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

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Abstract

Acoustic signals produced by caterpillars have been documented for over one hundred years, but in the majority of cases their significance is unknown. This study is the first to experimentally examine ‘clicking’ caterpillars from the superfamily Bombycoidea, with a focus on the silkmoth caterpillar, *Antheraea polyphemus*. Clicks produced by larvae measured on average 24.7 ms, displayed peak energy between 8 and 18 kHz, and varied in intensity between 58.1 and 78.8 dB peSPL. My hypothesis, that clicks function as acoustic aposematic signals, was supported by several lines of evidence. Experiments with forceps and chicks correlated sound production with attack. Sound production typically preceded or accompanied defensive regurgitation. Bioassays with invertebrates (ants) and vertebrates (mice) confirmed that the regurgitant is deterrent to would-be predators. Comparative evidence revealed that other Bombycoidea species, including *Actias luna* and *Manduca sexta*, also produce airborne sounds upon attack, and that these sounds precede or accompany regurgitation.

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1. Introduction

Acoustic communication in Lepidoptera has been an important area of scientific investigation, with over 200 published reports on the subject (reviewed in Minet and Surlykke, 2003). To date, research has focused primarily on the study of 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, which are used in the context of social communication and bat defense (e.g. 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 Noctuidae 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, a review of the literature on this topic indicates that there have been several preliminary and anecdotal reports suggesting that many species of larvae communicate acoustically (Table 1). These represent numerous species from at least 12 families, including for example, Tortricidae (Russ, 1969), Oecophoridae (Hunter, 1987), Notodontidae (Dumortier,

Table 1. Reports of sound- or vibration-producing Lepidoptera larvae.

Superfamily	Family	Species	Sound-producing mechanism	Suggested function	Reference(s)
Bombycoidea	Saturniidae	<i>Antheraea</i> (= <i>Telea</i>) <i>polyphemus</i>	Mandibles	Intimidation	Eliot and Soule, 1902; Federley, 1905; Wagner, 2005
		<i>Rhodias</i> <i>fugax</i>	Mandibles	-	Packard, 1904; Dumortier, 1963
	Sphingidae	<i>Smerinthus dissimilis</i>	-	Intimidation	Federley, 1905
		<i>Smerinthus geminatus</i>	Mandibles	-	Sanborn, 1868
		<i>Smerinthus excrucatus</i>	Mandibles	-	Sanborn, 1868
		<i>Sphecodina abbotti</i>	Mandibles	-	Dumortier, 1963; Wagner, 2005
		<i>Amorpha</i> (= <i>Cressonia</i>) <i>juglandis</i>	-	-	Sanborn, 1868; Wagner, 2005
		<i>Acherontia atropos</i>	Mandibles	-	Mead, 1869; Pearce, 1886
	Drepanoidea	<i>Drepana arcuata</i>	Scraping and drumming mandibles and scraping anal oars against the leaf	Territorial defense	Federley, 1905; Yack et al., 2001; Sen and Lin, 2002
		<i>Drepana bilineata</i>	Drumming mandibles and scraping anal oars against the leaf	Territorial defense	Yack, unpublished data
		<i>Drepana curvatula</i>	Scraping sound produced by rubbing two chitinous teeth from the anal segment against the leaf	-	Federley, 1905
		<i>Drepana lacertinaria</i>	"	-	Federley, 1905
		<i>Drepana falcata</i>	"	-	Federley, 1905
Gracillarioidea	Gracillariidae	<i>Nordstromia lilacina</i>	Scratching leaf surface with posterior portion of abdomen, and producing sharp clicking sounds by pounding mandibles against leaf	-	Sen and Lin, 2002
		<i>Caloptilia serotinella</i>	Scraping, plucking and vibrating against the leaf	Territorial defense	Fletcher et al., 2006

Table 1, continued

Superfamily	Family	Species	Sound-producing mechanism	Suggested function	Reference(s)
Papilionoidea	Lycaenidae	Several species	Stridulatory organ	Ant-larval mutualism	DeVries, 1991; Travassos and Pierce, 2000
	Riodinidae	Several species	Vibratory papillae	Ant-larval mutualism	DeVries, 1990; DeVries, 1991
	Pieridae	<i>Thyreus abbotii</i>	Sound created from friction by throwing head side-to-side	-	Packard, 1904
Tineoidea	Tineidae	Unknown species	Rustling sound created within dried leaf mines	-	Packard, 1904
Tortricoidea	Tortricidae	<i>Sparganothis pilleriana</i>	Head knocking	Territorial defense	Russ, 1969
Noctuoidea	Notodontidae	<i>Dicranura</i> sp.	Swinging head and pressing mouthparts against the leaf	-	Dumortier, 1963
		<i>Cerura</i> sp.	"	-	Dumortier, 1963
	Noctuidae	<i>Abablemma brimleyana</i>	Head knocking	-	McCabe, personal observation
Gelechioidea	Oecophoridae	<i>Diurnea fagella</i>	A hook, located on the distal end of a modified third thoracic leg, scrapes back and forth on the leaf's surface	Deterrent to intruding spiders and/or conspecifics	Hunter, 1987

1963), Saturniidae (Federley, 1905), and Sphingidae (Sanborn, 1868). In most of these cases, the acoustic signals have not been characterized and behavioural evidence for the context in which the signals are produced is generally absent.

