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Public use of olfactory information associated with predation in two species of social bees

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Recent studies have documented that social bees can use heterospecific information to find or avoid food resources, but little is known about whether bees gain information from heterospecifics about predation risk. We report the first detailed field tests in bees of hetero- and conspecific avoidance of olfactory information associated with predation. We determined whether *Apis mellifera* and *Bombus impatiens* would respond either to hetero- or conspecific haemolymph as an indication of a predation event, or to sting gland contents, which provide an alarm pheromone in honeybees and in many other social Hymenoptera. *Bombus impatiens* avoided their own haemolymph and *A. mellifera* haemolymph in foraging arena choice experiments. *Bombus impatiens* did not respond to *A. mellifera* alarm pheromone or to the odour of conspecific sting gland. In field experiments, *A. mellifera* avoided their own haemolymph and their own sting alarm pheromone, but did not avoid the haemolymph or sting gland contents of *B. impatiens* or native bumblebees (*Bombus vosnesenskii*) that regularly foraged around their hives. One factor behind the response of *B. impatiens* to heterospecific cues of predation may be its habit of solitary foraging, which may lead to more interactions with heterospecifics than would social foraging in which bees recruit nestmates to resources.

Information about predators is of vital importance to an animal's fitness and can be derived from an individual's own vigilance or from observing other individuals of the same or different species (Danchin et al. 2004; Caro 2005). Indeed, heterospecific-derived information can sometimes be as valuable as or more valuable than information from conspecifics (Seppänen et al. 2007), and the flow of information about predation between species may be an important factor organizing communities (Goodale et al. 2010). Perhaps the best studied network of communication about predation is that of aquatic fishes and amphibians, in which information about predators is obtained at many stages during the predator attack process. Prey species detect chemicals that come from the predator, other prey species detecting a predator, a predator attack, and even a predator ingesting prey (Wisenden & Stacey 2005; Ferrari et al. 2010). In this study, we investigated whether foraging social bees can obtain similar chemical information, specifically at the predator attack stage. Such information, by deterring floral visits, could have broader implications for plant pollination and fitness (e.g. Dukas 2005).

We have only learned relatively recently that social bees can respond to the risk of predation. Older work suggested that bees are not strongly influenced by sit-and-wait predators such as crab spiders (Morse 1979, 1986). However, more recent studies have shown that bees avoid flowers when there is visual or olfactory evidence of predators or dead conspecifics (Dukas 2001; Abbott 2006; Reader et al. 2006; Ings & Chittka 2008, 2009). Predation also affects recruitment behaviour at the nest. Honeybees reduce waggle dancing and recruit fewer nestmates to areas where there has recently been predation (Abbott & Dukas 2009). Honeybees also use a 'stop signal' that can be triggered by simulated predation attacks and that inhibits waggle dancing, thereby reducing recruitment to dangerous sites (Nieh 2010).

The study of heterospecific information flow among pollinators has also increased in the last decade. However, reports of heterospecific information use, thus far, remain limited to information about resource reward quality. For example, bumblebees can use the presence of honeybees as a cue of a good resource (Dawson & Chittka 2012). Bees also leave olfactory scent marks on flowers (Saleh et al. 2007; Wilms & Eltz 2008), cues which bees can learn to associate with reward or punishment (Saleh & Chittka 2006; Leadbeater & Chittka 2011), and which can be used by heterospecifics as well as conspecifics (Stout et al. 1998; Reader et al. 2005; Yokio et al. 2007). In addition, some species of stingless bees deposit chemical signals that guide nestmates to a rewarding

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food source and that can be intercepted by another species (Nieh et al. 2004; Lichtenberg et al. 2011). Thus, both signals and cues can be used by heterospecifics (Goodale et al. 2010).

