Why do caterpillars whistle at birds? Insect defence sounds startle avian predators

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ABSTRACT

Many insects produce sounds when attacked by a predator, yet the functions of these signals are poorly understood. It is debated whether such sounds function as startle, warning or alarm signals, or merely serve to augment other defences. Direct evidence is limited owing to difficulties in disentangling the effects of sounds from other defences that often occur simultaneously in live insects. We conducted an experiment to test whether an insect sound can function as a deimatic (i.e. startle) display. Variations of a whistle of the walnut sphinx caterpillar (*Amorpha juglandis*) were presented to a predator, red-winged blackbirds (*Agelaius phoeniceus*), when birds activated a sensor while feeding on mealworms (*Tenebrio molitor*). Birds exposed to whistles played back at natural sound levels exhibited significantly higher startle scores (by flying away, flinching, and hopping) and took longer to return to the feeding dish than during control conditions where no sounds were played. Birds habituated to sounds during a one-hour session, but after two days the startling effects were restored. Our results provide empirical evidence that an insect sound alone can function as a deimatic display against an avian predator. We discuss how whistles might be particularly effective 'acoustic eye spots' on avian predators.

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1. Introduction

Defence sounds, variously named distress, alarm, warning, or disturbance signals, depending on their purported functions, are widespread throughout the class Insecta (Alexander, 1967; Ewing, 1989; Masters, 1980). These acoustic signals, which can be transmitted as air- or solid-borne vibrations, are made in response to being handled or otherwise disturbed. They occur in species ranging in size from tiny beetles (Lewis and Cane, 1990) to giant wetas (Field, 1980) and are generated by a variety of mechanisms including stridulation, forced air, percussion and tymbalation (Ewing, 1989). Despite their prevalence and diversity, surprisingly little is known about the effects of these sounds on predators, and this subject has generated ongoing debate (Conner, 2014; Rowe and Halpin, 2013; Siddall and Marples, 2011). Proposed functions can be broadly categorized according to the target audience of the signals; those directed at conspecifics and some heterospecifics may func-tion as alarm signals to warn of impending danger or to recruit help (Cocroft, 1999; Hager and Kirchner, 2013). Alternatively, signals may be directed at predators, where proposed functions include but are not limited to, acoustic aposematism, startle, enhancement of visual signals, sonar jamming, and mimicry of something dangerous (Conner 2014; Masters, 1980; Rowe and Halpin, 2013). Our understanding of the protective value of defence sounds is limited because live insects may have multiple components to their displays, making it difficult to isolate the effects of the sounds alone (Rowe and Halpin, 2013; Siddall and Marples, 2011). Also, a defence sound may have overlapping functions; for example, in a live insect that is chemically defended, a sound could function as both an aposematic and a deimatic display (Ruxton et al., 2004; Skelhorn et al., 2016). In this study we test the hypothesis that an insect sound can function as a deimatic display, by isolating the effects of sound from the insect using a playback system.

Deimatic displays have long been characterized as a type of antipredator defence that functions by frightening or startling a predator (Edmunds, 1974; Ruxton et al., 2004; Sargent, 1990; Skelhorn et al., 2016). Classically cited examples include rapid eyespot displays on the hind wings of moths, or the sudden appearance of high contrast markings in cuttlefish (reviewed in Edmunds,

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1974). Three criteria are commonly used to assess the startling effects of a stimulus included in a deimatic display: (i) it evokes a startle response (e.g. fear, surprise, confusion) in a predator; (ii) it causes hesitation, resulting in a longer time to return to the prey; and (iii) it has a transitory effect on the predator, whose response should habituate over repeated exposure to the stimulus (Pilz and Schnitzler, 1996; Pomeroy and Heppner, 1977; Ruxton et al., 2004; Sargent, 1990; Skelhorn et al., 2016). Many studies have proposed that insect sounds function as deimatic displays against vertebrate predators including bats (Bates and Fenton, 1988; Mohl and Miller, 1976), rodents (Masters, 1979; Olofsson et al., 2012b; Smith and Langley, 1978), reptiles (Sandow and Bailey, 1978) and birds (Maldonado, 1970). However, few studies have empirically measured the startle response in predators (Umbers and Mappes, 2016), and a caveat must be placed on conclusions drawn from characteristics of the display alone (Skelhorn et al., 2016), or when live prey are used for previously stated reasons. To date, the deimatic function of insect defence sounds has not been subject to rigorous experimental testing.

There is current debate on what comprises a deimatic display, and whether in fact it is even a distinct form of defence (Rowe and Halpin, 2013; Skelhorn et al., 2016; Umbers et al., 2015; Umbers and Mappes, 2016). A deimatic function cannot be confirmed based solely on display characteristics (e.g. being evoked by a predator, or the sudden and conspicuous nature of the components), because such dynamic features could also function in aposematism, deflection of attack to an expendable body part (in the case of visual displays), or 'distress' calls to attract competing consumers (see Ruxton et al., 2004; Skelhorn et al., 2016). Also, defensive displays often have multiple components, and conceivably each component could have a different function (Skelhorn et al., 2016). Skelhorn et al. (2016) argue that instead of focusing on the display characteristics of the prey, we need to 'ask their predators'. Deimatic displays, they argue, should be defined based on the display's ability to exploit classic fear responses in predators, which can be empirically measured as a startle reflex; and ideally, such tests should be conducted using ecologically relevant predators. At present, they argue, there is no direct evidence that deimatic displays elicit fear responses in predators (Skelhorn et al., 2016). Furthermore, they advocate that it is particularly important to know the mechanism through which each component of the display operates. Umbers and Mappes (2016) concur that a predator's startle reflex should be elicited, but caution that this is difficult to measure directly. Our experiment addressed these concerns by empirically measuring startle responses elicited by one stimulus component- sound- in an ecologically relevant predator.

