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A bee or not a bee: an experimental test of acoustic mimicry by hoverflies

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Lay summary

Many plants and animals look, smell, or sound like something other than what they are in order to deceive and manipulate. We show that a small number of harmless hoverflies make sounds that computers cannot distinguish from the sounds of bumble bees, yet it seems that their bird predators are able to do learn to do so in the field.

A bee or not a bee: an experimental test of acoustic mimicry by hoverflies

Abstract

The degree of similarity between Batesian mimics and their models varies widely and occurs across a range of sensory modalities. We use three complementary experimental paradigms to investigate acoustic mimicry in hoverflies (Diptera: Syrphidae) which mimic stinging Hymenoptera. First, we analyse sounds made by 13 hoverfly species and 9 Hymenoptera species with and without simulated predation ("alarm" and "flight" sounds, respectively). We demonstrate that the bumblebees *Bombus terrestris, B. hortorum, and B. lucorum,* and the hoverfly *Cheilosia illustrata* exhibit alarm sounds that are significantly different to their respective flight sounds, and indistinguishable between species. We then demonstrate that the *B. terrestris* alarm sound reduces predation on artificial prey by wild birds, but that the hoverfly mimic alarm sound does not. Finally, we trained chicks to avoid distasteful food in the presence of different acoustic stimuli. Overall the chicks showed no difference in response to bee and hoverfly stimuli, demonstrating no innate aversion to the *Bombus* alarm sound. We therefore conclude that (i) similarity of acoustic signals exists among Hymenoptera and hoverflies, (ii) acoustic aposematic signals (but not the almost identical mimetic signals) are effective at reducing predation, and (iii) wild birds exhibit learned rather than innate aversion to certain acoustic stimuli.

Introduction

Aposematic insects advertise their defences to predators by presenting conspicuous warning displays (Poulton, 1890) and these displays can involve one or more sensory modalities. The signal may act in direct and indirect ways to influence the fitness of the signaller and the receiver. The signal may benefit individual prey exhibiting the signal if the defence is strong and immediate, through predator neophobia or startle displays. Benefits to prey may also be indirect, as aposematic signals serve to educate predators by forming an association between a strong sensory stimulus and a negative outcome (bad taste, sickness) such that the predator avoids similar prey individuals in the future (Gittleman and Harvey, 1980; Harvey and Greenwood, 1978). These honest signals can also be hijacked by mimics that reap the benefits of the predator’s learned aversion to a given signal without having to pay the cost of producing the defence to which it is linked. The result is that mimics converge towards their models, while models diverge from their mimics (Nur, 1970). Such species are known as Batesian mimics (Bates, 1862) and represent some of the most celebrated and intensively studied examples of evolution with an array of adaptations that render them, in some cases, almost indistinguishable from their model species. However, if there is such a clear benefit to close resemblance to an aposematic species then the question remains: why are there no perfect mimics? Indeed, while there are many examples of exceptional mimicry, there are far more species within the same taxonomic groups that exhibit little to no evidence of mimicry. A range of hypotheses have been put forward to explain why some species might exhibit closer mimetic similarity than others (for a review, see Penney et al., 2012). These include the simultaneous mimicry of multiple models (Edmunds, 2000), kin selection (Johnstone, 2002), observer failure to take into account the evolutionarily-relevant predator’s visual system (Cuthill and Bennett, 1993), and a relaxation of selection under certain circumstances (Sheppard, 1959; Sherratt, 2002).

A further possibility is that mimics resemble the same models in different sensory modalities, and recent analyses of the syrphid flies have suggested that there may be a role for behavioural mimicry alongside morphological mimicry (Penney et al., 2014). Several studies have also demonstrated classes of sound produced by hymenopteran models, involving a “hissing” noise that is distinct from flight noises (Kirchner and Roeschard, 1999; Sarma et al., 2002). Numerous suggestions have been made that syrphids mimic these acoustic signals. For example, some syrphid mimics have very similar wingbeat frequencies to their hymenopteran models (Gaul, 1952) and toads show greater avoidance of bees and syrphids with wings than of the same species with wings removed (Brower and Brower, 1965) . Under the assumption that these distinctive sounds represent an aposematic signal, and given that syrphids are already well-known to mimic other aspects of hymenopteran biology, it is a surprise, then, that a study that investigated acoustic mimicry in this group found equivocal evidence (Rashed et al., 2009). This is particularly surprising given the range of indirect observations that suggest acoustic mimicry occurs. Also, sounds are a common feature of aposematic displays (Masters, 1979) and acoustic Batesian mimicry has been described in field and laboratory experiments on a wide range of species from tiger moths to burrowing owls (Barber et al., 2009; Dowdy and Conner, 2016; Rowe et al., 1986) although some examples are disputed (Kardong, 1980; Sibley, 1955).

Many previous studies have taken a detailed, narrow approach to studying acoustic mimicry. This has involved removing acoustic cues (Brower and Brower, 1965), measuring wing beat frequencies (Gaul, 1952), or quantifying acoustic similarity (Rashed et al., 2009). What is needed is a comprehensive analysis that describes variation in acoustic signals within an evolutionary context and then tests empirically the potential benefits that such signals might confer to a mimic. To address this gap in the literature, a series of experiments were designed to search for acoustic mimicry in the syrphids, assess its impact on predator-prey interactions, and determine whether predator avoidance behaviours are learned or innate.