One interesting phenomenon is that of ‘clicking’ caterpillars from the superfamily Bombycoidea (Fig. 1). Several species belonging to the silkworm (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 also been 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 have been suggested to play a role in defense (Federley, 1905) or social communication (Wagner, 2005).

In this study, I explore the mechanism and function of caterpillar clicks by focusing primarily on one species, *Antheraea polyphemus* Cramer (Fig. 2). *A. polyphemus* is a silkworm that occurs throughout deciduous hardwood 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 of this species has been widely studied for its olfactory and pheromone system, however little is known about the behaviour and life history of the caterpillar. Sound production by late instar larvae has been reported in several publications (e.g. Eliot and Soule, 1902; Federley, 1905; Wagner, 2005) and by those rearing the larvae (Boettner, personal communication). Federley (1905) noted that third and fourth instars use their mandibles to produce a “tolerably

Fig. 1. Cladogram representing the nine families of the superfamily Bombycoidea (adapted from Minet, 1994).

Fig. 2. A fifth instar *Antheraea polyphemus* larva. Scale bar, 0.5 cm.

loud, tapping sound.” 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 an 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, I noted that clicking in *A. polyphemus* larvae is commonly associated with both disturbance and regurgitation. I 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, my objectives for this study are three-fold: (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, I have examined the distribution of this phenomenon in other Bombycoidea larvae by reviewing the literature and testing an additional 12 species. My results are discussed with respect to the general function and evolutionary significance of acoustic warning signals in caterpillars.

2. Materials and Methods

2.1 Animals

A. polyphemus larvae were obtained from three different sources. Between July and September 2005, gravid female moths were collected from the wild at mercury vapour and ultraviolet lights at the Mer Bleue Conservation Area in Ottawa, Ontario, Canada (NCC permit #3654). Eggs of wild females were donated by Dr. George Boettner (University of Massachusetts, Amherst, USA) and also purchased from Bill Oehlke (Montague, Prince Edward Island, Canada). 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 those in their third to fifth instars. However, a preliminary investigation of 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 belonged to the superfamily Bombycoidea. Gravid female *Actias luna*, *Dryocampa rubicunda*, *Pachysphinx modesta*, *Smerinthus cerisyi*, and *Smerinthus jamaicensis* moths were collected at lights between June and September 2005 and June and July 2006 at Mer Bleue. Eggs of *Manduca sexta* were donated by Shannon Meisner (Dalhousie University, Halifax, Canada) and purchased from the company LiveFood in Mercier, Quebec, Canada. In addition, 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 3 in the results section), as described above. All larvae used in comparative trials were those in their third to fifth instars.

2.2 Sound production

Since my preliminary observations corroborated earlier reports suggesting that the acoustic signals produced by the larvae are derived from the mandibles, 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 Sony TR7000 Digital Handicam 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 that had been stored in 70% ETOH were air dried, fixed to aluminum stubs with carbon paint, sputter-coated with gold-palladium, and examined with a JOEL JSM-6400 scanning electron microscope.

In order to gain insight into the function of sound production, airborne sounds produced by larvae were recorded to examine their temporal, spectral, and intensity characteristics. All recordings were made in an acoustic chamber (Eckel Industries Ltd.) located at Carleton University. For temporal analysis, airborne sounds were recorded using a Sony ECM-MS957 microphone (frequency response of 100 Hz-18 kHz) placed 10 cm from the heads of fifth instar larvae. Sounds were recorded onto a Sony DAT PCM-M1 at a sampling rate of 48 kHz, and subsequently digitized by a Mac computer (1 GHz PowerPC G4). Temporal qualities, such as the duration of clicks and the number of components within a click, were examined with Canary Bioacoustics Research Program (Cornell Laboratory of Ornithology, Ithaca, NY).