Our goal was to test the hypothesis that caterpillar whistles function as deimatic displays. The walnut sphinx caterpillar (Amorpha juglandis) (Sphingidae) (Fig. 1a) is a cryptically coloured species native to North America that when attacked, generates whistles from its spiracles (Bura et al., 2011) (Supplementary Video S1). Whistles produced upon attack are short in duration (~450 ms), broadband and multiharmonic with peaks ranging from 9 to 22 kHz, and are relatively loud (69–82 dB SPL at 5 cm) (Bura et al., 2011) (Fig. 1b and c). Previously it was proposed that whistles startle predators based on characteristics of the sounds (sudden, short duration and loud), the lack of an obvious associated chemical defence, and because in predatory trials with captive yellow warblers (Dendroica petechia, Parulidae), birds attacking live caterpillars jumped back or dove away (Bura et al., 2011). While these results support the hypothesis that whistles startle birds, the results are inconclusive, because responses of the three birds were not quantified or compared to controls, and the effects of sounds were confounded by the use of live caterpillars that also thrashed in response to attack (Bura et al., 2011) which may in itself have a startling effect. Here we test that whistles alone elicit a startle
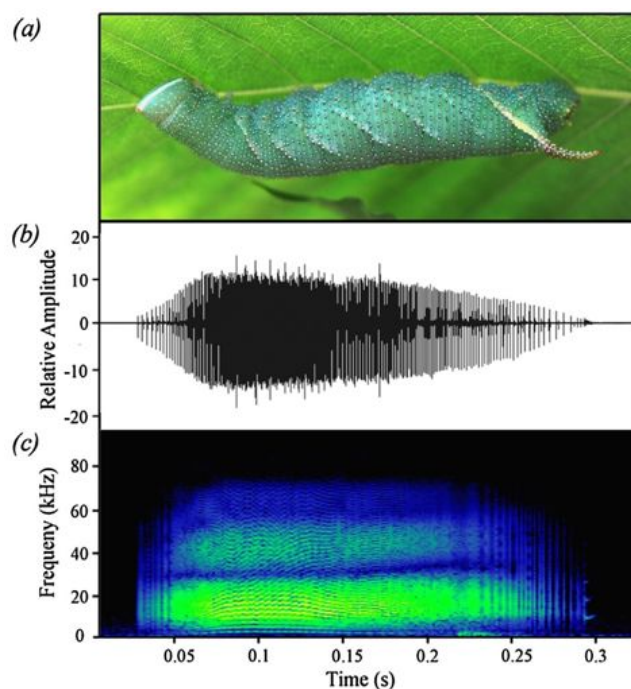

Fig. 1. (a) A late instar larva of the walnut sphinx moth (Amorpha juglandis) in its natural resting position on the underside of a leaf on its host plant. (b-c) Sound waveform and corresponding spectrogram of a single defensive whistle. The spectrogram was produced using a 512-point Fast Fourier Transform (Hann window, 50% overlap).

response in a native predator of Lepidoptera, the red-winged blackbird (Agelaius phoeniceus, Icteridae) (Robertson, 1973). To isolate the effects of the sound we used a sensor programmed to trigger sound playbacks when birds attacked mealworms. Following the criteria for deimatic displays described above, we predicted: (i) birds feeding on mealworms paired with sounds played at natural levels (60, 70 dB SPL) would exhibit higher startle scores compared to controls where no sounds were played; (ii) birds exposed to sound while feeding on mealworms would take longer to re-approach mealworms a second time compared to controls; (iii) birds would habituate to sounds upon repeated exposure within a trial.

2. Materials and methods

2.1. Animals and housing conditions

Twelve wild adult male red-winged blackbirds (Agelaius phoeniceus, Icteridae) were originally captured as controls for a previous experiment under a Canadian Wildlife Service permit to capture migratory birds (10771) and a Queen's University Animal Care Committee (UACC) protocol (2013-027) and subsequently used in our experiment under a UACC protocol (2014-1487) (Supplementary Data S1). All experiments were conducted at the Queen's University Biological Station (QUBS) near Chaffey's Lock, Ontario, Canada (44°33′55.34″N, 76°19′26.59″W) during June and July 2014 where birds were housed singly in individual flight cages in an outdoor aviary. The aviary consisted of 30 large (6 × 2.5 × 2.5m) cages arranged in two rows with an access hallway down the middle. Each cage was equipped with tree branches for perching, a sheltered area, shallow water bath, water dish, and a feeding platform comprising a plastic crate (60 × 60 × 30 cm) with a Petri dish (9 × 1.5 cm) affixed to the top (Fig. 2). Birds were offered a variety of foods daily including poultry starter, dragonflies, romaine
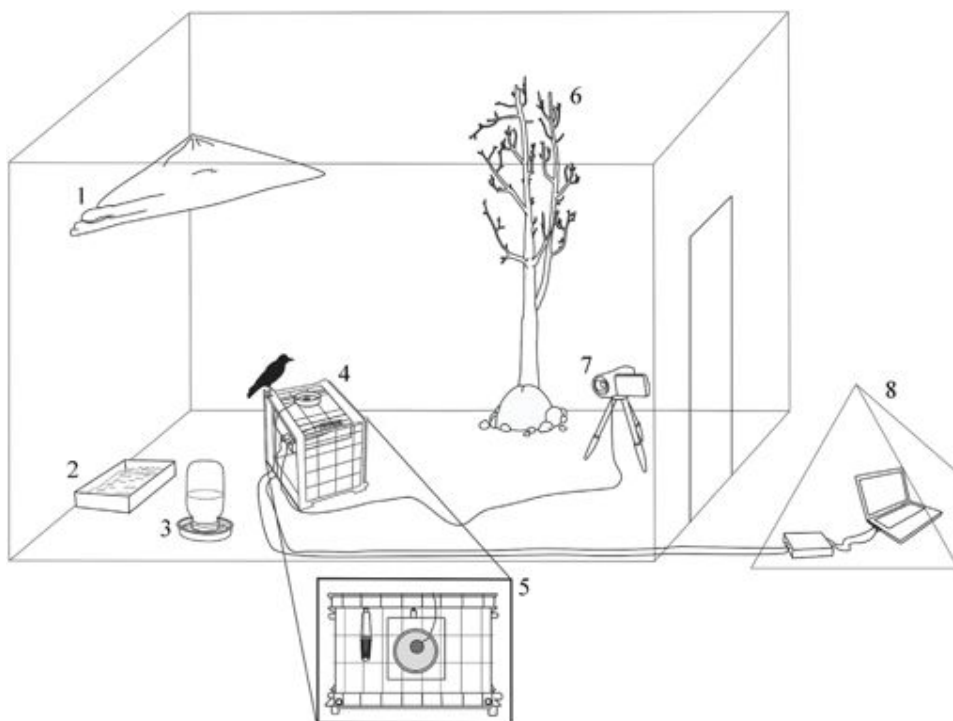
Fig. 2. Schematic representation of the experimental set up, showing the aviary cage and equipment used during control and experimental phases of the study: (1) tarp (2) bath (3) water dish (4) platform (5) top view of platform showing sensor feeding dish, speaker and microphone (6) branches (7) video camera (8) laptop and tent. The feeding platform used in training sessions was replaced with an identical feeding platform equipped with a sound activating sensor, and a speaker connected to a laptop to relay the sounds. A video camera was placed inside the cage and turned on in the experimental phases.