Methods

Experiment 1: Comparison of acoustic signals

Specimen collection: Recordings were made of 172 insects comprising 13 syrphid species, 9 Hymenoptera species, as well as 32 *Calliphora vomitoria* (Diptera: Calliphoridae) as a non-syrphid comparison. Individuals were collected using aerial nets between 12/06/2014 and 16/06/2014 at three locations in Leeds, UK (the University of Leeds West Campus, 53.807°N,-1.562°E; Meanwood Park, 53.840°N,-1.577°E; and Redcoat Lane, 53.808°N,-1.600°E) and stored in 30cm^3 sample tubes for transportation. Acoustic recordings were always taken within six hours of capture. Additionally *Episyrphus balteatus* pupae were purchased from Koppert Biological Systems (Koppert, Berkel en Rodenrijs, The Netherlands; product name Syrphidend) and *C. vomitoria* maggots were purchased from P&S Taylor (Sunny Bank Bait Farm, Halifax, UK). These were reared at 25^{o}C (±1.5^{o}C) and individuals were used within 24 hours of eclosion, Body mass was measured to ±0.01mg using a Mettler Toledo Micro Balance (Mettler Toledo, Greifensee, Switzerland; model AX26DR).

Acoustic recordings: Insects were dorsally tethered by the mesothorax to a 10cm length of rigid plastic coated wire (300μm diameter) using a non-toxic adhesive (Bostik). Reliable positioning of insects in front of the microphone was achieved using a retort stand, boss and clamp leaving 5cm of wire protruding on which the tethered insect was mounted. Recordings were taken using a Neumann mono microphone (Neuman, Berlin, Germany; model KM184, cardioid pick up) (response 20Hz to 20 kHz) positioned 10cm behind the tethered insect with a König and Meyer Popkiller (Wertheim, Germany; model 23956) exactly half way between the microphone and the insect to reduce the effects of the mechanical impact of air striking the microphone. Acoustic signals were recorded using AVID Pro-Tools11 digitising at 48 kHz and stored as waveform (.WAV) audio file format. All recordings were taken in a soundproof room at a temperature of 24°C (±1.5°C). Two recordings were made of each insect. First, insects were mounted in front of the microphone and allowed to attempt to fly. If after one minute an individual did not fly, flight was provoked by introducing a solid surface to the animal’s feet for 5 seconds then removing it again. These acoustic signals are hereafter referred to as “flight” sounds. Second, to simulate avian predation, insects were squeezed gently on the ventral side of the abdomen with a pair of flexible-tipped entomological forceps. Attacks were aimed at the insect's ventral side to avoid contract with the wings. Each insect was stimulated a minimum of three times in order to ensure that an alarm response had been elicited if the insect were capable of generating such a response (hereafter “alarm” sounds).

Acoustic analysis: The flight and alarm sounds produced by the insects were analysed using Avisoft-

SASLab Lite sound analysis software (Avisoft Bioacoustics, Glienicke, Germany). Fourier transformation was used to generate averaged power spectra for segments of the recordings. The target length of the analysed segments was 500ms but this varied due to variability in the length of the sustained acoustic response (range 118-500ms). Seven variables were extracted from these spectra: the frequency (P1) and power (P1dB) of the greatest amplitude, the frequency (P2) and power (P2dB) of the second greatest amplitude, the difference between the amplitudes (ΔdB) and frequencies (ΔHz) of the two peaks, the bandwidth of the peak power output measured as the difference between the high (BW1) and low frequencies (BW2) 6Db below the peak (ΔBW). BW1 was not used in the analysis because most power spectra lacked this feature. Variables are illustrated in Figure S1, with descriptions in Table S1. This cut-off point was chosen as 6Db represents a 50% decrease in power. Any data in the power spectra at frequencies above 12kHz were omitted as this is the high frequency limit generally attributed to avian hearing (Heffner and Heffner, 2007).

To evaluate the capacity of species to produce different flight and alarm sounds, sound files were analysed using two different techniques. First, the diffspec function in the Seewave package (Sueur et al., 2008) in R version 3.1.2 (R Core Team, 2014) was used to calculate the percentage difference between sounds based on their probability mass functions. We would predict that larger species would show greater capacity to produce warning sounds, as has been shown in morphological and behavioural modalities in hoverflies (Penney et al., 2012; Penney et al., 2014). To test this, the mean of the individual differences between flight and alarm sounds was tested against mean species mass using Pearson correlations. We performed a statistical hypothesis test for a difference between alarm and flight sounds within a species using a MANOVA. A set of seven out of nine acoustic characteristics were used, as some species exhibited spectra that did not allow the calculation of the remaining two parameters (P1dB and BW1). Data were extracted for each species individually and all variables were scaled to unit variance and mean-centred, then principal components analysis was used to extract orthogonal variables to avoid covariance in the raw data. A MANOVA was then performed with the sound type (alarm or flight) as the predictor and the first two principal components (which always explained >98% of the variance in the data) as the response.

To test for a difference between species, MANOVA was used as above but with all species together and the species as the predictor. The first four principal components were included as the response variable in the MANOVA, which explained 97.4% of the variance in the alarm sounds and 98.4% of the variance in the flight sounds. Two further groups of tests were performed. First, linear discriminant analysis (LDA) was conducted on the sounds using the lda function in the MASS package (Venables and Ripley, 2002) in R to attempt to discriminate among the species and to visualise the differences. Second, a pairwise comparison of species was carried out using the contrast function in the lsmeans package (Lenth, 2015) while accounting for multiple tests (n=66 pairwise comparisons) using the false discovery rate (FDR) in R.

