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RESEARCH ARTICLE

Hearing in caterpillars of the monarch butterfly (*Danaus plexippus*)

Chantel J. Taylor and Jayne E. Yack*

ABSTRACT

Many species of caterpillars have been reported to respond to sound, but there has been limited formal study of what sounds they hear, how they hear them and how they respond to them. Here, we report on hearing in caterpillars of the monarch butterfly (*Danaus plexippus*). Fourth and fifth instar caterpillars respond to sounds by freezing, contracting, and flicking their thorax in a vertical direction. Behavioural responses were evoked by sound frequencies between 50 and 900 Hz, with best sensitivity at 100–200 Hz. The lowest mean threshold was 79 dB SPL (particle velocity 605 μm s^{-1}) at 150 Hz. When presented with a repeated 200 Hz sound tone, caterpillars habituate by no longer responding. A series of ablation experiments confirmed that the primary sensory receptors are a pair of long hairs, called trichoid sensilla, located on the upper prothorax. These sensilla are ∼450 μm long, rest in a socket and are innervated by a single bipolar sensory neuron. Removal of these setae reduced responses significantly compared with controls. Other setae contributed minimally to hearing in response to 200 Hz tones, and tubercles and prothoracic shields played no apparent role in sound reception. We propose that hearing functions to prevent attacks by aerial insect predators and parasitoids, which produce flight sounds in the frequency range to which the caterpillars are sensitive. This research lays the foundation for further investigations on the function and evolution of hearing in caterpillars, and has significance for the conservation of threatened monarch butterfly larvae living near noisy urban environments and roadways.

KEY WORDS: Insect, Acoustic, Sound, Sensory, Lepidoptera, Trichoid sensilla

INTRODUCTION

Reports on caterpillar hearing date back more than two centuries (Bonnet, 1779, cited in Minnich, 1936). More than 30 species across diverse lepidopteran taxa have been noted to respond to a variety of sounds including the human voice, clapping, hovering insects, tuning forks, doors slamming, highway noise and jet aircraft (see Davis et al., 2018; Hogue, 1972; Johnson, 1893; Klots, 1969; Markl and Tautz, 1975; Minnich, 1925, 1936; Myers and Smith, 1978; Rothschild and Bergström, 1997; Tutt, 1893). Responses to these sounds can vary widely between species, and may include cessation of movement, contraction of the body, squirming, falling to the ground, flicking, waving the furcula and increasing heart rate. Despite the purported ubiquity of hearing in caterpillars (Minnich, 1936), little formal research has been undertaken to study the characteristics of sounds that induce a response, the hearing receptors or the functions of hearing. Detailed formal study of caterpillar hearing is limited to one species, the cabbage moth caterpillar, *Mamestra* (*Barathra*) *brassicae* (Lepidoptera: Noctuidae) (Markl and Tautz, 1975; Tautz, 1977, 1978, 1979). While comprehensively researched, hearing in *M. brassicae* is not representative of all other species, as behavioural responses to sound vary across species. It is likely that hearing in caterpillars has evolved more than once, and therefore it is important to identify hearing receptors and quantify sensitivity in other species. Our study investigated hearing in caterpillars of the renowned monarch butterfly, *Danaus plexippus* (Lepidoptera: Nymphalidae).

Monarch caterpillars have been previously reported to respond to sounds. Minnich (1936) noted larval *D. plexippus*' tendency to freeze, contract, and jerk their anterior ends in response to sounds produced by tuning forks. Rothschild and Bergström (1997) later revisited the phenomenon of monarch larval hearing, but focused primarily on volatile chemicals released by the larvae when agitated; however, they were unable to conclude that these volatiles were released in response to sound. Rothschild and Bergström (1997) noted that the caterpillars responded to aircraft passing overhead, and human shouting and 'buzzing' sounds, by making 'sudden ducking or twitching movements'. More recently, Davis et al. (2018) demonstrated that monarch caterpillars responded to sound playbacks of traffic noise by increasing their heart rate. They proposed that the effects of anthropogenic noise could have important implications for butterfly conservation. While these previous studies provide indirect support that monarch caterpillars react to airborne sounds, they lack formal testing on the acoustic stimuli required to evoke behaviours, the characteristics of sound-evoked behaviours and identification of the hearing organs. This study took an experimental approach to address these issues.

MATERIALS AND METHODS

Insects

Danaus plexippus (Linneaus 1758) were purchased as eggs or larvae from Gaïa Nature (Granby, QC, Canada), Monsieur Papillon (Chambly, QC, Canada) and Wish Upon a Butterfly (New Castle, PA, USA, permit no. P-2008-02240). Larvae were raised on potted milkweed, *Asclepias* spp., in a greenhouse at Carleton University, Ottawa, ON, Canada. Larvae used in experiments were in their fourth and fifth instars but third instars were also informally tested for their responses to sound, and in some cases used for morphology.

Sound playback, laser vibrometry and video recordings

Sound playbacks were conducted to assess and characterize: (i) behavioural responses to sound; (ii) sensitivity to different sound frequencies; (iii) responses to increasing sound levels; and (iv) responses to repeated sounds. Note that in this paper we use the word 'sound' to refer to airborne vibrations (both in the 'near' and 'far' field) and 'hearing' as the detection of airborne vibrations. We refer to vibrations through solids as 'vibrations' or 'solid-borne vibrations' (Yack, 2016). The nomenclature used to define acoustic stimuli is complex, and we refer the reader to Hill and Wessel (2016) or Windmill and Jackson (2016) for more detailed discussion of the

Department of Biology, Carleton University, Ottawa, ON, Canada K1S 5B6.

*Author for correspondence (jayneyack@cunet.carleton.ca)

J.E.Y., 0000-0001-9383-6437

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topic. The general set up for sound playback and video recording is described below, but specific details for each experiment are further explained under subsequent subheadings.

Individual larvae were tested for their response to sound while feeding, crawling or resting on a leaf of a host plant (*Asclepias* spp.). The potted plant was positioned on an antivibration table located in a sound chamber. Prior to any experiment, the larva was left undisturbed for a period of 15–30 min. A woofer (Sammi Sound, model CWR 200B50, range 24 Hz to 4 kHz; Samut Pakarn, Thailand) was placed 200–300 mm from the caterpillar on a separate structure. Pure tones (1–3 s duration, 25 ms rise/fall, linear ramp) between 100 and 1200 Hz were generated by a Tucker Davis Technologies digital signal processor (RX6 multifunction processor; Alachua, FL, USA) and shaped using a PC with Tucker Davis software (RPvdsEx, v.5.4). Sounds were recorded using an Earthworks (QTC40; Milford, NH, USA) condenser microphone placed adjacent to the caterpillar. The microphone output was connected to a Fostex Field Memory Recorder (FR-2; Tokyo, Japan), and the output from the data recorder was connected to the data acquisition system for the high-speed camera (see below).

