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Auditory encoding during the last moment of a moth's life

James H. Fullard[1,*], Jeff W. Dawson[1,†] and David S. Jacobs[2]

[1]*Department of Biology, Erindale College (University of Toronto), 3359 Mississauga Road, Mississauga, Ontario Canada L5L 1C6* and [2]*Department of Zoology, University of Cape Town, Cape Town, South Africa*

*Author for correspondence (e-mail: jfullard@utm.utoronto.ca)

†Present address: Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3EJ, UK

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Summary

The simple auditory system of noctuoid moths has long been a model for anti-predator studies in neuroethology, although these ears have rarely been experimentally stimulated by the sounds they would encounter from naturally attacking bats. We exposed the ears of five noctuoid moth species to the pre-recorded echolocation calls of an attacking bat (*Eptesicus fuscus*) to observe the acoustic encoding of the receptors at this critical time in their defensive behaviour. The B cell is a non-tympanal receptor common to all moths that has been suggested to respond to sound, but we found no evidence of this and suggest that its acoustic responsiveness is an artifact arising from its proprioceptive function. The A1 cell, the most sensitive tympanal receptor in noctuid and arctiid moths and the only auditory receptor in notodontid moths, encodes the attack calls with a bursting firing pattern to a point approximately 150 ms from when the bat would have captured the moth. At this point, the firing of the A1 cell reduces to a non-bursting pattern with longer inter-spike periods, suggesting that the moth may no longer express the erratic flight used to escape very close bats. This may be simply due to the absence of selection pressure on moths for auditory tracking of bat echolocation calls beyond this point. Alternatively, the reduced firing may be due to the acoustic characteristics of attack calls in the terminal phase and an acoustic maneuver used by the bat to facilitate its capture of the moth. Although the role of less sensitive A2 cell remains uncertain in the evasive flight responses of moths it may act as a trigger in eliciting sound production, a close-range anti-bat behaviour in the tiger moth, *Cycnia tenera*.

Key words: attacking bat, *Eptesicus fuscus*, noctuoid moth, acoustic, predator, echolocation, auditory defence.

Introduction

Many insects hear the ultrasonic echolocation calls of hunting insectivorous bats in time to allow them to escape predation (Hoy and Robert, 1996; Miller and Surlykke, 2001) and the ears of moths are amongst the most neurologically simple, containing up to four auditory receptors. The most intensively studied of these are the two-celled ears of owlet moths (Noctuidae) (for reviews, see Roeder, 1967, 1974; Spangler, 1988; Hoy and Robert, 1996; Fullard, 1998) and in a series of classic papers, Roeder and his colleagues described the physiological responses of the noctuid A1 and A2 auditory cells as well as the apparently non-auditory B-cell. They proposed that noctuids respond to the approach of bats with a bimodal defensive flight behaviour determined by the closeness of the bat as perceived by the moth (Roeder, 1962, 1964, 1974). Aerially foraging bats emit intense echolocation calls as they hunt and use a series of acoustic stages leading to prey capture (Griffin, 1958): (1) search, (2) approach, (3) tracking (Kick and Simmons, 1984), (4) terminal buzz (I) and (5) terminal buzz (II) (Surlykke and Moss, 2000). According to Roeder's (1974) model, the first stage (far-bat) of a flying moth's anti-bat response occurs when it directionally detects a distant bat in its search mode (i.e. emitting relatively faint and slowly repeated echolocation calls) with the most sensitive receptor, the A1 cell. Theoretically, the responses of the A1 cell then evoke controlled, directional flight that takes the moth away from the bat before the bat has detected the echo of the moth. The moth's second defensive mode (near-bat) occurs when it detects a close bat (i.e. emitting relatively intense and rapidly repeated echolocation calls). These sounds activate both the A1 cell and the less sensitive receptor, A2 cell, evoking erratic, non-directional flight as a 'last-ditch', anti-bat flight maneuver. In addition to the A2 cell, Lechtenberg (1971) suggested that the third noctuid receptor, the B cell, considered by earlier authors to be non-auditory (Roeder and Treat, 1957; Treat and Roeder, 1959), might identify the characteristic calls of the terminal stage of the bat's attack to evoke a sustained near-bat response in an escaping moth. The A2 cell has also been implicated in the activation of another near-bat defence, sound-production in the dogbane tiger moth, *Cycnia tenera* (Fullard, 1992; Dawson and Fullard, 1995). As a way of testing the bimodal theory, Roeder (1974) suggested observing the anti-bat behaviour of prominent moths (Notodontidae) whose ears each contain only the A1 receptor cell, and the subsequent study by Surlykke (1984) challenged the theory that near-bat

responses are evoked by the A2 cell by reporting that European notodontids appear to exhibit bimodal flight responses to simulated near *versus* far bats.

The inferential quality of our understanding of the neural control of the moth's anti-bat behaviour stems from the difficulty of neurally recording moth auditory responses to the sounds of real attacking bats, although such recordings have been done to searching bats (Roeder, 1966; Fenton and Fullard, 1979; Fullard and Thomas, 1981). As a bat approaches its target, it alters the duration, intensity, rate and frequency structure of its echolocation calls. A moth's avoidance flight in response to any one set of constant acoustic parameters does not reflect the changing conditions encountered during a real bat attack. Short of chronically recording the auditory responses of a free-flying moth under attack from an actual bat, the next best method would be to expose a moth's ear to an actual sequence of echolocation calls that it would hear as a bat performs its attack. Recently, Triblehorn and Yager (2002) performed a remarkable study in which they recorded the responses of an acoustically activated interneuron in a tethered praying mantis to the echolocation calls of a free-flying bat (*Eptesicus fuscus*). In this study, they discovered that the interneuron encoded the echolocation attack calls of the bat, but only until it was 272 ms (73 cm) from capturing the mantis, at which point it ceased firing. Recordings of auditory receptors in moths or mantids in the presence of free-flying bats are not currently possible due to the presence of the equipment required for recording the neural responses of these cells. Acoustically reproducing the echolocation attack sequence by using typical recordings of bat prey captures is also unsatisfactory, since bats recorded in the field are usually pointing in an unknown direction from the microphone, rendering the temporal structure of the recorded calls unusable as natural stimuli.

Fullard et al. (1994) proposed a method to circumvent this problem by using the echolocation sequence recorded from a laboratory-trained big brown bat (*Eptesicus fuscus*), a species known naturally to eat moths (Black, 1972), as it attacked a small microphone that it expected to be an edible target. Although differences exist between the (searching) calls of field *versus* laboratory-recorded bats (Surlykke and Moss, 2000), the *E. fuscus* recordings represent an excellent simulation of the terminal-phase echolocation calls of an attacking bat as perceived by a stationary target. We broadcast these recordings, as well as a noise-reduced, computer-generated digital replication, to five species of Nearctic noctuoid moths to observe the ear's responses to this, most crucial test of its survival role. We undertook these experiments for four reasons. First, if Roeder's theory of bimodal control of flight response (Roeder, 1974) is correct, we should see the onset of activity in the A2 cell at some point in this echolocation sequence in sufficient time to evade the bat. Second, if the sound-production behaviour of *C. tenera* is governed by the A2 cell as part of this moth's near-bat response, we should see its activity as a necessary, and perhaps sufficient, pre-requisite to that of the sound-producing structures (tymbals). Third, if the A1 cell alone is sufficient to evoke bimodal flight responses in notodontid moths (Surlykke, 1984), its encoding properties alone may simplify Roeder's theory. Fourth, if the proposal by Lechtenberg (Lechtenberg, 1971) that the B-cell contributes to the moth's hearing of a terminally attacking bat is correct, we should witness changes in its activity during the echolocation sequence leading up to the last moment of the moth's life.

Materials and methods

Animals

We used the following noctuoid moths (N=5 for all species) captured from wild populations during the summer months (June–September) in 2001 at the Queen's University Biological Station (QUBS) in southeastern Ontario, Canada (44°34′N, 79°15′W). Noctuidae: *Leucania pseudargyria* Guenée; Arctiidae: *Hyphantria cunea* (Drury), *Cycnia tenera* Hüber; Notodontidae: *Nadata gibbosa* J. E. Smith and *Symmerista albifrons* J. E. Smith. QUBS was also the site of all of the subsequent neurophysiological experiments. All species were identified using criteria of Ward et al. (1974), Covell (1984) and Riotte (1992).

Neural recordings and acoustic stimulation

We used standard extracellular electrophysiological techniques (Fullard et al., 1998) to expose the auditory nerve (IIIN1b) (Nüesch, 1957) of the various moths and record action potentials with a stainless steel hook electrode referenced to another placed in the moth's abdomen. Responses were amplified with a Grass Instruments P-15 pre-amplifier, digitized at a 20 kHz sampling rate (TL-2, Axon Instruments Ltd) and stored in a PC. All records were subsequently analysed using the programme, AxoScope 8.1 (Axon Instruments Ltd).