Experiment 2: Field study

Regardless of the interpretation of specific model-mimic relationships in these groups, it is then of interest to know whether there is an anti-predator role for the acoustic signal when all else is held equal. To quantify the evolutionary advantage of acoustic mimicry of bumblebees, a field study was carried out using wild birds as predators. Fieldwork was conducted in Hertfordshire, England, (51.855°N, -0.108°E) between 05/08/2014 and 18/08/2014. Pastry baits were made using 310g flour, 160g lard, 30ml water and 10ml Sainsbury's yellow food colouring (Easley and Hassall, 2014). Bait size was adjusted to 20mm length and 5mm diameter, with cross-sectional uniformity ensured by using a clay extruder with a 5mm aperture, in order to accommodate for the smaller birds in this experiment (e.g. European robin (*Erithacus rubecula*) and great tit (*Parus major*)) being unable to take baits compared to those in the previous study that include the Eurasian Magpie (*Pica pica*) and Rock Pigeon (*Columba livia*). Baits were deployed on 50cmx50cm wooden boards that were painted “Buckingham Green” with an exterior gloss (B&Q, Eastleigh, UK, product 5397007045949).

Four acoustic conditions were produced from the studio recordings: (i) post-attack *B. terrestris*, (ii) post-attack *Cheilosia illustrata,* (iii) *C. vomitoria* flight, and (iv) silence. The acoustic stimuli were sections of recordings between 657ms and 3537ms in length looped to generate a 6000ms waveform

(.wav) audio file using Avisoft-SASLab Lite (Avisoft Bioacoustics, Glienicke, Germany). Sound files were played on a constant loop using Alba MP3 players (Alba, Milton Keynes, UK: model 189/9935) and through Veho 360° capsule speakers (Veho, Southampton, UK: model VSS-001-360) positioned directly below the bait boards held in a plastic container lidded with cling film which prevented water damage but allowed unimpeded sound transmission. Suitable playback volume was determined by collecting a sample of 10 bumblebees (5 *B. terrestris*, 5 *B. lucorum*) and measuring the maximum volume produced by the insects during flight (mean 59.8Db ±8.6SE) and attack response (56.4Db±6.6SE) using a Tenma decibel meter (Tenma, China; model 72-947). Speaker volume was set so that the volume of acoustic stimuli was within this range across each board, this was checked using a Samsung Tablet (Samsung, Seoul, South Korea; model Tab2 10.1) running the Sound Meter application version 1.5.9a (Sound Meter, 2014).

Twenty pastry baits were presented on each of four 50x50cm wooden bait boards, set out in a square 2m from one another with a 50cm perimeter marked around each board. This distance between boards was chosen as it was calculated, using the inverse square law, that acoustic cues would be reduced to <1/32 the power on neighbouring conditions. This was decided to be sufficiently quiet as to not influence the birds feeding behaviours between conditions. Each board was randomly allocated one of the four acoustic treatments. Thirty minutes before sunrise, 20 baits were placed on each board and observations began 15 minutes before sunrise and continued for 4 hours separated into 8 x 30 minute periods. The period of time between a bird entering the 0.5m perimeter of a board and first pecking a bait was recorded. After each experimental run the remaining baits were removed and between each trial the acoustic conditions were changed so that over a 4 day period each condition was supplied in each location.

Field study data analysis: Cox proportional hazards models, implemented using the survival package (Therneau, 2014) in R (R Core Team, 2014), were used to test for a difference in survival rates between baits presented with different acoustic cues. Models included acoustic cue as a predictor and were stratified by date to account for variability in weather conditions between days of the experiment. Models were tested using cox.zph() to ensure that the data conformed to the assumptions of proportional hazards. Generalised Rank-Order MANOVA was then used to investigate significant difference between species (Thomas et al., 1999) and subsequent *post-hoc* Tukey's HSD analyses were conducted to further investigate the significant differences between the treatment levels.

Experiment 3: Laboratory study

Since the wild bird trial suggested that avian predators could distinguish between the acoustically-similar *B. terrestris* and *C. illustrata* alarm sounds, we conducted a third experiment using domestic chicks as a model system to explore the capacity of birds to learn to differentiate between the two stimuli. Day-old domestic chicken chicks (*Gallus gallus domesticus*, Ross 308 broiler strain, n=172) were purchased from a commercial hatchery and housed in groups of <24 in holding pens of dimensions 126cm x 84cm lined with sawdust. Holding pens were positioned so that chicks could get no closer than 2m from the experimental enclosures because at this distance, as with the wild bird experiment, experimental acoustics would be sufficiently quiet as to not influence birds while not being experimented upon. . Chicks were housed under a brightness of 40 lux under a light cycle of 23L: 1D for the first two nights with the dark period increasing by one hour on subsequent nights. Poultry shed temperature was 30°C for the first three days then reduced to 28°C. Water was always available and chick crumb feed (from Target Feeds Ltd.) was also constantly available in feeding trays except during experimental periods when food deprivation was imposed in accordance with Home Office regulations and authorised by the University of Leeds ethics committee. Chick crumb feed was used throughout the experiment to avoid introducing additional visual and taste stimuli that could interfere with responses to acoustic signals. Studies of multi-modal mimicry or more realistic experiments incorporating ecologically relevant combinations of stimuli should consider using pastry model prey.

