Naïve bats discriminate arctiid moth warning sounds but generalize their aposematic meaning

Jesse R. Barber[1],*, Brad A. Chadwell[1], Nick Garrett[1], Barbara Schmidt-French[2] and William E. Conner[1]

[1]Department of Biology, Wake Forest University, Winston-Salem, NC 27109, USA and [2]Bat Conservation International, Austin, TX 78746, USA

*Author for correspondence at present address: Colorado State University, Department of Fish, Wildlife and Conservation Biology, Fort Collins, CO 80523, USA (e-mail: barber.jesse@gmail.com)

SUMMARY

Naïve red (*Lasiurus borealis* Müller) and big brown (*Eptesicus fuscus* Beauvois) bats quickly learn to avoid noxious sound-producing tiger moths. After this experience with a model tiger moth, bats generalize the meaning of these prey-generated sounds to a second tiger moth species producing a different call. Here we describe the three-dimensional kinematic and bioacoustic details of this behaviour, first, as naïve bats learn to deal with an unpalatable model tiger moth and subsequently, as they avoid acoustic mimics. The tiger moths' first clicks influenced the bats' echolocation behaviour and the percentage of interactions that included terminal buzzes was associated with capture and investigatory behaviour. When the mimic was introduced, the bats decreased both their minimum distance to the tiger moth and the time at which they broke off their attack compared with their exposure to the model on the night before. These kinematic signatures closely match the bats' behaviour on their first night of experience with the model. Minimum distances and time of pursuit cessation increased again by the last night of the mimic's presentation. These kinematic and bioacoustic results show that although naïve bats generalize the meaning of aposematic tiger moth calls, they discriminate the prey-generated signals as different and investigate. Extrapolating to experienced bats, these results suggest that acoustic predators probably exert potent and fine-scaled selective forces on acoustic mimicry complexes.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/212/14/2141/DC1

Key words: kinematics, bioacoustics, bats, moths, Arctiidae, acoustic mimicry.

INTRODUCTION

The aerobatic spirals, loops and dives moths initiate when their simple ears detect the echolocation cries of a nearby bat are not the only defences they employ against their acoustic predators. Tiger moths (family: Arctiidae) answer the echolocation attack of bats with ultrasonic clicks broadcast from bilateral metathoracic structures called tymbals (for a review, see Miller and Surlykke, 2001). Active debate over the functions of these sounds has produced three main hypotheses: startle, jamming and warning. Although some evidence exists for both startle (Bates and Fenton, 1990; Miller, 1991) and jamming (Miller, 1991; Masters and Raver, 1996; Tougaard et al., 1998; Tougaard et al., 2004), acoustic aposematism appears to be the major function of tiger moths' acoustic reply to bat attack (Bates and Fenton, 1990; Hristov and Conner, 2005a; Ratcliffe and Nydam, 2008). A recent study (Hristov and Conner, 2005a) pitted naïve big brown bats (*Eptesicus fuscus*) against four naturally occurring species of tiger moths that varied in a pair of characters: presence or lack of a chemical defence, and ability or inability to produce sound. The learning profiles of capture success over seven nights showed that the bats failed to learn to avoid chemically protected moths unless those moths also provided an acoustic warning. Furthermore, moths that produced ultrasound in response to bat attack, but were not chemically protected, were captured and eaten by the bats. These results clearly support the warning model and demonstrate that the combination of chemical defence and acoustic warning is necessary to allow bats to associate moth-produced ultrasound with unpalatability.

We have extended this work by demonstrating that these moth warning sounds function in acoustic mimicry complexes (Barber and Conner, 2007). After experience with a noxious sound-producing model tiger moth species, naïve red (*Lasiurus borealis*) and big brown bats avoid a second sound-producing species of tiger moth offered to them, regardless of whether it is chemically protected or not, demonstrating both Müllerian and Batesian mimicry in this acoustical system. In other words, the bats generalize the meaning of these prey-generated sounds to a second tiger moth species producing a different call. A subset of the red bats in these experiments discovered the palatability of the Batesian mimic and began eating these moths. These same red bats displayed the ability to discriminate between the palatable acoustic mimic and the unpalatable acoustic model, when the model was reintroduced.

The metric used in the behavioural study discussed above (Barber and Conner, 2007) was the percentage of moths captured each foraging night. In order to elucidate the spatial and temporal specifics of decision making by the bats in these experiments we filmed each interaction with two synchronized high-speed video cameras that were calibrated with Direct Linear Transformation techniques to extract three-dimensional (3-D) kinematics from the trials (Abdel-Aziz and Karara, 1971; Chen et al., 1994; Hedrick, 2008). In addition, the acoustics of each bat–moth interaction were also recorded with an ultrasonic microphone. Here, we report that this detailed analysis of behaviour reveals that naïve red and big brown bats display a fine-scaled level of prey discrimination, despite generalizing the aposematic meaning of arctiid moth warning signals across multiple species.

MATERIALS AND METHODS
Animals and equipment

Nine naïve red bats (*Lasiurus borealis* Müller) and eight big brown bats (*Eptesicus fuscus* Beauvois) were raised in the laboratory to keep their insect-catching experience under experimental control (for details, see Barber and Conner, 2007). Once the bats were proficient at catching tethered, palatable, non-sound producing, control moths [*Galleria mellonella* (Linnaeus)], in an anechoic foam-lined indoor flight facility (5.8 m×4.0 m×3.0 m) for 2 weeks, experiments were initiated. Each bat was offered 16 moths a night sequentially and in random order for the 11–17 consecutive nights of each experiment. The moths were tethered to a fine monofilament line (40 cm) with a small surgical microclip. The tether was attached to a weighted mobile which, coupled with the moth's own erratic flight, allowed random prey movement within a defined interaction space of approximately 1 m^3. Eight *Galleria mellonella* were presented each night, along with four silent, palatable noctuids that served as size-matched novelty controls and four experimental sound-producing tiger moths.

All interactions were recorded with an ultrasonic microphone (Pettersson Elektronik© D940, Uppsala, Sweden) placed directly above the tethered moth. The microphone was matched in time to a pair of synchronized infrared-sensitive, high-speed cameras (Photron FastCam PCI©, Tokyo, Japan; 250 frames per second; 512×480 pixels resolution) by a custom-built calibration unit that simultaneously marked the sound record and triggered a pair of infrared LED lights, one facing each camera. The video files were recorded with Photron Fastcam Viewer v1.3© installed on a PC laptop. Infrared illumination was provided by four Wildlife Engineering© (Tucson, AZ, USA) LED arrays. This illumination was supplemented with a low-intensity deep red light for behavioural observation. The audio files were recorded in BatSound Pro© v3.8 (Pettersson Elektronik, Uppsala, Sweden) installed on a PC laptop connected to the bat detector *via* a National Instruments® (Austin, TX, USA) 6062E PCMCIA A/D sampling at 250 kHz. The acoustic behaviour of the bats and moths was monitored by the experimenter *via* a set of Sony® (Tokyo, Japan) 900 MHz wireless headphones linked to the bat detector.