We first exposed moth ears to acoustic pulses produced by a Hewlett-Packard function generator (model 3311A), shaped to a 1 ms rise/fall time (Coulbourn S84-04), amplified (National Semiconductor LM1875T) and broadcast at 2 pulses s^{-1} from a Technics EAS-10TH400B loudspeaker with a flat (±3 dB) frequency response from 15 to 70 kHz. The speaker was mounted 30 cm from the moth in a sound-absorbing, foam-filled Faraday cage. Intensities were recorded as mV peak-to-peak and later converted to dB sound pressure level (SPL) (rms re 20 μPa) from equal-amplitude continual tones using a Brüel and Kjær (B&K) type 4135 6.35 mm microphone and type 2610 B&K measuring amplifier. The system was regularly calibrated with a B&K type 4228 pistonphone. We first derived auditory threshold curves (audiograms) using 20 ms pulses at 5 kHz frequency increments randomly chosen from 5 to 100 kHz. We then constructed intensity–response plots using 25 kHz, 20 ms pulses at the following intensities: threshold (the dB SPL that evoked at least two auditory spikes per pulse), 60, 70, 80 and 90 dB SPL (depending upon the threshold of the moth; not all moths were exposed to all stimulus intensities).

We then exposed the same preparation to one of two bat echolocation sequences. For the relatively insensitive ears of notodontid and arctiid moths, we played the analog recording used by Fullard et al. (1994), which consists of 40 echolocation calls emitted by a flying bat (*Eptesicus fuscus*) as it attacked a microphone in the laboratory of Dr Jim Simmons (Department of Psychology, Brown University, USA) (for more details, see Fullard et al., 1994). We exposed the moths to five replicate sequences as played from a Racal Store 4D analog tape recorder running at 76.2 cm s^{-1}. To replicate the intensities used by the bat at the time of the recording, the call of the greatest amplitude was adjusted to equal 94 dB peSPL (peak equivalent SPL, compared to a 25 kHz continual tone) (Stapells et al., 1982). These recordings contain a background tape noise level of 35 dB SPL and could not be used with the more sensitive ears of the noctuid moth. Instead, we used a synthesized version of the sequence (courtesy of Mark Sanderson, Department of Psychology, Brown University, USA). The durations, emission rates and relative intensities of the calls were adjusted to match the original sequence and then time-expanded ×32. These calls were played as wave files from a Toshiba Satellite laptop (1710CDS) sound card (Crystal SoundFusion) into the Racal Store 4D tape recorder running at 2.4 cm s^{-1}. Upon playback at 76.2 cm s^{-1}, the flat acoustic spectra from 20 to 100 kHz of the original synthetic calls were attenuated at the higher frequencies, resulting in spectra that more closely resembled those of actual bat calls. We feel that frequency fidelity for these calls is not critical since the one- or two-celled moth ears used in this study do not frequency-discriminate (Roeder and Treat, 1957; Suga, 1961; Roeder, 1967). To test this, we compared the digital sequence to the original by broadcasting both recordings to the ear of the relatively insensitive notodontid *Symmerista albifrons*, and found no significant differences (P>0.05, Wilcoxon paired-sample test) in spike number/echolocation call between the two playbacks.

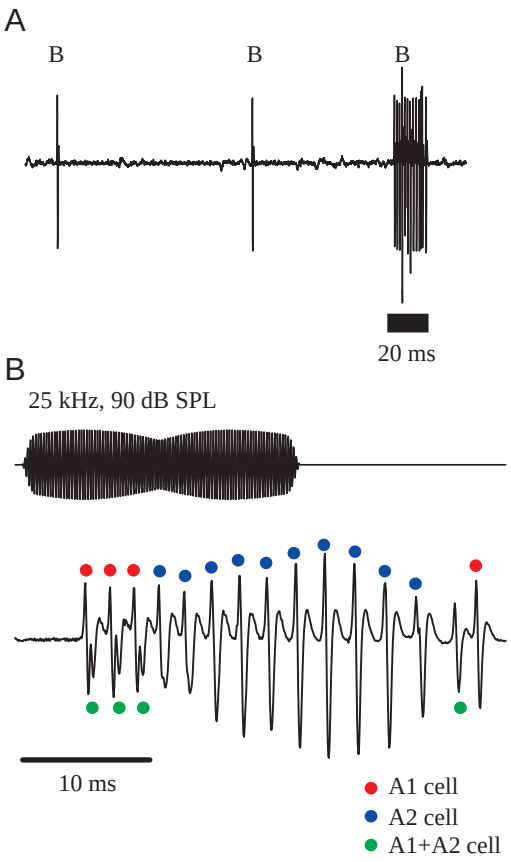

Fig. 1. Oscillograms of the auditory response of the arctiid, *Hyphantria cunea*, to single 20 ms bursts of ultrasound, illustrating how the three receptor cell action potentials were distinguished from each other. (A) A long-time sample reveals the regularity of the non-tympanal B cell as well as its higher amplitude. (B) A shorter time sample shows the variety of waveforms encountered when the two auditory receptors (A1 and A2) respond to an intense sound burst (the B cell is not present in this trace). The different amplitudes of the compound action potential formed by the firing of the A1 and A2 cells is due to the slightly changing phase relationship of the two cells as they fire. The apparent amplitude modulation of the 90 dB burst is due to the insufficient digital sampling used in creating the figure, but was not present in the stimuli used for the auditory trials.

Results

Auditory analyses

Spike discrimination

For every moth tested, we first used its own tympanic nerve's response to synthetic, pulsed stimuli to identify the individual receptor cells' extracellular action potentials. Depending upon the species, there are up to three receptor cells (B, A1 and A2) whose spikes are observed from the tympanic nerve during acoustic stimulation, and we used the following guidelines to discriminate their spikes.

The B cell is a multipolar, non-tympanic receptor (Treat and Roeder, 1959; Lechtenberg, 1971; Surlykke and Miller, 1982) and its spike was seen in most, but not all (e.g. those of *Cycnia tenera*) recordings. The B cell is traditionally identified by its large amplitude (Roeder and Treat, 1957; Suga, 1961; Lechtenberg, 1971; Surlykke and Miller, 1982; Norman et al., 1999), but after examining over 100 recordings we found that its extracellularly recorded spike amplitude is a variable trait for this cell and a more reliable characteristic to recognise the B cell was its firing regularity (Fig. 1A). Accordingly, we discriminated B cell spikes from other neural activity during acoustic stimulation by predicting when they would occur from their spike period.

In two-celled moth ears (e.g. Noctuidae) (Eggers, 1919; Suga, 1961), the A1 cell is traditionally identified as the cell with the lowest acoustic threshold and the A2 cell is that with the higher threshold (Roeder, 1966), and we have followed this practice. The A2 cell is not present in notodontid moths (Eggers, 1919; Treat and Roeder, 1959; Surlykke, 1984), so discerning receptor responses in these moths is relatively easy at all stimulus intensities. It can be difficult, however, to discriminate A1 and A2 action potentials in noctuid moths particularly when both cells fire simultaneously. To

discriminate A1 in each moth, we first observed its responses to pulsed stimulus at threshold intensity to characterize visually the shape of its spike in the absence of the A2 cell. During responses to high intensities when both cells were firing, we assumed that the first spike to appear was that of the A1 cell and then discriminated subsequent waveforms as being single unit A1, single unit A2 or some variation of the compound action potential consisting of both spikes (Fig. 1B).

Frequency sensitivity

We intentionally picked species with a range of auditory sensitivities to examine the different levels of auditory cell response to the echolocation attack sequence, and Fig. 2 illustrates the median audiograms for the species tested. A difference of maximum sensitivity (measured as the threshold dB at best frequency) of over 20 dB between the most sensitive moth (*Leucania pseudargyria*) and least sensitive moth tested (*Cycnia tenera*) is seen, with both of the notodontid species revealing insensitive ears compared to the noctuid. These curves indicate that the most sensitive frequency range for all of the species lies between 20 and 50 kHz, which is the echolocation bandwidth of most of the bats in this region (Fullard et al., 1983), and we used these results to select 25 kHz as the stimulus frequency for the pulsed stimulus trials.

Tympanic nerve response to pulsed stimuli

The intensity–response relationships of the A1 and A2 cells of our moths to pulsed 25 kHz ultrasound are illustrated in Fig. 3A. The values plotted in these graphs represent species medians of all the individuals tested and direct comparisons between threshold dB values will not necessarily result in values equal to two (the criterion used for threshold determination). In these and subsequent analyses we have calculated spike firing as each cell's instantaneous period (IP) (i.e. the time from the maximum amplitude of one action potential to the next). Since the purpose of our study was to observe the moth's auditory responses to the echolocation calls of an attacking bat, we did not expose their ears to sound intensities that represent distant, searching bats (e.g. less than 60 dB). For moths with A1 and A2 cells (*L. pseudargyria*, *H. cunea* and *C. tenera*), the firing of the A1 cell exhibits a short dynamic range attaining a minimum instantaneous period (i.e. maximum firing rate) by 70 dB. There is little subsequent decrease in spike periods up to a stimulus intensity of 90 dB, representing a close bat. For *L. pseudargyria* and *H. cunea* the A2 cell's spikes appear at intensities 20–30 dB higher than the A1 threshold and show a similarly short dynamic range to intensities of 70–90 dB, although this receptor firing does not plateau to the stimulus intensities we used. In *C. tenera* there was only sporadic appearance of the A2 cell in only one specimen at 90 dB (resulting in the median values of 0, as illustrated in Fig. 3A).