lettuce, corn, sunflower seeds, wild bird seed, thistle-based seed, eggs, strawberries, apples, kiwis, cuttlebone, and oyster shells. When experiments were completed, birds were observed for a minimum of two days to confirm that there were no signs of stress or illness, after which the birds were released at QUBS in a field ∼1 km from the aviaries during good weather conditions.

2.2. Experimental set up

Each bird experienced a training phase, control phase, and one or two experimental phases (see *Experimental Design*) and all phases were conducted within the bird's individual cage. During the training phase, only the equipment described above was present in the cage. During the control and experimental phases, the feeding platform used for training was replaced with an identical platform except that it contained a modified Petri dish with a piezoelectric sensor and speaker (Fig. 2; see *Sound Playback*). The sensor was connected to a Sony PCG 7185L personal computer (Sony; Tokyo, Japan) located in the aviary access hallway under a small tent ∼1 m outside the cage door and ∼2 m from the feeding platform, and the laptop in turn was connected to playback electronics (Fig. 2; see *Sound Playback*). A Sony camcorder (DCRTRV19) on a tripod (60 cm high) was placed inside the cage ∼1 m from the aviary door and ∼1 m from the feeding platform (Fig. 2). A Sony ECM-MS908C microphone connected to the camcorder was placed 10 cm from the playback speaker to monitor sound events for subsequent video analyses (Fig. 2). The set-ups used for the control and experimental phases were identical, except that in the control phase the sensor was disabled.

2.3. Sound playback

The playback stimulus was obtained from a previous study whereby defence sounds were elicited from a walnut sphinx cater-

pillar by administering a light pinch with forceps to its abdomen (Bura et al., 2011). Sounds were recorded using a Bruel & Kjaer (B&K; Naerum, Denmark) ¼ in. microphone (type 4943), amplified with a B&K Nexus conditioning amplifier (type 2690), and recorded to a Fostex FR-2 Field Recorder (Gardena, CA, USA) at a sampling rate of 192 kHz. The sound used for playback experiments was a typical representation of a defensive whistle elicited from a late instar caterpillar (Bura et al., 2011), with a dominant frequency of 9 kHz (bandwidth 14 kHz at −10 dB from peak) and a duration of 410 ms.

Sounds were played when contact was made with a piezoelectric sensor housed within a custom-made feeding dish unit (Fig. 2). The unit comprised a circular black Delrin® plastic receptacle (9.5 cm diameter x 3.5 cm high) with upper and lower compartments. The upper compartment held a clear plastic Petri dish (9 cm diameter × 1.6 cm high). The lower compartment was located inside the unit below the Petri dish, and contained the sensor's circuitry. The sensor was made of a Murata 7BB27-4L0 piezoelectric diaphragm (sensitivity 5 V/microstrain) (Murata Manufacturing Co., Ltd, Nagaokakyo, Kyoto) and a circuit with the output amplified to trigger on a deflection of 0.03 μm. The unit was powered by a USB adaptor connected to the laptop. The diaphragm was placed in the upper receptacle directly beneath the Petri dish so that contact made by the bird feeding on mealworms would trigger the playback. Sounds were broadcast by an Avisoft ScanSpeak (Avisoft Bioacoustics, Glienicke, Germany) (3–120 kHz) speaker concealed beneath the feeding dish (Fig. 2). The sensor dish and speakers were connected to an Avisoft playback system that included an Ultrasound Gate (USG) player 116, and Recorder USGH software.

Sounds were played at two levels, 70 and 60 dB SPL, in experimental phases one and two respectively (see *Experimental Design*) in order to assess the bird's response to a range of naturally occurring intensities of the walnut sphinx's whistle. Sound levels were measured 22 cm from the speaker, within range of the bird's head at the dish, using a B&K sound level meter (type 2239). To ensure

sound characteristics of playbacks represented those of the original recordings, sounds were played 20 times and dB levels were measured as described above to ensure that they fell within 5 dB of each other. Also, sounds were re-recorded 20 times using the B&K microphone and recording chain (see above) and analyzed using RavenPro Bioacoustics Research program 1.4 (Cornell Laboratory of Ornithology, Ithaca, NY, USA) to ensure that they matched the dominant frequency and bandwidth at −10 dB from peak of the original recording.

