**Alarm substances induce associative social learning in honeybees (*Apis mellifera)***

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**Abstract**

Alarm signals are widespread in the social insects. It is commonly accepted that such signals produce adaptive short-term aggressive or aversive responses in conspecifics, but the possibility that they could also lead to social learning about predator identity has not yet been addressed. Here we demonstrate that responses to alarm volatiles can lead to social learning about asocial stimuli in honeybees (*Apis mellifera*). Using a phototactic assay, we initially confirm previous findings that alarm volatiles deter individuals from approaching a coloured light. When the same individuals subsequently experienced the coloured light in the absence of alarm volatiles, the same deterrent effect was observed, suggesting that responses to alarm volatiles became conditioned to the coloured light. Previous experience with the light in absence of alarm cues did not induce this response, nor did previous association of alarm cues with a different coloured light. Our findings highlight that social insect signals can lead to social learning through a simple yet powerful associative mechanism.

**Keywords**

*Apis mellifera,* honeybees, predator avoidance, social cues, social learning, social signals

**Introduction**

Almost all social animals perceive and respond to social stimuli that provide information about the environment, which may be social cuesproduced incidentally by other animals, or *signals* that have evolved specifically to promote sharing of information. Signals are particularly common in the social insects ([Grüter & Leadbeater, 2014](#_ENREF_24); [Hölldobler & Wilson, 2009](#_ENREF_27); [Saleh, Scott, Bryning, & Chittka, 2007](#_ENREF_42); [Seeley, 1998](#_ENREF_44); [von Frisch, 1967](#_ENREF_48)), perhaps because senders benefit from sharing information with closely-related colony members, promoting ritualization and amplification of informative cues ([Leadbeater, 2015](#_ENREF_31); [Tinbergen, 1952](#_ENREF_46)). Alarm signals are especially frequent, often driving co-ordinated aggression towards predators ([Breed, Guzmán-Novoa, & Hunt, 2004](#_ENREF_6); [Jeanne, 1981](#_ENREF_28); [Parry & Morgan, 1979](#_ENREF_39); [Vinson & Sorenson, 1986](#_ENREF_47)), or avoidance of particular sites ([Balderrama et al., 1996](#_ENREF_2); [Sasaki, Hölldobler, Millar, & Pratt, 2014](#_ENREF_43)). Here, we show that alarm volatiles do not simply evoke immediate stereotyped response behaviour, but could also lead to social learning about stimuli that predict threat in the honeybee *Apis mellifera*.

Honeybee nests face robbing and predation from a variety of animals, such as mammals (e.g. badgers and humans) and predatory insects (e.g. wasps and hornets). Stings that are directed to such threats lead to the release of a distinctive alarm volatile, to which conspecifics respond by approaching and attacking the potential predators ([Blum, Fales, Tucker, & Collins, 1978](#_ENREF_4); [Boch, Shearer, & Petrasovits, 1970](#_ENREF_5)). Bees also encounter threats when alone and away from the nest, such as camouflaged crab spiders that wait for pollinators upon flowers ([Chittka, 2001](#_ENREF_8)), and there is evidence that the presence of recently-killed bees at forage sites is a deterrent ([Dukas, 2001](#_ENREF_18)). Goodale & Nieh (2012) have demonstrated that this effect can be elicited by sting gland contents and Balderrama *et al.* (1996) found that bees are deterred from entering areas where a conspecific has recently been disturbed, even when the conspecific is no longer present. Sting-gland pheromones may serve to dissuade relatives from visiting a dangerous location – an effect that can be amplified through signals within the hive (Nieh 2010). Although these immediate responses have obvious short-term adaptive functions for individuals responding to the deterrent, learning about the contextual cues that initially provoked their production might also prove useful. For example, through encountering alarm volatiles, individual bees might learn about the visual appearance of a predator, or the scent of a flower species that typically hosts high sit-and-wait predator densities.

