitats abut and often overlap (i.e. no physical barriers exist between the species).
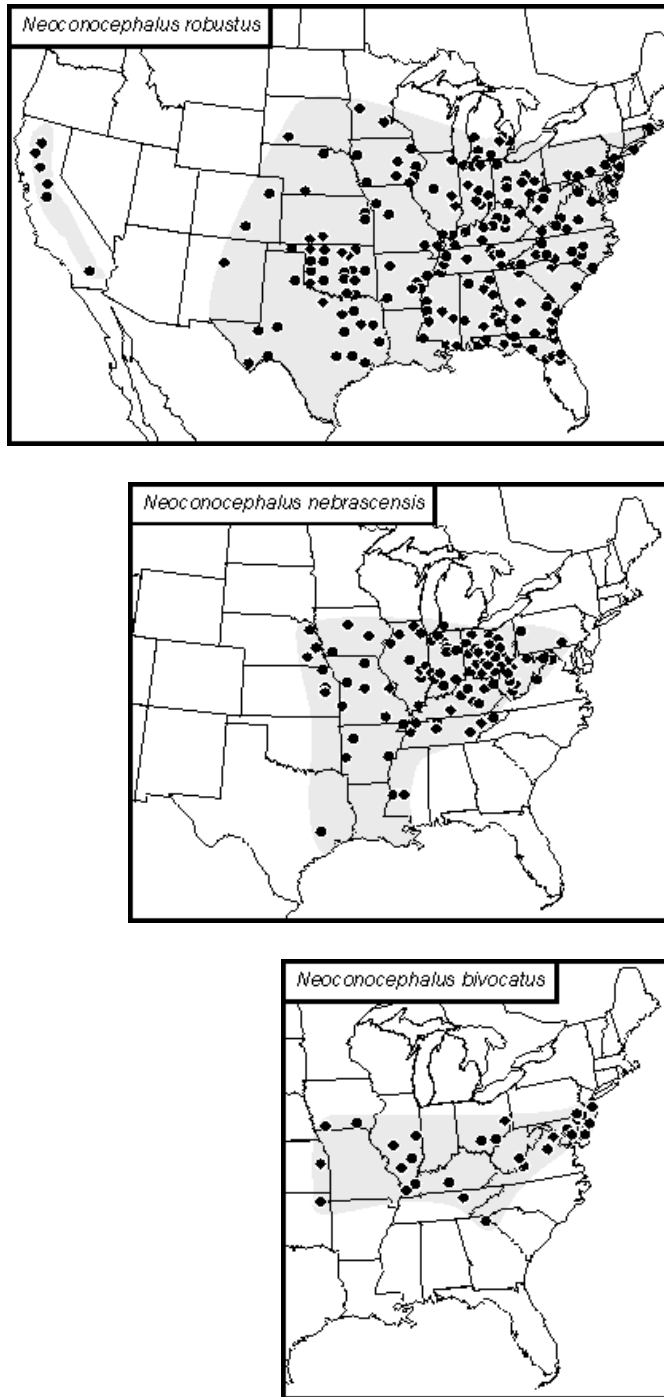
Figure 1

Maps showing the geographical distribution of *N. robustus*, *N. nebrascensis* and *N. bivocatus* in North America. Note that the ranges of *N. nebrascensis* and *N. bivocatus* are smaller than and occur within the range of *N. robustus*. From GrylTett database, compiled by Thomas J. Walker.

The breeding seasons of the three species also overlap. All three species may be heard calling in the same field on the same night (Walker et al., 1973; personal observation). While there has been no systematic study investigating the potential for hybridization among these three species, it appears to be a rare occurrence: during six years of field work, call recordings, morphological measurements and laboratory testing, we have found only a handful of potential hybrids (Büttner, 2002). The potential for hybridization, however rare, indicates that reproductive isolation via mechanical incompatibilities or post-copulatory barriers is unlikely.

Male *N. robustus*, *N. nebrascensis* and *N. bivocatus* produce stereotyped calls that differ significantly in temporal pattern and/or spectral content (Büttner, 2002; Schul and Patterson, 2003). The maintenance of the species-specific elements in each of these species calls, as well as the absence of obvious ecological or temporal isolation, suggests that the calls are important in species isolation. If the long-range calls effectively isolate these species, additional isolation mechanisms would be unnecessary (e.g., Gray, 2004).

Within the clade containing *N. robustus*, *N. nebrascensis* and *N. bivocatus*, the ancestral call probably consisted of a continuous single-pulse temporal pattern and a center-frequency of about 10 kHz (see Chapter 5 for detailed reasoning). Each species' call features one characteristic that distinguishes it from the calls of the other two species: In *N. bivocatus* and *N. nebrascensis* these are temporal characters (double-pulse pattern in *N. bivocatus* and verse structure in *N. nebrascensis*), while in *N. robustus* the center frequency is shifted significantly below 9 kHz (Büttner, 2002; Schul and Patterson,

2003). The 'unique' characteristic in each species' call are thus likely derived call traits (Deily and Schul, 2006). These derived call traits, however, are not analogous. While *N. robustus* simply shifted the value of an existing parameter (carrier frequency), *N. nebrascensis* and *N. bivocatus* introduced new call elements (double pulses and verses, respectively).

The presence and stereotyped production of these derived call traits indicates that they are important in call recognition in *Neoconocephalus*. However, how the female recognition system deals with such derived (in addition to the ancestral) call components is unknown. Is there a shift in (*N. robustus*) and/or a new recognition criterion (*N. bivocatus* and *N. nebrascensis*) that takes place in the female recognition system that parallels the changes in the calls?

Here, I study the acoustic communication systems of three closely related sympatric species of the Tettigoniid genus *Neoconocephalus*: *N. robustus*, *N. nebrascensis* and *N. bivocatus*. In Chapters 2-4 I consider all call parameters (temporal and spectral) that may be used in call recognition, then identify and fully characterize the mechanisms used by these species for call recognition. In Chapter 5 I summarize the results of Chapters 2-4, discuss the contributions of each mechanism to reproductive isolation, and relate both the calls and the recognition mechanisms to their likely ancestral states. Finally, I consider the data in *Neoconocephalus* as it relates to symmetrical versus asymmetrical divergence.

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Chapter 2

**Recognition of calls with exceptionally fast pulse rates:
Female phonotaxis in the genus *Neoconocephalus* (Orthoptera: Tettigoniidae)**

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Running title: Call recognition in *Neoconocephalus*

Abstract

Male *Neoconocephalus robustus* and *N. bivocatus* produce remarkably fast calls, with pulse rates of approximately175 - 200 Hz. The temporal call patterns differ significantly between the two species. Male *N. robustus* produce calls with a single pulse rate of 200 Hz. In *N. bivocatus*, pulses are repeated with alternating periods, resulting in distinct pulse pairs: approximately 175 pulses/s are grouped into 87 pulse pairs/s. In order to identify the temporal parameters used to recognize calls with such fast pulse rates, female call recognition was tested during phonotaxis on a walking compensator in both species. Female *N. robustus* were attracted to calls without amplitude modulation. Amplitude modulated signals were equally attractive, as long as the silent intervals were short enough. The maximally tolerated interval duration varied with pulse duriion. Female *N. bivocatus* did not require the paired-pulse pattern but were attracted to call models in which each pulse pair was merged into one long pulse. Females used the pulse rate to recognize such signals: pulse rates close to 87 Hz were attractive, largely

independent of the duty-cycle. Thus, females of the sibling species *N. robustus* and *N. bivocatus* used qualitatively different call recognition mechanisms.

Key words: Acoustic communication, temporal pattern recognition, hearing, amplitude modulation, phonotaxis

Introduction

The communication signals of insects and anurans often contain highly repetitive elements, which are either monotonously repeated or grouped into higher order patterns (reviews in Ewing, 1989; Gerhardt and Huber, 2002). The temporal pattern of these signals is often used by the receiver side of the communication system for call recognition. Several behavioral studies have identified the temporal characteristics used for this task. Specific temporal parameters that have been identified as important for call recognition include: (i) the rate (or period) of sound pulses (e.g. Hennig and Weber, 1997; Plewka, 1993; Thorson et al., 1982), (ii) the duty cycle of the signal (e.g. Helversen and Helversen, 1983; Schul, 1998), or (iii) absolute durations of sound pulses and/or the intervals between pulses (e.g. Helversen, 1972; Hennig, 2003; Schul and Bush, 2002), or combinations of such parameters (e.g. Doherty, 1985).

The pulse repetition rates of male calling songs of insects and anurans are predominantly in the range of 10 Hz to 60 - 80 Hz (e.g. Heller, 1988; for a notable exception see Heller, 1986). Accordingly, durations of pulses and intervals are typically 6 ms or longer. The auditory pathways of most insects and anurans are well able to encode temporal parameters of such rates and durations (review in Gerhardt and Huber, 2002).

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Male calls in the Tettigoniid genus *Neoconocephalus* are unusually fast. Pulse rates in the range of 200 to 250 pulses/s are common in this genus: 21 out of 25 species with described calls have pulse rates well above 100 Hz (Greenfield, 1990). It is questionable whether the sensory system is able to faithfully encode the temporal pattern of such fast calls: the temporal acuity of insect auditory receptor cells have been found to decrease for amplitude modulation rates above 100 Hz (e.g. Prinz and Ronacher, 2002; Surlykke at al., 1988). Additionally, the ascending auditory pathway will likely limit transmission to even lower modulation rates (e.g. Schildberger, 1984). Nevertheless, fine scale temporal patterns of the calls vary distinctly among *Neoconocephalus* species (Greenfield, 1990), especially between sibling species with overlapping ranges of occurrence. For example, the sibling species *N. robustus* and *N. bivocatus* have wide, overlapping ranges in eastern North America. The temporal patterns of their calls differ distinctively (Walker et al., 1973; Büttner, 2002). The calls of *N. robustus* consist of pulses monotonously repeated at a rate of 200/s (at 25ºC, Fig. 1a). In *N. bivocatus*, pulses are repeated with alternating periods, resulting in distinct pulse pairs (Fig. 1a). The repetition rate of these pulse pairs is 87 Hz (at 25ºC), i.e. about 175 individual pulses/s (Büttner, 2002). This strongly suggests that the temporal patterns are actually used for call recognition, despite their fast pulse rates.

Here, we study the selective phonotaxis in females of two closely related species, *N. robustus* and *N. bivocatus*. We focus on female selectivity for temporal call patterns to learn whether the fast temporal patterns of their calls contain useful information for call recognition, and if so, to identify the temporal call parameters actually used for call recognition, i.e. the recognition mechanism used by the females. Identifying such

behavioral recognition mechanisms, which classical ethology called 'innate releasing

mechanisms' (Lorenz, 1943; Tinbergen, 1953), is a prerequisite for the understanding of

the neuronal basis of call recognition.
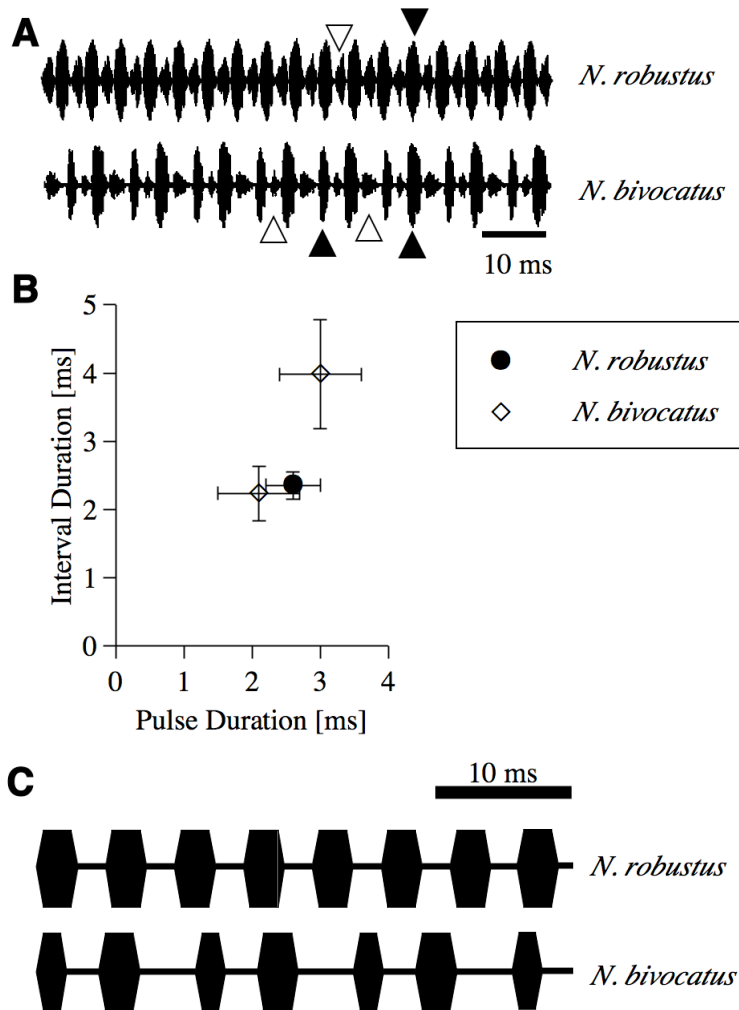
Figure 1

(A) Oscillograms of calls recorded from male *N. robustus* (top trace) and *N. bivocatus* (bottom trace) at 25°C. The filled arrowheads indicate sound produced during closing movements, open arrowheads the sound generated during the opening movements of the tegmina. (B) Durations of the pulses produced during the closing movements of the wings and of the intervals between them (Walker, 1975) in the calls of *N. robustus* and *N. bivocatus* (mean ± SD, n = 12 and 8, respectively). Due to the two alternating pulse periods of *N. bivocatus*, two combinations of pulse and interval are given for this species; pulse durations were combined with the duration of the following interval. (C) Oscillograms of the models of the conspecific calls of *N. robustus* (top trace) and *N. bivocatus* (bottom trace) used in this study. Note the different time scales in A and C.

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Materials and methods

Animals

We collected female *Neoconocephalus robustus* and *N. bivocatus* from the field as nymphs in Boone County, Missouri (USA), and identified them after Froeschner (1954) and Walker et al. (1973). The insects were kept at 20 - 25ºC and a light/dark cycle of 14/10 hours. The females were kept for at least two weeks after their adult molt before they were used in experiments. Females were tested for up to five weeks, during which we detected no changes in their selectivity.

Phonotaxis Experiments

We conducted behavioral tests on a walking compensator (Kramer treadmill; Weber et al., 1981) in an anechoic chamber at 25 ± 1 °C. In short, the insects were placed on top of a sphere, free to walk but kept in place by compensatory sphere rotations, while acoustic signals were presented from loudspeakers located in the insect's horizontal plane. The intended direction and speed of the animal were read out from the control circuitry The experiments were performed in the dark except for an infrared light used to monitor the movements of the animal on the sphere. For details see Weber et al. 1981, Schul 1998)

Stimulation

We generated synthetic signals using a custom developed DA-converter/amplifier system (16 bit resolution, 250 kHz sampling rate). The signals were delivered via one of two loudspeakers (EAS 10TH400C or Motorola KSN1218C) mounted at a distance of

150 cm in the horizontal plane of the insect and separated by an angle of 115°. We adjusted signal amplitude to 80 ± 1 dB peak SPL (re. 2 x 10^{-5} Pa) using a 1/4" condenser microphone (G.R.A.S. 40BF) positioned 1cm above the top of the sphere, and a Bruel and Kjaer sound level meter (B&K 2231). This amplitude is representative for a distance of 2 - 3 m from a calling male (Büttner, 2002; Schul and Patterson, 2003)

The calls of *N. robustus* and *N. bivocatus* have similar spectral composition (Schul and Patterson, 2003). Highest amplitudes are present in a narrow low-frequency band, and the frequency components at ultrasonic frequencies are at least 20 dB softer than the low frequency band. The center frequency of the low-frequency band differs significantly between the two species (*N. robustus* - 7 kHz, *N. bivocatus* - 10 kHz). We used pure tones of 7 kHz (for *N. robustus*) or 10 kHz (*N. bivocatus*) as carrier signals, to which we subsequently applied amplitude modulations. This simplification of the spectral call structure did not noticeably influence the attractiveness of the stimuli (see below).

The temporal patterns used as models of the natural calls were based on population mean values determined by Büttner (2002) at 25°C (Fig. 1B). The call model for *N. robustus* consisted of a continuous train of pulses of 3 ms duration, separated by silent intervals of 2 ms duration, resulting in a pulse rate of 200 Hz (Fig. 1C). The call model for *N. bivocatus* consisted of a continuously repeated train of paired pulses, each consisting of two pulses of 2.2 ms and 3.0 ms duration with an interval of 2.3 ms in between. These paired pulses were repeated after an interval of 4.0 ms, resulting in a paired-pulse rate of 87 Hz, (Fig. 1C). These call models were used as control stimuli throughout this study. For both species, female responses to these call models did not

differ significantly in walking speed or quality of orientation (= vector length) from responses to high quality recordings of conspecific calls.

In the first experiment, we tested the attractiveness of a continuous sine wave without amplitude modulation. In all other experiments, we varied pulse duration and interval duration independently. All stimuli were presented as continuous signals without a second-order time pattern modulating the pulse pattern.

Experimental Protocol

The experimental protocol is described in detail in Schul (1998) and Bush et al. (2002). All stimuli were presented twice for approximately 1.5 minutes, with loudspeakers switched between the two presentations. At the beginning of each series the control stimulus was presented, then two or three test stimuli, then another control, etc. Between stimuli a one-minute period of silence was imposed. Each experimental series lasted between 30 and 60 minutes, during which up to nine experimental stimuli (plus four controls) were presented. We varied the sequence of stimulus presentation among the individual females tested.

Data analysis

To evaluate the relative response of a female during a test situation, we calculated a Phonotaxis Score (Schul, 1998) which included measures for three criteria that positive phonotaxis should meet: (1) the relative walking speed, describing the locomotion activity elicited; (2) the vector length, describing the accuracy of orientation; (3) the orientation relative to the orientation during the control stimulus. Phonotaxis scores range

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from approximately +1 (perfect positive phonotaxis) to -1 (perfect negative phonotaxis). Phonotaxis scores close to 0 indicate either no response or random orientation. (for details of the data analysis and calculation of the phonotaxis score see Schul, 1998). Phonotaxis score for the control stimulus ranged between 0.8 and 0.95 for most females of both species.

We present all data as mean ± standard error of the mean (SEM). Female responses were considered significant if two criteria were met: (i) the mean phonotaxis score was significantly greater (Mann-Whitney u-test, $P < 0.05$; Zar, 1984) than a hypothetical population of responses of zero with identical sample size, and (ii) the average response was at least 50% of the response to the model of the conspecific call. Since the second criterion was always much more stringent than the first, we do not present the results of the u-tests in the text. Note that the application of a significance criterion merely emphasizes the shape of the response fields in order to clarify the mechanism used for call recognition, and was not meant to classify stimuli as 'recognized' and 'not recognized' (for a detailed discussion see Bush et al. (2002)).

Results

The first experiment was conducted to determine whether female *N. robustus* and *N. bivocatus* require the pulsed structure of their conspecific calls for call recognition (Fig. 2). We tested the attractiveness of an unmodulated signal (i.e. a continuous sine wave) relative to that of the call models. In *N. robustus*, the mean phonotaxis scores in response to the unmodulated sine wave (0.95 ± 0.04, n = 9, mean ± SEM) and in response

to the conspecific call model (0.91 ± 0.04, n = 9) were comparable (Mann-Whitney u-test, $U = 45$, n = m = 9, $P = 0.69$, *N.S.*). Thus, in *N. robustus,* the unmodulated signal was as attractive as the conspecific call model.

