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The neuroethology of song cessation in response to gleaning bat calls in two species of katydids, *Neoconocephalus ensiger* and *Amblycorypha oblongifolia*

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SUMMARY

We investigated whether the use of primary or secondary behavioural defences is related to prey sensory thresholds using two species of North American katydids, *Neoconocephalus ensiger* and *Amblycorypha oblongifolia*. Male katydids produce intense calling songs to attract mates, and many gleaning bat species are known to use these calls to locate them as prey. Low duty cycle calling (i.e. sporadic calls) is a primary defence against gleaning bats (prevents attacks), and song cessation is a secondary defence (enables survival of an attack), for which these two species show behavioural differences. Echolocation calls of *Myotis septentrionalis*, a sympatric gleaning bat species, were broadcast to singing katydids and to neural preparations of these katydids to test if differences in behavioural response were related to differences in auditory sensitivity. We measured thresholds and firing patterns of the T-cell, an auditory interneuron involved in predator detection. We hypothesized that low duty cycle calling is the best defence for species not sensitive enough to mount a secondary defence in response to predator cues; therefore, we predicted that *N. ensiger* (high duty cycle song) would have lower behavioural and T-cell thresholds than *A. oblongifolia* (low duty cycle song). Although more *N. ensiger* ceased singing than *A. oblongifolia*, the number and maximum firing rate of T-cell action potentials did not differ between species for echolocation call sequences. We suggest that the T-cell has divergent functions within the Tettigoniidae, including predator and mate detection, and the function could be context dependent in some species.

Key words: antipredator behaviour, echolocation, Tettigoniidae, calling song.

INTRODUCTION

Given the importance of predation as an agent of natural selection (Dawkins and Krebs, 1979), it is not surprising to find an immense diversity of antipredator defence strategies. Behavioural defences are related to the information available to the prey animal; therefore, it is necessary to consider the sensory capabilities of prey animals when exploring the evolutionary factors leading to variation in behavioural defences (Edmunds, 1974; Endler, 1991; Kavaliers and Choleris, 2001). Antipredator behavioural defences can be classified either as primary, those that occur regardless of the presence of a predator, or secondary, those that occur in response to the detection of a predator (Edmunds, 1974). In this paper, we investigate whether the use of primary or secondary behavioural defences is related to prey sensory thresholds.

Male katydids (Orthoptera: Tettigoniidae) produce calling songs to attract females, and these often intense and repetitive sounds can also attract the attention of predators. Thus, there is an apparent conflict between sexual and natural selection for these animals (Zuk and Kolluru, 1998). Although the majority of insectivorous bats hunt by catching prey on the wing, many species are known to glean (capture prey from surfaces) (Findley, 1993; Ratcliffe et al., 2006; Wilson, 1973). Several gleaning species use nocturnal orthopteran calling songs to locate these perched insects as prey (Bailey and Haythornthwaite, 1998; Belwood and Morris, 1987; Fenton et al., 1983; Tuttle et al., 1985). Belwood and Morris (Belwood and Morris, 1987) found that in a Neotropical katydid community, species that sing in habitats inaccessible to gleaning bats produced higher duty cycle songs (i.e. greater percentage of the time calling occupied by sound) compared to species singing in open mature forest where gleaning bats hunt. They suggested that this represented a trade-off in primary defences; either katydids sing in habitats that are refuges from bats, or they reduce the cues they provide to bats for locating them. In support of this argument, several experiments have demonstrated that gleaning bats either cannot, or take longer to, locate orthopterans that call from refuges (Bailey and Haythornthwaite, 1998) or have low duty cycle songs (Belwood and Morris, 1987; Hosken et al., 1994).

It is possible, however, that not all katydids rely entirely on primary defence against gleaning bat predation. Katydids have ears that are sensitive to ultrasound, and some species, such as *Neoconocephalus ensiger* (Libersat and Hoy, 1991) and *Tettigonia viridissima* (Schulze and Schul, 2001), have an in-flight diving response to ultrasound. The flight response is thought to be mediated by an interneuron located in the prothoracic ganglion called the T-cell (TN1) (Libersat and Hoy, 1991), which has one axon running anteriorly within the cervical connective and another running posteriorly within the prothoracic–metathoracic connective. This cell is broadly tuned, but more sensitive to ultrasound than audible sound in most species studied to date. This potentially gives katydids the ability to detect the ultrasonic echolocation calls of gleaning bats and cease singing as a secondary defence against these predators. Spangler (Spangler, 1984) reported that the katydid *Insara covilleae* paused singing when bats flew near it and also paused in response to continuous ultrasound. Faure and Hoy (Faure and Hoy, 2000a) discovered that the katydid *N. ensiger* paused and ceased singing in response to synthesized pulses of ultrasound in the lab, but rarely demonstrated this behaviour in response to audible sound. Song cessation, however, is a costly behaviour; females of the katydid

Requena verticalis abandon approaches to low intensity songs if the male stops singing (Bailey et al., 2003).

The purpose of our study was to investigate the relationship between predator detection threshold and the use of primary versus secondary defences in katydids. We chose two sympatric species of katydids inhabiting Eastern North America for comparison: *N. ensiger*, a species with a high duty cycle song, and *Amblycorypha oblongifolia*, a species with a low duty cycle song. Our first objective was to test the hypothesis proposed by Faure and Hoy (Faure and Hoy, 2000a), that song cessation in katydids could be elicited by the echolocation calls of a sympatric gleaning bat, *Myotis septentrionalis*, as a secondary defence. Our second objective was to determine if differences in behavioural responses between these two katydid species are related to differences in T-cell responses. We hypothesized that low duty cycle calling is the best defence for species not sensitive enough to mount a secondary defence in response to predator cues, and from this we predicted that *N. ensiger* would have a more pronounced singing acoustic startle response, and lower T-cell thresholds, than *A. oblongifolia*. Unlike *N. ensiger*, the presence of the T-cell has not been previously demonstrated in *A. oblongifolia*.

MATERIALS AND METHODS

Study area and animals

We conducted these experiments during July and August of 2004 to 2007 at the Queen's University Biological Station, near Kingston, Ontario, Canada. The protocols used in this study conformed to the guidelines of the Canadian Council on Animal Care and were approved by the animal care committees of the University of Toronto Mississauga and Queen's University. Permission to capture bats was obtained from the Ontario Ministry of Natural Resources (Wildlife Scientific Collector's Authorization). Bats (*Myotis septentrionalis* Trouessart) were captured in modified harp traps (Tuttle, 1974) positioned at the entrances of local abandoned mines, and were housed indoors in screened cages (60 cm×40 cm×40 cm; H×W×D). They were provided with water *ad libitum* and fed mealworms (*Tenebrio molitor* Linnaeus) after trials. Katydids (*Neoconocephalus ensiger* Harris and *Amblycorypha oblongifolia* De Geer) were collected in local fields by following the sound of their song and picking them off grass stems or shrubs. They were housed in plastic and metal mesh cages with water, cat food, grass and pieces of apple. For experiments requiring bats in flight, we used a large outdoor flight room (9.14 m×3.66 m×3.66 m; L×W×H) consisting of a wooden frame with fibreglass mesh panels for walls and ceiling, and an earthen floor.