The level of the sound (dB SPL, C-weighting) was calibrated for all frequencies using a Brüel & Kjær Type 2239A sound level meter (Naerum, Denmark), and best-fit lines were generated for each frequency using linear regression. Sound levels (dB SPL) played to the animal were calculated for each measured frequency and intensity combination using equations derived from the best-fit lines. Sounds were reported as pressure levels, as this is the traditional way to display tuning curves. However, these caterpillars are responding to airflow produced by the sounds, or so-called 'near-field' sounds (Barth, 2014; Tautz, 1979), similar to the cabbage moth (e.g. Markl and Tautz, 1975). Therefore, measurements in decibels were converted to approximate particle velocity (v) and particle displacement (ξ) in several steps. Particle velocity is proportional to sound pressure, but particle displacement is dependent on the frequency of the sound and the distance from the sound source (Beranek, 1954). First, the sound pressure p_{rms} in Pa was determined from the sound pressure level L_p, where the reference pressure p_{ref} is 20 µPa (0 dB SPL, human threshold of hearing) using the following equation:

$$p_{rms} = p_{ref} \cdot 10^{Lp/20}. \quad (1)$$

Particle velocity v and displacement ξ were calculated from the following equations:

$$v = p/(\rho \cdot c), \quad (2)$$

$$\xi_F = p/(\rho \cdot c \cdot 2 \cdot \pi \cdot f), \quad (3)$$

where p is the sound pressure obtained above; ρ is the density of air at 20°C (1.204 kg m^{-3}); c is the velocity of sound at 20°C (343.2 m s^{-1}); f is the frequency (Hz); r is the distance from the sound source (200 mm); λ is the wavelength of the sound that was measured; and γ is the phase angle between ξ and ξ_F. ξ was corrected by:

$$\xi = \xi_F/\cos(\gamma), \quad (4)$$

where:

$$\tan(\gamma) = \lambda/(2 \cdot \pi \cdot r), \quad (5)$$

because $r < \lambda$ for all frequencies tested (Tautz, 1979).

Behavioural responses to sound were recorded using a camcorder and, in some experiments, high-speed video. All trials were videotaped using a camcorder (Sony HDR-HC7 MiniDV or DCR-TRV140 Digital8 Handycam; Tokyo, Japan). Recordings began prior to the sound stimulus and continued until up to 10 s following the stimulus. Sounds were simultaneously recorded to the audio input of the camcorder with a microphone placed close to the caterpillar preparation. Videos were transferred to a computer for analysis, using either iMovie (v.6.0, Apple Inc., Cupertino, CA, USA) or KinoDV video editor (v.1.3, GNU General Public License). High-speed video recordings, in conjunction with sound and laser vibrometer recordings, were performed on a subset of animals. The sound playback set up was as described above, but the area was illuminated with a halogen light. A Lightning RDT (High Speed Imaging Inc., Markham, ON, Canada) camera captured 250 or 500 frames s^{-1}, using Xcitec MiDAS 2.0 software (Cambridge, MA, USA). Laser vibrometry was used to measure response latency by monitoring any movements of the caterpillar through leaf vibrations. An adhesive reflective disc (4 mm diameter) was placed directly on the leaf close to the caterpillar. Vibrations were recorded using a Polytec, PDV-100 portable digital vibrometer (Waldbronn, Germany). Data from the Earthworks microphone, laser vibrometer and high-speed video were digitized (PCI-6023E; National Instruments, Austin, TX, USA) to an A70 Toshiba Satellite (Tokyo, Japan) Notebook computer for further analysis with Xcitec MiDAS 2.0.

Responses to sound

Behaviour characteristics

Characteristics of behavioural responses to sound, including latency, duration, degree of movement and intervals between movements, were assessed using high-speed videography in conjunction with laser vibrometry and sound recordings. Flicks and contractions were analysed, but quantitative measurements on freezing were not included in the analysis, as freezing could only be assessed when the caterpillar was already moving. To characterize movements, a 200 Hz tone was selected based on tuning curve experiments (see Results). Contractions were measured for response latency, time to reach a contracted position and amount of displacement. Contraction latency was calculated by subtracting the time when the first movement of the caterpillar was detected with the laser vibrometer from the onset of the sound stimulus. Time to reach a contracted position was calculated from the laser and video frames. The amount of displacement was measured by taking body length measurements from the beginning to the end of the contraction. This was done by analysing frames before and during each contraction from the high-speed video in ImageJ (v.1.38; US National Institutes of Health, Bethesda, MD, USA). Flicks were measured for response latency, rise and fall time, and angle. Latency to the first flick following the onset of sound was calculated in the same manner as described above for contractions. The rise time of a flick was defined as the time it took the caterpillar to reach the peak position of a flick from the starting position, and the fall time was the amount of time it took to return to its starting position. Flick angle was measured as the angle between the portion of the caterpillar's body lifted from the substrate and that which was still attached, which usually meant that the fulcrum was located just anterior of the first pair of prolegs.

Mean, minimum, maximum and 95% confidence interval (CI) were calculated where possible, from contractions and flicks. Data were compared between individuals using unpaired t-tests, ANOVA or linear regression; paired t-tests were used to compare differences between two parameters among individuals. Statistical significance was interpreted as $\alpha \leq 0.05$. Statistical tests were performed using

MATLAB (v.7.0; The MathWorks, Natick, MA, USA) and Excel. MATLAB was also used to prepare figures from audio and laser data that were exported from MiDAS.

Tuning curves

Tuning curves (behavioural audiograms) were constructed to evaluate sensitivity to different sound frequencies. An individual larva was placed on a leaf of a potted plant placed 200–300 mm from a woofer in the set up as described above. The woofer and plant were enclosed in a cage lined with acoustic foam, which had one side unobstructed to allow observation and recording with a video camera. Pure tones 1 s in duration and between 100 and 1200 Hz were generated with a Tucker Davis Technologies digital signal processor. The first sound pulse of each frequency was delivered at a sub-threshold level and each successive pulse was increased in 3 dB steps; 10 s intervals of silence separated each pulse. Subsequent frequencies were presented at 50 or 100 Hz intervals, and were played in random order, with 3 min intervals of silence between frequencies. Audiograms were generated in this manner for a total of 32 larvae. All trials were videotaped for further analysis. A positive response to sound was noted when the caterpillar ceased movement (i.e. freezing, which was only detectable if the insect was feeding or crawling), contracted, or flicked within 1 s from the onset of the sound pulse.

Response to increasing sound levels

Behavioural responses to increasing sound levels were measured following playback of a 200 Hz stimulus. Each of 32 larvae was tested with eight sound levels played from low to high (72, 75, 78, 81, 84, 87, 89, 92 dB SPL). Each stimulus (1 s in duration, 25 ms rise/fall) was separated by a 10 s interval. The number of larvae that responded to a 200 Hz tone by contracting or flicking, as well as the mean number of times a caterpillar flicked following the stimulus onset, was recorded.

Habituation/sensitization

To assess how larvae responded to repeated stimuli, 1 s-long 200 Hz tones were played repeatedly at 10 s intervals. Each tone was played at 92 dB SPL, which corresponded to the loudest setting used in the audiograms, and was repeated until the larva stopped responding. The number of flicks at each *n*th tone was recorded for eight larvae.

Morphology

External morphology

To identify potential sound receptors, the external morphology of late instar (third to fifth) larvae was examined using an Olympus (Tokyo, Japan) SZX12 stereomicroscope. Based on these observations, the following structures were investigated using scanning electron microscopy (SEM): filiform trichoid sensilla, prothoracic shields and tubercles. To prepare specimens for SEM, 10 larvae preserved in 70% ethanol were critical point dried. In preparation for critical point drying, each larva was cut into segments of reasonable size for the procedure. Tissues were transferred to 95% ethanol for 8 h, and then to 100% ethanol for 16 h. Samples were critical point dried using a Polaron Critical Point Drying Apparatus (E3100 Jumbo Series II; Watford, UK) for 2 h, with a liquid carbon dioxide change every 15 min. Dried specimens were mounted on aluminium stubs, sputter-coated with gold–palladium, and examined using a JSM-6400 scanning electron microscope (JEOL, Tokyo, Japan). Guided by ablation studies (see Results), further morphological descriptions and measurements focused only on the filiform trichoid sensilla. Lengths of sensilla from fifth instar larvae preserved in 80% ethanol were measured by removing sensilla from the specimen using fine forceps, mounting them on a microscope slide and observing them with a Zeiss Axio Imager M1 compound microscope equipped with a Zeiss AxioCam MRm camera (1.4 megapixels, 1388×1040) and AxioVision (v.4.6.3.0) software.