Female *N. bivocatus* responded to the model of their conspecific call with a phonotaxis score of 0.88 ± 0.03 (n = 9). However, the unmodulated signal did not elicit significant responses in this species (Fig. 2B): the mean phonotaxis score in response to this stimulus (0.23 ± 0.10, n = 9) was significantly lower than that in response to the conspecific call (Mann-Whitney u-test, $U = 81$, n = m = 9, $P < 0.05$). Thus, female *N. bivocatus* require some temporal pattern for call recognition, while in *N. robustus* amplitude modulation is not required.

Experiments with N. robustus

Experiment 1 (Fig. 2) demonstrated that female *N. robustus* responded to an unmodulated signal as well as to their call model. In the next experimental series, we tested which amplitude modulations were attractive for this species by independently varying pulse duration and interval duration. We tested a total of 24 such combinations. The results of this experiment are given in Fig. 3.

Female *N. robustus* responded with high phonotaxis scores (between 0.87 and 0.96, n = 8 each) to signals with intervals of 2 ms or less, independent of pulse duration. With increasing interval duration, the signal became less attractive regardless of pulse duration (Fig. 3). At the pulse duration of the call model (3 ms) an interval duration of 4 ms resulted in a mean phonotaxis score of 0.13 ± 0.11 (n = 8). For longer pulses, female responses did not decline for such short intervals: As pulse length increased, the

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maximum interval duration that still elicited significant responses also increased. At a

pulse duration of 1 ms, females responded strongly to interval durations of 2 and 4 ms;

intervals of 6 and 8 ms did not elicit significant female responses (Fig. 3).
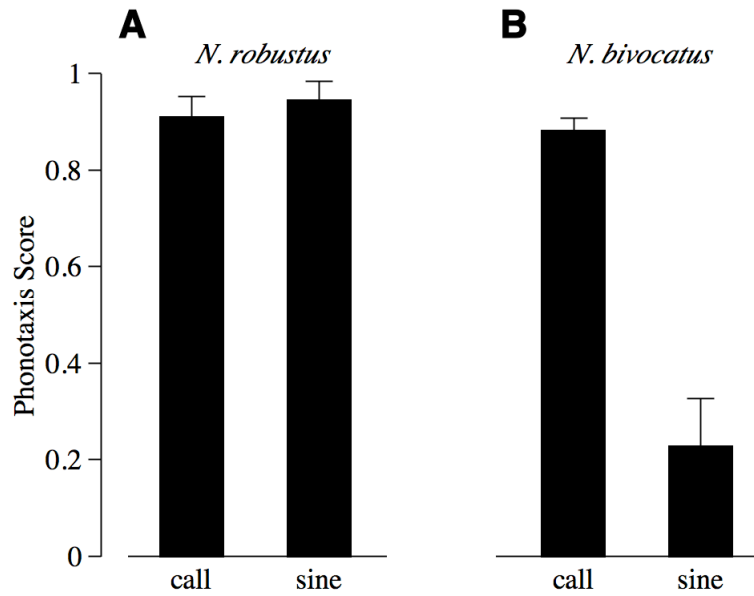
Figure 2

Phonotaxis score (mean ± SEM, n = 9 each) of female *N. robustus* (A) and *N. bivocatu*s (B) in response to the model of the conspecific call (left bar) and to an un-modulated sine wave (right bar). Asterisk indicates a significant difference (*P*< 0.05, Mann-Whitney u-test).
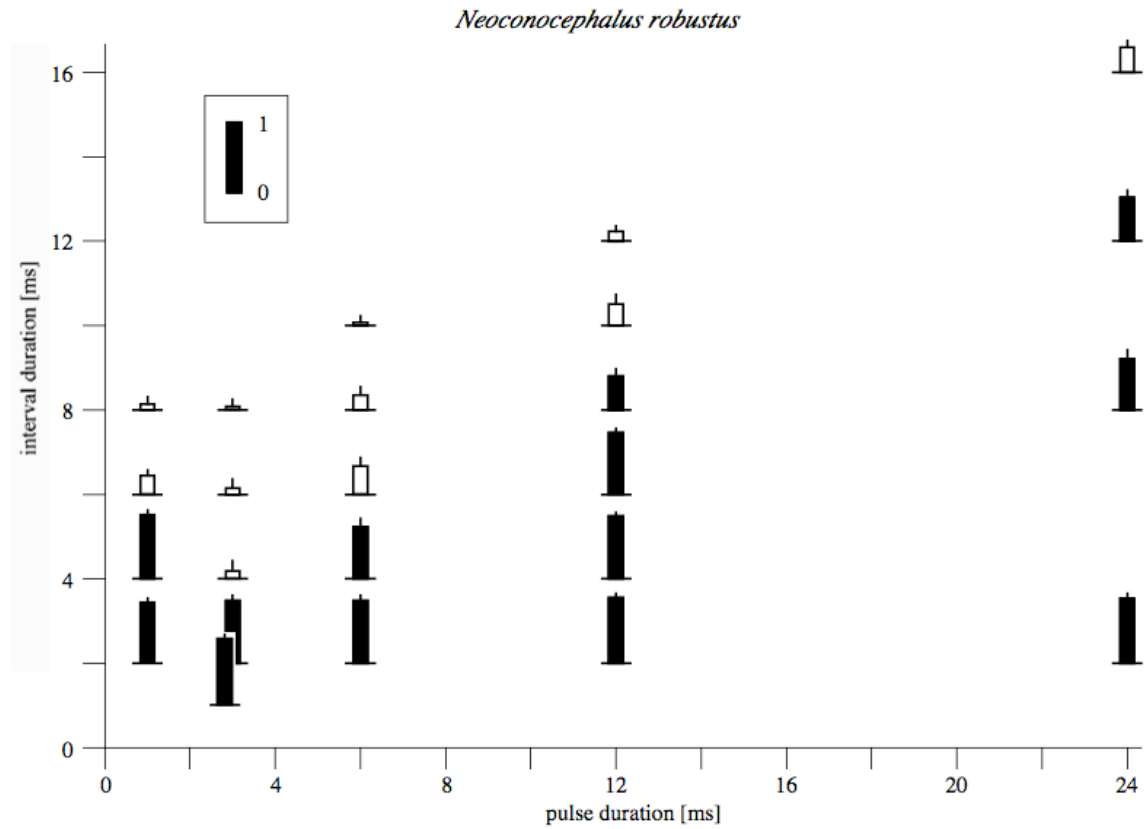
Figure 3

Importance of pulse duration and interval duration for phonotactic responses of *N. robustus*. The bars indicate the phonotaxis score (mean ± SEM, n = 8 - 9) for the respective parameter combination (see inset for the scale of the phonotaxis score). The baseline of each bar is positioned on the interval duration. Filled bars indicate significant responses and white bars non-significant responses.

Experiments with N. bivocatus

The first experiment (Fig. 2) demonstrated that an amplitude modulated signal was required for significant phonotaxis of female *N. bivocatus*. We designed the next experimental series to determine the simplest temporal pattern that would elicit significant phonotaxis. The model of the conspecific call served as control, while the pulse and interval durations occurring in the double pulse pattern of this signal (Fig. 1C, 4B top trace) were tested in various combinations (Fig. 4).

Three test stimuli used the duration of the first (= shorter) pulse of the double pulse (2.2 ms) in combination with three interval durations (Fig. 4, tests 1 - 3): (1) 2.3 ms, the interval within each double pulse; (2) 4.0 ms, the interval between double pulses; and (3) 9.3 ms, an interval duration resulting in a pulse rate equivalent to the double pulse rate of the call model. None of these stimuli elicited significant responses (Fig. 4).

Next, we tested three stimuli based on the duration of the second (= longer) pulse of the double pulse (3 ms), using interval durations corresponding to those used for tests 1 - 3: the interval durations used were 2.3 ms, 4.0 ms and 8.5 ms (Fig. 4, tests 4 - 6). Again, none of these three stimuli elicited significant responses from female *N. bivocatus*.

For the last two stimuli we substituted the double pulse of the call model with one longer pulse. One of the stimuli (Fig. 4, test 7) had a pulse duration of 5.2 ms, i.e. the duration of both pulses added (2.2 ms + 3.0 ms); the other stimulus (test 8) had a pulse duration of 7.5 ms, which was the duration of a double pulse including the silent interval (2.2 + 2.3 + 3.0 ms). Both of these temporal patterns elicited responses as strong as the

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control stimulus (phonotaxis scores of 0.88 ± 0.03 [test 7], 0.86 ± 0.02 [test 8], and 0.92 ±
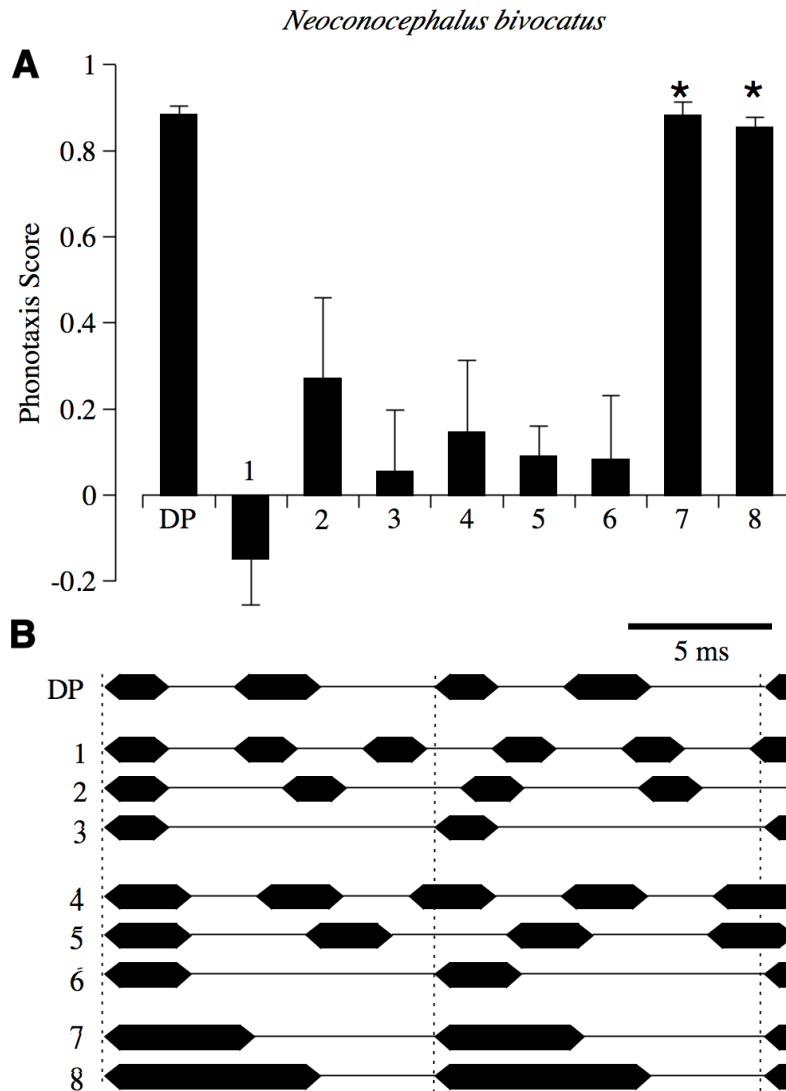
0.04 [control], n = 8).

Figure 4

(A) Phonotaxis scores (mean ± SEM, n = 8) of female *N. bivocatus* to different stimuli.

(B) Oscillograms of the stimuli used in A. These stimuli test the importance of the temporal parameters occurring in the pattern of the conspecific call. DP indicates the model of the conspecific call. For further description of the stimuli see text.

This experiment demonstrated that the more elaborate temporal structure of the natural call with two alternating pulse periods was not necessary to elicit phonotaxis in *N. bivocatus*. Rather, females responded to a pulse with the duration of the natural call's double pulse, repeated at the normal rate. This finding leads to the question of which combinations of pulse duration and interval duration are attractive for the females.

We designed the last set of experiments to determine the effective range of pulse durations (equivalent to a merged double pulse) and interval durations (equivalent to the interval between double pulses). We varied the duration of both parameters independently, and tested a total of 50 combinations. The results of this experiment are given in Fig. 5.

Female *N. bivocatus* showed significant phonotaxis to stimuli when the pulse period (i.e. the sum of pulse duration and interval duration) was close to 11.5 ms, which is the equivalent of a pulse rate of 87 pulses/s. This corresponds to the rate of double pulses in the male calls of this species. Such responses are located in the response field (Fig. 5) along a diagonal from top left to bottom right. Response magnitudes decreased sharply to both higher and lower pulse rates.

The attractiveness of stimuli with pulse rates close to 87 pulses/s was largely independent of the pulse duration: only for pulses of 3 ms and shorter did responses decline sharply. At the other end of the attractive field, an interval duration of 1ms was sufficient to maintain high phonotaxis scores (pulse 10 ms / interval 1 ms PS = 0.83 ± 0.06).

Only one stimulus outside of the above described response field elicited significant responses (pulse 4.5 ms / interval 1 ms, PS = 0.55 ± 0.1). This stimulus had a

pulse rate of 182 Hz, which is about twice the pulse rate of the other attractive stimuli.

Stimuli with half the pulse rate (43 Hz) were not attractive (pulse 15 ms / interval 8 ms

PS = -0.01 ± 0.12; 7.5 ms / 15.5 ms PS = 0.09 ± 0.16).

Figure 5

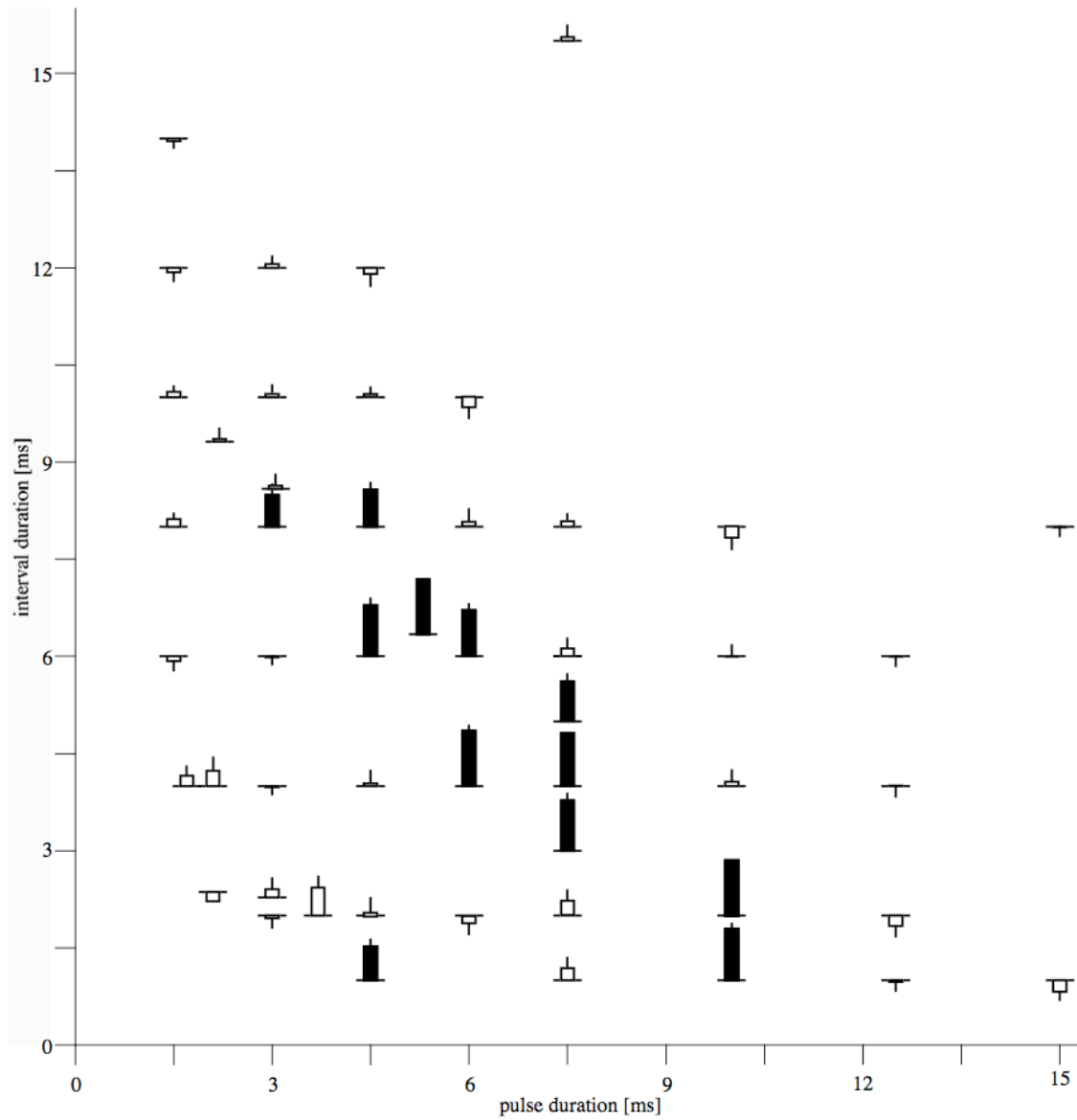Importance of pulse duration and interval duration for phonotactic responses of *N. bivocatus*. The bars indicate the phonotaxis score (mean ± SEM, n = 8 - 10) for the respective parameter combination (see inset for the scale of the phonotaxis score). The baseline of each bar is positioned on the interval duration. Filled bars indicate significant responses and white bars non-significant responses.

Discussion

Our results demonstrated that both *N. robustus* and *N. bivocatus* were highly selective for temporal call patterns (see below). However, the underlying recognition mechanisms were strikingly different. Female *N. robustus* responded to signals without amplitude modulation; at pulse durations typical for this species, interruptions of a few ms within a stimulus rendered the call unattractive. In contrast, female *N. bivocatus* required a distinct amplitude modulation for call recognition: females responded to calls containing a pulse rate of 87 Hz.

At this point, we want to emphasize that although *N. robustus* females responded to a signal without amplitude modulation, this does not mean that they are unselective for temporal pattern. Rather, such un-modulated signals have a highly specific pattern, and the mechanism to recognize it may be as specific or selective as a mechanism that detects specific amplitude modulations (e.g. a particular pulse rate). In light of our results, the common interpretation that responses to unmodulated noise represent responses to 'random signals' and indicate unselectivity (e.g. Morris and Fullard, 1983; Ryan and Rand, 1995) should be revisited: females might be highly selective and recognize the temporal pattern of 'no amplitude modulation'.

N. robustus

Female *N. robustus* responded to a continuous, un-modulated sine wave as well as to the model of the conspecific call. Amplitude modulated signals (such as the call model) were attractive, as long as the intervals were short (Fig. 3). The maximum interval duration tolerated by the females increased as pulse durations became either longer or

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shorter than that of the call model (3 ms). When pulses are longer, fewer intervals appear in the signal per unit time. An interval of a given duration will, over time, therefore have a smaller negative effect at long pulse durations than at shorter pulse durations. A different process is probably responsible for the increase in tolerated interval duration when pulse duration is only 1 ms. The sensory system probably cannot faithfully encode pulse durations in the range from 1 to 3 ms (Rössler and Schul, 1993). Accordingly, female responses at pulse durations of 3 ms and 1 ms decline once the sum of pulse and interval duration (i.e. the pulse period) surpasses 5 ms (see Fig. 3).

