RESEARCH ARTICLE

Whistling in caterpillars (*Amorpha juglandis*, Bombycoidea): sound-producing mechanism and function

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SUMMARY

Caterpillar defenses have been researched extensively, and, although most studies focus on visually communicated signals, little is known about the role that sounds play in defense. We report on whistling, a novel form of sound production for caterpillars and rare for insects in general. The North American walnut sphinx (*Amorpha juglandis*) produces whistle 'trains' ranging from 44 to 2060 ms in duration and comprising one to eight whistles. Sounds were categorized into three types: broadband, pure whistles and multi-harmonic plus broadband, with mean dominant frequencies at 15 kHz, 9 kHz and 22 kHz, respectively. The mechanism of sound production was determined by selectively obstructing abdominal spiracles, monitoring air flow at different spiracles using a laser vibrometer and recording body movements associated with sound production using high-speed video. Contractions of the anterior body segments always accompanied sound production, forcing air through a pair of enlarged spiracles on the eighth abdominal segment. We tested the hypothesis that sounds function in defense using simulated attacks with blunt forceps and natural attacks with an avian predator – the yellow warbler (*Dendroica petechia*). In simulated attacks, 94% of caterpillars responded with whistle trains that were frequently accompanied by directed thrashing but no obvious chemical defense. In predator trials, all birds readily attacked the caterpillar, eliciting whistle trains each time. Birds responded to whistling by hesitating, jumping back or diving away from the sound source. We conclude that caterpillar whistles are defensive and propose that they function specifically as acoustic 'eye spots' to startle predators.

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Key words: sound production, caterpillar, acoustic communication, defense, *Amorpha juglandis*, whistle, yellow warbler.

INTRODUCTION

Caterpillars are preyed upon heavily by a host of natural predators, including birds, wasps, shrews, mice, bats, snakes, frogs, lizards, ants, beetles and spiders (Holmes et al., 1979; Wagner, 2005; Kalka and Kalko, 2006). In response, they have developed a variety of anti-predator strategies, which have been studied extensively (e.g. Lederhouse, 1990; Bowers, 1993; Stamp and Wilkens, 1993). The primary defense of a caterpillar is to remain unseen (Stamp and Wilkens, 1993; Wagner, 2005). Morphological adaptations for concealment include crypsis through colour matching (e.g. Edmunds and Grayson, 1991; Canfield et al., 2009), counter-shading (e.g. Rowland et al., 2008) or camouflage (e.g. de Ruiter, 1952; Greene, 1989), whereas behavioural modifications include resting on the underside of leaves, restricting foraging times, removing evidence of their presence by cutting leaves (Heinrich, 1979) or throwing frass (Weiss, 2003).

Once a caterpillar has been detected by a predator, however, other defenses are often employed. These 'secondary' defenses can be chemical, physical or behavioural, including the presentation of traits that mimic unpalatable or potentially dangerous animals. Chemical defenses can be systemic, or emitted through regurgitation (Grant, 2006). In several cases, these are advertised visually through bright coloration. For example, the black and yellow stripes of caterpillars of the monarch (*Danaus plexippus*) butterfly advertise their sequestration of cardenolides (Bowers, 1993; Nishida, 2002), and

the colourful black, red and yellow spurge hawkmoth caterpillars (*Hyles euphorbiae*) broadcast their retention of phorbol esters in the gut (Hundsdoerfer et al., 2005). Physical defenses include spines and hairs, which can also be coupled with a chemical defense (Deml and Dettner, 2002; Lindstedt et al., 2008; Murphy et al., 2010), as well as thrashing, biting and dropping (Wagner, 2005). These spines can be exaggerated by bright colouration in some species (e.g. many Saturniidae) (Deml and Dettner, 2002) and are considered aposematic (Speed and Ruxton, 2005). Bluffing can involve non-chemically defended caterpillars mimicking the colours of defended species or resembling some other, more dangerous, animal. For example, some hawkmoth caterpillars possess elaborate eyespots on their anterior thoracic segments that can be enlarged by retracting the head into the thorax. This display is said to resemble the head of a bird, snake, lizard or small mammal (Morrell, 1969; Pittaway, 1993; Miller et al., 2006; Janzen et al., 2010).

The vast majority of studies on caterpillar defense mechanisms involve communication with predators in the visual realm (Lederhouse, 1990; Pittaway, 1993). Less is understood about exploiting other sensory modalities of predators, such as hearing. Defensive sounds in insects are widespread and have been well documented (Masters, 1979; Masters, 1980; Ewing, 1989) but are primarily reported in adults that have hard bodies and multiple mechanisms for producing sounds. However, there is increasing evidence that defensive sounds and vibrations might also be

widespread in soft-bodied larvae of holometabolous insects. In Lepidoptera, two recent studies (Brown et al., 2007; Bura et al., 2009) show that sounds made by the mandibles in silk-moth caterpillars are accompanied by chemical defenses, and these are thought to function as warning sounds. Other preliminary reports suggest that sound production is widespread in the superfamily Bombycoidea (reviewed in Brown et al., 2007). However, at present, little experimental work has been performed to validate the occurrence, mechanisms and functions of sound production in caterpillars.

