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Giant Asian honeybees use olfactory eavesdropping to detect and avoid ant predators

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POLLINATORS provide a key ecosystem service that can be influenced by predation and predator avoidance. However, it was unclear whether pollinators can avoid predators by eavesdropping, intercepting predator signals. Using a natural species assemblage, we show that a bee can eavesdrop on and avoid the trail pheromone of a sympatric ant, while foraging on a native plant. The giant Asian honeybee, Apis dorsata, avoided Calliandra haematocephala inflorescences with live weaver ants, Oecophylla smaragdina. Although few foraging bees were attacked, ants killed the bee in almost a third of attacks. Ant presence alone significantly reduced bee floral visits. Bees showed nearly equal avoidance of live ants and trail pheromone extracts, demonstrating that olfactory eavesdropping alone can elicit full avoidance. We then used GC-MS to analyse compounds deposited by ants walking and laying trail pheromone. The most abundant compounds were all trail pheromone components. However, bees did not avoid the most abundant and conspicuous trail pheromone component, heneicosane. Foragers may instead detect a mixture of different trail pheromone compounds. Our results contribute to a growing understanding of how public information about predators and competitors can shape food webs, and show that pollinators can tap into the private signals of predators and use this information to their advantage.

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workers create a sticky barrier that effectively isolates their nests from *O. smaragdina*, reinforcing this barrier upon detecting a weaver ant, but not after detecting another arboreal ant species (Duangphakdee, Koeniger, Koeniger, Wongsrirun, & Deownanish, 2005). Asian honeybees may therefore have evolved another defence, olfactory eavesdropping. Our goals were therefore to (1) determine whether ant presence (ant visual and olfactory stimuli) on an inflorescence could repel *A. dorsata* foragers, (2) test whether *A. dorsata* can use olfactory eavesdropping to avoid this ant’s trail pheromone, and (3) chemically analyse *O. smaragdina* trail pheromone and test bee eavesdropping on the trail pheromone’s most abundant chemical component.

**METHODS**

**Field Observations**

This research was conducted in full compliance with the laws of the People’s Republic of China. No specific permits were required for our field studies, which were conducted at Xishuangbanna Tropical Botanical Garden (XTBG), Chinese Academy of Sciences. Our study species: *A. dorsata* (bees), *O. smaragdina* (ants) and *C. haematocephala* (plant) are not endangered.

The field experiments were conducted from February 2013 to April 2014, during the blooming season of *C. haematocephala*, a species that we chose because *O. smaragdina* preys upon *A. dorsata* foraging on *C. haematocephala* inflorescences (Chen & Li, 2012). These shrubs were abundant at our field site and often contained weaver ants, which we observed attacking and killing *A. dorsata* foragers. Each inflorescence of *C. haematocephala* is globose and consists of an average of 40 flowers whose numerous long slender stamens (approximately 25 per flower) create the ‘powder puff’ appearance (Fig. 1a) that gives this plant one of its common names (Neveling & Elias, 1971). These inflorescences attracted *A. dorsata* and *O. smaragdina*. At our site, weaver ants were fairly common (we found 52 colonies at XTBG), and we observed them foraging for nectar, attacking and capturing *A. dorsata* (Fig. 1). On these inflorescences, we observed ants exhibiting typical trail pheromone deposition behaviour: dragging their abdomens and depositing small visible trail pheromone spots (Offenberg, 2007).

We created two patches (each 3 × 10 m), one with ants and one that was ant-free. Each patch contained 10 small trees that were approximately 3 m tall. None of these trees contained any ants, based upon thorough visual inspections. The patches were separated by 5 m, and trees from one patch did not have branches that touched trees from the other patch. In the ant-treated patch, we physically connected the branches of each pair of trees and released five queenright colonies of *O. smaragdina*, collected from mango and pomelo trees in the nearby botanical garden, one colony per tree pair. We waited 1 week after introducing the ants for their colonies to become established and then began the experiments. No ants were added to the ant-free patch, and we applied rings of sticky Tanglefoot resin around the trunks and branches to keep ants off of these trees. During our experiments, we continued to meticulously inspect the ant-free trees and confirmed that they were ant free. The *A. dorsata* foragers probably came from approximately 40 colonies located about 1 km away from our study site. We could not determine precisely how many different colonies came to our inflorescences because we used naturally foraging bees. However, we conducted our study over 15 months and used 20 different trees. We therefore probably used bees from multiple colonies.
However, our foragers did not avoid in coming to collect nectar from one in odour. In addition, because we did not detect the characteristic alarm pheromone counted these C pheromone left at choice (landed on an in aspirators to carefully capture all bees as soon as they made a A. dorsata began producing nectar, we removed the mesh and counted the (landed on an) ants attacking a bee while it visited the inflorescence. A successful attack was one in which ants killed the bee. We also counted the ants on inflorescences and within attack distance of bees. Based upon our attack observations, ants within a 2 cm length of the stem at the base of the inflorescence could easily attack bees. We therefore also counted these ‘stem’ ants. For these counts, we selected 30 different inflorescences, six from each of five trees with ants.