Sounds used for creating power spectra were recorded using a Brüel & Kjær 1/4 inch microphone type 4939 (frequency response of 4 Hz-100 kHz) placed 10 cm

from the mouth of larvae. Sounds were amplified with a Brüel & Kjær Nexus conditioning amplifier type 2690, and recorded onto a Fostex FR-2 Field Memory Recorder at a sampling rate of 88.2 kHz. Spectral characteristics of the signals were analyzed from wav files on a PC computer (AMD Athlon XP 2200⁺) using Raven Bioacoustics Research Program (Cornell Laboratory of Ornithology, Ithaca, NY). Spectra were produced using a 512-point fast Fourier transform (FFT) Hanning window. To decrease the high intensity shoulder produced as the result of background noise (Fig. 3), the first 300 Hz were filtered from each power spectrum.

Sounds used for determining peak frequency and bandwidth were recorded using a Brüel & Kjær 1/4 inch microphone type 4939 placed 10 cm from the mouth of larvae. Clicks were captured on a Tektronix THS720A oscilloscope 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 (Fig. 4). In some instances, a quality factor at -18 dB was not measured if the spectrum was extremely broadband.

The intensity of the acoustic signals was determined using the method outlined in Stapells et al. (1982). Clicks were captured from larvae placed 10 cm from a Brüel & Kjær 1/4 inch microphone type 4939. The amplitudes of the clicks were measured as voltages using a Tektronix THS720A oscilloscope. A continuous pure tone centered at the mean peak frequency of the clicks was generated with a Tabor Electronics 50MS/s Waveform Generator WW5061 coupled to a Brüel & Kjær Nexus conditioning amplifier type 2690, and broadcast through a Pioneer ART-54F Ribbon tweeter (frequency response of 2-30 kHz, maximum intensity 91 dB SPL). The

Fig. 3. Spectrum of the background noise produced while recording the frequency bandwidth of *Antheraea polyphemus* clicks. Note the high intensity shoulder (indicated with an arrow) that lies below 1 kHz.

Fig. 4. A representative power spectrum of an *Antheraea polyphemus* click displaying the two quality factors measured for analysis. The bandwidth (in kHz) was determined at -12 dB and -18 dB below peak frequency.

peak-to-peak intensity of the generated signal was adjusted until the output voltage was equal to that of the clicks emitted by the caterpillars. The dB peak equivalent SPLs (dB peSPLs) at 10 cm were then read from a Brüel & Kjær sound level meter type 2239 placed at the same location as the 1/4 inch microphone. The sound level meter was previously calibrated using a Brüel & Kjær acoustical calibrator type 4231.

2.3 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, *A. polyphemus* larvae were kept solitarily on either oak or birch leaf sprigs for a minimum of one hour. Using forceps, the head capsules of larvae were pinched either once, twice, or five times, with approximately five second intervals between each pinch. The defensive behaviours of the larvae were monitored with a Sony Mini-DV DCR-TRV19 Handicam, and a Sony audio ECM-MS907 microphone placed 3-4 cm away from the heads of larvae. Footage from each trial was 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 seconds 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.

I 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 x 2.4 m chicken coup in Carp, Ontario, Canada. The chicks were maintained at 20-25°C using heat lamps, and water and chick starter

crumb were provided ad libitum. On a bi-weekly basis starting on the second day after arrival, chicks were fed live 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 hours prior to testing. The testing apparatus (Fig. 5A), located on grass outside the chicken coop, consisted of a cardboard box measuring 0.5 x 0.5 x 0.6 m (l x w x h) with the floor of the box removed. A 0.1 x 0.1 m (l x w) 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 placed on a tripod. 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. Mealworms were offered to the chick to determine its willingness to feed. A single fifth instar larva was then placed on the platform (Fig. 5B). Video footage, noting the presence or absence of larval signaling and regurgitation during an attack, was analyzed using iMovie 3.0.3. The procedure outlined above was approved by Carleton University's Animal Care Committee (protocol I.D. #B05-8). Larvae were not re-used from trial to trial.

2.4 Invertebrate bioassay

Preliminary observations have correlated sound production to regurgitation in late instar *A. polyphemus* larvae. 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. To determine the palatability of *A. polyphemus* regurgitant to an invertebrate predator, I employed a bioassay modified from Peterson et al. (1987). An ant colony composed of two predatory *Formica* species

Fig. 5. (A) The testing apparatus used in the attack experiment with chicks. The video camera and clamped microphone are directed towards an empty platform. (B) The presentation of a fifth instar *Antheraea polyphemus* larva to a chick.

(Hymenoptera: Formicidae) was located on a grassy lawn in Ottawa, Ontario, Canada. In a clean Petri dish, mealworms were cut into small 2-4 piece segments measuring between 5-10 mm. Each segment was transferred with forceps to a small beaker containing freshly collected *A. polyphemus* regurgitant from larvae fed on red oak. Once the segment was coated in regurgitant, it was placed within 2-3 cm of an ant hole. Trials were videotaped with a Sony Mini-DV DCR-TRV19 Handicam, beginning the moment of first contact between the ant and mealworm segment. Control trials were performed whereby mealworm segments not covered in regurgitant were presented at ant holes. In addition, a second series of control trials were performed whereby mealworm segments covered in distilled water were presented at ant holes. Video footage was analyzed using iMovie 3.0.3.