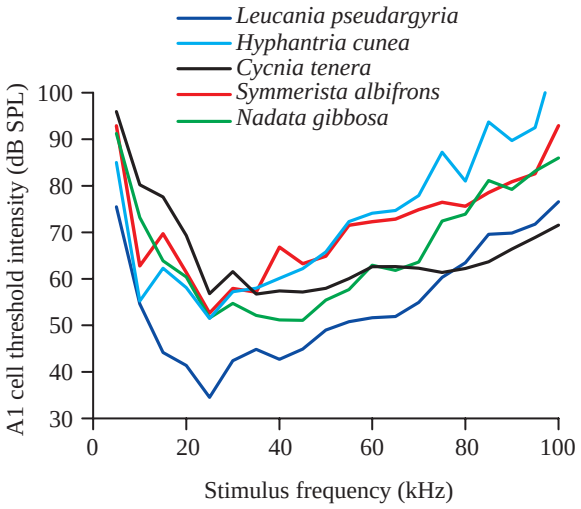

Fig. 2. Auditory sensitivity curves (audiograms) of the moths examined in this study. Each curve is the median of five individuals.

To obtain a measurement that we could use to compare spike periods amongst the species to the different stimulus pulse intensities, we normalised the median numbers of A1 and A2 spikes to each stimulus intensity (dB SPL). We report spike numbers as fractions of the maximum number attained. These curves reveal that, for the most sensitive moth tested (*L. pseudargyria*), A1 spike numbers reach a maximum at 80 dB, after which they decrease slightly. A2 spikes steadily increase to the most intense stimulus used (90 dB) and, unlike the A1 cell, do not reach a firing plateau. Similar responses are seen for the less sensitive arctiid, *H. cunea*. However, for the least sensitive moth tested, *C. tenera*, A1 spikes increase up to 90 dB without reaching a plateau. The responses of notodontids, moths whose ears possess only the A1 cell, indicate similar minimum spike periods with little change above 70 dB. Although the ear of the notodontid *S. albifrons* possesses a threshold almost 20 dB less sensitive than that of the two-celled noctuid ear of *L. pseudargyria*, it reaches a similar spike period minimum at the same intensity (60 dB), with a similar response plateau seen for spike number.

The IPs of the B cell to different stimulus intensities were measured for the same amount of time that the pulsed stimuli was delivered to the ear for the A1/A2 responses and are illustrated in Fig. 3B. Since only one of the seven *C. tenera* we tested exhibited identifiable B cell spikes we excluded this species. Although some moths exhibited very low B cell periods (e.g. *S. albifrons*), there were no significant differences observed in the firing periods of the B cell during the pulse trains amongst any of the moth species at any of the intensities used ($P>0.05$, Kruskal–Wallis one-way analysis of variance on ranks).

Tympanic nerve response to echolocation attack sequence

Fig. 4 illustrates the auditory response of one specimen of *H. cunea* to the recorded echolocation attack sequence of *E. fuscus*. From the start of the first echolocation pulse to the last, the sequence is 655 ms long and consists of 40 calls (Fig. 4A), with durations that remain relatively constant (median=2.8 ms) during the initial 465 ms, but with pulse periods that steadily

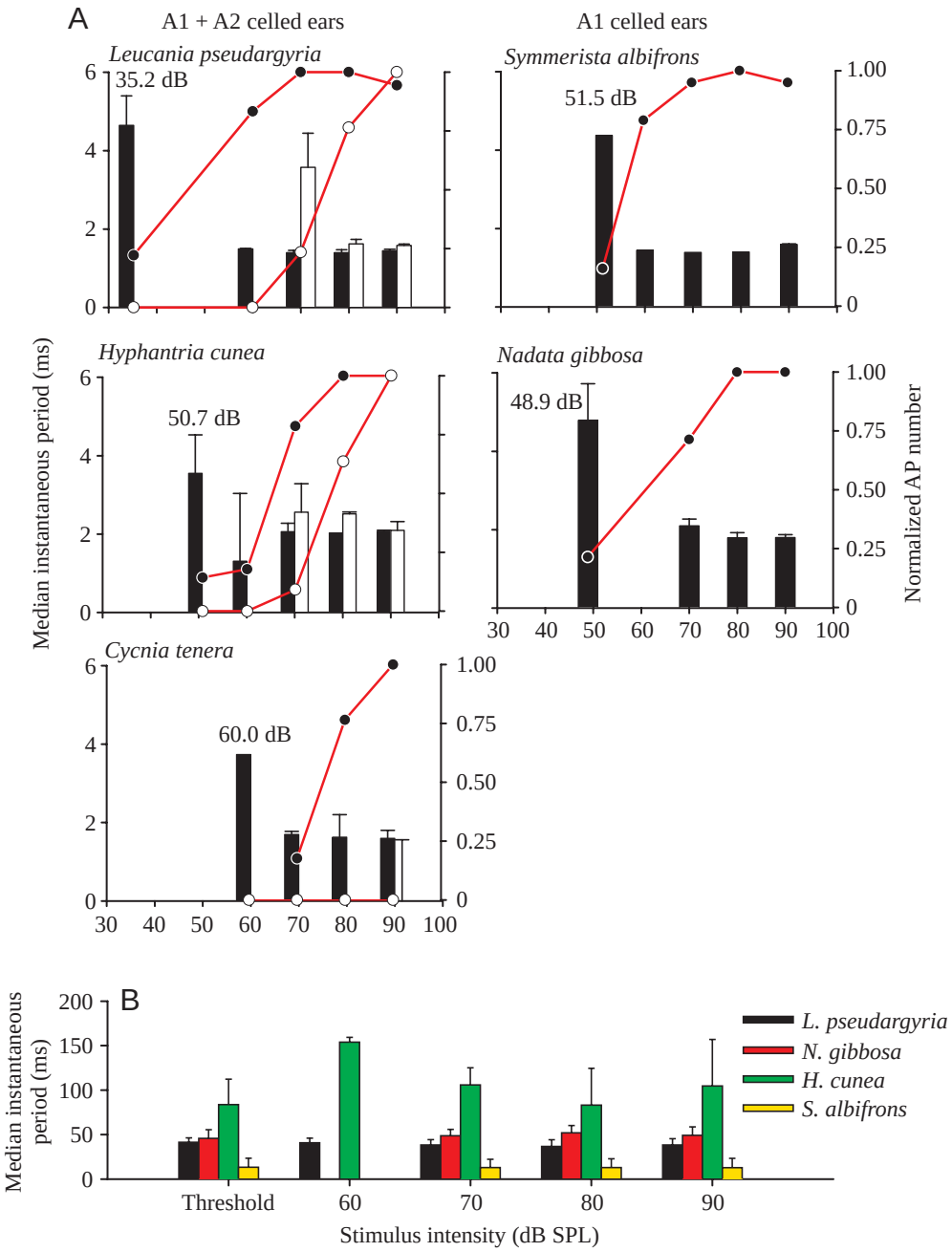

Fig. 3. Intensity response curves of the auditory nerve receptors for the species of moths in our study. (A) In each graph the median (+75% quartiles) A cell periods to pulsed stimuli at each intensity are illustrated, the median A1 threshold is indicated above the first bar. Filled bars, A1 cells; open bars, A2 cells. The total numbers (lines) of A1 (filled circles) and A2 (open circles) action potentials for each stimulus intensity (dB SPL) were normalised and plotted as a fraction of the intensity producing the most spikes. (B) Median B cell periods were measured during the pulsed stimulus exposures that were used to examine A1 and A2 cell responses.

decrease from 73 ms to 12 ms. At approximately 480 ms into the attack sequence, the durations and periods shorten to values of 0.6 ms and 6.0 ms, respectively, during the remainder of the sequence (for more acoustic details, see Fullard et al., 1994). Using the criteria of Kick and Simmons (1984), Surlykke and Moss (2000) and Triblehorn and Yager (2002) for this species of bat, we surmise that the attack sequence in our recording lasts for 480 ms in the 'approach' stage and ends in the 'terminal buzz II' stage, and represents a bat commencing its attack at a distance of approximately 3 m (Kick and Simmons, 1984).