Females of three other katydid species were reported to recognize signals without amplitude modulation: *Conocephalus nigropleurum* (Morris and Fullard, 1983), *C. brevipennis* (Guerra and Morris, 2002), and *Tettigonia caudata* (Schul, 1998) respond to continuous noise stimuli. Because male calls in *T. caudata* have a significantly longer duty cycle than the calls of congeners, the call recognition mechanism of this species was interpreted as filtering a minimum duty cycle – the un-modulated signal has 100% duty cycle and thus is attractive. However, duty cycle recognition does not adequately explain the data from *N. robustus*: A phonotaxis score of 0.5 occurred both when pulses and intervals were of equal duration (3 ms), and when the pulses (24 ms) had twice the duration of the intervals (12 ms)

Although female call recognition requires an unmodulated signal, male *N. robustus* produce a pulsed call with a pulse rate of 200 pulses/s. The call production mechanism prohibits un-modulated calls but the fast pulse rate ensures that the call is recognized as unmodulated by the females. Another feature of the *N. robustus* call

supports this interpretation: the pulses produced during the opening movement of the wings are significantly louder than in four other species of *Neoconocephalus*: in *N. robustus* their mean amplitude is 75% of the closing pulses, while in the other species their amplitude is between 20 and 40% (Büttner, 2002). These large opening pulses break up the interval between the closing pulses, so that effectively only gaps of 1 ms occur in the males' signals. This ensures that male calls are still recognized, even if the pulse rate is considerably lower, e.g. a call from a male at lower temperature.

N. bivocatus

In contrast to *N. robustus*, female *N. bivocatus* require a distinct amplitude modulation: they are attracted only to calls containing a pulse rate of 87 pulses/s. Additionally, pulses have to be longer than 3 ms. Male calls contain approximately 175 pulses/s, but by grouping the pulses into pairs they introduce the required 87 Hz component. Females ignore the interval within the pulse pair (2.3 ms duration), effectively merging the two pulses (2.2 and 3.0 ms duration) into one long pulse of sufficient duration for call recognition to occur. The duration of the interval ignored by *N. bivocatus* females is similar to the duration of the interval accepted by *N. robustus* females in 'un-modulated' signals.

Schul (1998) described a call recognition mechanism in *Tettigonia viridissima* that is similar to the one we describe in *N. bivocatus*. In *T. viridissima*, males produce a double pulse pattern and female call recognition merges the two pulses into one long pulse. Yet, the two species use different criteria to recognize these merged pulses. While in *N. bivocatus* call recognition is based on pulse rate, female *T. viridissima* rely on the

absolute durations of pulses and intervals, largely independent of the pulse rate (Schul, 1998).

Evolutionary implications

The calls of 25 *Neoconocephalus* species have been described qualitatively (Greenfield, 1990; Walker, 1975; Walker and Greenfield, 1983). The predominant temporal pattern is similar to that of *N. robustus*: pulses are repeated monotonously with a pulse rate of around 200 – 250 pulses/s (at 25ºC; Greenfield, 1990). The pulse rate in *N. robustus* of about 200 Hz is in the order of magnitude of maximum sustained firing rates of insect neurons (e.g. Franz and Ronacher, 2002). Such pulse rates are likely too fast to be encoded faithfully by the sensory system or to be analyzed by higher nervous centers. The absence of silent intervals, i.e., the absence of amplitude modulation, seems the only temporal characteristic that could be extracted from such a fast temporal pattern. Therefore, only a drastic reduction of pulse rate could lead to divergence of the communication system based on temporal cues. Because pulse rate is usually a 'static' call parameter (*sensu* Gerhardt, 1991), such large changes of pulse rate should be rare, as evidenced by the small number of species with pulse rates lower than 200 Hz.

N. bivocatus has circumvented this hurdle through a relatively small modification of the call generating mechanism. A delay introduced after every other closing movement of the forewings transforms a single pulse rate as in *N. robustus* into a double pulse rhythm. This modification introduces a new temporal component into the call, effectively halving the pulse rate. In the case of *Neoconocephalus*, it would transfer the (double)

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pulse rate from 200 Hz down to below 100 Hz, and thus into a range that could be encoded and recognized by the sensory system.

This study adds to a growing list of genera of acoustic insects and frogs in which call recognition mechanisms differ qualitatively between closely related species (Schul, 1998; Schul & Bush, 2002; Hennig, 2003). However, it is not clear which evolutionary mechanisms lead to this phenomenon (Schul & Bush, 2002), or if it is caused by the basic function of the nervous system underlying call recognition, as suggested by Hennig (2003). Broader comparative studies would be required to solve this problem.

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Chapter 3

Spectral selectivity during phonotaxis: a comparative study in *Neoconocephalus* (Orthoptera: Tettigoniidae)

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Running title: Spectral selectivity in *Neoconocephalus*

Abstract

The calls of male *Neoconocephalus* have most energy concentrated in a relatively narrow low-frequency band. In *N. robustus* this low-frequency band is centered around 7 kHz, while calls of *N. nebrascensis* and *N. bivocatus* have center frequencies close to 10 kHz. The importance of the position of the low-frequency band for female phonotaxis in these three species was determined using a walking compensator. Female *N. robustus* showed significant phonotaxis towards call frequencies from 5 to 10 kHz, and spectral selectivity towards higher frequencies did not change with stimulus amplitude. Significant responses in *N. nebrascensis* and *N. bivocatus* occurred at significantly higher frequency ranges than in *N. robustus*. In these species, spectral selectivity changed with stimulus amplitude; at 68 dB SPL, upper cutoff frequency was significantly lower than at 80 dB SPL in both species. Adding a higher harmonic to the conspecific carrier frequency had a strong inhibitory effect on phonotaxis in *N. robustus:* at higher relative amplitudes of the harmonic, phonotaxis was completely suppressed. Adding a higher harmonic to the conspecific carrier frequency had a much weaker but significant

inhibitory effect in *N. nebrascensis* and little, if any, effect in *N. bivocatus*. The processing of song spectrum in the sensory system is discussed with regard to the differences in spectral selectivity among the three species. The sharp spectral selectivity of *N. robustus* is interpreted as an adaptation for species isolation.

Key words: Acoustic communication, spectral processing, carrier frequency, call recognition, hearing, phonotaxis.

Introduction

The calls of most groups of Tettigoniids (katydids) have broadband spectral content that commonly extends well into the ultrasonic range (Gerhardt and Huber, 2002; Heller, 1988); only a few groups have narrow-band spectra in audible (e.g. Suga, 1966; Bailey, 1970) or ultrasonic (Morris et al., 1994) frequency ranges. In the genus *Neoconocephalus*, most of the call energy is concentrated in a narrow low-frequency band, with ultrasonic frequency components at least 20 dB softer than the low-frequency band (Fig. 1A; Greenfield, 1990). The characteristic frequency (center frequency) of the low-frequency band within the genus ranges from 7 to 16 kHz (Greenfield, 1990; Schul and Patterson, 2003). Based on measurements of hearing thresholds and the sound transmission properties of the habitats (tall grasslands and marshes), center frequencies close to 10 kHz are calculated to be most advantageous in this genus (Schul and Patterson, 2003).

Male calls of *N. robustus* have the lowest center frequency in the genus at 7 kHz (Schul and Patterson, 2003). This frequency is surprisingly low given the disadvantages of calling at 7 kHz: the hearing sensitivity in *N. robustus* is about 7 dB lower at 7 kHz than at 10 kHz, and there is no improvement in signal transmission between 10 kHz and 7 kHz to justify the use of a frequency that is mismatched with female sensitivity (Schul and Patterson, 2003). A potential explanation, however, is that calling in the low-frequency band provides this species with a 'private channel' (Narins, 1995) that is free of interfering calls of sympatric congeners. Alternatively, the low-frequency band may provide female *N. robustus* with an additional cue for call recognition beyond the temporal pattern, which is similar to the temporal pattern of several congeners (Deily and Schul, 2004).

In Tettigoniids, the hearing organ provides fine spectral resolution at the level of receptor cells (Kalmring et al., 1978; Römer, 1983). However, at the level of primary auditory interneurons, this frequency resolution is largely discarded as receptor cells converge on just a few interneurons with broad spectral sensitivity (e.g. Schul, 1997; Stumpner, 1999; review in Gerhardt and Huber, 2002). Accordingly, spectral selectivity of katydids is generally limited to detecting the absence or presence of energy in broad frequency bands (e.g. Latimer and Sippel, 1987; Bailey and Yeoh, 1988; Dobler et al., 1994; Jatho, 1995). Preferences based on fine-scale differences in call spectra (e.g. Bailey an Yeoh, 1988; Schul et al., 1998) are most likely based on differences in the perceived call amplitudes (Schul, 1999; review in Gerhardt & Huber, 2002). The more detailed spectral processing found in other groups of insects (e.g. Doolan & Young, 1989; Fonseca et al., 2000; Fonseca & Revez, 2002) has not been described in katydids.
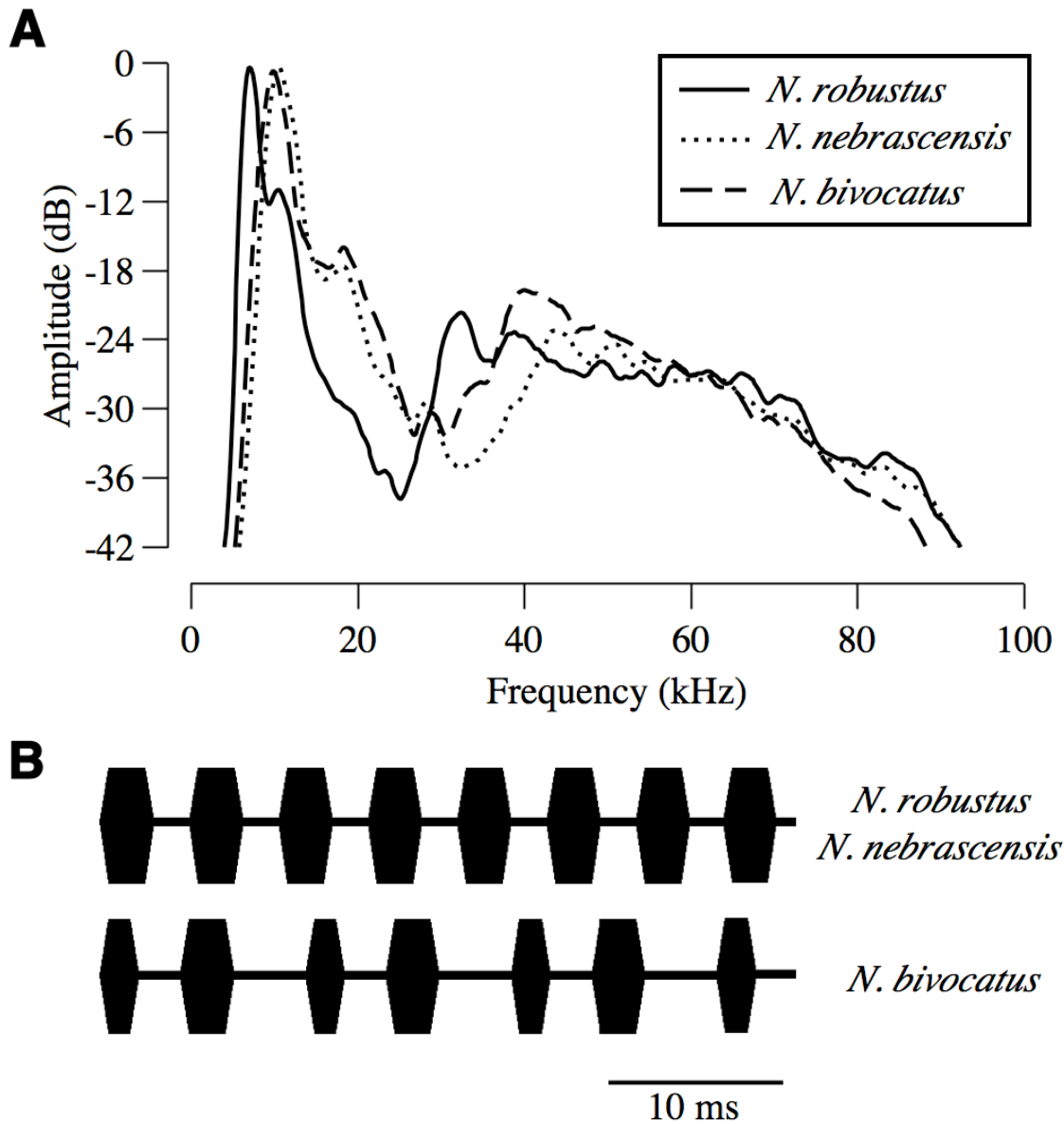
Figure 1

(A) Averaged spectra of male calls of *N. robustus* (solid line, n=10), *N. nebrascensis* (dotted line, n=10) and *N. bivocatus* (dashed line, n=8) at 25ºC. Adapted from Schul and Patterson (2003). (B) Pulse patterns of the conspecific call models used for *N. robustus* and *N. nebrascensis* (top trace), and *N. bivocatus* (bottom trace).

The small spectral difference between *N. robustus* and most of its congeners in the position of the low-frequency band (7 kHz *versus* 10 kHz) appears unlikely to be resolved by the spectral selectivity of the ascending pathway in katydids, and thus appears unlikely to serve an important function. However, the disadvantages that the use of the low carrier frequency entails for *N. robustus* (see above) suggest an adaptive function in this species, possibly for call recognition or masking avoidance. Here, we comparatively examine the spectral selectivity in *N. robustus* and two closely related species with sympatric occurrence (*N. nebrascensis* and *N. bivocatus*). We determine the importance of the position of the low-frequency band for female phonotaxis in *N. robustus*, and explore differences in spectral processing among the three species. Furthermore, we investigate potential mechanisms females may use to discriminate the carrier frequency of *N. robustus* from the higher carrier frequencies of its congeners.

Materials and Methods

Animals

We collected female *Neoconocephalus robustus* (Scudder, 1862), *N. nebrascensis* (Bruner, 1891) and *N. bivocatus* Walker, Whitesell and Alexander 1973 from the field as nymphs in Boone County, Missouri (USA), and identified them after Froeschner (1954) and Walker et al. (1973). *N. robustus* and *N. bivocatus* are considered sibling species (Walker et al., 1973; Greenfield, 1990). Preliminary results of a molecular phylogenetic analysis based on a mitochondrial locus support this assumption (R. L. Snyder and J. Schul, unpublished), and indicate that these species, together with *N. nebrascensis* and *N.*

ensiger, form a distinct clade within the genus *Neoconocephalus*. The insects were kept at 20 - 25ºC and a light:dark cycle of 14:10 hours. The females were held for at least two weeks after their adult molt before they were used in experiments. Females were tested for up to five weeks, during which we detected no changes in their selectivity.

Phonotaxis Experiments

We conducted behavioral tests on a walking compensator (Kramer treadmill; Weber et al., 1981) in an anechoic chamber at 25±1 °C. In short, the insects were placed on top of a sphere, free to walk but kept in place by compensatory sphere rotations, while acoustic signals were presented from loudspeakers located in the insect's horizontal plane. The intended direction and speed of the animal were read out from the control circuitry. The experiments were performed in the dark except for an infrared light used to monitor the movements of the animal on the sphere. For details see Weber et al. (1981) and Schul (1998).

Stimulation

We generated synthetic signals using a custom developed DA-converter/amplifier system (16 bit resolution, 250 kHz sampling rate). The signals were attenuated using a computer controlled attenuator and delivered via one of two loudspeakers (EAS 10TH400C) mounted at a distance of 150 cm in the horizontal plane of the insect and separated by an angle of 115°. We adjusted signal amplitude using a 1/4" condenser microphone (G.R.A.S. 40BF) positioned 1 cm above the top of the sphere, and a Bruel

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and Kjaer sound level meter (B&K 2231). All sound pressure levels are given as dB peak SPL (re 20μPa).

The temporal patterns of the call models used in this study were based on population mean values determined by Büttner (2002) at 25°C. All pulses used in the three call models had 0.5 ms rise and fall times, which are included in the durations of the pulses. Call models of *N. robustus* and *N. bivocatus* were identical to the control stimuli used in Deily and Schul (2004).

The temporal pattern for *N. robustus* (Fig. 1B) consisted of a continuous train of pulses of 3.0 ms duration, separated by silent intervals of 2.0 ms duration (i.e., a single-pulse pattern).

The temporal pattern for *N. bivocatus* consisted of a continuous train of paired pulses: the duration of these pulses was 2.2 ms and 3.0 ms, with an interval of 2.3 ms in between. These paired pulses were repeated after an interval of 4.0 ms (Fig. 1B). The call models of both *N. robustus* and *N. bivocatus* were presented as continuous signals, without a second order time pattern modulating the pulse pattern.

The call model of *N. nebrascensis* had the same pulse pattern as the *N. robustus* model (pulse duration of 3.0 ms and interval duration of 2.0 ms). However these pulses were not presented continuously, but grouped into verses of 1000 ms duration, which were repeated after a silent pause of 800 ms.

The calls of *N. robustus*, *N. nebrascensis* and *N. bivocatus* had similar spectral composition (see Introduction) but differed in the center frequency of the low-frequency band (Fig. 1A). The center frequency was at 7.0 kHz in *N. robustus*, 10.4 kHz in *N.*

nebrascensis, and 10.1 kHz in *N. bivocatus* (Schul and Patterson 2003). We used pure tone carriers of 7 kHz (*N. robustus*) and 10 kHz (*N. nebrascensis* and *N. bivocatus*) with the temporal patterns described above to construct conspecific call models for each species. This simplification of both the temporal and spectral structure did not noticeably reduce the attractiveness of these stimuli relative to natural calls (Deily and Schul, 2004). These call models were used as control stimuli throughout this study. We used the conspecific temporal pattern for each of the three species during all experiments.

Experiment 1: Here, we tested the effects of both carrier frequency and call amplitude on attractiveness. The carrier frequency of the call models varied from 5 to 60 kHz. Stimuli were presented at amplitudes of both 68 and 80 dB SPL.

Experiment 2: Here, we tested the effect of an additional high frequency component on the attractiveness of the call models by adding a second sinusoid to the conspecific carrier frequencies. Frequencies were chosen as integer multiples of the carrier frequencies (14, 28, and 42 kHz for *N. robustus*; 20 and 40 kHz for *N. nebrascensis* and *N. bivocatus*). Note that although up to three high frequency components were tested per species, only one high frequency component was added to the low-frequency band per trial stimulus, i.e. for *N. nebrascensis*, the three stimuli consisted of 10 kHz alone (control), 10 kHz + 20 kHz, and 10 kHz + 40 kHz. The absolute amplitude of the low-frequency component was set for each individual to the lowest amplitude at which it showed consistent phonotaxis when presented alone, and was held constant within each series of an individual. Amplitudes of the low-frequency component ranged from 50 to 56 dB SPL for *N. robustus*, 44 to 56 dB SPL for *N. nebrascensis*, and 44 to 62 dB SPL for *N. bivocatus*. The amplitude of the high frequency

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component was varied between 0 dB and +18 dB relative to the conspecific carrier. We conducted this experiment at amplitudes close to the behavioral threshold to detect weak effects of the high frequency component which would be masked by the strong excitation at higher stimulus amplitudes. Phonotaxis at these near-threshold amplitudes was comparable to that observed at 68 and 80 dB SPL.

Experimental Protocol

The experimental protocol is described in detail in Schul (1998) and Bush et al. (2002). Briefly, all stimuli were presented twice for approximately 1.5 minutes each (3 minutes in total), with the position of the loudspeaker changed between the two presentations. At the beginning of each series the control stimulus was presented, then two or three test stimuli, then another control, etc. Between stimuli a one-minute period of silence was imposed. Each experimental series lasted between 30 and 90 minutes, during which up to nine experimental stimuli (plus four controls) were presented. We varied the sequence of stimulus presentation among the individual females tested.

Data analysis

To evaluate the relative response of a female during a test situation, we calculated a Phonotaxis Score (Schul, 1998) which included measures for three criteria that describe the relative strength of phonotaxis: (1) the walking speed relative to the speed during the control stimulus (describing the locomotion activity elicited); (2) the vector length, describing the accuracy of orientation; and (3) the orientation relative to the orientation during the control stimulus. Phonotaxis scores range from approximately +1 (perfect

positive phonotaxis) to -1 (perfect negative phonotaxis). Phonotaxis scores close to 0 indicate either no response or random orientation (for details of the data analysis and calculation of the phonotaxis score see Schul (1998)). To facilitate comparison between species and between stimulus intensities, we normalized phonotaxis scores by setting the phonotaxis score to the control stimulus to 1.