The experimental arena was a sawdust lined cage measuring 42cm x 84cm containing a Veho 360° speaker (Veho, Southampton, UK: model VSS-001-360), an Alba MP3 player (Alba, Milton Keynes, UK: model 189/9935) and a feeding tray holding approximately 500g of chick crumb. On the first day post-hatch chick pairs were placed in the experimental arena and supplied with plain chick crumb twice during the day to acclimatise them to the experimental set-up (Skelhorn et al., 2010). Chicks remained in the arena until both chicks had pecked at the feed and were then allowed to remain in the arena for a further minute so that an association between feeding in the arena and being handled, a potentially stressful and negative experience, would not develop. Chicks were trained and tested in pairs since the “buddy method” involving separating chicks using mesh cages but retaining visual and auditory contact as described in Skelhorn et al. (2010) were found to be insufficient to prevent chicks from becoming distressed. Hence the paired trials were used due to ethical considerations to reduce the stress of the animals being separated from the rest of the group. Such stress is not only an animal welfare issue in and of itself, but would also influence the behaviour of the animals such that experimental data may not be reliable. Individual adult birds which are less stressed by solo foraging tasks may give more precise individual-level behavioural data (i.e. independent of social factors), but would not have been naïve at the start of the experiment.

On the second day post hatch chicks were divided randomly into four equal groups (n=43) and allocated to one of four feeding regimes. One training regime, representing aposematic prey, was provided with the acoustic stimulus of post-attack *B. terrestris* and provided chick crumb given a bitter flavour using Bitrex solution (6ml 5% Bitrex solution per 500g of crumb (Mostler, 1935)). The three other regimes were provided with unaltered chick crumb, representing undefended prey, presented with either a synthesised tone of 120Hz, post-attack *C. illustrata* sound or silence. Acoustic stimuli were supplied constantly while chicks were in the arena by the speaker at between 56.4 and 59.8Db, as established as an ecologically relevant level in the field study. Chicks were trained in this regime for three days before each group was further split into four groups ($n \geq 10$ in each group), each of which was tested once on one of the four treatments thus achieving a fully-factorial design of training vs. testing. During both the training and the testing phase chicks were deprived of food for 30 minutes before entering the arena, latency to peck at the food by each chick in a pair was recorded and chicks were allowed 30 seconds after pecking before being removed from the arena.

Laboratory study data analysis: Two-way ANOVAs were run using R to compare the latency to attack the baits during the testing phase, with the training treatment, testing treatment, and the interaction of training and testing as predictors. Using one-way ANOVAs, we further analysed the overall response to the different cues across training and testing phases, the response of bee-trained chicks to the four test treatments, and the response of chicks trained in each of the four cues to the bee test treatment. Tukey's HSD tests were used to investigate the data further where there was a statistically significant effect in the full model.

Results

Experiment 1: Comparison of acoustic signals

The Hymenoptera *Bombus terrestris* (MANOVA: $F_{2,35}$= 54.340, p<0.001, Pillai's trace=0.756), *B. hortorum* (MANOVA: $F_{2,9}$= 6.696, p=0.017, Pillai's trace=0.598), and *B. lucorum* (MANOVA: $F_{2,17}$= 44.797, p<0.001, Pillai's trace=0.841), and the Syrphidae *Cheilosia illustrata* (MANOVA: $F_{2,8}$=24.036, p<0.001, Pillai's trace=0.857) were the only species to generate significantly different flight and alarm sounds. A slow motion video showing the transition between flight and alarm sound production can be seen in the Supplementary Information, and Figure S2 shows the difference between acoustic waveforms from flight and alarm sounds in *B. terrestris*, *C. illustrata*, and the syrphid *Episyrphus balteatus*. When the percentage dissimilarity of flight and alarm sounds was analysed across the hoverflies (excluding singletons), there was a strong positive correlation with body size (Pearson correlation: R=0.741, p=0.014, n=10; Figure 1), indicating that larger animals produced alarm sounds that were more distinct from their flight sounds.

Linear discriminant analysis showed that the flight sounds of the species were clustered together with no clear separation of Hymenoptera and hoverflies (Figure 2A) but that the five hymenopteran *Bombus sp.*, and the three syrphids *C. illustrata*, *Eristalis tenax*, and *Leucozona lucorum* showed similar alarm sounds (Figure 2B; though note that *L. lucorum* is represented by a single individual). A MANOVA on the full flight dataset confirmed that while there were differences between species in flight sounds ($F_{88,536}$=2.578, p<0.001), there were no significant pair-wise differences after control for multiple comparisons using false discovery rates ($p>0.5$ in all cases; Figure 2A). A MANOVA on the full alarm dataset confirmed that there were much stronger differences between species in alarm sounds ($F_{88,540}=3.998$, $p<0.001$), and that *B. terrestris* produces an alarm sound that is significantly different to those of all other species apart from the other *Bombus* species, *C. illustrata*, *E. tenax*, and *L. lucorum* (as can be seen in Figure 2B).

Experiment 2: Field study

Baits presented with the post-attack sounds of *B. terrestris* experienced significantly lower attack rates than those presented in all other conditions (Cox proportional hazards analyses: *C. illustrata* alarm: $z=2.804$, $p<0.010$; *C. vomitoria* flight $z=2.656$, $p<0.010$; silence $z=2.115$, $p<0.050$, see Figure 3). These results demonstrate strong evidence for the evolutionary benefit of acoustic aposematism, but do not seem to support a role for mimicry. However, it is unclear as to the characteristics of the *Bombus* and *Cheilosia* signals that cause the birds to respond differently.