Fig. 4B is an expansion of *H. cunea*'s auditory response to the attack sequence during the first three calls of the bat's approach stage. The only receptor cell responding at this time is the A1 cell, which fires with 2 spikes/call while the B cell appears unaffected by the calls. At the initial part of the terminal buzz stage (Fig. 4C), both A1 and A2 cells are responding but there is no obvious encoding of either receptor to the preceding echolocation call. By the time the sequence is in the terminal buzz stage (Fig. 4D), the amplitudes of the echolocation calls are reduced by 7–16 dB relative to the most intense call in the sequence (even though the bat is closer to the microphone) as a result of the bat 'gain-controlling' its emitted calls (Kick and Simmons, 1984; Hartley, 1992; Boonman and Jones, 2002). This reduction in the call amplitudes corresponds with a disappearance of A2 spikes and

a change in the bursting firing pattern of the A1 cell, which now fires continually at higher spike IPs than those seen to the pre-terminal calls. To test whether the B cell changes its firing, we plotted the IPs of its spikes for all of the B cell spikes in the records of 19 moths for 400 ms before and 400 ms after the attack sequence (Fig. 5). Although individual moths exhibit variable responses in their B cell firing periods, there were no significant differences (P>0.05, Wilcoxon paired-sample test) in median instantaneous B cell periods 400 ms before compared to those during the first 400 ms of the attack sequence in the moths that exhibited B cell activity.

To examine the responses of the A1 and A2 cells during the attack sequence we plotted their IPs and number of spikes during the attack sequence. We have plotted only those IPs less than 10 ms, a value which is above that seen to pulsed stimuli at threshold intensity for all the moths. Fig. 6 illustrates this relationship for the A1-only ears of the notodontids, *N. gibbosa* and *S. albifrons*. In the top graph for each species, the A1 cell maintains a steady IP during the first 500 ms of the sequence, with most IPs shorter than those observed in response to 70 dB pulsed stimuli (Fig. 3). Approximately 500 ms into the sequence, the A1 IPs increase, with many rising above those observed to threshold intensity pulsed stimuli. At approximately 550 ms into the sequence (i.e. 105 ms before the bat would have captured the moth), all the IPs of the A1 cells have risen above threshold values. When examining A1 spikes per echolocation call in both species, the maximum number occurs during the first 300–400 ms, after which there is a reduction in the number of spikes/echolocation call until 500–550 ms into the sequence. At this point, even though the A1 and A2 cells continue to fire, there is no longer a discernible bursting firing pattern locked to the echolocation calls, rendering the

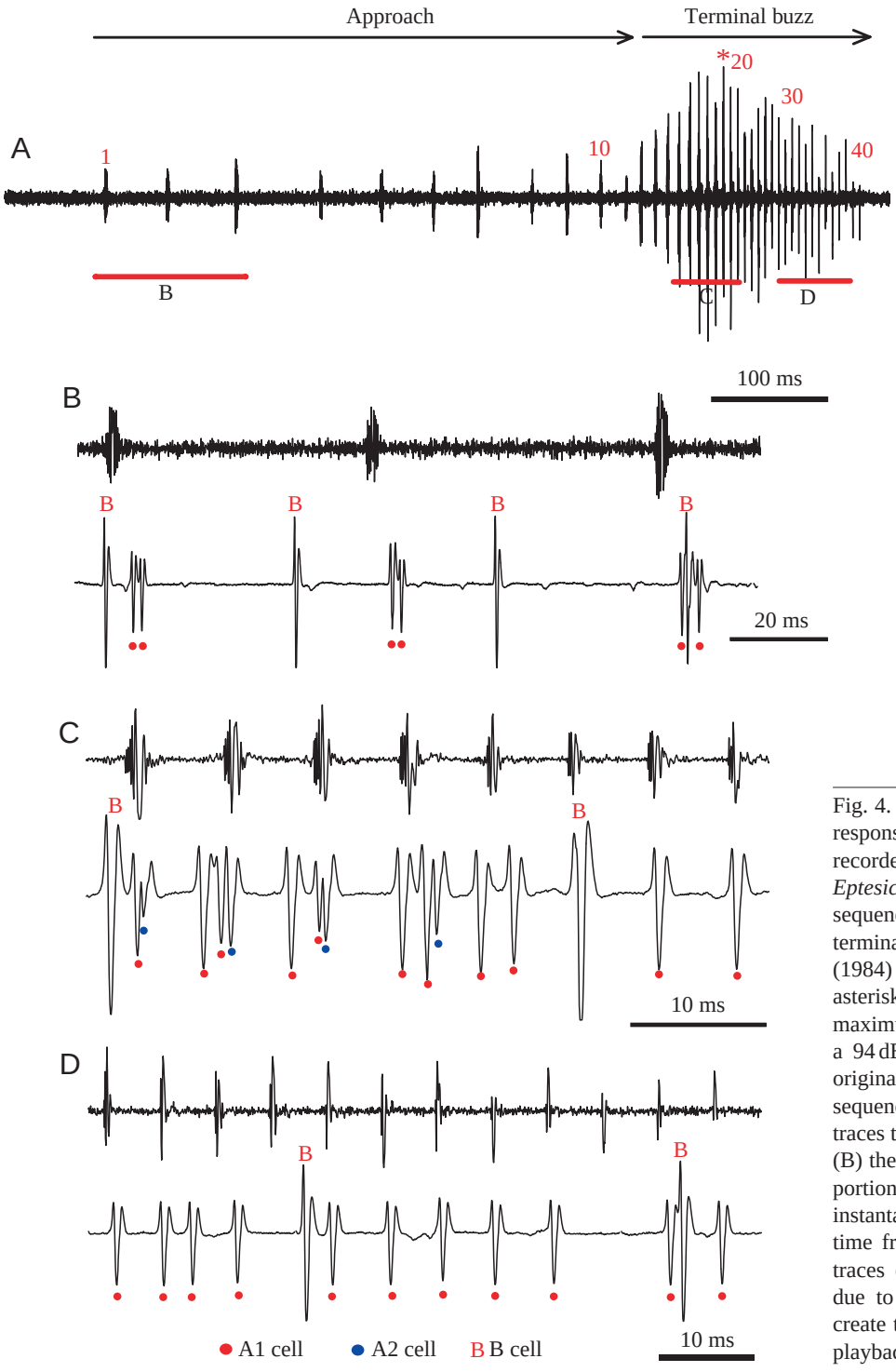

Fig. 4. Representative trace of an auditory response of the moth *Hyphantria cunea* to the recorded echolocation attack sequence of *Eptesicus fuscus*. (A) The entire playback sequence with the attack stages of approach and terminal buzz as defined by Kick and Simmons (1984) and Surlykke and Moss (2000). The asterisk over call 20 indicates the call of maximum amplitude that was adjusted to produce a 94 dB (peSPL) intensity, matching that of the original recording. (B–D) The lines beneath the sequence have been enlarged in the subsequent traces to illustrate the moth's auditory response to: (B) the approach stage, (C) the initial and (D) end portions of the terminal buzz stage. Spike instantaneous periods (IPs) were calculated as the time from one A1 or A2 spike to the next. The traces of the echolocation calls appear distorted due to the insufficient digital sampling used to create the illustration, but the signals used for the playback trials were analog and not distorted.

counting of spikes to stimulus impossible. To compare A1 spike numbers in the attack exposure to those measured during the pulsed stimulus trials we have converted spikes/pulse to fractions of the normalised maximum numbers reached during either the pulse trials or the echolocation sequence. For both *N. gibbosa* and *S. albifrons*, the A1 cell responds to echolocation calls with spike numbers exceeding those to 70 dB pulses for the initial 350 ms but drop below this at approximately 400 ms (*N. gibbosa*) and 475 ms (*S. albifrons*).

The A1 and A2 attack sequence responses for the most sensitive moth we tested, *L. pseudargyria*, are illustrated in Fig. 7. A1 cells from *L. pseudargyria* produce a more vigorous response than those of the notodontid moths for both IPs and spike numbers, but there is a similar loss of the bursting firing pattern to the bat's calls as the attack sequence enters the terminal stage. The A1 cell maintains IPs similar to the median value observed for 70 dB pulsed stimuli (Fig. 3) for the first 500 ms of the sequence. At this point, its IPs, like those of the notodontid A1, gradually increase until approximately 600 ms, when most of the specimens express A1 IPs longer than those seen to threshold intensity pulsed stimuli threshold. For most of the *L. pseudargyria*, the A2 cell fires from the beginning of the attack sequence but its IPs increase above the 70 dB pulsed stimuli level sooner than those of the A1 cell, and its spikes disappear in most moths approximately 550 ms into the sequence. Spike number/echolocation call counts in *L. pseudargyria* exhibit a similar pattern to that of the notodontids, with maximum numbers attained during the first 450 ms and then dropping off until the loss of bursting firing appears at approximately 500 ms.