We studied the reciprocal responses of *Apis mellifera* and *Bombus impatiens* to odours that are likely products of predator attack: bee haemolymph, midgut contents and sting gland contents. Haemolymph and midgut contents cannot be voluntarily released, but could be released upon damage by a predator or (for haemolymph) upon detachment of the honeybee sting, and thus may be reliable cues of the presence of a predator, analogous to epidermal cells released by fish predation (Ferrari et al. 2010). Sting gland is a source of alarm pheromone in many social Hymenoptera such as ants (Hölldobler & Wilson 1990), wasps (*Vespa*) (Moritz & Bürgin 1987; Bruschini & Cervo 2011) and honeybees (Blum 1969; Breed et al. 2004). Interestingly, bumblebee sting gland contents may not have alarm-pheromone-like properties (Maschwitz 1964, 1966); however, this has not been studied in detail or in the species that we used.

We assayed the response of foragers from laboratory colonies of *B. impatiens* to conspecific odours and odours derived from *A. mellifera*. Conversely, we also tested the responses of foragers from outdoor colonies of *A. mellifera* to conspecific odours and to those of two *Bombus* species, *Bombus impatiens*, which inhabits eastern North America, and *Bombus vosnesenskii*, a southern California native that forages at flowers also visited by our honeybee colonies. We hypothesized that social bees would respond to a heterospecific cue associated with predation, such as the odour of haemolymph, but would be less likely to respond to an alarm signal, such as that found in the sting gland of honeybees, because these alarm signals have evolved for a conspecific audience. However, because it is unknown whether the sting gland contents of bumblebees have an alarm pheromone effect, we had no a priori expectation of how bees would respond to these chemicals.

METHODS

Extractions

For any one type of extract, we ensured that the exemplars were made from bees of different colonies, except for extracts from *B. vosnesenskii*, which were made from wild bees collected at locations at least 500 m apart on the University of California San Diego (UCSD) campus, in La Jolla, CA, U.S.A.

Haemolymph was extracted using modified methods of Mayack & Naug (2010) to obtain haemolymph without puncturing internal organs and releasing glandular components (a possibility when using needle-extraction of haemolymph). Bees were freeze-killed and then the distil tips of their antennae were cut off. Cyanoacrylate adhesive was applied to mouthparts to avoid leakage of digestive tract contents. Bees were placed upside down in a filtered pipette tip placed inside a centrifuge tube, centrifuged at 8000 rpm for 1 min so that the haemolymph leaking from the antennae was gathered into the centrifuge tube. Tests showed that compounds with a molecular weight of at least 300 000 g/mole easily passed through this filter (high-range, protein ladder, run-through filter, separated and visualized using SDS-PAGE gel electrophoresis). We obtained an average $\pm$ SD of 5.0 ± 1.7 µl ($N = 10$) of haemolymph per *A. mellifera*, 14.0 ± 3.8 µl ($N = 10$) of haemolymph per *B. impatiens*, and 16.8 ± 2.8 µl ($N = 5$) of haemolymph per *B. vosnesenskii* (measured with a digital micropipette). After centrifugation, the bee was carefully removed and haemolymph was not used if there was any rupturing of the body or leakage from mouthparts. Pure haemolymph extracts were frozen at -70 °C until use and presented in the bioassay without mixing with water. In each assay trial, we used one bee-equivalent of haemolymph, comparing this with an equal volume of double-distilled water as a control.

We dissected the sting (poison) gland from *A. mellifera* ($N = 10$ bees), *B. impatiens* ($N = 10$ bees) and *B. vosnesenskii* ($N = 5$ bees) using a stereoscopic microscope to dissect freshly freeze-killed bees. To dissolve the fatty acid membrane of the sting gland, we placed the sting gland in hexane at room temperature (Nieh 2010). We used 20 µl of hexane (a standard bioassay gland solvent, Haynes & Millar 1998) to cover fully the sting gland in glass GC/MS vials with nonreactive septa caps (Agilent Technologies, Part No. 5182-0721, Santa Clara, CA, U.S.A.). In gland assays, we therefore used 20 µl of hexane as a control.

