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Clicking caterpillars: acoustic aposematism in *Antheraea polyphemus* and other Bombycoidea

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Summary

Acoustic signals produced by caterpillars have been documented for over 100 years, but in the majority of cases their significance is unknown. This study is the first to experimentally examine the phenomenon of audible sound production in larval Lepidoptera, focusing on a common silkmoth caterpillar, *Antheraea polyphemus* (Saturniidae). Larvae produce airborne sounds, resembling 'clicks', with their mandibles. Larvae typically signal multiple times in quick succession, producing trains that last over 1 min and include 50–55 clicks. Individual clicks within a train are on average 24.7 ms in duration, often consisting of multiple components. Clicks are audible in a quiet room, measuring 58.1–78.8 dB peSPL at 10 cm. They exhibit a broadband frequency that extends into the ultrasound spectrum, with most energy between 8 and 18 kHz. Our hypothesis that clicks function as acoustic aposematic signals, was supported by several lines of evidence. Experiments with forceps and domestic chicks correlated sound production with attack, and an increase in attack rate was positively correlated with the number of signals produced. In addition, sound production typically preceded or accompanied defensive regurgitation. Bioassays with invertebrates (ants) and vertebrates (mice) revealed that the regurgitant is deterrent to would-be predators. Comparative evidence revealed that other Bombycoidea species, including *Actias luna* (Saturniidae) and *Manduca sexta* (Sphingidae), also produce airborne sounds upon attack, and that these sounds precede regurgitation. The prevalence and adaptive significance of warning sounds in caterpillars is discussed.

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Key words: caterpillar, acoustic aposematism, sound production, regurgitation, Bombycoidea, Lepidoptera, *Antheraea polyphemus*.

Introduction

Acoustic communication in Lepidoptera has been an important area of scientific investigation, with over 200 published reports on the subject (Minet and Surlykke, 2003). To date, research has focused primarily on hearing and sound production in adults. In moths and butterflies, tympanal ears have evolved independently at least 7 times and function primarily for detecting the ultrasonic cries of insectivorous bats (Hasenfuss, 2000; Minet and Surlykke, 2003). Several species of adult Lepidoptera also produce sounds that are used in the context of social communication and bat defense (Spangler, 1986; Conner, 1999; Miller and Surlykke, 2001; Minet and Surlykke, 2003).

Comparatively little is known about the role of acoustics in larval Lepidoptera. Currently, there are only a few well-defined examples of sound production or reception (including air- and solid-borne vibrations) in caterpillars. Lycaenidae and Riodinidae butterfly larvae employ vibrational signals in mutualistic relationships with ants (DeVries, 1990; DeVries, 1991; Travassos and Pierce, 2000); some Drepanidae and Gracillariidae moth larvae use vibrations to dispute territorial ownership of leaf shelters with conspecifics (Yack et al., 2001; Fletcher et al., 2006); and some Noctuoidea and Gracillariidae moth larvae detect near-field sounds or seismic vibrations produced by insect predators and parasitoids (Meyhöfer et al., 1997; Tautz and Markl, 1978).

In addition to these experimentally tested examples, an extensive review of the literature on this topic has revealed many preliminary reports that caterpillars communicate acoustically. These represent species from at least 12 families including, for example, Tortricidae (Russ, 1969), Oecophoridae (Hunter, 1987), Notodontidae (Dumortier, 1963), Saturniidae (Federley, 1905) and Sphingidae (Sanborn, 1868). In most cases, the acoustic signals have not been characterized and behavioural evidence for the context in which the signals are produced is absent.

One interesting phenomenon is that of 'clicking' caterpillars from the superfamily Bombycoidea. Several species belonging

to the silkmoth (Saturniidae) and hawkmoth (Sphingidae) families have been described to produce airborne sounds audible to the human ear (e.g. Sanborn, 1868; Mead, 1869; Pearce, 1886; Packard, 1904; Eliot and Soule, 1902; Federley, 1905; Dumortier, 1963; Wagner, 2005). Although usually described as 'clicking', they have been also described as 'squeaking' or 'crackling'. In cases where the sound production mechanism has been postulated, it is generally believed that sounds originate from the mandibles. However, this has not been confirmed experimentally. Similarly, the function of these acoustic signals has not been studied, but has been suggested to play a role in defense (Federley, 1905) or social communication (Wagner, 2005).

In this study we explore the mechanism and function of caterpillar clicks by focusing primarily on one species, *Antheraea polyphemus* Cramer (Fig. 1), a silkmoth that occurs throughout deciduous forests, orchards and wetlands of North America. The large, cryptic larvae feed on a variety of tree leaves including oak (*Quercus*), maple (*Acer*), willow (*Salix*) and birch (*Betula*) (Milne and Milne, 1980). The adult has been widely studied for its olfactory system, but little is known about the behaviour and life history of the caterpillar. Sound production by late instar larvae has been previously reported (e.g. Eliot and Soule, 1902; Federley, 1905; Wagner, 2005). Federley noted that third and fourth instars use their mandibles to produce a "*tolerably loud, tapping sound*" (Federley, 1905). He continues, "*that here is question of a means of intimidation is not to be doubted, for if the larva is left in peace it keeps perfectly quiet, but when the larva cage is touched, or the larvae are taken out, they make this peculiar tapping sound, resembling the ticking of a watch*". This leads to a puzzling question as to why a cryptically coloured caterpillar would evolve the ability to produce sound, a trait which no doubt draws attention to itself.

Animal defense sounds are common in nature. They are often categorized as either startle or warning (Masters, 1979), although in some instances, these are not mutually exclusive. Startle sounds function to alarm a potential predator, causing it to hesitate momentarily. As a result, the delay of the attack helps increase the likelihood of a prey's escape. For example, torpid peacock butterflies *Inachis io* produce intense ultrasonic clicks that startle bats, providing opportunity for the butterflies to flee (Møhl and Miller, 1976). Conversely, warning sounds function to advertise the unprofitability of a prey item to a potential predator (Masters, 1980), the acoustic equivalent of aposematic colouration. For example, certain tiger moths (Arctiidae) produce sound that is effective in warning big brown bats that the moths are unpalatable (Hristov and Conner, 2005).

During preliminary investigations we noted that clicking in *A. polyphemus* larvae is commonly associated with both disturbance and regurgitation. We hypothesize that clicking functions as an acoustic aposematic signal to an impending regurgitant defense. If *A. polyphemus* larvae are acoustically aposematic, then the following predictions will be supported: (i) sound production will be associated with a predator attack, (ii) an increase in attack rate will be positively correlated with an increase in signaling, (iii) natural predators should be capable of hearing the acoustic signal, (iv) the regurgitant will be adverse to predators and (v) the acoustic signal will most often precede or accompany regurgitation. In investigating the acoustic behaviour of *A. polyphemus* larvae, our objectives for this study are threefold: (i) to identify the mechanism of sound production, (ii) to characterize the acoustic properties of these signals and (iii) to experimentally test the function of these sounds. In addition, we have examined the distribution of this phenomenon in other Bombycoidea by reviewing the literature and testing an additional 12 species. Our results are discussed with respect to the general function and evolutionary significance of acoustic warning signals in caterpillars.

Fig. 1. A fifth instar *Antheraea polyphemus* larva. Scale bar, 0.5 cm.

Materials and methods

Animals

Antheraea polyphemus Cramer larvae were obtained from several sources. In Canada, eggs were obtained from wild-caught females collected at the Mer Bleue Conservation Area in Ottawa, ON (NCC permit #3654), or purchased from Bill Oehlke (Montague, PEI). In the USA, eggs were obtained from wild-caught females captured in East Bridgewater, MA, or from second generation lab stock reared from females collected near Cape Cod, MA. Larvae were reared on cuttings of red oak (*Quercus rubra*), paper birch (*Betula papyrifera*) or sugar maple (*Acer saccharum*) maintained in indoor enclosures. All larvae used in experiments were in their third to fifth instars. However, a preliminary investigation on sound-producing ability was made on first and second instars as well.