We present all phonotaxis scores as mean ± standard error of the mean (SEM). Female responses were considered significant if two criteria were met: (i) the phonotaxis scores were significantly greater (Wilcoxon paired sample test, $P < 0.05$; Zar, 1984) than the phonotaxis scores obtained from the same females in response to silence; and (ii) the average response was at least 50% of the response to the model of the conspecific call. Both criteria agreed for most data points; in the few cases that only one was significant, the second criterion was usually more stringent than the first. Therefore, we do not present the results of the Wilcoxon paired sample tests in the text. Note that the application of significance criteria and cutoff frequencies (see below) merely emphasize the relative attractiveness of stimuli and are not meant to classify stimuli as 'recognized' or 'not recognized' (for a detailed discussion see Bush et al. (2002)).

For experiment one, we constructed frequency response functions; each function had a distinct roll-off towards higher frequencies above the conspecific call carrier frequency (Figs. 2, 3). We fit a sigmoidal function to the phonotactic response curve above the conspecific carrier frequency (see above) of each female by minimizing the sum of the squared errors. The frequency at which the sigmoid had an amplitude of 50% was defined as the upper cutoff frequency. We compared median upper cutoff

frequencies between the three species with a Mann-Whitney test, and at different

stimulus intensities within each species using a Wilcoxon paired sample test (Zar, 1984).

We tested the effect of the added high-frequency components during experiment 2

using a repeated measures analysis of variance (individual females as a random-effect),

using the Phonotaxis Score as the measure of performance. The Tukey test was used for

post-hoc pairwise comparisons between the call model and treatment groups (Zar, 1984).

We calculated ANOVA (General Linear Model) and post-hoc comparisons using Minitab

(Release 14.12.0, Minitab Inc., USA). We used a significance criterion (α) of 0.05.

Results

Experiment 1

In the first set of experiments, we tested female responses to call models with

varied carrier frequencies. Females of the three species showed significant phonotaxis in

the frequency range around the center frequency of their calls (Fig. 2). At 80 dB SPL

significant phonotaxis scores occurred in *N. robustus* from 5 to 10 kHz; in the two other

species significant responses occurred at higher frequencies (*N. nebrascensis*: 8 to 15

kHz, *N. bivocatus*: 7 to 15 kHz). The median upper cutoff frequency in *N. robustus* (10.3

kHz; Table 1) was significantly lower (Mann-Whitney U-test, $P < 0.002$) than in both *N.

nebrascensis* (14.9 kHz) and *N. bivocatus* (17.9 kHz). There was no significant difference

in upper cutoff frequencies between *N. nebrascensis* and *N. bivocatus* (Mann-Whitney U-

test, $P > 0.2$).
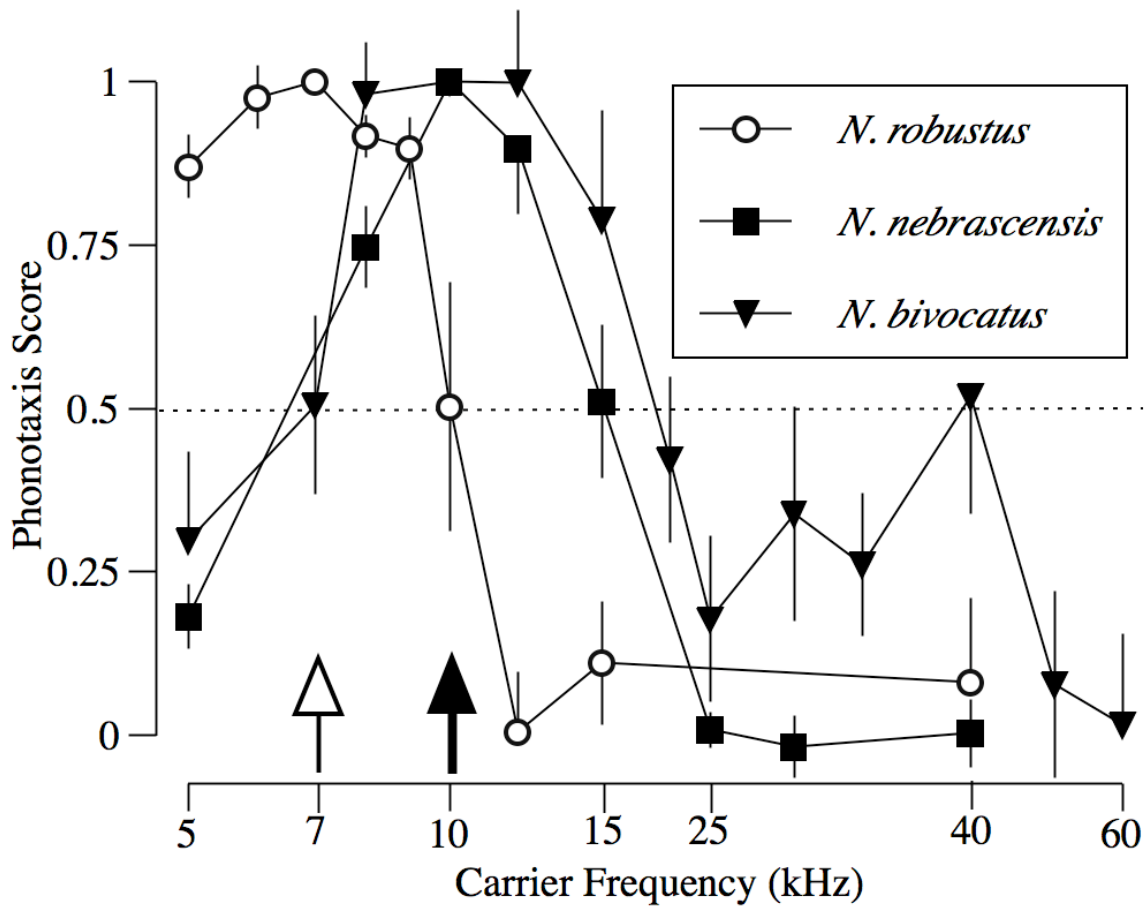
Figure 2

Importance of call carrier frequency for female phonotaxis of *N. robustus* (circles), *N. nebrascensis* (squares) and *N. bivocatu*s (triangles). Each point represents the mean phonotaxis score (± SEM) of 7 to 8 females. Phonotaxis scores were normalized relative to the phonotaxis score at the conspecific carrier frequencies (7 kHz for *N. robustus*, 10 kHz for *N. nebrascensis* and *N. bivocatus*). Arrows indicate the conspecific carrier frequencies. All responses above 0.5 (dashed line) were significant (see Methods) except for *N. bivocatus* at 40 kHz. All stimuli were presented at 80 dB SPL.

Table 1. *Median and range of cutoff frequencies in experiment 1.*

Species	Amplitude (dB SPL)	Cutoff Frequencies (kHz)		n
		Median	Range	
N. *robustus*	80	10.3	9.1 - 11.4	7
	68	10.2	9.0 - 12.5	8
N. *nebrascensis*	80	14.9	12.7 - 19.0	8
	68	12.3	10.1 - 15.0	8
N. *bivocatus*	80	17.9	12.1 - 19.4	7
	68	11.9	10.2 - 12.9	7

Table 1

For frequencies of 20 kHz or higher, mean phonotaxis scores of *N. robustus* and *N. nebrascensis* were below 0.1; in *N. bivocatus,* however, response strength remained above 0.1 for frequencies up to 40 kHz (Fig. 2). Although these responses were not significant, they suggest that frequencies between 20 and 40 kHz were somewhat attractive to *N. bivocatus* females.

Figure 3 compares the spectral selectivity of the three species at two stimulus amplitudes, 68 dB SPL and 80 dB SPL. In *N. robustus*, female selectivity did not change with stimulus amplitude (Fig. 3A); median cutoff frequencies (Table 1) did not differ between 68 dB SPL and 80 dB SPL (Mann-Whitney U-test, $P > 0.20$).

In both *N. nebrascensis* and *N. bivocatus*, spectral selectivity changed significantly with stimulus amplitude. Significant responses occurred over a narrower frequency range at 68 dB SPL than at 80 dB SPL in both species (Fig. 3B, C). Accordingly, median upper cutoff frequencies (Table 1) were significantly lower at 68 dB SPL than at 80 dB SPL (Mann-Whitney U-test, $P < 0.05$ for both species).

The amplitude independence of spectral selectivity in *N. robustus* is a typical signature of 'lateral inhibition', i.e. the spectral selectivity seems to be generated by low-frequency excitation and high frequency inhibition. Conversely, changes of spectral selectivity as seen in *N. nebrascensis* and *N. bivocatus* suggest that selectivity is generated by excitation only. We tested whether high frequencies have an inhibitory effect on female phonotaxis in the second set of experiments.

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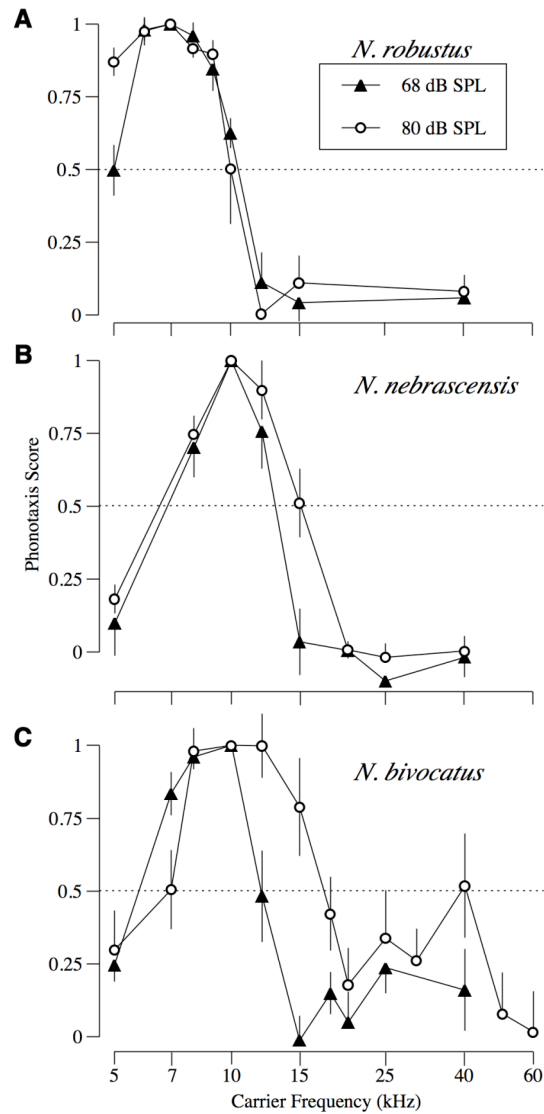
Figure 3

Importance of call carrier frequency for female phonotaxis at stimulus amplitudes of 68 dB SPL (triangles) and 80 dB SPL (circles) in (A) *N. robustus*, (B) *N. nebrascensis* and (C) *N. bivocatus*. Each point represents the mean phonotaxis score (± SEM) of 7 to 8 females. Phonotaxis scores were normalized relative to the phonotaxis score at the conspecific carrier frequencies (7 kHz for *N. robustus*, 10 kHz for *N. nebrascensis* and *N. bivocatus*). All responses above 0.5 (dashed line) were significant (see Methods) except for *N. bivocatus* at 40 kHz / 80 dB SPL.
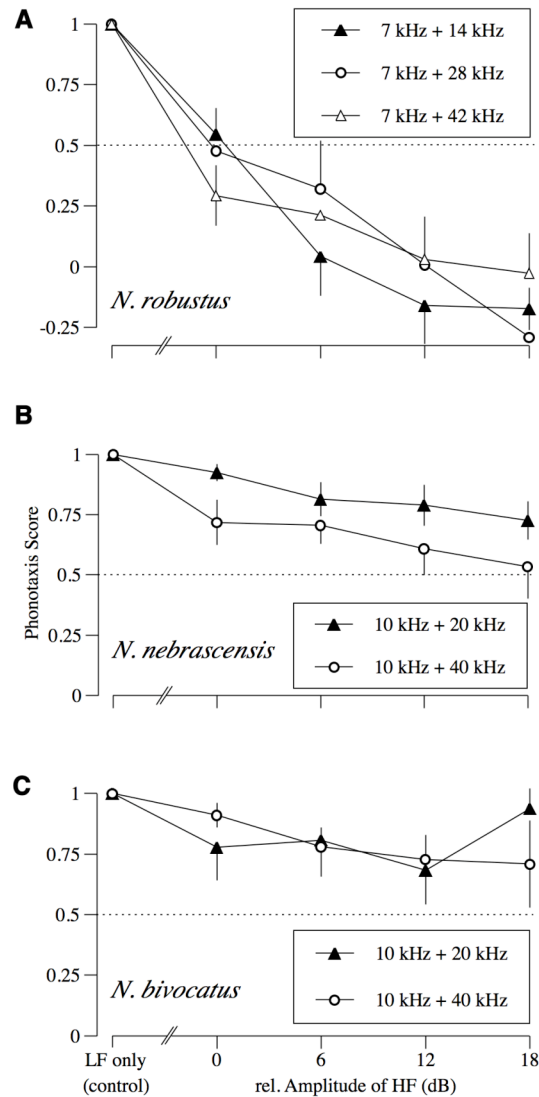
Figure 4

Effects of adding a high frequency (HF) component to call models on female phonotaxis of (A) *N. robustus*, (B) *N. nebrascensis* and (C) *N. bivocatus*. Each point represents the mean phonotaxis score (± SEM) of 7 to 8 females. Phonotaxis scores were normalized relative to the phonotaxis score to the conspecific call model (control), which comprised only the conspecific carrier frequency (LF; 7 kHz in *N. robustus*, 10 kHz in *N. nebrascensis* and *N. bivocatus*). HF components were added at amplitudes of 0 dB to +18 dB relative to the amplitude of the LF component. All responses above 0.5 (dashed line) were significant (for further details see Methods).

Experiment 2

In *N. robustus*, the inhibitory effect of adding a higher harmonic to the conspecific carrier frequency of 7 kHz (Fig. 4A) was highly significant (ANOVA, P < 0.001 for all three frequencies, see Table 2 for details). Post-hoc pairwise comparison demonstrated that responses to all stimuli that include a high frequency component were significantly weaker than to the control stimulus (Table 2). Females failed to show significant responses to any stimulus containing a high frequency component, except for 14 kHz at 0 dB relative amplitude (Fig. 4A).

In *N. nebrascensis*, adding either 20 kHz or 40 kHz to the conspecific carrier frequency (10 kHz) had significant effects on female responses (Fig. 4B; ANOVA: 20 kHz, P < 0.005; 40 kHz, P < 0.002; Table 2). Post-hoc pair-wise comparisons indicated that female responses to stimuli containing either frequency at +12 dB and +18 dB were significantly weaker than to the control stimulus (Tukey test, p<0.05 in all cases). However, all stimuli that included a high frequency component elicited significant responses in *N. nebrascensis* (Fig 4B).

In *N. bivocatus*, adding a higher harmonic to the conspecific carrier frequency of 10 kHz (Fig. 4C) had marginally significant effects (ANOVA: 20 kHz, P < 0.05; 40 kHz, P < 0.1; Table 2). Posthoc pairwise comparison to the control stimulus detected a significant reduction in response strength (P < 0.05) only for 20 kHz at +12 dB relative amplitude, while for all other stimuli with high frequency components these comparisons were not significant (Table 2). All stimuli that included a high frequency component elicited significant responses in *N. bivocatus* (Fig. 4C).

Table 2. *Results of ANOVA of experiment 2.*

Species	Frequency (kHz)	d.f.	F	P	P-values from post-hoc comparisons to control			
					0 dB	+6 dB	+12 dB	+18 dB
N. robustus	14	4	17.24	**<0.001**	**<0.05**	**<0.005**	**<0.001**	**<0.001**
	28	4	23.82	**<0.001**	**<0.05**	**<0.001**	**<0.001**	**<0.001**
	42	4	16.07	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
N. nebrascensis	20	4	4.91	**<0.005**	=0.809	=0.084	**<0.05**	**<0.01**
	40	4	5.92	**<0.002**	=0.091	=0.058	**<0.01**	**<0.001**
N. bivocatus	20	4	2.95	**<0.05**	=0.195	=0.375	**<0.05**	=0.960
	40	4	2.16	=0.100	=0.911	=0.343	=0.168	=0.144

Significant statistical results ($\propto < 0.05$) are shown in bold.

Table 2

Discussion

In *N. robustus*, *N. nebrascensis* and *N. bivocatus* female responses were limited to a narrow frequency range around the center frequencies of their conspecific calls. The addition of higher frequencies affected the three species to different degrees, strongly inhibiting phonotaxis in *N. robustus*, decreasing response strength in *N. nebrascensis*, and having only marginally significant effects in *N. bivocatus*.

The female responses curves in Figs. 2 and 3 were most likely a function of attractiveness of the different frequencies, rather than of their localizability. Analyzing the walking speed alone resulted in the same pattern of response functions as using the phonotaxis score. The walking speed indicates how enthusiastically females respond to a stimulus, independent of the available directional cues (i.e., it is thus influenced only by call attractiveness).

In *N. robustus* responses decreased steeply toward higher frequencies, and the upper cutoff frequency of female responses did not change with stimulus amplitude (Figs. 2, 3A). This suggests that 'lateral inhibition' (Gerhardt and Huber, 2002; Hennig et al., 2004) is involved in generating the spectral selectivity towards higher frequencies; frequencies below the upper cutoff frequency (10 kHz) have excitatory effect, while higher frequencies inhibit female responses. Experiment 2 directly demonstrates the inhibitory effect of frequencies above 10 kHz.

In *N. bivocatus* spectral selectivity changes significantly with stimulus amplitude (Fig. 3C), and the inhibitory effect of high frequencies during experiment 2 was marginal. This suggests that lateral inhibition plays only a minor role in the spectral selectivity towards higher frequencies in this species. Rather, an excitatory function alone seems to

sufficiently explain the selectivity found in experiment 1. The non-significant positive responses to frequencies from 20 to 40 kHz (Fig. 2) also indicate that high frequencies have little, if any, inhibitory effect in *N. bivocatus*.

Results in *N. nebrascensis* were intermediate between the two other species. High frequencies had a highly significant inhibitory effect during experiment 2, although considerably less than in *N. robustus* (Table 2, Fig. 4). Frequency selectivity towards higher frequencies changed with stimulus amplitude, albeit less than in *N. bivocatus,* and there was no positive trend for responses in the frequency range between 20 and 40 kHz as there was for *N. bivocatus* (Fig. 2). These results suggest that lateral inhibition plays a significant role in spectral selectivity in this species, but to a much lesser extent than in *N. robustus*.

Our results indicate that the influence of lateral inhibition on the spectral selectivity towards high frequencies differs significantly among the three species: inhibition is weakest in *N. bivocatus*, somewhat stronger in *N. nebrascensis*, and by far the strongest in *N. robustus*. Additionally, the border-frequency between excitation and inhibition was lower in *N. robustus* (approximately 10 kHz) than in the other two species (approximately 15-18 kHz).

Neuronal Processes Underlying Spectral Selectivity

Among Tettigoniids high hearing sensitivities occur in the broad range from below 5 kHz to above 80 kHz (Kalmring et al., 1990; in *Neoconocephalus*: Schul and Patterson, 2003). Auditory receptor cells project into the prothoracic ganglion where they converge onto a small number of auditory interneurons, which consequently have broad

spectral selectivity (review in Stumpner and Helversen, 2001). Sharpening of spectral selectivity through lateral inhibition occurs most prominently in one neuron: AN-1 receives excitation from frequencies below 20 kHz, but inhibition from frequencies above 20 kHz (Schul, 1997; Stumpner, 1997). Accordingly AN-1 thresholds increase steeply between 20 and 30 kHz (roll off >50-60 dB/octave: Schul, 1997; Stumpner, 1997). AN-1 is most likely involved in generating the spectral selectivity observed during phonotaxis in several Tettigoniid species (Schul, 1997; Stumpner, 1997).

