The Journal of Experimental Biology 208, 4159-4165
Published by The Company of Biologists 2005
doi:10.1242/jeb.01893

The contribution of tympanic transmission to fine temporal signal evaluation in an ultrasonic moth

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Accepted 21 September 2005

Summary

In lesser waxmoths *Achroia grisella*, pair formation and female mate choice involve very fine discrimination of male ultrasonic signals. Female *A. grisella* prefer male signals with longer pulses and longer 'asynchrony intervals', and evaluate differences in these characteristics in the range of 80–260 μs. The first step in the evaluation of these characteristics is the tympanic transmission of stimuli. We used laser vibrometry to describe the mode of vibration, frequency tuning and stimulus transmission of the tympana of *A. grisella*. The tympanic response consisted of a rotational mode of vibration, in which the anterior and posterior sections moved out of phase; the posterior section of the tympanum vibrated with all points moving in phase and maximum displacement at the attachment point of the scoloparium that contains the receptor cells. The tympana of *A. grisella* were tuned to high ultrasonic frequencies and had an estimated time constant (i.e. the limit to their temporal acuity) of about 20–50 μs. Pulse length and all but the shortest asynchrony interval were thus well resolved by the tympanum. We discuss implications for the evaluation of pulse length and asynchrony interval.

Key words: bioacoustics, mechanisms of mate choice, Lepidoptera, Pyralidae, *Achroia grisella*.

Introduction

Insect acoustic communication relies heavily on the temporal features of signals (Gerhardt and Huber, 2002; Greenfield, 2002). Some insects evaluate signal variation at very fine scales. In lesser waxmoths (*Achroia grisella* F., Lepidoptera: Pyralidae), females choose mates on the basis of very fine differences in their advertisement signals. Male *A. grisella* signal by fanning their wings, which causes tymbals at the base of the forewings to generate pairs of short ultrasonic pulses with each beat of the wings (Jang and Greenfield, 1996; Fig. 1A). In playback experiments (Jang and Greenfield, 1996), females preferred longer pulses, and evaluated differences in pulse length as small as 80 μs (Fig. 1B). Females also evaluated the 'asynchrony interval' of pulse pairs (Fig. 1A). They preferred longer asynchrony intervals when the shorter alternative was <250 μs, and they evaluated differences in asynchrony interval as small as 260 μs (Fig. 1C). The mean length of male pulses was 111±34 μs (± S.D.) and mean asynchrony interval of pulse pairs was 500±407 μs (Jang and Greenfield, 1996). Although the differences in pulse length and asynchrony interval evaluated by *A. grisella* are large relative to the mean values of these traits, they are very small in comparison with the temporal differences evaluated by most other insects (Gerhardt and Huber, 2002; Greenfield, 2002). *Achroia grisella* are capable of this level of discrimination even though they have tympana with four receptor cells each (Knopek and Hintze-Podufal, 1986; Scoble, 1992; Fig. 2A).

Here we begin to ask how *A. grisella* can perform these feats of evaluation. The first step in stimulus evaluation is the transmission of information to the receptor cells by the tympanum. In the present paper we describe tympanic transmission of stimulus characteristics in *A. grisella*. We focused on the temporal resolution of the tympana, i.e. the discrimination of small time differences that they are capable of, or their acuity (Green, 1985). There is a trade-off between temporal and spectral resolution. Fine temporal discrimination reduces the window over which precise frequency measurements can be obtained; conversely, a highly tuned system will vibrate for a long time and thus lose temporal information; a highly tuned system would also have higher sensitivity at the cost of temporal resolution (Fletcher, 1992). Further, the spectral resolution of a tympanum can be influenced by its mode of vibration; for example in locusts, complex tympanic vibration contributes to frequency evaluation (Stephen and Bennet-Clark, 1982; Breckow and Sippel, 1985; Windmill et al., 2005). We therefore described the mode of vibration, frequency tuning and mechanical transmission of temporal stimulus characteristics of the tympana of *A. grisella*, using laser vibrometry.