Species used for the comparative study were obtained from a variety of sources and were selected merely on the basis of their availability, and if they were Bombycoidea. Eggs were collected from wild-caught female *Actias luna, Dryocampa rubicunda*, *Pachysphinx modesta*, *Smerinthus cerisyi* and

Smerinthus jamaicensis moths at Mer Bleue Conservation Area. Eggs of *Manduca sexta* were donated by Shannon Meisner from a lab colony at Dalhousie University, NS, Canada, or purchased from LiveFood (Mercier, QC, Canada). Eggs of *Automeris io*, *Callosamia promethea* and *Hyalophora cecropia* were purchased from Bill Oehlke. Larvae of *Bombyx mori*, *Hyles euphorbiae* and *Mimas tiliae* were donated by Colleen Helferty, Naomi Cappuccino and Jacob Miall, respectively. Larvae were reared on their respective host plants (refer to Table 2). All larvae used in experiments were in their third to fifth instars.

Sound production

Video analysis and scanning electron microscopy of mouthparts were used to confirm and examine the mechanism of sound production. Head capsules of larvae were pinched with forceps to induce signaling. Images and sounds were acquired with a Digital Handicam (Sony TR7000, Tokyo, Japan) equipped with a zoom lens and a Sony audio ECM-MS907 microphone. The presence of sound in conjunction with mandibular movement was determined using iMovie 3.0.3. Dissected mandibles from fourth instar larvae were sputter-coated with gold–palladium and examined using a JOEL JSM-6400 scanning electron microscope (Tokyo, Japan).

Sounds were recorded to examine their temporal, spectral and intensity characteristics. All recordings were performed in an acoustic chamber (Eckel Industries Ltd., Cambridge, MA, USA) located at Carleton University. For temporal analysis, airborne sounds were recorded with a Sony DAT PCM-M1 at a sampling rate of 48 kHz, using a Sony ECM-MS957 microphone placed 10 cm from the heads of fifth instar larvae. Temporal qualities, such as the duration of clicks and the number of components within a click, were measured using Canary Bioacoustics Research Program (Cornell Laboratory of Ornithology, Ithaca, NY, USA).

For spectral analysis, sounds were recorded using a Brüel & Kjær 1/4″ microphone type 4939 (Naerum, Denmark) placed 10 cm from the mouth of larvae. Sounds were amplified with a Brüel & Kjær Nexus conditioning amplifier type 2690, recorded onto a Fostex FR-2 Field Memory Recorder (Gardena, CA, USA) at a sampling rate of 88.2 kHz, and subsequently analyzed with Raven Bioacoustics Research Program (Cornell Laboratory of Ornithology, Ithaca, NY, USA). Spectra were produced using a 512-point Fast Fourier Transform (FFT) (Hanning window). For determining peak frequency and bandwidth, sounds were captured on a Tektronix THS720A oscilloscope (Beaverton, ON, USA) and the spectral qualities were visualized using the FFT setting (Hanning window). In addition to measuring the peak frequency of clicks (arbitrarily defined as 0 dB), the bandwidth was characterized by measuring two quality factors at –12 dB and –18 dB below peak frequency. In some instances, a quality factor at –18 dB was not measured if the spectrum was extremely broadband.

Sound intensities were determined using the method outlined elsewhere (Stapells et al., 1982). Clicks were recorded from larvae placed 10 cm from a Brüel & Kjær 1/4″ microphone type 4939, and amplitudes measured as voltages on a Tektronix THS720A oscilloscope. A continuous pure tone centered at the mean peak frequency of clicks was generated with a Tabor Electronics 50MS/s Waveform Generator WW5061 (Tel Hanan, Israel) coupled to a Brüel & Kjær Nexus conditioning amplifier type 2690 and broadcast through a Pioneer ART-54F Ribbon tweeter (Pioneer Electronics, Long Beach, USA). The peak-to-peak intensity of the signal was adjusted until the output voltage was equal to that of the clicks emitted by the caterpillars. The dB peSPL values at 10 cm were then read from a Brüel & Kjær sound level meter type 2239 placed at the same location as the microphone.

Attack experiments

A pinch with forceps is commonly used to simulate an attack by a bird or the mandible bite of a predaceous insect (e.g. Stamp, 1986; Cornell et al., 1987; Bowers, 2003; Grant, 2006). Prior to the commencement of a simulated predator attack, single *A. polyphemus* larvae were kept on leaf sprigs for a minimum of 1 h. Using forceps, the head capsules were pinched either once, twice or five times, with approximately 5 s intervals between each pinch. The defensive behaviours of the larvae were monitored using a Sony Mini-DV DCR-TRV19 Handicam, and a Sony audio ECM-MS907 microphone placed 3–4 cm away from the heads of the larvae. Trials were analyzed using iMovie 3.0.3 to quantify (i) the mean number of clicks in a train, (ii) the mean length of a click train, (iii) the mean number of clicks in 60 s following one, two or five pinches, (iv) the prevalence of clicking and regurgitation with respect to the number of pinches administered and (v) the onset of signaling with respect to regurgitation.

We devised an additional experiment in which the defensive behaviour of the larvae could be documented when attacked by an avian predator. Sixteen newborn male domestic chicks (*Gallus gallus domesticus*) were obtained from a commercial hatchery and housed in a 1.5 m×2.4 m chicken coup in Carp, ON, Canada. The chicks were maintained at 20–25°C using heat lamps, and water and chick starter crumb were provided *ad libitum*. Twice a week chicks were fed mealworms (*Tenebrio molitor*) to accustom them to live prey. Trials were performed when the chicks were between 35 and 45 days old. Chicks were deprived of food 12 h prior to testing. The testing apparatus consisted of a cardboard box measuring 0.5 m×0.5 m×0.6 m (length×width×height) with the floor of the box removed. A 0.1 m×0.1 m (length×width) cut-out was made at 0.2 m from the base of the box to create a viewing hole for a Sony Mini-DV DCR-TRV19 Handicam. A Sony audio ECM-MS907 microphone was clamped approximately 5 cm from a styrofoam platform located adjacent to the viewing hole. At the beginning of each trial, a single chick was placed inside the testing apparatus. A single fifth instar larva was then placed on an oak sprig taped to the platform. Video footage, noting the presence or absence of larval signaling and regurgitation during an attack, was analyzed using iMovie 3.0.3. In addition, we quantified the mean number of clicks in 60 s produced by the larvae following a single attack. The procedure outlined

above was approved by Carleton University's Animal Care Committee (protocol I.D. #B05-8). Larvae or chicks were not re-used from trial to trial.

Invertebrate bioassay

Should the acoustic signals serve as a warning to an impending regurgitant defense, it is meaningful to demonstrate that the regurgitant is deterrent to would-be predators. In nature, *A. polyphemus* larvae are attacked by a diversity of predators including ants, praying mantids, spiders, birds and small mammals (Passoa, 1999). In addition, several species of wasps and flies have been identified as host-specific parasitoids of the larvae (Peigler, 1994). To determine the palatability of the regurgitant to an invertebrate predator, we employed a bioassay modified from Peterson et al. (Peterson et al., 1987). An ant colony composed of two predatory *Formica* species (Formicidae) was located on a grassy lawn in Ottawa, ON, Canada. In a clean Petri dish, mealworms were cut into segments measuring between 5–10 mm. Each segment was transferred with forceps to a beaker containing freshly collected *A. polyphemus* regurgitant (from larvae fed on red oak), and the coated segment was placed within 2–3 cm of an ant hole. Trials were videotaped with a Sony Mini-DV DCR-TRV19 Handicam. Two sets of control trials were performed whereby mealworm segments were not covered in regurgitant at all, or were covered in distilled water.