In this study, we introduce a novel form of sound production in caterpillars – whistling. While most insects and caterpillars that generate sound do so by rubbing body parts together or against a substrate, few generate sound through using air expulsion. The walnut sphinx *Amorpha juglandis* caterpillar is a cryptic species found throughout much of North America (Tuttle, 2007). Sounds produced by these caterpillars have been described in passing as '…a note resembling the sound tcêp or tceep' (Sanborn, 1868), 'whistles', 'hisses' and 'squeaks' (Wagner, 2005; Tuttle, 2007). Previous observers speculated that these sounds are generated by the spiracles (Wagner, 2005), but this has not been tested. In this study, we characterize the sounds produced by walnut sphinx caterpillars, test the hypothesis that they are produced by the spiracles and gain insight into their defensive function by performing experiments with simulated and natural-predator attacks.

MATERIALS AND METHODS
Animals
Larvae of the walnut sphinx, *Amorpha juglandis* (J. E. Smith 1797), were reared from eggs obtained from moths captured at ultraviolet lights at the Queen's University Biological Station (QUBS; Lake Opinicon, Ontario, Canada; 44°33′55.34″N, 76°19′26.59″W) in July 2008 and August 2009. Larvae were reared on cuttings of hop hornbeam (*Ostyra* sp.), alder (*Alnus* sp.), walnut (*Juglans* sp.) or beech (*Fagus* sp.). All experiments were performed on late (fourth or fifth) instar larvae. Caterpillars of *Paonias myops* (J. E. Smith), used for comparative purposes, were reared from eggs of moths captured at QUBS on cuttings of cherry (*Prunus* sp.).

Sound recordings and analysis
Sounds analyzed for spectral and temporal characteristics were recorded from 10 caterpillars using a Brüel & Kjær (B&K; Naerum, Denmark) 1/4 in microphone type 4939 (grid on), amplified with a Brüel & Kjær Nexxus conditioning amplifier type 2690 and recorded to a Fostex FR-2 Field Memory Recorder (Gardena, CA, USA) at a sampling rate of 192 kHz. Recordings were analyzed using RavenPro Bioacoustics Research Program 1.4 (Cornell Laboratory of Ornithology, Ithaca, NY, USA). Sound production was induced by placing an individual on a cutting of a host plant and delivering an attack to the abdomen with blunt forceps (see 'Attack trials' for details). All recordings were performed in an acoustic chamber (Eckel Industries, Cambridge, MA, USA).

Temporal characteristics, including train duration, number of pulses in a train, inter-pulse interval, pulse duration and the element (waveform) repetition rate, were measured from the first three trains of ten individuals. A train was defined as a series of sound pulses following an attack, until sound production ceased. A pulse is a group of uninterrupted waves, or elements. Mann–Whitney U tests were performed to compare the durations of the first and third trains of 10 individuals to determine whether sensitization occurred and to compare the repetition rate of waveforms in different sound types. Measured spectral characteristics included the fundamental and dominant frequencies and number of harmonics at –40 dB. Power

spectra and spectrograms were produced using a 1024-point Fast Fourier Transform (Hann window, 50% overlap). As pulses differed in their spectral qualities, they were categorized as types 1–3 (see Results for details). In order to determine where different pulse types occurred within a train, pulse trains of 11 animals were analyzed.

Sound pressure levels (SPLs) were measured from 10 sound pulses from five caterpillars by recording with a B&K 1/4 in microphone type 4939 (grid on), and measuring voltages on a Tektronix THS720A oscilloscope. Continuous pure tones centered at 10 and 15 kHz were generated using a Tabor Electronics 50MS/s Waveform Generator (WW5061, Tel Hanan, Israel) and broadcast through a Pioneer ART-54F Ribbon tweeter (Pioneer Electronics, Long Beach, CA, USA). Sounds were recorded using the B&K microphone set up (described above), and voltages were adjusted until the output was equal to those emitted by the caterpillars. The dB SPL values were then read from a B&K sound-level meter type 2239 placed at the same location as the microphone. Relative amplitudes of pulses occurring throughout a train were assessed from pulse trains in 10 caterpillars using the B&K recordings described above.

Sound production mechanism
We tested the hypothesis that sounds result from the expulsion of air through spiracles and identified the specific source of sound production using high-speed videography, selected reversible occlusions and laser vibrometry.

Experiments using high-speed video were performed to examine body movements associated with sound production in five caterpillars. A larva was placed on a wooden stick and induced to signal. A Lightning RDT (High Speed Imaging, Markham, ON, Canada) camera captured 500 frames per second using Xcitec MiDAS 2.0 software (Cambridge, MA, USA) on an A70 Toshiba Satellite (Tokyo, Japan) notebook computer. Sounds were recorded simultaneously using a B&K 1/4 inch microphone (see above) and a MiDAS DA-123 Data Acquisition Module (Cambridge, MA, USA). The relationship between body movements and sound production was analyzed using Xcitec MiDAS 2.0 software.

Reversible spiracular occlusions were performed to determine whether spiracles were involved in sound production and, if so, which ones. In the first occlusion experiment, five caterpillars that were tested and confirmed to produce sounds were anesthetized using carbon dioxide, and clear make-up latex (LR-1, Ben Nye Company, Los Angeles, CA, USA) was applied to all eight abdominal spiracles using the provided application brush. The latex would turn from white to clear when it had dried, which typically took less than 3 min. The larva was placed on its dorsal side once the latex had dried and tested for sound production 5 min after it had righted itself to ensure that all caterpillars tested were at the same level of alertness. To elicit sound production, a pinch was delivered to the abdomen, and the sound (or lack thereof) was recorded using a Sony ECM-MS908C microphone and Sony DCR-TRV19 camera (Tokyo, Japan). The latex was then gently removed from pairs of spiracles, and the caterpillar was re-tested for sound production. In a second experiment, 10 individuals were tested as above, but only the suspected sound-producing spiracles were covered. An additional control experiment was conducted on five separate individuals using the exact procedure as above but, instead, covering a non-sound-producing abdominal spiracle.