We found that transmission of pulse length was linear with

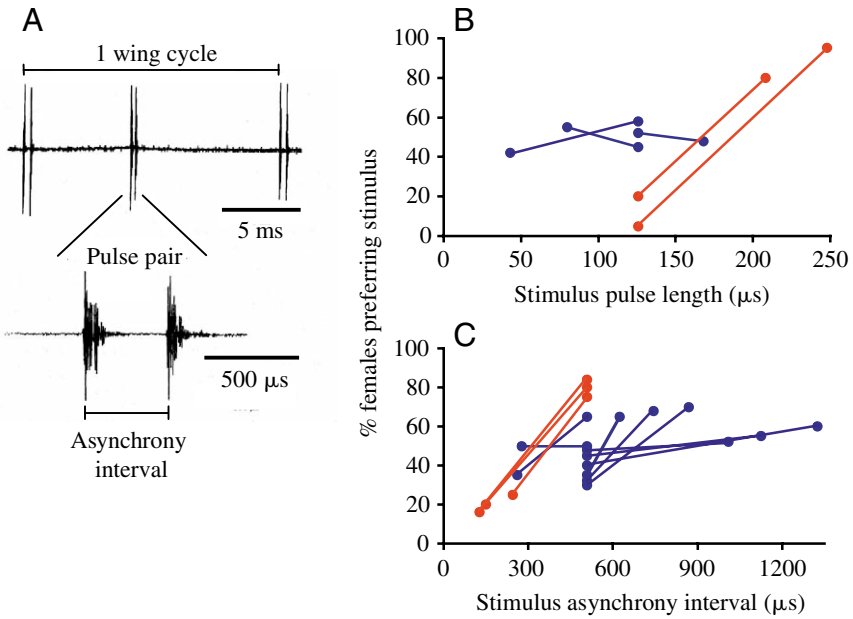

Fig. 1. (A) Advertisement signal of a male *A. grisella*. (B) Summary of two-speaker playback experiments showing female evaluation of pulse length in *A. grisella*. (C) Summary of two-speaker playback experiments showing female evaluation of asynchrony interval. Each line joins stimuli constrasted in a two-speaker experiment. Red lines indicate statistically significant preferences; blue lines, non-significant comparisons. Modified from Jang and Greenfield (1996).

responses only slightly longer than the stimuli, because of tympanic frequency tuning. Transmission of asynchrony interval was precise except for the shortest intervals. We discuss implications for possible mechanisms in the receptor cells that may explain the evaluation of these signal characteristics in *A. grisella*.

Materials and methods

Moths used in the experiments came from a captive colony established with over 200 individuals collected in Lawrence, Kansas, in 1999. Moths were reared on a diet of flour, glycerol, brewer's yeast, beeswax, honey and water (Jang and Greenfield, 1996), and maintained at ca. 24°C under a light cycle of 12 h:12 h light:dark. The experiments were conducted from November 2001 through March 2002, using 1-day-old adults.

Laser vibrometry

Achroia grisella have two tympana located ventrally on the first abdominal segment (Fig. 2A). Each tympanum consists of a sclerotized ring surrounding a membrane with a whitish anterior section and a transparent posterior section (Fig. 2A; Knopek and Hintze-Podufal, 1986). The attachment point of the scoloparium that contains the four receptor cells is in the middle of the posterior section (Fig. 2A, arrow; Knopek and Hintze-Podufal, 1986; Scoble, 1992).

To prepare the moths for observation, we cooled them at 5°C for 15 min, removed their legs, and fixed them with a mixture of resin and beeswax on a 2 cm×3 cm platform, with the thorax slightly pulled back to expose the tympana. We placed this preparation in a 50 cm×50 cm×50 cm box lined with acoustic foam with one open side. The box was on top of a vibration isolation table (Vibraplane, Kinetic Systems, Boston, MA, USA).

We monitored tympanic vibration using a laser vibrometer (Polytec Compact Laser Vibrometer CLV 1000, with a CLV M030 decoder module, at a sensitivity of 25 mm s^{-1} V^{-1}; flat frequency response up to 250 kHz; Polytec Inc., Auburn, MA, USA). The laser source was 17 cm from the moth, with the beam approximately perpendicular to the tympanum surface. We directed the beam with a micro-adjustable arm, monitoring its position on the tympanum with a dissecting scope. We estimated the accuracy of the positioning of the laser beam by measuring the distance from the edge of the tympanum to the center of the laser beam in repeated positionings of the laser beam on the scoloparium attachment point of the same individual. Accuracy was 4.4% of tympanum width (mean ± S.E.M.; tympanum width=0.51±0.01 mm, N=14; the width of the laser beam was ca. 0.05 mm; see Fig. 2A).