Analysis of trials determined (i) the number of mealworm segments rejected (i.e. left on the foraging grounds and not carried into an ant hole after more than 1 h following first contact), (ii) the mean time, following first contact, to carry mealworm segments into ant holes and (iii) the presence or absence of antennal preening during the first 60 s following first contact. Mann–Whitney *U* tests determined whether acceptance times differed significantly between experimental and control trial conditions.

Vertebrate bioassay

Because there is an acoustic component to the defensive response of *A. polyphemus* larvae, it can be reasoned that the signal is directed towards a hearing predator. Although many insects possess tympanal hearing organs (Hoy and Robert, 1996; Yager, 1999; Yack, 2004), hearing has not been reported for invertebrates known to attack *A. polyphemus* larvae (except for mantids; see Discussion). Therefore, we presume that a vertebrate predator such as a bird or mammal would most likely be the intended receiver of the acoustic signal. As a result, we devised an additional bioassay in which we could determine the palatability of the regurgitant to a vertebrate predator. Ten male domestic mice *Mus musculus* (strain CD-1), were obtained at 32-days old from a commercial supplier and housed in a vivarium located at Carleton University. Mice were kept individually in metal cages measuring 29.5 cm×18.2 cm×12.4 cm (length×width×height), and maintained on 5075 non-autoclave rodent chow (Charles River, Wilmington, MA, USA) on a 12 h:12 h light:dark cycle. Water was available *ad libitum*. Prior to experimentation, mice were housed in their respective cages for 7 days to ensure they had acclimated to their feeding and drinking stations. All animals were food deprived 6 h prior to testing.

Two glass food cups (7 cm in diameter and 4 cm tall) were placed 2 cm apart at one end of the cage, opposite the water spout. In one cup, a pre-weighed quantity of chow was coated in 6 ml of fresh regurgitant collected from larvae fed on red oak. In a second cup, a pre-weighed quantity of chow was coated in 6 ml of distilled water. In nine out of ten trials, both cups contained a quantity of food that approximated one another in terms of total mass (dry weight) by at least 96%. In one case, this value dropped to 87%. In half of the trials, the position of the cups was reversed to control for position preferences. Each mouse was subjected to an 18 h, two-choice test. The procedure outlined above was approved by Carleton University's Animal Care Committee (protocol I.D. #B06-10). Intake from the cups was quantified by mass at the completion of an experiment. For each cup, the amount of food consumed was divided by the total amount of food offered. This value was then expressed as percent consumption. To determine preference, a Mann–Whitney *U* test was used to examine whether consumption differed significantly between the two treatments.

Comparative study of other caterpillar species

Larvae acquired for the comparative study were observed for the ability to signal acoustically and/or regurgitate upon disturbance. Using forceps, the heads of larvae were pinched five times, with approximately 5 s intervals between each pinch. Defensive behaviours were recorded onto video using methods previously described. Trials were analyzed to examine the presence of acoustic signaling, the presence of regurgitation and the onset of signaling with respect to regurgitation. The temporal characteristics of the sounds, including the duration of clicks and the number of components within a click, were determined using Canary Bioacoustics Research Program. The method used for determining peak frequency of clicks was previously described for *A. polyphemus* larvae.

Results

Sound production

A variety of methods induced *A. polyphemus* larvae to produce sound, including jostling the enclosure in which the larvae reside, blowing on the larvae or pinching the body surface. The most reliable way to induce sound production, however, was by squeezing either side of the larvae's head capsules with forceps (supplementary material, Movie 1). Video footage acquired from sound-producing larvae confirmed earlier suggestions (Eliot and Soule, 1902; Federley, 1905) that clicks are produced by the mandibles (Fig. 2). When the left and right mandibles are opened and closed, contact between the two surfaces during closing creates a distinguishable 'click'. Scanning electron micrographs revealed that the surface of the mandibles is serrated, with several tooth-like ridges occurring on the upper plane.

Clicks are distinct and intentional sounds created only upon disturbance, and can readily be distinguished in their temporal and intensity patterns from passive sounds made during feeding, for example (Fig. 3A,B). Clicks ranged in intensity from 58.1–78.8 dB peSPL at 10 cm (determined from 10 larvae, from which we measured the intensity of their loudest and quietest clicks). Although most prominently heard in late instars, sound production was also noted in a second instar larva. Presumably, the ability to produce sound is not limited to late instars, but due to the small size of early instars, their sounds generally cannot be perceived.

The temporal characteristics of click trains were determined from larvae that produced sound following a single pinch to the head capsule with forceps. The duration of click trains and hence the number of clicks in a train varied between individuals. Some larvae clicked only twice after a single attack, while others clicked hundreds of times. On average, the duration of a click train lasted over 1 min and included 50–55 clicks (Table 1). Individual clicks within a train were on average 24.7±17.2 ms, and typically comprised multiple components (Fig. 3C, Table 1). The variability in the surface of the mandibles may account for the diversity in click structure, whereby multiple components are produced with varying degrees of contact between mandibular ridges.

Spectral analysis revealed that clicks are broadband, with most energy between 8 and 18 kHz (mean peak frequency 13.8±7.7 kHz, N=30; Fig. 3D). At –12 dB below peak frequency, the bandwidth was characteristically broad (26.9±14.5 kHz, N=30). At –18 dB below peak frequency, nearly half of the clicks examined were characterized by bandwidths that extended beyond 40 kHz (37.5±15.5 kHz, N=23).

Attack experiments

A simulated predator attack with forceps induced a variety of defensive behaviours in the larvae, including thrashing the head from side to side, sound production and regurgitation (supplementary material, Movie 1). We performed 52 one-pinch trials, 48 two-pinch trials and 50 five-pinch trials. In one-pinch trials, 63.5% of larvae produced sound. This value increased to 83% and 82% for two- and five-pinch trials, respectively. The number of acoustic signals produced by larvae also increased with the degree of disturbance (Fig. 4). During the first 60 s after an attack, larvae in one- and two-pinch trials signaled 20.6±35.5 times and 25.1±22.5 times, respectively, whereas larvae in five-pinch trials signaled on average 54.3±46.1 times. The amount of clicking in one- and two-pinch trials differed significantly from five-pinch trials (Mann–Whitney U test, P=0.00002 and P=0.003, respectively, two-tailed test).

Regurgitation was also positively correlated with an increase in the number of attacks (Fig. 5A,B). In one-pinch trials, 9.6% of larvae regurgitated. In two- and five-pinch trials, this value increased to 41.7% and 68%, respectively (Fig. 5C). In five-pinch trials, larvae first regurgitated most often with the second pinch (Fig. 5D). Many of the larvae directed their regurgitant towards their ‘attacker’ by wiping their mouthparts on the forceps. In addition, all larvae re-imbibed the fluid once an attack was terminated.

The relationship between sound production, regurgitation and number of attacks is illustrated in Fig. 6A. In one-pinch trials, larvae predominately responded by clicking without regurgitating (C+R–). As the number of pinches increased to five pinches, the proportion of (C+R–) larvae decreased. Conversely, few larvae in one-pinch trials responded by clicking and regurgitating (C+R+). However, as the number of pinches increased, so did the proportion of (C+R+) larvae. In five-pinch trials, the first click preceded regurgitation significantly (χ^2=35.4, P<0.001; Fig. 6B).