Laser Doppler vibrometry was used to test further the hypothesis that sound was coming out of a specific pair of spiracles, as well as to provide a visual record of air movements associated with these spiracles. A caterpillar was placed on a wooden stick so that the spiracle of interest or a control spiracle was positioned perpendicular

to a thin (2 mm) strip of lens paper (Ross Optical, Ladd Research Industries, Burlington, VT, USA) suspended across a wooden frame at a distance of 1 cm from the spiracle. The beam of a laser vibrometer (PDV-100 Polytec, Irvine, CA, USA) was reflected off the tissue paper using a reflective disc. Signaling was induced, and vibrations created by the sounds (or lack thereof) detected by the laser (Velocity 22 mm/s; high-pass-filter off; low-pass-filter 20 kHz) were recorded to a Marantz data recorder (PMD 671, D&M Professional, Itasca, IL, USA) at a sampling rate of 44.1 kHz.

Anatomy of spiracles

The external anatomy of the abdominal spiracles of *A. juglandis* was examined to assess size differences between sound- and non-sound-producing spiracles. Spiracles of five preserved caterpillars were photographed using an Olympus SZX12 (Olympus Corporation, Tokyo, Japan) light-microscope equipped with an AxioCam MRc5 (Carl Zeiss Micro Imaging GmbH, Göttingen, Germany) camera. Images were captured using AxioVision 4.6, and the length and width of spiracles were measured using ImageJ 1.42q (NIH, USA). Mean sizes of the sound-producing spiracle were compared with all others using an ANOVA, and *post hoc* analyses were conducted with a Tukey–Kramer honest significant difference test using JMP8 statistical software. For comparison, spiracles in *P. myops*, a similarly sized but silent species from the same subfamily as *A. juglandis*, were examined as outlined above.

Attack trials

Attack trials were performed to examine the relationship between attack, sound production and other defenses, and to assess the response of a predator to the sounds. Simulated attacks using forceps and natural attacks with an avian predator were conducted. All experiments were performed on caterpillars resting on a sprig of host-plant and videotaped using a Sony DCR-HC85 Handycam equipped with a Sony ECM-MS957 microphone. Analysis of the trials was performed using iMovie 7.1.4.

During simulated attack trials, larvae were isolated on sprigs at least 30 min before experimentation, and behaviours were recorded for 60 s before attack and 60 s following the commencement of the first attack. Attacks with blunt forceps were conducted to simulate an attack by a bird or the bite of a predatory insect (e.g. Bowers, 2003; Grant, 2006). Ten trials were performed where the resting caterpillar was attacked once near the head or abdomen and then quickly released. To assess further how an animal responded to multiple attacks, eight additional trials were performed where attacks were repeated five times, with an interval of ~5 s between successive attacks. Trials were analyzed for the number of animals that produced sounds when attacked, and comparisons (Mann–Whitney *U*) were made with respect to the number of pulses produced 60 s before, and 60 s following, a one- or five-pinch attack. Videos were also analyzed to document other defensive behaviours associated with sound production.

Predator trials were conducted with three captive yellow warblers (Parulidae, *Dendroica petechia*) at Queen's University in Kingston, Ontario, Canada. Yellow warblers are a likely predator of the walnut sphinx as this bird frequently eats caterpillars (Lowther et al., 1999), and overlaps in distribution with the walnut sphinx (American Ornithologists' Union, 1998; Tuttle, 2007). All yellow warblers were housed in individual cages (45.7 cm×45.7 cm×91.4 cm) with access to food (Exact Rainbow Canary/Finch diet, Kaytee Products, Chilton, WI, USA) and water *ad libitum*. The birds were accustomed to searching foliage in their cage for prey items and previously had been fed live caterpillars of various species. The birds were born in the wild but were raised in captivity from ~7 days old. For each trial, a caterpillar was placed on a twig inside the enclosure of the bird amidst the other vegetation and close to at least one perch. Trials began when the experimenter had moved away from the cage (~2 m) and ended when the bird no longer showed an interest in the caterpillar. One trial was conducted for each bird, and trials lasted 6–16 min. This work was approved by the Queen's University Animal Care and Use Committee (protocols 100340 and 100168) on birds that were held in captivity under Canadian Wildlife Service permit CA0243. Trials were analyzed for the defensive behaviour of the caterpillars (including sound production), the number of times the birds attacked, the latency to the first attack from the beginning of the trial, and the reactions of the birds to the sounds.

RESULTS

Walnut sphinx caterpillars consistently generated audible 'whistles' when attacked (see supplementary Movie 1), and sounds were sometimes accompanied by other defensive behaviours (see below). During our rearing and handling of the caterpillars, we noted no evidence of caterpillars responding acoustically to the presence of conspecifics, and sound production was not detected in early instars (1–3).