Bat groups

To test acoustic mimicry we presented an unpalatable, sound-producing, model tiger moth species for 5 nights and then on night 6, introduced a second, mimic tiger moth species producing a different sound for 5 nights. Five *E. fuscus* and two *L. borealis* were run in a Müllerian experiment where the model (either *Cycnia tenera* Hübner or *Syntomeida epilais* Walker) and mimic (either *S. epilais* or *C. tenera*) moths were noxious [see Barber and Conner (Barber and Conner, 2007) for hostplant details]. As both species of bats in this group performed nearly identically, they were combined for analysis. Seven *L. borealis* and three *E. fuscus* were run in a Batesian experiment where the model (*C. tenera*) was noxious but the mimic (*Euchaetes egle* Drury) was entirely palatable (for palatability data, see Hristov and Conner, 2005b). This group of bats was subsequently separated in accordance with how each bat handled the mimic. Bats that were deceived (defined as capture of ≤5% of the mimic on the last night of the mimics' presentation) for the duration of the mimics' presentation (four *L. borealis* and all three *E. fuscus*) will hereafter be referred to as the Batesian deceived group. As both species of bats performed nearly identically in the Batesian deceived group, they were combined for analysis. Three *L. borealis* discovered the palatability of the mimic (defined as capture and consumption of ≥50% of the mimic on the last night of its presentation) and will

be referred to as the Batesian discovered group. All bat groups were presented with tiger moths with tymbals (sound-producing structures) removed, at the end of each experiment, to examine the role of other sensory modalities (such as olfactory and echoic cues) in these behaviours. For the Batesian discovered group, the model tiger moth (*C. tenera*) was reintroduced before moths with ablated tymbals were presented.

3-D kinematic analysis

The interaction space was calibrated by placing a 40-point, 160 cm×160 cm×160 cm calibration frame within the views of both high-speed cameras. Each of the 40 calibration points was digitized in both camera views in a custom MatLab® (Natick, MA, USA) program (Hedrick, 2008) which fit a set of direct linear transformation (DLT) coefficients to the data. Video files from both cameras for each bat–moth interaction were then imported into a second custom MatLab® digitization program (Hedrick, 2008) calibrated *via* this set of DLT coefficients, providing 3-D coordinates for any point that could be seen in both camera views. The 'centre-of-object' of the bat and moth were then manually digitized over the duration of each interaction when both animals were within view of each camera.

A third custom MatLab® program (BATracker.m; coded by B.A.C.) smoothed the raw 3-D coordinates using a quintic smoothing spline [MatLab® spaps routine; *sensu* Walker (Walker, 1998)]. Using these smoothed 3-D coordinates, the vector from the bat to the moth at each time point was calculated by subtracting the moth's position from the bat's position. The distance between the bat and moth was also calculated for each position in time (i.e. each video frame). The first time derivative of the bat's spline function is the velocity vector (magnitude and direction) of the predator. Bat flight speed (the magnitude of the bat's velocity vector) and closing rate (change in bat–moth distance over time) were averaged over the last 200 ms of the bat–moth interaction prior to minimum bat–moth distance. To estimate the closing rate, the first time derivative of the bat–moth distance was calculated using a five-point piecewise quadratic polynomial regression implemented in MatLab® with no modifications from the algorithm described by earlier workers (Lanczos, 1956). Phi (ϕ) represents the deviation (in degrees) of the bat's velocity vector from the bat–moth vector. If $\phi=0$ deg., the bat is flying directly at the moth and if $\phi=180$ deg., the bat is flying directly away from the moth. Time of veer (T_{veer}) is defined as the time from minimum bat–moth distance that ϕ becomes greater than 45 deg. and does not return below that threshold (Fig. 1). One avoidance flight was analyzed per bat, per night (usually the first bat–moth interaction). Video files were selected for analysis to maximize the amount of time the bat and moth were in view of both cameras and to maximize the signal-to-noise ratio of both the video and corresponding audio recordings.

Bioacoustic analysis

Sound files were analyzed in BatSound Pro© v3.8. Files were digitally high-passed filtered above 12 kHz using a fourth order Butterworth filter. Temporal parameters were measured from the oscillogram (voltage×time) window. Frequency parameters were quantified from power spectra created with a Fast Fourier Transform (FFT) of 1024 using a Hanning window. The parameter, period change, was quantified by measuring the time period between the two echolocation cries just prior to the first tiger moth clicks (period 1) and the following period that contained the first clicks (period 2). Period 2 was then normalized to a percentage of period 1. Values reported for period change are thus in
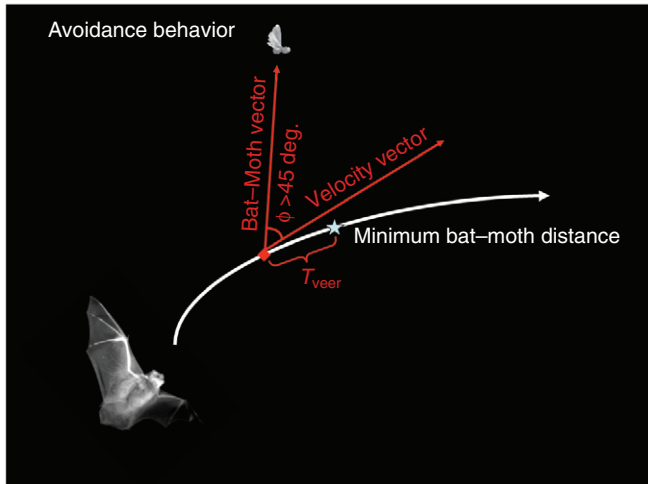
Fig. 1. Illustration of kinematic parameters. T_{veer} is defined as the elapsed time interval in ms between the time of minimum bat–moth distance (star) and the time when the angle (ϕ) between the bat–moth bearing vector and the bat's velocity vector became greater than 45 deg. and did not return below that threshold (diamond). The illustration depicts a red bat (*Lasiurus borealis*) avoiding a noxious, sound-producing *Cycnia tenera* tiger moth, with the white arrow representing the bat's flight trajectory over time.

percentage change between period 1 and period 2. We also quantified the spectral properties of the bats' echolocation calls before and after the first tiger moth clicks. A 15 dB bandwidth was calculated on either side of the peak frequency of the echolocation call just prior to the first tiger moth clicks (pulse 1) and the echolocation call following the clicks (pulse 2). These bat

calls formed the ends of temporal periods 1 and 2. Bandwidth change was then calculated by subtracting the spectral values of pulse 1 from pulse 2. These temporal and spectral parameters were quantified as percentage change to minimize the effects of analyzing biosonar calls from different attack sequences, from different bats, from different points in the attack sequence (depending on when the moth clicked), and that were recorded at varying distances and orientations to the microphone.

