



## Social but not solitary bees reject dangerous flowers where a conspecific has recently been attacked

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Social bees are known to avoid inflorescences marked with dead conspecifics or their smell. The avoidance response could be triggered by alarm signals actively given by attacked bees or by substances passively released through injuries as a by-product of the attack. To discriminate between these two options we note that both social and solitary bees are expected to react to nonsignalling cues associated with predation risk, while only social bees are expected to give alarm signals. We simulated risky inflorescences by pinching a landing bee with forceps, and compared the rate at which bees visited these experimental inflorescences and unmanipulated control inflorescences. We conducted the experiment with four species of social bees, *Apis mellifera*, *Apis dorsata*, *Apis florea* and *Bombus terrestris* and with three species of solitary bees, *Eucera* sp., *Panurgus* sp. and *Nomia strigata*. We found that while the three species of solitary bees responded similarly to control and experimental inflorescences, all four species of social bees strongly rejected inflorescences where we simulated a predation attempt. The finding that only social species avoided landing on dangerous inflorescences strongly suggests that the release of the alarm cue has been selected for its signalling value in social bees.

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Animals rely on cues to detect their predators and avoid fatal encounters (Lima & Dill 1990). Such cues can belong to different sensory domains, such as the visual, auditory or chemical domains (Chivers & Smith 1998; Barbosa & Castellanos 2005). The cues to which prey respond can be produced by the predator itself or by other prey (Kats & Dill 1998; Wyatt 2003; Barbosa & Castellanos 2005). Prey-produced alarm cues can, at least in principle, be divided into two groups, according to whether their release is merely a by-product of the predation attempt (i.e. body fluids that have escaped through skin injuries) or has been selected for its signalling value (Chivers & Smith 1998; Kats & Dill 1998; Wyatt 2003). In this study, we investigated the use of alarm signals by foraging bees.

Aggressive alarm pheromones, which trigger attacks to intruders during colony defence, have been well described in species with a high level of social development, such as bees from the tribes Apini and Meliponini. (Koeniger et al. 1979; Roubik et al. 1987; Schmidt 1998; Schorkopf et al. 2009). In contrast, the role of evasive alarm pheromones, triggering an escape response, remains less clear in social bees. It is known that certain substances, such as

2-heptanone, a pheromone released through the mandibular glands of *Apis mellifera* bees, repel bees at the foraging site (Butler 1966; Simpson 1966; Rieth et al. 1986; Vallet et al. 1991). Although it has been suggested that foragers may deposit this compound on visited flowers to signal nectar depletion, this hypothesis has not been conclusively demonstrated (Balderrama et al. 1996; Stout & Goulson 2001; Gawleta et al. 2005). In particular, it seems likely that evasive alarm pheromones play a role in the predator avoidance response of foraging bees. Thus, evasive alarm pheromones are used by some Asian *Apis* species (Suwannapong et al. 2011a) which do not appear to mark visited flowers (Suwannapong et al. 2011b) and, in *A. mellifera* and certain species of Meliponini bees, these evasive substances are released in response to an experimental disturbance (Lindauer & Kerr 1960, page 31; Balderrama et al. 1996). Furthermore, honeybees and bumblebees reject flowers with a crushed conspecific or its smell (Stout et al. 1998; Dukas 2001; Abbott 2006), and honeybees, *A. mellifera*, reject flowers where a crab spider has previously struggled with another honeybee (Llandres & Rodríguez-Gironés 2011).

Available evidence therefore suggests three nonexclusive interpretations: bees mark visited flowers to increase colony foraging efficiency, bees use alarm pheromones to mark dangerous flowers, or bees can detect, and avoid, the smell of a crushed conspecific. To discriminate between these hypotheses, we note that they make contrasting predictions for social and solitary bees.