Hyphantria cunea represents a relatively insensitive two-celled noctuoid ear whose A1/A2 attack sequence responses are illustrated in Fig. 8. While the A1 cell of this species faithfully responds to each echolocation call for the first 500 ms of the attack sequence, as for the preceding moths, its IPs also increase to those above in response to 70 dB pulsed stimuli by the time the sequence is approximately 550 ms old. For the remaining 100 ms of the attack sequence, the A1 cell loses its bursting firing pattern and exhibits longer IPs than those to threshold intensity pulsed stimuli. A surprising observation for this moth was its extremely reduced A2 activity compared to *L. pseudargyria* for most of the attack sequence. At approximately 500 ms into the sequence, the A2 appears briefly in only two of the five moths tested but with IPs above those to 70 dB pulsed stimuli. One of the five moths had brief A2 firing that was above that of 70 dB but two of the five moths showed no A2 activity at all. Spike numbers/echolocation call reveal a similarly reduced responsiveness to the attack sequence, with maximum numbers less than those for *L. pseudargyria* reached for the first 375 ms of the sequence and then rapidly dropping off until bursting firing disappears at 525 ms. The A2 cell briefly fires with its highest numbers at 450–525 ms into the sequence, after which it loses its bursting firing response to the echolocation calls.

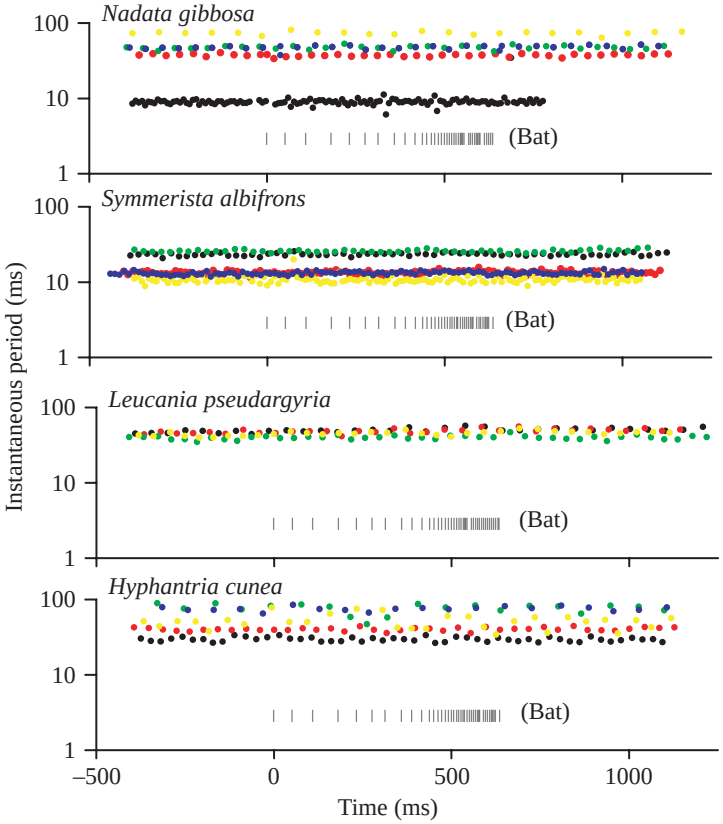

Fig. 5. The instantaneous B cell periods before, during and after exposures to the recorded bat attack sequence. Each colour represents a different individual.

Cycnia tenera phonoresponse

The arctiid *Cycnia tenera* emits trains of ultrasonic clicks from paired structures (tymbals) when either touched or exposed to ultrasonic pulses, especially those resembling the calls of the terminal phase of the bat's attack (Fullard, 1984; Fullard et al., 1994). Fig. 9A illustrates one specimen's response to a stimulus pulse that is subthreshold for evoking a tymbal response (the tymbal nerve motor spikes associated with sound production in the intact moth) (Dawson and Fullard, 1995). In the neural trace of Fig. 9A, the B cell (the only specimen that exhibited this cell) fires in a characteristically regular fashion and the auditory response consists of only the A1 cell. The neural trace in Fig. 9B reveals two new spikes: first, the A2 cell is seen as additional spikes within the rapidly firing A1 cell train and second, the rhythmic firing of the tymbal nerve (IIIN2a) (Nüesch, 1957) is seen superimposed on the tympanic nerve trace (in *C. tenera*, the spikes that activate the tymbal exist as large compound action potentials that can be indirectly monitored at some distance from the tymbal nerve (Fullard, 1992). We are confident that the spikes monitored in Fig. 9 originate from the tymbal nerve, based on their spike periods and bilateral rhythmicity (Dawson and Fullard, 1995). Fig. 10 illustrates the response of the A1 and A2 cells in *C. tenera* to attack sequence intensities below and above those required to evoke a tymbal response. In

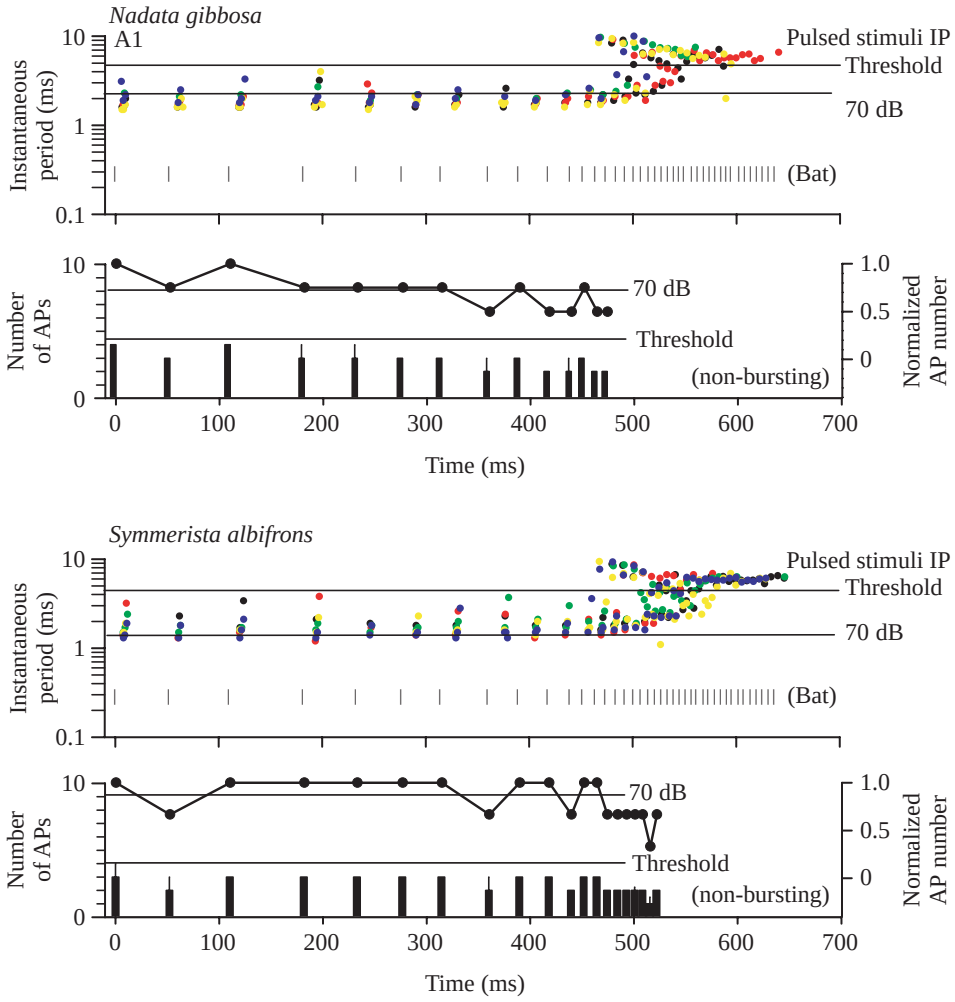

Fig. 6. Auditory receptor responses for the single A1 cell in the two notodontid moths of our study. For each species, the top graph shows all of the instantaneous A1 cell periods for each individual plotted (as separate colours) to the recorded bat attack sequence (marked as vertical lines). The time scale begins at the beginning of the first bat echolocation call. The median A1 periods measured for the pulsed intensity response trials at both threshold and 70 dB stimulus intensities (Fig. 3) are indicated as horizontal lines. For each species the bottom graph shows the median number of A1 cell spikes (APs) to each of the bat echolocation calls up to the point where encoding ceases (determined visually). After this point firing continues but in a non-bursting, continual pattern. The median spike (AP) numbers are also represented as normalised fractions of the maximum counts (thick lines).