Analysis of trials consisted of determining (i) the number of mealworm segments rejected (i.e. left on the foraging grounds and not carried into an ant hole after more than one hour 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 seconds following first contact. Mann-Whitney *U* tests examined whether mean acceptance times differed significantly between experimental and control trial conditions.

2.5 Vertebrate bioassay

Because there is an acoustic component to the defensive response of *A. polyphemus* larvae, it can be reasoned that the signal is likely directed towards a hearing predator. The larvae are attacked by a diversity of predators including ants, praying mantids, spiders, birds, and small mammals (Passoa, 1999). Although many insects possess tympanal hearing organs (Hoy and Robert, 1996; Yager, 1999; Yack,

2004), hearing has not been reported for invertebrates attacking *A. polyphemus* larvae (except for mantids- see discussion). Therefore, I 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, I devised an additional bioassay in which I 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 x 18.2 x 12.4 cm (l x w x h), and maintained on 5075 non-autoclave rodent chow (from the company Charles River) on a 12:12 light-dark cycle. Water, provided in a spout positioned at one side of the cage, was available ad libitum. Prior to experimentation, mice were housed in their respective cages for seven days to ensure they had acclimated to their feeding and drinking stations. All animals were food deprived six hours before testing to ensure an eagerness for foraging.

Two glass food cups, each measuring 7 cm in diameter and 4 cm tall, were placed 2 cm apart at one end of the cage, opposite the water spout (Fig. 6). In one cup, a pre-weighed quantity of chow was coated in 6 ml of fresh regurgitant collected from *A. polyphemus* 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-hour, 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 weight at the completion of an experiment.

Fig. 6. Schematic representation of the testing apparatus used in the vertebrate bioassay. Two glass food cups, one containing water-covered chow and the other containing regurgitant-covered chow, were presented to mice in a two-choice test. A water spout was fitted into the lid of the cage, opposite the food cups.

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 mean percent consumption differed significantly between the two treatments.

2.6 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 five second intervals between each pinch. Defensive behaviours were recorded onto video using methods previously described. Trials were analyzed to examine (i) the presence of acoustic signaling, (ii) the presence of regurgitation, and (iii) 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. The results of the comparative study were tabulated alongside examples of sound-producing larvae from the literature.

3. Results

3.1 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. Video footage acquired from sound-producing larvae confirmed earlier suggestions (Eliot and Soule, 1902; Federley, 1905) that clicks are produced by the mandibles (Fig. 7). 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 be readily distinguished in their temporal and intensity patterns from passive sounds made during feeding, for example (Fig. 8A,B). Clicks ranged in intensity from 58.1-78.8 dB peSPL at 10 cm (determined from 10 larvae in which I measured the intensity of their loudest and quietest clicks; Fig. 9). Although most prominently heard in late instars, sound production was also audible in a second instar larva. Presumably, the ability to produce sound is not limited to late instars, however due the small size of early instars, 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

Fig. 7. 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 (indicated with arrows) lie below the labrum. (C and D) Scanning electron micrographs of the inner surface of the left and right mandibles, respectively. Scale bars, 250 μm .

Fig. 8. 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. The duration of a single click is typically 24.7 ms. (D) Click spectra from five different individuals. The bandwidth varies, but in all clicks most energy is between 8 and 18 kHz.

Fig. 9. Sound intensity curve for clicks produced by late instar *Antheraea polyphemus* larvae at 10 cm. Clicks vary in intensity from 58.1 to 78.8 dB peSPL.

hundreds of times. On average, the duration of a click train lasted over a minute and included 50-55 clicks (Table 2). Individual clicks within a train were on average 24.7 ± 17.2 ms (mean $\pm$ S.D.). A closer inspection at high resolution revealed that almost all clicks consisted of multiple components (Fig. 8C). Although clicks consisting of two components were most frequently encountered, clicks ranged from single-component clicks to nine-component clicks. The variability in the surface of the mandibles may account for the diversity in click structure, whereby multiple component clicks are produced when there is varying degrees of contact between the mandibles.

Spectral analysis revealed that clicks are broadband in structure, with most energy between 8 and 18 kHz (mean peak frequency 13.8 ± 7.7 kHz, mean $\pm$ S.D., $N=30$) (Fig. 8D). At -12dB below peak frequency, the bandwidth was characteristically broad (26.9 ± 14.5 kHz, mean $\pm$ S.D., $N=30$). Furthermore, 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 , mean $\pm$ S.D., $N=23$).