The laser vibrometer output was high-pass filtered (10 kHz, KH 3202, Krohn-Hite, Avon, MA, USA) and recorded using a custom-made analog–digital (A-D) converter system (16-bit, 250 kHz sampling rate, flat frequency response up to 100 kHz). A trigger signal indicating the timing of stimulus presentation was generated by the playback system (see below) and recorded on a second channel. We used this trigger signal to synchronize recordings of the stimuli and of the tympanic response in the analysis of transmission of temporal characteristics (see below).

To describe the vibration of the tympanum, we initially recorded at six points on the membrane (Fig. 2A, minus the points adjacent to that indicated by the arrow) for ten females and five males. We then increased the number of points to eight (Fig. 2A) for nine additional females. To describe frequency tuning and transmission of stimulus temporal characteristics, we recorded at the attachment point of the scoloparium on the posterior section of the tympanum (N=42 females; Fig. 2A, arrow).

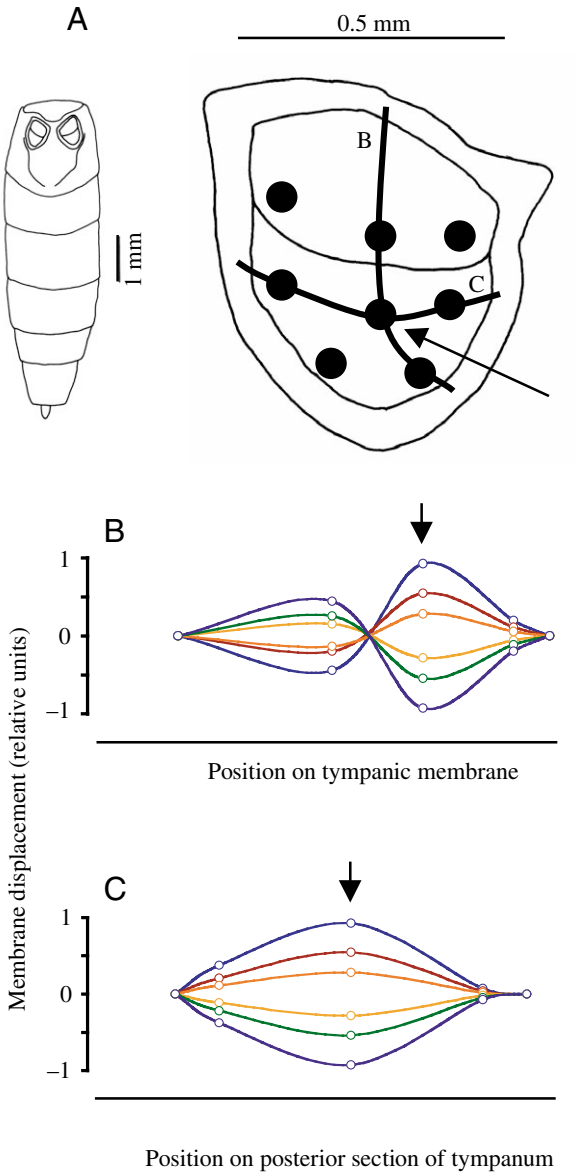

Fig. 2. Mode of vibration of the tympanum of *A. grisella*. Arrows indicate the attachment point of the scoloparium in the posterior section of the tympanum. (A) Ventral view of the abdomen of a female, thorax and legs removed, and detail of the right tympanum. Dots indicate the position and diameter of the laser beam. Lines labeled B and C indicate the lateral and cross-section views of the tympanum shown in (B) and (C). A typical example of the displacement of points in the tympanum in one period of vibration of one female is shown in (B) and (C). Displacement was measured from freeze-frames of the animation of a vibrating tympanum at 97.5 kHz. Points on the sclerotized ring surrounding the tympanic membrane were assigned zero displacement. (B) Lateral section along the longitudinal axis of the tympanum, showing the rotational mode of vibration of the tympanum. (C) Cross-section of the posterior section of the tympanum, showing the mode of vibration with all points moving in phase and maximum displacement at the attachment point of the scoloparium.

Stimulus generation

To describe the tympanic mode of vibration and frequency tuning, we used 41-ms pulses of broad-band ultrasonic noise (20–100 kHz) with a rise time of 0.5 ms. This stimulus permitted measuring the output of the tympanum over most of the frequency range relevant to the moths for detecting bat echolocation cries and conspecific signals (Rodríguez and Greenfield, 2004).