Statistical analysis

Statistics were performed in SPSS® (Chicago, IL, USA) v.14.0. To investigate the bioacoustics and kinematics of avoidance behaviour in this acoustic mimicry experiment we focused on three comparisons between different foraging nights. Nights 1 (introduction of the model moth) and 5 (last night of the model's presentation) were compared to look at the effect of aposematic learning, nights 5 and 6 (introduction of the mimic moth) were contrasted to examine mimicry and nights 6 and 10 (last night of the mimics presentation) were compared to evaluate the lasting effects of mimicry on these parameters (for details, see Barber and Conner, 2007). Kinematic and bioacoustic parameters for each bat group were analyzed using a series of repeated-measure ANOVAs followed by paired *t*-test comparisons. Owing to heteroscedasticity, the percentage of bat–tiger moth interactions that included terminal buzzes were analyzed using three nonparametric Friedman's ANOVAs, followed up by Wilcoxon's pairwise comparisons.

To control for familywise and experimentwise multiple comparison errors we adjusted our *P*-values both within each omnibus test (i.e. within a repeated-measure ANOVA) and across all tests reported, using the False Discovery Rate method (Benjamini and Hochberg, 1995) in QVALUE (familywise: smoother method, lambda range 0.0 to 0.9; experimentwise: bootstrap method, $\lambda=0$)

Table 1. Repeated-measure ANOVAs for 3-D kinematic and bioacoustic parameters

Parameter	Bat group	F	d.f.	P-value	Adj. P (Q)	Night 1*	Night 5*	Night 6*	Night 10*
Minimum bat–moth	**Müllerian**	**8.32**	**3,18**	**0.01**	**0.02**	**7.6±8.6**	**35.8±14.6**	**14.0±9.1**	**28.9±12.8**
distance (cm)	**Batesian deceived**	**17.92**	**3,18**	**0.001**	**0.003**	**10.9±9.8**	**32.6±11.4**	**16.4±6.3**	**27.4±7.0**
	Batesian discovered	4.77	3,6	0.05	0.07	5.9±1.9	28.7±2.1	15.5±18.8	1.4±0.09
T_{veer} (ms)	**Müllerian**	**13.82**	**3,18**	**0.01**	**0.02**	**−28.9±21.8**	**−103.8±27.2**	**−46.9±26.7**	**−94.0±51.7**
	Batesian deceived	**16.22**	**3,18**	**0.03**	**0.05**	**−14.1±33.0**	**−123.7±36.2**	**−46.0±29.0**	**−74.4±25.9**
	Batesian discovered	1.71	3,6	0.26	0.22	−52.0±42.3	−144.0±38.2	−106.7±164.0	13.8±24.4
Bat flight speed (m s⁻¹)	Müllerian	0.68	3,18	0.47	0.30	2.0±0.7	2.2±0.5	2.2±0.7	2.5±0.8
	Batesian deceived	1.7	3,18	0.37	0.26	2.8±0.4	2.6±0.7	2.3±0.5	3.0±1.1
	Batesian discovered	0.19	3,6	0.90	0.40	2.1±0.8	2.6±0.6	2.3±1.0	2.3±1.0
Closing rate (m s⁻¹)	Müllerian	0.66	3,18	0.73	0.37	0.8±0.7	0.6±0.4	1.0±0.5	0.7±0.3
	Batesian deceived	1.34	3,18	0.35	0.26	1.0±0.5	0.9±0.7	1.6±0.6	1.4±1.2
	Batesian discovered	0.30	3,6	0.82	0.39	0.9±0.7	1.2±1.0	1.2±1.1	1.5±1.0
Time from first click to	Müllerian	0.46	3,18	0.67	0.37	2150±400	2025±619	2094±456	1680±704
T_{veer} (ms)	Batesian deceived	0.38	3,18	0.13	0.15	1619±946	2035±71.6	1443±708	1898±546
	Batesian discovered	1,12	3,6	0.41	0.28	1856±213	2508±1313	2811±1128	2320±198
Period change (%)	Müllerian	0.23	3,18	0.85	0.39	262±149	243±203	199±103	214±111
	Batesian deceived	2.33	3,18	0.16	0.17	153±45	137±18	174±39	175±22
	Batesian discovered	0.77	3,6	0.55	0.34	181±24	291±281	165±42	122±46
Bandwidth change (%)	Müllerian	2.21	3,18	0.12	0.15	−1.2±3.8	0.7±6.7	−0.1±9.5	5.3±5.5
	Batesian deceived	1.18	3,18	0.58	0.34	−2.6±3.9	−2.7±8.1	2.1±5.1	0.1±4.5
	Batesian discovered	0.46	3,6	0.72	0.37	−0.7±3.1	−2.0±7.4	−2.1±11.4	2.6±3.7
% Interactions with	**Müllerian**	$\chi^2=32.94$	**10**	**0.0001**	**0.001**	**85.4±20.8**	**17.9±18.9**	**34.4±30.1**	**35.7±37.7**
terminal buzz	**Batesian deceived**	$\chi^2=36.50$	**10**	**0.0001**	**0.001**	**64.3±24.4**	**11.9±20.9**	**24.9±26.6**	**8.3±14.4**
	Batesian discovered	$\chi^2=28.13$	**12**	**0.005**	**0.01**	**75.0±25.0**	**16.7±28.9**	**91.7±14.4**	**91.7±14.4**
Terminal buzz	Müllerian	0.26	4,8	0.26	0.23	260±31	228±90	219±81	258±33
duration (ms)	Batesian deceived	0.42	4,8	0.79	0.39	197±72	133±80	166±92	164±87
	Batesian discovered	2.26	4,4	0.23	0.22	180±60	192±11	162±62	150±20

d.f., degrees of freedom; Adj. P (Q), false discovery rate adjusted P-value; T_{veer}, time of veer.
*Values are mean ± s.d. Statistically significant analyses are in bold type.

Table 2. Paired *t*-test comparisons of minimum bat–moth distance and time of veer for the Müllerian and Batesian deceived bat groups

| | Bat–moth minimum distance | | | | T_{veer} | | | |
| | Müllerian | | Batesian deceived | | Müllerian | | Batesian deceived | |
Nights	*P*-value	Adj. *P* (Q)	*P*-value	Adj. *P* (Q)	*P*-value	Adj. *P* (Q)	*P*-value	Adj *P* (Q)
1–5	0.001	**0.003**	0.001	**0.002**	0.0001	**0.004**	0.0001	**0.004**
5–6	0.03	**0.03**	0.009	**0.009**	0.001	**0.002**	0.008	**0.01**
6–10	0.004	**0.006**	0.001	**0.002**	0.05	**0.05**	0.04	**0.04**

T_{vee}, time of veer; Adj. *P* (Q), false discovery rate adjusted *P*-value; Nights, pairwise foraging night comparisons.
Statistically significant comparisons are in bold type.