3.2 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. I 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. 10). During the first 60 seconds after an attack, larvae in one and two pinch trials signaled on average 20.6 ± 35.5 times (mean $\pm$ S.D.) and 25.1 ± 22.5

Table 2. 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	79.73	52.76	24.73	2.88
S.D.	76.64	82.18	17.16	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. 10. (A) Oscillograms of late instar *Antheraea polyphemus* sounds obtained during one, two, and five pinch trials. The time in which each larva is attacked is indicated with arrows. (B) The mean number of clicks in 60 seconds produced by late instar larvae following the first attack in one, two, and five pinch trials. The total amount of signaling is positively correlated with the number of attacks.

times (mean $\pm$ S.D.) respectively, whereas larvae in five pinch trials signaled on average 54.3 ± 46.1 times (mean $\pm$ S.D.). 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; refer to Appendix I for raw data).

Regurgitation was also positively correlated with an increase in the number of attacks (Fig. 11A,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. 11C; refer to Appendix II for raw data). In five pinch trials, larvae regurgitated most often with the second pinch, and rarely regurgitated after the fourth or fifth pinch (Fig. 11D). Many of the larvae directed their regurgitant towards their 'attacker' by wiping their mouthparts on the forceps. In addition, all larvae recovered their regurgitant by re-imbibing the fluid once an attack was terminated.

The relationship between sound production, regurgitation, and number of attacks is illustrated in Fig. 12A. 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 (chi-square=35.4, $P<0.001$; Fig. 12B).

Grasping and pecking by the beak of a chick was sufficient to induce sound production in 100% of larvae ($N=16$; Fig. 13). 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 12.5% of trials, the precise timing of regurgitation in relation to clicking was not

Fig. 11. (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 (denoted by the letter 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.

Fig. 12. (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.

Fig. 13. The percent distribution of sound production and regurgitation in fifth instar *Antheraea polyphemus* larvae in response to attacks by chicks. Sound production occurred most often in conjunction with regurgitation, and secondly preceding regurgitation.

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 6 cases where chicks attacked only once, larvae signaled 39.3 ± 27.3 times (mean $\pm$ S.D.). 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 one to four 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.

3.3 Invertebrate bioassay

Of the control trials in which untreated mealworm segments were offered to ants, 15 of 15 trials were accepted within 11 minutes or less. Similarly, of the control trials in which mealworm segments were coated in water, 11 of 11 trials were accepted within 10 minutes or less. Of the experimental trials in which mealworm segments were coated in regurgitant, 14 of 16 trials were accepted within 45 minutes or less, while in two trials, the segments were completely rejected. On average, and excluding the 2 trials that were not accepted at all, ants took significantly longer to accept segments coated in regurgitant (975.6 ± 661.1 s, mean $\pm$ S.D., $N=14$), than untreated segments (260.8 ± 151.1 s, mean $\pm$ S.D., $N=15$, Mann-Whitney *U* test, $P=0.00008$, two-tailed test) or water-covered segments (210.7 ± 132.3 s, mean $\pm$ S.D., $N=11$, Mann-Whitney *U* test, $P=0.00003$, two-tailed test; Fig. 14). There was no difference between average acceptance times of the 2 controls (Mann-Whitney *U* test, $P=0.19$, two-tailed test).

In addition to monitoring acceptance rates, preening behaviour of the ants was

Fig. 14. The amount of time taken for ants to carry untreated mealworm segments, segments covered with distilled water, and segments covered in *Antheraea polyphemus* regurgitant from larvae fed on *Quercus rubra*, 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.

noted. To control for trials with short duration times, preening was only monitored during the first 60 seconds 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 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 only once (1 of 11 trials).

3.4 Vertebrate bioassay

Under choice test conditions, mice preferentially consumed chow coated in distilled water over chow coated in regurgitant (Mann-Whitney *U* test, $P=0.0039$, two-tailed test, Fig. 15). On average, mice consumed $46.0 \pm 10.6\%$ (mean $\pm$ S.D.) of water-covered chow compared to only $28.5 \pm 11.2\%$ (mean $\pm$ S.D.) of regurgitant-covered chow. Data were examined for left and right position preferences between control and experimental diets, however no observable trend was detected.

3.5 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 additional sound-producing species, *A. luna* (Saturniidae) and *M. sexta* (Sphingidae) (Fig. 16). Both species produced audible clicking sounds when pinched with forceps. Similar to the

Fig. 15. Mean consumption of mouse chow coated in distilled water (control) versus mouse chow coated in *Antheraea polyphemus* regurgitant (experimental) from larvae fed on leaves of *Quercus rubra*.