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According to Hamilton's (1964a, b) rule, upon detection of a potential predator an individual will benefit from alerting group members if the cost to the sender is smaller than the cumulative benefit to group members, discounted by the appropriate coefficients of relatedness. We should therefore expect the evolution of alarm signals in social, but not in solitary insects (Wyatt 2003). Indeed several studies have shown that the active release of alarm signals has evolved in eusocial and group-living animals (reviewed in Blum 1969, 1974a, b; Verheggen et al. 2010), while we are not aware of any study that has specifically looked for the presence of alarm signals in solitary insects. As mentioned above, eusocial bees are known to use aggressive alarm pheromones (Koeniger et al. 1979; Roubik et al. 1987; Schmidt 1998; Schorkopf et al. 2009), which have not been found in social species with small colonies such as bumblebees (Maschwitz 1966). In contrast, alarm signals or predation cues are involved in the predator avoidance response of honeybees and bumblebees (Stout et al. 1998; Dukas 2001; Abbott 2006).

While the release of alarm signals is more likely to evolve in social than in solitary insects, both social and solitary species are expected to respond to any cue that signals the presence of a predator. Indeed, in the particular case of bees, it has been shown that solitary bees should accept lower levels of predation risks while foraging than social species (Clark & Dukas 1994; Rodríguez-Gironés & Bosch 2012). We should therefore expect solitary bees to respond more strongly than social bees to the presence of cues signalling the proximity of predators, but only social bees to give alarm signals to warn their conspecifics. A similar argument would suggest that social, but not solitary bees should mark visited flowers to increase colony foraging efficiency. To discriminate between marks left to inform about danger or resource depletion, we note that in the former case bees should only avoid flowers where a conspecific has been attacked, while in the latter flower rejection should depend on the number and duration of previous visits.

The main aim of this study was to determine whether social bees release deterrent substances as alarm signals to communicate the presence of a dangerous flower. Of particular interest is the possible use of alarm signals by bumblebees, which do not appear to use aggressive alarm pheromones for nest defence (Maschwitz 1966). A subsidiary aim was to confirm that solitary bees make no use of such alarm signals. To do this, we compared the number of bees, from different social and solitary species, visiting and rejecting control inflorescences that had been visited by a bee and inflorescences where we had simulated a predator attack by pinching a bee with forceps. If bees marked all flowers they exploited, control and experimental inflorescences should be treated alike. If bees release some volatile cues as a side-effect of the attack, there should be no association between the release of such compounds and the sociality status of the bees: we would expect no differences in the response of social and solitary bees to the manipulation. If, on the other hand, the substance is actively released as a warning signal, only social bees should respond to the manipulation.

## METHODS

### Study Sites and Species

We conducted the experiments in six different geographical areas: Baza (Granada, Spain), Almeria (Spain), Villuercas-Ibores (Extremadura, Spain), Cannonvale (Queensland, Australia), MacRitchie Reservoir Park (Singapore) and Xishuangbanna (Yunnan province, China) between May 2009 and May 2011. In each locality we conducted trials in patches as distant as possible in order to avoid pseudoreplication. The distance used between patches for each species was approximately 20, 50, 100, 100, 1000, 5000 and 5000 m for *Nomia strigata*, *Panurgus* sp., *Apis dorsata*, *Apis florea*, *Bombus terrestris*, *Eucera* sp. and *A. mellifera* bees, respectively. Our data set comprised a total of 233 experimental and 236 control trials from seven species of bees foraging at different flower species (see Table 1). We selected *A. mellifera*, *A. dorsata*, *A. florea* and *B. terrestris* as species representative of social bees and *Eucera* sp., *N. strigata* and *Panurgus* sp. as species representative of solitary bees. We selected our species from three different families: Apidae, Halictidae and Andrenidae (see Table 1).