Testing Bee Avoidance of Ants

To obtain our test inflorescences, we randomly selected five trees in each patch and used a fine nylon mesh that excluded bees and ants to enclose one randomly selected immature inflorescence (not producing nectar) per tree. Two days later, when the flowers began producing nectar, we removed the mesh and counted the A. dorsata foragers visiting this inflorescence for 5 min. We used aspirators to carefully capture all bees as soon as they made a choice (landed on an inflorescence), and therefore recorded each bee’s choice only once. Aspiration did not release alarm pheromone because we did not detect the characteristic alarm pheromone odour. In addition, A. dorsata foragers will avoid A. dorsata alarm pheromone left at C. haematocephala flowers (Chen & Li, 2012). However, our foragers did not avoid inflorescences at which the most bees were captured (see Results, Fig. 2a, b). Captured bees were subsequently frozen to eliminate potential pseudoreplication.

Because of the large number of ants on the ant-treated trees, ants began visiting inflorescences in the ant-present patch almost as soon as we removed the bag. On ant-trees, each of our focal inflorescences contained an average of five ants. There were no ants on the ant-free trees. We only made observations on sunny days from 1100 to 1430 hours. We repeated these observations five times, conducting 25 ant-free and 25 ant-present trials. Because we immediately removed landing bees, they were not attacked by ants on our focal inflorescences during our trials and thus did not deposit alarm pheromone or other bee odours associated with predation that could have influenced bee choices.

Observing Ants Attacking Honeybees

For 30 days (from 1100 to 1430 hours each day, a total of 1050 h) observers watched inflorescences on five different trees with weaver ants and counted the (1) bee visits, (2) bees attacked by ants and (3) bees successfully killed by ants. We defined a bee visit as one bee coming to collect nectar from one inflorescence. An attack consisted of ants attacking a bee while it visited the inflorescence. A successful attack was one in which ants killed the bee. We also counted the ants on inflorescences and within attack distance of bees. Based upon our attack observations, ants within a 2 cm length of the stem at the base of the inflorescence could easily attack bees. We therefore also counted these ‘stem’ ants. For these counts, we selected 30 different inflorescences, six from each of five trees with ants.

Testing Bee Eavesdropping on Ant Trail Pheromone

To determine whether bee foragers could eavesdrop on ant trail pheromones, we used paired-choice assays to test forager responses to (1) natural odour extracts and (2) synthetic heneicosane, the most abundant compound that we identified in the ant odour trail pheromone (Table 1). In this paired-choice assay, one inflorescence was the control treatment and the other was the experimental treatment.

We collected the trail pheromones from six different ant colonies by cutting off the branches holding the queenright nest of each colony. This nest was then suspended by a clean, 20 cm long, thin, Teflon-coated, metal wire (modified from Choe, Villafuerte, & Tsutsui, 2012) from the tree originally occupied by the ant colony. Ants soon began to move up the wire back into their home tree and deposited trail pheromone by visibly dragging their abdomen. They also left small spots that characterize odour trail marking by this species (Offenberg, 2007). We allowed approximately 30 ants to walk along the wires and deposit trail pheromone and odours naturally associated with pheromone deposition, such as CH cues deposited by walking, for 10 min. We then gently removed any remaining ants with soft forceps and washed each wire with 300 μl of hexane into a clean gas chromatography vial (one vial per wire). We slowly reduced the volume of this extract to 200 μl with a N2 stream, sealed the vials, and set them aside in the freezer at −20 °C for later use.