To describe the transmission of temporal characteristics, we used stimuli modeled after male *A. grisella* signals (Jang and Greenfield, 1996): short pulses with a trapezoid shape, rise and fall times of 8 μs, and plateaus of length that we varied in the experiments. Recordings of these stimuli show peak energy at 90–100 kHz with frequencies down to 25 kHz within 6 dB of the peak. To describe pulse length transmission, we used single pulses and varied their length (100–330 μs). For asynchrony interval transmission, we used pairs of 100 μs pulses and varied asynchrony interval (120–1120 μs). These values cover the range of pulse lengths and asynchrony intervals tested in playback experiments (Jang and Greenfield, 1996).

Stimulus presentation

The stimuli were presented with a speaker (Technics 10TH400C) placed 28 cm from the moth. The speaker membrane was approximately parallel to the ventral surface of the moth. The stimuli were generated using a custom-designed D-A converter system (16-bit, 250 kHz sampling rate). The trigger signal (see above) was generated simultaneously by this system. For each moth, we presented the stimuli 200 times and averaged the recordings to improve the signal-to-noise ratio.

Stimulus amplitude was calibrated at the preparation site with no moth present using a Brüel and Kjaer (Naerum, Denmark) 2231 sound level meter and a $^{1}/_{4}$-inch free-field microphone (40 BF, GRAS, Vedbaek, Denmark). Stimulus amplitude was similar to that of a male signaling at 10 cm (mean=86 dB peSPL re: 0 dB=20 μPa, range=73–92 dB peSPL, N=25 males). We set the RMS-amplitude of the long-pulse (noise) stimulus to 86 dB SPL, using the fast setting of the sound level meter. For the short pulses, we set peak amplitude to 84 dB peSPL.

We obtained reference recordings of the loudspeaker output using the $^{1}/_{4}$-inch microphone with its protective grid, placed at the position of the moths, and amplified by the sound level meter and high-pass filtered (10 kHz, KH 3202, Krohn-Hite, Avon, MA, USA). The protective grid caused the frequency response of the microphone to decrease by 13 dB octave^{-1} from 40 to 100 kHz; we corrected for this frequency response before performing the frequency tuning analysis.

Signal analysis

To improve the accuracy of our temporal measurements, we increased the sampling rate of our recorded files to 1 MHz in CoolEdit 2000 (Syntrillium Software Corporation, Phoenix, AZ, USA). Analyses of mode of vibration and frequency tuning were performed with custom computer programs written in Matlab (Mathworks, Inc., Natick, MA, USA).

For the analysis of the tympanic mode of vibration, we used

a program provided by Quang Su (Watson School of Engineering and Applied Science, SUNY Binghamton, USA). This program calculated the phase relationships between points on the tympanum by calculating transfer functions (fft size=8192 points) between a reference file (stimulus recording) and the laser recordings. The transfer function describes the amplitude ratio and relative phase between the laser and stimulus recordings at the frequencies evaluated (Bendat and Piersol, 1986). Because the amplitude of sound pressure as recorded with a microphone is proportional to particle displacement, while the laser recordings are proportional to velocity, we converted the transfer function magnitude to displacement-relative units by dividing by $2\times\pi\times$frequency. (Note that sound pressure is in phase with particle velocity, but its amplitude is proportional to displacement.) The program then generated an animation of the tympanic vibration for each individual. We used only recordings with high coherence (>0.8 across 20–100 kHz, coherence calculated with fft size=8192); this reduced our sample of females to five moths whose tympana were monitored on six points and four moths whose tympana were monitored on eight points. Coherence is a function in the frequency domain, with values between 0–1 that indicate how well the input corresponds to the output at each frequency. Coherence can thus be used to measure signal quality, i.e. the extent to which the signal is linearly related to the stimulus and free from unrelated noise (Kates, 1992). We used the magnitude-squared coherence function:

$$|C(f)|^2 = |C_{xy}(f)|^2 / C_{xx}(f)C_{yy}(f)\,, \qquad (1)$$

where $C_{xy}(f)$ is the cross-power spectrum between the signal and the stimulus recording, $C_{xx}(f)$ is the auto-power spectrum of the signal, and $C_{yy}(f)$ is the auto-power spectrum of the stimulus recording (Kates, 1992; Robert et al., 1998).