(Storey et al., 2004). These adjusted *P*-values were compared against an α-value of 0.05 to determine statistical significance. All data are presented as means ± standard deviation (s.d.).

RESULTS
3-D kinematics of avoidance behaviour

On night 1, as the bats were learning the aposematic signal of the sound-producing model tiger moth, the minimum distances between the bats and arctiids during avoidances were small (Table 1; Müllerian group, Fig. 2; Batesian deceived group, Fig. 3; Batesian discovered group, Fig. 4; see Movie 1 in supplementary material, for an example

of this behaviour). By night 5 of the model's presentation minimum distances had significantly increased (compared with night 1) for the Müllerian and Batesian deceived groups (repeated-measure ANOVAs followed by paired *t*-tests, Tables 1 and 2; see Movie 2 in supplementary material, for an example of this behaviour). Upon introduction of the mimic on night 6, minimum bat–moth distances decreased significantly (compared with night 5; Movie 1 in supplementary material) for these two groups and by night 10 (contrasted with night 6) distances had increased again (Tables 1 and 2; Movie 2 in supplementary material). The Batesian discovered group showed a similar pattern except for extremely small minimum distances on night 10, but a repeated-measure ANOVA revealed no statistical differences among nights (Table 1).

Nearly the same configuration of response was seen in T_{veer}, which we define as the time from minimum bat–moth distance that the angle (φ) between the bat's velocity vector and the bat–moth vector exceeds 45 deg. and does not return below that value. On night 1, T_{veer} occurred shortly before the time of minimum distance for all three bat groups (Table 1; Müllerian group, Fig. 2; Batesian deceived, Fig. 3; Batesian discovered, Fig. 4; Movie 1 in supplementary material). Comparing night 1 to the last night of the model's presentation on night 5, T_{veer} increased significantly for the Müllerian and Batesian deceived
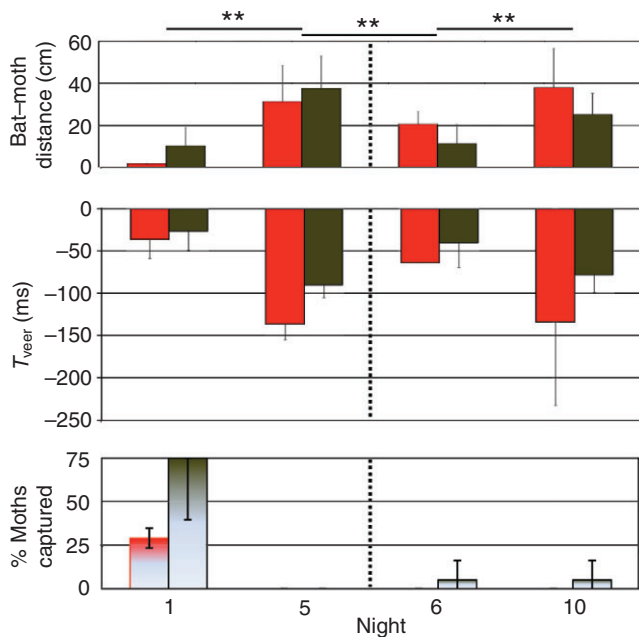

Fig. 2. 3-D kinematics of avoidance behaviour: Müllerian group. Three behaviours are plotted on the *y*-axis against experimental nights 1, 5, 6 and 10 on the *x*-axis. The top graph shows minimum bat–moth distance. The middle graph, T_{veer} (see Fig. 1). T_{veer} values are negative as they are anchored to the time of minimum bat–moth distance and this temporal parameter consistently occurred before the bats reached their closest 3-D position to the moth. The bottom graph shows the mean percentage of tiger moths captured; depicted with fade-coloured bars (for details, see Barber and Conner, 2007). The vertical dashed line between nights 5 and 6 indicates the change from the model tiger moth to the mimic. Lines with asterisks indicate statistical significance for the comparison (nights) they unite. In each pair the first asterisk indicates significance for minimum bat–moth distance and the second asterisk for T_{veer}. Bat species were combined for statistical analysis within bat groups (see Materials and methods) but are presented separately here. Data from red bats (*Lasiurus borealis*, N=2) are in red and those from big brown bats (*Eptesicus fuscus*, N=5) are in brown. Data are means ± s.d.
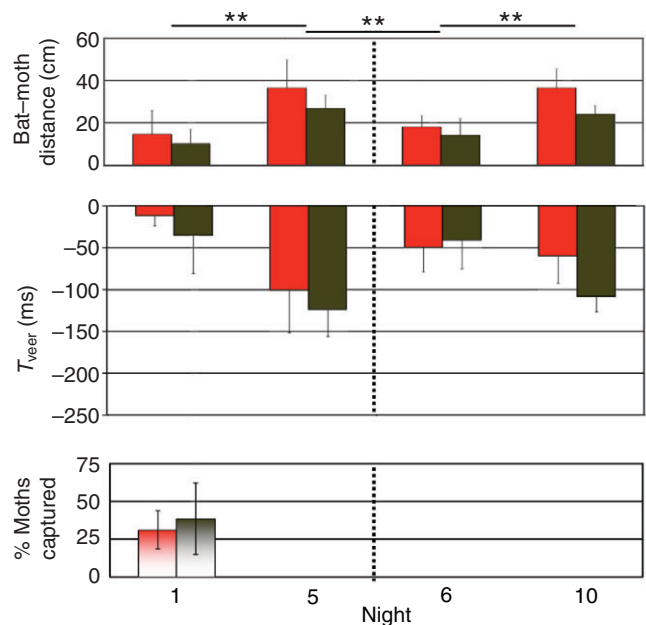

Fig. 3. 3-D kinematics of avoidance behaviour: Batesian deceived group. See Fig. 2 legend for details. Red bats (*Lasiurus borealis*, N=4) are in red and big brown bats (*Eptesicus fuscus*, N=3) are in brown. Data are means ± s.d.

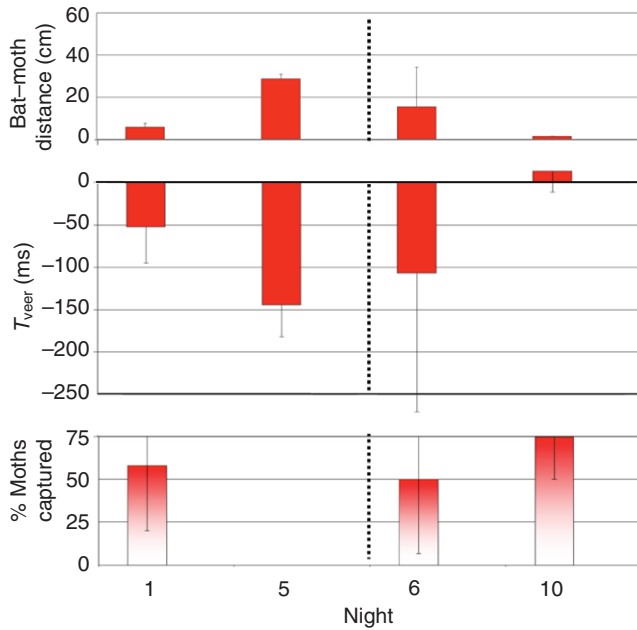

Fig. 4. 3-D kinematics of avoidance behaviour: Batesian discovered group. See Fig. 2 legend for details. No comparisons were significant for this group (*Lasiurus borealis*, N=3). Data are means ± s.d.

increased again (contrasting night 6 and 10; Tables 1 and 2; see Movie 2 in supplementary material). T_{veer} did not change significantly across nights for the Batesian discovered group (Table 1).