Fig. 16. Fifth instar *Actias luna* (A) and *Manduca sexta* (B) larvae. Scale bars, 1 cm. (C and 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 and F) Clicks from the trains in C and D respectively, with an expanded time scale showing that clicks are generally composed of two components.

sound-producing mechanism of *A. polyphemus* larvae, clicks were produced by the mandibles.

Clicks produced by *A. luna* larvae were on average 69.8 ± 8.4 ms (mean $\pm$ S.D., $N=9$), with a peak frequency of 21.5 ± 9.7 kHz (mean $\pm$ S.D., $N=14$). Upon closer inspection at high resolution, almost all clicks consisted of two components (Fig. 16E). Clicks produced by *M. sexta* larvae were on average 32.6 ± 10.3 ms (mean $\pm$ S.D., $N=10$), with a higher peak frequency of 38.0 ± 7.4 kHz (mean $\pm$ S.D., $N=15$), and also generally consisted of two components (Fig. 16F). Five consecutive pinches with forceps induced all *A. luna* larvae to click ($N=10$). Regurgitation occurred in 6 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 3). *A. luna* and *M. sexta* clicked and regurgitated, but 8 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 2 species, *A. io* and *B. mori*, neither produced sound nor regurgitated.

Table 3. 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	<i>Bombyx mori</i> Linnaeus	<i>Morus rubra</i>	No	-	No	-
Saturniidae	<i>Actias luna</i> Linnaeus	<i>Betula papyrifera</i>	Yes	Mandibles	Yes	-
	<i>Automeris io</i> Fabricius	<i>Betula papyrifera</i>	No	-	No	-
	<i>Callosamia promethea</i> Drury	<i>Betula papyrifera</i>	No	-	Yes	-
	<i>Dryocampa rubicunda</i> Fabricius	<i>Acer saccharum</i>	No	-	Yes	-
	<i>Hyalophora cecropia</i> Linnaeus	<i>Betula papyrifera</i>	No	-	Yes	-
Sphingidae	<i>Rhodia fugax</i> Butler*	-	Yes	Mandibles	?	Packard, 1904; Dumortier, 1963
	<i>Acherontia atropos</i> Linnaeus*	-	Yes	Mandibles	?	Mead, 1869; Pearce, 1886
	<i>Amorpha juglandis</i> Smith*	-	Yes	-	?	Sanborn, 1868; Wagner, 2005
	<i>Hyles euphorbiae</i> Linnaeus	<i>Euphorbia</i> sp.	No	-	Yes	-
	<i>Manduca sexta</i> Linnaeus	<i>Solanum lycopersicum</i>	Yes	Mandibles	Yes	-
	<i>Mimas tiliae</i> Linnaeus	<i>Alnus</i> sp.	No	-	Yes	-
	<i>Pachysphinx modesta</i> Harris	<i>Populus tremuloides</i>	No	-	Yes	-
	<i>Smerinthus excacatus</i> Smith*	-	Yes	Mandibles	?	Sanborn, 1868
	<i>Smerinthus dissimilis</i> Bremer*	-	Yes	-	?	Federley, 1905
	<i>Smerinthus cerisyi</i> Kirby	<i>Populus tremuloides</i>	No	-	Yes	-
	<i>Smerinthus jamaicensis</i> Drury**	<i>Populus tremuloides</i>	Yes (Sanborn, 1868) No (this study)	-	Yes (this study)	Sanborn, 1868
	<i>Sphecodina abbottii</i> Swainson*	-	Yes	Mandibles	?	Dumortier, 1963; Wagner, 2005

*Information from the literature only

**Reclassified from *Smerinthus geminatus* Say

4. Discussion

In this study, I investigated the phenomenon of clicking caterpillars in the superfamily Bombycoidea and focused primarily on larvae of the silkmoth species, *A. polyphemus*. I demonstrated that upon disturbance, *A. polyphemus* larvae produce airborne sounds using their mandibles that often precede defensive regurgitation. My hypothesis, that larvae are producing sounds to warn predators of an impending regurgitant defense, was supported by several lines of experimental evidence. In the following discussion, I will: (i) begin by defining the term aposematism, (ii) examine my results with respect to the testable predictions outlined in my introduction, (iii) propose alternative hypotheses for the function of signaling in *A. polyphemus*, (iv) discuss, in general, the concept of sound production in caterpillars by investigating the evolution of acoustic, rather than visual, aposematic signals, and (v) provide future directions for this important area of research.

4.1 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 (see reviews in 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, 2005). Therefore, in the context of this discussion, I will use the term acoustic aposematism synonymously with warning sounds.

4.2 Are *A. polyphemus* clicks acoustic aposematic signals?

My 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 by several lines of evidence. 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 an increase in 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 second period than did larvae that were pinched only once or twice.

