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### Olfactory eavesdropping of predator alarm pheromone by sympatric but not allopatric prey



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Eavesdropping is predicted to evolve between sympatric, but not allopatric, predator and prey. The evolutionary arms race between Asian honey bees and their hornet predators has led to a remarkable defence, heat balling, which suffocates hornets with heat and carbon dioxide. We show that the sympatric Asian species, Apis cerana (Ac), formed heat balls in response to Ac and hornet (Vespa velutina) alarm pheromones, demonstrating eavesdropping. The allopatric species, Apis mellifera (Am), only weakly responded to a live hornet and Am alarm pheromone, but not to hornet alarm pheromone. We observed typical hornet alarm pheromone-releasing behaviour, hornet sting extension, when guard bees initially attacked. Once heat balls were formed, guards released honey bee sting alarm pheromones: isopentyl acetate, octyl acetate, (E)-2-decen-1-yl acetate and benzyl acetate. Only Ac heat balled in response to realistic bee alarm pheromone component levels (<1 bee-equivalent, 1  $\mu$ g) of isopentyl acetate. Detailed eavesdropping experiments showed that Ac, but not Am, formed heat balls in response to a synthetic blend of hornet alarm pheromone. Only Ac antennae showed strong, consistent responses to hornet alarm pheromone compounds and venom volatiles. These data provide the first evidence that the sympatric Ac, but not the allopatric Am, can eavesdrop upon hornet alarm pheromone and uses this information, in addition to bee alarm pheromone, to heat ball hornets. Evolution has likely given Ac this eavesdropping ability, an adaptation that the allopatric Am does not possess.

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Olfactory eavesdropping occurs when an unintended recipient exploits a signal to its own advantage, and it can influence animal community structure (Goodale, Beauchamp, Magrath, Nieh, & Ruxton, 2010). Eavesdropping can be detrimental, neutral or beneficial to the signaller (Lichtenberg, Zivin, Hrncir, & Nieh, 2014). Thus, the definition of a kairomone intersects with the concept of olfactory eavesdropping because a kairomone is a chemical signal or cue that is detected by an unintended receiver to the consistent detriment of the emitter (Ruther, Meiners, & Steidle, 2002; Wyatt, 2014). Mammalian prey can avoid predators by using predator

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chemical signals such as kairomones (Garvey, Glen, & Pech, 2016; Jones et al., 2016). Many parasitoids use kairomones, exploiting host chemical signals (Louapre & Pierre, 2014), sometimes in surprisingly complex ways (Elgar, Nash, & Pierce, 2016). In honey bees, cell-capping pheromone is attractive to the parasite Varroa jacobsoni (Trouiller, Arnold, Chappe, Le Conte, & Masson, 1992). The stingless bee, Tetragonisca angustula, has defensive responses that are triggered by the raiding pheromone of robber bees, Lestrimelitta limao (Kärcher & Ratnieks, 2015; Wittmann, Radtke, Zeil, Lübke, & Francke, 1990).

Honey bees can eavesdrop upon the alarm pheromones produced by foragers of other bee species, resulting in a predator avoidance benefit for the overall pollinator assemblage (Li, Wang, Tan, Qu, & Nieh, 2014b; Wang et al., 2016; Wen et al., 2017). However, predators have evolved other strategies. The European beewolf, Philanthus triangulum, a sphecid wasp, preys upon bees, which they may locate based upon honey bee olfactory signals (Schmitt, Herzner, Weckerle, Schreier, & Strohm, 2007). Vespa velutina hornets are attracted to geraniol, a component of

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honey bee aggregation pheromone, and use it to find and attack nests (Couto, Monceau, Bonnard, Thiéry, & Sandoz, 2014).

It was not clear, however, whether honey bees can use olfactory eavesdropping to detect hornet pheromones. Vespa velutina produces venom gland volatiles that are an alarm pheromone: these volatiles strongly attract hornet nestmates near hornet nests and elicit nest defence (Cheng, Wen, Dong, Tan, & Nieh, 2017). However, given that this alarm pheromone is produced both defensively and during hornet attacks, it could also serve as a kairomone for prey that have evolved with these hornets. Since conspicuous signals are often used in competition and defence (Bradbury & Vehrencamp, 2011), bees may detect the most abundant components in hornet alarm pheromone to mount a stronger defence. Apis cerana cerana (Ac) is sympatric with V. velutina throughout the hornet's entire range in China (Akre, 1978). The introduction of the allopatric European honey bee *Apis mellifera ligustica* (Am) throughout large areas in China therefore provides an opportunity to test this evolutionary hypothesis.

*Vespa velutina* hunt for bees on flowers (Tan et al., 2007), but most commonly attack bee nests, where they can devastate weak colonies by killing up 20–30% of Ac workers and even higher percentages of Am workers (Tan et al., 2005). These hornets can also 'hawk' and capture flying foragers at bee nest entrances (Tan et al., 2007). Hawking *V. velutina* have a three-fold higher rate of capturing Am as compared to Ac (Tan et al., 2007). Because Am has no strong defences against *V. velutina* (Arca et al., 2014), this hornet has caused severe problems in areas of Europe where it has invaded, leading some beekeepers to abandon apiculture (Villemant et al., 2011).