The elapsed time from first tiger moth click to T_{veer} did not change for any of the three bat groups (repeated-measure ANOVA; Table 1). There were also no significant changes in bat flight speed or closing rate for any of the groups across experimental nights (repeated-measure ANOVAs; Table 1). The red bats did achieve the highest flight speeds measured (5.5 ms^{-1}) but the overall averages for both reds (2.5±0.9 ms^{-1}) and browns (2.3±0.5 ms^{-1}) were very similar.

Bioacoustics

Although the bats approximately doubled the echolocation call period (i.e. interpulse interval in milliseconds) that contained the first tiger moth clicks compared with the period just prior to these moth clicks in all conditions, this parameter (period change) did not vary across the course of the experiment for any of the three bat groups (repeated-measure ANOVA; Table 1; see Fig. 5). A comparison of the percentage change in spectral bandwidth between the biosonar pulse just prior to moth clicks and the next biosonar cry (i.e. the first call after the initial tiger moth clicks) did not change and showed no significant differences across foraging nights for any of the three bat groups (repeated-measure ANOVA, bandwidth change; Table 1).

Friedman's ANOVAs of the percentage of bat–tiger moth interactions that contained a terminal buzz revealed statistical differences across nights for all three bat groups (Table 1, Fig. 6). Planned Wilcoxon's comparisons between nights 1 and 5 revealed significantly less buzzing on the last night of the model's presentation for the Müllerian and Batesian deceived groups (Table 3, Fig. 6). No difference was found comparing nights 5 and 6 (when the mimic was introduced) or nights 6 and 10 (last night of the mimic's presentation)
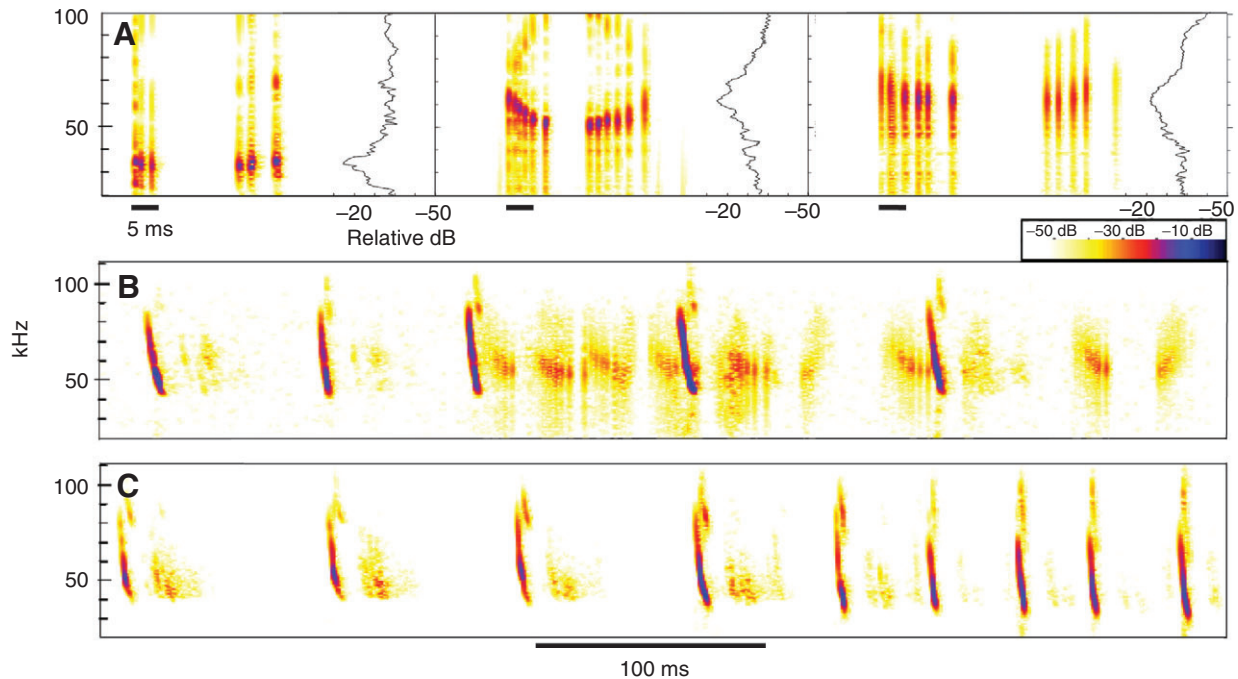
groups (repeated-measure ANOVAs followed by paired *t*-tests; Tables 1 and 2; Movie 2 in supplementary material). Introduction of the mimic resulted in a significantly shorter T_{veer} (comparing night 5 with night 6; see Movie 1 in supplementary material) for these two groups and by the final night of the mimic's presentation, T_{veer}

Fig. 5. (A) Spectrograms (frequency in kHz × time) and power spectra (frequency in kHz × relative intensity in decibels) of the ultrasonic warning sounds of the tiger moths *Syntomeida epilais*, *Cycnia tenera* and *Euchaetes egle* (left to right). (B) A spectrogram of a *C. tenera* tiger moth responding to a red bat (*Lasiurus borealis*) echolocation attack. The moth begins to click just after the third biosonar cry. (C) A spectrogram of a *L. borealis* attacking a silent, palatable, control noctuid moth presented for comparison purposes. Notice how the two echolocation attack sequences are in similar stages initially, based on interpulse interval but in (B), after the tiger moth's ultrasonic response, the bat's interpulse intervals elongate.