Sound characteristics

Temporal characteristics were measured from the first three pulse trains from 10 animals. Trains ranged from 90 to 8764 ms (mean 2626±2339 ms; median 1907 ms) in duration and contained 1–8 pulses (Fig. 1A) (*n*=30). Within a given trial, no significant differences were observed in the durations of the first and third trains (Mann–Whitney *U*, *P*=0.88). Pulse durations ranged between 44 and 2060 ms (mean 440±272 ms; median 420 ms) (*n*=101), and the mean inter-pulse interval was 501±282 ms (*n*=72). Not all pulses were the same in their spectral characteristics, and therefore we divided them into one of three categories (Fig. 1; see supplementary Movie 1 and supplementary Audio 1). Type 1 pulses (Fig. 1B) contain one or two multiharmonic series occurring near the beginning or middle of the pulse, with 16–48 harmonics per series. Type 2 pulses (Fig. 1C) were more broadband than type 1 pulses throughout the entire pulse, often (67%) having a short whistle at the very beginning or end of the pulse. Type 3 pulses (Fig. 1D) were characterized as pure whistles, with one or two harmonic series with two to five harmonics per series. There was a trend with respect to where each type occurred during a train, with all type 1 pulses occurring in the first third of a train, whereas 42.6% and 68.9% of types 2 and 3 occurred in the middle and final third of the train, respectively (*n*=34 trains; *N*=11). Characteristics of the broadband, multi-harmonic and pure whistle sounds from the various pulse types are displayed in Fig. 1B–D and Table 1. Waveforms differed between multi-harmonic, pure whistles and broadband sounds, with pure whistles having a significantly higher element-repetition rate than the other two sounds (*P*=0.0001; Mann–Whitney *U*). Multi-harmonic and more broadband sounds had more complex waveforms, with repetition rates that also differed significantly from one another (*P*=0.0001; Mann–Whitney *U*) (see Table 1).

Sound pressure levels of 10 pulses selected at random from five animals ranged from 69 to 82 dB SPL at 5 cm. The relative amplitude of pulses in a typical train declined from the beginning to the end, with the first being 12.9 dB greater on average than the last (*N*=10).

Sound production mechanism

Initial investigations into the mechanism of sound production involved videotaping body movements that accompanied sound
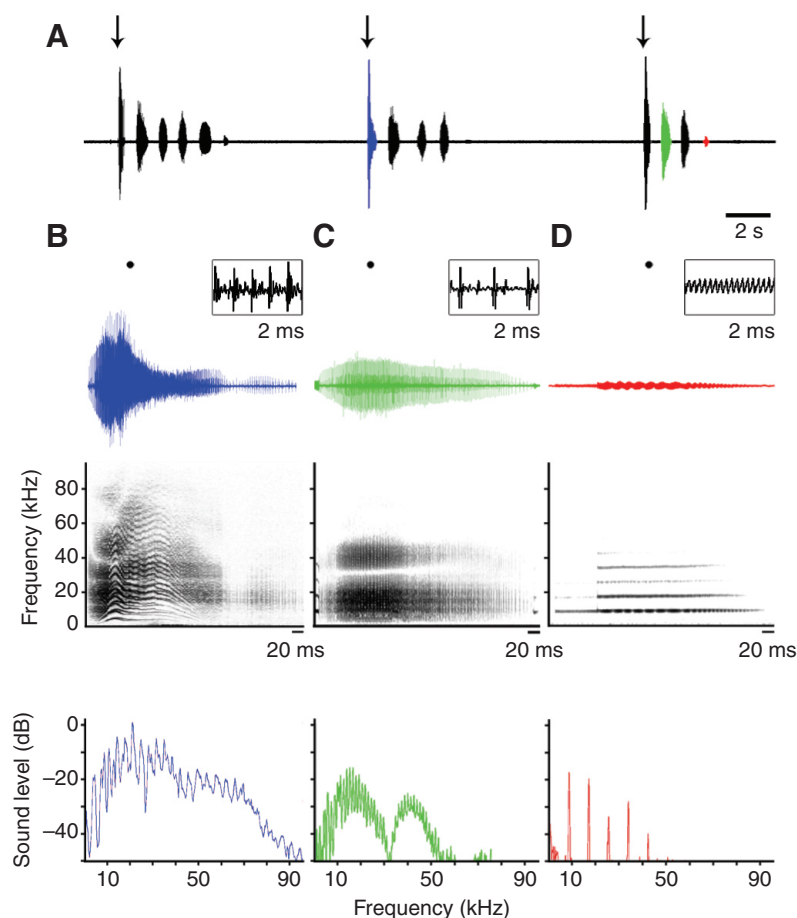
Fig. 1. *Amorpha juglandis* sounds recorded from fifth-instar larvae. (A) Oscillogram of three pulse trains following three consecutive attacks. Attack onset is marked with an arrow. (B–D) Oscillograms and corresponding spectrograms of time-expanded pulses from A, showing pulse types 1 (B), 2 (C) and 3 (D). Insets represent an expanded time-scale of the area represented by the black circle. Spectrograms and power spectra represent segments of the oscillogram marked by the black circle.

production. Sound production was always accompanied by contraction of the anterior body segments (thorax and first two abdominal segments) (Fig. 2). The contraction lasted 404±193 ms and began 98±111 ms before the onset of the sound (*N*=5). Following the contraction, it took 156±36 ms to return to full extension.

Occlusion experiments were conducted to determine, first, whether spiracles were involved in sound production and then to identify which spiracles were involved. When all abdominal spiracles were obstructed, sound production was eliminated in 100% of caterpillars, but, when the spiracles were uncovered, sound production resumed in 100% of the individuals (*N*=5). In a second experiment, we applied latex to only the A8 spiracles, and sound production was eliminated in all trials (*N*=10). Once the latex was removed, sound production returned in all trials. The ability to signal was never lost in animals where another set of spiracles had been blocked (A7, *N*=5).