2.4. Experimental design

Each bird experienced up to four consecutive phases: a training phase, a control phase (no sound), experimental phase one (sounds were played at 70 dB SPL), and experimental phase two (sounds were played at 60 dB SPL). This order of presentation was necessary to allow measurement of habituation of the startle response over time within a phase (see *Statistical Analyses*).

The purpose of the training phase was to familiarize the birds with consuming mealworms from a Petri dish on a feeding platform. Each bird was provided with six mealworms each day for four consecutive days. The dishes were checked after one hour for the number of mealworms consumed. A bird was considered to have been successfully trained if it removed any of the mealworms from the dish each day. Birds that successfully completed the training phase (11/12) served as subjects in the control phase two days later.

During the control phase, birds fed on mealworms from the disconnected sensor feeding dish. One hour prior to testing, all food was removed to increase the bird's motivation to eat. Equipment was then placed in the bird's cage and six mealworms were placed in the feeding dish. Behaviours were video recorded for up to one hour, or until all mealworms were consumed, after which time the equipment was removed from the cage.

Two days later, all birds that completed the control phase (9/11) were given the opportunity to participate in experimental phase one, where all conditions were the same as for the control phase except the sensor dish was activated, triggering sound playbacks at 70 dB SPL when the bird fed from the dish. Two days following phase one, all birds that completed the control phase (9/11) were given the opportunity to participate in experimental phase two. In phase two all conditions were the same as for phase one except the sound volume was decreased to 60 dB SPL. Some birds successfully participated in both experimental phases while others only participated in phase one (see *Results*).

2.5. Behavioural assessment

The following measurements were obtained from video recordings of the control and experimental phases: (i) Startle scores were assigned to each behaviour based on modifications to a previous grading systems for avian startle responses, where behaviours that caused predators to retreat from the prey (i.e. fly away) were given a higher score (Olofsson et al., 2012a; Schlenoff, 1985) (Table 1). Scores were quantified for each bird by recording the highest score that occurred within 500 ms following each contact with the feeding dish (e.g. if a bird flew away and hopped it was assigned a score of 2, but if it hopped and ruffled feathers it was assigned a score of 1); (ii) Latency to return to the feeding dish was measured by noting the times between up to five consecutive contacts with the feeding dish. Behaviours throughout each experimental phase were first scored with the sound turned off (blindly), and then with the sound turned on to mark the onset of the stimulus. All behavioural analyses were conducted using JWatcher 0.9 (D.T. Blumstein et al. UCLA & Macquarie University).

Table 1
Avian startle behaviours and scoring.

Behaviour	Description of behaviour	Score
No reaction	No discernible reaction to consuming mealworm with or without sound	0
Shoulder flinch	Flinching one or both wings, wings do not extend	1
Wing flap	Full extension of one or both wings without flying away from platform	1
Ruffles feathers	Shaking/trembling feathers raised up from skin	1
Body flinch	A whole body sudden short movement	1
Startle hop	A sudden upward hop where both feet go up in the air and back down to the ground	1
Fly away	Flying off platform, flapping both wings	2

Adapted from Olofsson et al., 2012a; Schlenoff, 1985.
A list of avian startle behaviours recorded, definitions of the behaviours and how each behaviour was scored for analysis. Avian Startle Scores were assigned to behaviours based on the following criteria: A score of 0 was given for no discernible reaction to prey; a score of 1 was given for behaviours that caused hesitation to consume prey; and a score of 2 was given for behaviours that caused the predator to retreat away from prey.

2.6. Statistical analyses

To test whether birds startled in response to the sound within 500 ms after first contact with the feeding dish, the startle score of each bird in its control phase was compared with its startle score in the experimental phases that they participated in using a Fisher-Pitman permutation test for paired data. As a directional hypothesis was being tested, a one-tailed test was used (Ruxton and Neuhauser, 2010). A non-parametric test was chosen because the sample size was small (Ryan, 2013). To test whether birds took longer to return to the dish following their first exposure to sound (in experimental phases one and two) compared to no sound (control), the latencies between first and second contact with the dish were log transformed and then compared for each bird using a Fisher-Pitman permutation test for paired data.

To test whether birds habituated to the sounds (or lack thereof) within a phase, we measured the change in startle scores over up to six consecutive contacts with the feeding dish. To quantify habituation, we measured the slope of the least squares line expressing the bird's startle score as a function of the discrete time of dish contact, where the first dish contact is a time of 1, the second dish contact is a time of 2, and so on. Under the assumption of no habituation, a bird's startle scores are exchangeable and on average the slope of the startle score against time is zero. To test the significance of the mean slope of the startle score against time, we used a permutation test with within-bird resampling. Since the number of permutations is too large, we used a Monte-Carlo simulation with 5000 samples to estimate the p-value (Motulsky and Christopoulos, 2004). By using 5000 samples, the maximum error of the estimate will be less than 1.5% at a 95% confidence level.

As an additional measure of habituation, we assessed whether birds took less time to return to the feeding dish over time by measuring latencies between up to five contacts with the feeding dish within a phase. The latencies were log transformed and then we measured the slope of least square line of the latencies. To test the significance of the mean slope against time, we used the same tests as described above.

We also examined how long it took birds to consume mealworms over the course of six contacts with the feeding dish, and how the types of startle behaviours changed during this period. We measured the time difference between the first and sixth contact

with the dish for each bird in each experimental phase and compared this to the difference in the control phase using a two-tailed Fisher Pitman permutation test for paired measurements. Changes in startle behaviours over time were documented by charting the proportion of each behaviour type that contributed to the mean startle score for each dish contact.