Table 3. Wilcoxon pairwise comparisons of the percentage of bat–tiger moth interactions that contained a terminal buzz, for all three bat groups

Nights	Müllerian		Batesian deceived		Batesian discovered	
	P-value	Adj. P (Q)	P-value	Adj. P (Q)	P-value	Adj P (Q)
1–5	0.02	**0.05**	0.03	**0.05**	0.11	0.19
5–6	0.20	0.26	0.41	0.41	0.11	0.19
6–10	0.89	0.89	0.10	0.14	1.00	1.00
10–11	0.03	**0.05**	0.01	**0.05**	0.11	0.19
12–13	N/A	N/A	N/A	N/A	0.18	0.23

Adj. P (Q), false discovery rate adjusted P-value; Nights, pairwise foraging night comparisons; N/A, not applicable.
Statistically significant comparisons are in bold type.

for any of the bat groups (Table 3). On night 11, for the Müllerian and Batesian deceived groups, the mimic's sound-producing structures were removed to examine the role of the moth-generated sounds in these avoidance behaviours (for details, see Barber and Conner, 2007). A comparison between night 10 and 11 for these groups showed that silencing the moths significantly increased the percentage of terminal buzzes (Table 3, Fig. 6). For the Batesian discovered group, on night 11, the model was reintroduced and whereas the percentage of buzzing decreased, no statistical differences were found; nor were differences revealed on night 13 (compared with night 12) when the model's sound-producing structures were removed (Table 3, Fig. 6) (for details, see Barber and Conner, 2007). An examination of terminal buzz duration showed no significant changes across the experiments for any of the bat groups (Table 1).

DISCUSSION
3-D kinematics of avoidance behaviour
After the first few captures of a noxious, model tiger moth our sample of naïve bats began to avoid these sound-producing arctiids. The Müllerian and Batesian deceived groups' first avoidances resulted in minimum bat–moth distances of approx. 10 cm and our measure of when the bats broke off their pursuit (T_{veer}; Fig. 1) was small (approx. –20 ms). Following 5 days of experience with the model these parameters increased markedly. Bat–moth minimum distances increased by over 25 cm to ~35 cm and T_{veer} increased by almost 100 ms to ~115 ms. This same kinematic pattern was seen in the Batesian discovered group but the differences were not statistically significant. These kinematic signatures mirror the capture data (Barber and Conner, 2007), confirming that with experience naïve bats learn to avoid sound-producing tiger moths
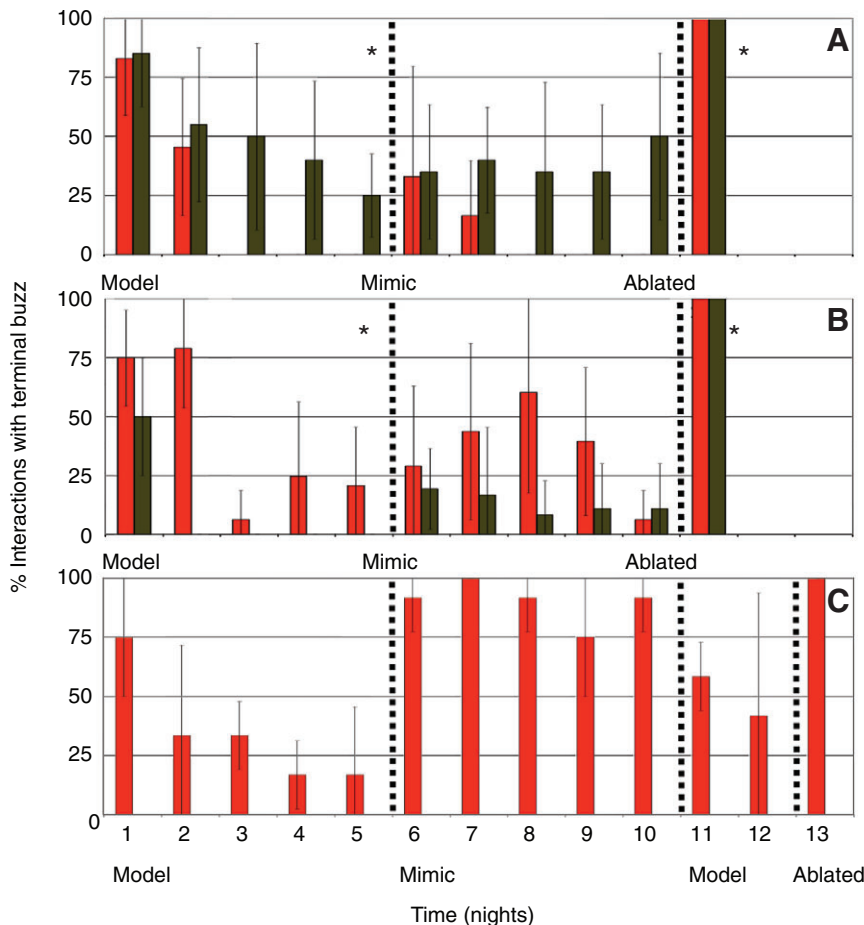

Fig. 6. Percentage of bat–tiger moth interactions with a terminal buzz. (A) Müllerian group (*E. fuscus*, N=5; *L. borealis*, N=2); (B) Batesian deceived group; (*E. fuscus*, N=3; *L. borealis*, N=4); (C) Batesian discovered group (*L. borealis*, N=3). Vertical dashed lines indicate when the tiger moth species presented to the bats was changed or when the moths' sound-producing structures were ablated, which is also noted in text below the x-axis in each panel. Asterisks above night 5 indicate a significant difference between night 1 and 5 and those above night 11 denote significance between nights 10 and 11. Bat species were combined for statistical analysis but are presented separately here. Data for red bats are shown in red, those for big brown bats, in brown. Data are means ± s.d.

that pair their warning with a chemical defence (Hristov and Conner, 2005a).

These same two kinematic measures also suggest that our sample of naïve bats, while generalizing the aposematic meaning of moth clicks across two different arctiid species (Barber and Conner, 2007), discriminate the signals as acoustically different. First, on night 6, upon introduction of the mimic both the Müllerian and Batesian deceived group of bats flew almost 20 cm closer to the mimic than they did to the model on the previous evening. These bats' minimum distances to the newly introduced mimic (approx. 15 cm) were very similar to the small minimum distances the bats displayed during their first avoidances of tiger moths on night 1. Second, when the mimic was presented, bats in the Müllerian and Batesian deceived group decreased the time period when they broke off their attack (T_{veer}) by almost 70 ms when compared with their behaviour with the model on the night before. As with minimum bat–moth distance, these short T_{veer} values (approx. –45 ms) were comparable to T_{veer} during their first avoidances. The Batesian discovered group did not show a statistically significant decrease in minimum distance or T_{veer} after introduction of the mimic, during the few avoidance flights they exhibited. Limitations imposed by the large variance in kinematic parameters and the small sample size are conceivably responsible for this negative finding. Regardless, this group of bats exposed the Batesian mimic's palatability.