For the analysis of tympanic frequency tuning, we calculated transfer functions between the laser and stimulus recordings (fft size=8192 points). We converted the transfer functions to displacement-relative units (see above). We then $\log_{10}$-scaled the transfer functions and calculated a sliding average three times over a window of 3 kHz. This procedure did not change the overall shape of the curves, but removed spikes in the individual measurements that were coincident with phase discontinuities between the laser and stimulus recordings. At the high frequencies involved in the experiment, small differences in subject placement create differences in the sound field. To account for this variation, we repeated this analysis for two separate recordings of the stimulus, averaged the two resulting transfer functions for each moth, and calculated a grand mean and standard deviation. We used only recordings with high coherence (>0.8) across 20–100 kHz (this reduced our sample of females to 29).

To evaluate the transmission of stimulus temporal characterisics we measured pulse length and asynchrony interval in CoolEdit. We defined the beginning of pulses at the point in which the amplitude reached 20% of the maximum of the pulse; we defined the end of pulses at the point in which amplitude fell below 30% of the maximum of the pulse and

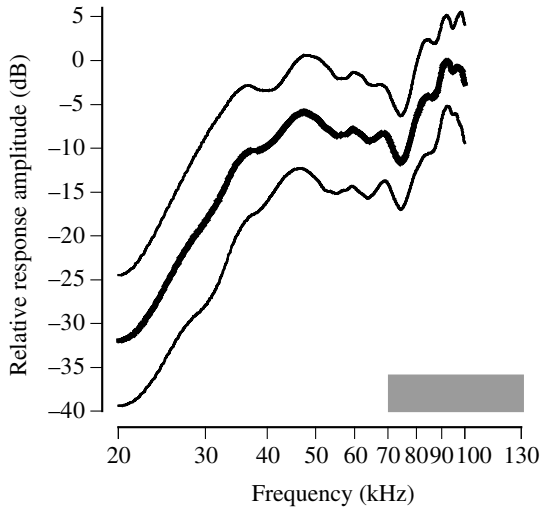

Fig. 3. Frequency tuning of the tympanum of *A. grisella*. The curves show the mean ±1 S.D. of 29 transfer functions (in displacement-relative units) between the microphone recordings of the noise stimulus and the laser vibrometer recordings of the tympanic response at the attachment point of the scoloparium in the posterior section of the tympanum. The gray bar indicates the frequency range of male *A. grisella* signals (Jang and Greenfield, 1996).

remained low. Because the tympanum did not respond to the lower frequencies present in the stimuli (Fig. 3; recordings of the stimuli showed energy down to 25 kHz, see above), we discarded the low-frequency energy from the stimulus recordings before the analysis of transmission of temporal characteristics. To discard the low-frequency energy we high-pass filtered the stimulus recordings at 70 kHz. This filtering did not alter the laser recordings of the tympanic response (data not shown), because most of their energy was at high frequencies (see Fig. 3). We therefore used the unfiltered laser recordings for this analysis. Finally, we used a custom computer program written in Borland Pascal 7.0 (Borland, CA, USA) to measure the effect of asynchrony interval on the RMS amplitude of the tympanic response to stimulus pulse pairs varying in asynchrony interval. We measured RMS amplitude over a window of 1350 μs from the beginning of the response. This window included the pulse pair in its entirety. For this analysis we used the files resulting from averaging the 200 recordings for each stimulus presentation to each individual, to improve the signal-to-noise ratio (see above). The data showed homogeneity of variance, and we performed one-way analyses of variance (ANOVAs) and Model I regressions.

Results

Mode of vibration

The tympanum of *A. grisella* vibrated in a rotational (or rocking) mode, with a nodal line between the anterior and posterior sections, which moved out of phase with each other (Fig. 2B). This mode was predominant across the range of frequencies assayed (20–100 kHz) in all nine females whose

recordings had high coherence. The posterior section of the tympanum vibrated with all points moving in phase and maximal displacement at the attachment point of the receptor cells (Fig. 2C). The animations of vibrating tympana showed no obvious difference between the right and left tympana of females, or between males and females.

Frequency tuning

The tympana of *A. grisella* were most sensitive at high ultrasonic frequencies (Fig. 3). Highest response amplitude occurred at 90 kHz. From 90 to 71 kHz, response amplitude decreased at a rate of 35 dB octave^{-1}. Between 71 and 35 kHz, response amplitude was within –6 to –12 dB relative amplitude, with a secondary peak at 45 kHz. Finally, between 35 and 20 kHz, response amplitude decreased at a rate of 28 dB octave^{-1}.