Laser recordings (*N*=5) confirmed that sound is produced by the movement of air through the A8 spiracles (Fig. 3). In all five

Table 1. Spectral and temporal characteristics of different sound categories (mean ± s.d.)

	Multi-harmonic	Broadband	Whistle
Dominant frequency (kHz)	22.1±7.4	15.3±2.4	9.5±3.1
Fundamental frequency (kHz)	3.5±0.9	–	8.8±1.4
Number of elements in 10 ms	33.4±10.0	7.2±2.2	88.5±14.9
N	10	10	10

individuals, the laser registered a large-amplitude signal when placed in front of A8 but not when placed in front of the control spiracle (A5). There was evidence of a small amount of vibration when recording over A5 (Fig. 3), but this occurred regardless of whether that spiracle was exposed or covered with latex. Therefore, we conclude that these vibrations were being picked up indirectly from sounds produced by A8.

Spiracle anatomy
External measurements of the spiracles in *A. juglandis* revealed that A8 was longer than the other seven abdominal spiracles (A8 and A7, *P*=0.0007; A8 and all other spiracles *P*<0.0001) (Fig. 3A), and wider than one spiracle (A8 and A1, *P*=0.001; *N*=5). This pattern was not repeated in *P. myops*, where there was only a significant difference between the length and width of spiracles A8 and A1 (*P*=0.0007 and 0.0056, respectively; *N*=5). See supplementary Table S1 for spiracle measurements in both species.

Attack experiments
Simulated attacks consisted of either one or five pinches being delivered with blunt forceps (see supplementary Movie 1). In one-pinch attacks, sound production occurred in 90% of the trials (*N*=10), with an average of 5.2±3.9 pulses over a 60 s interval. Sound production was elicited in 100% of the five-pinch trials (*N*=8), with an average of 9.3±7.8 sound pulses over a 60 s interval. There was a significant difference in the number of pulses produced in the 60 s interval preceding and following a one-pinch attack (*P*=0.0002) and preceding and following a five-pinch attack (*P*=0.0003). The number of pulses during the 60 s interval following a one- or five-
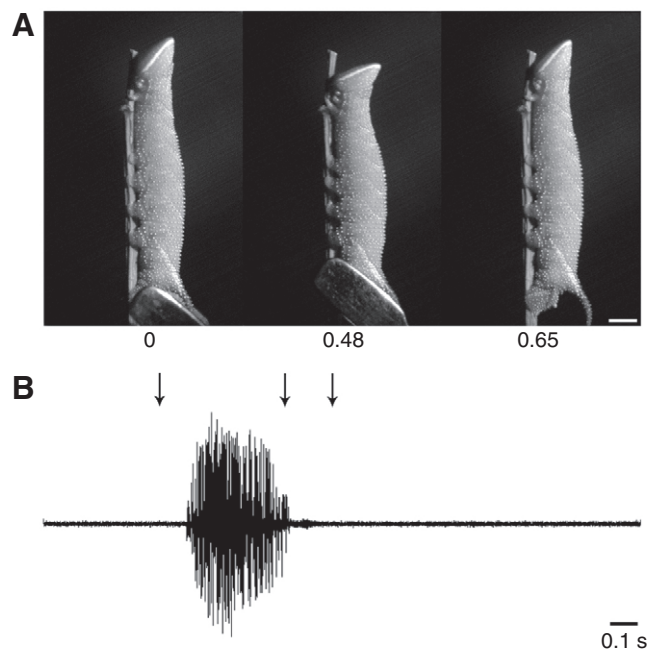
Fig. 2. Body movements associated with sound production. (A) High-speed video frames of a larva during sound production showing, from left to right, the beginning of compression, full compression and full extension (scale bar, 2.5 mm). Times of occurrence for each frame are indicated below each frame. (B) Oscillogram of the associated sound, with arrows marking the occurrence of the frames in A.
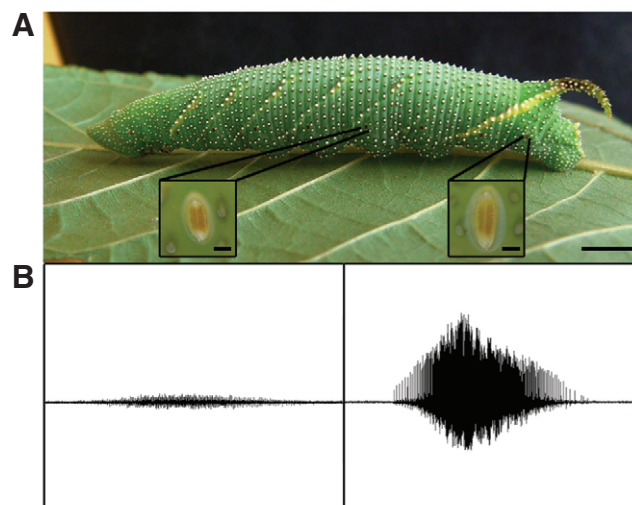

Fig. 3. Spiracle anatomy and air expulsion. (A) A fifth-instar caterpillar (scale bar, 5 mm) showing the fifth and eighth abdominal spiracles magnified in the inset (inset scale bars, 0.25 mm). (B) Oscillograms obtained from laser recordings showing the movement of air produced by a sound pulse when either positioned above the fifth (left) or eighth (right) spiracles.

pinch attack was not significant (P=0.3488; Mann–Whitney U). When attacks were directed to the posterior abdomen, the caterpillar would release its first whistle while thrashing once towards the site of attack, presumably intending to bite the attacker. The caterpillar would then return to its resting position and continue its sound pulse train coupled with the telescoping body movements. Anterior attacks elicited the same series of behaviours except that the caterpillar would flick its anterior body away from the pinch instead of towards it. Regurgitation was observed only once during a one-pinch trial and was not observed in any of the five-pinch trials or any of the multi-attack B&K trials conducted for sound recordings.