To determine whether birds that had previously experienced the sound in experimental phase one remained habituated to the stimulus in experimental phase two, and to test whether birds responded differently to sound levels in experimental phases one (70 dB SPL) and two (60 dB SPL), we conducted two separate types of analyses. First, we compared startle scores (up to 500 ms after first exposure to sound) and latencies (between first and second contact with dish) of birds that had participated in both phases using a two-tailed paired samples Fisher-Pitman permutation test. We also compared the startle scores (up to 500 ms after first exposure to sound) and latencies (between first and second contact with dish) of all birds regardless of whether they had participated in both phases, using a combined paired and two-sample data permutation test (Einsporn and Habtzghi, 2013). For the second analysis to combine the paired and unpaired observations, the test statistic generated was a weighted combination of the mean differences of the unpaired and paired observations. All statistical analyses were conducted using the R Project 3.0.1 (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1. Participation in experimental phases

Eleven of the original 12 birds successfully completed the training phase, but only nine (Birds 2,4,5,8,9,11,18,21,30) participated in the control phase. All nine birds were given the opportunity to participate in each of the following two experimental phases. Of these nine birds, six participated in phase one (Birds 2,9,11,18,21,30), and eight in phase two (Birds 2,4,5,8,9,18,21,30). All behaviours identified *a priori* as startle responses (Fig. 3, Table 1) were observed over the course of this study.

3.2. Do sounds startle birds?

Startle scores measured within 500 ms of first contact with the dish were significantly higher in both experimental phases (with sound) than in control phases (no sound) (Phase one: $X \pm SE = 1.83 \pm 0.41$, $N = 6$; Control: $X \pm SE = 0.33 \pm 0.82$, $N = 6$, Fisher Pitman test: $t6 = -4.39$, $P = 0.016$), (Phase two: $X \pm SE = 1.63 \pm 0.74$, $N = 8$; Control: $X \pm SE = 0.5 \pm 0.93$, $N = 8$; Fisher Pitman test: $t8 = -3.21$, $P = 0.027$) (Fig. 4). Of the startle behaviours listed *a priori* (Table 1), fly away was the most common upon first contact with the dish in all phases (Supplementary Fig. S1, Supplementary Video S2). Latencies to return to feed following first contact with the feeding dish were higher for experimental conditions compared to controls but these differences were not significant (Phase one: $X \pm SE = 673 \pm 1154$ s, $N = 6$; Control: $X \pm SE = 3.6 \pm 5.83$ s, $N = 6$; Fisher Pitman test: $t6 = -2.8$, $P = 0.147$), (Phase two: $X \pm SE = 36.63 \pm 60.94$ s, $N = 8$; Control: $X \pm SE = 5.36 \pm 7$ s, $N = 8$; Fisher Pitman test $: t8 = -1.14$, $P = 0.281$).

3.3. Do birds habituate to sounds within a phase?

Habituation was assessed by evaluating the changes in startle scores, and latencies to return to the dish across six consecutive contacts within a phase. Our results show that startle scores for both experimental phases decreased over time; in experimental phases one and two the slopes across all birds were −0.23 and −0.18 respectively. While startle scores decreased over time, this
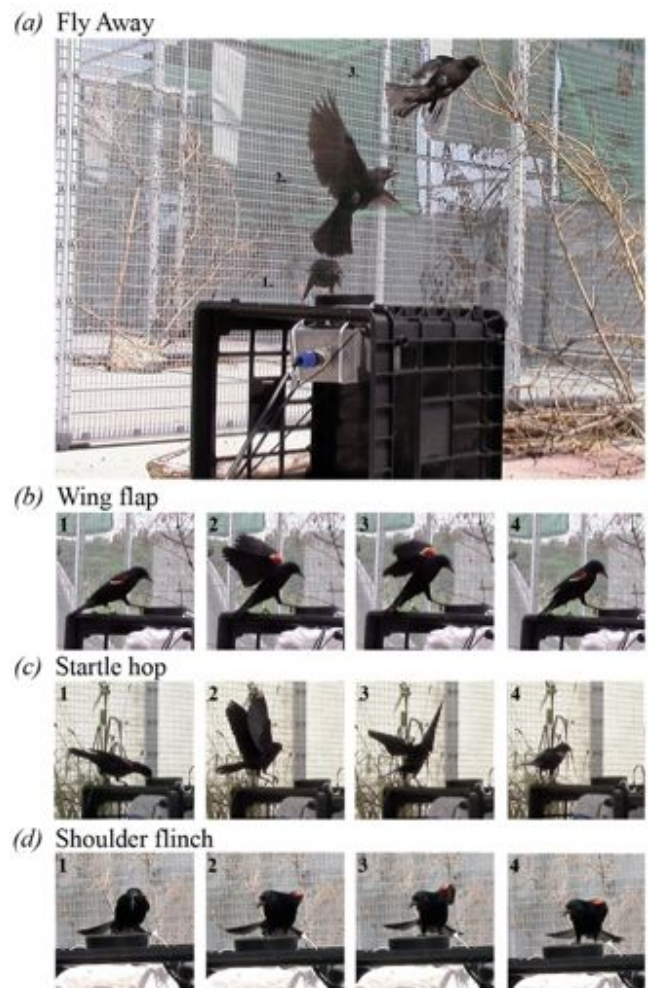

Fig. 3. The four most frequent startle behaviours occurring 500 ms after exposure to the sounds in experimental phases one and two were (a) fly away (b) wing flap (c) startle hop and (d) shoulder flinch. The numbers indicate consecutive screen captures of the first to fourth positions of the bird during each response.

change was not statistically significant for experimental phase one ($P = 0.11$), but was for phase two ($P = 0.007$) (Fig. 5a and b, Supplementary Video S2). In the control condition, the startle scores also decreased significantly over time ($P = 0.005$), although the slope of the line (Slope = −0.12) was small relative to the experimental phases (Fig. 5c). The change in latencies between successive contacts with the dish decreased over five contacts with the feeding dish for both experimental phases (Supplementary Fig. S2). In phase one this decrease was significant (Slope = −1.07, $P = 0.0002$). In experimental phase two there was a negative but non-significant decrease in latency over time (Slope = −0.63, $P = 0.069$). As expected, in the control phase there was no significant positive or negative change in latency over time (Slope = −0.01, $P = 0.491$) (Supplementary Fig. S2).