### Experimental Procedure

For each trial we selected and marked one inflorescence (hereafter referred to as the focal inflorescence) and assigned it to the experimental or control treatment in pseudorandom order: treatment was allocated randomly to odd inflorescences, and even inflorescences were assigned to whatever treatment had not been used for the previous observation. For the control treatment, we waited until a bee landed on the inflorescence and left it. For the experimental treatment, we waited until a bee landed on the selected inflorescence and carefully held it on the inflorescence for 2–10 s, grasping the bee with forceps over the thorax. We did not visibly harm bees: they left the area flying as soon as we released them. Social bees emitted a strong distinctive smell when pinched (a similar observation was reported by Abbott 2006), although one of the authors of this study was unable to detect it.

Once the bee left, we recorded the number of bees approaching and visiting the focal inflorescence during the trial. After a bee approached the inflorescence, we distinguished two bee responses: visits and rejections. We considered that a bee visited an inflorescence when it approached and landed on it, and that the bee rejected the inflorescence when it approached it, hovered for a few seconds in front of it (sometimes touching it with its forelegs) and then left without landing. Trials lasted 10–30 min depending on the visit rate of each bee species (see Table 1). All trials were conducted during sunny weather, at the peak time of bee activity. In most trials, several bees approached the focal inflorescence. The total number of bees approaching control and experimental inflorescences for each bee species is given in Table 1.

**Table 1**

Bee species, bee family, flower species, duration of trials (min) and sample size of experimental and control trials performed for each bee species

Bee species	Family	Flower species	Experimental treatment (N)	Control treatment (N)	Duration of trials (min)	Site of collection
<i>Apis mellifera</i>	Apidae	<i>Bidens alba</i>	35 (105)	35 (96)	30	Australia
<i>Apis dorsata</i>	Apidae	<i>Calliandra emarginata</i>	35 (116)	35 (102)	15	China
<i>Apis florea</i>	Apidae	<i>Alchornea tiliifolia</i>	30 (84)	30 (80)	15	China
<i>Bombus terrestris</i>	Apidae	<i>Teucrium fruticans</i>	40 (104)	40 (112)	20	Almeria, Spain
<i>Eucera</i> sp.	Apidae	<i>Lavandula stoechas</i>	5 (10)	7 (17)	20	Extremadura, Spain
<i>Panurgus</i> sp.	Andrenidae	<i>Launaea pumila</i>	40 (48)	40 (45)	20	Granada, Spain
<i>Nomia strigata</i>	Halictidae	<i>Melastoma malabatricum</i>	48 (152)	49 (140)	10	Singapore

The total number of bees that approached experimental and control inflorescences in each experiment is given in parentheses after the sample size.

## Statistical Analyses

### Treatment effect for each bee species

For each combination of bee and flower species used, we performed generalized linear models with quasi-Poisson (where we had significant overdispersion) or Poisson error distribution and log link function to determine whether bees approached control and experimental inflorescences at the same rate. The models included the number of bee approaches to inflorescences as the dependent variable, and treatment (experimental versus control) as the independent variable.

Upon approach, bees could either visit or reject the inflorescence. To determine whether the probability that bees visited inflorescences after approaching them was the same for control and experimental treatments, we used generalized linear models with binomial error distribution and logit link function. In these analyses, the dependent variable was the pair (number of visits, number of approaches) for each trial and the independent variable was treatment (experimental versus control).

We used likelihood ratio tests to assess whether treatment had a statistically significant effect on bee response (Dobson & Barnett 2008) and applied the sequential Bonferroni correction whenever the results were statistically significant (Hochberg & Tamhane 1987).

### Effect of sociality

Owing to shared ancestry, species are not statistically independent data points. Therefore, to test the hypothesis that a life history trait, sociality, affects the evolution of alarm signals, we performed an additional statistical analysis to correct for phylogenetic distances from the species used in our study. We used Mesquite 2.75 (Maddison & Maddison 2009) to assemble the phylogenetic tree and get the phylogenetic correlation structure to correct for this phylogenetic dependence (Paradis 2006). The basic tree structure (from species or genus to family level, Fig. 1) was built using the information available in the literature (Danforth et al. 2006; Raffiudin & Crozier 2007; Cardinal et al. 2010). For testing our hypothesis we used a variable called ‘repellence of the dangerous flower’ as the dependent variable. This variable was the difference between the mean proportion of visits (total number of visits/total number of approaches) to control flowers and the mean proportion of visits to experimental flowers for each species of bees. If bees rejected most experimental flowers and thus the proportion of visits to experimental flowers was much lower than the proportion of visits to control flowers, the variable ‘repellence’