Trials with yellow warblers were performed to assess the behaviours of both the caterpillar and an avian predator. An attack event was defined as the bird pecking at the caterpillar one or more times in a row following an inspection. Each of the three birds attacked the caterpillar more than once (2.7±0.6 times; N=3), and each attack provoked the caterpillar to make sounds. Acoustic responses were similar to those in the simulated attacks, with train durations of 2800±2250 ms, and 1–8 pulses per train (N=3; n=11). The larva never produced sound before the initial attack in a trial. However, once each bird provoked the caterpillar to produce sound by attacking it, the caterpillar became sensitized and subsequent sound trains could be evoked simply by leaf movements produced by the approaching bird. Thrashing responses by the caterpillar were similar to those observed in forceps attacks, whereby attacks to the anterior caused the caterpillar to flick its head away from the bird, and posterior attacks caused the caterpillar to arc its head dorsally towards the site of attack. As far as we could determine, in each trial, there was no evidence of caterpillar regurgitation.

Following the onset of a trial, the average latency to the initial attack by birds was 140±112 s. The birds responded to the first train of sounds by flinching, cocking their heads and inspecting, and eventually moving away from the caterpillar by hopping or flying away. Each bird attempted a second attack 223±98 s after it had moved away from the caterpillar, and responded to the sounds of the caterpillar by again moving away. In one case, the response was dramatic, with the bird diving down and away from the caterpillar into thicker vegetation, as if attacked by a predator (Gaddis, 1980; Lima, 1993) (see Fig. 4 and supplementary Movie 2). In each case, the caterpillar remained unmolested in the cage with the bird for an average of 8 min 59 s ± 5 min 49 s after the last attack, and trials were terminated when the bird no longer showed an interest in the caterpillar. Caterpillars involved in our trials survived the attacks by yellow warblers and exhibited no obvious wounds from the attacks.

In summary, during all of the attack trials, sound production was the most consistent response to attack. Upon the first attack during a trial, sound production was accompanied by directed thrashing, but, upon subsequent attacks, sound production was not necessarily accompanied by thrashing. Regurgitation was rare during attack trials, occurring only in 3% (1/31) of all trials (1 of 18 forceps trials, none of three bird trials, and none of 10 multi-attack trials conducted for the B&K sound trials). However, we noted that, outside of formal attack trials, regurgitation was observed in situations when it was necessary to handle the caterpillars continually for extended periods, such as when they were being set up for occlusion trials or when changing their food in their enclosures. During these times, caterpillars would regurgitate more readily and try to bite, but such behaviours, typically, were not accompanied by sound production.

DISCUSSION

Sound production and hearing have evolved multiple times in adult Lepidoptera (moths and butterflies), and research on the topic has contributed to the fields of neuroethology, ecology and animal behaviour (Conner, 1999; Minet and Surlykke, 2003; Yack, 2004). However, comparatively little is known about the roles that sounds and vibrations play in larval Lepidoptera, although a growing body of literature suggests that acoustic communication is widespread (Tautz and Markl, 1978; Travassos and Pierce, 2000; Yack et al.,

A

Inspection (*t*=0 s) Attack (*t*=0.15 s) Dive (*t*=0.33 s) Dive continues (*t*=0.43 s)
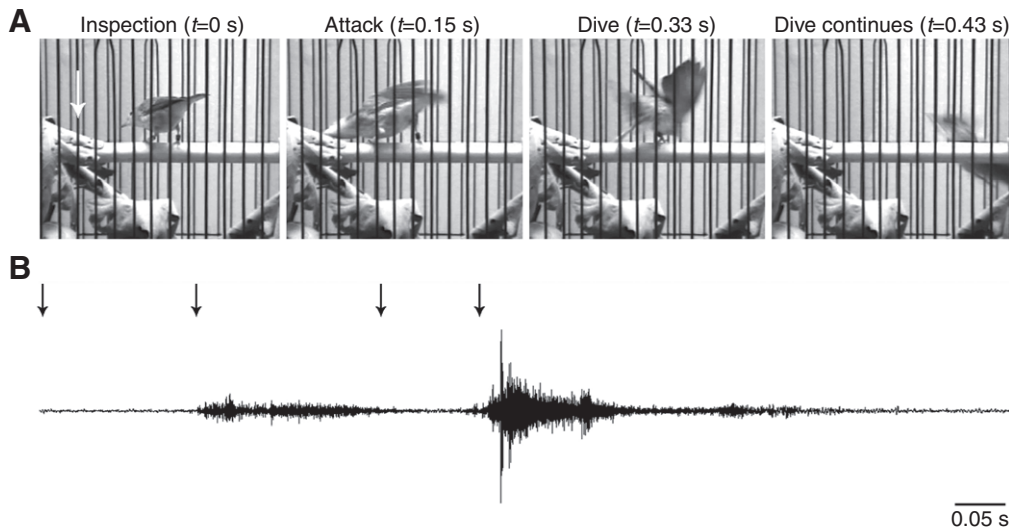
B

0.05 s

Fig. 4. Reaction of a yellow warbler to the defensive sounds of the walnut sphinx caterpillar. (A) Video frames of the encounter, with the time of occurrence appended in parentheses. The white arrow in frame 1 indicates the position of the caterpillar. (B) Oscillogram of the caterpillar sounds, with arrows corresponding to the onset of each video frame shown in A. The sound pulse following the fourth arrow is the noise produced by the retreating bird.