Social learning about predation cues can often be explained by a simple associative mechanism ([Griffin, 2004](#_ENREF_23)). Under this framework, an animal produces a (learnt or unconditioned) response to a social stimulus that it encounters (such as an alarm call, evasion behaviour or chemical cue produced by a conspecific), and this response becomes conditioned to novel stimuli in the vicinity ([Griffin, 2004](#_ENREF_23); [Heyes, 1994](#_ENREF_25); [Mineka & Cook, 1993](#_ENREF_35)). For example, damselfly larvae reduce their movement in the presence of cues from injured conspecifics. This response becomes associated with other, asocial cues that are simultaneously present (such as cues from an unfamiliar predator species), which can later evoke reduction in movement in the absence of conspecific cues ([Wisenden, Chivers, & Smith, 1997](#_ENREF_49)). The same mechanism may underlie social learning about predators across many other taxonomic groups, including primates ([Mineka, Davidson, Cook, & Keir, 1984](#_ENREF_36)), birds ([Curio, Ernst, & Vieth, 1978](#_ENREF_15)), some fish species ([Chivers & Smith, 1998](#_ENREF_9)), and amphibians ([Ferrari, Messier, & Chivers, 2007](#_ENREF_19)). Indeed, there is growing evidence to suggest that the mechanisms underlying many other social learning processes are also the result of simple associations between social cues or signals and asocial stimuli, such as food site characteristics, flavours or habitats (for reviews see Griffin, 2004; Heyes, 1994, 2012; Leadbeater, 2015; Leadbeater & Chittka, 2007). Given that honeybee associative learning abilities have been widely documented ([Avarguès-Weber & Giurfa, 2013](#_ENREF_1); [Bitterman, Menzel, Fietz, & Schäfer, 1983](#_ENREF_3); [Couvillon & Bitterman, 1980](#_ENREF_14); [Giurfa, 2007](#_ENREF_22); [Takeda, 1961](#_ENREF_45)), it seems likely that honeybee responses to alarm volatiles could be rapidly conditioned to novel asocial stimuli that are experienced concurrently.

Balderrama and colleagues (1996) have previously developed a means to measure the deterrent effect of honeybee alarm volatiles, which capitalizes upon this species’ strong phototactic response when captured. These authors confined individual bees in a dark vial which was connected via a tunnel to a second vial, where a light could be seen. Bees consistently took longer to approach the light when a stressed conspecific had recently been in the lit vial. The deterrent effect was not observed when the stressed bee’s sting chamber and mandibular gland were sealed with wax, or when the head was removed and the sting chamber sealed, but removing the head without sealing the sting chamber elicited a strong effect. Thus, the deterrent effect cannot be explained simply by the odour of another bee, nor by the presence of haemolymph, and is most likely produced by a volatile substance that is under central inhibition (Balderrama *et al.* 1996).

Here, we use the same phototactic assay as Balderrama *et al.* (1996) to investigate whether the deterrent effect of alarm volatiles can become conditioned to asocial cues that are experienced concurrently. In phase 1, individual honeybees were exposed to alarm volatiles together with a coloured light. In phase 2, they experienced the light alone. We hypothesised that bees would take longer to approach the coloured light in phase 2, only after it had been experienced in parallel with alarm volatiles.

**Methods**

Each honeybee in our study (*N=*80) underwent two experimental phases and both phases were simple assays of the same phototactic response. In Phase 1, we exposed individual honeybees in our focal experimental group (E1) to a coloured light together with alarm volatiles collected from stressed conspecifics. In Phase 2 we assayed the latency of the same individuals to approach the same coloured light in the absence of alarm volatiles. Control group C1 experienced the same protocol, but in the absence of alarm volatiles throughout. Our hypothesis predicted that subjects in group E1 will be slower than those in group C1 to approach the light in Phase 2, because it has previously been associated with alarm volatiles.

Two alternative hypotheses might also explain such a result ([Rescorla, 1967](#_ENREF_41)), each of which generates testable predictions. Firstly, control bees (C1) experience exactly the same conditioned stimulus (the coloured light) twice, and thus may become habituated or sensitised to it (“Novelty hypothesis”). Experimental bees may not experience this effect because the coloured light is presented together with alarm volatiles in Phase 1 but not in Phase 2; they may treat the light as a more novel stimulus in Phase 2 than control bees do. Secondly, experimental bees are exposed to alarm volatiles, while controls are not. Alarm volatiles might simply lead individuals to become more risk averse, and consequently slower to approach the light on their second encounter (“Risk Aversion hypothesis”). To overcome these concerns, we incorporate a second experimental group (E2), and a corresponding control group (C2). These subjects experience the same protocols as groups E1 and C1, but encounter a different coloured light in Phase 2. The “Novelty” hypothesis predicts that group E2 should be as slow to approach the light as group E1, or slower, because both experience a stimulus that is perceived as novel. For group E1, this stimulus is the coloured light in the absence of alarm volatiles, and for group E2, it is a differently-coloured light. The “Risk Aversion” hypothesis predicts that both groups E1 and E2 should be slower than controls to approach the light in Phase 2, because both have been exposed to alarm volatiles. In contrast, only our focal hypothesis predicts that solely Group E1 should be slow to approach the light relative to all other treatment groups.