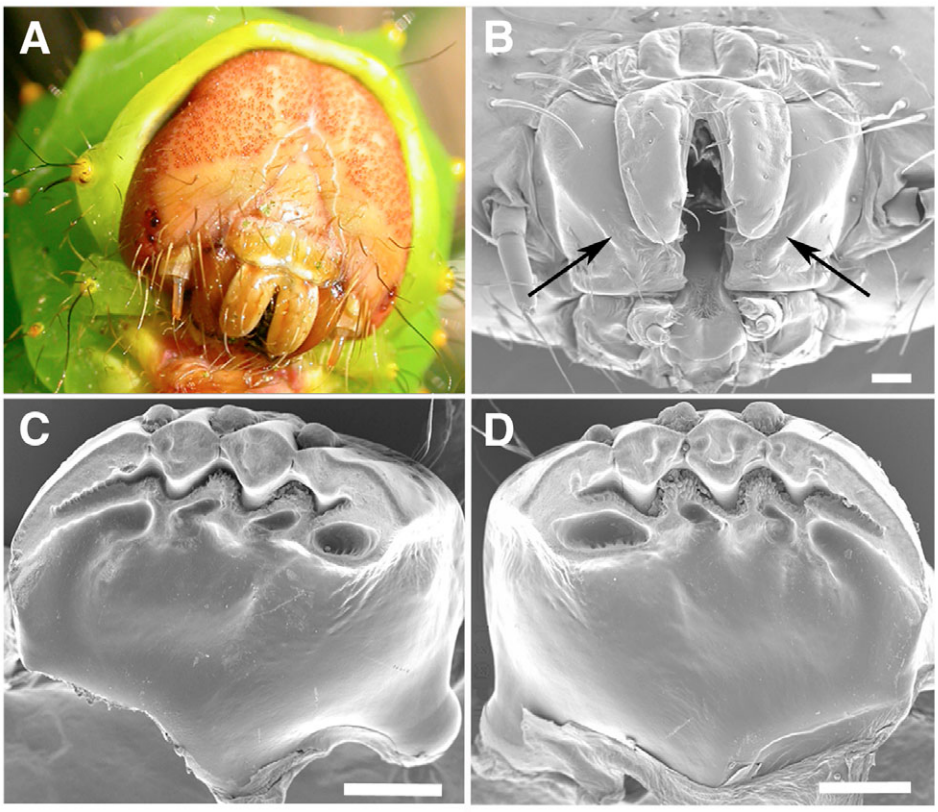

Fig. 2. Sound-producing structures of *Antheraea polyphemus* larvae. (A) Close-up of the head region. (B) Scanning electron micrograph of the mouthparts. The left and right mandibles (arrows) lie below the labrum. (C,D) Scanning electron micrographs of the left and right mandibles, respectively. Scale bars, 250 μm.

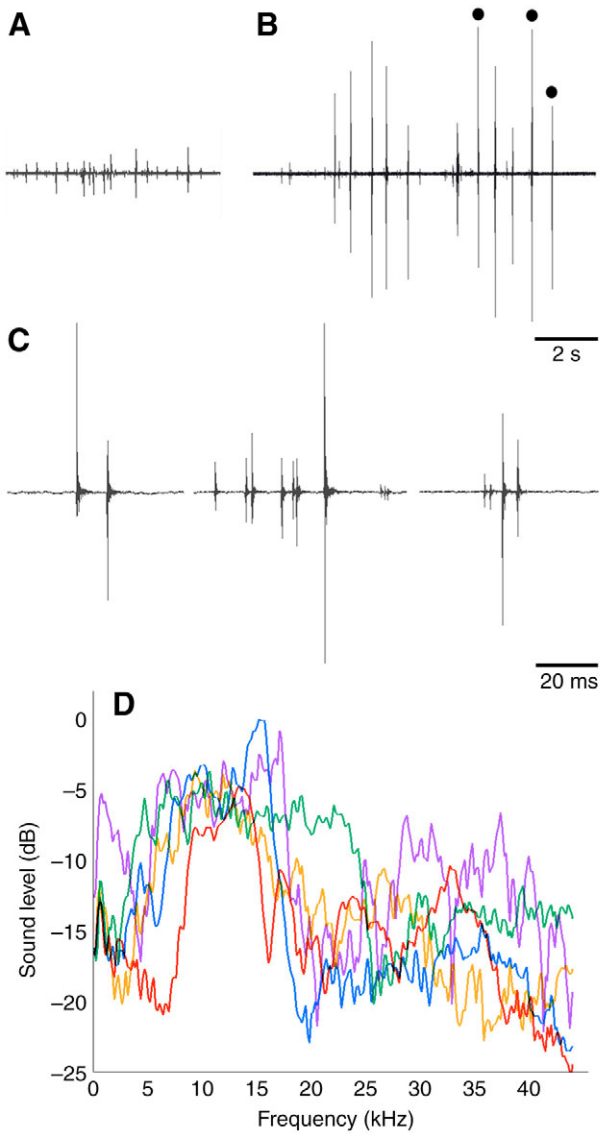

Fig. 3. Oscillograms of *Antheraea polyphemus* sounds recorded from fifth instar larvae. (A) Sounds recorded from an individual feeding on *Quercus rubra* leaves at 10 cm. (B) A click train, showing the typical click pattern of a larva after being pinched with forceps at 10 cm. (C) Three clicks from the train in B (denoted by black circles) with an expanded time scale showing the multiple components of clicks. The most typical pattern, a double-component click, is shown on the left. (D) Click spectra from five different individuals. The bandwidth varies, but in all clicks most energy is between 8 and 18 kHz.

Grasping and pecking by the beak of a chick was sufficient to induce sound production in 100% of larvae (N=16) (supplementary material, Movie 2). Regurgitation occurred in 87.5% of trials. In these trials, sound production and regurgitation occurred simultaneously 43.8% of the time, and sound production preceded regurgitation 25% of the time. In the remaining 12.5% of trials, the precise timing of regurgitation in relation to clicking was not caught on film because the heads of larvae were directed away from the camera lens. In one trial (representing 6.2%), sound production followed regurgitation. In six cases where chicks attacked only once, larvae signaled 39.3±27.3 times. Larvae did not produce more signals when attacked once by chicks than when 'attacked' once with forceps (Mann–Whitney U test, P=0.29, two-tailed test). All of the larvae survived the attacks by chicks. A typical attack sequence was characterized by an approach, an attack that consisted of 1–4 pecks and a withdrawal once sound production and/or regurgitation was induced. In only three trials did the chick return for an additional attack once the larvae had regurgitated.

Invertebrate bioassay

Of the control trials in which untreated mealworm segments were offered to ants, 15 of 15 trials were accepted within 11 min or less. Similarly, of the control trials in which mealworm segments were coated in water, 11 of 11 trials were accepted within 10 min or less. Of the experimental trials in which mealworm segments were coated in regurgitant, 14 of 16 trials were accepted within 45 min or less, while in two trials, the segments were completely rejected. On average, and excluding the two trials that were not accepted at all, ants took significantly longer to accept segments coated in regurgitant (975.6±661.1 s, N=14), than untreated segments (260.8±151.1 s, N=15, Mann–Whitney U test, P=0.00008, two-tailed test) or water-covered segments (210.7±132.3 s, N=11, Mann–Whitney U test, P=0.00003, two-tailed test) (Fig. 7). There was no difference between average acceptance times of the two controls (Mann–Whitney U test, P=0.19, two-tailed test).

In addition to monitoring acceptance rates, preening behaviour of the ants was noted. To control for trials with short duration times, preening was only monitored during the first 60 s of each trial following first contact with the mealworm segment. One reason that it took longer for ants to accept mealworm segments coated in regurgitant is that ants would consistently preen their antennae when they came into contact

Table 1. *Temporal characteristics of clicks produced by late instar Antheraea polyphemus larvae when pinched once with forceps*

	Train duration (s)	Number of clicks per train	Duration of clicks (ms)	Number of components
Mean ± s.d.	79.73±76.64	52.76±82.18	24.73±17.16	2.88±2.05
Minimum	<1	2	3.63	1
Maximum	257.5	426	50.71	9
Number of clicks	–	–	25	25
Number of animals	33	33	5	5