2001; Castellanos and Barbosa, 2006; Fletcher et al., 2006; Scott et al., 2010). Although naturalists have described the defensive sounds produced by silk and hawkmoth (Bombycoidea) caterpillars (reviewed in Brown et al., 2007), the function and mechanism of sound production in these caterpillars remain largely unknown. We describe a novel form of sound production – whistling – and discuss its possible function.

Sound production mechanism

Our results support the hypothesis that the sounds of walnut sphinx caterpillars are produced by air expulsion. Reports on this mode of sound production in insects are extremely rare (Haskell, 1974; Ewing, 1989), with few studied examples. The death's-head hawkmoth, *Acherontia atropos*, produces a two-part cry by the movement of air in and out of the pharyngeal cavity (Busnel and Dumortier, 1959; Dumortier, 1963). The first part of the cry occurs as air moves through the proboscis and past the epipharynx, causing it to vibrate. The second part is caused when the epipharynx is raised and the air is forced out through the proboscis. Madagascar hissing cockroaches of the genera *Gromphadorhina* and *Elliptorhina* produce sounds by forcing air through an area of modified trachea and a pair of specialized spiracles (Nelson, 1979; Sueur and Aubin, 2006). In the walnut sphinx, we show that sounds are produced by air being forced out of the last abdominal spiracle (A8). Occlusion of the A8 spiracles (but not other spiracles) eliminates sound production, and air movements are observed when tissue is placed over these spiracles. We propose that the sound-producing mechanism operates similarly to that reported for hissing cockroaches (Nelson, 1979). In the cockroach, hisses are generated when the animal closes all spiracles, except the enlarged fourth abdominal spiracle, and forces air out the latter by contracting the abdominal expiratory muscles. In the walnut sphinx, we propose that sounds are produced by air being forced out of the enlarged A8 spiracles during successive contractions of the anterior dorso- and ventro-longitudinal muscles, while all other spiracles are maintained in a closed position. The variation in frequency structure observed between pulse types is probably related to blowing pressure as changing forces have an effect on frequency properties of sounds (Bradbury and Vehrencamp, 1998). In the walnut sphinx, multiharmonic type 1 pulses, with their complex waveforms, occur near the beginning of the pulse trains, where the force is greatest, whereas pure whistles occur at the end of trains, where the pulses

are less intense. The size of the spiracle opening might also affect the frequency structure. Broadband hisses produced by cockroaches occur when the spiracle is completely open, whereas pure whistles occur when the spiracle is partially closed (Fraser and Nelson, 1982). Based on the resemblance of types 1 and 2 pulses to the hisses, and type 3 to the whistles, of cockroaches, a similar mechanism is possibly being employed. The specific mechanisms underlying sound production in the walnut sphinx could be further explored using anatomical and neurophysiological studies of the respiratory system, as well as visualization of internal trachea during sound production using X-ray imaging in live animals (Westneat et al., 2003).

Function

We conclude that sound production in the walnut sphinx functions in defense because an acoustic response is elicited consistently following attack by an artificial or natural predator. Defense sounds are commonly reported in insects (Masters, 1979) and are hypothesized to serve a variety of different functions, including startle [e.g. peacock butterfly (Mohl and Miller, 1976); tiger beetles (Yager and Spangler, 1997)], warning or aposematism [e.g. stridulation in many insects (Masters, 1979); hissing in bumblebees (Kirchner and Röschard, 1999)], mimicry [e.g. Batesian, drone flies, hoverflies and Hymenoptera (Brower and Brower, 1965; Rashed et al., 2009); Mullerian, tiger moths (Barber and Conner, 2007)], jamming [e.g. tiger moths (Conner, 1999; Corcoran et al., 2009)] and recruitment of conspecifics [e.g. hissing in honey bees (Sen Sarma et al., 2002)]. In the following discussion, we argue in favour of the hypothesis that sounds made by walnut sphinx caterpillars function to startle vertebrate predators.

Before further discussing the function of defensive sounds, it is important to consider which predators the signals are directed towards. To the best of our knowledge, there are no reports describing the predators of walnut sphinx caterpillars. However, we assume that they are consumed by the same predators as those of other hawkmoth caterpillars, including birds as a major predator, as well as bats, mice, shrews, wasps, ants, beetles and spiders (Pittaway, 1993; Tuttle, 2007). The whistles produced by *A. juglandis* overlap with the frequency range of hearing in most birds (Dooling, 1991), and our results show that yellow warblers, a likely predator of the walnut sphinx, react to the caterpillar sounds. Caterpillar whistles also have significant energy in the ultrasound

range and, thus, could be directed at gleaning bats and mice. There is evidence that bats eat large caterpillars (Kalka and Kalko, 2006; Wilson and Barclay, 2006), and mice are repelled by sounds produced by insects with similar sound frequencies [e.g. bee hissing (Kirchner and Röschard, 1999)]. Common arthropod predators that attack caterpillars, including wasps, ants, carabid and coccinellid beetles, bugs and spiders (Stamp and Wilkens, 1993), lack tympanal ears (Yack, 2004), and we therefore conclude that the defensive sounds of caterpillars target primarily vertebrate predators.