Histology

Larvae were preserved in 3% glutaraldehyde in 0.2 mol l^{-1} dibasic phosphate buffer (Humason, 1997) and stored at 4°C. Tissue surrounding filiform sensilla was excised, dehydrated in an ethanol series, and cleared in Histo-Clear (National Diagnostics, Atlanta, GA, USA). Cleared tissue was infiltrated with Paraplast X-tra paraffin (McCormick Scientific, St Louis, MO, USA) at 60°C for 30 min, then embedded in the same. Thick (5–10 µm) sections were cut with a Leitz 1512 (Wetzlar, Germany) microtome, transferred to Fisherbrand Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA, USA), and warmed on a slide warmer overnight at 40°C. They were deparaffinized through a xylene/Histo-Clear and ethanol series, and stained with 3% Multiple Stain Solution (Polysciences, Inc., Warrington, PA, USA). Cover slips were affixed with Permount (Fisher Scientific), and observed and measured with a Zeiss microscope as described above.

Experimental ablations

Four types of structures were tested for their possible role in hearing: prothoracic tubercles, abdominal tubercles, prothoracic shields and selected trichoid sensilla. To ablate the prothoracic tubercles, the larva was first tested for a response to a 200 Hz stimulus by flicking. It was then anaesthetized with carbon dioxide, and a fine hair was used to ligature the two prothoracic tubercles at their bases. With micro-dissection scissors, both tubercles were removed and the caterpillar was retested for a response to sound. The two abdominal tubercles were tested in the same fashion but in different larvae. To 'ablate' the prothoracic shields, the caterpillar was similarly tested for a response to sound and anaesthetized as described above. Using a stereomicroscope, a small drop of entomological grade paint (Shannon Luminous Materials, Inc., Santa Ana, CA, USA) was daubed onto the pair of prothoracic shields. These larvae were then tested for a response to sound. Preliminary ablation experiments of the tubercles and prothoracic shields were performed on only two larvae each as these were quickly ruled out as being involved in sound reception (see Results).

Trichoid seta ablations were subdivided into full ablations (all target setae removed), partial ablations (selected setae removed) and controls (no setae removed). Larvae for all experiments were third to fifth instars, and chosen based on their response by flicking to a 1 s 200 Hz tone played at 80 dB SPL. Larvae were randomly assigned to different experimental and control groups. Prior to ablation, the subject was anaesthetized with the minimum amount of carbon dioxide required to keep it immobile until the setae were removed (or not removed as in the control). For full ablations (n=27 individuals), each of the two pairs of sensilla on the prothorax, and the single pair of rear (10th) abdominal sensilla were plucked out using a pair of fine forceps. Removal of all six sensilla took approximately 3 min. The type of treatment that the larvae received was recorded on video and each larva was painted with different colours of entomological grade paint to later identify which individuals had received which treatment. As soon as they regained mobility, larvae were placed back on the host plant, and following a rest period (15–30 min) played a series of 1 s sound pulses. Larvae that did not respond were tested again in 15–30 min.

Journal of Experimental Biology (2019) 222, jeb211862. doi:10.1242/jeb.211862

For partial ablations, the same protocol was followed, except only one pair of sensilla was ablated. A control experiment was performed on 15 caterpillars whereby the caterpillars were subjected to the same procedure as above, but no sensilla were removed. Proportions of ablated and control larvae were compared with chi-square tests.

To further assess whether trichoid sensilla were responsible for an acoustic response, five of the individuals used in full ablations that were third or fourth instars were isolated and tested again following their next moult. Following this, they were inspected under a stereomicroscope to confirm that they had regrown the sensilla that had previously been removed.

RESULTS

Responses to sound

Monarch caterpillars typically responded to sound with one or more of the following: (i) freezing, comprising a cessation of movement; (ii) contraction, where the caterpillar contracts the anterior portion of its body; and (iii) flicking, where the caterpillar vertically flicks the anterior portion of its body. While each of these behaviours can occur in isolation, they may not be entirely independent of one another. In order to contract, the caterpillar must first cease other activities such as feeding or crawling; when it flicks, its head is tucked into the head collar region, as it is during a contraction. Therefore freezes, contractions and flicks may be different magnitudes of the same behaviour. Nonetheless, because each of these can occur independently in response to sound, we describe them separately. Occasionally, a larva flicked so vigorously that it lost its grip with its prolegs and fell off the leaf, curled up into a ball, and remained motionless for a minute or more. As dropping was observed rarely in fourth and fifth instars and was not reported in formal trials, we do not describe it further.

Freezing is the cessation of all movement, which is sustained for a variable period of time, ranging from 1 to 60 s. The nature of this type of response is such that it can only be scored when the caterpillar is already engaged in movement, such as feeding or crawling. Therefore, freezes were not used in assessing thresholds or responses to different sound levels.

A contraction arises from the contraction of longitudinal muscles, resulting in a small decrease in body length, and the anterior tubercles moving backwards (Fig. 1A). Most of the reduction in length results from the head capsule retracting. Contractions in response to a 200 Hz tone observed in high-speed videography trials showed a small (1.88%) but significant decrease in body length (paired *t*-test, t=3.97, P=0.0106, n=7). The mean latency from the onset of the sound stimulus was 580 ms (95% CI: 71.5–1090 ms, minimum 40 ms, maximum 1.51 s, n=7) and the time it took to reach a contracted position was 78.6 ms (95% CI: 10.9–147 ms, minimum 35.8 ms, maximum 240 ms, n=7).

During a flick, the caterpillar releases its thoracic legs from the substrate, and rapidly raises and then lowers the anterior portion of its body, holding on to the leaf with its prolegs (Fig. 1B; Movies 1 and 2). Flicks occurred in isolation, or in trains of up to 13 flicks at an average rate of 2.1 flicks s^{-1} (Fig. 1B; Movies 1 and 2). In response to a 200 Hz tone, the mean flick angle was 26 deg (95% CI: 17.83–34.91 deg; n=13, 33 flicks). The upward movement was the fastest, taking an average of 42.6 ms to complete, and significantly shorter than the 73.4 ms duration of the downward stroke (paired *t*-test, t=6.93, P<0.0001, n=13, 45 flicks). The mean latency from the onset of the 200 Hz tone to the onset of the flick was 471 ms when all flicks were measured (95% CI: 305–637 ms, minimum 100 ms, maximum 1526 ms; n=13, 23 flicks), or 290 ms when the fastest flick for each individual was measured (95% CI: 152–427 ms, minimum 100 ms, maximum 729 ms, n=13). There was a relationship between flick order and flick angle, whereby flick angle increased from first to second and third flick, remained steady for the fourth flick, and decreased for the fifth flick (Fig. S1). However, this variation was not significantly different when all flick numbers were compared (ANOVA, f=2.34, P=0.08, n=33).