Birds took longer to consume all mealworms during the course of the trial in both experimental phases compared with the control. This time difference was significantly higher in experimental phase two (Phase two: $X + SE = 378.57 \pm 524.6$ s, $N = 8$; Control: $X + SE = 9.99 \pm 8.88$ s, $N = 8$; Fisher Pitman test: $t8 = -1.77$, $P = 0.03$), but not for experimental phase one (Phase one: $X \pm SE = 531.75 \pm 579.41$ s, $N = 6$; Control: $X + SE = 9.18 \pm 10.1$ s, $N = 6$; Fisher Pitman test: $t6 = 1.78$, $P = 0.13$) (Supplementary Table S2).

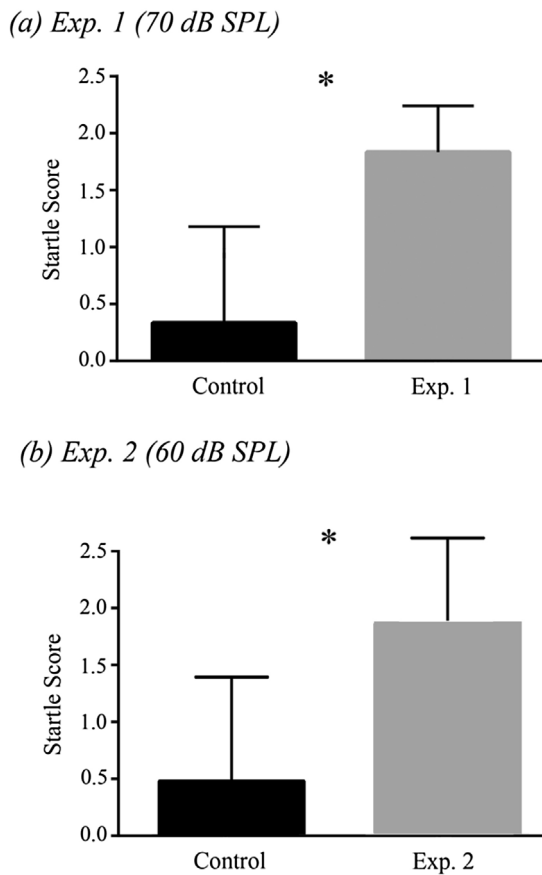(a) Exp. 1 (70 dB SPL)

(b) Exp. 2 (60 dB SPL)

Fig. 4. Birds exposed to playbacks of walnut sphinx whistles show significantly higher mean startle scores up to 500 ms after first contact with the feeding dish than in their respective control conditions in (a) experimental phase one (Phase one: $X \pm$ SE = 1.83 ± 0.41, N = 6; Control: $X +$ SE = 0.33 ± 0.82, N = 6; P = 0.016); and (b) experimental phase two (Phase two: $X \pm$ SE = 1.63 ± 0.74, N = 8; Control: $X +$ SE = 0.5 ± 0.93, N = 8; P = 0.027).

The types of startle behaviours changed over the course of all phases. Upon first contact with the dish within a phase, flying away comprised the highest percentage of all behaviours observed: phase one (83%), phase two (66%), and control (100%). However, as the number of contacts with the dish increased, a greater variety of behaviours was observed (i.e. wing flapping, hopping, flinching, ruffling feathers) (Supplementary Fig. S1, Supplementary Table S1).