My third prediction stated 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 permits 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 invertebrate predators, such as 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 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 the 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 mealworms 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. Although some preliminary research was initiated to determine the contents of *A. polyphemus* regurgitant, no results were obtained (for more information, refer to Appendix III). Thus, the chemical composition of the 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 my 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.

4.3 Alternative hypotheses

My 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.

From the experimental evidence obtained in this study, it seems certain that sound production serves a defensive function, however there is a remaining possibility regarding the mechanism through which the clicks exert their function. A third alternative hypothesis is that the clicks produced by larvae 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. In this study, I 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 (Boettner, personal communication).

4.4 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 in *M. sexta*. In 2001, Walters et al. provided a detailed account of the defensive responses of laboratory-reared and wild *M. sexta* larvae following a series of simulated attack experiments. 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 that are on average 21.5 kHz and 38.0 kHz, respectively. Both values are considerably higher than the mean peak frequency of clicks produced by *A. polyphemus* larvae (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 I tested did not produce sound. In fact, in one instance, sound production was not present in a species previously reported as sound-producing. In his letter to the Canadian Entomologist, Sanborn (1868) wrote, "... my valued friend, Mr. Philip S. Sprague, of this city (Boston), has recalled the fact of a similar sound being produced by larvae of ... *Smerinthus geminatus*, Say (reclassified as *S. jamaicensis*), when irritated, in the breeding cage. Mr. S. has, in his own mind, attributed this sound to the motion of the mandibles upon each other ...". In my 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 in Walters et al. (2001). However, sound production is not a regional characteristic for *A. polyphemus*, since larvae from Ontario, Prince Edward Island, and Massachusetts all produce sound. It could be that *S. jamaicensis* was misidentified, or that the taxonomic reclassification of *S. jamaicensis* from *S. geminatus* is incorrect. Either way, the incongruence of my

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 my 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 families, possibly because larvae from the other families are too small to make audible sounds.

4.5 The evolution of acoustic, rather than visual, aposematic signals

Many animals employ the use of acoustic cues 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 put forth by Ratcliffe and Fullard (2005), who have suggested 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 my reasoning to be upheld, it must be shown that *A. polyphemus* (and other sound-producing larvae) are, in fact, visually cryptic to their predators.

4.6 Future directions

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 my 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 also 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 in caterpillars.

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6. Appendices

6.1 Appendix I: Click data

Raw data for the number of clicks in 60 seconds produced by late instar *Antheraea polyphemus* larvae following the first attack in one, two, and five pinch trials.

Trial #	One pinch	Two pinch	Five pinch
	Number of clicks in 60 seconds:		
1	3	8	72
2	0	54	0
3	0	45	0
4	0	3	53
5	27	10	0
6	0	26	0
7	2	19	0
8	0	20	0
9	0	20	25
10	13	25	0
11	62	4	107
12	57	24	85
13	24	26	8
14	0	47	0
15	29	49	22
16	138	0	29
17	182	26	0
18	97	2	13
19	33	51	89
20	39	0	46
21	0	34	28
22	18	0	42
23	69	47	73
24	4	0	17
25	0	0	26
26	0	0	96
27	0	3	16
28	10	86	97
29	0	30	166
30	14	31	93
31	14	22	108
32	0	2	135
33	59	79	58
34	1	0	83
35	8	7	94
36	0	40	22
37	0	54	45
38	0	39	74
39	8	61	38
40	16	45	71
41	21	42	139
42	0	55	61
43	19	0	12
44	34	26	66
45	0	17	77
46	14	6	82
47	2	6	18

48	4	12	143
49	19		36
50	0		152
51	6		
52	26		
Average:	20.62	25.06	54.34
Standard deviation:	35.48	22.48	46.07

6.2 Appendix II: Regurgitation data

The number of late instar *Antheraea polyphemus* larvae that regurgitated for the first time following one, two, and five pinches with forceps.

Trial #	One pinch	Two pinch	Five pinch
	Regurgitation? Y or N		
1	N	N	Y
2	Y	N	N
3	N	N	Y
4	N	Y	Y
5	N	N	Y
6	N	N	N
7	N	Y	N
8	N	N	N
9	N	N	N
10	N	N	Y
11	N	Y	N
12	N	N	Y
13	Y	N	Y
14	N	Y	Y
15	N	N	Y
16	N	Y	Y
17	N	N	Y
18	N	N	N
19	Y	N	Y
20	N	Y	Y
21	N	Y	N
22	N	Y	Y
23	N	Y	N
24	N	N	N
25	N	N	Y
26	N	N	Y
27	N	N	Y
28	N	N	Y
29	N	Y	N
30	N	Y	Y
31	N	Y	Y
32	N	N	N
33	N	N	Y
34	N	Y	Y
35	N	N	N
36	N	Y	N
37	N	Y	N
38	Y	Y	N
39	N	Y	Y
40	N	Y	Y
41	N	N	Y
42	N	N	Y
43	N	Y	Y
44	N	N	Y
45	N	Y	Y
46	Y	N	Y
47	N	N	Y
48	N	N	Y
49	N		Y