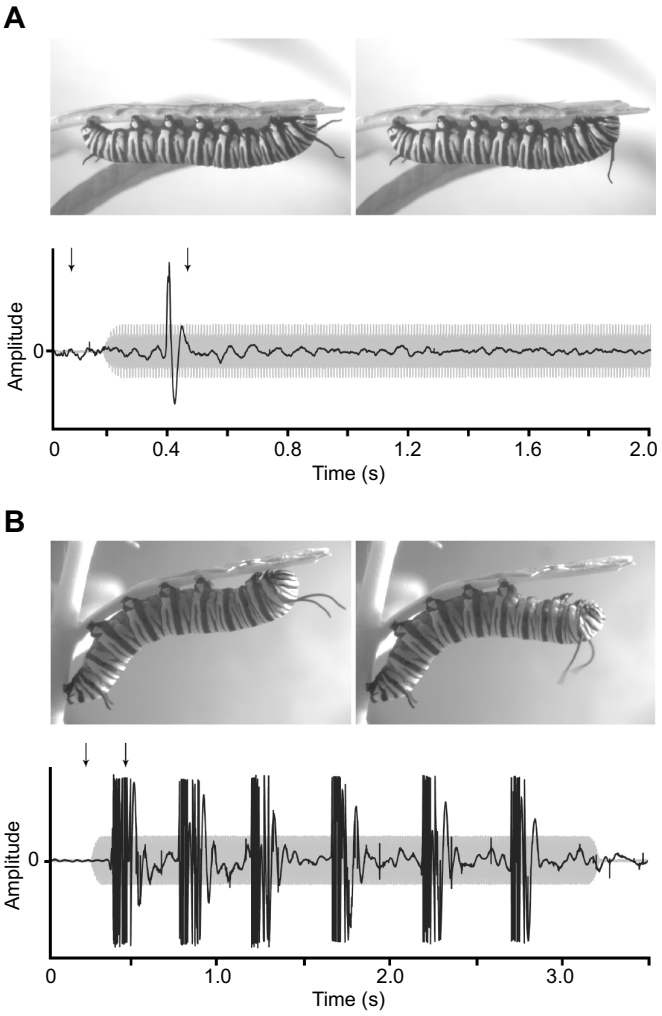

Fig. 1. Monarch caterpillars respond to sound. Fifth instar caterpillars respond to sound by contracting (A) and flicking (B) in response to a 200 Hz tone. Top images show high-speed video frames of the caterpillar at time points before and after the sound onset, indicated by arrows on the graph below. Graphs show the sound stimulus (grey trace) with caterpillar movements (black trace) recorded from the leaf surface using a laser vibrometer.

Tuning curves

Larvae responded by contracting or flicking (freezing was not included in threshold assessments) in response to pure tones between 50 and 900 Hz (Fig. 2A). The tuning curve was similarly shaped for particle velocity values (Fig. 2B). Particle velocity values are included in parentheses in the following description. Larvae were most sensitive to low-frequency sounds between 100 and 200 Hz. The lowest mean threshold was 79 dB SPL (605 µm s^{-1}) at 150 Hz, but this was not significantly different for thresholds at 100 Hz (80 dB SPL or 679 µm s^{-1}; unpaired *t*-test, t=0.468, P=0.642, n=32) or 200 Hz (80 dB SPL or 668 µm s^{-1}; unpaired *t*-test, t=0.303, P=0.763,

Journal of Experimental Biology (2019) 222, jeb211862. doi:10.1242/jeb.211862

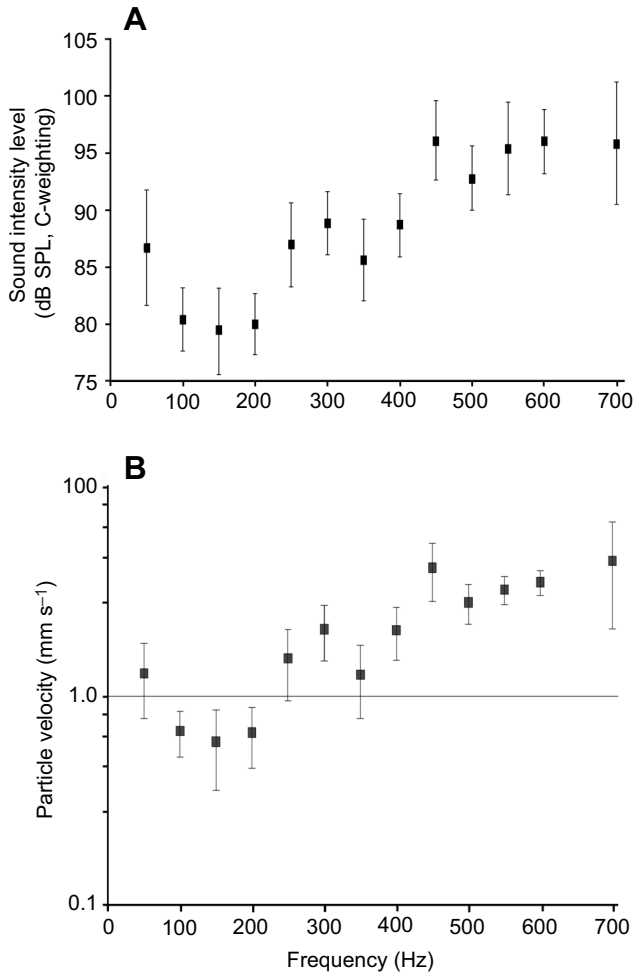

Fig. 2. Behavioural tuning curves. (A) Behavioural response (contraction or flicking) thresholds at different sound frequencies. Caterpillars were most sensitive to sounds between 100 and 200 Hz. Error bars indicate 95% confidence interval (CI) of the mean for each frequency (n=32). (B) As above, but plotted as particle velocity.

n=32). The minimum threshold for an individual was 68 dB SPL (202 µm s^{-1}) at 100 Hz. A second tuning curve based on flicks only was similarly shaped but showed higher thresholds (Fig. S2). The similarity of audiograms for all responses (Fig. 2A) and for only flicks (Fig. S2) was expected, because the majority of responses were flicks. A tuning curve was constructed using the same data as shown in Fig. 2, but recalculated to yield particle displacement. These data show that particle displacement was highest at 50 Hz (22.7 µm), but remained approximately constant between 150 and 700 Hz, at approximately 1.12 µm.

Response to increasing sound amplitude

More caterpillars responded as sound levels increased, and those that did respond produced more flicks (Fig. 3) (n=32). Fig. 3A shows that contractions comprised a much smaller proportion of all responses from 32 caterpillars at all sound levels, but this proportion decreased at higher sound levels. Fig. 3B shows that the number of individuals that responded by flicking increased steadily from six responders at 72 dB SPL, to 23 at 92 dB SPL (left axis), and the mean number of times an individual flicked per sound stimulus also increased with higher sound levels (right axis). Responding larvae flicked only once at 72 dB SPL, and an average of 3.22 times at 92 dB SPL.

Response to repeated sound stimuli

Results of the habituation/sensitization experiment for eight caterpillars are presented in Fig. 4. When presented with a repeated 200 Hz sound at 10 s intervals, approximately the same number of flicks was observed between the first and fifth tones presented, but with a small decrease in response to the second tone. Following the fifth tone, the number of flicks declined steadily with each subsequent tone.

Morphology

Based on external morphology, three types of structures were identified as possible hearing organs: prothoracic shields, tubercles and filiform setae. These are described below, with emphasis on the prothoracic filiform setae, as these were later confirmed to function in sound reception based on ablation experiments.

Prothoracic shields

A prothoracic shield occurs dorsally on each side of the first thoracic segment, dorsal and anterior to the thoracic trichoid sensilla and anterior to the thoracic tubercle (Fig. 5A). Prothoracic shields are more heavily sclerotized than the surrounding cuticle, with a smooth surface, and six thick tapered setae originating from it (Fig. 5A,C). Prothoracic shields were investigated only in preliminary ablation trials (see below).