We tested the responses of foragers from two *B. impatiens* and two *A. mellifera* colonies to the contents of the ventriculus (midgut). To extract this fluid, we dissected out the ventriculus under a stereoscopic microscope, on a clean glass slide, cut it open, and harvested the interior fluid. We obtained an average $\pm$ SD of 21.2 ± 3.9 µl ($N = 4$) and 42.3 ± 13.1 µl ($N = 4$; measured with a digital micropipette) per *A. mellifera* and *B. impatiens*, respectively. Pure gut contents were used without addition of water during the bioassay. Controls consisted of an equal volume of double-distilled water.

General Assay Methods

We conducted paired feeder choice tests using visually identical feeders. Each feeder consisted of a large 9 cm diameter petri dish with a small 3.5 cm diameter dish placed inside and filled with 3 ml of unscented 2.0 M sucrose solution (enough to fill the petri dish approximately halfway with sucrose). A circle of filter paper 3.5 cm in diameter was slipped under the smaller, inner petri dish. At the beginning of the trial, the experimental treatment was dispensed on the filter paper of the experimental feeder, and the control substance (water or hexane, as appropriate) was placed on the filter paper of the other feeder. The haemolymph and sting gland extracts were largely clear, and thus, the experimental filter paper did not present a different colour from the control filter paper, nor did we detect differences in the UV reflectance of these substances from controls when viewed with UV photography (UV-Nikkor 105 mm f4.5 lens, Baader Venus filter transmitting 320–390 nm, and a Nikon SB-14 UV-E flash). We tested each extract type separately. We conducted only one trial per day. Each trial lasted 20 min, with feeder positions switched every 5 min to correct for potential site bias.

Bumblebee Responses

To study bumblebee responses to odours at food sources, we successively used five laboratory colonies trained to foraging arenas. Native California bumblebees are no longer commercially available, and thus, we sequentially worked with five laboratory colonies of *B. impatiens*. Colonies were ordered from Biobest, Inc. (Leamington, ON, Canada), and all individuals were marked with numbered bee tags (www.beeworks.com). The nest was contained in a $32 \times 29 \times 15$ cm wooden box, which was connected via a tube to a foraging arena of clear plastic, $54 \times 33 \times 26$ cm. Immediately before each trial, the arena was cleaned of odours with laboratory detergent and dried. Colonies were kept for 2 weeks before experiments began, and given 2.5 M unscented sucrose (65% w/w) ad libitum in 9 cm diameter petri dishes inside the foraging arena. Pollen obtained from honeybees was freshly ground and supplied inside the nestboxes.

In a *B. impatiens* trial, two clean feeders were placed side by side, 6 cm apart, in a clean foraging arena, approximately 1.5 m from the nest. Bees were allowed into the foraging arena one at a time, to

avoid potential social facilitation effects. A choice was defined as a bee that placed all six legs into the outer petri dish. Bees that made a choice were removed with an aspirator and kept in a holding tank, and only returned to the colony at the end of the trial. An average ± SD of 15.0 ± 8.5 bees chose per trial ($N = 20$ trials). We conducted trials in May–December of 2010 and January–March 2011.

Honeybee Responses

We consecutively used five *A. mellifera* colonies established in an outdoor apiary near the UCSD campus when testing extracts of *B. vosnesenskii* and another five colonies when testing extracts of *B. impatiens*. During an *A. mellifera* trial, bees were trained to feed at the same kind of artificial feeder used with *B. impatiens*, placed on top of a rectangular plastic platform (13 × 38 cm) mounted on a 1 m high tripod. Only honeybees from the focal colony were allowed to visit the feeder. We verified the colony identity of the foragers by marking bees with paint marks on their thoraces and observing their return to the nest. The tripod feeder was slowly moved away from the hive until it was 10 m away. We removed the feeder, cleaned the tripod with laboratory detergent and dried it. The test feeders were then set out 6 cm apart. *Apis mellifera* individuals were considered to have made a choice if they landed on the outer petri dish. Bees that made a choice were immediately removed with an aspirator and choices were only counted if they occurred in the absence of other bees. An average ± SD of 23.8 ± 13.6 bees chose per trial ($N = 30$ trials). We conducted trials during May–November 2010, June–July 2011 and January–March 2012.