Experiment 3: Laboratory study

Our laboratory study tested three predictions. First, that chicks trained on distasteful food with *B. terrestris* sound would show greater latency to peck at both *C. illustrata* and *B. terrestris* food in the test phase, indicating a generalisation of the acoustic cue. The results showed that chicks trained on the *B. terrestris* food treatment exhibited no difference in latency to attack any of the four food treatments in the test phase (ANOVA: $F_{3,40}=0.766$, $p=0.520$; Figure 4A). Second, that chicks trained on palatable (i.e. not *B. terrestris*) foods would show lower latency to attack when tested on *B. terrestris* food, indicating that they had learned that the sounds were associated with palatable food. Here, we found that chicks' latency to attack the *B. terrestris* food treatment during the testing phase was not significantly influenced by their food treatment during the training phase (ANOVA: $F_{3,43}=0.319$, $p=0.812$; Figure 4B). Finally, that chicks respond to acoustic cues without discrimination, in which case we would expect a greater latency to peck at foods presented with acoustic stimuli vs. silence, irrespective of trials, testing, and training. We found a significant difference in latency to attack across all encounters (training and testing) between acoustic stimuli (ANOVA: $F_{3,667}=5.578$, p=0.001; Figure 4C), and that this involved significantly shorter latency to attack the silent food compared to *C. illustrata* (p=0.002) or *B. terrestris* food (p=0.014), and borderline significance in the shorter latency to attack the tone food compared to *C. illustrata* food (p=0.051). These results suggest that while training did not appear to have an effect on predator interactions there may be a general heightened latency to attack when food is presented with an acoustic cue (as has been suggested previously; Rowe and Guilford, 1999), with some suggestion that it is insect sounds specifically rather than sound *per se* that delay predation.

Discussion

This study presents three complementary experiments: (i) a comparative analysis of the presence of acoustic mimicry demonstrating the mimetic links between several *Bombus* species (a Müllerian complex) and a subset of Syrphidae. Specifically, the similarity of alarm sounds indicate that *Bombus* species represent a group of Müllerian acoustic mimics, and that *C. illustrata, E. tenax,* and possibly *L. lucorum* are Batesian acoustic mimics of the *Bombus* group; (ii) a field based predation experiment under ecologically-relevant conditions that demonstrates a benefit to the aposematic signal using naturally-foraging birds but not for the acoustically-similar mimetic signal produced by a hoverfly; and (iii) a tightly-controlled laboratory study using a model avian visual system to demonstrate that there is a general aversion to sound (and insect sounds in particular) but no innate avoidance of the *Bombus* alarm sound. This allows us to conclude that some hoverflies produce sounds that are indistinguishable from those of Hymenoptera based on acoustic analysis, that there is no innate avoidance to Hymenoptera sounds in particular, and that aposematic sounds enhance survival (but mimicry of those sounds by syrphids may not). The findings complement previous work on morphological and behavioural mimicry in this system (Penney et al., 2012; Penney et al., 2014).

As bumblebees use pheromones to warn conspecifics of predators (Goodale and Nieh, 2012) and are unable to detect airborne sounds (Hunt and Richard, 2013) it is reasonable to assume that the observed acoustic change from flight to alarm sound has evolved as an aposematic alarm signal. While our findings from Experiment 1 are consistent with an explanation of Müllerian mimicry for the shared alarm sounds of the *Bombus* sp., this pattern could also be explained simply through shared evolutionary history. Despite experimental work demonstrating the benefits of Batesian mimicry of *Bombus* sp. (e.g. Brower et al., 1960), experimental work demonstrating generalised avoidance of *Bombus* congeners is lacking. All other Hymenoptera examined here immediately began biting and stabbing their abdomen at the simulated predator suggesting that in these species conditioning the predator not to attack similar individuals in the future is preferable to the survival of individual workers. Considering the significantly smaller size of *Bombus* colonies compared to other social hymenopterans (*Bombus* <100, *Apis* <100 000 and *Vespidae* <1000; data from (Bourke, 1999)) and the greater energetic investment needed to produce larger workers it is considerably more costly to lose an individual worker and therefore preferable to facilitate escape. Therefore there is greater selective pressure for *Bombus* species to develop additional modes of signalling to their predators and facilitate escape. Moreover, bumble bees are more robust, accumulating terminal levels of damage more slowly than other insects when attacked (Krebs and Avery, 1985) allowing them more time to escape during an attack. These factors could allow for the evolution of an aposematic alarm signal that could encourage the predator to release the individual, and which could be exploited by mimics. As far as we know there have been no experimental studies prior to our own that have attempted to move beyond conjecture with respect to the alarm sounds. There are a number of studies that have proposed a role for the sounds as acoustic aposematic signals, such as Kirchner and Roeschard (1999) who described a “hissing” of bees in response to nest invasion, air currents, and the presence of mice (which showed aversion to the sound). Those hissing responses were never associated with escape behaviour. In addition, our own high speed video work (see Supplementary Information for a link to the video online) demonstrates that the alarm sound in *Bombus terrestris* is associated with a change in wing stroke amplitude which does not seem biomechanically appropriate for an escape behaviour. We suggest that it has more in common with wing whirring (as used during behavioural thermoregulation), with the wings decoupled from the flight muscles, to produce an acoustic aposematic signal.