Tubercles

Long, fleshy appendages called tubercles are located on the second thoracic and eleventh abdominal segments (Figs 5A,B and 6A). Tubercles are covered with many short setae (~20 µm in length) mostly erected perpendicular to the tubercle, except near the tubercle's tip, where they aligned toward the tip (Fig. 5B). Tubercles were investigated only in preliminary ablation trials (see below).

Filiform sensilla

Seven pairs of filiform sensilla were identified: two pairs on the prothorax, four pairs on the abdominal segments that bear prolegs, and one pair on the posterior abdominal segment (Fig. 6A). Two anterior pairs are located on each side of the prothoracic segment, along the lateral midline of the larva, with one positioned dorsal to the other (Fig. 6A–C). The lower anterior sensilla are located just anterior to the spiracle, while the upper sensilla are located dorsal and slightly anterior to the spiracle (Fig. 6B,C). The upper anterior setae are curved slightly upwards and anteriorly, while the lower anterior sensilla are curved slightly downwards and anteriorly (Fig. 6C). One pair occurs on each of the four abdominal segments that bear prolegs, one on each proleg, located below the spiracle (Fig. 6A); however, these were not always present. These abdominal sensilla are curved downwards. The paired posterior sensilla occur on each side of the tenth abdominal segment, behind the abdominal tubercles (Fig. 6A), and are curved slightly downwards and posteriorly.

Sensilla from fifth instar larvae were measured. The mean length of the anterior upper prothoracic sensilla was 452 µm (95% CI: 413–492 µm, minimum 343 µm, maximum 551 µm, n=10). These sensilla were 112 µm longer than the lower front sensilla, which had a mean length of only 340 µm (95% CI: 320–362 µm, minimum 302 µm, maximum 400 µm, n=11; unpaired t-test, t=5.72, P<0.0001, n=21). Posterior abdominal sensilla, which had a mean length of 336 µm (95% CI: 286–387 µm, minimum 208 µm, maximum 455 µm, n=10), were 115 µm shorter than anterior upper sensilla (unpaired t-test, t=4.06, P=0.0007, n=21), but were not significantly shorter than lower prothoracic sensilla (unpaired t-test, t=0.179, P=0.860, n=21). Sensilla on abdominal segments

Journal of Experimental Biology (2019) 222, jeb211862. doi:10.1242/jeb.211862

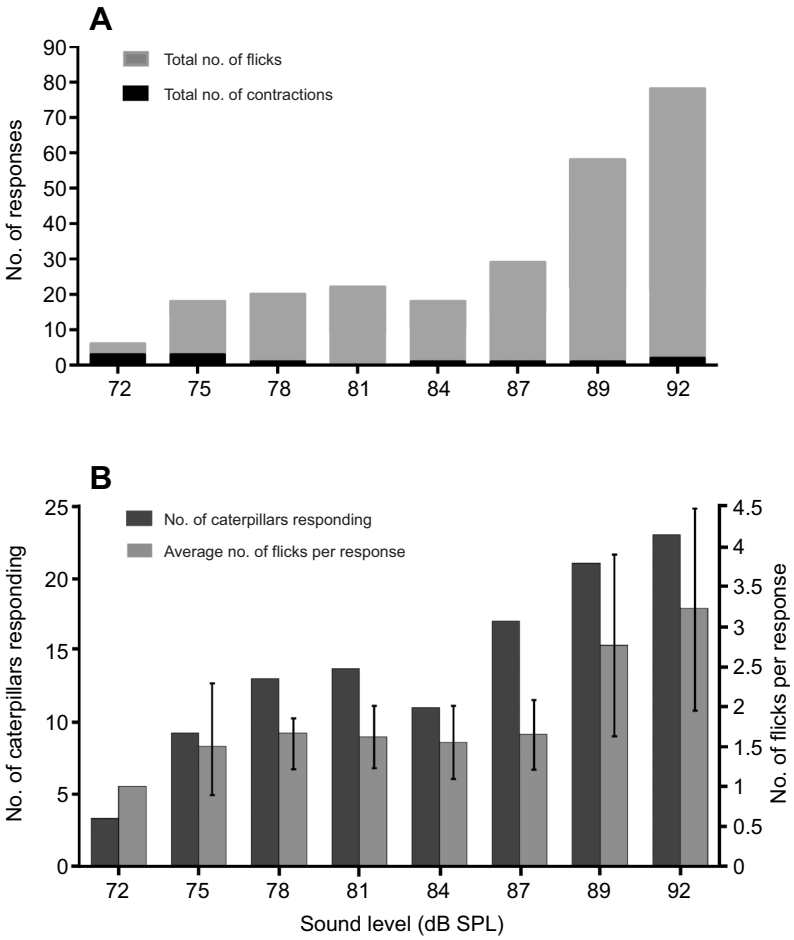

Fig. 3. Responses to increasing sound levels. (A) The effect of sound level (dB SPL) on the total number of responses (contractions and flicking) to a 200 Hz tone (*n*=32). Higher levels resulted in more flicks but not more contractions. (B) Distribution of flicks in response to a 200 Hz tone as sound levels increased. Left axis shows the number of individuals responding by flicking to increasing sound levels (*n*=32). Right axis shows the mean number of flicks per caterpillar. Error bars represent 95% CI.

bearing prolegs were shorter than the other sensilla, at approximately 250 µm long, but they were not always present for measurement. All filiform sensilla were tapered slightly at both ends, and inserted into a dome-shaped socket that was raised above the surface of the cuticle (Fig. 6D–F). Each sensillum was innervated by a bipolar sensory neuron (Fig. 6F).

Ablations

Individuals with tubercles removed, or prothoracic shields covered in paint, all responded readily to sound shortly following the procedure. Therefore, these structures were ruled out as receptors and were not tested further. The abdominal sensilla on segments bearing prolegs were also not investigated further, because they were relatively short compared with the prothoracic and posterior sensilla, and because during preliminary investigations, several larvae that responded to sound were observed to lack these setae as a result of breakage. Thus, subsequent experiments were conducted on the anterior thoracic and rear abdominal sensilla.

All three pairs of sensilla (prothoracic and rear abdominal) were removed to first test whether any were involved in sound reception.

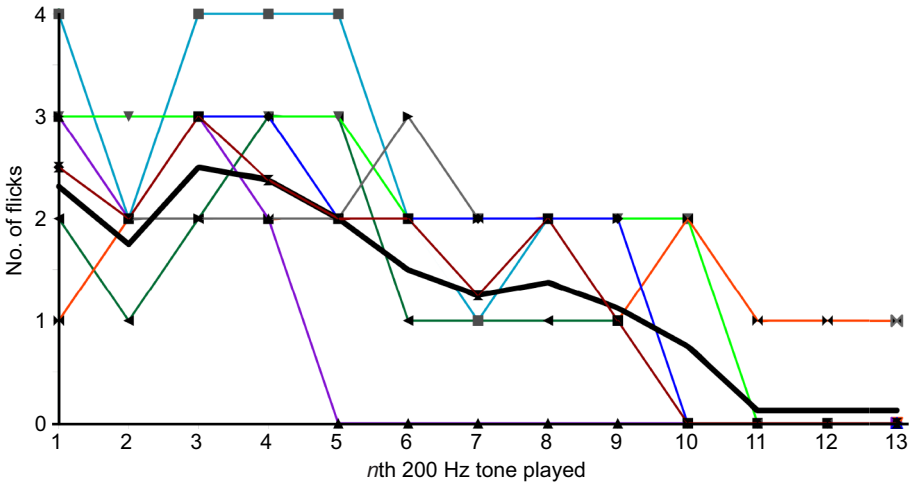

Fig. 4. Caterpillars habituate to repeated sounds. Mean number of flicks by *n*=8 fifth instar caterpillars in response to repeated 1 s tones (200 Hz) played at 10 s intervals. Bold line represents mean over all individuals and coloured lines indicate individual caterpillars.