Transmission of temporal characteristics

Pulse length transmission was linear but with responses 20–30 μs longer than the stimuli (Fig. 4). Transmission of asynchrony interval was precise for asynchrony intervals ⩾170 μs (Fig. 5A). For 120-μs asynchrony intervals there was overlap between the responses to the pulses in a pulse pair (according to our criterion of 30% of the peak pulse amplitude to define the end of a pulse), due to the 20–30 μs longer response to individual stimulus pulses (Fig. 5A). Variation in the mean RMS amplitude of the tympanic response according to stimulus asynchrony interval was under 2 dB and statistically non-significant (Fig. 5B).

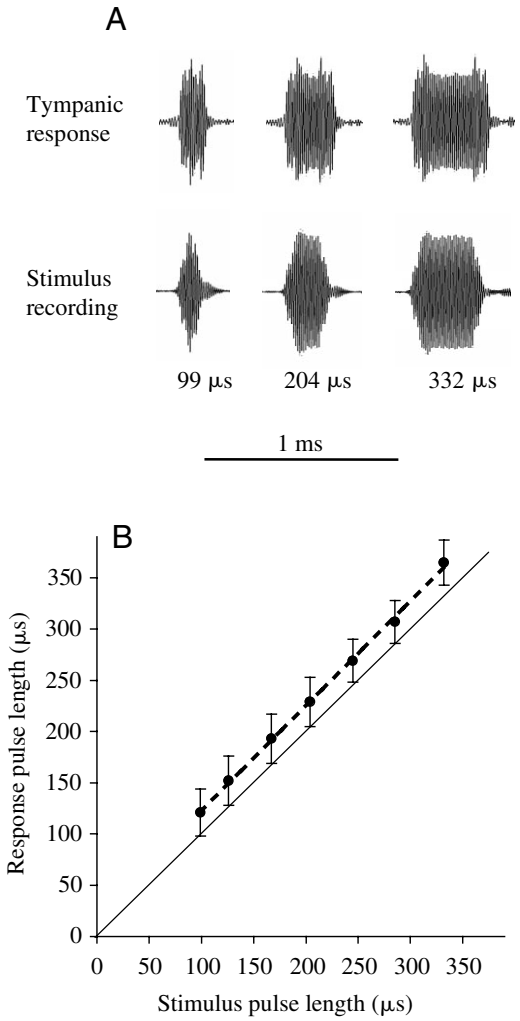

Fig. 4. Transmission of pulse length by the tympanum of *A. grisella*. (A) Recordings of the tympanic response and stimulus pulses. (B) Regression of response pulse length on stimulus pulse length (r^2=0.999, $F_{1,5}$=7334.4, P<0.001, slope=0.97; dotted line). Symbols indicate mean response length ±1 S.D. The solid line indicates a 1:1 relationship.

Discussion

Our experiments provide a first step towards explaining the evaluation of pulse length and asynchrony interval in *A. grisella*. We discuss the tympanic mode of vibration and frequency tuning before turning to the transmission of pulse length and asynchrony interval and the implications for possible mechanisms for their evaluation.

The tympanic response in *A. grisella* consisted of a rotational mode of vibration, in which the anterior and posterior sections moved out of phase. The posterior section of the tympanum vibrated with all points in phase and maximum displacement at the attachment point of the scoloparium that contains the receptor cells. This relatively simple mode of vibration agrees with most studies on the vibration of insect tympana (Larsen and Michelsen, 1978; Michelsen and Larsen, 1978; Schiolten et al., 1981), except for locusts, where the tympanic membrane and associated Müller's organ sclerites vibrate in a complex pattern (Stephen and Bennet-Clark, 1982; Breckow and Sippel, 1985; Windmill et al., 2005).

The tympanum of *A. grisella* was most sensitive at high ultrasonic frequencies. Peak response occurred at the frequency range of male signals and there was a pronounced roll-off. However, response amplitude was within 12 dB of the peak for frequencies as low as 35 kHz. This response reflects the mechanical tuning of the tympanic membrane and of the scoloparium itself (see Adams, 1972). In noctuid moths, the mechanical response of the scoloparium can consist of a resonant vibration with harmonics (Adams, 1972). The two peaks of high sensitivity in our results may thus reflect such harmonics in the resonant vibration of the scoloparium.