Acoustic stimuli used in behavioural and neurological experiments

We recorded three stimuli for playback experiments with katydids: two types of echolocation call sequences of *M. septentrionalis* (gleaning and searching calls, both predator cues) and the calling song of a cricket (non-predatory sound). Recordings of *M. septentrionalis* echolocation call sequences were made in August 2004. Our initial interest in *M. septentrionalis* stemmed from observations of several bats gleaning singing *N. ensiger* in the flight room. We subsequently discovered that naïve *M. septentrionalis* released into the flight room would land on a speaker broadcasting this insect's calling song. To obtain a suitable gleaning attack sequence, we released four *M. septentrionalis* individually into the flight room and broadcast the calling song of *N. ensiger* at 93 dB peak equivalent sound pressure level (peSPL) (Stapells et al., 1982), with reference to a 13 kHz continuous tone, from a laptop *via* a data acquisition card (DAQCard 6062E; National Instruments, Austin, TX, USA), ultrasonic amplifier (70101; Avisoft Bioacoustics, Berlin, Germany) and speaker (ScanSpeak 60102; Avisoft Bioacoustics). The speaker was positioned on a shelf 1.1 m above ground facing toward the centre of the flight room. An array of 60 infrared-sensitive diodes spaced 3 cm apart on a pole was placed opposite and two metres away from two near infrared light sources (51 W, λ=890 nm) on tripods. The array was positioned such that the axis of light to receivers was perpendicular to the axis of sound originating at the speaker, and the point between the receivers and sources was 2 m away from the speaker. The diodes registered the amount of light received as voltage, and thus when a bat flew between the light sources and the diodes it caused a decrease in the voltage produced by the diode(s) in the shadow of the bat. This signal was transformed by a custom built converter into a 1 s 12 V DC pulse, which was recorded onto one channel of a RACAL Store 4D tape recorder at 76 cm s^{-1}. A second channel recorded the echolocation calls of the bat from a 6.35 mm microphone (2200C; Larson Davis, New York, USA) positioned directly above the speaker. This provided a recording of the attacking bat's echolocation calls with a marker at the time it crossed the 2 m light line in front of the speaker. A near-infrared-sensitive CCD camera (VCB-3524; Sanyo, San Diego, CA, USA) placed at the back of the room facing the speaker and recording onto a VHS tape provided information about the horizontal and vertical position of the bat relative to the speaker when it broke the light beam. From these three relative measurements, we could calculate the exact distance of the bat to the speaker when it triggered the light array. We recorded one attack sequence per bat.

To estimate intensity of echolocation calls produced during a gleaning attack, we used the same equipment to record a 60 kHz continuous tone (a typical peak frequency for the recorded echolocation calls) generated by a custom-built MATLAB (Version R2006b; The MathWorks, Natick, MA, USA) application on a laptop and broadcast from an Avisoft speaker. The volume was increased until the voltage recorded by the RACAL tape recorder was the same as that recorded for the bat call at the time of the IR marker. This was then broadcast to a 6.35 mm condenser microphone (Type 4135; Brüel and Kjær, Nærum, Denmark) and measuring amplifier (Type 2610: Brüel and Kjær) to obtain the peak equivalent sound intensity for that call. Two of the four bats triggered the light array during their approach to the speaker. We calculated a source level call intensity (i.e. at 10 cm) of 104 dB peSPL for one bat and 105 dB peSPL for the other bat (equivalent to 78 and 79 dB peSPL at the microphone, i.e. intensity at the target). These sequences were digitized and one representative sequence was selected for playback to singing katydids.

We recorded search calls of *M. septentrionalis* from four light-tagged bats released in a forest clearing. Recordings were made using a 6.35 mm microphone (2200C: Larson Davis), anti-aliasing filter (150 kHz; Pettersson Elektronik AB, Uppsala, Sweden), data acquisition card (DAQCard 6062E; National Instruments), and a laptop running BatSound Pro (version 3.31, Pettersson Elektronik AB) at a sampling rate of 500 kHz. Echolocation calls produced at intervals greater than 50 ms were considered search phase calls (Surlykke and Moss, 2000) and measurements were taken of spectral and temporal characters (duration, peak frequency and bandwidth). One representative call (peak frequency: 43 kHz, duration: 2.1 ms) was selected and repeated within a single file using BatSound Pro. This call was repeated 16 times with a period (time from the start of one call to the start of the next call) of 97 ms (the average search call period of the recorded bats). We made the total duration of this

sequence (1.46 s) approximately equal in length to the chosen gleaning attack sequence (1.40 s) to keep the stimuli similar.

One second of silence was added to the start of each sequence to ensure that any observed katydid responses could be attributed to the echolocation calls, not any initial speaker noise. Both sequences were bandpass filtered in BatSound Pro (Butterworth, filter order 8) between 25 and 200 kHz to remove background katydid calls and any high frequency incidental noise. Periods of time between echolocation calls were silenced. We created a series of eight files that each decreased by 5 dB from the original using the "change volume" feature in SASLab Pro (Avisoft Bioacoustics). For both behavioural and neurophysiological playback experiments (see below), the voltage required to produce 90 dB peSPL at 30 cm (the distance between speaker and katydid) for the search calls or the most intense gleaning call was recorded. We calibrated the speaker prior to each playback experiment by adjusting the voltage output from the amplifier to this value for the highest amplitude file for each sequence. This meant that the nine files per sequence were broadcast from 50 to 90 dB peSPL at the katydid at 5 dB increments.

In addition to bat echolocation calls, a single file of cricket calling song (*Teleogryllus oceanicus* Le Guillou), an Australian species to which the katydids in our study would never have been exposed, was played to each singing katydid at an intensity of 85 dB peSPL. This song was recorded from one lab colony male within a sound attenuating chamber with the same equipment used for recording bat search calls. We created a file consisting of one long chirp (five pulses) followed by nine short chirps (two pulses each, 31 ms mean duration of pulses, 5.0 kHz peak frequency) for a total duration of 1.40 s (similar duration to the echolocation call sequences). We included this stimulus to test if the acoustic startle response of katydids is specific to bat calls or can also be elicited by a novel, but non-threatening, sound.

Katydid behavioural responses to acoustic stimuli

We placed individual katydids within cylindrical metal mesh cages (72 mm×150 mm diameter×height) for playback experiments. Four cages were placed on a table, each with the centre of the cage 30 cm from the edge and separated by 5 cm, in a dark, quiet room. The surface of the table and the wall behind was lined with sound attenuating foam. We used a laptop running Avisoft Recorder to playback echolocation and cricket calling song sequences to the katydids and record the calling song and responses of the katydids. Echolocation playback files were broadcast to the katydids at a sampling rate of 214 kHz *via* the high-speed DAQ card, ultrasonic amplifier and an ultrasonic speaker (Technics leaf tweeter, EAS 10TH400B), which was positioned at the edge of the table (hence 30 cm from centre of the cage) using a tripod. A condenser microphone (CM16: Avisoft Bioacoustics) positioned within 1 m of the katydids and connected to one channel of a USB ultrasound acquisition board (Avisoft Ultrasound Gate 416) recorded the calling song of the katydids at a sampling rate of 214 kHz. A direct line from the output of the high speed DAQ card was recorded onto a second channel of the Avisoft USG thus providing a stimulus trace for each recording.

When one katydid began singing, we placed the speaker directly in front of and 30 cm away from that individual and removed the other cages. First, we recorded 30 s of singing as an example of calling song for that individual. We then started playing back one of the echolocation sequences (either search or gleaning calls, alternating which sequence was tested first for each katydid) starting with the least intense file (50 dB peSPL). If the katydid did not cease singing, we waited 30 s and then proceeded to the next more intense file. This protocol was followed until the katydid stopped singing. The intensity of this file was recorded as the threshold for song cessation for this individual. For *A. oblongifolia*, we timed the echolocation sequences to occur between katydid song emissions (see below) but, because of the high repetition rate, this was not possible for *N. ensiger*. We used a functional definition for song cessation that considers its effectiveness as a defence against gleaning bats. Song cessation was defined as a period greater than 9.5 s, the mean duration of time five *M. septentrionalis* investigated a speaker broadcasting katydid calls after it went silent, plus three standard deviations (H.M.t.H., personal observation). This represents the amount of time in which for 99.7% of the cases the bat would have left the vicinity. We feel this is an appropriate measure for an antipredator response considering the gravity of the situation if the katydid resumes singing with a predator nearby. After the echolocation sequences, the single 85 dB peSPL cricket calling song file was broadcast to the singing katydid.