All the bats in these experiments showed greater interest in the mimic than they did to their last exposure of the model. Minimum distance and T_{veer} appear to be tapping into the same kinematic manifestation of decision making in these bats; an earlier T_{veer} results in a greater minimum bat–moth distance. However, these two parameters were only moderately correlated (0.5–0.6, Spearman's ρ) on two of the comparison nights (nights 6 and 10). Regardless, these obviously related kinematic variables are likely reflections of the bat's interest in the target constrained by both inherent biomechanical flight limitations (e.g. Norberg and Rayner, 1987) and experimental design restrictions (i.e. pursuing a tethered moth in the corner of a 'small' flight room). These constraints perhaps also explain why no differences across groups or conditions were found in either overall bat flight speed or in the rate that the bats decreased their distance to the moth, which could theoretically provide different information than flight speed, by incorporating the bat's trajectory towards the moth.

Minimum distances and T_{veer} increased again for the Müllerian and Batesian deceived groups to almost 30 cm and –85 ms by the last night of the bats' exposure to the mimic. Thus, demonstrating that the investigatory behaviour exhibited either convinced the bats or did not dissuade them from inferring that the new moth was sending the same message as the model.

An alternative explanation of these kinematic patterns is that the tiger moths simply clicked at different times to the bat attacks on different experimental nights and this was the primary impetus behind the avoidance behaviour. However, the relationship between when the tiger moths first clicked and when the bats broke off their pursuit (T_{veer}) did not change over the course of the experiments. The tiger moths consistently clicked approximately 2 s before T_{veer}, when the bats were well outside of the calibrated interaction space, probably reflecting the common response threshold that arctiids appear to share (Barber and Conner, 2006).

Bioacoustics

Although the bats approximately doubled the biosonar pulse period between the echolocation calls that contained the first tiger moth clicks and the period just prior to these moth clicks, this relative measure did not change across experimental conditions for our sample of bats. This temporal change has been noted before in adult *Myotis septentrionalis* interacting with tethered tiger moths (Ratcliffe and Fullard, 2005). This change could be interpreted as support for jamming, as evidence for increasing listening time between biosonar echoes (Barber et al., 2003), or synthetically, as a combination response, where inherently negative (e.g. disruptive) tiger moth clicks are more easily associated with a negative consequence, thus eliciting increased listening behaviour (*sensu* Ratcliffe and Fullard, 2005). It is important to note that this increase in pulse period was seen both in attacks that contained a terminal buzz and those that did not. In other words, the increased pulse interval was, often, more than just a reflection of an aborted echolocation attack sequence.

The lack of change in biosonar pulse bandwidth across this same interval (i.e. before and after the first tiger moth click) provides evidence against a direct jamming interpretation of this temporal finding. Theoretically, changing bandwidth is one method that a sonar receiver could use to circumvent jamming (Simmons and Stein, 1980). Recently, *Tadarida brasiliensis* has been shown to shift its search call frequencies upward when dynamically challenged with the playback of a conspecific echolocation sequence (Gillam et al., 2007) experimentally corroborating previous correlational field studies (Orbist, 1995; Surlykke and Moss, 2000; Ratcliffe et al., 2004; Ulanovsky et al., 2004). The tiger moths used in these experiments and an assemblage of tropical tiger moths (Barber and Conner, 2006) respond during the end of the approach phase of the echolocation attack, when the spectral bandwidth of the biosonar cries has reached a near maximum (Simmons et al., 1978; Schnitzler and Kalko, 2001). Thus, the lack of change in bandwidth reported may represent a constraint of echolocation attack behaviour.

In the bat groups deceived by the mimic, the percentage of bat–tiger moth interactions that contained a terminal buzz significantly decreased over the course of aposematic learning. Although introduction of the mimic increased the percentage of terminal buzzes in all three bat groups, these differences were not statistically significant. The percentage of terminal buzzes significantly increased again for the deceived groups when the sound-producing structures were removed from the mimetic tiger moths, and the bats subsequently caught almost all of these silenced moths. It seems that the percentage of interactions with terminal buzzes is directly related to both capture and investigatory behaviour (i.e. to precisely localizing where the moth is in 3-D space). However, the duration of the terminal buzz did not change across the experiments for any of the bat groups, suggesting that terminal buzz duration may be a hard-wired attribute of echolocation behaviour (Wilson and Moss, 2004).

Conclusions

The increase in percentage of terminal buzzes upon introduction of the mimic, although not statistically significant, highlights an apparent discordance between the kinematic data presented here and the capture data presented earlier (Barber and Conner, 2007). When the sound-producing organs are removed from tiger moths, they are captured by bats. We have interpreted this result to mean that it is the prey-generated sounds that are driving the mimicry, not olfactory cues, the wingbeat frequency of the moths or other information obtained from the echolocation stream. If this is true, why do the bats decrease their minimum distance and break off their attack later to the newly introduced acoustic mimic compared with their last experience with the model? There are at least three possibilities. First, the bat's behaviour could be attributed solely to acquiring more information about the different prey-generated sounds the mimic is producing. Second, perhaps the bats are gathering information from

chemical cues and the echolocation stream but their use of those cues is synergistically dependent on the tiger moth sounds. In other words, the bats do not extract the necessary discriminatory information from these other modalities unless cued to do so. The third, and related possibility, is that these lab-raised naïve bats are still learning to use other cues and that perhaps with experience (or teaching from adult bats) they would employ such sensory abilities.

Regardless of these caveats, the data presented here clearly show that while bats generalize the aposematic meaning of tiger moth sounds (Barber and Conner, 2007) they discriminate the sounds as different and investigate. That our sample of bats can make this acoustic discrimination is not surprising given the remarkable spectral (Roverud, 1999; Stebut and Schmidt, 2001) and temporal (Simmons et al., 1990; Masters et al., 1997) resolution that bats display in laboratory paradigms. Many questions remain to be answered concerning how bats categorize insect warning sounds. The naïve bats used in these experiments generalized the meaning of tiger moth sounds across calls that varied markedly in temporal and spectral characteristics (see Fig. 5) [for species' averages see Conner (Conner, 1999) and Fullard and Fenton (Fullard and Fenton, 1977)]. However, the discrimination behaviour these bats displayed suggests that mimics may be under strong selection to adhere to the signal constraints of their model.

REFERENCES

Abdel-Aziz, Y. I. and Karara, H. M. (1971). *Direct Linear Transformation From Comparator Coordinates into Object Space Coordinates in Close-Range Photogrammetry: Proceedings of the Symposium on Close-Range Photogrammetry*, pp. 1-18. Falls Church, VA: American Society of Photogrammetry.

Barber, J. R. and Conner, W. E. (2006). Tiger moth responses to a simulated bat attack: timing and duty cycle. *J. Exp. Biol.* **209**, 2637-2650.

Barber, J. R. and Conner, W. E. (2007). Acoustic mimicry in a predator-prey interaction. *Proc. Natl. Acad. Sci. USA* **104**, 9331-9334.

Barber, J. R., Razak, K. A. and Fuzessery, Z. M. (2003). Can two streams of auditory information be processed simultaneously? Evidence from the gleaning bat *Antrozous pallidus. J. Comp. Physiol. A* **189**, 843-855.