For the assay, we randomly selected two immature inflorescences that were not yet providing nectar on an ant-free tree and bagged them to exclude bees and allow nectar to build up. Two days later, when the flowers had fully bloomed and offered nectar, we cut off two inflorescences and placed each on a 1 m high tripod 40 cm from the tree. The tripods were 40 cm apart. This placement allowed us to give bees a choice between a control and a treatment inflorescence, a classic paired-choice test, which would not have been possible on a tree on which multiple inflorescences surrounded the control and treatment inflorescences.

With a micropipette, we added 20 μl of the ant pheromone (equivalent to the trail pheromone produced by 30 ants, 1/10th of extract) at ambient air temperature onto the experimental inflorescence and 20 μl of pure hexane to the control inflorescence. To avoid visually altering the inflorescence’s appearance, we added the treatments deep inside its thick ball of stamens.
We chose an extract corresponding to 30 ants because this is the average number of ants that we found within the attack distance of a forager on an inflorescence (see Results). We waited 2 min to allow the volatile hexane to evaporate and then counted bees landing on the experimental and control inflorescences over 10 min. We used hexane because it is an excellent solvent commonly employed in olfactory bioassays (Millar & Haynes, 1998). Hexane also rapidly evaporates. At the ambient air temperatures of our trials (>30°C), hexane quickly dissipated because it has a vapour pressure of >187.11 mmHg, nearly six times greater than the vapour pressure of water under the same conditions (Beyer, 1988). In addition, honeybees (A. mellifera) are not disturbed by hexane in foraging choice assays (Goodale & Nieh, 2012). We only recorded bees that made choices when no other bees were on the inflorescences to avoid potential local enhancement, since bees can be attracted or repelled by the presence of other bees. We performed 20 trials, using inflorescences from 10 different trees. Each pair of inflorescences was used for only one trial.

To test the effect of heneicosane, we followed the same methods as above, and applied 86.6 ng of heneicosane in 20 ml of hexane (equivalent to the amount in trail pheromone from 30 ants, Table 1).

### Testing the Potential Effect of Bee Cuticular Hydrocarbons

Although we immediately captured bees with aspirators as soon as they landed, it is possible that they deposited a small amount of CH cues when they made contact with the inflorescence. CH cues could have influenced subsequent forager choices. To test this possibility, we placed an inflorescence, obtained as described above, on a 1 m high tripod, 40 cm from its tree. For the control treatment (no CH), each bee approached the inflorescence but was captured with an aspirator before it could land. For the experimental treatment (with CH), a bee was allowed to land on the inflorescence, walk on it, and feed for 20 s before it was captured. We chose 20 s because this is longer than the average total contact time by bees on an inflorescence during a 10 min paired-choice trial (see above). Over the next 10 min, we then counted the bees that landed on the inflorescence and captured each bee so that it would not be recounted. These subsequent bees could have left CH cues, but the experimental trials would still have had a far greater amount of CH cues than the control trials. We ran 25 experimental trials and 25 control trials, using inflorescences from five ant-free trees.

### Chemical Analysis and Bioassay

To identify the pheromone components, we separately analysed one trail pheromone sample from each of six different ant colonies. Each sample contained the trail pheromone of approximately 30 ants (total of six samples corresponding to 180 ants) and was
collected as described above. We also obtained six separate control samples. To obtain each control, we placed a clean, 20 cm long, thin, Teflon-coated, metal wire on a tree without ants, waited for 10 min, and then washed the control wire with hexane as described above. For all extracts, we gently reduced the volume to 50 μl with an N2 stream, and added octane as an internal standard for gas chromatography retention times and mass spectrometry spectra with those of the authentic compounds, and matching mass spectra.

Statistics

We used chi-square goodness-of-fit tests and performed calculations with Microsoft Excel v14.4.3.

RESULTS

Bee Avoidance of Ants and Trail Pheromone

Weaver ants hunted A. dorsata foragers on inflorescences. On average, 5.3 ± 1.1 ants waited, largely hidden, in an inflorescence (Fig. 1a) with another 24.9 ± 1.8 ants on the stem at the base of the inflorescence. A total of 30.2 ± 2.0 ants were therefore in close range of a bee foraging at an inflorescence. Ants attacked by biting the bee on its body and appendages. Although the bees fought back, biting and attempting to sting the ants, it was usually difficult for the bee to insert its stinger into the ants and to fend off the multiple ant attackers. As soon as the ants began attacking a bee forager, nearby ants joined the attack (Fig. 1b). Of 980 bee foraging visits to an inflorescence, 3.3% of foragers (N = 32) were attacked by ants, and 1% of foragers (N = 10) were killed by ants. Thus, attack by ants had a 31.2% chance of successfully killing a bee (Fig. 1c), although significantly more attacks were unsuccessful (X² = 4.50, P = 0.03, N = 32 attacked bees).