The recordings for each katydid were analyzed in SASLab Pro. We describe calling song in both species using the term 'emission' to refer to the repeated element of song. In *N. ensiger*, this refers to the syllable produced by a single closing of the wings, whereas in *A. oblongifolia* this refers to the single call produced at regular intervals by more complex wing movements. To compare calling song between species, we measured emission duration (ms), emission period (ms; the time from the beginning of one syllable/call to the beginning of the next) from the oscillogram and peak frequency (kHz; frequency with the most energy) and bandwidth (kHz; the difference between low and high frequencies 15 dB less intense than the peak frequency) from the power spectrum (1024-point fast Fourier transform, Hamming window) for ten pre-stimulus emissions per individual. Duty cycle was calculated as emission duration divided by emission period multiplied by 100%. To determine if song pausing occurred, we measured the periods of calling song for 15 s prior to and 15 s after echolocation call playback for *A. oblongifolia* and for 1.4 s prior to, during, and 1.4 s after playback for *N. ensiger*. This difference was due to the difference in repetition rate between the two species. Song pausing was defined as a period that is greater than the mean period of emissions from that individual produced before the start of each playback sequence plus four standard deviations (Faure and Hoy, 2000a).

Katydid neurological responses to acoustic stimuli:

After behavioural testing, each katydid was tested for their neural responses to synthetic ultrasonic pulses and the same echolocation sequences that they were exposed to during singing. Katydids were secured to modelling clay, ventral side up using metal struts and the forelegs held in a natural position by securing the tarsi to the head of a pin using modelling clay or wax. The cuticle overlying the neck connectives was removed and that between the prothoracic and mesothoracic sternites was cut. Preparations were placed 30 cm from an ultrasonic speaker (Technics leaf tweeter, EAS 10TH400B) at 90° to the longitudinal axis of the katydid within a grounded Faraday cage lined with sound attenuating foam. The cervical connective ipsilateral to the presentation speaker, a connective that carries one of the axons of the T-cell (Faure and Hoy, 2000b; Nebeling, 2000; Rheinlaender and Römer, 1986), was hooked by a stainless steel electrode and a reference electrode was inserted into the abdomen. The connectives anterior to the recording electrode and posterior to the prothoracic ganglion were cut to reduce extraneous nervous activity. The signal from the electrodes was

amplified (P15 AC amplifier: Grass Technologies, West Warwick, RI, USA), digitized using a computer data acquisition board (Pico Technology, St Neots, Cambridgeshire, UK), and displayed online with an oscilloscope-emulating program (Picoscope 5.10.7). The stimuli and nerve signals were also recorded using the Avisoft USG and recorder software on the same computer.

Neural responses to two types of acoustic stimuli were tested. First, we generated threshold response curves (audiograms) by broadcasting 10 ms pure tone pulses at a repetition rate of 2 s^{-1} and a sampling rate of 500 kHz using a custom-built MATLAB application, data acquisition board (BNC 2110, National Instruments), and ultrasonic amplifier. Frequencies from 5 to 100 kHz in 5 kHz increments were broadcast in random order, and the sound intensity was increased until threshold was reached, defined as a single T-cell spike in at least four of five consecutive pulses (Faure and Hoy, 2000b). Second, we broadcast the 19 files used in the behavioural experiments (gleaning and search call sequences from 50 to 90 dB peSPL at the preparation at 5 dB intervals and cricket calling song at 85 dB peSPL) and simultaneously recorded the nerve response and stimuli on two separate channels of the Avisoft USG using Avisoft recorder at a sampling rate of 214 kHz. Ten seconds of silence separated each playback presentation to prevent adaptation of the T-cell between stimulus presentations. We measured three variables in these recordings: total number of spikes during the stimulus sequence, latency to the first spike for the first call in each search phase sequence (ms) and the instantaneous spike rate (inverse of the time between spikes; s^{-1}) (Nabatiyan et al., 2003). Only the mean of the five greatest instantaneous spike rates was used in statistical analyses to get a more accurate estimate of spike rate in response to the stimuli. We also measured these variables for 1.4 s of the recording before playbacks to get values of background T-cell spike number and rate.

We tested in one animal that the large spike responding to sound in *A. oblongifolia* was a T-cell using a double electrode recording, one recording electrode hooked onto the ascending connective and another hooked onto the descending connective of the prothoracic ganglion [as previously described for *N. ensiger* (Faure and Hoy, 2000b); various species (Suga, 1966)]. Given the morphology of the T-cell (one branch extending anteriorly and another extending posteriorly from the prothoracic ganglion), we expected to see high amplitude action potentials following ultrasonic stimuli on both electrode traces in *A. oblongifolia*. We broadcast the same synthetic pulses as used for the audiograms and gradually raised the intensity for each frequency to record responses.

Statistical analysis

For statistical analysis, we used *R* (version 2.3.1) for tests on categorical data (e.g. Cochran's *Q* test and log-likelihood ratio) and SPSS (version 15.0) for tests on continuous variables (*t*-tests, repeated measures ANOVAs). Recognizing that data was collected from the same individuals in different experiments, and as a compromise between the strictest concept of experiment-wide error correction and treating each statistical test as independent, we chose to use Bonferroni corrections for groups of tests of the same data type (i.e. song measurements, behavioural response data, neural threshold curves, T-cell responses to entire echolocation and cricket sequences).

RESULTS

Song characteristics of katydids

Calling song characteristics for *A. oblongifolia* and *N. ensiger* are summarized in Table 1 and oscillograms are provided in Fig. 1. *Amblycorypha oblongifolia* produced song of longer emission duration (*t*-test: t_{17}=15.4, P<0.001), lower duty cycle (t_{17}=–21.5, P<0.001), lower peak frequency (t_{17}=–7.3, P<0.001), and greater bandwidth (t_{17}=5.3, P<0.001) than *N. ensiger* after Bonferroni correction for four tests (α=0.0125). *Amblycorypha oblongifolia* produced longer emissions but at proportionately longer intervals resulting in a lower duty cycle calling song than *N. ensiger*.