Statistics

We first tested whether foragers would avoid the various types of bodily fluids. Foragers chose between two feeders, and we therefore calculated the two-tailed binomial probability ($p = q = 0.5$) to determine whether they showed a preference ($\alpha = 0.05$). For this analysis, all bee choices, including those from different colonies, were pooled together.

Neither *B. impatiens* nor *A. mellifera* showed any response to gut contents. We therefore focused our next analysis on whether a forager's decision (experimental or control feeder) was influenced by (1) treatment (haemolymph or sting), (2) source of treatment (*A. mellifera* or *B. impatiens*) and (3) colony. We ran a nominal logistic regression model using JMP 9.0.2 software (SAS Institute, Cary, NC, U.S.A.), after confirming that our data met parametric assumptions. Because we used the same data set for the second analysis, we chose a conservative route and applied the Bonferroni sequential correction ($k = 2$) on all tests, and only considered tests significant if they passed this correction (Zar 1999).

RESULTS

Bombus impatiens significantly avoided haemolymph from conspecifics (binomial test: $P = 0.0003$; Fig. 1) and from *A. mellifera* ($P = 0.0033$). *Bombus impatiens* showed no avoidance of sting gland extract from conspecifics ($P = 0.90$) or from *A. mellifera* ($P = 0.46$). *Bombus impatiens* also showed no avoidance of the gut contents from conspecifics ($P = 0.48$) or from *A. mellifera* ($P = 0.50$).

For *B. impatiens*, the species identity from which the scents were obtained did not significantly influence response (nominal logistic regression, effect likelihood ratio test: $\chi^2_1 = 0.34$, $P = 1.0$). However, avoidance was higher for haemolymph than for sting gland extract ($\chi^2_1 = 11.00$, $P < 0.002$). There was no significant colony effect ($\chi^2_4 = 9.22$, $P = 0.11$) and no significant interaction effect between species identity and extract type ($\chi^2_1 = 1.12$, $P = 0.56$).

Apis mellifera significantly avoided conspecific haemolymph (binomial test: $P = 0.0006$) and sting gland extract ($P < 0.0001$). However, *A. mellifera* did not avoid extracts obtained from either bumblebee species. Honeybees did not avoid the haemolymph ($P = 0.91$) or sting gland extract ($P = 0.13$) of *B. impatiens*, or the haemolymph ($P = 1.0$) or sting gland extract ($P = 0.34$) of *B. vosnesenskii*. Likewise, honeybees did not avoid the gut contents of conspecifics ($N = 46$, $P = 0.88$) or of *B. impatiens* ($N = 58$, $P = 0.51$).

For *A. mellifera*, the source (conspecific versus heterospecific) of the olfactory information was crucial (nominal logistic regression, effect likelihood ratio test: $\chi^2_2 = 25.77$, $P < 0.0001$), because the bees only avoided conspecific odours (haemolymph and sting extract). Both conspecific sting gland extract and haemolymph were equally repulsive to *A. mellifera* ($\chi^2_1 = 1.03$, $P = 0.62$). There was no colony effect ($\chi^2_9 = 12.98$, $P = 0.22$) and no significant interaction between species identity and extract type ($\chi^2_2 = 2.63$, $P = 0.53$).

DISCUSSION

Here we demonstrate that two species of bees will avoid conspecific haemolymph, a likely cue of predation. In addition, *B. impatiens* avoided the haemolymph of *A. mellifera*, although the reciprocal response was not found. To our knowledge this is the first finding of the use by bees of information about predation derived from a heterospecific. This response was innate (*B. impatiens* had no experience with *A. mellifera*) and is particularly interesting because the two species, although related (both members of the family Apidae) have not extensively coevolved, since *A. mellifera* is not native to North America. Below, we discuss why *B. impatiens* may have responded to heterospecific haemolymph while *A. mellifera* did not. We also examine the implications for the lack of response by *B. impatiens* to the sting gland contents of *A. mellifera*, and for the lack of both species' responses to sting gland contents of *B. impatiens*. First, however, we summarize what is known about predation of bees on flowers and what fluids might be released in a predatory attack.