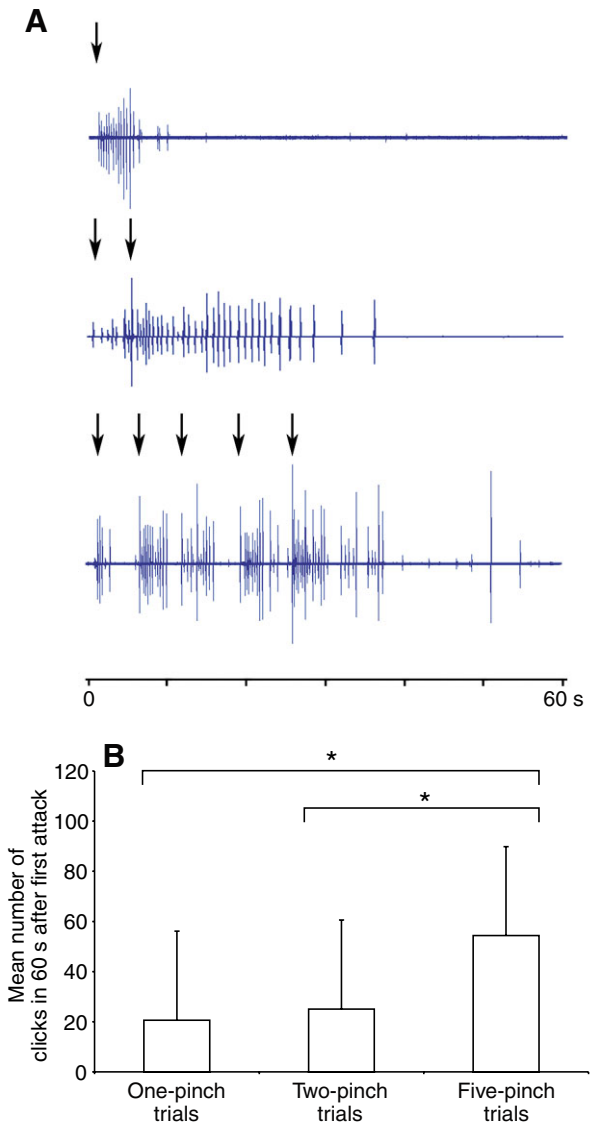

Fig. 4. (A) Oscillograms of late instar *Antheraea polyphemus* sounds obtained during one-, two- and five-pinch trials. Arrows indicate the times when each larva was attacked. (B) Number of clicks in 60 s produced by late instar larvae following the first attack in one- (20.6±35.5), two- (25.1±22.5) and five-pinch (54.3±46.1) trials (means ± s.d.). The total amount of signaling is positively correlated with the number of attacks (*P<0.001).

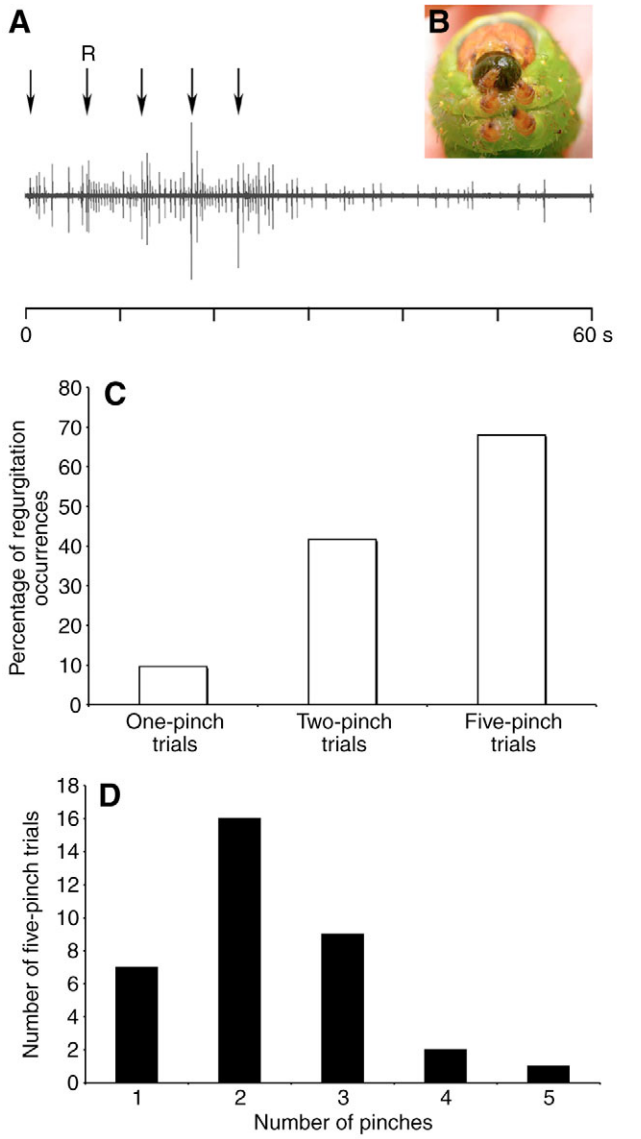

Fig. 5. (A) A representative oscillogram of sounds produced by a fifth instar *Antheraea polyphemus* larva when pinched five consecutive times with forceps. The larva first regurgitated (R) after the second pinch was administered. (B) Defensive regurgitation in a fifth instar larva in response to a simulated predator attack. (C) The percentage of regurgitating late instar larvae in one-, two- and five-pinch trials. (D) The number of pinches required to elicit regurgitation in late instar larvae. Larvae most commonly regurgitated after the second pinch.

with the regurgitant. In all 16 experimental trials, at least one ant per trial was observed preening its antennae. Ants did not preen (0 of 15 trials) while in contact with untreated mealworm segments. A similar pattern was observed with ants contacting mealworm segments covered in water, where preening was observed once (1 of 11 trials).

Vertebrate bioassay

Mice preferentially consumed chow coated in distilled water over chow coated in regurgitant (Mann–Whitney U test, P=0.0039, two-tailed test; Fig. 8). On average, they consumed 46.0±10.6% of water-covered chow compared to only 28.5±11.2% of regurgitant-covered chow. Data were examined for left and right position preferences between control and experimental diets, but no observable trend was detected.

Comparative study of other caterpillars

An additional 12 species were surveyed for the ability to produce sound. The larvae were distributed among three Bombycoidea families (Bombycidae, Saturniidae and Sphingidae). This study resulted in the identification of two

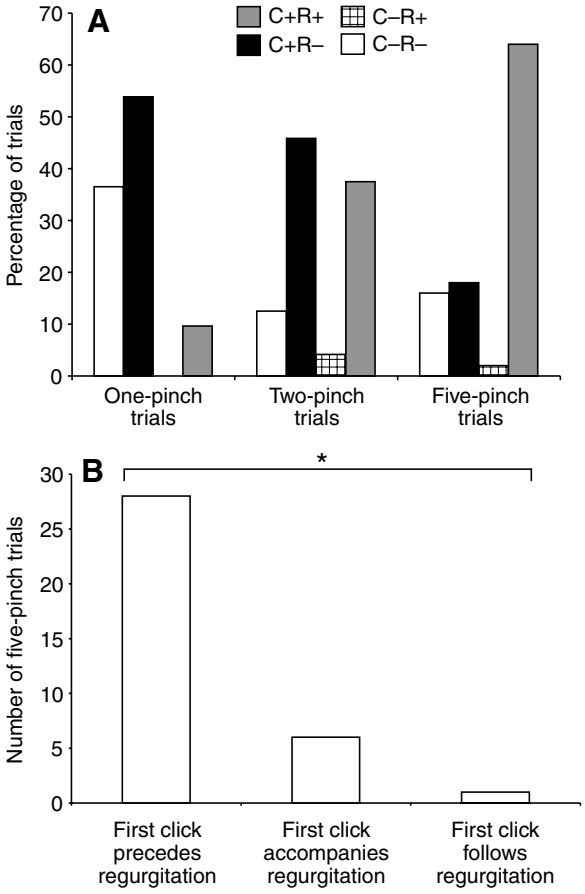

Fig. 6. (A) The escalation in behavioural responses of late instar *Antheraea polyphemus* larvae to increasing levels of disturbance. C–R–, neither clicking nor regurgitation; C+R–, clicking only; C–R+, regurgitation only; C+R+, clicking and regurgitation. C+R+ increased between one-, two- and five-pinch trials, whereas C+R– decreased between one-, two- and five-pinch trials. (B) The temporal relationship between clicking and regurgitation. In almost all five-pinch trials, the acoustic signaling occurred before regurgitation. *Significant difference (P<0.001).