Bates, D. L. and Fenton, M. B. (1990). Aposematism or startle? Predators learn their responses to the defenses of prey. *Can. J. Zool.* **68**, 49-52.

Benjamini, Y. and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Series B Stat. Methadol.* **57**, 289-300.

Chen, L., Armstrong, C. W. and Raftopoulos, D. D. (1994). An investigation on the accuracy of three-dimensional space reconstruction using the direct linear transformation technique. *J. Biomech.* **27**, 493-500.

Conner, W. E. (1999). 'Un chant d' appel amoureux' acoustic communication in moths. *J. Exp. Biol.* **202**, 1711-1723.

Fullard, J. H. and Fenton, M. B. (1977). Acoustic and behavioural analyses of the sounds produced by some species of Nearctic Arctiidae (Lepidoptera). *Can. J. Zool.* **55**, 1213-1224.

Gillam, E. H., Ulanovsky, N. and McCracken, G. F. (2007). Rapid jamming avoidance in biosonar. *Proc. Biol. Sci.* **274**, 651-660.

Hedrick, T. L. (2008). Software techniques for two- and three-dimensional kinematic measurements of biological and biomimetic systems. *Bioinspir. Biomim.* **3**, 034001.

Hristov, I. H. and Conner, W. E. (2005a). Sound strategy: acoustic aposematism in the bat-tiger moth arms race. *Naturwissenschaften* **92**, 164-169.

Hristov, I. H. and Conner, W. E. (2005b). Effectiveness of tiger moth (Lepidoptera, Arctiidae) chemical defenses against an insectivorous bat (*Eptesicus fuscus*). *Chemoecol.* **15**, 105-113.

Lanczos, C. (1956). *Applied Analysis.* London: Isaac Pitman (reprinted 1988. New York: Dover).

Masters, W. M. and Raver, K. A. (1996). The degradation of distance discrimination in big brown bats (*Eptesicus fuscus*) caused by different interference signals. *J. Comp. Physiol. A* **179**, 703-713.

Masters, W. M., Raver, K. A. S., Kornacker, K. and Burnett, S. C. (1997). Detection of jitter in intertarget spacing by the big brown bat *Eptesicus fuscus. J. Comp. Physiol. A* **181**, 279-290.

Miller, L. A. (1991). Arctiid moth clicks can degrade the accuracy of range difference discrimination in echolocating big brown bats, *Eptesicus fuscus. J. Comp. Physiol. A* **168**, 571-579.

Miller, L. A. and Surlykke, A. (2001). How some insects detect and avoid being eaten by bats: tactics and countertactics of prey and predator. *Bioscience* **51**, 570-581.

Norberg, U. M. and Rayner, J. M. (1987). Ecological morphology and flight in bats (Mammalia: Chiroptera): wing adaptations, flight performance, foraging strategy and echolocation. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **316**, 335-427.

Obrist, M. K. (1995). Flexible bat echolocation: the influence of individual, habitat and conspecifics on sonar signal design. *Behav. Ecol. Sociobiol.* **36**, 207-219.

Ratcliffe, J. M. and Fullard, J. H. (2005). The adaptive function of tiger moth clicks against echolocating bats: an experimental and synthetic approach. *J. Exp. Biol.* **208**, 4689-4698.

Ratcliffe, J. M. and Nydam, M. L. (2008). Multimodal warning signals for a multiple predator world. *Nature* **455**, 96-99.

Ratcliffe, J. M., ter Hofstede, H. M., Avila-Flores, R., Fenton, M. B., McCracken, G. F., Biscardi, S., Blasko, J., Gillam, E., Orprecio, J. and Spanjer, G. (2004). Conspecifics influence call design in the Brazilian free-tailed bat, *Tadarida brasiliensis. Can. J. Zool.* **82**, 966-971.

Roverud, R. (1999). Frequency discrimination of brief tonal steps as a function of frequency in the lesser bulldog bat. *J. Comp. Physiol. A* **185**, 247-253.

Schnitzler, H. U. and Kalko, E. K. V. (2001). Echolocation by insect-eating bats. *BioScience* **51**, 557-569.

Simmons, J. A. and Stein, R. A. (1980). Acoustic imaging in bat sonar: echolocation signals and the evolution of echolocation. *J. Comp. Physiol. A* **65**, 61-84.

Simmons, J. A., Lavender, W. A., Lavender, B. A., Childs, J. E., Hulebak, K., Rigden, M. R., Sherman, J., Woolman, B. and O'Farrell, M. J. (1978). Echolocation by free-tailed bats (*Tadarida*). *J. Comp. Physiol. A* **125**, 291-299.

Simmons, J. A., Ferragamo, M., Moss, C. F., Stevenson, S. B. and Altes, R. A. (1990). Discrimination of jittered sonar echoes by the echolocating bat, *Eptesicus fuscus. J. Acoust. Soc. A* **167**, 589-616.

Stebut, B. and Schmidt, S. (2001). Frequency discrimination threshold at search call frequencies in the echolocating bat, *Eptesicus fuscus. J. Comp. Physiol. A* **187**, 287-291.

Storey, J. D., Taylor, J. E. and Siegmund, D. (2004). Strong control, conservative point estimation, and simultaneous conservative consistency of false discovery rates: A unified approach. *J. R. Stat. Soc. Series B Stat. Methodol.* **66**, 187-205.

Surlykke, A. and Moss, C. F. (2000). Echolocation behaviour of big brown bats, *Eptesicus fuscus*, in the field and the laboratory. *J. Acoust. Soc. Am.* **108**, 2419-2429.

Tougaard, J., Cassedy, J. H. and Covey, E. (1998). Arctiid moths and bat echolocation: broad-band clicks interfere with neural responses to auditory stimuli in the nuclei of the lateral lemniscus of the big brown bat. *J. Comp. Physiol. A* **182**, 203-215.

Tougaard, J., Miller, L. A. and Simmons, J. A. (2004). The role of arctiid moth clicks in defense against echolocating bats: interference with temporal processing. In *Advances in the Study of Echolocation in Bats and Dolphins* (ed. J. A. Thomas, C. F. Moss and M. Vater), pp. 365-371. Chicago, IL: University of Chicago Press.

Ulanovsky, N., Fenton, M. B., Tsoar, A. and Korine, C. (2004). Dynamics of jamming avoidance in echolocating bats. *Proc. Biol. Sci.* **271**, 1467-1475.

Walker, J. A. (1998). Estimating velocities and accelerations of animal locomotion: a simulation experiment comparing numerical differentiation algorithms. *J. Exp. Biol.* **201**, 981-995.

Wilson, W. W. and Moss, C. F. (2004). Sensory-motor behaviour of freeflying FM bats during target capture. In *Advances in the Study of Echolocation in Bats and Dolphins* (ed. J. A. Thomas, C. F. Moss and M. Vater), pp. 22-27. Chicago, IL: Chicago University Press.