was close to 1. On the other hand, if the proportion of visits to experimental flowers was similar to the proportion of visits to control flowers, the variable ‘repellence’ was close to the value 0. A statistical model was constructed to test our hypothesis, that is, to test whether there was a significant effect of sociality on the repellence of bees towards the dangerous flower. We analysed our model by means of phylogenetic generalized least squares (PGLS) including the phylogenetic correlation structure as a random factor. We used three evolutionary models, Brownian, Pagel and Ornstein–Uhlenbeck (Martins & Hansen 1997; Pagel 1999), and selected the evolutionary model that best described the evolution of our trait along the phylogeny using the AIC criterion (Akaike 1973). Once the best evolutionary model was selected, we tested for the effect of sociality in our dependent variable.

We used R 2.8.1 (R Development Core Team 2008) for statistical analyses.

## RESULTS

### Bee Approaches

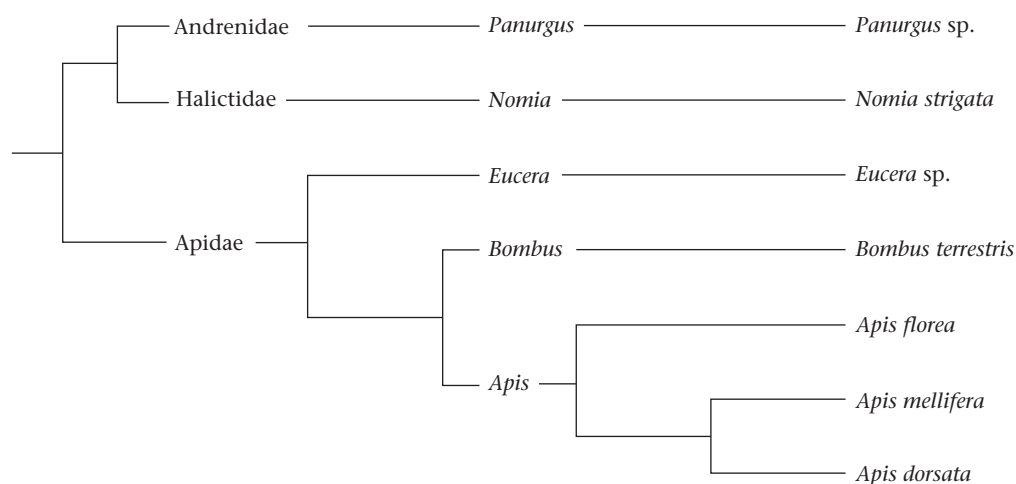
All species of social and solitary bees approached control and experimental inflorescences at similar rates. The difference was not statistically significant for any of the species (all  $P > 0.34$  in the absence of Bonferroni corrections; see Table 2).

### Bee Visits

Social bees typically landed on the control flowers they approached, but rejected experimental flowers (Fig. 2). As a result, the difference between the probability of landing at control and experimental flowers was statistically significant for all social bee species we tested (all  $P < 0.001$  after applying the Bonferroni correction; Table 3). In contrast, solitary bees were equally likely to visit control and experimental inflorescences after an approach (Fig. 2), and the difference between the probability of landing at control and experimental flowers was not statistically significant for any of the solitary species (in the absence of Bonferroni correction, all  $P > 0.25$ ; Table 3).