Behavioural responses

Behavioural trials were run on 10 *A. oblongifolia* and 15 *N. ensiger*. Individuals were categorized as either demonstrating a response (song pausing and/or song cessation) or no response to the acoustic stimuli (Table 2). Ten statistical tests were run on the categorical data presented in Table 2 using a critical alpha value of 0.005 after Bonferroni correction. For *A. oblongifolia*, there were no differences among the three playback stimuli in the number of individuals that

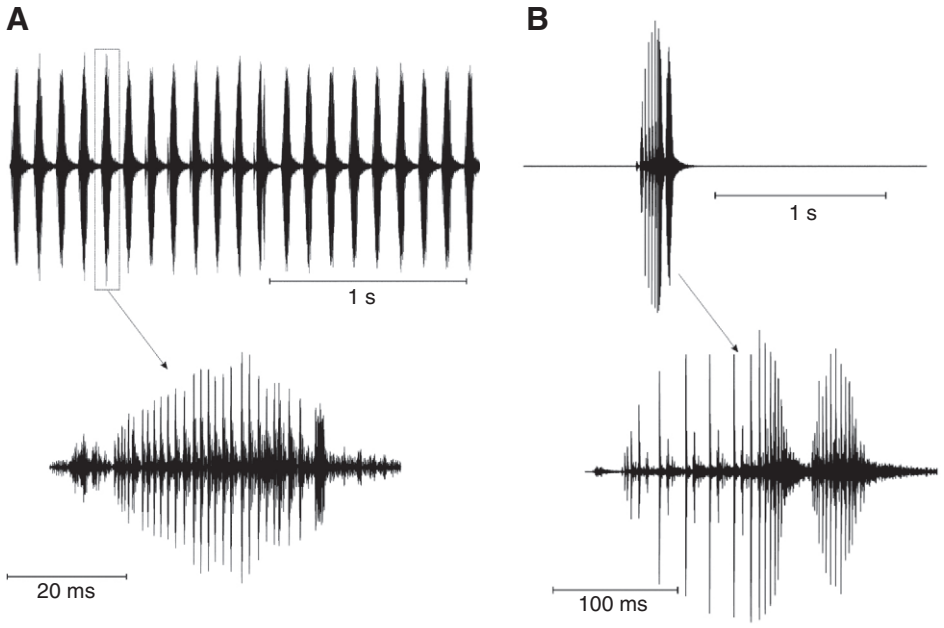

Fig. 1. Oscillograms of the calling song of (A) *Neoconocephalus ensiger* and (B) *Amblycorypha oblongifolia* at a time scale typical for the period of *A. oblongifolia*.

Table 1. Calling song characteristics of *Amblycorypha oblongifolia* and *Neoconocephalus ensiger*

Song characteristic	*Amblycorypha oblongifolia* (*N*=9)	*Neoconocephalus ensiger* (*N*=10)
Emission duration (ms)	345.6±20 (call)	50.2±1.6 (syllable)
Emission period (ms)	2392.7±100.4 (call)	89.2±4.0 (syllable)
Duty cycle (%)	14.7±0.7	56.7±1.7
Peak frequency (kHz)	9.9±0.3	13.7±0.4
Bandwidth (kHz)	21.6±2.7	7.8±0.3

Values are means ± s.e.m.

Table 2. Number of individuals within each treatment demonstrating song pausing only, song pausing and cessation (i.e. a pause occurred at a lower intensity stimulus than song cessation), song cessation only (no pausing occurred), or no response to playback of *Myotis septentrionalis* echolocation calls (one gleaning attack and one series of 16 search calls) and *Teleogryllus oceanicus* calls

	Amblycorypha oblongifolia (*N*=10)			*Neoconocephalus ensiger* (*N*=15)		
	Gleaning sequence	Search call sequence	Cricket calls	Gleaning sequence	Search call sequence	Cricket calls
Responses						
Song pausing only	5	4	2	0	0	6
Song pausing and cessation	2	2	0	5	5	0
Song cessation only	1	2	1	10	10	8
No response	2	2	7	0	0	1

responded (Cochran's Q test: Q=8.3, d.f.=2, P=0.016) or ceased singing (Q=3.5, d.f.=2, P=0.174). For *N. ensiger*, there were no differences among the three stimuli in the number of individuals that responded (Q=2, d.f.=2, P=0.368), but more individuals ceased singing in response to the two bat stimuli than in response to the cricket stimulus (Q=14, d.f.=2, P<0.001). *Amblycorypha oblongifolia* and *N. ensiger* did not differ in the number of individuals responding to the bat stimuli (log-likelihood ratio: gleaning sequence, G=1.1, d.f.=1, P=0.294; search call sequence, G=1.1, d.f.=1, P=0.294), but significantly more *N. ensiger* ceased singing than *A. oblongifolia* (gleaning sequence, G=12.3, d.f.=1, P<0.001; Search call sequence, G=9.4, d.f.=1, P=0.002). There were significantly more responses by *N. ensiger* than *A. oblongifolia* to cricket calls (G=8.6, d.f.=1, P=0.003), but the two katydid species did not differ in the number demonstrating song cessation to this stimulus (G=3.4, d.f.=1, P=0.064).

Threshold sound intensities to pause and cease singing were generally lower for *N. ensiger* than *A. oblongifolia*, although this was more apparent for song cessation than song pausing (Fig. 2). The mode for song cessation (i.e. the sound intensity level at which the most individuals of the species ceased singing) was "no threshold" for *A. oblongifolia*, meaning most individuals never ceased singing up to and including at 90 dB peSPL, whereas the mode for *N. ensiger* ranged from 60–70 dB peSPL. Pause periods were longer for *A. oblongifolia* than *N. ensiger* (Table 3), reflecting the longer period of this species (Table 1).

Neural responses

Double electrode recordings confirmed the presence of a putative T-cell in one *A. oblongifolia*. The largest spike in response to sound could be recorded from both the ascending and descending connectives from the prothoracic ganglion (Fig. 3A). When this characteristic spike occurred, it was present on both traces. Although there were cases of spontaneous firing, it generally only occurred after the presentation of a pulse of sound, and responded more reliably to ultrasound than audible sound. That other nerve cell spikes could be detected on one connective and not the other rules out the possibility that electrical cross-talk was producing an artefactual double trace. Although two T-cells have been reported in katydids, TN1 and TN2 (Schul, 1997), we believe the cell we recorded in both species is TN1 for several reasons: (1) Schul (Schul, 1997) reported that TN2 could not be recorded using extracellular methods because of the small axonal diameter, (2) the large spikes we observed (often five times greater in amplitude than any other neural activity on the cervical connective) are typical for TN1 because of its giant axonal diameter (Faure and Hoy, 2000b; Rheinlaender and Römer, 1986; Schul, 1997), and (3) the shape of the threshold response curves are similar to those reported for TN1 in other species (see Discussion).

Threshold T-cell response curves (audiograms) were similar for *A. oblongifolia* and *N. ensiger* (Fig. 3B), but *N. ensiger* had significantly lower thresholds for frequencies greater than 40 kHz, whereas *A. oblongifolia* had significantly lower thresholds for 10 and 15 kHz (t-tests between species for thresholds at each frequency with a critical alpha value of 0.0025 after Bonferroni correction for 20 tests). Latencies to the first search call were significantly lower for *N. ensiger* than *A. oblongifolia* for all stimulus intensities except for 50 and 75 dB peSPL (t-tests, N=10, P<0.006).