Bees experience predation from sit-and-wait ('ambush') predators on flowers. Crab spiders (family Thomisidae) are frequent attackers of bees as large as bumblebees, although they are often not successful (Morse 1979, 1986); larger ambush spiders such as lynx spiders (family Oxyopidae) may regularly include bees in their diet (Nyffeler et al. 1994). Other important ambush predators include assassin bugs (family Reduviidae, Greco & Kevan 1995) and mantids (order Mantodea, Caron 1990). Predator interactions are clearly important enough that honeybees will avoid flowers with spiders or dead bees, or on which they have been attacked (Dukas 2001; Reader et al. 2006), and decrease their recruitment to dangerous flowers (Abbott & Dukas 2009; Nieh 2010). Bumblebees also avoid flowers where there are dead bees or odours of dead bees (Abbott 2006), or where they have been attacked (Ings & Chittka 2008, 2009).

What cues or signals may be associated with a predatory attack on bees? Fluids from bees may be left on flowers where ambush predators have attacked (Abbott 2006). Any wound might release haemolymph, the fluid that flows in the open circulatory system of bees (Dade 1994). Indeed, haemolymph is important in wound healing in insects (Theopold et al. 2004). Haemolymph consists of both the haemocytes, the blood cells, and a fluid plasma, which is a complex mixture of water, ions and proteins that functions to maintain the body tissues (Chapman 1998). In addition, a deep cut to the abdomen of a bee can lead to the release of the contents of