The putative acoustic mimics, *C. illustrata* and *E. tenax*, are two of the three largest mimics in this study (Figure 1). This finding, along with the observation that there is a significant positive correlation between size and the capacity to generate alarm sounds, is consistent with the relaxed selection hypothesis that suggests that larger species that are more profitable prey and suffer higher predation rates should develop greater levels of mimetic fidelity than smaller, less rewarding species (Penney et al., 2012; Sherratt, 2002). Furthermore, we can confirm that this behaviour is not a feature of all large syrphids as *Volucella pellucens* was the second largest species but lacked obvious acoustic mimetic qualities. While flight and alarm sounds of *V. pellucens* were different according to their probability mass functions (Figure 1), there was no significant difference between the two (MANOVA: $F_{2,10}$=0.146, p=0.864) and LDA grouped *V. pellucens* with the larger group of non-mimetic species (Figure 2B). It is possible that this species has evolved other means of predator avoidance, and observations by the authors of the fly's considerable speed and agility during flight have recently been supported by laboratory comparisons which show that *V. pellucens* is among the fastest syrphid fliers and can generate considerable aerodynamic force (Belyaev et al., 2014). Our results imply that, as has been suggested for behavioural mimicry (Penney et al., 2014), acoustic mimicry may be restricted to large, high-fidelity mimics. The results therefore provide the novel suggestion that mimics may resemble different models in different mimetic modalities, although previous work has interpreted a similar pattern as a lack of acoustic mimicry (Rashed et al., 2009). However, it is interesting to note that *C. illustrata* is considered to be a morphological mimic of *Bombus pratorum*, while *E. tenax* is considered to be a morphological mimic of the honeybee, *A. mellifera* (Howarth and Edmunds, 2000). *L. lucorum* is easily confused with *C. illustrata* (Ball and Morris, 2013), but is not considered to be mimetic and lacks the colour patterns of *B. pratorum*. Indeed, we argue for a re-evaluation of Rashed et al.'s work, the results of which are broadly consistent with those of the present study, which also demonstrated similarity between *Bombus* alarm sounds and those of some hoverflies. We propose that where morphological and acoustic mimicry appear to rely upon different models this constitutes a "multi-model" mimicry system (Edmunds, 2000).

The fact that just two of the species investigated exhibit acoustic mimicry, and that Rashed et al.

(2009) were unable to find the phenomenon despite investigating a similar number of hoverflies of arguably higher mimetic fidelity, such as *Spilomyia sayi*, demonstrates that this behaviour is not a feature of all visually mimetic species. Our findings are consistent with a previous systematic survey of behavioural mimicry in hoverflies, which revealed mimetic behaviours in only six species (belonging to only two genera, *Temnostoma* and *Spilomyia*) out of 57 species that were assayed (Penney et al., 2014). Our identification of two species which do appear to exhibit acoustic mimicry from a relatively small sample, and which are found in two different tribes within the subfamily Eristalinae, opens the possibility that acoustic mimicry may be more common than behavioural mimicry in the Syrphidae, of which there are over 6000 identified species worldwide (Rotheray and Gilbert, 2011).

Of particular interest in this study is the apparent lack of effect of the *Cheilosia illustrata* alarm sound when presented to wild birds in the field, despite its acoustic similarity to that of *Bombus terrestris*. There are two explanations for this pattern, which are not mutually exclusive. The first is that the birds are able to discriminate between the sounds based on an aspect of the acoustic signal that was not measured during the acoustic analysis. The second explanation is that the benefits of the acoustic signal are dependent upon some other aspect of the mimic phenotype that is not represented in the pastry prey. For instance, higher morphological mimicry is associated with behavioural mimicry, suggesting that the benefits of morphological and behavioural mimicry may be contingent on one another (Penney et al., 2014). This notion of the interdependence of the multiple sensory modalities through which mimicry manifests poses a problem for experimental researchers, as it is a complex task to remove one modality without affecting others. Previous work with invertebrate sensory modalities has reduced the sensory perception of the signal receiver (Bretman et al., 2011) or removed the capacity of a signaller to produce certain signals (Olofsson et al., 2012). However, such manipulations would have serious ethical implications and low ecological relevance in this study.

While a substantial body of work now exists on the ecology and evolution of mimicry, combining comparative, lab and field studies, there has been far less study of the mechanisms by which mimetic traits are produced and the lability of the genes underlying those traits. The video in the supplementary information suggests that the alarm sound in *Bombus* is produced by decoupling the wings from the flight muscles to produce low amplitude, high frequency movements, similar to the thermoregulatory behaviour known as “wing-whirring” (May, 1979). Interestingly a previous study of thermoregulation also suggested that hoverflies make a high pitched noise during warm-up (Morgan and Heinrich, 1987), and so it is possible that thermoregulation and acoustic mimicry are linked in some way. That sound production identified in *Bombus* accompanies reduced wing movement suggests these acoustics satisfy an alternative function to movement during an attack, supporting the suggestion of an aposematic signal which *Syrphidae* were able to mimic due to a shared physiology. What is needed to test this hypothesis is a survey of the internal anatomy during sound production using a technique such as high speed cineradiography to compare the musculature of models and mimics (Betz et al., 2008).

The findings described here represent the first empirical evidence linking the acoustic similarity of Hymenoptera and Syrphidae acoustic signals to survival benefits in the field. However, further work involving a larger array of acoustic signals is required to test these field patterns comprehensively. If the survival benefits of acoustic mimicry in Syrphidae were to be confirmed, it would constitute the third mode of mimicry in the Syrphidae alongside visual (Penney et al., 2012) and behavioural (Penney et al., 2014), and all three modes suggest a role for body size in the evolution of mimetic traits. Larger hoverflies generate sounds when attacked that are indistinguishable from those produced by bumblebees immediately after attack while smaller and non-mimetic syrphid species are less able to exhibit this behaviour as predicted by the relaxed selection hypothesis. A subsequent field experiment demonstrated that avian predators preferentially avoided prey presented with post-attack *B. terrestris* acoustic stimulus, confirming the presence of a selective pressure for the evolution of acoustic aposematism and, potentially, for the evolution of mimicry in this predator-prey complex. An experiment using naive predators demonstrated that the predator’s aversion is not intrinsic but is developed over an extensive learning period and at considerable expense to the aposematically signalling population. It is therefore suggested that further investigations into the interaction between acoustic and visual mimicry using a multi-modal experimental design would yield interesting results (Skelhorn et al., 2015). The discovery of a novel mode of mimicry in such a highly studied system suggests that such acoustic mimetic complexes are likely to be present across the natural world, particularly with acoustically dominant predators.