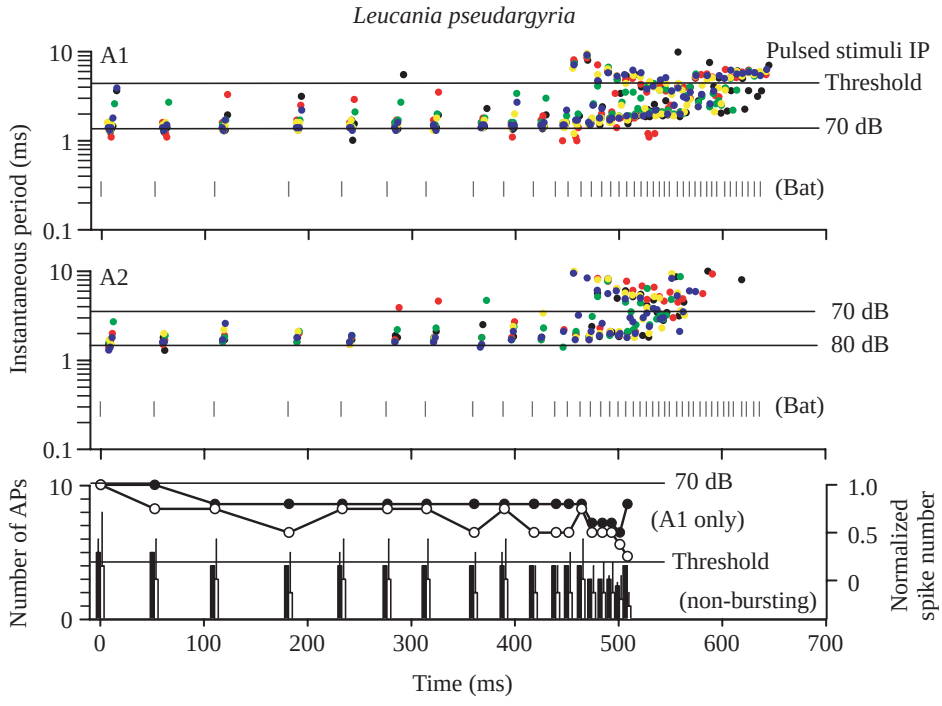

Fig. 7. The instantaneous A1 and A2 spike periods and total number of spikes elicited to the recorded bat attack sequence in the most sensitive moth we tested, the noctuid *Leucania pseudargyria*. Filled bars, A1 cell spikes; open bars, A2 cell spikes. For further details, see Fig. 6.

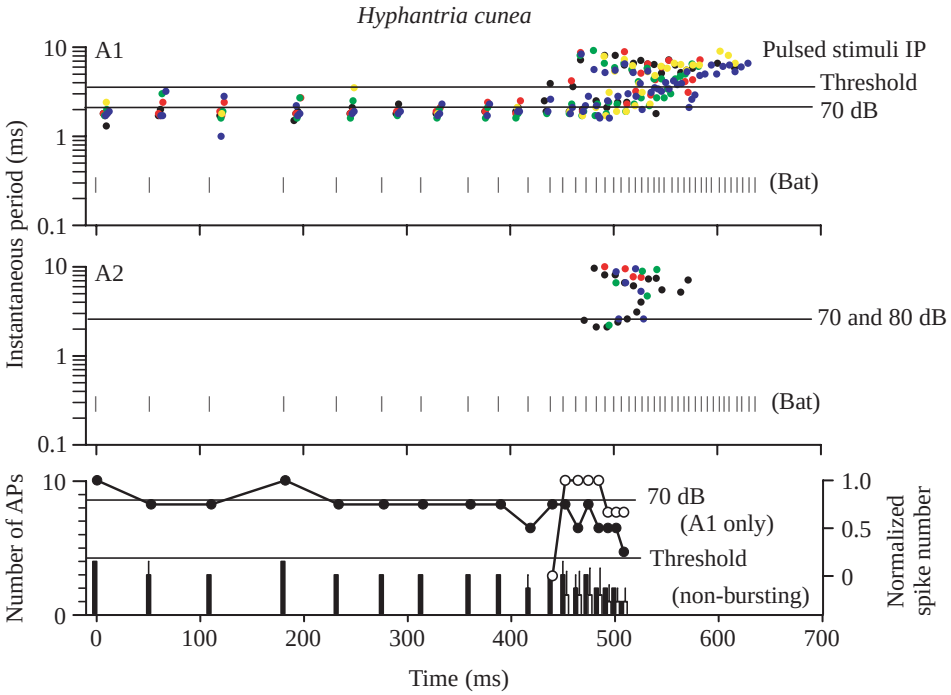

Fig. 8. The instantaneous A1 and A2 spike periods and total number of spikes elicited to the recorded bat attack sequence in the arctiid moth, *Hyphantria cunea*. Filled bars, A1 cell spikes; open bars, A2 cell spikes. For further details, see Fig. 6.

Fig. 10A, the sequence is played at normal dB and evokes an A1 response similar to that observed in *H. cunea*. A1 burst fires with shorter IPs than those to 70 dB pulsed stimuli to approximately 550 ms from the start of the sequence. As with the other moths tested, after this point the A1 loses its bursting pattern and fires with increasing IPs until approximate 575 ms, when the IPs are less than those evoked from threshold pulsed stimuli. At normal dB levels, the A2 cell is present in only two specimens, appearing approximately 525 ms into the sequence and quickly disappearing. Since the tymbal response is labile and usually difficult to evoke in dissected specimens, we ran another series of sequence exposures at a higher intensity where we set the most intense call in the sequence to a level of 100 dB peSPL, which allowed us to evoke the tymbal response in three specimens (Fig. 10B). While there was no obvious difference in the A1 cell's response at the higher stimulus intensity there was considerably more activity in the A2 cell, which fired to the first echolocation call in the sequence and continued 550–600 ms later. Tymbal spikes were observed in these specimens, commencing at approximately 475–550 ms into the sequence and persisting until the end of the attack sequence.

Discussion

Assuming that, for most moths, bat-detection is the only function of their ears (for a discussion of this assumption, see Fullard, 1998), moths exist in three auditory conditions as they fly throughout the night. Before a bat appears, the moth is in the 'no-bat' condition, where the only activity of the ears arises from the spontaneous firings of the A1 cell with long IPs (Roeder, 1967; Fullard, 1987). When the moth first detects a bat (i.e. its calls exceed A1 threshold), the A1 fires with more

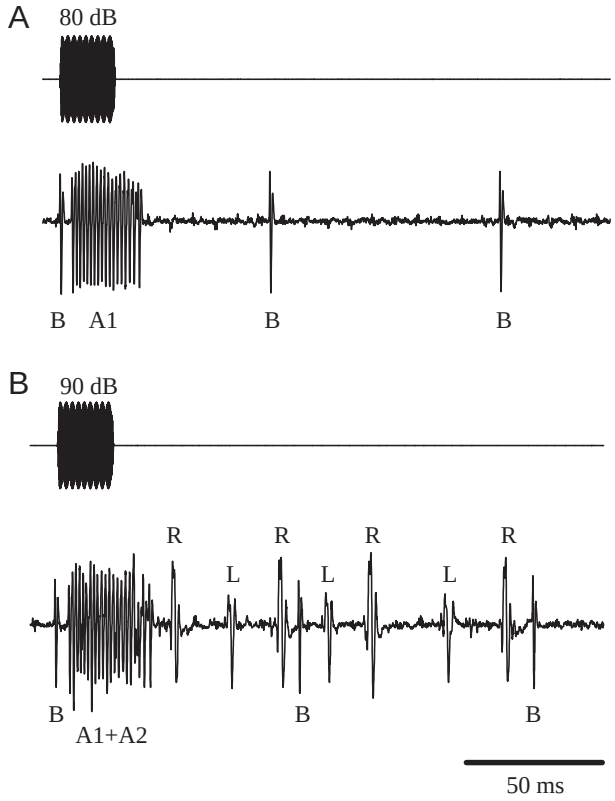

Fig. 9. Oscillogram of the auditory (top trace) and tymbal response (bottom trace) in the sound-producing moth, *Cycnia tenera*. (A) At a pulsed stimulus of 80 dB, only the A1 auditory cell is evoked to the sound; (B) at 90 dB, both A1 and A2 receptors fire. B, B cell. In extracellular recordings the large compound action potentials of the tymbal nerve can be indirectly recorded at the tympanic nerve and are seen as alternating spikes produced by the right (R) and left (L) tymbal motor nerves.

spikes of shorter IPs, indicative of the 'far-bat' condition, and when the bat is very close its more intense calls place the moth into a 'near-bat' condition. With only three sensory cells per ear (two in the case of notodontids) it has been tempting to ascribe specific flight responses to control by these individual cells. However, the results of Surlykke (1984) and the present study suggest that the picture is not as simple as first envisaged by Roeder (1974).

All of the following interpretations of our results are strongly dependent on the emitted echolocation intensity of bats in natural conditions. While there have been many estimates of the intensities of searching bats (*Eptesicus* spp: 94–140 dB, at 10 cm) (Roeder, 1966; Griffin, 1971; Jensen and Miller, 1999; Surlykke and Moss, 2000), there have been only two reports of the intensities emitted during the attack sequence of free-flying bats as received by a stationary target, for *E. fuscus* (Kick and Simmons 1984) and *Myotis daubentonii* (Boonman and Jones, 2002). Boonman and Jones (2002) report *M. daubentonii* emitting a dB SPL of approximately 85 dB at approximately 1 m from the target. We chose an intensity of 94 dB, which was the value emitted by *E. fuscus* when it was approximately 1 m from its target, where our recordings originated (Kick and Simmons, 1984). Surlykke and Moss (2000) have demonstrated that bats emit more intense searching calls when hunting in the wild compared to the laboratory, but differences (if any) in call intensities during the terminal phase are not known. We believe that our interpretations are conservative, since the echolocation intensities received by a moth exposed to a real attacking bat will be constantly changing due to the movement of the moth as well as the muffling of the bat's sounds by the moth's wings as they obscure the ears during flight (Payne et al., 1966).