Journal of Experimental Biology (2019) 222, jeb211862. doi:10.1242/jeb.211862

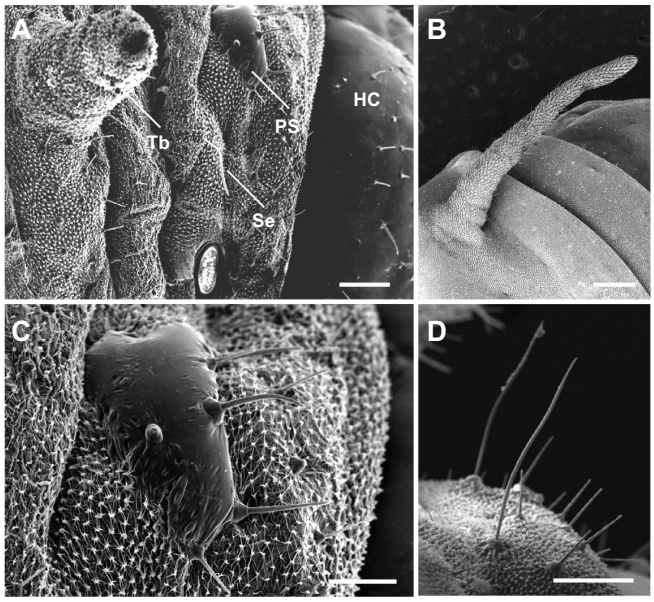

Fig. 5. Scanning electron micrographs of putative receptors tested in ablation experiments. (A) Anterior region of a third instar larva showing the three types of structures examined in ablation studies: Tb, tubercle; Se, seta; PS, prothoracic shield. The head capsule (HC) is also labelled for orientation. Scale bar: 100 µm. (B) Whole anterior tubercle of third instar larva. Scale bar: 500 µm. (C) Right prothoracic shield of third instar larva. Scale bar: 50 µm. (D) Thoracic trichoid sensilla from third instar larva. Scale bar: 100 µm. Note that early instars were used for scanning electron micrographs to show several structures together. However, experiments were conducted on late instars.

In total, 27 individuals were ablated in this manner, and none responded to sound within 1 h or 2 days of the procedure (Table 1). In contrast, 93% of controls responded within 1 h of the procedure, and 100% responded within 2 days. No larvae responded after having both anterior thoracic pairs of sensilla removed, and only a single individual responded following removal of its anterior upper sensilla. Both of these findings differed significantly from the expected proportion obtained from the controls (Table 1). Removal of either the lower anterior or posterior pair of sensilla had no significant effect on the proportion of larvae that responded (Table 1). Five individuals that had all three pairs of setae (both thoracic and posterior abdominal) ablated and were third or fourth instars at the time of ablation were retested following their next moult. All five individuals responded readily to the sound stimuli following their moult. It was confirmed that all five larvae had regrown each of the three pairs of sensilla.

DISCUSSION

Our results show that monarch caterpillars respond to low-frequency (100–900 Hz) sounds by freezing, contracting, and flicking dorsally, and that these responses varied with sound levels. The primary hearing receptors were identified as a pair of trichoid sensilla located on the upper prothorax. The following discussion focuses on the sensory mechanism, the behavioural responses to different sound characteristics, the adaptive significance of hearing in caterpillars, and the broader implications of this research.

Near-field hearing reception

In this study, caterpillars responded to sounds played from a loudspeaker, and in previous studies, caterpillars responded to the presence of passing aircraft (Rothschild and Bergström, 1997), traffic noise (Davis et al., 2018) and tuning forks (Minnich, 1936).

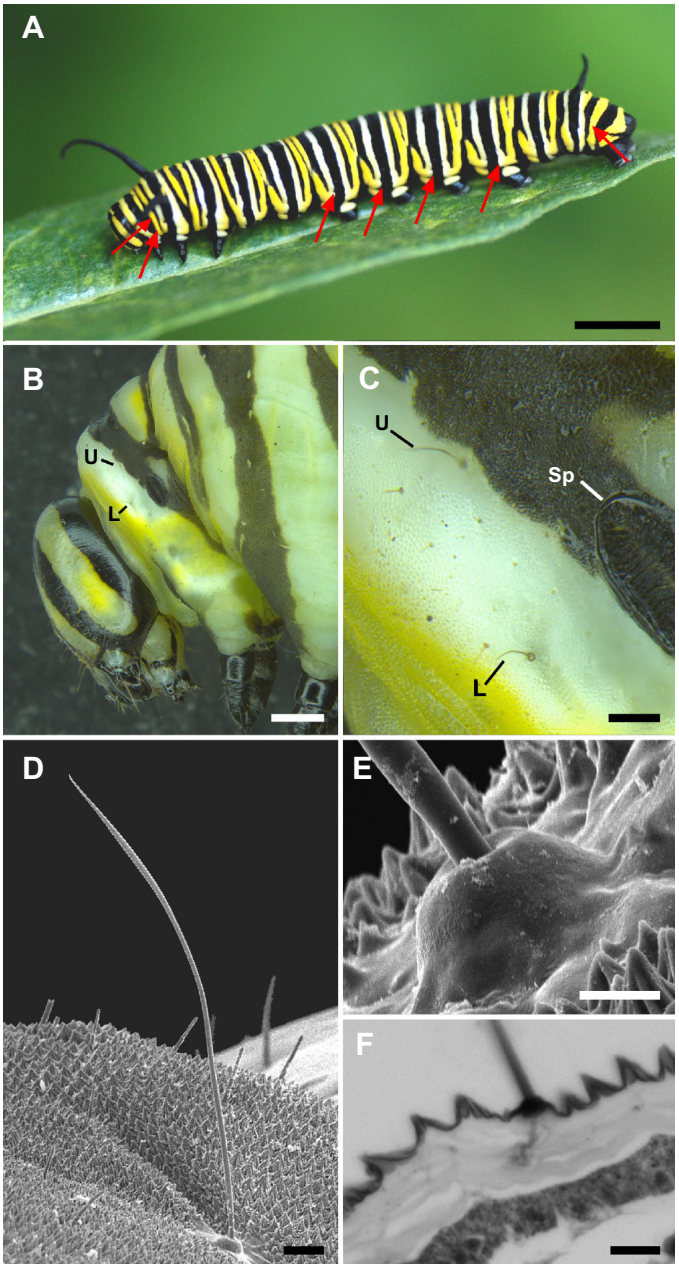

Fig. 6. Trichoid sensilla on monarch caterpillars. (A) Fifth instar caterpillar with arrows indicating the location of sensilla on the prothorax, and abdominal segments 3–6 and 10. The head of the caterpillar is on the left. Scale bar: 1 cm. (B) Anterior region of a fourth instar larva showing the location of the upper (U) and lower (L) prothoracic trichoid sensilla. Scale bar: 1 mm. (C) Close ups of the prothorax of a fourth instar larva showing the upper (U) and lower (L) sensilla, and prothoracic spiracle (Sp). Scale bar: 200 µm. (D) Scanning electron micrograph of an upper prothoracic sensillum. Scale bar: 40 µm. (E) Scanning electron micrograph of the socket base. Scale bar: 10 µm. (F) Section through the socket of a sensillum showing the sensory neuron innervation. Scale bar: 20 µm.