### Effect of Sociality

We found a significant effect of sociality on the level of repellence of bees towards the dangerous flowers after correcting for phylogenetic distances from the bee species used in our study



**Figure 1.** Phylogeny rebuilt using published information for the different taxa used for our experiments.

**Table 2**

Results of likelihood ratio tests comparing the approach rate (per min) between control and experimental flowers for each bee species

Species	Approach rate $\pm$ SE		Deviance	P
	Control	Experimental		
<i>Apis mellifera</i>	0.11 $\pm$ 0.01	0.12 $\pm$ 0.01	0.40	0.58
<i>Apis dorsata</i>	0.29 $\pm$ 0.02	0.33 $\pm$ 0.02	0.90	0.34
<i>Apis florea</i>	0.26 $\pm$ 0.03	0.28 $\pm$ 0.03	0.10	0.76
<i>Bombus terrestris</i>	0.14 $\pm$ 0.01	0.13 $\pm$ 0.01	0.29	0.58
<i>Eucera</i> sp.	0.12 $\pm$ 0.03	0.10 $\pm$ 0.02	0.24	0.62
<i>Panurgus</i> sp.	0.07 $\pm$ 0.01	0.08 $\pm$ 0.01	0.09	0.76
<i>Nomia strigata</i>	0.29 $\pm$ 0.02	0.31 $\pm$ 0.03	0.77	0.38

Sample sizes for each bee species are reported in Table 1.

(Table 4). While social bees were highly repelled from flowers where we simulated the predator attack, solitary bees did not show any repulsion towards the dangerous flowers (see Fig. 2).

## DISCUSSION

Our results provide clear evidence that the social species but not the solitary species avoided flowers where a conspecific had recently been attacked. Whereas solitary bees treated similarly control and experimental inflorescences, treatment had a strong effect on the response of the social bees to the inflorescences they approached. Social bees approached both inflorescence types at the same rate but they landed on most of the control inflorescences they approached and rejected most inflorescences where we had simulated a predation attempt (Fig. 2). A phylogenetically corrected test confirmed that repulsion of flowers where we had simulated a predation attempt was stronger for social than for solitary bees.

### Limitations of the Study

Logistic constraints prevented us from running all tests in the same place and at the same time, or with the same plant species. As

**Table 3**

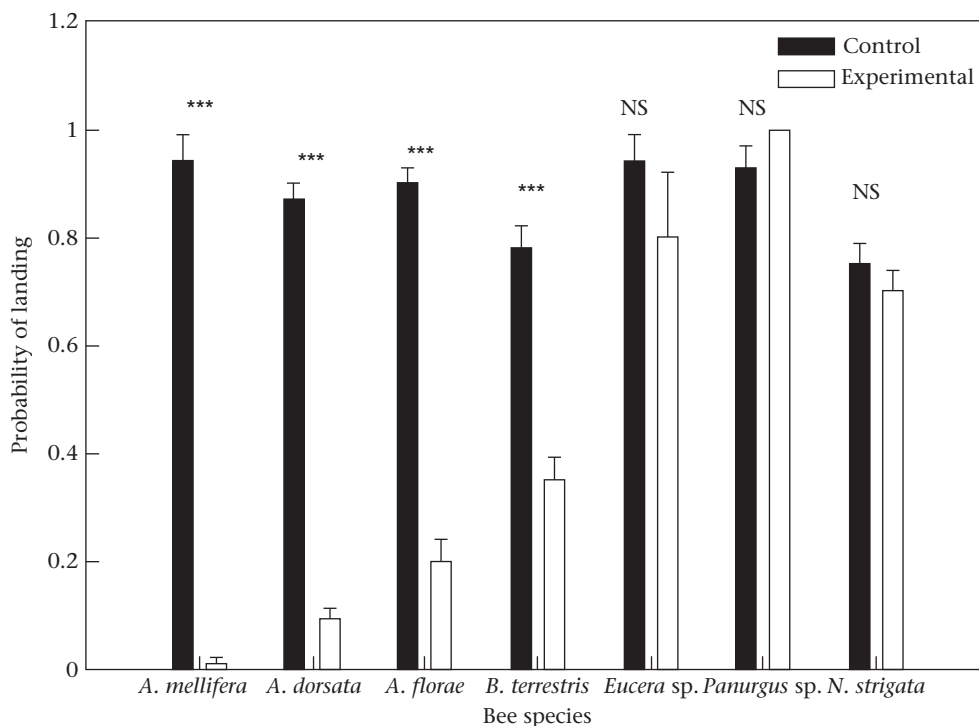
Results of likelihood ratio test comparing the probability of landing after an approach between control and experimental flowers for each bee species