Bee foragers strongly avoided live ants on an inflorescence: 60% of bees landed on the inflorescence without ants (X² = 40.20, P < 0.0001, N = 661 bees; Fig. 2a). When we tested the effects of ant odour trail extracts alone, a similar majority (63%) of bees chose to land on the inflorescence without ant pheromone (X² = 24.78, P < 0.0001, N = 349 bees; Fig. 2b).

Our control showed no CH effect on bee visits. Over all trials, 79 bees landed on the control inflorescence and 81 on the inflorescence with CH cues (X² = 0.06, P = 0.81, N = 160 bees).

Ant Trail Pheromone Analysis and Bioassay

GC–MS analyses revealed the presence of several compounds (Table 1, Fig. 3) that have all been previously identified (Table 2) as compounds found in the Dufour gland of O. smaragdina, a gland that produces trail pheromone. None of these compounds were present on control wires. We identified three hydrocarbons (heneicosane, docosane and tricosane) that may be present in worker ant cuticles but that are known to be present in significant quantities in the Dufour gland. All other compounds, including potential CH cues, were present only at trace levels (Fig. 3). The most abundant components were heneicosane (40.6% of the average sample) and tricosane (30.1%). However, bees were not repulsed by heneicosane; 53% landed on the inflorescence with heneicosane and 47% on the hexane control (N = 179 bees; X² = 0.45, P = 0.50; Fig. 2c).

DISCUSSION

We present the strongest evidence, to date, that a bee can eavesdrop on and thereby avoid the trail pheromone of a sympatric predatory ant in a natural setting while collecting nectar from a native plant species. Bees showed equal avoidance of live ants and trail pheromone extracts, suggesting that olfactory eavesdropping alone is sufficient to elicit full avoidance. We also provide the first data on the attack and success rates of O. smaragdina ants preying upon foraging A. dorsata foragers. The weaver ants exhibited behaviour typical of how they attack, kill and dispatch large prey items (Hölldobler, 1983). The ant attack rates that we observed (3.3% of bee foragers were attacked) are similar to those reported by other studies examining crab spider predation on bees (4–11%, Dukas & Morse, 2003; Morse, 1986). Although such attack rates...
may seem low, they can affect floral visits and pollination (Dukas, 2005). Moreover, the mere presence of predators on flowers reduces visits by insect pollinators by 36% (Romero et al., 2011). Thus, O. smaragdina probably reduces pollinator visits. The 31.2% success rate of the weaver ants is much higher than that reported for spiders (9.4–10.8%, Dukas & Morse, 2003; Morse, 1986), probably because multiple ants typically attacked a single bee forager (Fig. 1). This 2.9–3.3 fold higher rate of successful weaver ant predation should provide a strong impetus for bees to recognize and avoid weaver ants.

Bees alarm pheromone probably did not play a role in our results. No bees were attacked on the focal inflorescence during our observations. These inflorescences had been previously bagged before they bloomed and produced nectar. As such, they did not attract bee foragers and had no bee odours (CH or alarm pheromone) prior to use. In addition, our use of aspiators to capture bee foragers evidently did not release alarm pheromones that altered the overall trend. We captured the most bees on the control inflorescence, which was, none the less, more attractive than the inflorescence with live ants or ant odour trail (Fig. 2a, b).

Thus, bee foragers probably responded only to the odours and visual presence of the ants. However, given that ants tended to hide at the base of an inflorescence (Fig. 1b), bees probably detected ant presence by smell rather than by sight. In fact, live ants (Fig. 2a) and ant odour trail extract (Fig. 2b) elicited very similar levels of aversion: 60% and 63%, respectively, landed on control inflorescences. Exposure to ant odour trails repulsed bee foragers just as much as live ants.

We obtained O. smaragdina trail pheromone that ants had deposited on a clean substrate. Our extracts probably included CH cues and compounds from rectal glands, Dufour glands and ant anal sacs. From these extracts, we identified heneicosane (40.6%) and tricosane (30.1%) as the two main components. Keegans, Billen, and David Morgan (1991) used a different technique, dissecting out the Dufour glands of ants (a source of O. smaragdina trail pheromone), and identified heneicosane (13.8%) and undecane (41.4%) as major components. Unlike Keegans et al. (1991), we did not identify undecane in our sample, but this may arise from methodological differences.