Each of these sound sources could potentially be received by the caterpillar as 'near-field' sound (particle-displacement component), far-field sound (pressure component) or as solid-borne vibration through the plant (see Caldwell, 2014; Ewing, 1989; Yack, 2004). We propose that caterpillars are responding primarily, if not exclusively, to particle displacement rather than to solid-borne vibrations or far-field sounds. First, the results of the ablation study confirm that filiform sensilla are the primary receptors. Filiform

Journal of Experimental Biology (2019) 222, jeb211862. doi:10.1242/jeb.211862

Table 1. Number and proportion of *Danaus plexippus* larvae that responded to sound following removal of various filiform setae

Setae removed	*N*	Responders	Proportion	*P*-value
All	27	0	0	<0.0001*
Both prothoracic	10	0	0	0.0023*
Upper prothoracic	10	1	0.1	0.0084*
Lower prothoracic	10	8	0.8	0.73
Rear abdominal	10	9	0.9	0.93
None (control)	15	14	0.93	–

P-values were obtained by comparison with control (no setae removed).
*Statistically significant.

sensilla are not sensitive to changes in pressure, because air pressure is equal around the entire sensillum, and as such cannot exert any force on it (Barth, 2014; Tautz, 1979). In this way, monarch caterpillar trichoid sensilla resemble other 'near-field' sound detectors in other insects and spiders (Ewing, 1989; Shamble et al., 2016; Tautz, 1979). Second, the caterpillars are most sensitive to sounds below 1 kHz, which is typical of near-field sound receptors (Greenfield, 2002; Tautz, 1979). Third, it is unlikely that caterpillars were responding to vibrations transmitted through the leaf, as there was no discernible vibration on the leaf when sounds were played (see Fig. 1; Movie 2). Also, the sensilla identified as being key for sound reception were not in direct contact with the leaf. We therefore conclude that caterpillars are responding to the 'near-field' component of sound. It is important to note, however, that this does not mean that caterpillars may only respond to very close sound sources. While the particle displacement component of a sound source was previously thought to occur at short distances, typically within one wavelength from the sound source, more recent studies show that 'near-field' receptors respond to sounds from much greater distances (e.g. Menda et al., 2019; Shamble et al., 2016; Zhou and Miles, 2017), which could explain why monarch caterpillars have been noted to respond to anthropogenic sound sources such as planes and road noise (Davis et al., 2018; Rothschild and Bergström, 1997).

The upper thoracic trichoid sensilla were identified as being the primary sound receptors. When the upper sensilla were removed either solely or in combination with the other focal pairs of trichoid sensilla (37 trials in total), only one larva responded. Ablation of the lower prothoracic and rear abdominal sensilla had minimal effects on hearing. Although sensilla on abdominal segments three to six were not removed, their presence or absence (when naturally broken) had no effect on hearing. Minnich (1936) conducted a series of experiments to locate possible regions of the body responsible for sound reception in late instar *D. plexippus* larvae. When larvae were trisected, the anterior (thorax and head) and middle (from the thorax to between the second and third prolegs) portions of the body continued to respond to a 256 Hz tuning fork; however, this response was faint and inconsistent and required very loud sounds, according to Minnich (1936). Minnich (1936) proposed that diffuse hairs on the body or, alternatively, chordotonal organs inside the body might be responsible for sound reception. While our results show strong support for the upper thoracic sensilla playing a key role in sound reception, we do not discount the possibility that other trichoid sensilla contribute to sound reception under different stimulus parameters. For example, the fact that the abdominal setae are all on segments bearing prolegs might indicate that at particularly high sound intensities, they may stimulate the prolegs to relax their grip on the substrate, causing the falling behaviour observed in some larvae. Also, other trichoid sensilla may be sensitive to other sound characteristics outside of those used in our ablation studies.

Morphological features of the upper prothoracic setae in *D. plexippus* may be related to the acoustic responses reported here. Their location on the dorsal prothorax, close to the prothoracic ganglia and muscles, is optimally situated for anterior flicking. Their shape and length are also of significance, as the physical structure of sensilla is important in determining their resonant properties (Barth, 2000; Tautz, 1979). A near-field receptor must be sufficiently long to extend past the boundary layer of air around the larva's body at biologically relevant frequencies (Barth, 2014), and this boundary layer ranges between 12.5 and 106 µm for the best frequency range of *D. plexippus* (see Tautz, 1979). However, it must not be so long that the displacement angle decreases to the point where the sensory cell is unable to transduce the stimulus. A hair length of ∼450 µm is in the ideal range to oscillate at the best sensitivity range of the larvae. The lower prothoracic sensilla and rear abdominal setae are shorter than the upper prothoracic sensilla, and the four pairs of abdominal sensilla are shorter still. But, they are all still longer than the boundary layer. These other sensilla may contribute to hearing in some way such as broadening the frequency or dynamic range, and their potential contribution to hearing could be further investigated.

Behavioural responses to sound

Monarch caterpillars responded to low-frequency (100–900 Hz) tones by freezing, contracting, flicking their anterior segments, and occasionally dropping from the plant. The greatest sensitivity and most vigorous responses occurred between 100 and 200 Hz. These results agree in part with previous studies. Rothschild and Bergström (1997) reported that late instars responded to flying bumblebees and wasps, human buzzing and shouting sounds, and aircraft by 'sudden ducking or twitching movements of the head and a simultaneous agitation of the two anteriorly placed filiform tubercles'. While the sounds in that study were not recorded, and sound may not have been isolated from visual or other cues, it is likely that such sounds would include frequencies below 1 kHz (e.g. Raboin and Elias, 2019; Rashed et al., 2008). Davis et al. (2018) played road noises to caterpillars and noted increases in heart rate. While the road noise characteristics were not described in Davis et al. (2018), an examination of the supplementary sound files revealed that most energy was below 1 kHz. Minnich (1936) reported flicking and freezing in response to tuning forks with frequencies of 256, 512 and 1024 Hz, with the highest percentage of responses at 512 Hz. Minnich (1936) did not play sounds between 100 and 200 Hz, the range at which we report best sensitivity. Interestingly, our results also show moderate sensitivity around 350 Hz (see Fig. 2A). These anomalies may be explained by the resonant properties of the sensilla. For example, the sensilla responsible for sound reception likely have a resonant frequency at or near 175 Hz, because this value falls near the middle of the best frequency range. If sensilla resonate at 175 Hz, then they would resonate harmonically at ∼350 Hz, explaining the small increase in sensitivity at this frequency.

Function of hearing in monarch caterpillars

We propose that hearing in monarch larvae functions to protect against aerial insect parasitoids and predators. The following predictions would support this hypothesis: (i) larvae should be under selective pressure from aerial insects; (ii) flight sounds should match the frequencies to which *D. plexippus* are most sensitive; and (iii) larvae should respond in a way that would be effective in defending themselves against these insects. These predictions are supported, as discussed below.