50	N		Y
51	N		
52	N		
Frequency of regurgitation:	5/52	20/48	34/50
Percentage of trials in which larvae regurgitated:	9.62%	41.67%	68.00%

6.3 Appendix III: Chemical composition of the regurgitant

Currently, the source of the deterrent properties of *A. polyphemus* regurgitant is unknown. It could be that the sticky nature of the regurgitant is sufficient to repel predators, or the regurgitant may contain chemicals that render it distasteful. Should the latter be true, it is important to determine whether the chemicals are synthesized de novo by the caterpillar or sequestered from the leaves of the caterpillar's host plants. Some preliminary research was conducted to help resolve some of this uncertainty, however the experiment was not completed in full and no results were obtained. Time constraints and machinery breakdown contributed to the lack of completion. However, although the experiment remains incomplete, the reasoning behind its design is worth examining.

Many species of Lepidoptera are associated with chemically-defended plants. Some sequester materials from the plants rather than manufacturing their own defensive compounds (reviewed in Nishida, 2002). Plant secondary chemicals sequestered by Lepidoptera include many terpenic, phenolic, and nitrogen-containing compounds. Conversely, some species of Lepidoptera obtain their defensive quality by synthesizing toxins (reviewed in Blum, 1987). Various larval species fortify their bodies with spines or urticating hairs containing synthesized histamine (e.g. Rothschild, 1985), while some that are strongly associated with cyanogenic plants are capable of synthesizing cyanogenic glycosides (e.g. Nahrstedt, 1996). Currently, there are no examples of larvae that synthesize chemicals for use in defensive regurgitation. As a result, it seems reasonable to propose that *A. polyphemus* regurgitant acquires its defensive quality through the sequestration of host plant chemistry.

If *A. polyphemus* larvae are given free reign to feed on leaves of different tree species such as red oak, white oak, pin oak, sugar maple, Norway maple, white birch, ironwood, and walnut, they will preferentially select red oak (Fleishman, personal communication). The reason for this preference is currently unknown, although it is possible that consuming leaves from red oak bestows an enhanced defensiveness to the caterpillar's regurgitant. The leaves of the tree species fed to *A. polyphemus* larvae in this study contain a diverse array of polyphenols. They are among the most widely studied plant secondary compounds and their production is often regarded as an indication of chemical defense (Riipi et al., 2002). One sub-class of plant polyphenols are tannins, broadly defined as water soluble, high molecular weight polymers that have the ability to bind proteins (Bate-Smith and Swain, 1962). Tannins may be classified into two functional groups, hydrolyzable and condensed tannins (proanthocyanidins). Generally, tannins induce a negative response when consumed. These effects can be instantaneous such as a bitter or unpleasant taste, or can have a delayed response related to anti-nutritional and toxic effects (Forkner et al., 2004). High mortality and morbidity have been observed in sheep and cattle fed oak and other tree species containing more than 20% hydrolyzable tannins. In poultry, levels from 0.5-2.0% can cause depression in growth and egg production; levels from 3-7% can cause death (Reed, 1995).

Given that sufficient information exists to support the idea that host plant chemistry may be responsible for the defensive nature of *A. polyphemus* regurgitant, I designed an experiment which would help to resolve its composition. Using high performance liquid chromatography (HPLC), I planned to examine the similarity in regurgitant profiles to those of the caterpillars' host plant leaves. In addition, I

planned to compare the HPLC profiles of the regurgitant to the profiles of chemical standards known to be present in the leaves of the caterpillars' host plants.

The following is a brief summary of the preliminary work undertaken. The heads of *A. polyphemus* larvae were pinched with forceps to induce regurgitation. Using sterile pipettes, samples were collected from larvae fed on red oak, paper birch, and sugar maple and emptied into 1.5 ml pre-weighed vials. Acetone was added to each vial in a 10:1 ratio, and the solutions were sonicated for 30 minutes using a Branson 5210 sonicator. The solubilized fractions were filtered into 1.5 ml vials using 0.2 µm filters attached to 1 mL syringes. Similarly, leaves from the three host plants were excised from their petioles and ground with liquid nitrogen using a mortar and pestle. Acetone was added to each vial in a 10:1 ratio, and the solutions were sonicated for 30 minutes. All samples were placed at 4°C. As was previously mentioned, analysis of the solutions was to be conducted using HPLC, however the equipment required repair with only a short period of time remaining in this study.

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