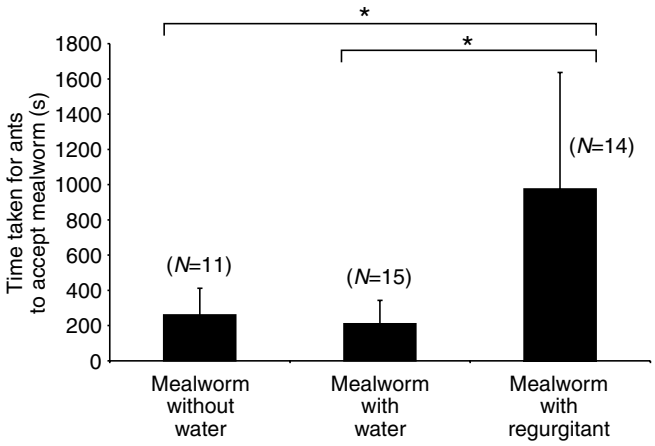

Fig. 7. The amount of time taken for ants to carry untreated mealworm segments, segments covered with distilled water, and segments covered in regurgitant, into one of their foraging holes. Note that two trials in which mealworms were coated in regurgitant were not included for analysis, because the mealworms were never accepted by ants. Values are means ± s.d. *Significant difference (P<0.001).

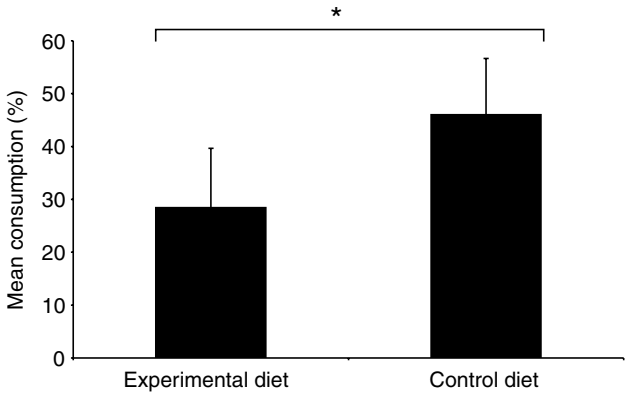

Fig. 8. Mean consumption (± s.d.) of mouse chow coated in distilled water (control diet) *versus* mouse chow coated in regurgitant (experimental diet). (N=10). *Significant difference (P<0.005).

additional sound-producing species: *Actias luna* (Saturniidae) and *Manduca sexta* (Sphingidae) (Fig. 9). Both species produced audible clicking sounds when pinched with forceps. Similar to the sound-producing mechanism of *A. polyphemus* larvae, clicks were produced by the mandibles.

Clicks produced by *A. luna* were on average 69.8±8.4 ms (N=9), with a peak frequency of 21.5±9.7 kHz (N=14). Upon closer inspection at high resolution, almost all clicks consisted of two components (Fig. 9E). Clicks produced by *M. sexta* were on average 32.6±10.3 ms (N=10), with a higher peak frequency of 38.0±7.4 kHz (N=15), and also generally consisted of two components (Fig. 9F). Five consecutive pinches with forceps induced all of *A. luna* larvae to click (N=10). Regurgitation occurred in six of these trials, where sound production either preceded or accompanied regurgitation. Similarly, sound production was observed in all trials with *M. sexta* larvae (N=7). Three of these animals regurgitated, and in all cases sound production preceded or accompanied regurgitation.

Regurgitation appears to be widespread in both sound-producing and non-sound-producing Bombycoidea larvae (Table 2). *A. luna* and *M. sexta* clicked and regurgitated, but eight other species examined, including *C. promethea*. *D. rubicunda*, *H. cecropia*, *H. euphorbiae*, *M. tiliae*, *P. modesta*, *S. cerisyi* and *S. jamaincensis*, regurgitated without producing sound. Only two species, *A. io* and *B. mori*, neither produced sound nor regurgitated.

Discussion

In this study, we investigated the phenomenon of clicking caterpillars in the superfamily Bombycoidea and focused primarily on one silkmoth species, *A. polyphemus*. Upon

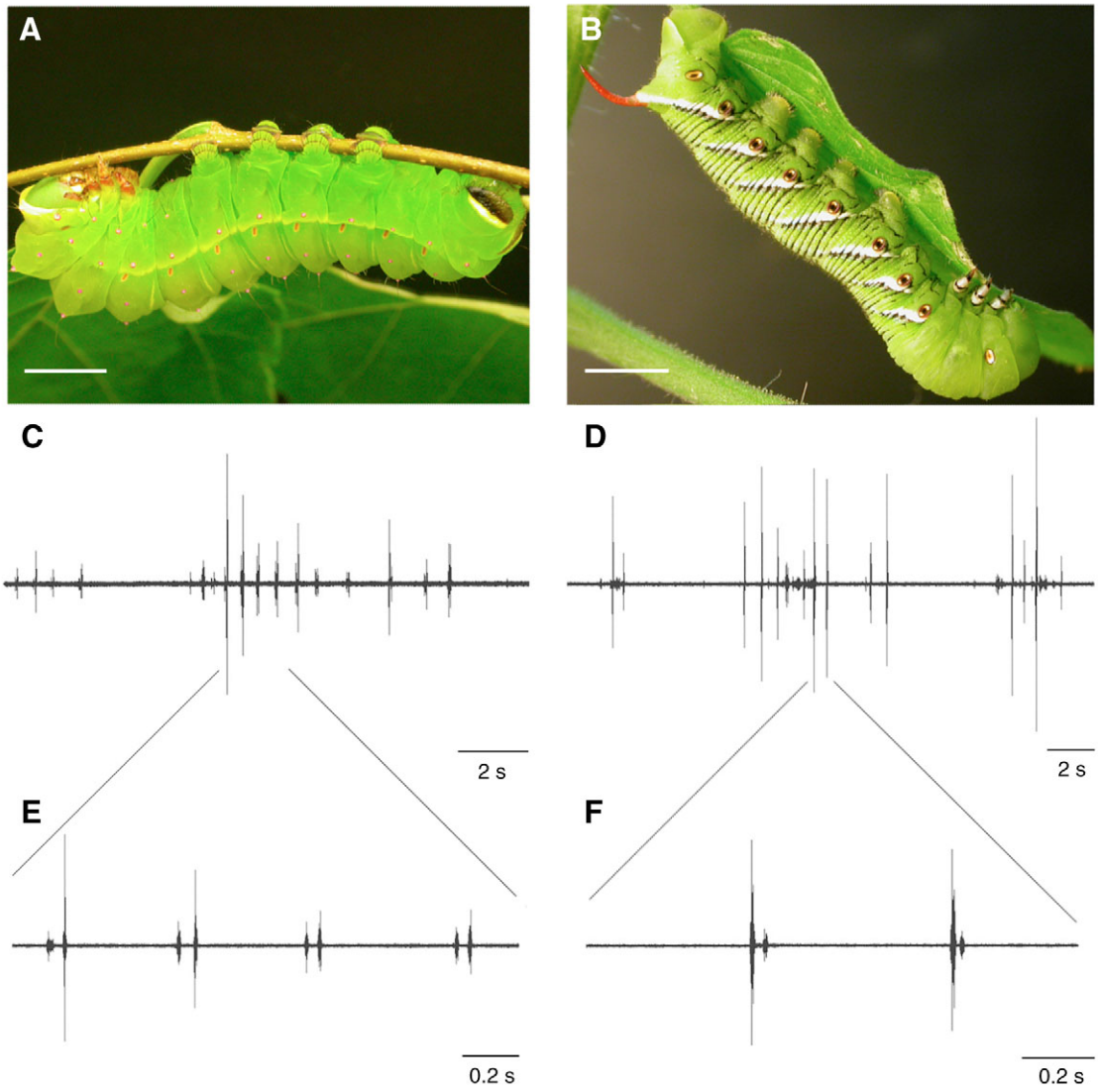

Fig. 9. Fifth instar (A) *Actias luna* and (B) *Manduca sexta* larvae. Scale bars, 1 cm. (C,D) Oscillograms of sounds produced by *A. luna* and *M. sexta*, respectively, showing the typical click patterns of larvae after being pinched with forceps. (E,F) Clicks from the trains in C and D, respectively, with an expanded time scale showing that clicks generally have two components.

disturbance, *A. polyphemus* produce airborne sounds that often precede or accompany defensive regurgitation. Our hypothesis that sound production warns predators of an impending regurgitant defense was supported by several lines of experimental evidence. In the following discussion we examine our results with respect to the testable predictions outlined in the introduction, propose alternative hypotheses for the function of signaling in *A. polyphemus* larvae, and discuss the concept of sound production in caterpillars by examining the advantages of acoustic, rather than visual, aposematic signals.