3.4. Do birds respond differently between experimental phases?

There were no significant differences between experimental phases with respect to the birds' startle scores or latencies to return to feed upon first approach, regardless of experience or sound level. Startle scores for birds that participated in both phases did not differ between experimental phase one ($X \pm$ SE = 1.8 ± 0.44, N = 6) and phase two ($X \pm$ SE = 1.8 ± 0.44, N = 6) (X Difference = 0; Fisher Pitman test: $t4 = -1.63$, P = 1), nor did latencies differ between phase one ($X \pm$ SE = 226.86 ± 413.48 s, N = 5) and phase two ($X \pm$ SE = 17.86 ± 20.54 s, N = 5) (X Difference = 209; Fisher Pitman test: $t4 = 1.45$, P = 0.302). These results suggest that even though birds habituated in phase one, they startled to the sound when reintroduced in phase two, and startle scores and latencies did not differ by sound level. When considering all birds combined, regardless of their previous experience, startle scores (Combined Paired Permutation Test: Difference[w] = 0; P = 1) and latencies between first and second contact with the feeding dish (Combined Paired Permutation Test: Difference[w] = 921.1, P = 0.08) were also not found to
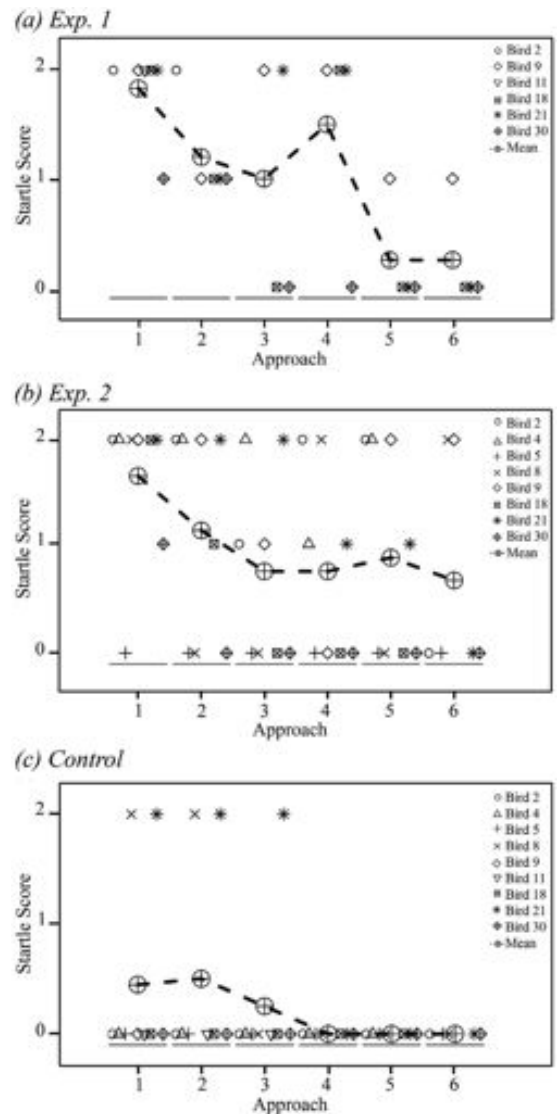

Fig. 5. Change in startle score over the course of 6 contacts with the feeding dish amongst individuals in (a) experimental phase one (b) experimental phase two and (c) control phase. The dotted line represents the overall mean within each phase.

be significantly different between phases one and two. This result suggests that startle scores and latencies were the same at the two sound levels regardless of experience.

4. Discussion

4.1. Are caterpillar whistles deimatic displays?

Our results show that caterpillar whistles evoke startle reflexes in an avian predator. Captive red-winged blackbirds exhibited significantly higher startle scores when exposed to whistles played at naturally occurring sound levels (60–70 dB SPL) compared to control conditions (no sound). Birds responded by flying away from the platform, ruffling their feathers and flinching, all direct measures of the avian startle reflex (Morris, 1956 ; Olofsson et al., 2012a; Pomeroy and Heppner, 1977). While these measures of startle in avian predators are indicators of fear (Hebb, 1946), supporting Skelhorn et al.'s (2016) argument that deimatic displays should be defined by their ability to evoke fear responses, we concur with Umbers and Mappes (2016) that fear responses are physiological states that are difficult to measure in predators, particularly in the

field. As a second measure of startle we predicted that birds would take longer to re-approach the feeding dish following exposure to sound. Latencies were higher in both experimental phases compared to the control, but these differences were not significant. We propose that the lack of a significant difference may be due to the small sample size, or, because foraging strategies that animals use in a lab setting are not necessarily the same as they would use in the field (Shettleworth, 1989). Birds foraging in our semi-natural aviary experiments would have been accustomed to obtaining food from a feeding dish, and therefore less reluctant to return, even after being startled, than following an encounter with a suspicious prey in the field. We also noticed inter-individual variability in latencies, with some birds continuing to return to the dish promptly after being startled (<50 s), while others took longer (>2500 s) perhaps due to personality differences. Perhaps these birds were less reluctant to return to eat because of their familiarity with the feeding conditions, compared to what they might experience in the wild when presented with many novel foods. Also, unlike wild birds, they had nowhere else to find food.

Predators are expected to habituate to a deimatic display over time (Pomeroy and Heppner, 1977; Sargent, 1990) particularly in the lab where there is repeated exposure to startle stimuli (Ingalls, 1993; Sargent, 1990). This measure allows one to differentiate between alternative hypotheses such as aposematism, or that the sounds themselves are aversive (i.e. harmful to the ear). Our results showed that predators habituated to the sounds based on a decrease in startle score following repeated exposure. The slope was more gradual in experiment one (not significant) and steeper in experimental phase two (significant). In the control phase, two birds flew away upon first pecking at the dish. We observed that the birds that flew away in the control phase were skittish in all phases when feeding, and perhaps were more cautious when feeding, causing them to fly away. Due to the fact that the rest of the birds did not startle at all in the control phase, the difference in response in these two birds was pronounced resulting in a decrease in startle score (significant), although the slope was much smaller than in both experimental phases. As a second indicator of habituation within a trial, we found that latencies to return to the dish decreased over time in both experimental phases, although not significantly so in experimental phase two. As predicted, there was no significant positive or negative change in latency over time in the control phase where no sound stimulus was present. Collectively these results demonstrate that within a given trial, birds habituated to the stimulus, indicating that the sound itself was not aversive to the predator.

Birds that habituated to the sounds in experiment one became dishabituated to these sounds two days later, underscoring the importance of novelty for a deimatic display to be effective (Sargent, 1990). It is likely that encounters with these caterpillars in the wild would be infrequent, because they are cryptically coloured, and occur solitarily on their host plants (Bura et al., 2016). Even birds that have evoked a deimatic display in a caterpillar are unlikely to take the risk to return to the potentially dangerous source (Janzen et al., 2010). Therefore, re-sensitization of the startle response after a few days is likely to occur in nature as well as in the captive condition.