The ascending pathway of *Neoconocephalus* has not been studied in detail. However, it is likely that the differences in spectral selectivity described here result from differences in AN-1 properties among the three species. The strength of the high-frequency inhibition on AN-1 should vary dramatically among them, being strongest in *N. robustus* and weakest in *N. bivocatus*. Furthermore, the border between excitation and inhibition should be shifted towards lower frequencies in *N. robustus* compared to the two other species. Among closely related species of the Tettigoniid subfamily Phaneropterinae, differences of AN-1 properties occur in a similar order of magnitude as suggested by our experiments (Stumpner, 2002).

The sharp decline in response strength of female *N. robustus* between 9 and 12 kHz and the amplitude independence of this decline are exceptional among ensiferans. In Tettigoniids, behavioral tuning is usually amplitude dependent (*N. bivocatus* and *N. nebrascensis* in this study; Hardt, 1988; Dobler et al., 1994); preferences based on small-scale spectral differences (within frequency ranges of a few kHz) are overridden by small changes in amplitude (Bailey and Yeoh, 1988; Schul et al. 1998). In some crickets,

behavioral tuning does exhibit steep roll-offs (e.g. Hennig and Weber, 1997). However, this selectivity is caused by the tuning of the hearing organ, and in this respect is also amplitude dependent. In contrast, *N. robustus* responds to 9 kHz, but not to 12 kHz, largely independent of call amplitude.

Although spectral selectivity in *N. robustus* appears to have attained a 'new quality' among Tettigoniids in steepness and amplitude independence, it is instead most likely based on quantitative changes in the sensory system: high frequency inhibition on AN-1 shifted towards lower frequencies, and its synaptic weighting increased (see above). The spectral selectivity of *N. robustus* is most likely the result of evolution from less selective ancestors. Given that 7 kHz is less suited than 10 kHz for long range communication in *Neoconocephalus* (Schul and Patterson, 2003), the question arises: What evolutionary forces caused the shift in call frequency and call processing in *N. robustus*?

Evolutionary Influences on Call Spectrum

The three species studied here are likely sibling species (see methods); each species' call features one characteristic that distinguishes it from the calls of the other two species: In *N. bivocatus* and *N. nebrascensis* these are temporal characters (double-pulse pattern in *N. bivocatus* and verse structure in *N. nebrascensis*), while in *N. robustus* the center frequency is shifted significantly below 9 kHz (Büttner, 2002; Schul and Patterson, 2003). These three call characteristics (double-pulses, verse structure, and center frequency below 9 kHz) are uncommon in this genus: of 23 described calls, 18 have single-pulse pattern, 17 are continuous (Greenfield, 1990), and most species' calls are

limited to frequencies above 9 kHz (Schul and Patterson 2003; Greenfield, 1990). This pattern suggests that within the clade containing *N. robustus*, *N. nebrascensis* and *N. bivocatus*, the ancestral call consisted of a continuous single-pulse temporal pattern and a center-frequency of about 10 kHz. The 'unique' characteristic in each species' call thus are likely derived call traits.

In both *N. nebrascensis* and *N. bivocatus*, the derived temporal characteristics provide cues for the females to recognize their conspecific calls: *N. bivocatus* females recognize the double pulse rate of approximately 87 Hz, and *N. nebrascensis* females require a distinct verse structure (Deily and Schul, 2004; in prep.). Neither of these two species shows significant phonotaxis to signals with the temporal pattern of its congeners; i.e. their species-specific temporal call pattern ensures species isolation (Deily & Schul, 2004; in prep.). In contrast, the presence or absence of these derived temporal characteristics is not a reliable cue for female *N. robustus*, which show significant phonotaxis to the temporal pattern of at least one congener (*N. bivocatus*, Fig. 5). Thus, temporal pattern recognition is insufficient for *N. robustus* to avoid mismatings. Furthermore, temporal selectivity in *N. robustus* could not be higher without rejecting the conspecific temporal pattern (Deily and Schul 2004). However, in this species, the spectral difference provides a more reliable cue for species recognition, especially when combined with the temporal cues (Fig. 5).

The calls of *N. robustus'* ancestors most likely had the same temporal pattern (and the same temporal call recognition mechanism) as *N. robustus*, but a higher center frequency. After the appearance of species with derived temporal patterns, the temporal call recognition of this ancestral population would not have enabled reliable rejection of
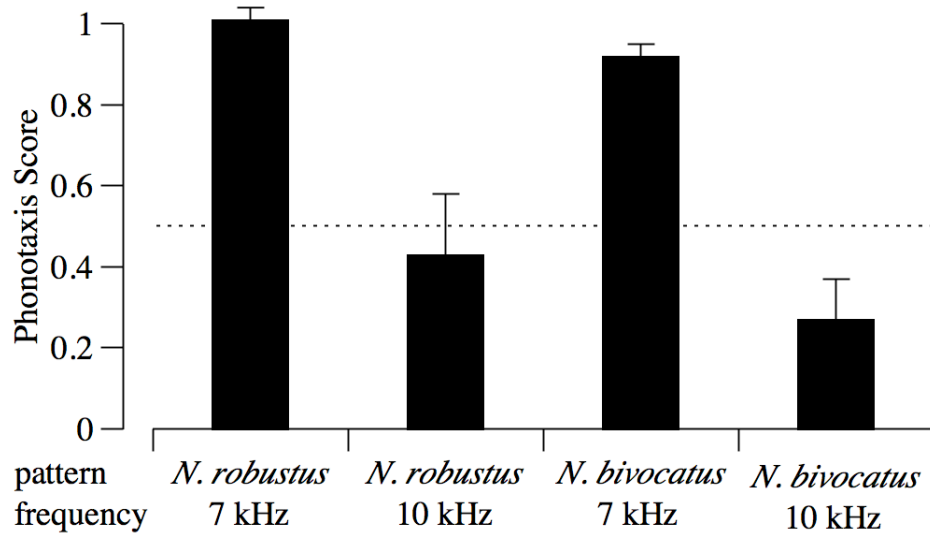
Figure 5

Responses of female *N. robustus* to stimuli with the temporal pattern and carrier frequency of calls of *N. robustus* (7 kHz) or *N. bivocatus* (10 kHz). Bar height indicates the mean phonotaxis score (+ SEM) of 8 females. Stimulus amplitude: 80 dB SPL. All responses above 0.5 (dashed line) were significant (see Methods).

the 'new' calls. Thus, selection would have favored any traits that reduced the risk of hybridization. Because temporal selectivity could not be sharpened enough to reject the new temporal pattern (see above) we suggest that a lower call frequency evolved in response to the appearance of new call patterns. Thus a reinforcement-like process (Dobzhansky, 1937) could have gradually shifted the call center frequency towards lower frequencies and concomitantly sharpened spectral selectivity in *N. robustus*.

Hearing sensitivity of *N. robustus* females is considerably lower at 7 kHz than at 10 kHz and therefore the shift to the lower call center frequency resulted in a reduction of communication distance (Schul and Patterson, 2003). Also, as the ears of Tettigoniids usually function as pressure receivers rather than pressure gradient receivers, 7 kHz likely provides less peripheral directionality than 10 kHz (Gerhardt and Huber, 2002). These disadvantages of the derived center frequency in *N. robustus* support our view that *N. robustus* was 'pushed' by congeners to the lower center frequency, rather than that the low center frequency provides an advantage in itself such as a 'private' communication channel free of masking signals. Due to the strong inhibitory effect of higher frequencies on female phonotaxis, calls of congeners should inhibit phonotaxis in *N. robustus* and therefore interfere with intraspecific communication in this species. The adaptive value of the spectral selectivity of *N. robustus* seems not to be interference avoidance, but rather species isolation.

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Chapter 4

(Submitted to The Journal of Experimental Biology (June, 2006))

Pulse pattern, verse structure, and their role in call recognition in *Neoconocephalus nebrascensis* (Orthoptera: Tettigoniidae)

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Running title: Call recognition in *N. nebrascensis*

Abstract

The calls of many Orthopteran species are comprised of a simple trill of pulses, the temporal pattern of which is often important for call recognition. Male *N. nebrascensis* produce pulses with a temporal structure typical for the genus. However, they modify this pattern by grouping their pulses into verses, thereby creating a higher order temporal structure. The importance of the pulse pattern and the verse structure for call recognition in *N. nebrascensis* was determined using a walking compensator. Females required the pulse pattern for call recognition, responding only when the intervals between pulses were short or absent. Females also required the verse structure for call recognition. Furthermore, females recognized the verse structure only when the amplitude modulation depth between verses and pauses exceeded 18 dB. Only when both the pulse pattern and the verse structure were in the appropriate ranges did females recognize the calls. We discuss the hypothesis that the pulse pattern recognizer in this species is ancestral, and that a derived verse recognition mechanism was added. The verse recognition mechanism

in *N. nebrascensis* is interpreted as a trait adapted for pre-mating isolation. We further

discuss the hypothesis that the properties of the female verse recognition mechanism

force males to synchronize (cooperate) in order to preserve an attractive pattern.

Key words:　Acoustic communication, temporal pattern recognition, call recognition,
　　　　　　　phonotaxis, amplitude modulation depth, phonotaxis.

Introduction

Male calls of many Orthopteran species are simple trills which often last from

several minutes to hours (e.g., Walker, 1957; Alexander, 1960; Pipher and Morris, 1974;

Gwynne and Morris, 1983; Greenfield, 1990). These trills are comprised of a single

element ('pulse') repeated with a constant rate. In such species, females rely on the

temporal pattern of these trills to recognize conspecific calls, evaluating parameters such

as pulse rate, duty cycle, or duration of the pulses (Walker, 1957; Morris et al., 1978;

Schul, 1998; Deily and Schul, 2004).

Among closely related species, such simple call patterns have often diverged

distinctly, either by changing the value of one temporal parameter (e.g. the pulse rate), or

by qualitatively changing the temporal pattern of the trill (e.g. Alexander, 1960; Walker

and Greenfield, 1983; Heller, 1988). Examples of the latter include many katydid species,

where a delay is inserted after every other pulse, resulting in a distinct double pulse

pattern (Heller, 1988). During such divergence of the call patterns, the recognition

mechanism of females changed accordingly, now recognizing the new temporal feature

(i.e. double pulses) of the derived call pattern (Schul, 1998; Deily and Schul, 2004).

Besides changing the pulse pattern, another common line of divergence is to add a second order time structure to the trill, resulting in distinct verses or echemes (Walker, 1957; Heller, 1988; Greenfield, 1990; Morris et al., 1994). Here, the pulse pattern of the trill is usually maintained within the verses. In such cases the receiver can, in principle, change in two ways. First, a new mechanism that recognizes the verse structure could be added to the existing pulse pattern recognizer. In this case the female would be selective for both the pulse pattern and the verse structure (e.g. Walker, 1957). Alternatively, the existing pulse pattern recognizer could be modified to recognize the verse structure. This would result in a loss of selectivity for the pulse pattern (e.g. Walker, 1957). It is also possible that the verse structure is not used for call recognition (e.g. Pollack and Hoy, 1981; Schul, 1998), but functions in a context other than call recognition (e.g. male-male competition: Greenfield, 1990).

The calls of most species of the katydid genus *Neoconocephalus* are comprised of a continuous trill with a pulse rate of approximately 200 pulses/s (Greenfield, 1990). Both lines of divergence described above (i.e. modification of pulse pattern and introduction of verse structure) occur within this genus (Greenfield, 1990). In species with modified pulse patterns, female call recognition relies on the new temporal parameters (Deily and Schul, 2004).

In *N. nebrascensis*, the pulses comprising the male call are grouped into distinct verses of 1 s duration, which are regularly repeated after somewhat shorter periods of silence (Greenfield, 1990; Büttner, 2002; Fig. 1). Within these verses, the ancestral pulse pattern was preserved (Deily and Schul, 2006). Here, we study the selective phonotaxis

of female *N. nebrascensis*. We test whether the ancestral pulse pattern and/or the derived verse structure are important for call recognition, and identify and characterize the mechanisms used in call recognition.

In species with versed calls, males often synchronize or alternate their verses with the verses of neighboring males (Alexander, 1960; Walker, 1969; reviews in Greenfield, 2002, 2006). These timing interactions are often considered to result from a female preference for leading males (e.g., Greenfield, 1994). An alternative explanation is that males synchronize their calls to preserve the conspecific verse structure that females use for call recognition (Greenfield, 2002).

In *N. nebrascensis*, the verses of neighboring males exhibit remarkable synchrony during which leader-follower relationships often remain unchanged over extended periods of time (Meixner and Shaw, 1979; Meixner and Shaw, 1986; pers. observation). We discuss potential causes of call synchronization in *N. nebrascensis* based on our investigation of the call recognition mechanisms.
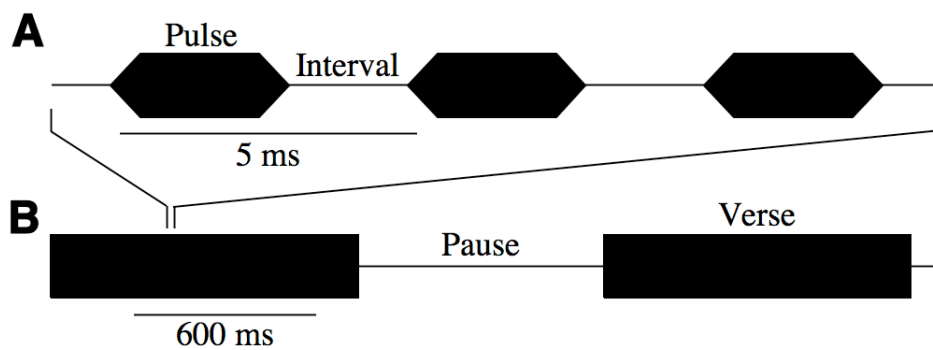
Figure 1

Oscillogram of the call model, showing: (A) the pulse pattern, which was modulated by a verse structure (B). This temporal pattern was based on population mean values of recorded males. Note different time scales in A and B.

Materials and Methods

Animals

We collected female *Neoconocephalus nebrascensis* (Bruner, 1891) from the field as nymphs in Boone County, Missouri (USA), and identified them after Froeschner (1954). The insects were kept at 20 - 25ºC and a light: dark cycle of 14:10 hours. The females were kept for at least two weeks after their adult molt before they were used in experiments. Females were tested for up to four weeks, during which we detected no changes in their selectivity.

Phonotaxis Experiments

We conducted behavioral tests on a walking compensator (Kramer treadmill; Weber et al., 1981) in an anechoic chamber at 25±1 °C. In short, the insects were placed on top of a sphere, free to walk but kept in place by compensatory sphere rotations, while acoustic signals were presented from loudspeakers located in the insect's horizontal plane. The intended direction and speed of the animal were read out from the control circuitry. The experiments were performed in the dark except for an infrared light used to monitor the movements of the animal on the sphere. For details see Weber et al. (1981) and Schul (1998).

Stimulation

We generated synthetic signals using a custom developed DA-converter/amplifier system (16 bit resolution, 250 kHz sampling rate). The signals were attenuated using a

computer controlled attenuator and delivered via one of two loudspeakers (Motorola KSN1218C) mounted at a distance of 150 cm in the horizontal plane of the insect and separated by an angle of 115°. We adjusted signal amplitude using a 1/4" condenser microphone (G.R.A.S. 40BF) positioned 1 cm above the top of the sphere, and a Bruel and Kjaer (Naerum, Denmark) sound level meter (B&K 2231). All sound pressure levels are given as dB peak SPL (re 20μPa).

In the call spectrum of *N. nebrascensis*, highest amplitudes are present in a narrow low-frequency band centered around 10 kHz. Frequency components at ultrasonic frequencies are at least 20 dB softer than the low-frequency band (Schul and Patterson, 2003). In this study, we used pure tones of 10 kHz as carrier signals, to which we subsequently applied amplitude modulations.

We constructed a model of the conspecific call, which we used as a control stimulus throughout this study. The temporal pattern of this call model was based on population mean values determined by Büttner (2002) at 25ºC. The temporal pattern of the call model consisted of a train of pulses of 3.0 ms duration, separated by silent intervals of 2.0 ms duration (Fig. 1A). These pulses were then grouped into 1000 ms verses, which were repeated after a silent pause of 800 ms duration (Fig. 1B). Female responses to this call model were comparable to responses to natural conspecific calls (Deily and Schul, 2006).

The amplitude modulation envelopes used in this study had 0.5 ms rise and fall times, which are included in the pulse durations given. All stimuli were presented at 80 dB SPL.

In the first set of experiments, we tested the importance of the pulse pattern for female phonotaxis. We varied the duration of the interval for pulse durations of 3 ms and 10 ms. The stimulus with 0 ms interval duration had no amplitude modulation within the verses, i.e. each verse was a continuous sinusoid. All stimuli used in this set of experiments had the standard verse structure (1000 ms verse duration, 800 ms pause) (Fig. 2).

In the second set of experiments, we tested the importance of the verse structure for female phonotaxis. We independently varied verse duration and pause duration. All stimuli used in this set of experiments had the standard pulse pattern (3 ms pulse duration, 2 ms interval). We also tested a stimulus with no verse structure, i.e. a continuous train of pulses (Fig. 3).

In the third set of experiments, we determined the modulation depth females required for recognition of the verse structure (Fig. 4A). We used two types of stimuli: (1) We applied the conspecific verse structure to a continuous train of pulses, but rather than having silent pauses, we attenuated the pulses within the pauses by 6 to 30 dB relative to the amplitude of the verses (Fig. 4B, Stimulus 1). (2) In the second stimulus, we combined the call model with a continuous train of pulses to mask the verse structure of the call model. The amplitude of the masking signal was varied from -6 to -30 dB relative to the amplitude of the call model. Here, the pulses of the call model and the masking signal alternated; to avoid signal overlap, we used pulse and interval durations of 2.5 ms in both signals (Fig. 4B, Stimulus 2).

Experimental Protocol

The experimental protocol is described in detail in Schul (1998) and Bush et al. (2002). Briefly, all stimuli were presented twice for approximately 1.5 minutes each (3 minutes in total), with the position of the loudspeaker changed between the two presentations. At the beginning of each series the control stimulus was presented, then two or three test stimuli, then another control, etc. Between stimuli a one-minute period of silence was imposed. Each experimental series lasted between 30 and 90 minutes, during which up to nine experimental stimuli (plus four controls) were presented. We varied the sequence of stimulus presentation among the individual females tested.

Data analysis

To evaluate the relative response of a female during a test situation, we calculated a Phonotaxis Score (Schul, 1998) which included measures for three criteria that describe the relative strength of phonotaxis: (1) the walking speed relative to the speed during the control stimulus (describing the locomotion activity elicited); (2) the vector length, describing the accuracy of orientation; and (3) the orientation relative to the orientation during the control stimulus. Phonotaxis scores range from approximately +1 (perfect positive phonotaxis) to -1 (perfect negative phonotaxis). Phonotaxis scores close to 0 indicate either no response or random orientation (for details of the data analysis and calculation of the phonotaxis score see Schul (1998)).

We present all phonotaxis scores as mean ± standard error of the mean (SEM). Female responses were considered significant if two criteria were met: (i) the mean phonotaxis score was significantly greater (Wilcoxon paired sample test, $P < 0.05$; Zar,

1984) than the mean phonotaxis score obtained from the same females in response to silence; and (ii) the average response was at least 50% of the response to the model of the conspecific call. Since the second criterion was typically more stringent than the first, we do not present the results of the Wilcoxon paired sample tests in the text. Note that the application of significance criteria merely emphasize the relative attractiveness of stimuli and are not meant to classify stimuli as 'recognized' or 'not recognized' (for a detailed discussion see Bush et al. (2002)).