Aposematism

The term aposematism was coined by Edward Poulton in 1890. In its original context, he defined 'aposematic colouration' as "*an appearance which warns off enemies because it denotes something unpleasant or dangerous; or which directs the attention of an enemy to some specially defended, or merely non-vital part; or which warns off other individuals of the same species*" (Poulton, 1890). In recent years, it has been shown that aposematism evolved because predators learn to avoid brightly patterned or otherwise conspicuous prey more rapidly than cryptic prey (Gittleman and Harvey, 1980; Gittleman et al., 1980; Sherratt, 2002). Many experimental studies on aposematism have focused primarily on systems involving brightly patterned visual displays (for reviews, see Cott, 1940; Wickler, 1968; Guilford, 1990). However, the displays of aposematic animals do not always rely on colouration. In fact, the term aposematism has often been used to describe warning odours (e.g. Nishida et al., 1996; Schmidt, 2004) and sounds (e.g. Dunning and Krüger, 1995; Kirchner and Röschard, 1999; Hristov and Conner,

Table 2. *Comparative study of sound production and regurgitation in larvae from the superfamily Bombycoidea, including data from the current study and examples from the literature*

Family, Species	Host plant	Sound production?	Mechanism	Regurgitation?	Reference(s)
Bombycidae					
Bombyx mori Linnaeus	*Morus rubra*	No	–	No	–
Saturniidae					
Actias luna Linnaeus	*Betula papyrifera*	Yes	Mandibles	Yes	–
Automeris io Fabricius	*Betula papyrifera*	No	–	No	–
Callosamia promethea Drury	*Betula papyrifera*	No	–	Yes	–
Dryocampa rubicunda Fabricius	*Acer saccharum*	No	–	Yes	–
Hyalophora cecropia Linnaeus	*Betula papyrifera*	No	–	Yes	–
Rhodia fugax Butler[1]	–	Yes	Mandibles	?	(Packard, 1904; Dumortier, 1963)
Sphingidae					
Acherontia atropos Linnaeus[1]	–	Yes	Mandibles	?	(Mead, 1869; Pearce, 1886)
Amorpha juglandis Smith[1]	–	Yes	–	?	(Sanborn, 1868; Wagner, 2005)
Hyles euphorbiae Linnaeus	*Euphorbia* sp.	No	–	Yes	–
Manduca sexta Linnaeus	*Solanum lycopersicum*	Yes	Mandibles	Yes	–
Mimas tiliae Linnaeus	*Alnus* sp.	No	-	Yes	–
Pachysphinx modesta Harris	*Populus tremuloides*	No	–	Yes	–
Smerinthus excæcatus Smith[1]	–	Yes	Mandibles	?	(Sanborn, 1868)
Smerinthus dissimilis Bremer[1]	–	Yes	–	?	(Federley, 1905)
Smerinthus cerisyi Kirby	*Populus tremuloides*	No	–	Yes	–
Smerinthus jamaicensis Drury[2]	*Populus tremuloides*	Yes (Sanborn, 1868) No (this study)	– –	Yes (this study)	(Sanborn, 1868)
Sphecodina abbottii Swainson[1]	–	Yes	Mandibles	?	(Dumortier, 1963; Wagner, 2005)

[1]Information from the literature only.
[2]Reclassified from *Smerinthus geminatus* Say.

2005). Therefore, we will use the term acoustic aposematism synonymously with warning sounds.

Are clicks emitted by A. polyphemus acoustic aposematic signals?

Our experimental results support several predictions designed to test the acoustic aposematism hypothesis. The first prediction, that sound production is associated with a predator attack, was supported. Many forms of disturbance, including blowing on the larvae or jarring their enclosures, caused the larvae to produce sound. In addition, simulated predator attacks with forceps and attacks by chicks strongly associated sound production with physical disturbance. The second prediction, that escalation in attack rate is positively correlated with the amount of signaling, was also supported. An increase in the number of pinches administered to the larvae was significantly associated with an increase in the number of acoustic signals produced. On average, larvae that were administered five consecutive pinches produced more than twice as many clicks over a 60 s period than did larvae that were pinched only once or twice.

Our third prediction states that natural predators should be capable of hearing the acoustic signal. The clicks produced by *A. polyphemus* larvae are broadband in structure, with an upper frequency limit that extends into ultrasound. A broad bandwidth is a distinguishing feature of insect disturbance sounds (Masters, 1979). Warning sounds typically display average bandwidths of approximately 40 kHz at 10 dB below peak frequency (Masters, 1980). Several clicks analyzed in this study had bandwidths at –12 and –18 dB that spanned greater than 40 kHz. The broad nature of *A. polyphemus* clicks permit them to be perceived by a diversity of predators whose optimal hearing ranges may not coincide. Larval Lepidoptera are common prey items of gleaning bats (e.g. Kalka and Kalko, 2006; Wilson and Barclay, 2006). Thus, the high frequency component of clicks (20 kHz and above) may be perceived by bats whose best hearing range extends into the ultrasound spectrum (e.g. Neuweiler, 1989). Likewise, the lower frequency component of clicks (20 kHz and below) is within the optimal hearing range of avian predators (e.g. Schwartzkopff, 1955; Frings and Cook, 1964; Dooling, 1991). It is also possible that praying mantids can hear clicks. Mantid hearing, believed to function primarily in bat detection, is most acute at ultrasonic frequencies, generally between 25 and 50 kHz (Yager, 1999). The sound intensity of clicks was determined to be 58.1–78.8 dB peSPL at 10 cm. Upon attack,

most predators would be even closer to the larvae than 10 cm. It is therefore reasonable to assume that clicks are well within the hearing threshold of their natural predators.

The fourth prediction, which states that regurgitant is adverse to predators, was supported by results obtained from the invertebrate and vertebrate bioassays. Mice preferentially consumed control diet over diet containing regurgitant. Similarly, ants were quicker to accept control mealworms than those coated in regurgitant, and were more likely to preen following contact with regurgitant. These results demonstrate that the regurgitant does afford some degree of protection against natural enemies. In addition, the fact that two predators as distantly related as ants and mice were deterred to some degree suggests that the regurgitant is effective against a range of predators. Both mice and ants did accept a portion of food items containing regurgitant, suggesting that regurgitating larvae may still experience moderate levels of predation. Two possible reasons account for the adverse quality of the regurgitant, which are not mutually exclusive. The regurgitant itself may gum up the mouthparts of attacking predators (like ants), or it may contain chemical compounds that render it distasteful. The composition of *A. polyphemus* regurgitant is currently unknown. If the adverse nature of the regurgitant is related to chemistry, it remains to be seen whether the defensive compounds are synthesized *de novo* or acquired through host plant secondary chemistry.