Danaus plexippus larvae face high mortality from invertebrate predators and parasitoids (de Anda and Oberhauser, 2015; Oberhauser et al., 2015). Common aerial invertebrate natural enemies include tachinid flies and paper wasps. *Lespesia archippivora* (Diptera: Tachinidae) is the most common parasitoid that attacks monarch larvae in North America (Oberhauser et al., 2007), although 12 species of other tachinid flies and one species of braconid wasp have been reported on *D. plexippus* as well (Arnaud, 1978). One study of parasitism rates in North America found that an average of 13% of fourth and fifth instar larvae were parasitized by tachinids, but this number reached up to 90% for some sites (Oberhauser et al., 2007). Vespid wasps are common aerial predators as well (Oberhauser and Solensky, 2004). This shows that there is selective pressure on *D. plexippus* to hear and effectively respond to aerial enemies. Our results show that larvae were most sensitive to sound frequencies that fall within the range of a number of flying Diptera and Hymenoptera (e.g. Rashed et al., 2008; Sotavalta, 1963; Tautz and Markl, 1978).

The behavioural responses of monarch larvae to sound are consistent with defensive reactions against aerial insect predators or parasitoids. Many parasitoids and predators use visual cues for short-range location of prey (e.g. Nakamatsu and Tanaka, 2005; Oliai and King, 2000), and may require movement, detected either visually or as solid-borne vibrations, before they will attack (e.g. Bushbeck and Strausfeld, 1997; Stireman, 2002; Vet and Bakker, 1985). Therefore, freezing may serve a cryptic function in monarch larvae once they detect an approaching enemy. It is not immediately clear how contracting is adaptive, but possibly this behaviour engages antagonist muscle pairs that are held in an isometric contraction, keeping the caterpillar still. Because contractions occur more at low sound levels, they may be more likely to occur when aerial enemies are in the area, but not close enough for the caterpillar to flick and risk drawing attention to itself. Flicking probably functions to knock away aerial predators, or prevent oviposition by parasitoids. Observations of other larval Lepidoptera support this hypothesis, as flicking has been experimentally (Tautz and Markl, 1978) or anecdotally (Hogue, 1972; Myers and Smith, 1978) reported to confer protection. Some tachinids try to lay the egg on the larva's head capsule, where the larva cannot bite it off (Iwao and Wellington, 1970), and flicking creates a moving target that probably makes oviposition difficult. Although *D. plexippus* only flicks dorsally in response to sound, lateral flicking in other larval Lepidoptera has been shown to be effective against some terrestrial predators (Evans, 1982). An alternative explanation for dorsal flicking is the dispersal of volatile repellent chemicals, which may be released from an area near the head collar region in *D. plexippus*, as proposed by Rothschild and Bergström (1997). However, it has not been confirmed that volatiles are released in response to sound. Future research should focus on behavioural experiments with aerial predators and parasitoids to test the efficacy of hearing in avoiding natural enemies.

Caterpillar hearing: diversity and evolution

In 1936, Minnich stated, 'the response [to sound] is so widespread as to indicate a very general, perhaps universal, characteristic of lepidopterous larvae'. Until now, formal studies of hearing in caterpillars have focused on the cabbage moth caterpillar, *Mamestra* (*Barathra*) *brassicae* (Lepidoptera: Noctuidae). *Mamestra brassicae* larvae respond to near-field sounds between 40 and 1000 Hz, with best sensitivity at 100–150 Hz (Markl and Tautz, 1975; Tautz, 1977). Reactions to sound are described as cessation of locomotion, contraction of the thorax, and squirming that normally leads to dropping from the substrate (Markl and Tautz, 1975). Four pairs of filiform sensilla were identified as responding to sound: two pairs on the prothoracic segment, and one pair on each of the mesothoracic and metathoracic segments (Markl and Tautz, 1975; Tautz, 1977). Further experiments showed that these caterpillars use their hearing to detect approaching insect predators, as significantly more intact larvae survived wasp attacks compared with those with ablated receptors (Tautz and Markl, 1978). Comparisons between hearing in *D. plexippus* and *M. brassicae* revealed some similarities and differences. They are tuned to similar sound frequencies, with best sensitivity between 100 and 200 Hz. Both respond to sounds by stopping movement when crawling and contracting thoracic segments, but they differ in that *M. brassicae* does not flick dorsally, which is the dominant response of *D. plexippus*. Our results indicate that hearing in *D. plexippus* was mostly affected by the removal of a single pair of sensilla on its upper prothorax. In *M. brassicae*, four pairs of filiform sensilla on the thorax are important in sound reception (Markl and Tautz, 1975). It is unknown whether the sensilla in *M. brassicae* and *D. plexippus* are homologous. To determine homology, chaetotaxy of first instar *M. brassicae* and *D. plexippus* must be compared, although at this taxonomic level, homology of some setae is disputed (Ballmer and Wright, 2008). Behavioural responses to sound in caterpillars have been reported for several distantly related taxa, including the Bombycoidea, Noctuoidea, Papilionoidea and Geometroidea, and these responses vary between species (e.g. Minnich, 1936; Myers and Smith, 1978; White et al., 1983). Selection pressures on hearing and responses to sound are likely to vary with the relative proportions and different species of predators and parasitoids that attack lepidopterous larvae (Hawkins et al., 1997). Given the simplicity of filiform sensilla as hearing organs, it is likely that they evolved from a mechanoreceptor seta, such as one of the many that cover the body of larvae. Just as the pre-existence of chordotonal organs facilitated multiple independent origins of tympanal ears in adult Lepidoptera (Yack, 2004), the presence of mechanoreceptive setae on the body of larval Lepidoptera may be a similar exaptation. We recommend further formal studies on different species to gain a better understanding of the importance that sound plays in the sensory ecology of larval Lepidoptera.

Research on hearing in caterpillars has implications for conservation, pest control and biotechnology. Populations of monarchs have been in decline for the past two decades and causes of their decline have been attributed to many factors, including loss of overwintering sites, widespread use of pesticides and climate change (Stenoien et al., 2018). Another factor that may affect populations of monarchs and other terrestrial invertebrates is anthropogenic noise. Acoustic noise from roadways, railways, wind turbines and other anthropogenic sources can have a negative impact on insect populations (Raboin and Elias, 2019). Monarch larvae have been shown to respond to road noise and aircraft (Davis et al., 2018; Rothschild and Bergström, 1997). Our results show that monarch larvae habituate after repeated exposure to sound, and therefore in the presence of anthropogenic noise they may be more at risk of parasitism. A better understanding of hearing in caterpillars also has implications for pest control. For example, intermittent playbacks of bee buzzing sounds in a greenhouse setting significantly reduced consumption of foliage by the beet army worm, *Spodoptera exigua* (Tautz and Rostás, 2008). Finally, sensory organs that respond to near-field sounds (i.e. airflow) are prominent and widespread in invertebrates. These structures include filiform hairs and lightweight antennae of insects, and trichobothria and silk of spiders (Barth, 2014; Casas and Dangles, 2010; Tautz, 1979; Zhou and Miles, 2017). Such structures are highly sensitive

and research on their form and function provides great promise for inspiring miniature sensing devices (Barth, 2014; Zhou and Miles, 2017). We encourage further research into comparative studies on hearing 'hairs' in caterpillars, including characterization of the natural air flow patterns that stimulate them, the diversity of responses that they evoke, and understanding how they function to detect and localize sounds.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: C.J.T., J.E.Y.; Methodology: C.J.T., J.E.Y.; Formal analysis: C.J.T., J.E.Y.; Investigation: C.J.T., J.E.Y.; Resources: J.E.Y.; Data curation: C.J.T., J.E.Y.; Writing - original draft: C.J.T., J.E.Y.; Writing - review & editing: C.J.T., J.E.Y.; Visualization: C.J.T., J.E.Y.; Supervision: J.E.Y.; Project administration: J.E.Y.; Funding acquisition: J.E.Y.

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