The range of relatively high sensitivity of *A. grisella* tympana (peak at 90 kHz and response amplitude within 12 dB down to 35 kHz) encompasses the frequencies of the echolocation cries of many bat species (Neuweiler, 1989; Miller and Surlykke, 2001). The tympana of *A. grisella* are thus most sensitive to the frequencies of male signals, but not much less sensitive to the frequencies typical of their natural enemies. This tuning agrees with behavioural evidence of sensitivity to the frequencies of male signals and bat echolocation cries (Jang and Greenfield, 1996; Greenfield and Baker, 2003; Rodríguez and Greenfield, 2004).

The tympana of *A. grisella* were also capable of fine temporal resolution. The transmission of pulse length was linear, but responses were 20–30 μs longer than the stimuli, which led to overlap between pulses separated by 120 μs asynchrony intervals, but not for longer asynchrony intervals. This level of resolution is comparable to that of noctuid moths (Schiolten et al., 1981) and finer than in katydids and crickets (Larsen, 1981).

The combination of frequency tuning and high temporal resolution in *A. grisella* tympana may be explained because high temporal resolution may stem from sensitivity to ultrasound *per se* (Schiolten et al., 1981). A system that vibrates over *n* cycles will take less time to do so at higher frequencies than at lower frequencies.

Our results suggest a likely range for the tympanic time constant in *A. grisella*. The time constant of a tympanum refers to its ability to follow brief changes in the temporal pattern of a stimulus, i.e. its acuity (Green, 1985). This time constant can be estimated by calculating the impulse response of the tympanum with a very brief stimulus (much shorter than the time constant one wishes to estimate), to observe how long the tympanum continues to vibrate after the end of the stimulus (Schiolten et al., 1981). Although we did not use this method, our results give an indication of the tympanic time constant in *A. grisella*. According to our criterion of 30% of peak amplitude to define the end of pulses, tympanic responses were 20–30 μs longer than the stimuli. The shortest asynchrony interval faithfully transmitted by the tympanic response was of 170 μs with 120 μs tympanic responses, so the briefest gap resolved was of 50 μs. Thus, the time constant of *A. grisella* tympana may be 20–50 μs. This estimate is shorter, by as much as a third, than the tympanic time constant of noctuid moths (ca. 60 μs; Schiolten et al., 1981), which is in accord with the tuning of noctuid tympana to lower frequencies (Schiolten et al., 1981).