We propose that defensive whistles function to startle predators and thereby reduce the risk of predation. Startle displays typically have three features (Edmunds, 1974; Sargent, 1990; Ruxton et al., 2004). First, they are employed by the prey only after being disturbed. In support of this, walnut sphinx caterpillars whistled only after being attacked, relying on crypsis as a primary defense. Second, the display is conspicuous, causing the predator to hesitate momentarily while the prey has an opportunity to escape. The intimidating nature of the sounds produced by walnut sphinx was evident from the reaction of the yellow warblers. The sounds were loud (i.e. conspicuous) and caused the birds to hesitate or abandon their attacks on the caterpillar. In one case, the bird dived away from the caterpillar into thicker vegetation in a manner similar to how it would react to a predator (Gaddis, 1980; Lima, 1993). Contrary to what is predicted for startle sounds, however, none of the caterpillars attempted to escape. This could be explained by the fact that escape is not a viable option for large caterpillars. They cannot run or fly away, and, although dropping from the leaf on silk is a common defense strategy for some caterpillars, this has not been observed in the Bombycoidea (Sugiura and Yamazaki, 2006). Presumably, large caterpillars do not drop on silk strands because a silk strand cannot support their weight, and dropping to the ground is both energetically costly and dangerous (Dethier, 1959). Finally, the prey is not otherwise defended, and therefore predators will habituate to the signals.

During attack trials with forceps or birds, regurgitation was extremely rare – during all 31 trials (including 18 formal attack trials with forceps, 10 forceps attacks during the B&K trials and three bird trials), a caterpillar regurgitated only once (3.2%). Grant (Grant, 2006) categorizes caterpillars according to how much they regurgitate in response to attacks with forceps: primary regurgitators regurgitate soon after attack (1–3 pinches), can control the amount released and the direction it is aimed and re-imbibe the bubble when attacks cease. Secondary regurgitators (4–6 pinches) will regurgitate after thrashing, biting or trying to escape, cannot control the amount released and do not re-imbibe. Finally, non-regurgitators will regurgitate only after long-lasting attacks (8–10 pinches, if at all), and this might be a result of exhaustion or stress rather than a true defense. According to this classification scheme, the walnut sphinx would be classified as a non-regurgitator. This is in contrast to previous studies of sound production in caterpillars, where sound is strongly correlated with the occurrence of a chemical defense and believed to be aposematic (Brown et al., 2007; Bura et al., 2009).

In the absence of a chemical or physical defense, it is argued that attackers will become habituated to the signals and eventually eat the caterpillar. However, the yellow warblers in this study were clearly deterred by the sounds and did not habituate during the course of the trial sufficiently to return and eat the caterpillar. Although certain predators might become habituated to startle displays in experimental studies, the type of predator and the frequency of encountering the display will dictate how the predator responds (e.g. Bates and Fenton, 1990; Vallin et al., 2005; Vallin et al., 2007). In nature, yellow warblers are active foragers,

constantly moving through vegetation in their territories in search of food [average territory size across their breeding range varied from 0.04 to 0.39 ha (Lowther et al., 1999)]. Thus, if a warbler is deterred from eating a caterpillar during an attack, the same bird is unlikely to encounter the caterpillar again soon afterwards. In addition, foraging time is frequently limited for birds, and they must weigh the costs and benefits of potentially dangerous prey while being vigilant for predators themselves (Kaby and Lind, 2003). Thus, we suggest that, if foraging yellow warblers encountered prey that produce a startling sound in the wild, these birds would move on to other food rather than risk a prolonged attack on a potentially dangerous food item. A similar argument has been used recently to explain the efficacy of eye-spot displays in tropical caterpillars (Janzen et al., 2010).

In conclusion, caterpillars of the walnut sphinx produce whistles by forcing air through the eighth abdominal spiracles. We hypothesize that caterpillars have specific control over individual spiracles such that all but the eighth pair are closed during body contractions. Further studies on the respiratory system will lend insights into the specific modifications to the nervous, tracheal and musculature systems that have accompanied the evolution of sound production. We conclude that whistles function as part of the defensive repertoire of the larvae and propose, based on our results to date, that these sounds protect the caterpillar by startling predators. This hypothesis could be further tested with predator trials on muted caterpillars to determine whether sound alone is sufficient to deter predation, or with predator trials on undefended and palatable food items while broadcasting walnut sphinx sounds. Future studies with natural predators of different species, including arthropods, will help to clarify the specific role, or roles, that whistling plays in the survival of these caterpillars.

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	A1	A2	A3	A4	A5	A6	A7	A8
A. juglandis length	0.59±0.05	0.63±0.06	0.65±0.06	0.67±0.06	0.67±0.05	0.69±0.06	0.76±0.08	0.95±0.07
A. juglandis width	0.23±0.03	0.27±0.05	0.30±0.04	0.29±0.06	0.31±0.03	0.32±0.03	0.36±0.03	0.34±0.02
P. myops length	0.53±0.04	0.60±0.04	0.62±0.04	0.61±0.06	0.61±0.05	0.64±0.05	0.68±0.07	0.70±0.06
P. myops width	0.24±0.02	0.29±0.04	0.26±0.02	0.27±0.02	0.27±0.02	0.28±0.03	0.27±0.03	0.31±0.04

Table S1. Abdominal spiracle sizes in mm (means ± s.d.) for *A. juglandis* and *P. myops*