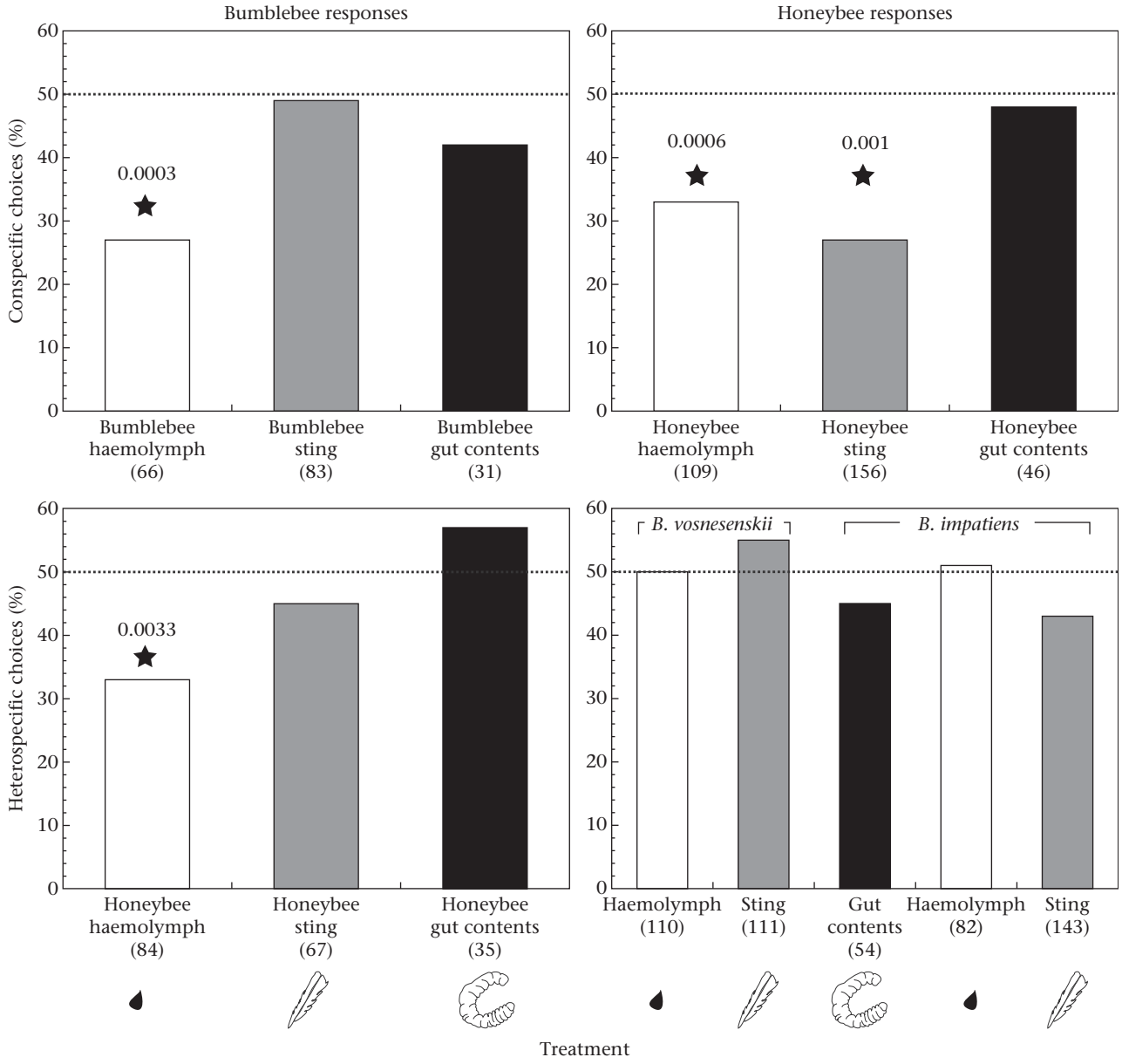

Figure 1. Percentages of bumblebee and honeybee foragers that chose the experimental feeder over the control feeder (numbers of individuals are given in parentheses). A level of 50% indicates no choice (dashed lines). Results that differed significantly from 50% are indicated by a star (two-tailed binomial probability is shown above the bar). A significant low percentage corresponds to avoidance of the experimental compound (haemolymph, sting gland extract or gut contents).

the midgut (ventriculus), which composes much of the mass of the abdomen (Dade 1994). Finally, if bees attempt to sting the predator, they will release the contents of the sting gland, which include a variety of venoms (Schmidt 1982) and pheromones (Breed et al. 2004). Observations by colleagues and ourselves indicate that both haemolymph and sting gland contents may be released in attacks by ambush predators. For example, observations of mantids have shown that smaller individuals pull off limbs of bees, whereas larger ones rip apart the exoskeleton, usually near the thorax, with their sharp mandibles, releasing haemolymph (A. Bray, personal communication). In observations of spiders attacking bees at feeders, bees occasionally attempt to sting spiders and sometimes leave their sting apparatus inside the predator when they fly away, releasing both sting alarm pheromone and haemolymph (T. Jack & J. C. Nieh, personal observation).

We hypothesized that alarm pheromone, a chemical signal evolved to elicit colony defences, would elicit conspecific, not heterospecific, responses. We found evidence for this hypothesis. *Apis mellifera* showed aversion to conspecific sting pheromone, but *B. impatiens* did not respond to *A. mellifera's* sting pheromone. In contrast, we hypothesized that haemolymph, an olfactory cue, would elicit heterospecific and conspecific aversion. This hypothesis was supported by *B. impatiens*, which responded to heterospecific haemolymph. However, *A. mellifera* did not respond to heterospecific haemolymph (or to heterospecific sting gland content, which may lack alarm-pheromone-like properties, see below). *Apis mellifera* also showed no response to odours of co-occurring native bumblebees (*B. vosnesenskii*) at our field site.

What potential factors may explain the response to heterospecific haemolymph by *B. impatiens* and not by *A. mellifera*? First, there might be some cue of predation that we did not include in our

presentation, for example, material in the fat body, to which both species would respond symmetrically. However, both species could detect and avoid conspecific haemolymph. Second, *A. mellifera* could be physiologically incapable of detecting heterospecific haemolymph. We find this unlikely because honeybees are olfactory generalists and a substantial body of research demonstrates that they can detect a wide variety of volatile compounds (Sandoz 2011). Third, there could be historical and biogeographical reasons for our result. The haemolymph of *A. mellifera* could smell to *B. impatiens* like the haemolymph of a native North American bee. While this could be true, if the odour of bee haemolymph is phylogenetically conserved, we would have expected the reverse pattern, as *Bombus* species naturally occur in the range of *A. mellifera*, whereas *B. impatiens* did not encounter *Apis* species until relatively recently.