Vespa have evolved thick exoskeletons that are difficult for bee stings or mandibles to penetrate. However, Ac has evolved a remarkable social strategy, heat balling, in which a large mob of bees surrounds the hornet and essentially suffocates it by rapidly increasing the temperature and the level of carbon dioxide inside the ball (Matsuura & Yamane, 1990; Ono, Igarashi, Ohno, & Sasaki, 1995; Sugahara, Nishimura, & Sakamoto, 2012; Sugahara & Sakamoto, 2009). Heat balling can also kill defending bees (Tan et al., 2016) and therefore has some cost for the colony. As in multiple other animal signalling systems, this cost has led to the evolution of a warning signal (Bradbury & Vehrencamp, 2011). When Ac guards visually detect an approaching hornet, they produce a visual 'I see you' (ISY) signal consisting of wing shimmering and body shaking that warns the hornet of imminent heat balling if it moves closer (Tan et al., 2012a) and is also similar to pursuitdeterrence signals (Caro, 1995). In contrast, the allopatric Am has very weak defences against this hornet. Am does not possess the ISY signal (Tan et al., 2012a) and forms smaller and less effective heat balls that achieve significantly lower internal temperatures than Ac heat balls (Tan et al., 2005).

Ac and Am therefore provide an excellent opportunity to test the hypothesis that Ac, but not Am, have evolved effective hornet detection strategies. Our goals were to determine the proximate factors that cause both species to heat ball hornets and, based upon the recent finding that *V. velutina* uses its sting venom volatiles as an alarm pheromone (Cheng et al., 2017), to test whether these bee species can use olfactory eavesdropping to detect hornets.

#### METHODS

We conducted our experiments in an apiary with *A. c. cerana* (Ac) and *A. m. ligustica* (Am) colonies at Yunnan Agricultural University, Kunming, China during July–November 2016, when both species actively forage and are naturally preyed upon by hornets at our apiary. We used a total of 12 Ac colonies and 12 Am colonies, all healthy based upon careful visual inspection of combs and bees,

and that had been established at our apiary for more than 2 years. Each Ac and Am colony was chosen to be approximately the same size (6000–8000 workers) and consisted of four combs housed inside a wood box. All colonies had 20–30 guard bees at their nest entrances during our trials. Colonies were separated by at least 5 m, which was sufficient to prevent hornet attacks at one colony from eliciting any alarm behaviour at nearby colonies.

For the bioassays, we presented a *V. velutina* hornet that was either (1) alive and intact or (2) dead and de-scented. Live hornets were captured with an insect net while they foraged and each was tied around its petiole with fine wire at the end of a 1 m long wood stick (Tan et al., 2016). We used a different hornet per trial and, after each trial, carefully washed the wire and wood sticks with laboratory detergent, rinsed them with 100% ethanol, and then dried them for several hours in the full sun to remove potential odours.

To prepare dead and de-scented hornets, we froze live hornets, rinsed them three times with 100% dichloromethane, and dried them in the sun for several hours. To determine whether this descenting procedure was effective, we presented de-scented dead hornets and dead hornets with intact odours at the entrances of Am and Ac nests. De-scented dead hornets elicited almost no approaches from bees when presented at nest entrances (Fig. 1).

To count the number of bees that heat balled a hornet, we recorded heat balling with a Sony<sup>™</sup> HDR-PJ790 video camera. To ensure accurate counts, we played back each video in slow motion and counted the number of bees. All trials were conducted between 0900 and 1500 hours on clear, sunny days.

Every Am and Ac colony was naturally attacked by *V. velutina* at least once per week. We therefore monitored colonies to ensure that they had not been attacked at least 1 h before the start of an experimental trial to ensure that colony responses were not due to natural attacks. In our preliminary trials with tethered hornets presented to colonies (see below), we found that colonies regained normal, nonalarmed guard and forager activity less than 20 min after a hornet attack. For all experiments, between tests of each treatment, we waited 30 min (see experiment 1).

None of the three species used is endangered and we designed our experiments to minimize the adverse impacts on our subjects.

## Experiment 1: Effect of Hornet Distance on the Heat-balling Response

Colonies do not heat ball a hornet until it approaches and begins to fly close to the colony entrance because heat balling is costly: it results in bee deaths (Tan et al., 2016). To simulate a hornet approaching a colony and to consistently measure heat-balling responses from different colonies and species, we placed a live hornet 10 cm from the focal colony entrance and successively moved it 1 cm closer, each 30 s, until we reached 3 cm, a distance at which the heat balls reached their maximum sizes (total trial duration of 4 min; Fig. 1). In this experiment, each trial therefore reflected the cumulative response of the colony over time and distance to an approaching hornet. We chose 10 cm because this was similar to the distances at which freely flying, hawking hornets naturally approach Ac and Am colonies (Tan et al., 2012b). We used 10 Ac and 10 Am colonies in this experiment.

## Experiment 2: Effect of Natural Olfactory and Visual Stimuli on Heat Balling

We next compared the effects of multiple natural stimuli (olfactory and visual) on heat balling. Tan et al. (2016) demonstrated that Ac sting alarm pheromone and the presence of a live hornet are important for heat balling. We therefore tested four treatments positioned at the end of a wood stick (see experiment 1): (1) a dead Download English Version:

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