Results

In the first experiment, we tested female selectivity for pulse pattern within verses (Fig. 2). Females responded with highest phonotaxis scores when the duration of the interval was 0 or 2 ms. With increasing interval durations, the signal became less attractive, independent of the pulse duration. Females did not show significant phonotaxis when the interval duration was 8 ms or higher. Thus, females responded when the interval was short enough, largely independent of pulse duration. This mechanism is the same as the call recognition mechanism in *N. robustus* (Deily and Schul, 2004).

Next we tested female selectivity for the verse structure. First, to determine whether the verse structure was required for female call recognition, we presented a continuous train of pulses (i.e. no verse structure). Females showed no significant responses to this signal (PS = -0.03; Fig. 3 inset).

Since females required the verse structure for call recognition, we independently varied the durations of the verse and pause to determine the effective range of these parameters (Fig. 3). Females showed significant responses to verse durations of 500-8000 ms and to pause durations of 100-4000 ms. With increasing duration of the verse, the effective range of the pause duration also increased. For example, at a verse duration of 1000 ms, significant responses occurred at pause durations of 100-1600 ms, while at a verse duration of 4000 ms, significant responses occurred at pause durations of 400-4000 ms.

The previous experiment demonstrated that a verse structure was crucial for call recognition in *N. nebrascensis*. In the final experiment, we tested the amplitude modulation depth between verses and pauses which was required by females to recognize the verse structure (Fig. 4). Both signal types used (see methods) yielded comparable results. For modulation depths up to 18 dB, the stimuli failed to elicit significant phonotaxis; significant responses occurred only when the modulation depth was 24 to 30 dB (Fig. 4). That is, significant responses occurred only when the sound energy in the pauses was less than 1% that of the verses.
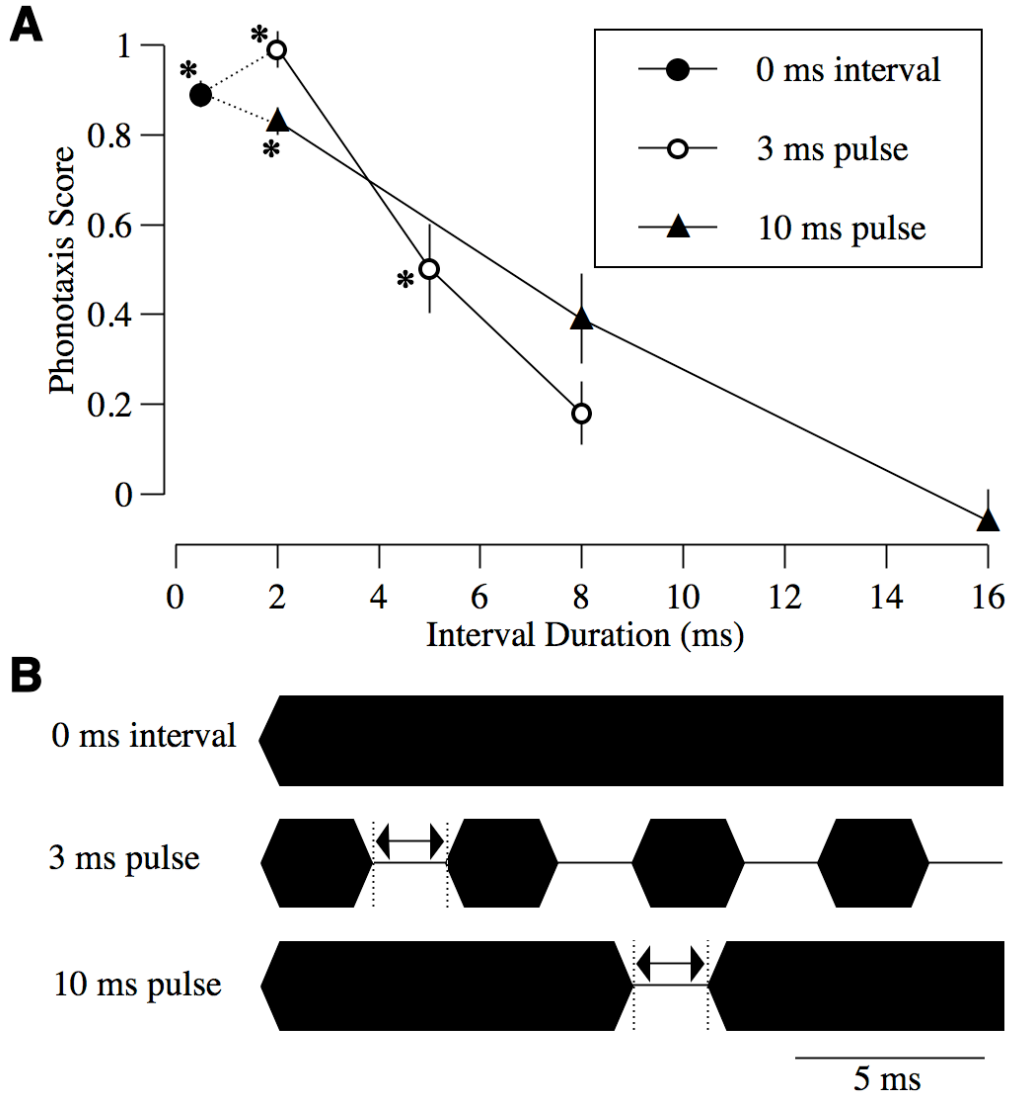
Figure 2

Importance of the pulse pattern for phonotactic responses of female *N. nebrascensis*. (A) Phonotaxis score (mean ± s.e.m., n = 8 or 9 each) as a function of interval duration for two different pulse durations. 0 ms interval indicates a sinusoid with no amplitude modulation within verses. Asterisks indicate significant responses. (B) Oscillograms illustrating the pulse pattern of the stimuli used. All stimuli were presented using the conspecific verse structure.
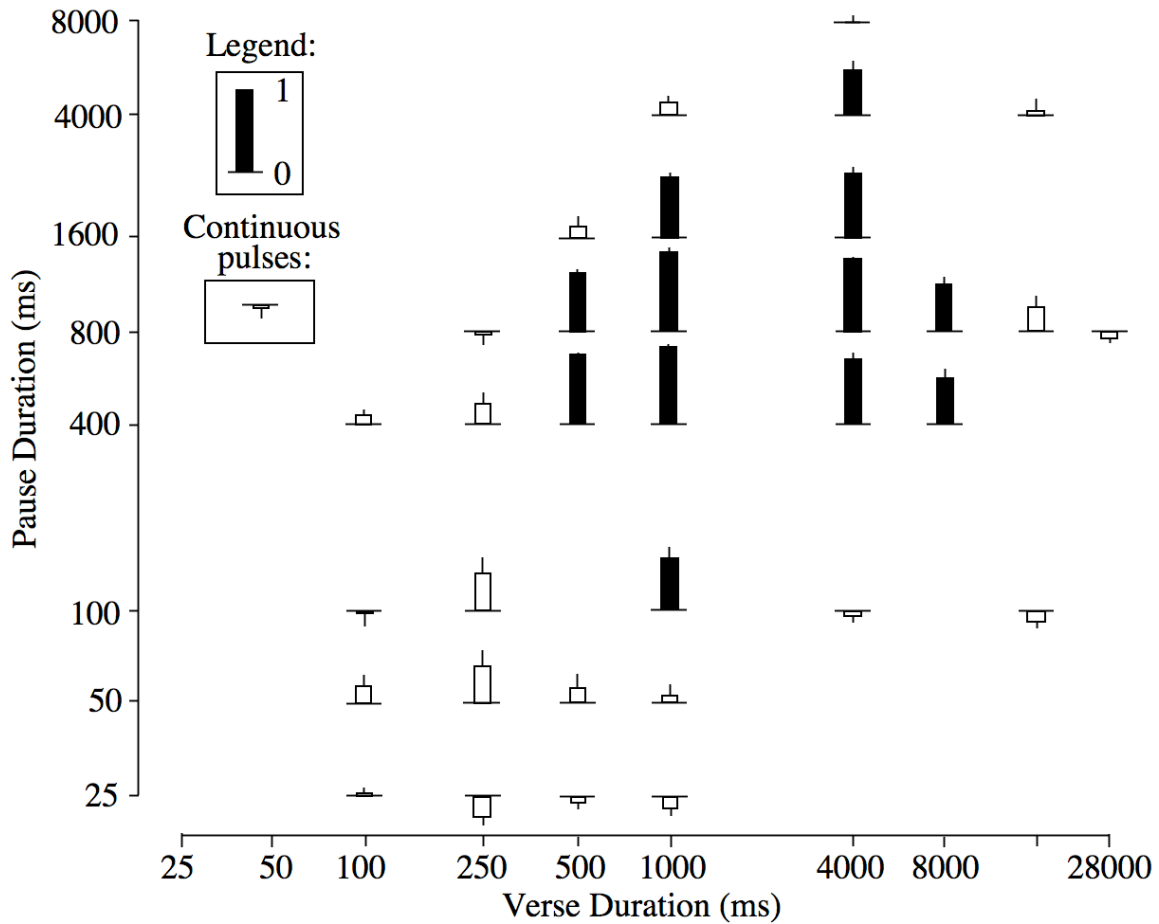
Figure 3

Importance of the verse structure for phonotactic responses of female *N. nebrascensis*.
The bar-height indicates the phonotaxis score (mean ± s.e.m., n = 8 each) for the
respective parameter combination (see legend for the scale of the phonotaxis score). The
baseline of each bar is positioned on the pause duration. The inset labeled 'continuous
pulses' represents the response to a continuous stimulus without a verse structure (i.e. 0
ms pause duration). All stimuli were presented using the conspecific pulse pattern. Filled
bars indicate significant responses and white bars non-significant responses. Note
logarithmic axes.
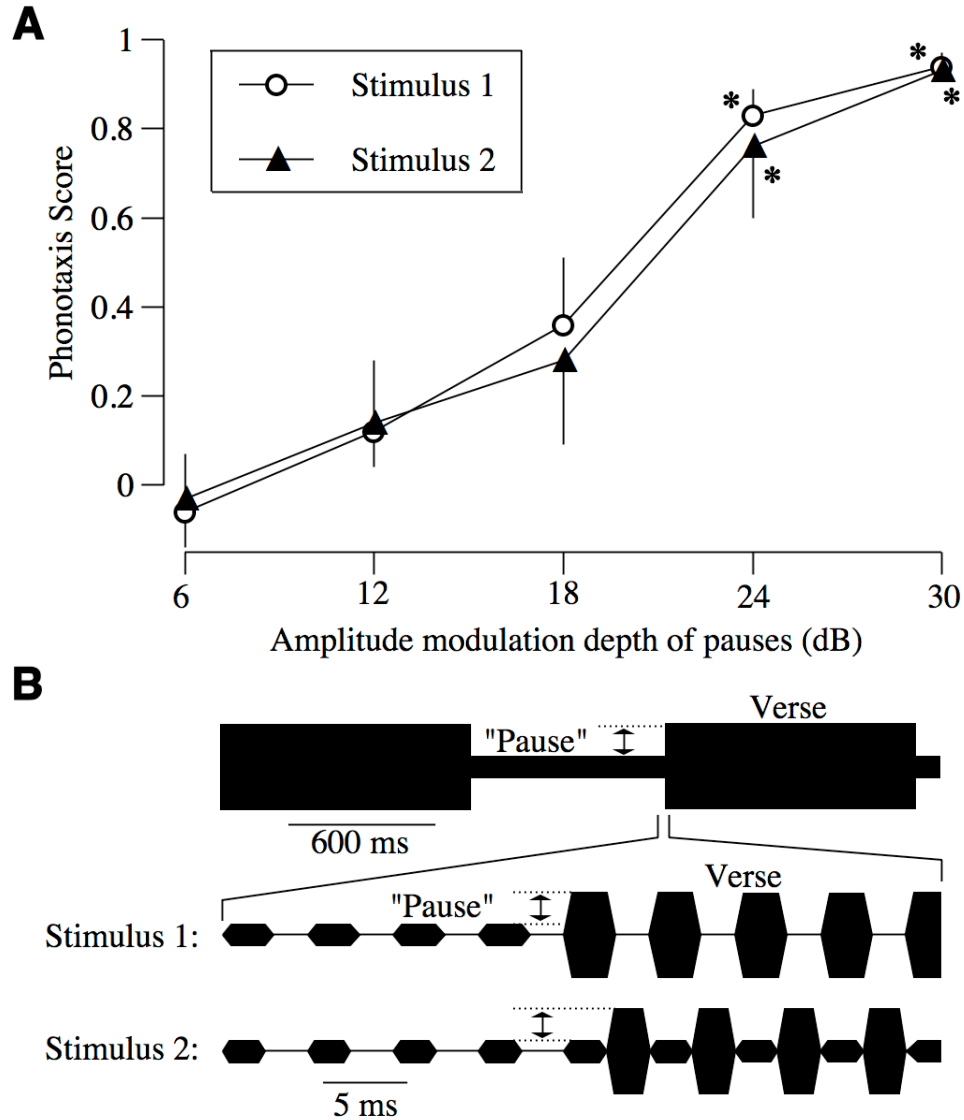
Figure 4

(A) Phonotaxis scores (mean ± s.e.m., n = 8 each) of female *N. nebrascensis* to call models in which the amplitude modulation depth between verses and pauses was varied from 6 to 30 dB. Asterisks indicate significant responses. (B) Oscillograms illustrating the stimuli used in A (12 dB amplitude modulation depth shown). All stimuli were presented using the conspecific pulse pattern and verse structure. For further description of the stimuli, see text.

Discussion

During phonotaxis, female *N. nebrascensis* were selective for the pulse pattern and the verse structure of the calls. Females responded only when both call parameters were in the appropriate ranges. To recognize the verse structure females required an amplitude modulation depth between verses and pauses in excess of 18 dB.

Within the verses of the *N. nebrascensis* call, the pulse pattern (first order time structure) has the same structure as the pulse pattern in the continuous *N. robustus* calls; this pulse pattern is likely the ancestral state in *Neoconocephalus* (Greenfield, 1990; Deily and Schul, 2006). Temporal selectivity for this pulse pattern in *N. nebrascensis* is based on the same mechanism as in *N. robustus*: females of both species evaluated the duration of the intervals, responding only when intervals were short or absent (Fig. 2; Deily and Schul, 2004). This mechanism to recognize pulse pattern is likely ancestral in *Neoconocephalus* (Deily and Schul, 2006). Thus, *N. nebrascensis* retained the ancestral selectivity for pulse pattern recognition.

The second order time structure (verses) in the *N. nebrascensis* call is likely a derived call trait in this genus (Greenfield, 1990). Female *N. nebrascensis* require an appropriate verse structure for call recognition; continuous calls were not attractive (Fig. 3). This indicates that, concomitantly with the evolution of the verse structure, female *N. nebrascensis* added a verse recognition mechanism to the ancestral pulse pattern recognizer.

In several species of field and tree crickets, females assess both the pulse pattern and the verse ("chirp") structure of male calls. However, male calls in these species are attractive if either of these parameters are in the correct range, i.e. the two mechanisms are 'or-gated' (Walker, 1957; Tschuch, 1976, 1977; Doherty, 1985). Such 'or-gating' might be used to increase the chances of signal recognition if, for example, the pulse pattern is degraded during transmission in the habitat. However, such a recognition mechanism also results in a reduced selectivity, as a wider range of signals are attractive.

Female *N. nebrascensis* responded only to calls containing both an appropriate pulse pattern and an appropriate verse structure; calls which lacked either of these parameters were not attractive. This 'and-gating' of the two recognition mechanisms results in higher selectivity than either mechanism alone. The "and-gating" in *N. nebrascensis* suggests that adding a new call trait (verses) and an appropriate recognition mechanism aides species isolation rather than signal detection. The added selectivity for verse structure enables females to reliably discriminate between conspecific calls and calls of closely related species without verse structure (e.g., *N. robustus* and *N. bivocatus*).

In order to recognize the presence of the verse structure, female *N. nebrascensis* required an amplitude modulation depth of 18 to 24 dB between verses and pauses (i.e. a continuous background signal at -18 dB effectively masked the call model). This was a surprising finding, as other Tettigoniids are able to detect a pulse pattern modulation depth of less than 4 dB (Schul and Fritsch, 1999). In the sensory pathway of Tettigoniids

and crickets, the temporal pattern of a call is reliably transmitted if the call is 4 to 6 dB louder than background signals (Pollack, 1988; Römer and Krusch, 2000). Similarly, anurans are able to detect individual calls when they are 3 to 6 dB above the background chorus (e.g. Wollerman, 1999; Murphy and Gerhardt, 2000). In this light, the 18-20 dB modulation depth required by *N. nebrascensis* females seems not only surprising but disadvantageous, as background signals are likely to mask the conspecific pattern. One may speculate whether this is due to a neural constraint, or simply a property of an 'unsophisticated' verse recognizer.

Causes of male synchrony in N. nebrascensis

In choruses of calling male *N. nebrascensis*, call synchronization is commonly observed across large distances (Meixner and Shaw, 1979; 1986) . Such call synchronization is often interpreted as a consequence of female preferences for leading calls: competition for the leading position may result either in call synchronization of alternation (Greenfield, 2002).

Whether female *N. nebrascensis* have a preference for leading calls (e.g. Greenfield, 2002) remains to be tested. Male calling behavior in *N. nebrascensis*, however, suggests the absence of a leader preference in females of this species. First, the leader-follower relationships of verses of neighboring *N. nebrascensis* often remain unchanged over extended periods of time, and upon joining a chorus, males are unlikely to drop even a single verse (Meixner and Shaw, 1979; 1986). If males were competing to be the leader, the role of leader should occasionally shift among males, and males should occasionally skip individual verses to gain the leader position (Greenfield, 1994; 2002;

2006). Second, leader preferences are usually associated with open-ended preference for faster verse repetition rates (Greenfield, 2006), rather than with a closed response field as in *N. nebrascensis*.

The large required modulation depth of *N. nebrascensis* females, on the other hand, may cause call synchronization. In order to preserve the verse structure, male *N. nebrascensis* should synchronize their verses. Because of the large modulation depth required by females, this synchronization should not only occur among neighbors, but also with more distant males. This is because an unsynchronized male eight times as far away as a female (i.e. -18 dB) would destroy both his own and all other males' verse structure within that radius (assuming 6 dB attenuation/double distance). Thus synchronization of the verse structure should occur among all males within a chorus, as it is commonly observed. Thus, in *N. nebrascensis* call synchronization seems to be consequence of the female call recognition mechanism, which requires a distinct verse structure of large modulation depth. In this light, male synchronization in *N. nebrascensis* could be interpreted as cooperation among calling males rather than the outcome of competition (e.g. Greenfield, 1994; 2002).

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Chapter 5

Summary of Results and Abstract

Neoconocephalus robustus, *N. nebrascensis* and *N. bivocatus* were each able to reliably reject the calls of the other two species. Hence, reproductive isolation in these species is probably achieved using the communication system. The mechanisms responsible for call recognition and reproductive isolation, however, were strikingly different.

Male calls of *N. nebrascensis* and *N. bivocatus* contain the derived temporal features of double-pulses and verses, respectively. Females of these species used unique recognition mechanisms to detect the presence of these derived temporal features. Females did not respond when the conspecific derived temporal pattern was absent. Thus, for *N. nebrascensis* and *N. bivocatus*, the derived male calls along with the derived female recognition mechanisms ensured reproductive isolation (Figs. 2B and 2C).