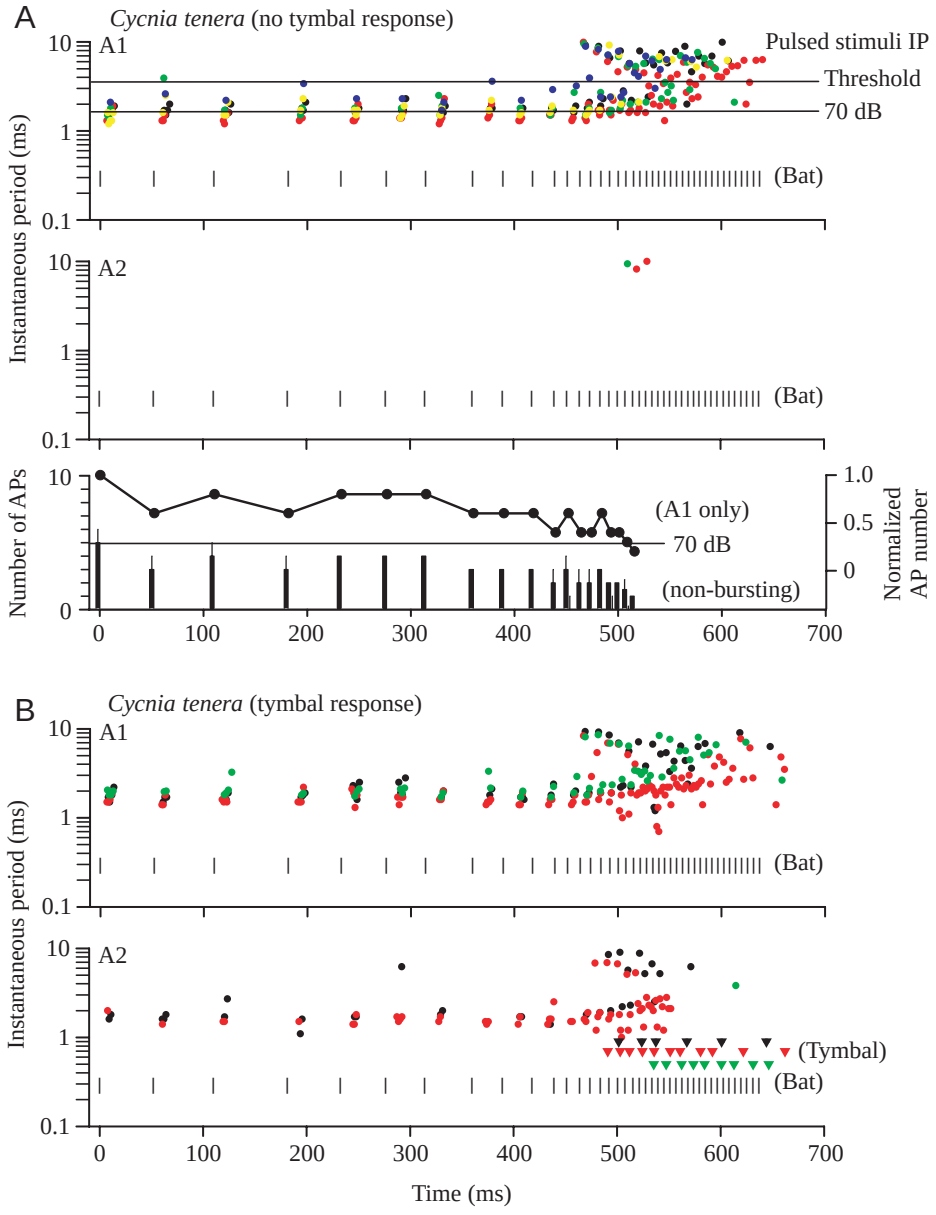

Fig. 10. Instantaneous A1 and A2 cell spike periods for the sound-producing arctiid moth *Cycnia tenera*, when acoustically stimulated by the bat attack sequence delivered at an intensity that: (A) does not evoke a tymbal response and, (B) does evoke a tymbal response (tymbal motor nerve compound action potentials are indicated in B as inverted triangles). For further details, see Fig. 6.

B cell

The first reports of the B cell in noctuoid moths discounted its role as an auditory receptor (Roeder and Treat, 1957; Treat and Roeder, 1959) and this conclusion has been supported in subsequent studies (Surlykke, 1984; Yack and Fullard, 1990). Lechtenberg (1971), however, observed that the firing of the B cell in a number of North American noctuids, including one used in our study, *Leucania pseudargyria*, was inhibited by pulsed ultrasonic stimuli. He used these results to suggest that moths might be able to identify the terminal stage of a bat's attacks to sustain its near-bat response. None of our attack sequence playbacks produced any significant change in the B cell activity for any moth we tested, including *L. pseudargyria*, and we conclude that, during an attack of natural durations, repetition rates and intensities, the B cell plays no auditory role. Lechtenberg (1971) exposed his moths to sound pulses that were more powerful (77–102 dB SPL combined with longer durations) than those encountered by a moth during a natural attack sequence. Yack and Fullard

(1993) point out studies demonstrating that proprioceptive sensory cells in a variety of insects can be activated by unnaturally intense sounds, but these do not constitute adaptively functional auditory responses. We suggest that the sounds used by Lechtenberg (1971) artifactually elicited (*via* the A cells) auditory-evoked muscular changes in his moths that were secondarily encoded as proprioceptive responses by the B cell, which is known to change its firing pattern under sustained skeletal stresses (Treat and Roeder, 1959). It is unlikely, however, that these responses play any role in the natural avoidance behaviour of the flying moth since the acoustic conditions required to elicit them would not be encountered in an attacking bat. It has been suggested that the B cell in noctuoid moths is the evolutionary vestige of a homologous proprioceptor in thoracically earless moths (Treat and Roeder, 1959; Yack and Fullard, 1990). We suggest that its persistence in eared noctuid moths is simply a reflection of the low evolutionary 'cost' that simple nervous sensory systems present to their owners, e.g. auditory systems in moths released from bat predation (Surlykke, 1986; Surlykke and Treat, 1995; Fullard et al., 1997; Surlykke et al., 1998; Rydell et al., 2000).

A1 cell

Roeder (1964) proposed that bat-evoked activity in the noctuid A1 cell was responsible for a moth initiating its far-bat flight responses with the directionality of this response arising from the differential activity of the ear closest to the bat. In analyzing our auditory neural responses, we consider instantaneous periods (inter-spike intervals) (Roeder, 1964) to be a more useful variable than averaged firing characteristics (e.g. spikes s^{-1}), since it is the number of receptor spikes combined with their instantaneous periods that determine the degree to which they will excite and possibly activate postsynaptic interneurons. Our counts of the numbers of receptor spikes per echolocation pulse (Figs 6–8, 10) was only taken to the point where there was no longer a discernible bursting response to individual pulses, although spiking continues beyond this point. Nevertheless, there is a decrease in the total number of spikes $pulse^{-1}$ evoked by the attack sequence to a point where bursting firing is replaced by a long-IP, continual-firing response. By comparing the responses of moth auditory cell IPs to bat attack sequence calls to those of synthetic pulses of known intensities (Fig. 3), we can model the anti-bat behaviours that should be expressed during the bat's attack. Our results indicate that the A1 cell in noctuoid moth ears encodes the calls of a pre-terminal, attacking bat with IPs shorter than those to pulsed sounds of 70 dB. Depending upon which estimate of the in-flight intensities of bats we choose, a received intensity of 70 dB represents a 35–55 dB drop in the emitted output of a searching bat, and would represent an echolocating bat that was 3–10 m away (Lawrence and Simmons, 1982) and should elicit far-bat, controlled flight in the moth (Roeder, 1964). For A2-less notodontid moths, we suggest that far-bat responses are evoked by A1 IPs that match those observed to pulses at threshold to 70 dB (i.e. 4.5–2 ms) while A1 IPs shorter than those to 80 dB pulses (i.e. less than 2 ms) will evoke evasive near-bat flight responses. Our results reveal that the A1 cell in two notodontid moth species encodes the approaching echolocation calls with near-bat IPs for approximately 500 ms after the start of the attack sequence. However, approximately 100–200 ms before the bat captures the moth, its firing decreases in spike numbers, increases IPs to higher values than those at threshold and is ultimately reduced to non-bursting, continual firing at threshold or longer IPs. We call this degradation in the bursting, short IP nature of the A1 cell firing pattern a partial drop-out, to discern it from the total drop-out seen for A2 cell firing. A1 cell partial drop-out (Fig. 6) occurs at similar times in both of the notodontids, and we suggest that it is caused by the combination of short durations and reduced intensities of the echolocation calls of the terminal buzz stage of the attack. Roeder (1964) concluded that '*tones are much less effective in eliciting turning-away than are pulses of the same intensity*', and Boyan and Fullard (1988) demonstrated that interneuron (501) in the noctuid, *Agrotis infusa* was activated by A1 spike rates of 256 Hz (i.e. IPs of 3–4 ms) and suggested that continual firing at low IPs may be rejected as noise by the moth CNS. If A1 IPs, combined with a tone-like, non-bursting firing pattern exceed those to 70 dB pulsed stimulus intensities, it implies that the moth would revert to a condition of far-bat and no longer express erratic flight. While far-bat responses are appropriate against a distant bat that is unaware of the moth's presence, such flight would be maladaptive when faced with a close *Eptesicus fuscus* that has targeted on the moth and is closing in for the final attack.

