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

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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.

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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 (Lasiusus 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 (Linnæus)], in an anechoic foam-lined indoor flight facility (5.8 m x 4.0 m x 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 m². 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 x 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 Cyriace tenera Hübner or Syntomoida 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 x 160 cm x 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 φ=0 deg., the bat is flying directly at the moth and if φ=180 deg., the bat is flying directly away from the moth. Time of veer (Tveer) 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 x 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
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 mimic 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, λ=0)

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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Bat group</th>
<th>F</th>
<th>d.f.</th>
<th>P-value</th>
<th>Adj. P (Q)</th>
<th>Night 1*</th>
<th>Night 5*</th>
<th>Night 6*</th>
<th>Night 10*</th>
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</thead>
<tbody>
<tr>
<td>Minimum bat–moth distance (cm)</td>
<td>Müllerian</td>
<td>8.32</td>
<td>3,18</td>
<td>0.01</td>
<td>0.02</td>
<td>7.6±8.6</td>
<td>35.8±14.6</td>
<td>14.0±9.1</td>
<td>28.9±12.8</td>
</tr>
<tr>
<td></td>
<td>Batesian deceived</td>
<td>19.72</td>
<td>3,18</td>
<td>0.001</td>
<td>0.003</td>
<td>10.9±9.8</td>
<td>32.6±11.4</td>
<td>16.4±6.3</td>
<td>27.4±7.0</td>
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<tr>
<td></td>
<td>Batesian discovered</td>
<td>4.77</td>
<td>3,6</td>
<td>0.05</td>
<td>0.07</td>
<td>5.9±1.9</td>
<td>28.7±2.1</td>
<td>15.5±18.8</td>
<td>1.4±0.09</td>
</tr>
<tr>
<td>Tveer (ms)</td>
<td>Müllerian</td>
<td>13.82</td>
<td>3,18</td>
<td>0.01</td>
<td>0.02</td>
<td>28.9±21.8</td>
<td>103.8±27.2</td>
<td>49.6±27.6</td>
<td>94.0±51.7</td>
</tr>
<tr>
<td></td>
<td>Batesian deceived</td>
<td>16.22</td>
<td>3,18</td>
<td>0.03</td>
<td>0.05</td>
<td>14.1±3.0</td>
<td>123.7±36.2</td>
<td>46.9±29.0</td>
<td>74.4±25.9</td>
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<td></td>
<td>Batesian discovered</td>
<td>1.71</td>
<td>3,6</td>
<td>0.22</td>
<td>0.22</td>
<td>52.0±4.3</td>
<td>144.0±38.2</td>
<td>106.7±16.0</td>
<td>13.8±24.4</td>
</tr>
<tr>
<td>Bat flight speed (m s⁻¹)</td>
<td>Müllerian</td>
<td>0.68</td>
<td>3,18</td>
<td>0.47</td>
<td>0.30</td>
<td>2.0±0.7</td>
<td>2.2±0.5</td>
<td>2.2±0.7</td>
<td>2.5±0.8</td>
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<tr>
<td></td>
<td>Batesian deceived</td>
<td>1.7</td>
<td>3,18</td>
<td>0.37</td>
<td>0.26</td>
<td>2.8±0.4</td>
<td>2.6±0.6</td>
<td>2.3±0.5</td>
<td>3.0±1.1</td>
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<tr>
<td></td>
<td>Batesian discovered</td>
<td>0.19</td>
<td>3,6</td>
<td>0.40</td>
<td>0.40</td>
<td>2.1±0.8</td>
<td>2.6±0.6</td>
<td>2.3±1.0</td>
<td>2.3±1.0</td>
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<tr>
<td>Closing rate (m s⁻¹)</td>
<td>Müllerian</td>
<td>0.66</td>
<td>3,18</td>
<td>0.73</td>
<td>0.37</td>
<td>0.8±0.7</td>
<td>0.6±0.4</td>
<td>1.0±0.5</td>
<td>0.7±0.3</td>
</tr>
<tr>
<td></td>
<td>Batesian deceived</td>
<td>1.34</td>
<td>3,18</td>
<td>0.35</td>
<td>0.26</td>
<td>1.0±0.5</td>
<td>0.9±0.7</td>
<td>1.6±0.6</td>
<td>1.4±1.2</td>
</tr>
<tr>
<td></td>
<td>Batesian discovered</td>
<td>0.30</td>
<td>3,6</td>
<td>0.82</td>
<td>0.39</td>
<td>0.9±0.7</td>
<td>1.2±1.0</td>
<td>1.2±1.1</td>
<td>1.5±1.0</td>
</tr>
<tr>
<td>Time from first click to Tveer (ms)</td>
<td>Müllerian</td>
<td>0.46</td>
<td>3,18</td>
<td>0.67</td>
<td>0.37</td>
<td>2150±400</td>
<td>2025±619</td>
<td>2094±620</td>
<td>1866±700</td>
</tr>
<tr>
<td></td>
<td>Batesian deceived</td>
<td>0.33</td>
<td>3,18</td>
<td>0.13</td>
<td>0.15</td>
<td>1619±946</td>
<td>2035±716</td>
<td>1443±708</td>
<td>1898±546</td>
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<tr>
<td></td>
<td>Batesian discovered</td>
<td>1.48</td>
<td>3,6</td>
<td>0.41</td>
<td>0.28</td>
<td>1856±213</td>
<td>2508±1313</td>
<td>2811±1128</td>
<td>2320±198</td>
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<tr>
<td>Period change (%)</td>
<td>Müllerian</td>
<td>0.23</td>
<td>3,18</td>
<td>0.85</td>
<td>0.39</td>
<td>262±149</td>
<td>243±203</td>
<td>199±103</td>
<td>214±111</td>
</tr>
<tr>
<td></td>
<td>Batesian deceived</td>
<td>2.33</td>
<td>3,18</td>
<td>0.16</td>
<td>0.17</td>
<td>153±45</td>
<td>137±18</td>
<td>174±39</td>
<td>175±22</td>
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<tr>
<td></td>
<td>Batesian discovered</td>
<td>0.77</td>
<td>3,6</td>
<td>0.55</td>
<td>0.34</td>
<td>181±24</td>
<td>291±281</td>
<td>165±42</td>
<td>122±46</td>
</tr>
<tr>
<td>Bandwidth change (%)</td>
<td>Müllerian</td>
<td>2.21</td>
<td>3,18</td>
<td>0.12</td>
<td>0.15</td>
<td>142.3±8.9</td>
<td>278±7.1</td>
<td>21.5±11</td>
<td>2.6±3.7</td>
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<tr>
<td></td>
<td>Batesian deceived</td>
<td>1.18</td>
<td>3,18</td>
<td>0.58</td>
<td>0.34</td>
<td>6.2±3.9</td>
<td>7.2±8.1</td>
<td>2.1±5.1</td>
<td>0.1±4.5</td>
</tr>
<tr>
<td></td>
<td>Batesian discovered</td>
<td>0.46</td>
<td>3,6</td>
<td>0.72</td>
<td>0.37</td>
<td>0.7±3.1</td>
<td>0.7±4.7</td>
<td>2.1±11.4</td>
<td>2.6±3.7</td>
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<tr>
<td>% Interactions with terminal buzz</td>
<td>Müllerian</td>
<td>χ²=32.94</td>
<td>10</td>
<td>0.0001</td>
<td>0.001</td>
<td>85.4±20.8</td>
<td>17.9±18.9</td>
<td>34.4±30.1</td>
<td>35.7±37.7</td>
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<tr>
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<td>Batesian deceived</td>
<td>χ²=36.50</td>
<td>10</td>
<td>0.0001</td>
<td>0.001</td>
<td>64.3±24.4</td>
<td>11.9±20.9</td>
<td>24.9±26.5</td>
<td>8.3±14.4</td>
</tr>
<tr>
<td></td>
<td>Batesian discovered</td>
<td>χ²=28.13</td>
<td>12</td>
<td>0.005</td>
<td>0.01</td>
<td>75.0±25.0</td>
<td>16.7±28.9</td>
<td>91.7±14.4</td>
<td>91.7±14.4</td>
</tr>
<tr>
<td>Terminal buzz duration (ms)</td>
<td>Müllerian</td>
<td>0.26</td>
<td>4,8</td>
<td>0.26</td>
<td>0.29</td>
<td>260±51</td>
<td>228±90</td>
<td>219±91</td>
<td>258±33</td>
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<tr>
<td></td>
<td>Batesian deceived</td>
<td>0.42</td>
<td>4,8</td>
<td>0.79</td>
<td>0.39</td>
<td>197±72</td>
<td>133±80</td>
<td>166±92</td>
<td>164±87</td>
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<tr>
<td></td>
<td>Batesian discovered</td>
<td>2.26</td>
<td>4,4</td>
<td>0.23</td>
<td>0.22</td>
<td>180±60</td>
<td>192±11</td>
<td>162±62</td>
<td>150±20</td>
</tr>
</tbody>
</table>