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Figures

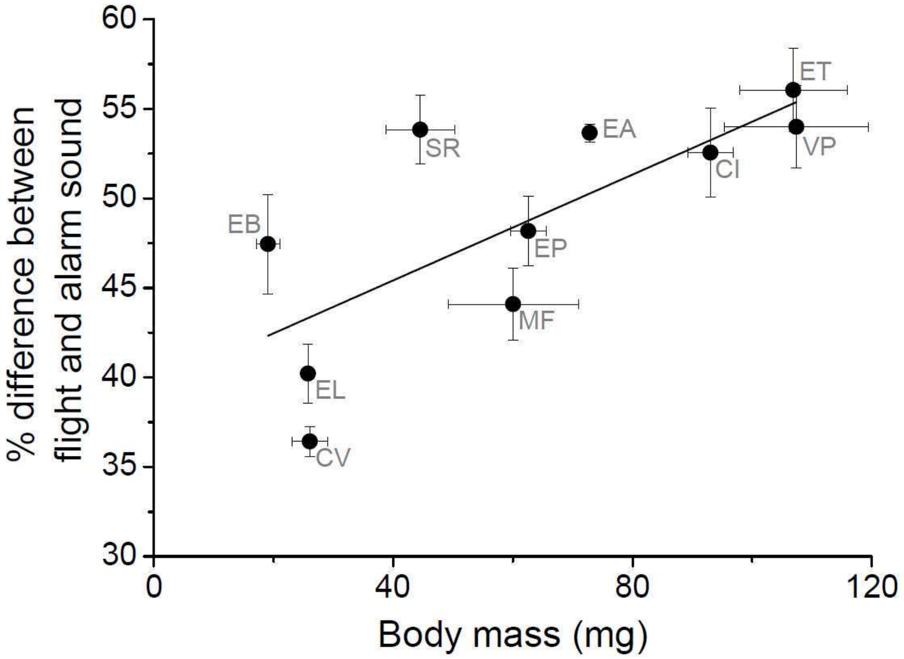

Figure 1: Larger-bodied hoverflies (Diptera: Syrphidae) exhibit a greater variation between their routine flight sound and their alarm calls, which resemble those of Hymenoptera. This finding follows the expectation of the relaxed selection hypothesis, whereby larger prey species are under greater selective pressure to evolve anti-predator defences. Abbreviations are: *Apis mellifera* (AM), *Bombus terrestris* (BT), *Calliphora vomitoria* (CV), *Cheilosia illustrata* (CI), *Episyrphus balteatus* (EB), *Eristalis arbustorum* (EA), *Eristalis pertinax* (EP), *Eristalis tenax* (ET), *Eupeodes luniger* (EL), *Myathropa flora* (MF), *Syrphus ribesii* (SR), and *Volucella pellucens* (VP).

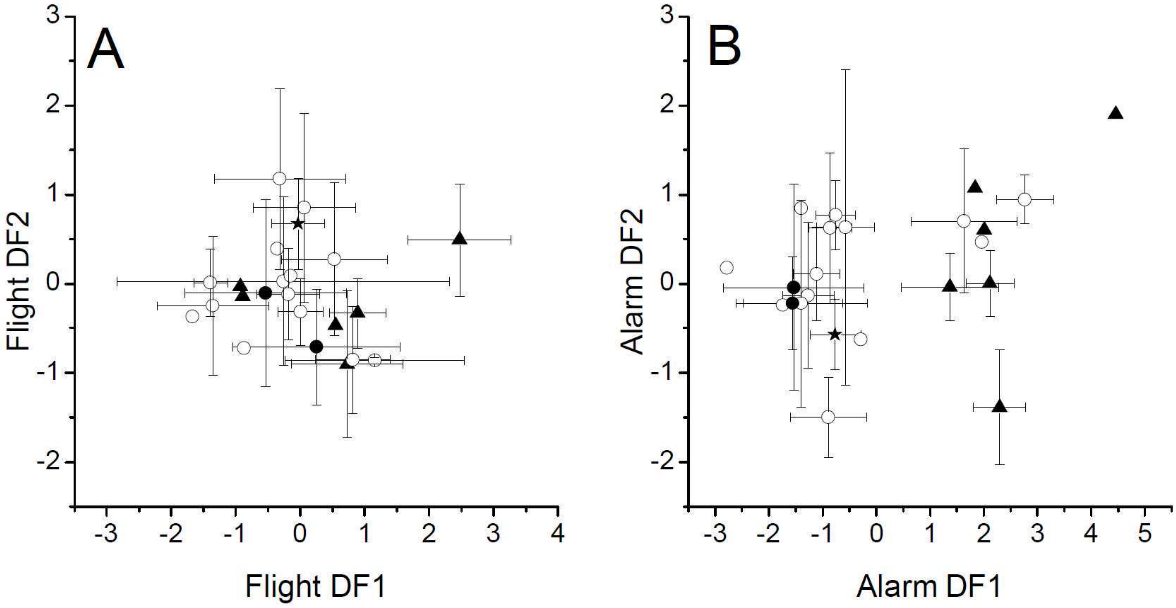

Figure 2: Linear discriminant analysis showed that while (A) flight sounds were very similar across hoverflies and their Hymenopteran models, (B) there were two discrete groups of species that produce different types of alarm call. This latter group includes all six *Bombus* species and the mimics *Eristalis tenax*, *Cheilosia illustrata*, and *Leucozona lucorum*. Points are species means (open circles = hoverflies, filled circles = Vespidae, triangles = *Bombus* sp., star = *Apis mellifera*) and error bars are 95% confidence intervals.