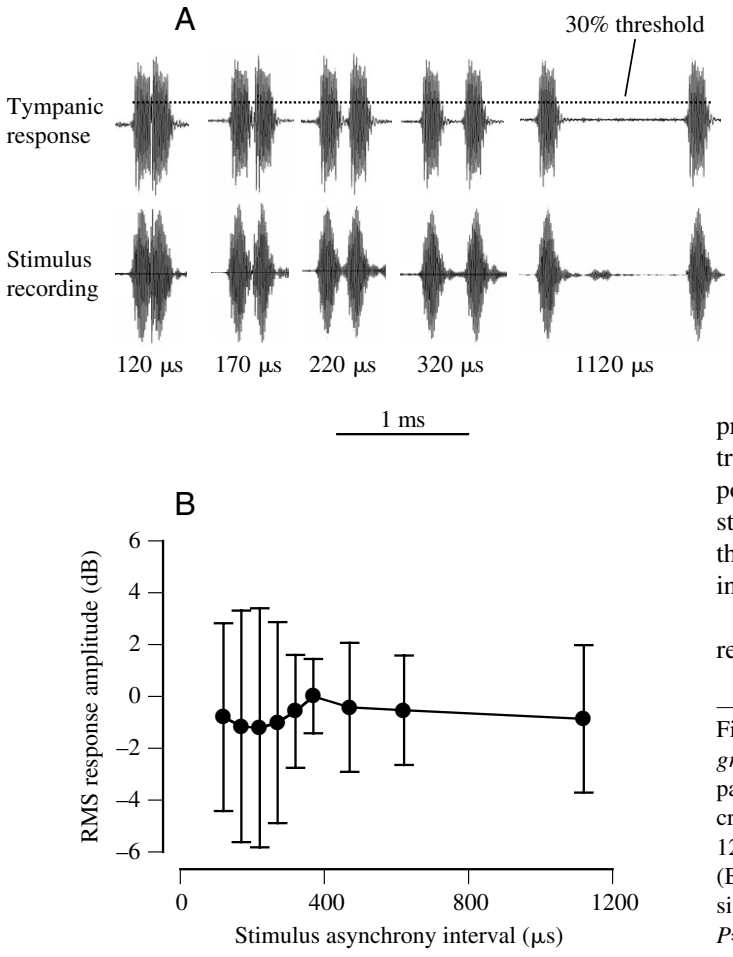

Fig. 5. Transmission of asynchrony interval by the tympanum of *A. grisella*. (A) Recordings of the tympanic response and stimulus pulse pairs varying in asynchrony interval. The dotted line marks the criterion of 30% of peak amplitude for defining the end of pulses. For 120 μs asynchrony intervals the pulses in the response overlapped. (B) Variation in tympanic response RMS amplitude was not significantly affected by stimulus asynchrony interval ($F_{8,369}$=0.60, P=0.78). Values are mean ±1 S.D.

Evaluation of pulse length

Tympanic transmission of pulse length was linear, with responses slightly longer than the stimuli. The stimulation transmitted by the tympanic response can probably be encoded by integration of energy over time by the receptor cells. The requirement for this mechanism to obtain is that male pulses be shorter than the integration time of the receptor cells. The integration time is the interval over which stimuli are summated (Green, 1985; Tougaard, 1998, 1999). Summation involves integration of energy over time, and integration of the probability of detection over time (Green, 1985; Tougaard, 1998, 1999). Integration of energy occurs at shorter time scales than integration of probability of detection, and obtains if the integration time is longer than the stimuli (Green, 1985; Tougaard, 1998, 1999). We do not have estimates of the integration time for *A. grisella*, but estimates for other moths are in the range of 2–5 ms (Tougaard, 1998). If the integration time of *A. grisella* receptor cells has similar values, it is an order of magnitude longer than the male pulses (Fig. 1). The estimate of a short time constant for *A. grisella* tympana (20–50 μs) indicates that tympanic responses to male pulses will fall within the likely integration time of their receptor cells. Thus, longer pulses can evoke stronger responses in the receptor cells and increase the likelihood that an action potential will occur.

Evaluation of asynchrony interval

The evaluation of asynchrony interval is a more problematic question. Our results showing precise tympanic transmission of asynchrony intervals ⩾170 μs rule out the possibility that non-linear tympanic transmission influences the strength of the stimulation delivered to the receptor cells. Thus, the mechanism responsible for evaluation of asynchrony interval must reside at the neural level.

Experiments with noctuid moths suggest that summation of receptor potentials is maximized when the interval separating

two short pulses lasts 1–5 ms; single pulses, and pulses separated by intervals longer than the integration time of the receptor cell (2–5 ms), result in weaker spiking activity (Tougaard, 1996, 1998). Extrapolating these findings to the time scale of *A. grisella* asynchrony intervals does not offer an explanation for the evaluation of asynchrony interval. On the one hand, all the asynchrony intervals discriminated by females are shorter than the likely integration time of the receptor cells (Fig. 1C), so that they may deliver the same amount of energy to the receptor cells. On the other hand, if the amount of energy delivered varies with asynchrony interval, it would be the shorter intervals that deliver the stronger stimulation, because summation of sequential receptor potentials would be higher when the receptor potentials are separated by smaller intervals. Thus, the potential effect would be for a preference for shorter asynchrony intervals, instead of the observed pattern (Fig. 1C). The mechanism responsible for evaluation of asynchrony interval may therefore involve non-linear summation of receptor potentials. Features of *A. grisella* signals that may influence non-linear summation include the very short length of their pulses, and the very high amplitude at which they are produced (Jang and Greenfield, 1996).

In conclusion, we found no mechanical limit to temporal resolution in the transmission of ultrasonic stimuli by the tympana of *A. grisella*. Pulse length can be encoded by the receptor cells. Evaluation of asynchrony interval probably occurs at other levels of the nervous system.

We thank Ron Miles and Quang Su (Watson School of Engineering and Applied Science, SUNY Binghamton) for advice in experimental design and for providing analysis software. Karthik Ramaswamy and Holly Shugart provided help in the laboratory. Gerlinde Höbel commented on the manuscript and provided very helpful discussion and technical assistance. Financial support came from Hungerford awards (University of Kansas) and a Grant-in-Aid of Research from Sigma Xi to R.L.R., and National Science Foundation grants IBN 0132357 to R.B.C. and IBN 9807915 to M.D.G.

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