We conducted 12 statistical tests on T-cell responses to entire stimulus sequences, reducing the critical alpha value to 0.004 after

Table 3. Mean period of the first observed song pause for two katydid species in response to three playback stimuli

	Amblycorypha oblongifolia		*Neoconocephalus ensiger*	
Treatment	*N*	Mean duration of first pause ± s.e.m. (ms)	*N*	Mean duration of first pause ± s.e.m. (ms)
Gleaning sequence	7	4528.1±397.4	5	86.8±4.1
Search call sequence	6	6337.2±692.4	5	102.4±11.0
Cricket calls	2	4077.8±534.1	6	176.9±21.3

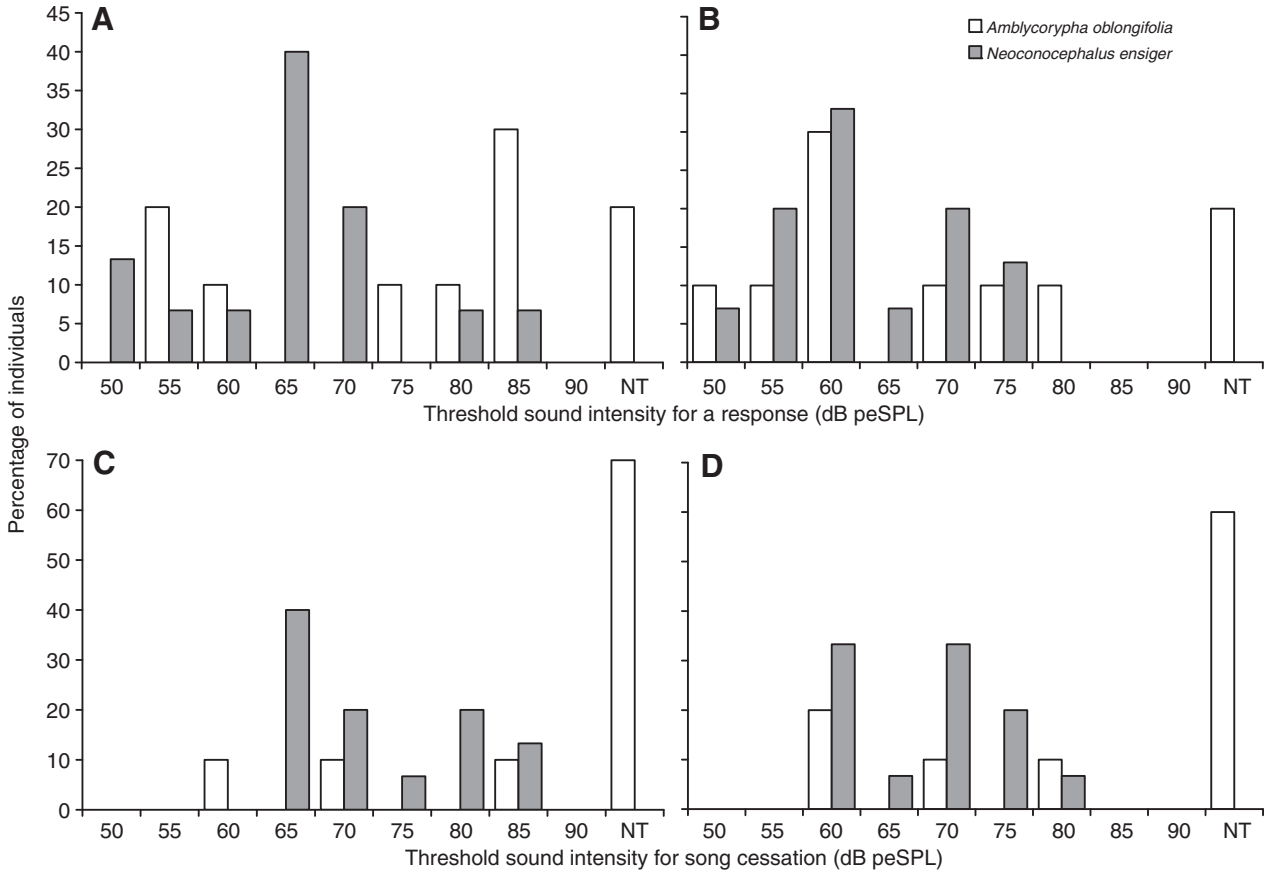

Fig. 2. Percentage of individuals per threshold sound intensity producing a response (song pausing or cessation) for (A) a gleaning echolocation sequence and (B) a search call echolocation sequence, and song cessation for (C) a gleaning echolocation sequence and (D) a search call echolocation sequence. NT, no threshold, indicating that the katydid continued to sing after the final 90 dB peSPL stimulus.

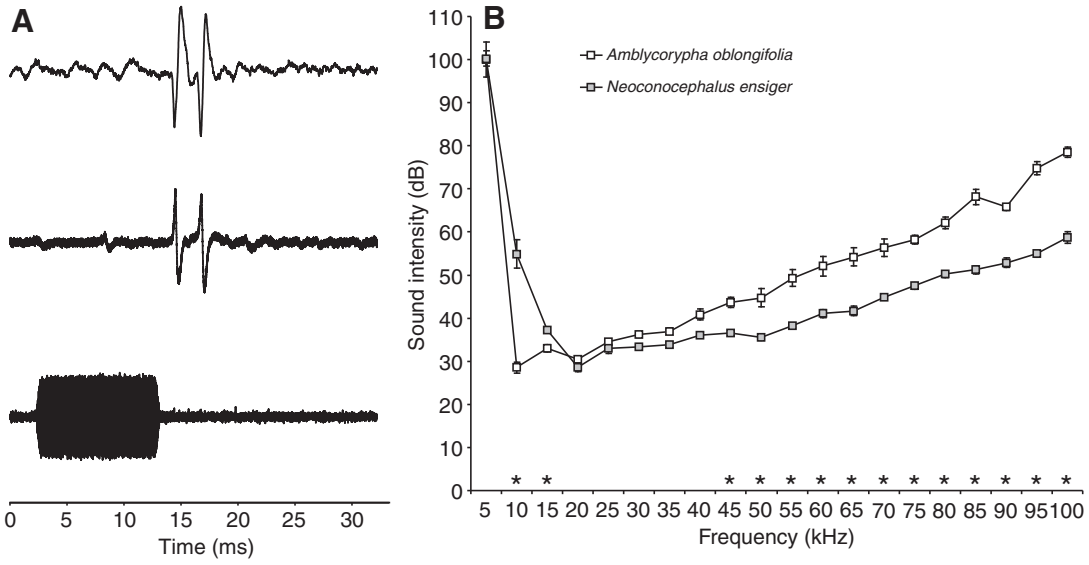

Fig. 3. T-cell responses to pulsed sound in katydids. (A) Synchronized oscillograms of a double electrode recording from *Amblycorypha oblongifolia*. Top trace: descending ipsilateral connective from the prothoracic ganglion; middle trace: ascending ipsilateral connective from the prothoracic ganglion; bottom trace: microphone recording of the sound stimulus (a 10 ms 25 kHz pulse). (B) Audiogram curves of threshold T-cell responses to 10 ms pulses for *Amblycorypha oblongifolia* (N=12) and *Neoconocephalus ensiger* (N=17). Asterisks indicate significant differences between species (t-tests, P<0.0025).

Bonferroni correction. We used a two-factor repeated measures ANOVA design to test for significant differences in T-cell responses between the two echolocation sequences (gleaning and search calls) and between intensities of each sequence (10 intensity levels: background, and 50–90 dB peSPL). Separate tests were run for each species and each variable (number of spikes per sequence and mean of the five greatest instantaneous spike rates). In all cases, the assumption of sphericity was met for factor 1, echolocation sequence (Mauchly's W=1), but was violated for factor 2, intensity (Mauchly's W<0.001, P<0.001) and the interaction factor, echolocation sequence × intensity (Mauchly's W<0.001, P<0.01). Therefore, the Greenhouse–Geisser corrected degrees of freedom were used for intensity and the interaction component.