Finally, heterospecific information could be less valuable for *A. mellifera* than for *B. impatiens*. Honeybees are group foragers that can encounter olfactory information produced by nestmates at the same foraging patch and, in general, get a large proportion of their foraging information from nestmates (Seeley 1995), not heterospecifics. However, bumblebees are solitary foragers (Dornhaus & Chittka 2004), and thus, they may be more likely to encounter honeybees than other bumblebees while foraging, especially since honeybees are generally more abundant than native bumblebees (in both nests per square metre and in individuals per nest, Goulson 2003).

Neither *B. impatiens* nor *A. mellifera* responded to the odour of bumblebee (*B. impatiens* and *B. vosnesenskii*) sting gland contents. It is possible that some component of sting pheromone in bumblebees is only activated by stinging itself, although the chemical mechanism of such activation has not, to our knowledge, been reported. This result supports the preliminary reports by Maschwitz (1964, 1966) that sting gland extract does not elicit alarm in *Bombus lucorum*, *Bombus hypnorum* or *Bombus hortorum*. Similarly, Dukas (2005, page 1405) observed (primarily in *B. rufocinctus*) that 'bumblebees struggling with beewolves released large amounts of alarm pheromone, which I could readily smell. This, however, did not result in fleeing of the other bumblebees from the patch'. The lack of an alarm effect by bumblebee sting glands is surprising because the sting glands of many other social Hymenoptera have alarm-pheromone-like effects (Blum 1969; Moritz & Bürgin 1987; Hölldobler & Wilson 1990; Bruschini & Cervo 2011). Bumblebees are highly sensitive to vibrational disturbances near their nest, and this perhaps may be the primary mode for alarm activation (Kirchner & Röschard 1999).

Neither species responded to midgut contents. It is possible, and would be interesting for future researchers, to examine aversion to hindgut contents. Bumblebees and honeybees sometimes expel hindgut contents in aggressive or defensive contexts (Bernasconi et al. 2000; Tarpy & Fletcher 2003). We used midgut contents because of the larger volume of fluid contained in this area that might be released during wounds inflicted by a predator.

The discovery of *B. impatiens*' aversion to *A. mellifera* haemolymph is analogous to the well-established finding that aquatic prey species are able to recognize the odours that other prey species may release upon being injured by a predator (Wisenden & Stacey 2005; Ferrari et al. 2010). It is possible that *B. impatiens* were not able to distinguish between their own and *A. mellifera* haemolymph, given their relatively close relatedness within the Apidae (Cardinal et al. 2010). For example, fish respond to semiochemicals released by injured heterospecifics that are closely related (Mirza & Chivers 2001). Extending the analogy of the aquatic system (Ferrari et al. 2010), it would be interesting to determine whether bees can detect predator odours (kairomones), or whether they are averse to the faeces of predators that have recently fed on conspecific or heterospecific bees.

To our knowledge, this is the first report of the use of heterospecific 'cues' of predation in which the relationship is evidently asymmetrical. Asymmetry in the sharing of information about predation between species has been demonstrated, although it usually occurs because one species is more inclined to produce information in the form of a signal than is the other species (Goodale et al. 2010). For example, downy woodpeckers, *Picoides pubescens*, eavesdrop on heterospecific alarm calls of black-capped chickadees, *Parus atricapillus* (Sullivan 1984). In the case of an alarm cue, the production of the cue is usually not under the control of the animal that produces it. Asymmetry might still develop, however, because of some characteristic of the heterospecific receivers (Goodale et al. 2010). Here we argue that differences in the value or likelihood of encountering such information might underlie the response differences between *A. mellifera* and *B. impatiens*. Such asymmetrical use of cues, not signals, by heterospecifics may be more common and deserves further study in a variety of animal systems.

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