We suggest two possible reasons for A1 cell partial drop-out. The first is that, since presumably few moths survive past the A1 partial drop-out point of a bat's attack, there has never been sufficient selection pressure to maintain a vigorous A1 response for the final milliseconds leading up to the moth's capture. There are acoustical reasons (e.g. avoidance of pulse-echo overlap) for why bats change the structure and intensities of their approach-terminal phase calls (Simmons and Stein, 1980; Hartley, 1992; Kalko and Schnitzler, 1989; Kalko, 1995; Boonman and Jones, 2002) and the effects on moth ears may simply be coincidental. However, a second explanation for the A1 partial drop-out suggests an adaptive tactic used by a bat to facilitate its capture of eared moths. By reducing the intensities and durations of its terminal buzz calls and thereby reducing the moth's A1 response, a bat may be able to prematurely halt the moth's near-bat flight responses long enough to get its final target bearings before it contacts its prey. Kalko (1995) has shown that wild European pipistrelle bats reduce their flight speed to as low as 1 m s^{-1} during the terminal phase of their attack and this could give a bat additional time to orient toward the moth, especially if it became less responsive to the bat's calls. Certain bats emit allotonic echolocation calls at dominant frequencies that are either too high or too low for moths to detect (for a review, see Fullard, 1998). It has been argued that this type of echolocation represents an acoustic counter-strategy against moth auditory

defences (Fenton and Fullard, 1979; Rydell and Arlettaz, 1994; Pavey and Burwell, 1998; Bogdanowicz et al., 1999; Jacobs, 2000; Norman and Jones, 2000). Whether the moth's final flight is altered to the benefit of the bat due to A1 partial drop-out could be tested, using detailed video analyses of bats and moths during the final 150 ms of the bat's attack to reveal if the moth prematurely terminates its evasive flight, prior to itself being terminated.

A2 cell

Whereas the near-bat responses of notodontids are dependent solely on the A1 cell, the A2 cell of noctuids has been suggested to provide for auditory 'insurance' (Roeder, 1964). This cell's command role in triggering near-bat responses (Roeder, 1974) seems unlikely in the light of Surlykke's report (Surlykke, 1984) of A2-less notodontids also expressing near-bat responses. Our notodontid A1 results suggest that this cell exhibits responses similar to those of the A2 cell in noctuids and it is possible that the firing pattern of the A1 cell is all that is required to evoke near-bat flight defences, as originally suggested by Roeder (1964). It is difficult to link a particular behaviour to the activity of the A2 cell since there has been no clear demonstration of when the moth begins its near-bat flight. In addition, in previous studies the responses of the A2 cell have been evoked to pulsed sounds that do not simulate the full suite of call characteristics emitted by attacking bats (Suga, 1961; Roeder, 1964, 1974; Coro and Pérez, 1983). Our results reveal that while the A2 cell exhibits a rigorous response in the noctuid *L. pseudargyria*, its significance is less apparent for the two arctiids tested, moths whose ears also possess A2 cells. In both of these arctiid moths, the A2 appears either not at all or only sporadically during the last 100 ms of the terminal stage, presumably when the bat is into its final attack flight. The A2 cell also exhibits partial drop-out during the terminal stage of the bat's attack, but it occurs *sooner* than that of the A1 cell. If the A2 cell was solely responsible for evoking near-bat responses it would be extremely maladaptive for this cell to stop firing at the critical time when the bat is commencing its final attack. The fact that the A2 cell is lacking in certain moth taxa combined with its labile characteristics in moths that do possess it, supports the suggestion (Lewis and Fullard, 1996) that this cell is vestigial and not used in the flight responses of moths.

Cycnia tenera represents a unique opportunity to examine what anti-bat behaviours the A2 cell might control. Since sound production is a reliable response to bat calls that is easily evoked in *C. tenera*, this behaviour provides a convenient substitute for the difficult-to-quantify responses of flight. Behavioural studies of sound production in *C. tenera* suggest that this moth emits its clicks late into the attack sequence, possibly to induce a phantom-echo-jamming effect in the bat (Fullard et al., 1994). Our results confirm these observations and partially support the hypothesis that the A2 cell serves a command role in this behaviour (Fullard, 1982). While our neural results suggest the necessity of A2 activity for sound production behaviour, we cannot demonstrate the sufficiency of the A2 cell since the A1 cell is also firing during sound production [although the intensity response curves of *C. tenera* (Fig. 3) indicate no obvious change in A1 firing between sub- and suprathreshold stimulus dBs]. The precise mechanism of the A2 cell's effect on sound production is not simple, however, as the attack sequences in Fig. 10 illustrate. Although sound-production only appears when A2 fires, this behaviour shows a long latency from the onset of A2 (at least 500 ms), suggesting a more complicated interneuronal network for this response (Fullard, 1992; Dawson and Fullard, 1995).

Comparison with interneuron responses in the praying mantis

The description by Triblehorn and Yager (2002) of the responses of a praying mantid's interneuron to the echolocation calls of an attacking (real) bat provides a fortuitous opportunity to compare afferent and interneuronal auditory processing to naturally significant sounds. Although mantids and moths represent phylogenetically distant insect groups (Wheeler et al., 2001), there appears to be considerable conservation in central nervous system auditory processing centres in a wide diversity of insects (Boyan, 1993). This suggests that a common selection pressure (e.g. the echolocation calls of foraging bats) has shaped the auditory processing of different insects in a similar fashion.

As with our receptor responses, Triblehorn and Yager (2002) observed a firing drop-out of the interneuron they were monitoring (501-T3) to the terminal buzz calls emitted by the bat that attacked the mantid. The mantid drop-out is total (i.e. the cell completely stops firing) and occurs at an earlier point (average 272 ms before capture) in the attack sequence than that of the A1/A2 partial drop-outs seen in our trials (50–90 ms before 'capture'). This is expected because it is the cumulative firing of the afferents that drives the activity of higher-order interneurons. Our observation that A1 and A2 spike numbers decrease while their IPs increase during the terminal buzz suggests that moth and mantid receptors would be less likely to drive post-synaptic cells past their thresholds. Changes to the behaviours during the terminal buzz may therefore occur sooner than our results suggest. Triblehorn and Yager (2002) propose that there are adaptive mechanisms underlying the total drop-out in 503-T3 (e.g. active inhibition arising from other interneurons), which could allow for other neurally evoked near-bat responses to be expressed. Our receptor response results, however, suggest that it is the partial drop-out of the peripheral encoding system that results in the total drop-out of interneurons and that the adaptive value, if any, of this shut-down may be only for the bat. Triblehorn and Yager (2002) discount afferent encoding failure, citing studies that show the ability of mantid, moth and lacewing receptors to encode for rapidly repeated pulses. However, these studies used synthetic pulses of high intensities and/or long durations and these responses may not reflect auditory receptor encoding during actual bat attacks.

Conclusions

From our study, we conclude the following: (1) the B cell

provides no auditory function during the attack sequence of a bat's approach and may in fact be vestigial, as first proposed by Treat and Roeder (1959); (2) the A1 cell encodes the approach calls of an attacking bat up to approximately 100–200 ms before the bat would capture the moth but then reduces its firing to that representative of a far-bat, which may result in disactivation of interneurons and a premature cessation of near-bat responses; (3) the A2 cell is activated as the bat enters its attack sequence but also experiences a partial drop-out at a point before the bat terminates its attack, and that while this cell may serve no function in the flight responses of moths in general, it may activate the near-bat response of sound-production in tiger moths. Finally, our results and those of Triblehorn and Yager (2002) stress the need to appreciate the differences that exist between simulated and real acoustic conditions when extrapolating laboratory findings of neural data to events occurring in the real world. As Roeder (1964) stated with characteristic foresight, '*quantitative comparisons between behavioural and neurophysiological observations must be treated with some reservation*'.

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