Each treatment group contained 20 bees, and within groups, half of the subjects underwent a “green light” protocol, and half a “blue light” protocol. The experimental design is summarised in Table 1.

 *Phase 1: Conditioning*

Honeybee foragers (*Apis mellifera*) were caught on returning to a hive located at Queen Mary University of London throughout the month of September, 2013. Individual bees were placed in glass vials (‘vial A’; 5.5cm X 2.5cm X 2cm). Each vial was connected to a second vial (‘vial B’) via a transparent plastic tube (15cm x 2.5cm; Fig. 1). For honeybees in groups E1 and E2 (Table 1), vial B contained conspecific alarm volatiles, obtained by gently pressing a conspecific (from the same colony) in the vial with a 1cm3 piece of sponge for one minute before removing both the sponge and the bee ([Balderrama et al., 1996](#_ENREF_2)). At the end of vial B, the test bee could see a blue or green light (“True Utility Flexi Lite + laser” LED; True Brands Ltd, covered with 3 layers of green or blue cellophane; Bright Ideas Marketing Limited). The light source (5mm in diameter) appeared as a point stimulus rather than bathing the vial in colour. Subjects in control groups, C1 and C2 (Table 1), experienced the same set-up and light stimuli, but in the absence of any alarm volatiles. Once both vials were connected, a light-restricting black sheet was placed over vial A to encourage the honeybee to move towards the experimental vial through a phototactic response. We measured how long it took test subjects to move from vial A to vial B. A timer was initiated when the subject’s head crossed a line between vial A and the connecting tube and was stopped once the bee had fully entered vial B.

*Phase 2: Test*

Immediately after phase 1, vials were swapped with clean replacements. Bees in groups E1 and C1 were presented with a vial illuminated by the same colour experienced in phase 1, but in absence of alarm cues (Table 1). Bees in groups E2 and C2 were presented with an alternative light colour (either green or blue) to the one they had experienced in phase 1. Again, the time to enter vial B was recorded.

*Statistical analysis*

All analyses were carried out in R v. 2.12.0. We modelled the log-transformed time it took bees to enter vial B using general linear models. We selected models by comparing Akaike’s information criterion (AIC) values between different models. A model was considered to be a significantly better fit if the AIC value was lower by two units or more ([Johnson & Omland, 2004](#_ENREF_29)).

First, we aimed to confirm earlier findings that alarm volatiles are a deterrent to the phototactic response in our protocol ([Balderrama et al., 1996](#_ENREF_2)). If true, the best model to predict latency to approach the light in Phase 1 should include the presence of alarm volatiles. We compared a basic model, which contained the intercept, with candidate models that included the presence of alarm volatiles, the colour of the light stimulus and the interaction between them as predictors (Table 2a).

Secondly, we tested whether latency to approach the light in Phase 2 was influenced by exposure to the same light accompanied by alarm volatiles in Phase 1. Here, our response variable was latency to enter the vial in Phase 2. The basic model incorporated the colour of the light stimulus presented in phase 2 (since we found that light colour had an influence on latency in phase 1) and was compared to candidate models which included whether bees were exposed to alarm volatiles in phase 1, whether the presented light colour was familiar (i.e. the same light bees experienced in phase 1), and their interaction (Table 2b). We predict that a full model containing the interaction term should be a better fit than one excluding the interaction term, because latency to approach the light following exposure to alarm volatiles should be conditional upon the familiarity of the light (i.e. observed only for group E1).

*Ethical note*

Test subjects were caught upon returning to their hive using a net and were quickly put in glass vials and placed in a dark bag to minimise stress. We marked all test subjects with a non-toxic acrylic paint so they could later be released. As soon as an experiment was finished (average time = 30 mins), individuals were released next to their hive. We tried to minimise the stress experienced by bees that were used to produce the alarm volatiles by pressing them using a soft piece of foam to extract the alarm volatiles which limited any physical injury to individuals. Alarm volatile-producing bees were also released after an experiment had finished.

**Results**

In Phase 1, bees took longer to enter the vial when it contained alarm volatiles (Fig. 2a (i) and (ii)). Accordingly, the best model to predict latency to enter the vial included whether the vial contained alarm volatiles (estimate: 0.74 ± 0.15 [mean ± standard error, [SE]]) and the colour of the light stimulus (estimate: 0.31 ± 0.15 [SE]; Table 2a; ∆AIC to next best-fitting model = 2.0).