We did not exhibit any significant avoidance of heneicosane (Fig. 2c), although we used the quantity (86.6 ng) that in natural trail pheromone elicited bee aversion (Fig. 2b). Bee foragers may therefore be eavesdropping on a different compound or a blend of compounds in weaver ant trail pheromone. If bees recognize this pheromone by learning a multicomponent olfactory blend (Reinhard et al., 2010), multiple compounds in the right proportions will be necessary to trigger recognition and avoidance. A future study examining the efficacy of the 10 individual compounds that we identified and a synthetic blend (Table 1) should elucidate whether A. dorsata avoidance is elicited by eavesdropping on a single compound or multiple compounds. Our results with heneicosane provide preliminary data for such a study.

Bees probably eavesdropped on the odour trail pheromone and not CH cues deposited by the tarsi or other body parts of walking ants. Our method collected all compounds associated with trail pheromone deposition, including CH. However, we did not detect these CH cues in our GC–MS analysis (Table 1, Fig. 3) because the chemical signal, trail pheromone, was far more abundant. Heneicosane, docosane and tricosane are CH cues found on ants (Elmes, Akino, Thomas, Clarke, & Knapp, 2002; Errard, Hefetz, & Jaisson, 2006) but also occur in the Dufour gland, a major source of O. smaragdina trail pheromone (Keegans et al., 1991). All unidentified peaks in our analyses for compounds heavier than nonane (see Fig. 3) were smaller than the peak for nonanal (4.1%, Table 1), a compound 10 fold less abundant than heneicosane. Any CH cues would have been present at even lower levels (see peaks in Fig. 3). Although honeybees have excellent olfaction and can detect low concentrations of many compounds, they most easily detect, process and learn the most abundant odour components (Reinhard et al., 2010). Thus, if bees learned to associate trail pheromone odours with weaver ants, they are unlikely to have learned CH cues over the much more abundant trail pheromone compounds. If bees evolved to innately recognize ant trail pheromone, they should likewise detect the most conspicuous signals that reliably indicate predator presence, trail pheromone. To be conspicuous, signals should stand out against the environment. None of the compounds that we identified (Table 1) were found on any of the control wires placed on ant-free trees. Thus, these compounds (Table 1) were only associated with ant odour trails and were conspicuously different from background odours in the environment. It is unclear whether A. dorsata has an innate or a learned avoidance of O. smaragdina pheromone. However, O. smaragdina was common at
our field site and abundant on the inflorescences of *C. haematocephala* with ant colonies. Thus, bee foragers could have learned to associate odour trail odours with the threat of ant predation.

Public information about predators therefore contributes to an information web that helps shape food and interaction webs (Schmidt, Dall, & van Gils, 2010). Our results support the major role of olfaction in the ecology of information, influencing how pollinators obtain food and how plants are pollinated. Moreover, other components of this food web, such as herbivores, can use public information about ant presence. The herbivorous beetle *R. wallacei* can detect and avoid *O. smaragdina* pheromone (Offenberg et al., 2004). Herbivores are also repelled by *Azteca* and *Camponotus* ant odours (Gonthier, 2012). Fruit flies are repelled from ovipositing on mangos bearing odours deposited by the African weaver ant, *Oecophylla longinoda* (van Mele, Vayssieres, Adamou, & Sinszogan, 2005). In thinking about pollination, we may therefore contemplate a broader olfactory landscape that includes detection of predators, all of this information weighing in to influence floral visits and, ultimately, pollinator and plant fitness.

In addition, it is interesting to consider how eavesdropping may shape the evolution of weed ant trail pheromones. To disguise their pheromones, flies such eavesdropping, ants could evolve pheromones with larger, less volatile compounds that are mainly detected through direct contact. However, bees seem very adept at detecting larger compounds, such as those contained in cuticular hydrocarbon ‘footprints’, from a distance (Goulson et al., 2000; Witjes & Eltz, 2009; Yokoi & Fujisaki, 2008). Future studies examining the extent to which bees can detect heavier compounds from a distance would be useful for understanding bee detection of such public information. A comparative analysis of odour trail pheromones from ant species that do and do not prey upon bee foragers would also be illuminating. We expect predatory species to face stronger selective pressures to make their pheromones less conspicuous.

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