Male calls of *N. robustus* are comprised of a relatively low carrier frequency (a derived trait). Again, females use a derived mechanism to recognize the presence of the derived call trait. However, in contrast to *N. nebrascensis* and *N. bivocatus*, this mechanism is not unique to this species, and is not sufficient to prevent responses to heterospecifics. In addition to a sharpened spectral selectivity, female *N. robustus* are more selective for the pulse pattern and the verse structure than their congeners. Thus, instead of using one highly effective and qualitatively new mechanism, female *N.*

robustus appear to have sharpened their selectivity for the pulse pattern, verse structure, and carrier frequency in order to prevent responses to heterospecifics (Fig. 2A).

Below, I first describe the calls and detail the mechanisms of call recognition and reproductive isolation used by each species. I then provide rational for considering certain traits to represent the ancestral condition in *Neoconocephalus*. I use knowledge of the ancestral traits to discuss the asymmetry in the response of species using different combinations of ancestral and derived call traits. Finally, I relate my data from *Neoconocephalus* to the concepts of symmetrical versus asymmetrical divergence and to the patterns found in several other groups of acoustically communicating taxa.

A

Pulse Pattern   Verse Structure

N. robustus — SP / C

N. nebrascensis — SP / V — Pause, Verse

N. bivocatus — DP / C

Interval, Pulse, 10 ms, 1000 ms

B

Spectral Structure

— *N. robustus* (7 kHz)

······· *N. nebrascensis* (10 kHz)

– – *N. bivocatus* (10 kHz)

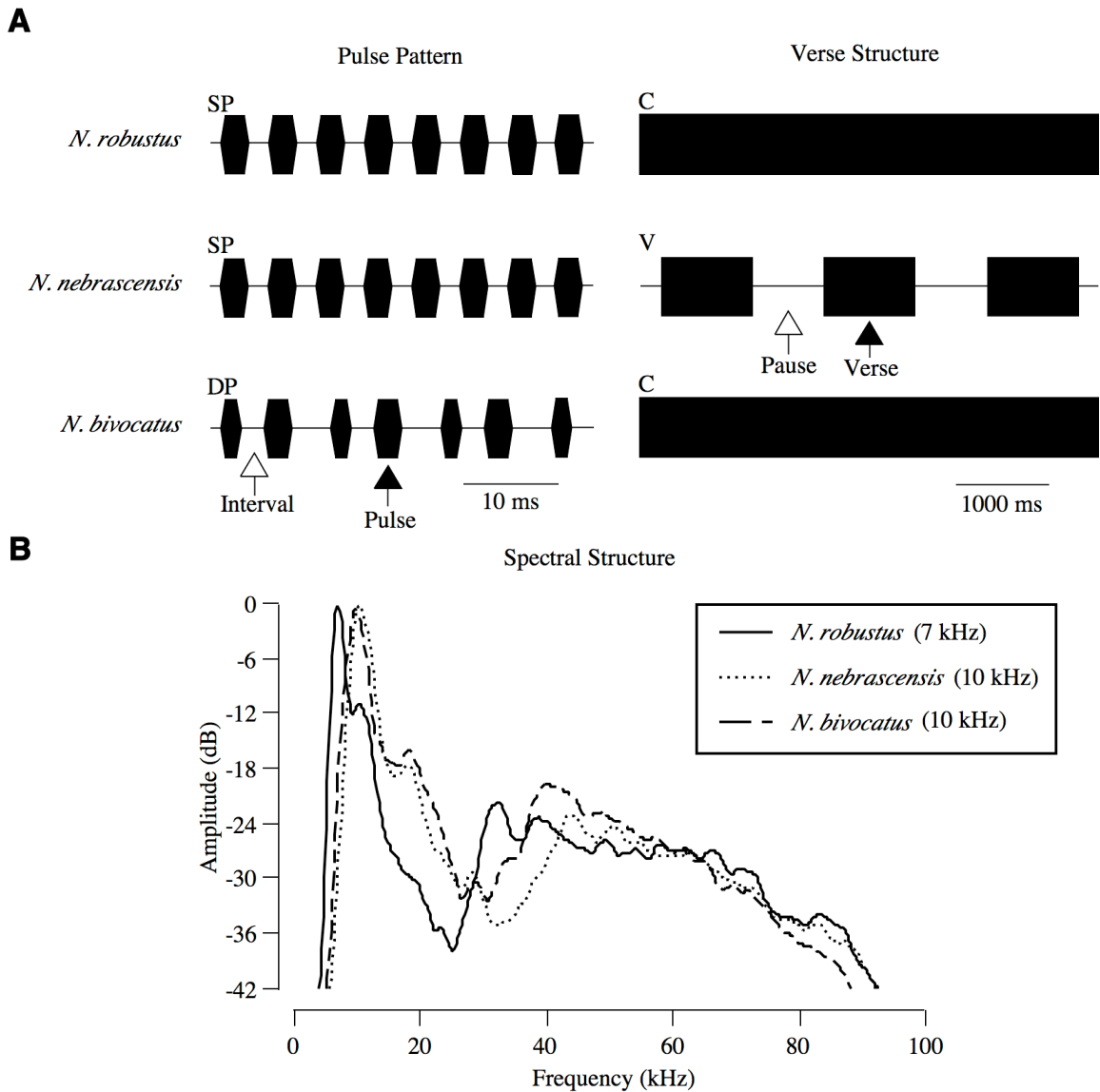Amplitude (dB) vs Frequency (kHz)

Figure 1

(A) Oscillograms of the conspecific call models used throughout Chapters 2-4. All parameters were based on population mean values determined by Büttner (2002) at 25°C.. Oscillograms on the left show the pulse pattern of each species (SP = Single pulse pattern; DP = Double pulse pattern). Oscillograms on the right indicate whether the call is produced continuously (C) or whether it is modulated by a verse structure (V). Note different time scales. (B) Averaged spectra of male calls of *N. robustus* (solid line, n=10), *N. nebrascensis* (dotted line, n=10) and *N. bivocatus* (dashed line, n=8) at 25°C. Adapted from Schul and Patterson (2003).
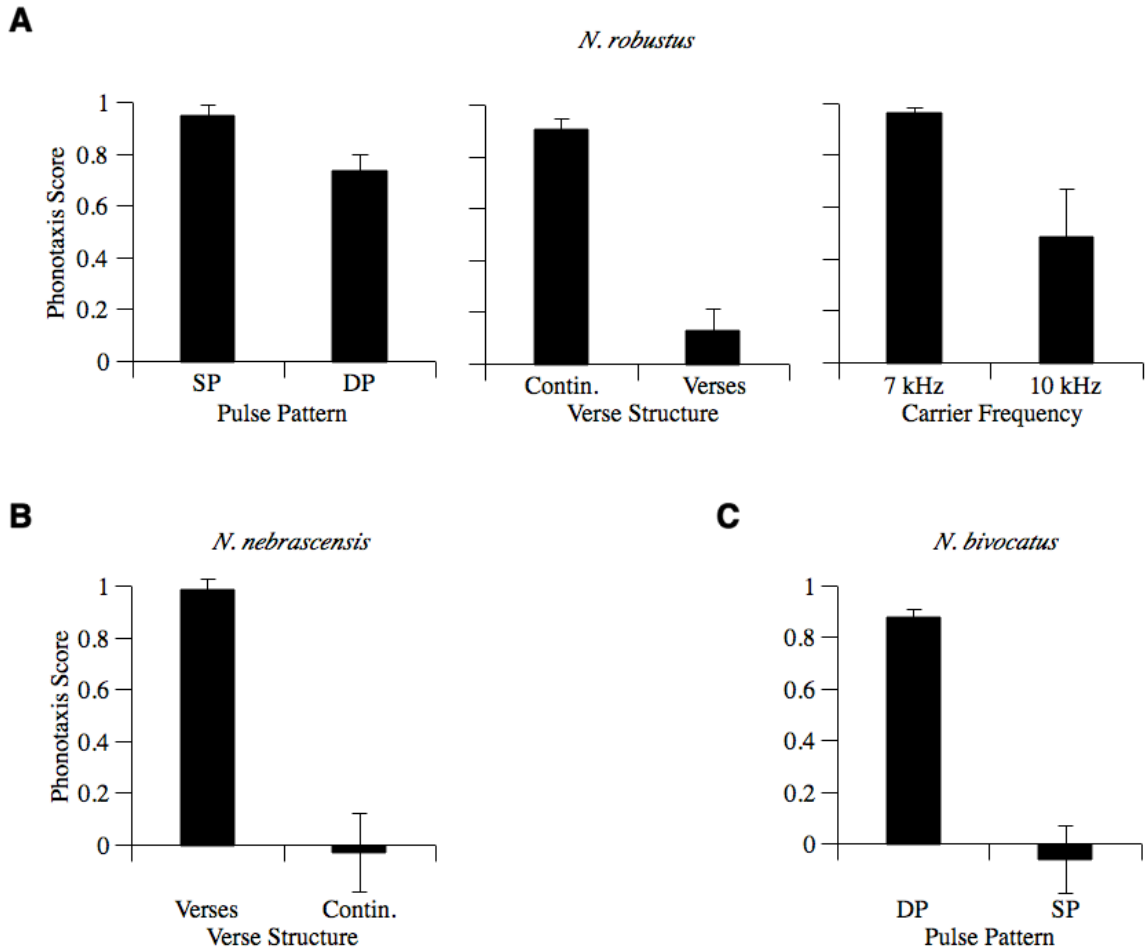
Figure 2

Comparison of the mechanisms responsible for reproductive isolation in (A) *N. robustus*, (B) *N. nebrascensis* and (C) *N. bivocatus*. Each bar represents the phonotaxis score (mean ± s.e.m.; n = 7-9 each) elicited by conspecific (left bar) and heterospecific (right bar) values of the indicated call parameter.

A *N. robustus*

B *N. nebrascensis*

C *N. bivocatus*

Phonotaxis Score

Pulse Pattern

Conspecific   Sine

Figure 3
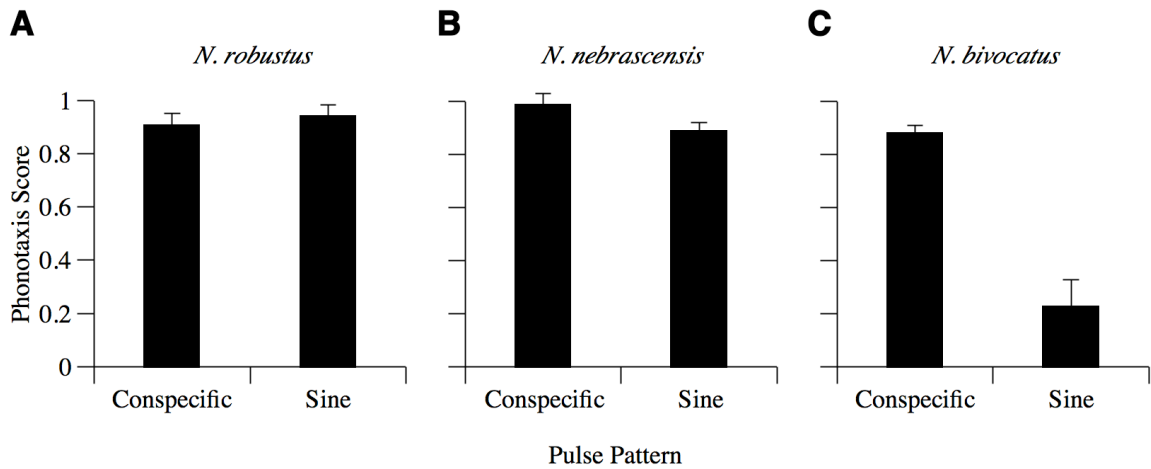
Comparison of the importance of the pulse pattern for call recognition in *N. robustus*, *N. nebrascensis* and *N. bivocatus*. Each bar represents the phonotaxis score (mean ± s.e.m.; n = 8-9 each) in response to the model of the conspecific call (left bar) and to an un-modulated sine wave (right bar in A and C) or to a sine wave presented in the conspecific verse structure (right bar in B).
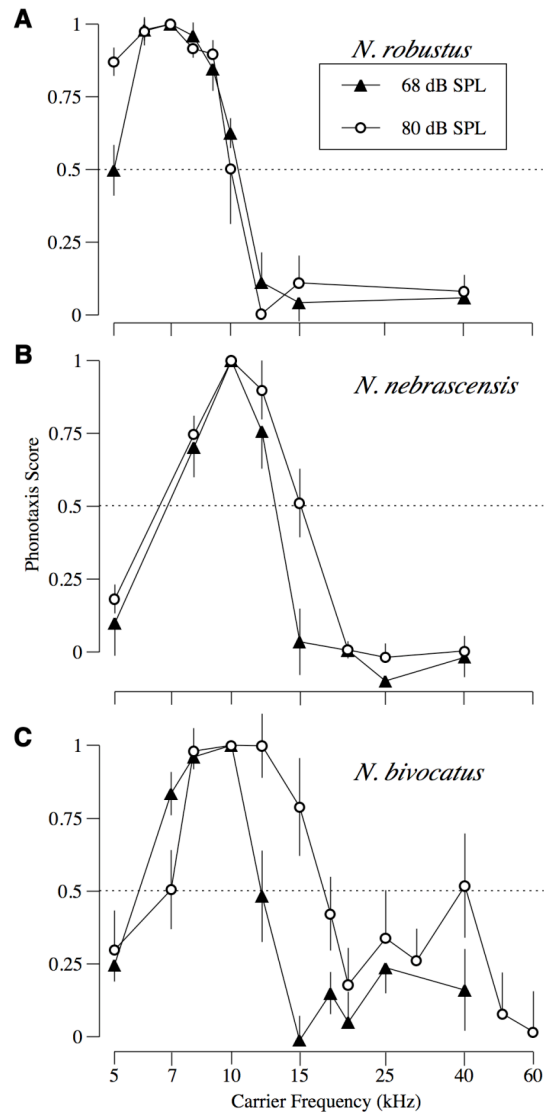
Figure 4

Importance of call carrier frequency for female phonotaxis at stimulus amplitudes of 68 dB SPL (triangles) and 80 dB SPL (circles) in (A) *N. robustus*, (B) *N. nebrascensis* and (C) *N. bivocatus*. Each point represents the mean phonotaxis score (± SEM) of 7 to 8 females. Phonotaxis scores were normalized relative to the phonotaxis score at the conspecific carrier frequencies (7 kHz for *N. robustus*, 10 kHz for *N. nebrascensis* and *N. bivocatus*). All responses above 0.5 (dashed line) were significant (see Methods in Chapter 3) except for *N. bivocatus* at 40 kHz / 80 dB SPL.

Figure 5 (following page)

Comparison of call recognition mechanisms between *N. robustus* (circles) *N. nebrascensis* (squares) and *N. bivocatus* (triangles). 1A summarizes the role of the pulse pattern in call recognition, 1B summarizes the role of the verse structure in call recognition, and 1C summarizes the role of carrier frequency in call recognition. Each point represents the mean phonotaxis score in response to the indicated stimulus ($\pm$ s.e.m.; n = 7-10 each in A and C, 4-9 each in B; 21 of the 25 points in B had sample sizes of 7 or more). The pulse durations used in A were those used in the conspecific call models of each species. Asterisks indicate significant responses.
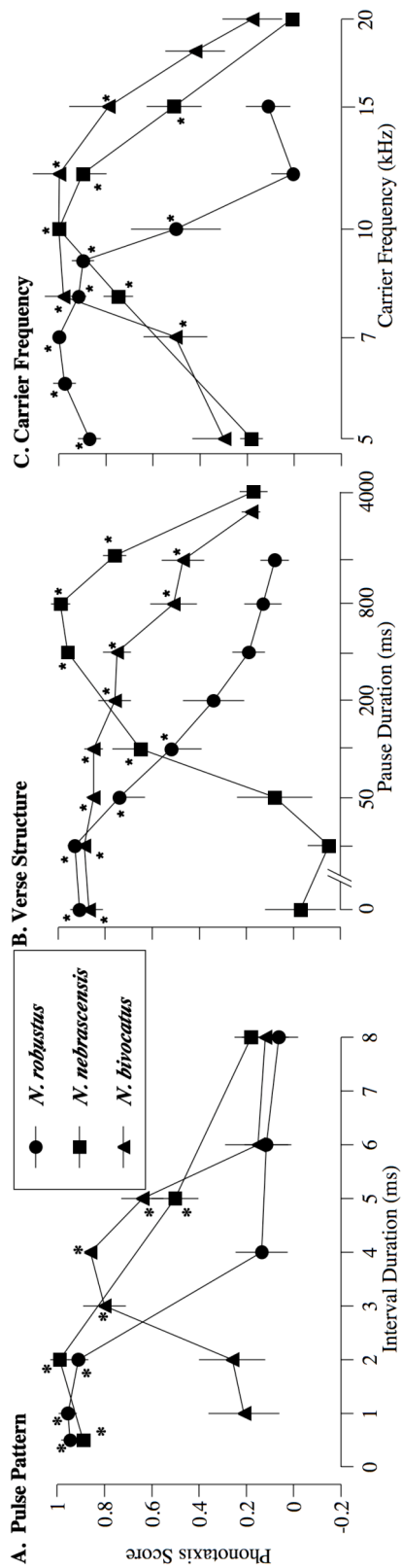
Figure 5

Table 1A. Male call traits.

Call parameter	*N. robustus*	*N. nebrascensis*	*N. bivocatus*
Pulse pattern	Single pulse	Single pulse	**Double pulse**
Verse structure	Continuous	**Verses**	Continuous
Carrier frequency	**7 kHz**	10 kHz	10 kHz

Bold indicates derived call trait.

Table 1B. Female call recognition mechanisms.

Call parameter	*N. robustus*	*N. nebrascensis*	*N. bivocatus*
Pulse pattern	Maximun interval duration*	Maximun interval duration	**Pulse rate***
Verse structure	Continuous*	**Verses***	Continuous
Carrier frequency	**Strong HF inhibition***	Weak HF inhibition	Very Weak HF Inhibition

Bold indicates derived recognition mechanism.
* indicates mechanism used for species isolation.

Table 1

Call recognition in *N. robustus*

Male *N. robustus* produce a continuous trill of pulses at a rate of about 200/s, and use a carrier frequency of about 7 kHz (Fig. 1; Table 1A).

Female *N. robustus* were attracted to a continuous 7 kHz sine wave. Calls containing a pulse pattern (e.g. the conspecific call) were attractive, as long as the intervals were short enough (Fig. 3A; Chapter 2).

In response to versed calls, such as those produced by male *N. nebrascensis*, responses of female *N. robustus* declined relatively sharply. Significant responses occurred only when the pause duration was 100 ms or less. Pauses of 800 ms (i.e., as used by *N. nebrascensis*) failed to elicit significant phonotaxis in *N. robustus* (fig. 5B; Chapter 4).

Female *N. robustus* responded with significant phonotaxis to call models using carrier frequencies from 5 to 10 kHz at 80 dB SPL, although responses declined precipitously between 9 and 12 kHz. Selectivity toward high frequencies did not change when stimuli were presented at 68 dB SPL (Fig. 4A). Such intensity-independent response functions are a typical signature of lateral inhibition (Gerhardt and Huber, 2002; Hennig et al., 2004). Accordingly, the selectivity towards high carrier frequencies in *N. robustus* is caused by a high-frequency inhibition of phonotaxis (Fig. 5C), and is interpreted as an adaptation for species isolation. In effect, this inhibitory mechanism produces an intensity-independent spectral selectivity that reduces female responses toward closely related congeners using slightly higher carrier frequencies (e.g. *N. nebrascensis* and *N. bivocatus* at 10 kHz) (Chapter 3).

Call recognition in *N. nebrascensis*

Male *N. nebrascensis* produce a continuous trill of pulses at a rate of about 200/s (as in *N. robustus*), and use a carrier frequency of about 10 kHz (as in *N. bivocatus*). However, *N. nebrascensis* differs from the other two species by grouping its pulses into 1s verses, thereby modulating the pulse pattern with a higher order verse structure (Fig. 1; Table 1A).