In Phase 2, bees were slower to approach the coloured light only if they had experienced alarm volatiles associated with that specific light colour in Phase 1 (Fig. 2b). Accordingly, latency to approach the light in Phase 2 was best predicted by the model that incorporated whether bees had previous exposure to the alarm volatiles in phase 1, whether the colour of the light stimulus was familiar or new, and the interaction between them (Table 2b). This model received significantly more support than that predicted by the “Risk Aversion hypothesis”, in which the effect of volatiles is not conditional upon the familiarity of the light (ΔAIC=13.3). In other words, bees avoided the coloured light only if they had previously experienced it in Phase 1, *and* if that experience was accompanied by exposure to alarm volatiles.

A model containing the interaction term cannot distinguish between our focal hypothesis and the “novelty” hypothesis, because both predict a potential difference between groups E1 and E2. The focal hypothesis predicts that E2 should be more similar to the control groups than E1, because the light that they encounter has not been associated with alarm volatile, but a significant interaction effect might also be detected because E2 were slower than E1, in accordance with the “Novelty” hypothesis. Thus, we used a third general linear model to compare latency in Phase 2 between groups E1 and E2 directly: we found that E2 were significantly quicker than group E1 to approach the light (Fig 2b; effect size for E2 relative to E1: -0.67+ 0.19 [SEM]).

**Discussion**

Our results support the hypothesis that volatile alarm cues could facilitate the learning of predator-related cues in honeybees. We found that bees were deterred from approaching a coloured light only after the light had been experienced in contiguity with conspecific alarm cues. Previous experience of the light alone did not induce this response, nor did previous association of volatile alarm cues with a different light colour. The association between the coloured light and alarm volatiles was integral in facilitating the deterrent effect, implying a key role for associative learning in this process ([Griffin, 2004](#_ENREF_23); [Heyes, 1994](#_ENREF_25); [Leadbeater, 2015](#_ENREF_31); [Leadbeater & Chittka, 2007](#_ENREF_32)). Our findings highlight that associative learning can be a simple and efficient process for predator avoidance learning in a wide array of different taxonomies ([Chivers & Smith, 1994](#_ENREF_10); [Mineka & Cook, 1993](#_ENREF_35)), including insects ([Grüter & Leadbeater, 2014](#_ENREF_24); [Wisenden et al., 1997](#_ENREF_49)).

Associative learning is a taxonomically widespread, domain-general process that is common to social and solitary species alike, raising the question of whether social learning about predator-associated cues involves any form of adaptive specialization ([Heyes, 1994](#_ENREF_25), [2012](#_ENREF_26)). There is nothing specifically “social” about the learning process that we describe, since the same effect should be observed with any deterrent odour. Likewise, there is nothing specifically social about related examples of social learning about predators, such as observational conditioning of fear in rhesus macaques ([Cook & Mineka, 1989](#_ENREF_12), [1990](#_ENREF_13)), or conditioning of alarm responses to novel predators in many fish species ([Brown & Laland, 2003](#_ENREF_7)). Social learning (which is classically defined as “learning that is influenced by interaction with, or observation of, another animal (typically a conspecific) or its products; ([Heyes, 1994](#_ENREF_25)) often arises from simple associative processes ([Griffin, 2004](#_ENREF_23); [Heyes, 1994](#_ENREF_25), [2012](#_ENREF_26); [Leadbeater, 2015](#_ENREF_31); [Leadbeater & Chittka, 2007](#_ENREF_32)), which predate social life but are co-opted to produce adaptive use of social information.

It is generally considered that responses to social stimuli, such as the deterrent effect of alarm volatiles in bees ([Balderrama et al., 1996](#_ENREF_2)) observed here, are hard-wired. Yet, the ontogeny of most responses to social cues and signals has rarely been studied sufficiently to rule out a role for learning in their development ([Galef, 2013](#_ENREF_20)). For example, it is conceivable that social insect responses to alarm signals might be partly learnt ([Collins, 1980](#_ENREF_11)), just as frightened conspecifics might trigger a fear response in rhesus monkeys simply through previous association with danger ([Heyes, 2012](#_ENREF_26)), and rats could acquire positive associations concerning conspecific breath components (that are later conditioned to food flavours, Galef, Jr, Mason, Preti & Bean, 1988) through maternal licking. Growing evidence shows that responses to social information can indeed be learnt ([Dawson, Avarguès-Weber, Chittka, & Leadbeater, 2013](#_ENREF_17); [Katsnelson, Motro, Feldman, & Lotem, 2008](#_ENREF_30); [Leadbeater & Chittka, 2009](#_ENREF_33); [Mottley & Giraldeau, 2000](#_ENREF_37); [Reader, 2014](#_ENREF_40)). When learnt (rather than unconditioned) responses become conditioned to neutral stimuli (such as predators), the social learning process is akin to a taxonomically general associative mechanism termed second-order conditioning ([Dawson et al., 2013](#_ENREF_17); [Leadbeater & Chittka, 2007](#_ENREF_32)).