Species	Probability of landing $\pm$ SE		Repulsion	Deviance	P
	Control	Experimental			
<i>Apis mellifera</i>	0.94 $\pm$ 0.05	0.01 $\pm$ 0.01	0.94	220.66	<0.001
<i>Apis dorsata</i>	0.87 $\pm$ 0.03	0.09 $\pm$ 0.02	0.75	150.15	<0.001
<i>Apis florea</i>	0.90 $\pm$ 0.03	0.20 $\pm$ 0.04	0.79	89.52	<0.001
<i>Bombus terrestris</i>	0.78 $\pm$ 0.04	0.35 $\pm$ 0.04	0.45	42.28	<0.001
<i>Eucera</i> sp.	0.94 $\pm$ 0.05	0.80 $\pm$ 0.12	0.06	1.22	0.27
<i>Panurgus</i> sp.	0.93 $\pm$ 0.04	1.00 $\pm$ 0.00	-0.06	1.27	0.26
<i>Nomia strigata</i>	0.75 $\pm$ 0.04	0.70 $\pm$ 0.04	0.08	1.00	0.31

The variable 'repulsion' refers to the difference between the mean proportion of visits (total number of visits/total number of approaches) to control flowers and the mean proportion of visits to experimental flowers for each bee species. Sample sizes for each bee species are reported in Table 1.

a result, some of the differences that we recorded might also reflect variations in other factors, such as temperature or resource availability, between the different sites where we performed our experiments. Nevertheless, it is very unlikely that the behavioural differences between social and solitary bees could be explained solely by differences in environmental factors between sites, since there was no association between degree of sociality and experimental site (see Table 1).

Another caveat that we must point out is that, although experimental inflorescences were not visibly damaged and all bees flew away from the area as soon as we released them, it is possible that bees were injured or inflorescences damaged as a result of the experimental manipulation, and that approaching bees were responding to volatile cues that had escaped from inflorescences or injured bees. While acknowledging this possibility, we point out that there is no reason why social but not solitary bees should have responded to cues correlated with flower damage or bee injury. Furthermore, honeybees, *A. mellifera*, do not avoid foraging sites marked with crushed bee thorax (Butler 1966), and should



**Figure 2.** Average probability of landing following an approach of *Apis mellifera*, *A. dorsata*, *A. florea*, *Bombus terrestris*, *Eucera* sp., *Panurgus* sp. and *Nomia strigata* bees to control (black bars) and experimental (white bars) inflorescences. Error bars represent SEs. \*\*\* $P < 0.001$ .

**Table 4**

Results of the phylogenetic generalized least squares analyses for each evolutionary model

Model	df	AIC	t	P
Brownian	5	4.532	3.011	0.029
Pagel	5	6.529	2.934	0.032
Ornstein–Uhlenbeck	5	5.639	2.915	0.033

therefore not avoid experimental inflorescences in response to volatiles released through injuries produced in the bee thorax.

#### Resource Depletion or Predation Risk?

One may argue that social bees could be responding to ‘footprints’: scent marks deposited by the attacked bee during its normal foraging activity. It is known that honeybees, bumblebees and some species of solitary bees avoid visiting inflorescences that have recently been exploited by a bee of the same or a different species (Stout et al. 1998; Gawleta et al. 2005; Wilms & Eltz 2008). When simulating the predation attempt we held bees on the inflorescences for 2–10 s, so that, in principle, experimental inflorescences might have contained stronger ‘footprints’ than control inflorescences. However, data collected on the duration of the visits of the species used revealed that the duration of undisturbed visits was 0.4–20.75 s (mean  $\pm$  SD = 6.70  $\pm$  5.83 s). Moreover, we rarely observed bees rejecting inflorescences following visits lasting 10 s or more. Thus, we can safely conclude that social bees were not rejecting experimental inflorescences because attacked bees had left unusually strong scent marks on them.