4.2. How do whistles protect caterpillars?

Whistles played at natural levels caused birds in an aviary to startle, but by which mechanisms might these sounds protect caterpillars in the wild? It has been postulated that deimatic displays protect prey in a number of different ways: the predator might drop the prey, give the prey time to escape, or be sufficiently frightened that it abandons the prospective prey altogether (Edmunds, 1974; Sargent, 1990), although there is limited empirical support for any

such mechanisms because few studies monitor the responses of predators (Skelhorn et al., 2016). The primary defence of the walnut sphinx is crypsis, and only when disturbed, does it produce whistles (Bura et al., 2011). Following attack, caterpillars hang on to the host plant tightly with their terminal abdominal prolegs, and sometimes thrash their anterior body segments while producing sound. However, sound production occurs with or without the accompaniment of thrashing. In a previous study involving both simulated and live predator attacks, caterpillars did not attempt to escape, similar to the responses of most other Bombycoidea caterpillars tested to date (Brown et al., 2007; Bura et al., 2009; Bura et al., 2012; Bura et al., 2011; Bura et al., 2016). The large body size of these larvae might make it difficult to drop from silk strands, which are likely to snap, and dropping to the ground is costly (Sugiura and Yamazaki, 2006). Therefore, the main protective value of the deimatic display in this species is not likely to lead to prey escape. We also rule out the likelihood that sounds provoke the predator to drop the prey, because caterpillars hold steadfast to their host plant while producing sound. Instead, we argue that whistles are effective because they elicit an innate startle response in the predator, causing it to abandon the prey. To a foraging bird in the wild, further pursuit of a potentially lethal threat may not be worthwhile, particularly when the bird is capable of moving on in search of more profitable prey (Janzen et al., 2010). While the birds in our experiments startled to sound alone, we expect that if exposed to live caterpillars, the effects of thrashing would have a synergistic effect as a multi-component display. Also, we cannot rule out the possibility that walnut sphinx caterpillars are chemically defended, and that whistles could function as both deimatic and aposematic displays. However, we argue against aposematism because chemicals are not expelled during sound production. All things considered, we argue that the primary function of caterpillar whistles is to frighten predators.

4.3. Why do whistles startle birds?

Deimatic displays are proposed to evoke a startle or fear response in a predator by the following non mutually exclusive mechanisms: (i) by mimicking the appearance of the predator's own predator, (ii) by exploiting the predator's innate startle reflexes, or (iii) by causing the predator to mistaken the prey for something else that could inflict harm (Skelhorn et al., 2016). We offer an alternative hypothesis: that the whistles mimic a bird alarm call. Alarm calls in passerine birds are widespread and function to alert conspecifics or heterospecifics to the presence of danger (Caro, 2005). Such calls elicit behaviours not unlike startle reflexes; in birds, these including escape, diving for cover and increased vigilance (Fallow et al., 2013; Griesser, 2013; Marler, 1955). Alarm calls across different bird species have common features including short duration and high frequency (Fallow et al., 2011). For example, the *seet* call of the American Robin is 1.8 s and 9.5 kHz (see Vanderhoff and Eason, 2009). Some of the whistles of the walnut sphinx are also of short duration (400 ms to 2 s), with the same dominant frequency as *seet* calls (9.5 kHz) (Bura et al., 2011). Both the red-winged blackbirds in our experiments and the yellow warblers from a previous experiment (Bura et al., 2011) exhibited flight responses and diving behaviours in response to the caterpillar sound. Therefore, it is conceivable that caterpillars might mimic bird alarm calls. This intriguing hypothesis could be tested by comparing the responses of birds to bird alarm calls with those of walnut sphinx whistles. Even then, it may be difficult to disentangle the 'deimatic' from the 'alarm call mimicry' hypotheses, because the latter response may have evolved from the same neural circuitry involved in the innate startle reflex (Hollen and Radford, 2009).

5. Conclusion

Defence sounds have been reported in almost every insect order (Alexander, 1967). Onomatopoeically called hisses, clicks, buzzes, chirps, rattles, taps, knocks, whistles, squawks and squeaks, among other things (Alexander, 1967; Ewing, 1989; Masters, 1980; Rowe and Halpin, 2013) defence sounds can differ significantly in their temporal, spectral and amplitude characteristics. Yet, there has been little rigorous experimental investigation of the functions of these signals on predators. Currently, studies are mostly restricted to the unique relationship between tiger moths and their echolocating bat predators (Corcoran et al., 2010; Conner and Corcoran, 2012; Conner, 2014). It has been recently argued that bio-acousticians fall behind visual ecologists in understanding the complexity between predator-prey interactions involving acoustic defence signals (Conner, 2014). Our study provides the first empirical evidence that an insect sound alone can evoke a fear response in an avian predator; thus functioning as an acoustic 'eyespot'. Caterpillar whistles are but one type of defence sound reported for Bombycoidea caterpillars (Bura et al., 2016). It is proposed that in caterpillars, different sound types, including whistles, clicks, chirps and vocalizations, convey different messages to predators, with some functioning better to reinforcing learning, while others are more suited to frightening predators (Bura et al., 2016). We propose that Bombycoidea caterpillars are excellent models for studying the functions of defence sounds, because the sounds are less likely to serve overlapping functions in social or sexual contexts as they would in adult insects such as tiger moths. Future studies should focus on the selection pressures for different defensive 'vocabularies' by considering the effects of phylogeny as well as predator psychology.

Animal welfare

This experiment was carried out with permission from the Queen's University Animal Care Committee (2014-1487).

Declaration of interest

We have no competing interests.

Fundings

Authors contributions

A.L.D and J.E.Y. conceived and designed the experiment. A.L.D and C.A.Y conducted the experiment and collected the data. A.L.D. interpreted the data and G.L conducted statistical analysis. A.L.D drafted the manuscript. A.L.D. and C.A.Y created the figures. L.S provided the animals, maintenance of the aviaries and consultations regarding the experimental set-up and sound playback. All authors gave final approval for publication.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.beproc.2017.02.002.

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