We demonstrate that anti-predator responses may become conditioned even to cues that are not naturally relevant to predation, such as a coloured light, highlighting the flexibility of this learning process in honeybees. In a scenario where predators are cryptic or undetectable (such as crab spiders, sit-and wait ambush predators that lurk on flowers), this flexibility may be of benefit, allowing other features, such as flower location, to be learnt in order to avoid revisiting predator-infested foraging patches. Conversely, an unselective approach to learning all predator-associated cues could also result in maladaptive behaviour, with beneficial information potentially being misconstrued or used in the wrong context. Yet many animals demonstrate an inherent preparedness, or “filter”, for learning relevant predator cues more efficiently than arbitrary ones ([Chivers & Smith, 1994](#_ENREF_10); [Cook & Mineka, 1990](#_ENREF_13); [Curio et al., 1978](#_ENREF_15); [Davies & Wellbergen, 2009](#_ENREF_16); [Magurran, 1989](#_ENREF_34)). Thus, although we found that honeybees were capable of learning an arbitrary stimulus, a natural predator, such as a spider, may elicit a stronger or longer response. Note that there is no evidence that such biases are specific to social (rather than asocial) learning. For example, although rhesus macaques can learn socially to fear snakes but not flowers ([Cook & Mineka, 1989](#_ENREF_12)), it seems likely that the monkeys might be more likely to learn to fear snakes than flowers in general, irrespective of whether through observing others or direct interaction ([Leadbeater, 2015](#_ENREF_31); [Öhman, Flykt, & Esteves, 2001](#_ENREF_38)).

In conclusion, we demonstrate that stereotyped social insect responses to alarm cues can form the basis for social learning about predator-associated cues. Our study opens questions regarding the extent to which natural selection shapes social learning about predators, which could be addressed by studying the ontogeny of responses to social information and biases in the types of stimulus/response relationships that can be learnt socially.

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Designed the experiments: EL, ED

Collected the data: ED

Performed the statistical analyses: EL

Wrote the manuscript: EL, ED, LC

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**Table and Figure Legends**

**Table 1**

**Summary of experimental treatments and predictions.**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Treatment | *N* | Light colour Phase 1 | Light colour Phase 2 | Alarm volatiles in phase 1? |
| E1 | 10 | Blue | Blue | Yes |
| C1 | 10 | Blue | Blue | No |
| E2 | 10 | Blue | Green | Yes |
| C2 | 10 | Blue | Green | No |
| E1 | 10 | Green | Green | Yes |
| C1 | 10 | Green | Green | No |
| E2 | 10 | Green | Blue | Yes |
| C2 | 10 | Green | Blue | No |

Table displays sample sizes (*N*) and experimental conditions for Experimental (E1, E2) and Control groups (C1, C2). Groups below the horizontal line mirror the experimental conditions above the line, except that the colours have been reversed (i.e. green lights instead of blue).

**Table 2**

Candidate models investigating the effect of various variables on the latency time to approach vial B for phase 1 and phase 2.

|  |  |  |  |
| --- | --- | --- | --- |
| Phase | Model | AIC | ΔAIC |
| 1 | basic | 192.2 | 20.4 |
| volatiles | 173.9 | 2.1 |
| light colour | 191 | 19.2 |
| **volatiles + light colour** | **171.8** | **0** |
| volatiles \* light colour | 173.8 | 2 |
|  |  |  |  |
| 2 | basic | 156.3 | 23.2 |
| volatiles | 146.6 | 13.5 |
| familiar | 156.3 | 23.2 |
| volatiles + familiar  | 146.4 | 13.3 |
| **volatiles \* familiar**  | **133.1** | **0** |

The best models (shown in bold) were selected based on the lowest AIC value. ΔAIC indicates the difference between that model and the best model.

 **Figure 1:** Diagram of experimental set-up. Individual honeybees, placed in vial A,were timed entering vial B. Black material was placed over vial A (shown by the grey dotted box) to encourage movement towards vial B. Vial B contained alarm volatiles and/or a coloured blue or green light.

**Figure 2:** Mean (± SEM) latency times (s) for honeybees to enter vial B (a & b) in phase 1, when alarm volatiles were either absent or present and (c & d) in phase 2, when bees, that had or had not been previously exposed to alarm volatiles, were presented with a familiar or novel light colour. Colours of bars indicate light colours in (a & c) blue light replicate set and (b & d) green light replicate set. Hatched lines indicate trials where alarm volatiles were presented with the light stimulus.