The interaction term for the number of spikes per sequence was not significant for both species (*N. ensiger*: $F_{3.6,32.7}$=4.976, P=0.004; *A. oblongifolia*: $F_{2.6,23.6}$=5.828, P=0.005; Fig. 4), allowing for the interpretation of main effects. There were no significant differences in the number of spikes between echolocation sequences for either species (*N. ensiger*: $F_{1,9}$=0.892, P=0.370; *A. oblongifolia*: $F_{1,9}$=5.392, P=0.045), but there were significant differences between intensity levels (*N. ensiger*: $F_{2,18}$=43.247, P<0.001; *A. oblongifolia*: $F_{1.8,16.5}$=18.688, P<0.001). *Post hoc* pairwise LSD comparisons revealed which intensity levels differed from others, represented as different letters on Fig. 4. The number of spikes appears to increase steadily for *N. ensiger*, but increases very abruptly at 55 and 60 dB peSPL and then plateaus or even decreases with intensity for *A. oblongifolia* (Fig. 4). The interaction term was not significant for the mean of the five greatest instantaneous spike rates for both species (*N. ensiger*: $F_{3.2,29.2}$=0.322, P=0.824; *A. oblongifolia*: $F_{3.1,28.2}$=1.172, P=0.339; Fig. 4), allowing for the interpretation of main effects. There were no significant differences in the mean of the five greatest instantaneous spike rates between echolocation sequences for either species (*N. ensiger*: $F_{1,9}$=0.182, P=0.680; *A. oblongifolia*: $F_{1,9}$=0.760, P=0.406), but there were significant differences between intensity levels (*N. ensiger*: $F_{2.5,22.3}$=24.695, P<0.001; *A. oblongifolia*: $F_{2.5,22.6}$=21.016, P<0.001). As with the number of spikes per sequence, instantaneous rate increases continuously and gradually for *N. ensiger*, but abruptly increases and plateaus at 55 dB peSPL for *A. oblongifolia* (Fig. 4). For both species, the mean of the five greatest instantaneous rates was greater for 50 dB peSPL (the lowest intensity playback) than background rate.

To test for differences in T-cell responses between species, we used *t*-tests on the greatest differences observed within each level of factor 1 (echolocation sequences). The greatest difference in number of spikes between the two katydid species for the gleaning sequence occurred at 60 dB peSPL, and this was significantly different (t_{18}=2.998, P=0.008). The greatest difference in number of spikes between the two katydid species for the search sequence occurred at 55 dB peSPL, and this was not significantly different (t_{18}=2.281, P=0.035). The greatest differences in instantaneous rate between the two katydid species for both echolocation sequences were not significant (gleaning sequence 60 dB peSPL: t_{18}=1.321, P=0.203; search sequence 55 dB peSPL: t_{18}=1.192, P=0.249).

To test for T-cell response differences between echolocation calls and cricket calls for each species, we ran four repeated measures ANOVAs on data for the three 85 dB peSPL treatments (gleaning calls, search calls, and cricket calls). There were no significant differences in the number of spikes per treatment for *A. oblongifolia* ($F_{2,26}$=1.2, P=0.323) or *N. ensiger* ($F_{2,26}$=0.9, P=0.406). Likewise, for the mean of the five greatest instantaneous rates, there were no significant differences for *A. oblongifolia* ($F_{2,26}$=0.4, P=0.682) or *N. ensiger* ($F_{2,26}$=0.2, P=0.862). We plotted the maximum spike rate over 100 ms bins for the first 1 s of the playback and found that although the initial spike rates were similar between *T. oceanicus* and search calls, the rate then adapted to a lower value for the *T. oceanicus* calls (Fig. 5).

DISCUSSION

Our data provides the first field-collected support for the hypothesis proposed by Faure and Hoy (Faure and Hoy, 2000a) that song cessation can be elicited by the echolocation calls of gleaning bats

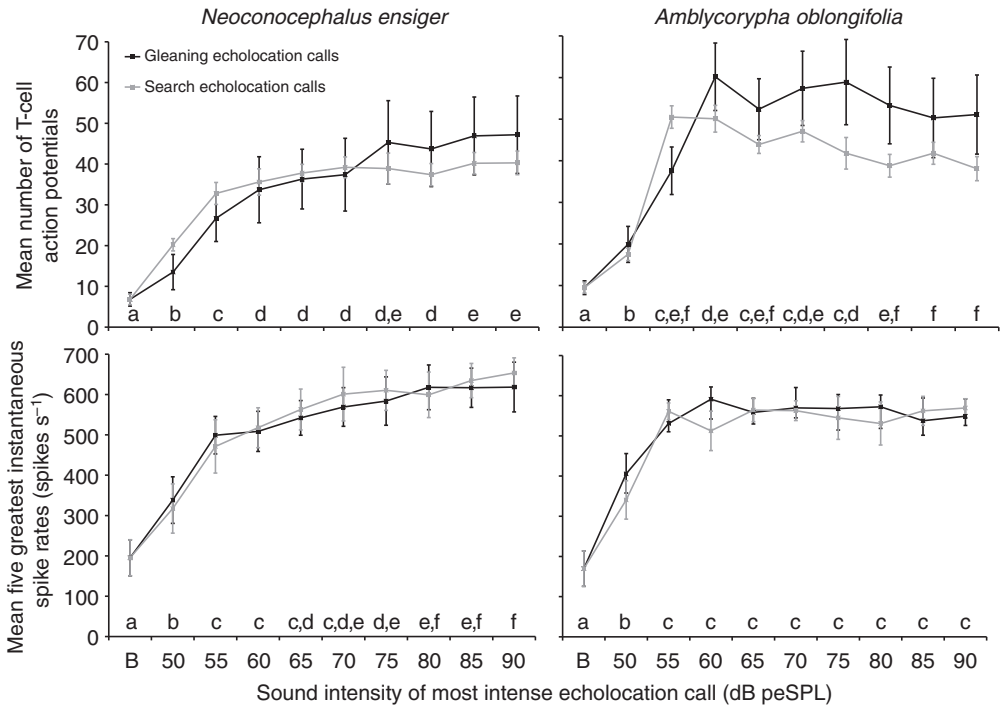

Fig. 4. Mean number of T-cell action potentials for each echolocation sequence and mean of the five greatest instantaneous spike rates per intensity for *Neoconocephalus ensiger* and *Amblycorypha oblongifolia*. Black lines: *Myotis septentrionalis* gleaning attack echolocation sequence; grey lines: *M. septentrionalis* search call sequence. Bars indicate ± s.e.m. B, background activity (see Materials and methods). Different letters indicate significant differences between activity levels.

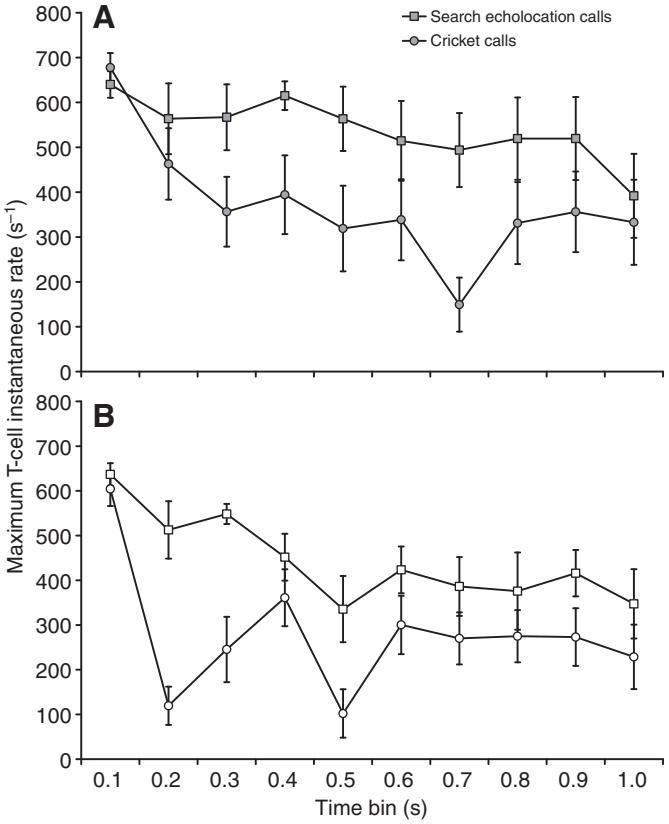

Fig. 5. Maximum T-cell instantaneous rate (s^{-1}) for 100 ms time bins across the first 1 s of the 85 dB peSPL *Myotis septentrionalis* search calls (squares) and *Teleogryllus oceanicus* calls (circles) for (A) *Neoconocephalus ensiger* (*N*=10) and (B) *Amblycorypha oblongifolia* (*N*=10). Bars indicate ± s.e.m.