#### Alarm Cue or Signal?

A number of previous studies have shown that social bees avoid cues associated with dead conspecifics. For example, bumblebees avoided inflorescences containing either a freshly killed bumblebee or its smell (Abbott 2006) and inflorescences treated with extract of conspecific body parts (Stout et al. 1998). Furthermore, Dukas (2001) offered 20 *A. mellifera* honeybees a choice between an artificial nectar source with a dead bee (killed by pressing it gently inside a test tube) and a control feeder, and found that 19 of the 20 bees sampled chose to land on the control feeder. These results are consistent with the hypothesis that social bees, upon encountering a predator, produced an alarm signal to warn conspecifics. However, because experimental inflorescences were marked with dead, and often crushed bees, conspecifics might be reacting to the presence of substances the release of which has not been selected for its signalling value. Indeed, there are several species of animals, such as cockroaches, isopods, caterpillars and springtails among others, that use fatty acids released from body fluids of conspecifics to recognize and avoid their predators (Rollo et al. 1994; Nilsson & Bengtsson 2004; Yao et al. 2009). Moreover, some animals when injured by predators passively release fluids that induce an alarm response in neighbouring individuals of the same and other species. For example, fishes, sea urchins, sea snails, crustaceans and other aquatic animals respond in an alarmed manner to chemicals released passively from injured conspecifics (e.g. Smith 1992; Jacobsen & Stabell 2004; Fleming et al. 2007). Crayfish, *Orconectes virilis*, responded similarly to cues from injured conspecifics, sympatric heterospecifics and novel heterospecifics (Pecor et al. 2010). Likewise, exposure to cues released by damaged individuals of their own or other species triggered a predator avoidance response in the freshwater gastropod *Lymnaea stagnalis*, a response that was strongest to cues from sympatric species (Dalesman et al. 2007).

The presence of an avoidance response in social but not solitary bee species suggests that social species release, and respond to, an alarm pheromone to warn nestmates about the presence of a hidden predator. If the avoidance response was triggered by a volatile cue emitted as a by-product of the simulated attack, and the release of this cue had not been selected for its signalling value, we should expect social and solitary bees to release and respond to the cue. Indeed, everything else being equal solitary bees should be expected to show stronger predator avoidance responses than social bees. Essentially, this is because the loss of a bee has a relatively minor impact on the reproductive output of a social bee colony, but a very strong impact on the reproductive output of a solitary bee (Clark & Dukas 1994; Rodríguez-Gironés & Bosch 2012). The use of alarm signals is expected to evolve only when signaller and receiver(s) are close kin (Hamilton 1964a, b), a condition that is met among social bees but not among solitary species. In this respect, the use of alarm signals by bumblebees suggests that, despite their primitive recruitment system (Dornhaus & Chittka 2001), bumblebees are normally surrounded by their sisters while foraging.

Although collective defence has been previously reported as a characteristic feature in different species of eusocial and group-living animals (reviewed in Blum 1969, 1974a, b; Verheggen et al. 2010), our study is the first that specifically compares behavioural responses to potential alarm pheromones in different species of social and solitary species from the same taxonomic group. Moreover, as far as we know, our study is the first to show that a bee species with a low level of social development, *B. terrestris*, may also use alarm signals to inform conspecifics of a nearby danger. Although in the absence of the chemical determination of the compounds released by social bees we should interpret our results with caution, our findings give strong support to the view that social but not solitary bees emit evasive alarm pheromones to communicate the presence of danger.

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