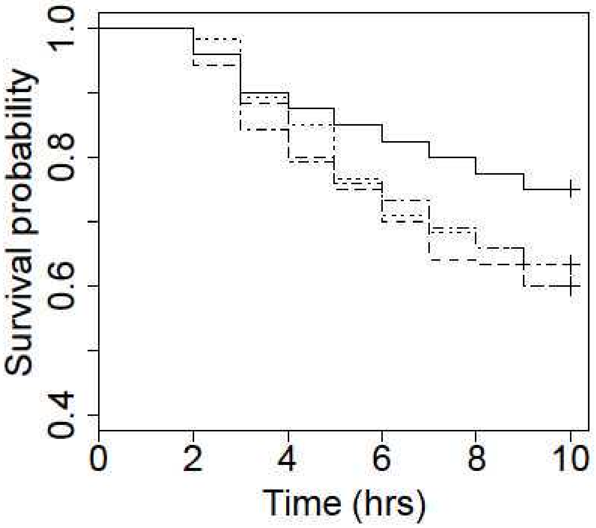

Figure 3: Survival of identical baits presented to wild birds with four acoustic stimuli: post-attack *Bombus terrestris* (solid line), post-attack *Cheilosia illustrata* (dotted line), *Calliphora vomitaria* flight (dashed line), and silence (dot-dash line).

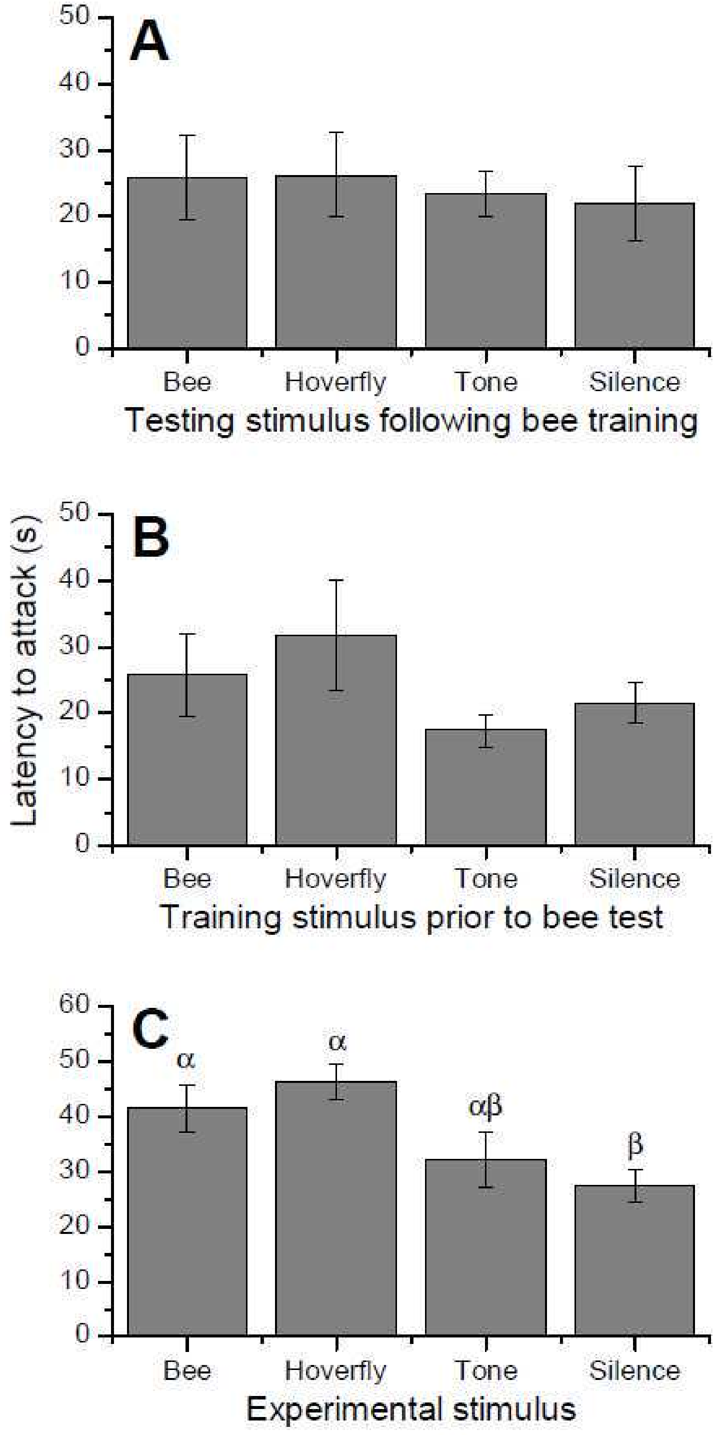

Figure 4: Results of experimental training of domestic chicks to recognise acoustic cues: (A) latency to attack between four types of food following training on distasteful food presented with a *Bombus terrestris* alarm sound, (B) latency to attack distasteful food presented with a *B. terrestris* alarm sound following training on four types of food, and (C) overall latency to attack different food types across all training and testing phases in the experiment. Bars are means and error bars represent 1SE.