in a defensive manner in an orthopteran insect. Both katydid species were as likely to respond (i.e. pause or cease singing) to the echolocation call presentations in our experiments, but song pausing may not be an effective defence considering that some gleaning bats relying on prey-generated sounds hover near the prey before landing and cease echolocating during this time [e.g. *Plecotus auritus* (Swift and Racey, 2002)]. Song cessation would be a more likely deterrent considering observations that bats abort gleaning attacks when fluttering moths stop fluttering [*M. evotis* (Faure and Barclay, 1992), *M. septentrionalis* (Ratcliffe and Dawson, 2003)] *Neoconocephalus ensiger* was more likely to cease singing (100% of individuals) than *A. oblongifolia* (30 or 40% of individuals, depending on the treatment) in response to *M. septentrionalis* echolocation calls. These data support our prediction that low duty cycle katydid species do not rely on secondary defence against gleaning bats. The lack of difference in responses to gleaning or searching echolocation calls suggests that these katydids can activate this defence before bats initiate an attack, improving the possibility of surviving an encounter (Endler, 1991). We choose to interpret the two tests with *P*-values close to significance with caution because of our sample sizes and the use of Bonferroni correction, but neither of these cases strongly influence the final conclusions of this study (e.g. *A. oblongifolia* may be responding more to the cricket song than bat calls, and the two species may differ in their likelihood of song cessation to cricket song).

These results are reflected in the sound intensity thresholds for responses and cessation: *N. ensiger* had a lower modal response threshold for gleaning calls and a lower modal cessation threshold for both echolocation sequences. *Neoconocephalus ensiger* cease singing in response to echolocation calls of *M. septentrionalis* from 60 to 85 dB peSPL at the katydid. These values correspond with the range of source level call intensity estimates for various gleaning bats: *M. evotis* [77.3±2.9 dB peSPL (Faure and Barclay, 1994)], *M. septentrionalis* [78±1.9 dB peSPL (Faure et al., 1993); 102±6 dB peSPL for search calls, and approximately 65 dB peSPL at the microphone for gleaning calls (Miller and Treat, 1993); upper limit of approximately 105 dB peSPL, this study], *P. auritus* [88.6 and 97.1 dB peSPL (Waters and Jones, 1995)]. In a direct measure of hearing distance, Schul et al. (Schul et al., 2000) demonstrated that the katydid *Phaneroptera falcata* can hear search calls of the gleaning bat *Myotis myotis* at a range of 13–30 m. Hearing can be impaired during self-generated sound; cricket auditory interneurons are inhibited during song emissions by a corollary discharge, which helps maintain sensitivity between emissions (Poulet and Hedwig, 2002; Poulet and Hedwig, 2003). Faure and Hoy (Faure and Hoy, 2000a) provide some behavioural evidence that a similar mechanism may be at work in *N. ensiger*; they found that these katydids only ceased singing when a pulse of ultrasound fell between call emissions.

Interestingly, many individuals of both katydid species responded to our control stimulus, the *T. oceanicus* calls, a sound to which the katydids in our study would never have been exposed. There were no significant differences in T-cell spike numbers or rate over entire sequences between echolocation and *T. oceanicus* calls, despite the fact that the cricket calls are at 5 kHz, a frequency for which the T-cell has a high threshold in audiograms (Fig. 3B). This could be due to the greater duty cycle of the cricket calls than the bat calls (*T. oceanicus* calls 48%, gleaning bat calls 2.3%, searching bat calls 2.4%) and the presence of harmonics in the cricket calls at 10 and 15 kHz, frequencies to which the T-cell is most sensitive (Fig. 3B). Although these harmonics were 40–45 dB less intense than the peak 5 kHz frequency, the katydids were 45–71 dB more sensitive to these frequencies (Fig. 3B). The maximum rate of T-cell firing decreased more over time for the *T. oceanicus* calls than the bat search calls, however, and it is possible that in the case of cricket calls, T-cell firing quickly dips below the required firing rate for a behavioural response. Significantly fewer *N. ensiger* ceased singing in response to cricket calls than bat calls, but there was no difference in the number of *A. oblongifolia* responding to bat calls or cricket calls. We suggest that this indicates *N. ensiger* may possess the ability to differentiate between predator and non-predator sounds, whereas *A. oblongifolia* exhibits a more generalized response to sounds.

The neural audiograms for *N. ensiger* and *A. oblongifolia* were similar in shape to each other and to previously recorded T-cell audiograms for other species (Faure and Hoy, 2000b; Forrest et al., 2006; Hill and Oldfield, 1981; McKay, 1969; Suga, 1966; Suga and Katsuki, 1961), but they were significantly different from each other at certain frequencies. The T-cell of *A. oblongifolia* demonstrates low threshold sensitivity to the frequencies of its own calling song (10 kHz), which is not found in *N. ensiger*. Likewise, *N. ensiger* has significantly lower thresholds for high frequencies that are typically emitted by gleaning bats (e.g. 45–55 kHz in this study). This result, however, did not correspond with significant differences in the number or rate of T-cell spiking to the specific echolocation call sequences presented to these two species. This suggests that the difference in behavioural responses between these two species is not linked to T-cell activity, especially spike rate, which is important in eliciting the flight acoustic startle response in crickets (Nolen and Hoy, 1984). Two alternative hypotheses for our results,

to be tested in future research are, (1) the T-cell is not involved in the singing acoustic startle response, or (2) the function of the T-cell is not conserved within the Tettigoniidae and could be context dependent in some species. We suggest that the latter hypothesis is most likely and discuss our reasons for this belief below.

Suga and Katsuki (Suga and Katsuki, 1961) first described the T-cell in a katydid, and subsequent studies reported T-cells in other katydid species and described characteristics that suggested its role in predator avoidance, such as sensitivity to high frequencies, large axonal diameter (hence fast conduction velocity), and a phasic response that encodes short pulses of sound better than long pulses (Faure and Hoy, 2000b; Faure and Hoy, 2000c; Kalmring et al., 1979; McKay, 1969; Schul, 1997). Although katydids have other ascending interneurons sensitive to ultrasound, the T-cell has a lower threshold, higher firing rate, and larger axonal diameter than these other interneurons (Stumpner and Molina, 2006). Behavioural audiograms in *N. ensiger* for the flight acoustic startle response (Libersat and Hoy, 1991) and the singing acoustic startle response (Faure and Hoy, 2000a) are similar in shape, tuning and threshold and could be controlled by the same neural mechanism considering that similar muscles are involved in these two responses (Faure and Hoy, 2000a). These authors suggest that the T-cell may be involved in the acoustic startle response of *N. ensiger* since the pattern of the tuning curve of the T-cell closely matches these behavioural audiograms, but with lower thresholds (by about 30 dB). Schul and Sheridan (Schul and Sheridan, 2006) recently demonstrated that the T-cell in *N. retusus* can function in auditory stream segregation. The T-cell rapidly adapts and stops spiking in response to pulses of sound at high repetition rates (i.e. calling song), but will fire if pulses at a slower rate and different frequency interrupt this pattern (Schul and Sheridan, 2006). This property makes this cell ideal for listening for predators, especially bats, in noisy environments. Therefore, we believe that the T-cell remains the most likely candidate for evoking the singing acoustic startle response.