Female *N. nebrascensis* were attracted to a 10 kHz sine wave presented in the conspecific verse structure. Verses containing a pulse pattern (e.g. the conspecific call) were attractive as long as the intervals were short enough (Fig. 3B; Chapter 4). This is the same pulse pattern recognizer as is used by *N. robustus* (Fig. 3A; Chapter 2).

In addition to the pulse pattern, female *N. nebrascensis* required a verse structure for call recognition (Fig. 2B). Females responded over a relatively wide range of verse and pause durations, although responses decline towards both long and short verse and pause durations (Chapter 4). Female selectivity for the duration of long pauses is similar to that of *N. bivocatus* (significant responses occur up to pause durations of 1500 ms; fig. 5B).

To recognize the verse structure female *N. nebrascensis* required an amplitude modulation depth between verses and pauses in excess of 18 dB. This requirement is interpreted to force males to synchronize their verses with those of their neighbors (Chapter 4).

Female *N. nebrascensis* responded with significant phonotaxis to call models using carrier frequencies from 8 to 15 kHz at 80 dB SPL. Selectivity toward high frequencies was dependent on stimulus amplitude: significant responses occurred over a

narrower frequency range at 68 dB SPL than at 80 dB SPL (Fig. 4B). However, several lines of evidence indicate that there is a weak inhibitory effect of high frequencies, albeit much less than was found in *N. robustus* (Fig. 5A; Chapter 3). For *N. nebrascensis*, carrier frequency is not necessary or sufficient for reproductive isolation from the species considered here.

That *N. nebrascensis* has a weak high frequency inhibition suggests that the strong high frequency inhibition present in *N. robustus* is not a unique trait to that species.

Call recognition in *N. bivocatus*

Male *N. bivocatus* produce a continuous train of pulses. However, in contrast to *N. robustus* and *N. nebrascensis* (single pulses), pulses are repeated with alternating periods, resulting in distinct pulse pairs (double pulses): approximately 175 pulses/s are grouped into 87 pulse pairs/s. This species uses a carrier frequency of about 10 kHz (Fig. 1; Table 1A).

Female *N. bivocatus* required a distinct pulse pattern: they were attracted only to calls containing a pulse rate of about 87 pulses/s, largely independent of pulse and interval durations. Using the duration of the merged double-pulse corresponding to the conspecific pattern, pulse rates from 80-95 Hz elicited significant responses, while pulse rates of 74 and 105 Hz failed to elicit significant responses. This pulse rate corresponds to the repetition rate of pulse-pairs in the conspecific call (Figs. 2C and 3C; Chapter 2).

In response to versed calls, such as those produced by male *N. nebrascensis*, responses of female *N. bivocatus* declined. However, responses did not decline as sharply

as in *N. robustus*. Significant responses occurred up to pause durations of 1500 ms. Pauses of 800 ms (i.e., as used by *N. nebrascensis*) elicited significant phonotaxis in *N. bivocatus* (fig. 5B; Chapter 4).

Female *N. bivocatus* responded with significant phonotaxis to call models using carrier frequencies from 7 to 15 kHz at 80 dB SPL. Selectivity toward high frequencies was dependent on stimulus amplitude: significant responses occurred over a narrower frequency range at 68 dB SPL than at 80 dB SPL (Fig. 4C). This intensity-dependent response reflects the absence (or negligible contribution) of a high-frequency inhibition in this species during phonotaxis (Fig. 5C; Chapter 3). For *N. bivocatus*, carrier frequency is not necessary or sufficient for reproductive isolation from the species considered here.

Reproductive Isolation

The calls of *N. robustus*, *N. nebrascensis* and *N. bivocatus* each feature one distinguishing characteristic: a low carrier frequency in *N. robustus*, a verse structure in *N. nebrascensis*, and double-pulses in *N. bivocatus* (Fig. 1; Table 1A). Each of these call characteristics are likely derived in this clade (see next section).

In *N. robustus*, *N. nebrascensis* and *N. bivocatus*, the properties of the female recognition system allow pre-mating isolation of each of these species from the other two. The underlying mechanisms responsible for this isolation, however, are different for each of the three species. In both *N. nebrascensis* and *N. bivocatus*, the derived temporal characteristic in each is necessary for call recognition and sufficient for species isolation. (Fig. 2B and 2C).

In contrast, while the derived call trait (low carrier frequency) of *N. robustus* is important for call recognition (Chapter 3), this characteristic is not sufficient to wholly prevent responses to *N. nebrascensis* or *N. bivocatus* (Fig. 2A). Thus, in addition to having a sharpened spectral selectivity, *N. robustus* uses sharpened temporal selectivity for pulse pattern and verse structure to minimize responses to the calls of *N. nebrascensis* and *N. bivocatus* (Figs. 2A and 5).

Call recognition (and reproductive isolation) thus has a different signature in *N. robustus* than in *N. nebrascensis* and *N. bivocatus*. In *N. robustus*, several partially effective mechanisms are used for call recognition, each of which is demonstrably sharpened relative to congeners using similar mechanisms (Fig. 5). In contrast, the derived temporal patterns of *N. nebrascensis* and *N. bivocatus* allow unambiguous identification of conspecific males. Accordingly, females of these species have not sharpened other mechanisms of call recognition relative to their congeners (Fig. 5).

The Ancestral Call in *Neoconocephalus*

The assumed ancestral call for the clade of *Neoconocephalus* containing *N. robustus*, *N. nebrascensis* and *N. bivocatus* consists of a continuous single-pulse temporal pattern and a center-frequency of about 10 kHz (Table 1A). Support for this assumption comes from the following:

1. The ancestral call traits (single pulse pattern, continuous, and center frequency of about 10 kHz) are common in this genus: of 23 described calls, 18 have single-pulse pattern, 17 are continuous (Greenfield, 1990), and most species' calls are limited to frequencies above 9 kHz (Schul and Patterson 2003; Greenfield, 1990).

2. Variation in this genus is such that species typically modify the pulse pattern or add a verse structure while leaving the rest of the parameters unchanged (Greenfield, 1990). This suggests that the continuous single-pulse temporal pattern is the ancestral condition from which species subsequently diverged.

3. Hearing sensitivity of *Neoconocephalus* females is considerably lower at 7 kHz than at 10 kHz (Schul and Patterson, 2003). The shift to a lower call frequency would therefore result in a reduction of communication distance. Also, as the ears of Tettigoniids usually function as pressure receivers rather than pressure gradient receivers, 7 kHz likely provides less peripheral directionality than 10 kHz (Gerhardt and Huber, 2002). These disadvantages of a low carrier frequency, as well as the transmission properties of the habitat (Schul and Patterson, 2003) suggest that 10 kHz is the most effective carrier frequency for *Neoconocephalus*. That *N. robustus* was likely 'pushed' by congeners to this lower center frequency (Chapter 3) suggests that in the ancestral state (i.e. without the interfering calls of congeners) this species used a carrier frequency of 10 kHz.

4. Preliminary results of a molecular phylogenetic analysis based on a mitochondrial locus support our assumptions of the ancestral call (R. L. Snyder and J. Schul, unpublished data).

The Ancestral Call Recognition Mechanism in *Neoconocephalus*

For most *Neoconocephalus* species, which produce a continuous trill of pulses at a constant rate of about 200/s (Greenfield, 1990), the temporal recognition mechanism used by *N. robustus* (i.e. maximum interval duration) is probably the only mechanism

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that would function. Essentially, it is doubtful whether the sensory system is able to extract any more detailed information from the temporal pattern of such fast calls: the temporal acuity of insect auditory receptor cells have been found to decrease for amplitude modulation rates above 100 Hz (e.g. Prinz and Ronacher, 2002; Surlykke at al., 1988). Additionally, the ascending auditory pathway will likely limit transmission to even lower modulation rates (e.g. Schildberger, 1984).

Given that the ancestral call was likely continuous, the mechanism of verse recognition used by *N. nebrascensis* is likely a derived trait, as is the sharpened selectivity for the verse pattern found in *N. robustus*.

Finally, given the disadvantages of using a low carrier frequency, spectral processing as found in *N. robustus* is probably a derived trait. That *N. nebrascensis* and *N. bivocatus* both call at 10 kHz and lack such a pronounced inhibitory mechanism supports this interpretation.

Thus, we suggest that the ancestral recognition system consisted of a maximum interval duration selectivity (unsharpened as in *N. nebrascensis;* Fig. 5A), a verse selectivity as in *N. bivocatus* (unsharpened; Fig. 5B), and a spectral selectivity as in *N. nebrascensis* or *N. bivocatus* (Fig. 5C) (Table 1B).

Ancestral Versus Derived Communication Systems

The calls and recognition mechanisms differ among *N. robustus*, *N. nebrascensis* and *N. bivocatus* in terms of their relation to the ancestral state. The calls of *N. nebrascensis* and *N. bivocatus* contain features not present in the ancestral call. Females of these species recognize these features using derived recognition mechanisms (Table 1;

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Figs. 2B and 2C). In contrast, the calls of male *N. robustus* contain no new features relative to the ancestral call; the low carrier frequency is derived, but a carrier frequency does exist in the ancestral call. Similarly, female *N. robustus* have sharpened both temporal and spectral recognition mechanisms, but they do not use mechanisms that differ qualitatively from the ancestral state (Table 1; Fig. 5).

The calls of *N. robustus'* ancestors most likely had the same temporal pattern and the same temporal call recognition mechanisms (albeit probably less selective) as *N. robustus*, but a higher carrier frequency. After the appearance of species with derived temporal patterns (e.g. double-pulses or verses), the temporal call recognition of this ancestral population would not have enabled reliable rejection of the 'new' calls. Thus, selection would have favored any traits that reduced the risk of hybridization. One such trait might be the relatively sharp rejection by *N. robustus* of the versed temporal pattern (Fig. 5B). This mechanism alone is highly effective at preventing female *N. robustus* from responding to the calls of *N. nebrascensis*. *N. bivocatus*, which is able to reject the *N. nebrascensis* call using the pulse pattern, has no such sharpened selectivity for the verse structure.

However, in terms of rejection of heterospecific calls, the derived pulse pattern used by *N. bivocatus* was perhaps more problematic for the ancestors of *N. robustus*. Pulse pattern selectivity in *N. robustus* is sharpened relative to other species using this same mechanism, and could not be more selective without rejecting the conspecific temporal pattern (Fig. 5A; Chapter 2). While this increased selectivity for pulse pattern does have a significant effect, it does not allow reliable rejection of the *N. bivocatus* call

(Fig. 2A). Because of this, we suggest that a lower call carrier frequency evolved in *N. robustus* (in tandem with increased temporal selectivity) in response to the appearance of one or more derived temporal patterns (e.g. double-pulses) (Chapter 3).

While the neuronal mechanisms responsible for the increased spectral and temporal selectivities in *N. robustus* are unknown, each appears to be simply a strengthening or sharpening of mechanisms present in congeners (Fig. 5). Thus a reinforcement-like process (Dobzhansky, 1937; sensu Blair, 1955) could have been responsible for a step-wise increase in selectivity for low frequencies, pulse pattern, and verse structure in *N. robustus*. This situation is what classical sexual selection and reinforcement models would predict, with the caveat that it appears to function across several parameters (multi-dimensional) at the same time.

In contrast to the situation in *N. robustus*, species isolation in *N. nebrascensis* and *N. bivocatus* occurs using only the derived temporal pattern in each species. As discussed in Chapter 1, this may reflect the prerequisite of a small divergent population to make a clear and immediate distinction between derived and ancestral males. Without such a distinction, they might be quickly reabsorbed or replaced by the parental population. While such effective mechanisms using qualitatively novel mechanisms may be a rare occurrence, those populations that were not able to make such a sharp distinction might not have survived. Only for species with wide distributions (i.e. large gene-pool), might the opportunity for a reinforcement-like mechanism be afforded (e.g. *N. robustus*; Fig. 1 in Chapter 1). Since such large widely distributed populations are less likely to evolve new mechanisms than are smaller isolated populations, the larger population might often use ancestral traits.

There are no clear intermediaries between the proposed ancestral communication system in *Neoconocephalus* and the derived qualitatively different communication systems used by *N. bivocatus* and *N. nebrascensis*. Questions of the origins and evolutionary mechanisms leading to this situation are beyond the scope of this work. However, as more studies are examining the intricacies of communication and divergence, this pattern seems to be common (review in Gerhardt and Huber, 2002). The evolutionary mechanisms responsible for such patterns are unknown, although they seem not to be adequately explained by current models of signal–receiver coevolution (Schul and Bush, 2002).

Symmetric versus Asymmetric Interactions

As discussed above, reproductive isolation of *N. robustus* from *N. nebrascensis* and *N. bivocatus* relies in parallel on several different reproductive isolation mechanisms. In this species, all of these mechanisms are associated with the communication system. However, the response presumably could have taken a different form (e.g. an ecological separation or a temporal isolation).

Reproductive isolation of *N. nebrascensis* and *N. bivocatus* from the other two species each use a single mechanism. Each of these mechanisms is different from the mechanisms of the other species. As in *N. robustus*, this response presumably could have taken a different form.

In *Neoconocephalus*, divergence is symmetric in that each species is effectively reproductively isolated from its congeners. However, from a more limited knowledge of

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this system, we might conclude that divergence and/or displacement in *Neoconocephalus* is asymmetric. Each species investigated here uses a unique mechanism (or set of mechanisms) of reproductive isolation not present in the other two. For these species of *Neoconocephalus*, acoustic cues are sufficient for isolation. However, the use of other isolation mechanisms is likely in other groups.

Relatively little data exist on how ecological, temporal, geographic, and/or behavioral isolation (in addition to gametic and physical incompatibilities) contribute to reproductive isolation in groups where a single mechanism is not sufficient, or where the interactions are viewed to be asymmetric. However, many studies suggest that reproductive isolation is asymmetric based on data from only a subset of these mechanisms (e.g., Kaneshiro, 1980; Wasserman and Koepfer, 1980; Vigneault and Zouros, 1986; Sperling and Spence, 1991; Emms et al., 1996; Shapiro, 2001; Coyne et al., 2002; Smadja et al., 2004; review in Gerhardt and Huber, 2002). One conclusion repeatedly drawn from this result is that the two species are in the process of merging, or that one is being replaced by the other. However, this is not necessarily so. The experimental design of these studies often take place under laboratory conditions, and so may frequently underestimate the contributions of certain isolation mechanisms that may function in nature (e.g. behavioral or fine-scale spatial differences). Experiments designed to test a wider variety of potential reproductive isolation mechanisms might dispel the notion of asymmetric reproductive isolation (or at least provide a better estimate of its occurrence). Rather than asymmetric reproductive isolation, we might consider the alternative of a multi-dimensional and symmetric reproductive isolation.

Speculations on Other Interactions in *Neoconocephalus*

Preliminary observations suggest that another congener (*N. retusus*) shows what might be a temporal mechanism of reproductive isolation. This species produces a continuous pulse pattern that is intermediate between *N. robustus* and *N. bivocatus*. Its temporal recognition system is the same as that used by *N. robustus*, although not as sharply tuned (Fig. 5A; J. A. Deily, unpublished data). Thus, this species would not be able to reject the calls of *N. robustus* or *N. bivocatus* on the basis of temporal pattern, and may or may not reject the temporal pattern of *N. nebrascensis* on the basis of the verse structure. In addition, while *N. retusus* uses a higher than average carrier frequency of 15 kHz (Schul and Patterson, 2003), this species responds well to signals using 10 kHz (M. Talwar, unpublished data). Thus, *N. retusus* almost certainly would not reject the call of *N. bivocatus*, and would possibly fail to reject the calls of *N. robustus* and *N. nebrascensis*. However, at least in Missouri, *N. retusus* is active later in the year than these three species (personal observation), and so potentially avoids them not through acoustic mechanisms but through temporal isolation.

Evidence of Multi-Dimensional Reproductive Isolation Outside of *Neoconocephalus*

Tettigonia

Male katydids of the genus *Tettigonia* produce calls containing similar patterns of variation as found in *Neoconocephalus*. The call of *T. cantans* is comprised of a continuous trill of pulses (single pulse pattern). The call of *T. viridissima* is also continuous, but uses a double pulse pattern. The call of *T. caudata* is comprised of a single pulse pattern that is modulated by a verse structure (Heller, 1988; Schul, 1994).

Females of these species use qualitatively different mechanisms to recognize their conspecific calls. In *T. caudata* and *T. viridissima*, the temporal pattern is sufficient to isolate these species from the other two (i.e., neither responds to the temporal patterns of heterospecifics). In *T. cantans*, temporal cues are not sufficient to avoid calls of heterospecifics. However, this species also uses differences in the spectra of the calls to aid in call recognition (Schul, 1998; Schul et al., 1998; Schul, 1999).

Teleogryllus

In the closely related crickets *Teleogryllus commodus* and *T. oceanicus*, females can discriminate between males of the two species based on the temporal patterns alone (Pollack and Hoy, 1979). However, while female *T. commodus* require the pulse rates corresponding to the conspecific trills and chirps, female *T. oceanicus* require only the pulse rate corresponding to the conspecific chirp (Pollack, 1982; Hennig and Weber, 1997). The carrier frequency is also important for reproductive isolation in *T. oceanicus*. Females of this species responded to calls using the conspecific carrier frequency, but not to calls using the carrier frequency of *T. commodus*. Female *T. commodus* made no such distinction on the basis of carrier frequency (Hennig and Weber, 1997).

Chorthippus

Male *Chorthippus* produce a series of pulses that they group into verses ("syllables"). The verses in these species are further grouped into phrases. Among *C. brunneus*, *C. biguttulus* and *C. mollis*, one important mechanism females use to recognize conspecific males is through assessment of the durations of the verse and pause. These

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parameters are important in each of these species, although they prefer slightly different values. The phrase duration is also important for call recognition: while *C. biguttulus* and *C. mollis* require only that the phrase have a minimum duration (e.g. female *C. biguttulus* respond 50% of the time to phrases with a 1000 ms duration), *C. brunneus* responds only to phrases lasting between 50 and 300 ms. Thus, *C. brunneus* may discriminate among the calls of the other two species on the basis of phrase length (Helversen and Helversen, 1994).

Magicicada

Some periodical cicadas of the genus *Magicicada* are isolated from one another based solely on life cycle characteristics (e.g. 13-year and 17-year species). Between these groups, differences in the calls are uncommon: the life cycle is frequently the only distinguishing characteristic. Within synchronously breeding groups, however, different species frequently produce calls containing species-specific characteristics (Marshall and Cooley, 2000). The 13-year *M. neotredicem* discriminates conspecific calls from those of *M. tredicem* in areas of sympatry using differences in the call carrier frequency. This discrimination is reported as an instance of reproductive character displacement (Marshall and Cooley, 2000).

That different species of *Magicicada* have different life cycles (e.g. 13-year and 17-year species) is usually interpreted to indicate allochronic speciation (e.g. West-Eberhard, 2003). That is, the two species were temporally isolated and then diverged. Alternatively, is it possible that, analogous to the acoustic displacement in *M. neotredicem*, the two species diverged and then were displaced temporally?

Conclusions

Individual studies of reproductive isolation frequently concentrate on only one of several possible mechanisms. While one mechanism (or one class of mechanism; e.g. acoustic cues) may be responsible for reproductive isolation, other mechanisms may also be important. Studies considering only a subset of potential mechanisms frequently find an 'asymmetry' between groups, resulting in conclusions potentially based on incomplete information (e.g. that one species is being replaced by another).

It is clear that mechanisms of reproductive isolation can differ markedly between closely related species, and that several mechanisms may function together to ensure reproductive isolation. It is therefore plausible that a combination of isolation mechanisms (e.g. temporal, spectral, behavioral, geographic, etc.) is responsible for reproductive isolation in a given species. Further, a completely different set of reproductive isolation mechanisms may be used by closely related species. This would result in asymmetric displacement on the scale of individual traits, but in an overall symmetric displacement on the scale of individual animals.

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