To our knowledge, regurgitation had not been previously reported in *A. polyphemus* larvae. Defensive regurgitation is widespread in insects (Eisner, 1970; Blum, 1981), but is not necessarily a ubiquitous defense strategy of caterpillars (Grant, 2006). Despite this lack of ubiquity, several studies have demonstrated the effective use of regurgitation by certain species of larvae in interactions with natural enemies (e.g. Gentry and Dyer, 2002; Peterson et al., 1987; Cornelius and Bernays, 1995; Theodoratus and Bowers, 1999). Because regurgitation can be an energetically costly defensive response (Bowers, 2003), *A. polyphemus* larvae attempt to reduce the cost by re-imbibing their regurgitant and accurately directing their mouths towards their attacker.

The final prediction states that the acoustic signal will most often precede or accompany regurgitation. In attack experiments with forceps, larvae predominantly produced sound prior to regurgitation. When attacked by chicks, many larvae responded with simultaneous sound production and regurgitation. Although force was not quantitatively measured in attack experiments, it was evident that chicks attacked the larvae much more forcibly than the pinches administered by forceps. Presumably, a forceful attack might result in a more aggressive defensive response, thereby necessitating larvae to produce sound in conjunction with, rather than, preceding defensive regurgitation.

Alternative hypotheses

Our hypothesis has been strongly supported in this study. However, it is prudent to consider alternative hypotheses, since airborne sound production by caterpillars has never been experimentally examined before. What are other possible functions for clicking by *A. polyphemus* larvae? First, they may be producing sounds in social interactions with conspecifics. However, in this study, sound production was not observed during any interactions between caterpillars. In addition, *A. polyphemus* larvae are insensitive to airborne sounds and appear to lack hearing organs, which strongly suggests they would be unable to detect the clicks of nearby caterpillars. Furthermore, *A. polyphemus* larvae are not gregarious as late instars, casting further doubt that the intended receiver of the acoustic signals would be a conspecific.

Second, sound production may be an incidental sound caused by regurgitation. However, as was demonstrated in the attack experiments with forceps, larvae are capable of regurgitating without clicking, and clicking without regurgitating. Furthermore, results from the comparative study indicate that several species of Bombycoidea readily regurgitate without producing sound. Since the ability to produce sound is independent from the ability to regurgitate, this hypothesis has little merit. While it is evident that clicking is not a by-product of regurgitation, an interesting possibility remains that sound production may have evolved from movement of the mouthparts while regurgitating or biting in response to an attack.

A third alternative hypothesis is that the clicks function as startle sounds. In fact, the first three predictions discussed here also provide support for this hypothesis. However, an important prediction to support the startle hypothesis would be that larvae attempt to escape following sound production. We did not observe any form of dispersal behaviour following attacks with forceps or by chicks, casting doubt on the validity of the startle hypothesis. In numerous field studies with *A. polyphemus*, larvae tend to move very little, even when attacked by multiple parasitoids (G.H.B., unpublished observation).

Comparative study

Comparative evidence suggests that the phenomenon of clicking caterpillars is widespread. In addition to *A. polyphemus* larvae, mandibular clicking has been reported in a number of species from the families Saturniidae and Sphingidae, two of which were identified for the first time in this study. *A. luna* and *M. sexta* produced broadband clicks with their mandibles when disturbed, clicking typically preceded defensive regurgitation, and no form of escape behaviour followed sound production. These observations provide additional support that clicks function as acoustic aposematic signals. It is surprising that other studies have not previously reported on sound production in these two species, particularly for *M. sexta*. In 2001, a detailed account of the defensive responses of laboratory-reared and wild *M. sexta* larvae following a series of simulated attack experiments was published (Walters et al., 2001). Although thrashing, striking and defensive regurgitation were reported, no mention was made regarding sound production.

One interesting difference between *A. polyphemus* clicks and the clicks produced by *A. luna* and *M. sexta* larvae is the spectral qualities of the signals. *A. luna* and *M. sexta* produce clicks with most energy at 21.5 kHz and 38.0 kHz,

respectively. Both values are considerably higher than the peak frequency produced by *A. polyphemus* (13.8 kHz). The high frequency component of clicks produced by *A. luna* and *M. sexta* larvae may be an incidental result of structural differences in mouthparts, or may lend additional support to the idea that clicks are directed towards gleaning bats.

Several species of Bombycoidea that we tested did not produce sound (Table 2). In fact, in one instance, sound production was not present in a species previously reported as sound-producing. Mandibular clicking was described in *Smerinthus geminatus* (reclassified as *S. jamaicensis*), when disturbed (Sanborn, 1868). In our study, sound production could not be induced in any of the *S. jamaicensis* larvae. It is possible that sound production is a regional characteristic in certain populations. This might account for the exclusion of sound production as one of the defensive responses of *M. sexta* larvae (Walters et al., 2001). However, sound production is not a regional characteristic for *A. polyphemus*, since larvae from Ontario and Prince Edward Island, Canada, and Massachusetts, USA all produce sound. The incongruence of our results with the observations of Sanborn necessitates that more *S. jamaicensis* larvae be tested for sound production in the future.

Currently, airborne sound production has been reported (including our data) for at least nine species belonging to the superfamily Bombycoidea. However, sound production does not occur in all species. Although it may be too early to make generalizations about why some larvae produce sound while others do not, possible explanations include the size of larvae and their mouthparts, the degree of warning colouration (see below) and taxonomy. To date, sound production has only been reported in species from two of the nine Bombycoidea families, namely the Saturniidae and Sphingidae, possibly because larvae from the other families are too small to make audible sounds.

The evolution of acoustic, rather than, visual aposematic signals

Many animals employ the use of sounds in conjunction with aposematic colouration. It is thought that additional signal components act to reinforce the association between colouration and unpalatability, a strategy referred to as multicomponent or multimodal signaling (Partan and Marler, 1999; Rowe, 1999). The use of multiple signals increases the efficacy of information transfer by acting on several sense modalities in the predator (e.g. Rowe and Guilford, 1999). However, if pairing a visual cue with an acoustic one helps to reinforce the message of unprofitability to potential predators, it begs the question: why are *A. polyphemus* and other sound-producing larvae cryptically coloured? One reason might be that clicks produced by Bombycoidea larvae are primarily directed towards auditory predators, rather than visual ones. This is similar to the argument (Ratcliffe and Fullard, 2005) that the brightly coloured dogbane tiger moth, *Cycnia tenera*, produces ultrasonic clicks that serve as defensive signals against vision-poor insectivorous bats. However, it is possible that the use of acoustic signals without conspicuous colouration is advantageous because it does not compromise the caterpillars' ability to remain camouflaged. Acoustic warnings, unlike visual ones, are not 'on' all the time. Rather, they are only employed once an attack by a predator has been initiated, presumably because the continuous production of sound, unlike the continuous display of colour, is energetically costly. Cryptic colouration permits vulnerable larvae to remain as inconspicuous as possible up until the moment of attack. This allows for protection against an entire range of predators that vary in their degree of visual acuity. However, for our reasoning to be upheld, it must be shown that *A. polyphemus* (and other sound-producing larvae) are, in fact, visually cryptic to their predators.

Conclusion

Upon discovering that *A. polyphemus* larvae produce sound, the aim of this study was to identify the mechanism of sound production, to characterize the acoustic signals and to test the hypothesis that *A. polyphemus* larvae and several other species of Bombycoidea are producing sounds that function as acoustic aposematic signals. Several lines of experimental evidence were provided to lend support to our hypothesis. In the future, it will be important to demonstrate the effectiveness of sound production and regurgitation at deterring natural predators from attacking larvae. An experiment that monitors the behaviours of experienced predators who have previously encountered the regurgitant will be significant in lending support to the acoustic aposematism hypothesis. Furthermore, chemical analysis of the regurgitant with bioassay-guided fractionation might help to resolve its deterrent qualities. Lastly, an investigation into additional sound-producing species will assist in providing insight into the evolution of this interesting phenomenon.

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