d.f., degrees of freedom; Adj. P (Q), false discovery rate adjusted P-value; Tveer, time of veer.

*Values are mean ± s.d. Statistically significant analyses are in bold type.

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**Fig. 1. Illustration of kinematic parameters.** Tveer 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.
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 $T_{\text{veer}}$, which we define as the time from minimum bat–moth distance that the angle ($\phi$) between the bat’s velocity vector and the bat–moth vector exceeds 45 deg. and does not return below that value. On night 1, $T_{\text{veer}}$ 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, $T_{\text{veer}}$ increased significantly 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). 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).

Statistically significant comparisons 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

<table>
<thead>
<tr>
<th>Nights</th>
<th>P-value</th>
<th>Adj. P (Q)</th>
<th>P-value</th>
<th>Adj. P (Q)</th>
<th>P-value</th>
<th>Adj. P (Q)</th>
<th>P-value</th>
<th>Adj. P (Q)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Müllerian</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1–5</td>
<td>0.001</td>
<td>0.003</td>
<td>0.001</td>
<td>0.002</td>
<td>0.0001</td>
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$T_{\text{veer}}$, time of veer; Adj. P (Q), false discovery rate adjusted P-value; Nights, pairwise foraging night comparisons.
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 \( T_{\text{veer}} \) (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, \( T_{\text{veer}} \) increased again (contrast night 6 and 10; Tables 1 and 2; see Movie 2 in supplementary material). \( T_{\text{veer}} \) did not change significantly across nights for the Batesian discovered group (Table 1).

The elapsed time from first tiger moth click to \( T_{\text{veer}} \) 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)
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

<table>
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<th>Nights</th>
<th>Müllerian P-value</th>
<th>Müllerian Adj. P (Q)</th>
<th>Batesian deceived P-value</th>
<th>Batesian deceived Adj. P (Q)</th>
<th>Batesian discovered P-value</th>
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<td>N/A</td>
<td>N/A</td>
<td>0.18</td>
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</table>

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.

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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 $\rho$) 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; Suryk 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

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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.

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