How can the T-cell be responsible for the singing acoustic startle response when it demonstrates almost identical spike number and rates in two katydid species that show different behavioural responses to bat calls? Neurophysiological studies have revealed more ascending auditory interneurons in katydids (e.g. Stumpner and Molina, 2006) than crickets, which appear to have only two: AN1 responsive primarily to low frequencies and AN2 responsive primarily to high frequencies (Stumpner and von Helversen, 2001). This is not surprising given that most crickets sing within a comparatively restricted low-frequency range and can therefore discriminate between conspecifics and predators on the basis of frequency alone (Moiseff et al., 1978). Katydids, on the other hand, vary greatly in calling song frequencies and often produce broadband calls that include ultrasound frequencies. Some authors have suggested that in many species of katydids, T-cell function might vary with behavioural context in a manner similar to that seen in crickets (Schul and Schulze, 2001; Schulze and Schul, 2001; Stumpner and Molina, 2006): AN2 in crickets functions in ultrasound avoidance in flight (Nolen and Hoy, 1984), but in mate localization during phonotaxis (Schildberger and Hörner, 1988). Female katydids of *Tettigonia viridissima* require both audible and ultrasonic calling song components for phonotaxis, and TN1 faithfully encodes the double pulse structure of the calling song in this species (Schul, 1997). This species also demonstrates an in-flight acoustic startle response to ultrasound (Schulze and Schul, 2001). Schul and Schulze (Schul and Schulze, 2001) manipulated the intercall interval and found that there was a minimum interval required for walking phonotaxis, but not flying phonotaxis. This is consistent with the hypothesis that the T-cell functions in coding the syllable structure of the song during walking, but functions in bat avoidance during flight (Schul and Schulze, 2001).

It has also been suggested that the amount of ultrasound in the calling song of katydids could provide information to females about the distance to the male, since ultrasound attenuates more than audible sound (Römer and Bailey, 1990). Our recordings of *A. oblongifolia* reveal greater bandwidth extending up into ultrasonic frequencies, compared to the narrowband calls of *N. ensiger*. Species in the genus *Neoconocephalus* have pure-tone calls in the audible range with few ultrasonic components (Schul and Patterson, 2003). The T-cell in *Neoconocephalus* spp. also adapts out to repetitive signals that represent their calling song (Faure and Hoy, 2000c; Schul and Sheridan, 2006), suggesting that this cell would be ineffective at mate localization for these species. Therefore, one factor that may influence the ability of katydids to react to predator-specific ultrasound on the ground is the presence or absence of ultrasonic components in their calling songs.

An additional complication is found in the subfamily Phaneropterinae (e.g. *Amblycorypha* spp.) in which males and females both sing in a duet; the female usually produces a short tick in response to the male song, which the male then uses for phonotaxis (Bailey, 2003). The curves presented in Fig. 4 can be considered equivalent to intensity–response curves, which measure the response of a neuron to a given stimulus at increasing intensities. Although the number and rates of T-cell spiking do not differ greatly, the shapes of these curves differ between species. For *N. ensiger*, the T-cell had a large dynamic range with the number and rate of spikes increasing in a continuous manner over a range of 40 dB, typical of the intensity–response curves for ultrasound reported for *N. ensiger* by Faure and Hoy (Faure and Hoy, 2000b). For *A. oblongifolia*, however, the T-cell had a small dynamic range with the number and rate of T-cell spikes increasing up to 55 or 60 dB peSPL and then remaining constant. This latter pattern is similar to that seen for *Amblycorypha rotundifolia* (Forrest et al., 2006) and *Ancistrura nigrovittata*, Phaneropterinae (Stumpner and Molina, 2006), and the T-cell response of *N. ensiger* to audible sound

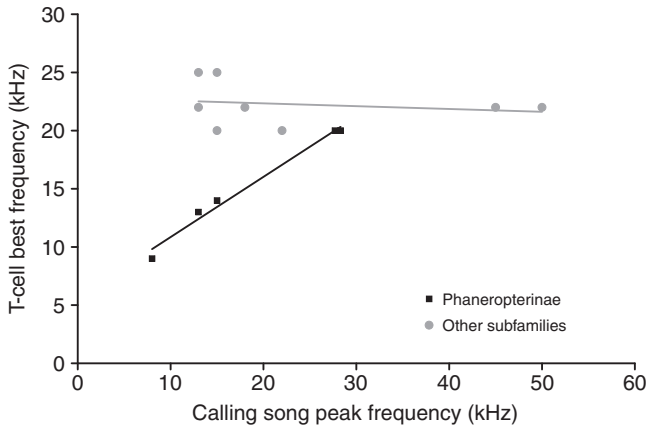

Fig. 6. Relationship between T-cell best frequency (frequency with the lowest intensity threshold) and peak frequency of the calling song for 13 species of Tettigoniidae. Sources: *Neoconocephalus ensiger* (Faure and Hoy, 2000b); *Amblycorypha rotundifolia* (Forrest et al., 2006); *Acripeza reticulata*, *Caedicia longipennis*, *Mygalopsis marki* (Hill and Oldfield, 1981); *Decticus* sp. (Nebeling, 2000); *Neoconocephalus retusus* (Schul and Sheridan, 2006); *Phaneroptera falcata* (Schul et al., 2000); *Ancistrura nigrovittata* (Stumpner and Molina, 2006); *Conocephalus saltator*, *Drepanoxiphus modestus*, *Phlugis* sp. 1 and sp. 2 (Suga, 1966).

(Faure and Hoy, 2000b). Females of *An. nigrovittata* demonstrate a similar behavioural response, with peak response rates to male calling song at 50–60 dB (Dobler et al., 1994a). We plotted the best frequency for the T-cell as a function of calling song frequency for 13 katydid species published in the literature, and female calling song frequency was used for phaneropterines (Fig. 6). We suggest that phaneropterine katydids may have T-cell responses that track calling song whereas katydid species of other subfamilies do not. The response latency of female Phaneropterine katydids can be extremely short [15–50 ms (Dobler et al., 1994b; Heller and von Helversen, 1986)] and is comparable to latencies for ultrasound acoustic startle responses in various insect groups (reviewed in Faure and Hoy, 2000a). It could be that the same neural circuit has been adapted to different functions in different groups of katydids.

Given the great diversity of calling songs and mate finding strategies in the Tettigoniidae, it would not be surprising to find variety in the function of auditory interneurons as well (Faure and Hoy, 2000c; Schulze and Schul, 2001). Our prediction of lower sensitivity to predator cues in a low duty cycle katydid was not borne out by our results, but if the T-cell has adapted for use in mate location during singing for *A. oblongifolia*, this may equate to the same thing. Differences in the apparent role of the T-cell as either a predator or mate detector across a variety of taxa could reflect gaps in our knowledge of how this cell functions across such a diverse taxon. Research on the response of the T-cell to predatory and conspecific cues in phylogenetically diverse katydid species is necessary to elucidate the evolution of function in this neuron and we should not expect uniformity in that function. We suggest that the ability of katydids to mount a secondary defence against gleaning bats (i.e. song cessation) depends on factors specific to the lifestyle of each species such as the amount of ultrasound in the calling song, calling song duty cycle, mate finding strategy and gleaning bat predation pressure.

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