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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 μg) 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.

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 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 pursuit-deterrence 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 de-scenting 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™ 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

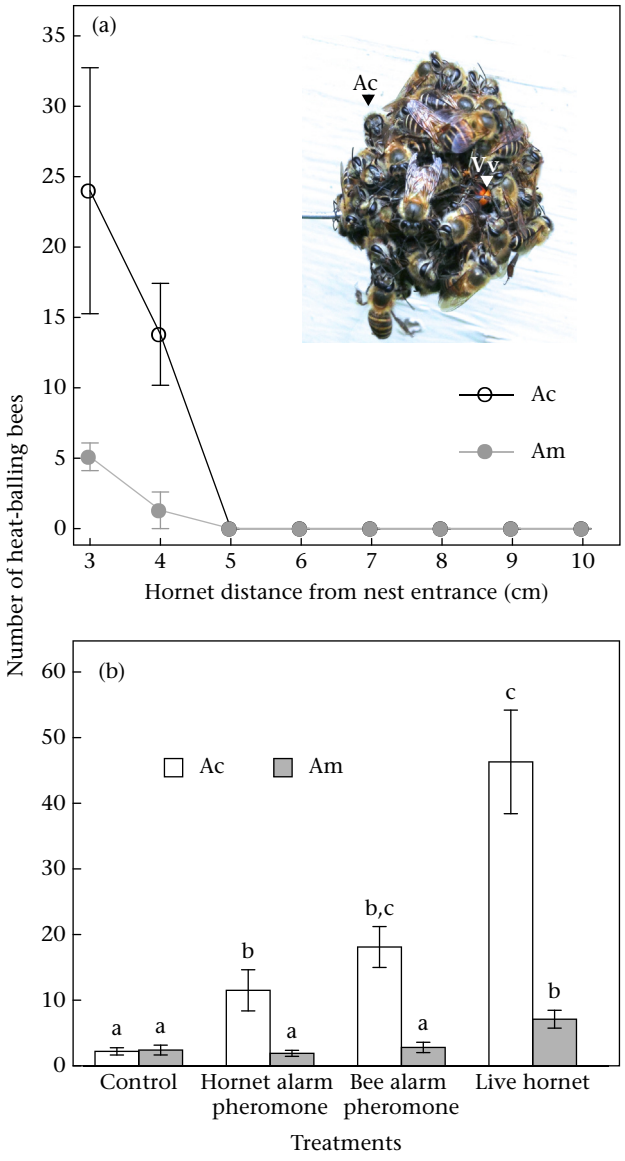

Figure 1. Critical distance and triggers for heat balling. (a) Colony heat-balling responses of *A. cerana* (Ac) and *A. mellifera* (Am) to hornets at different distances from the nest entrance. The photo shows an Ac heat ball around a hornet (Vv) whose orange abdomen is momentarily visible. Means and standard errors are shown. Samples sizes are as follows: $N_{colonies} = 10$ Ac and 10 Am, $N_{trials} = 1$ trial per colony at each test distance, $N_{attackers\ from\ all\ trials} = 210$ Ac and 64 Am. (b) The effectiveness of different pheromones and stimuli for eliciting heat balling. The control was a dead, de-scented hornet, and the hornet alarm pheromone and bee sting pheromone treatments consisted of these pheromones added to dead and de-scented hornets. Ac and Am sting pheromones were, respectively, used to test Ac and Am responses. Mean and standard error bars are shown. Significant differences are indicated with different letters (Tukey's HSD test: $P < 0.05$). Samples sizes are as follows: $N_{colonies} = 10$ Ac and Am, $N_{trials} = 1$ trial per colony with each of the four treatments and $N_{attackers\ from\ all\ trials} = 781$ Ac and 142 Am.

and de-scented hornet (control hornet), (2) hornet alarm pheromone (Cheng et al., 2017) consisting of one freshly dissected hornet venom gland added to a control hornet, (3) bee sting alarm pheromone composed of one freshly dissected bee venom gland (taken from a guard bee of the focal colony <10 s before presentation) added to a control hornet and (4) a live worker hornet. To obtain the venom glands, we carefully captured hornets with a net as they foraged on plants and caught individual guard bees with glass vials at their nest entrances immediately before the start of a trial. The insects were then anaesthetized with carbon dioxide gas before venom gland dissection. The dissected gland was then torn open with forceps to release its contents and rubbed over the thorax of a dead and de-scented hornet. In comparison with a solvent extraction, this method has the benefit of providing all of the glandular components if some compounds are not equally soluble in the chosen solvent.

We used 3 cm as the test distance for this experiment because experiment 1 demonstrated that nearly all Ac and Am colonies would attack a hornet placed 3 cm from the colony entrance. Each trial lasted 3 min, because preliminary observations at our apiary showed this time interval was sufficient for the heat ball to reach its maximal size with both species at this distance. We used 10 Ac and 10 Am colonies, and each colony was tested with each of the four treatments only once. Treatment presentation order was randomized.

Experiment 3: Volatile Compounds Produced During Heat Balling

Sample collection

Tan et al. (2016) demonstrated that Ac sting alarm pheromones can stimulate heat balling. However, it was unclear what odours are produced during natural heat balling by Ac and Am. We therefore analysed volatiles released during heat balling at two time points: during the initial approach of guard bees attacking a hornet (immediately upon hornet presentation) and once a substantial heat ball had formed (3 min after hornet presentation). We tied a live anaesthetized hornet to a clean soft wire and placed it 3 cm from the focal bee colony entrance (see experiment 2). Guard bees began the heat-balling process by immediately landing on and attacking the hornet once it revived and began to move. We then gently removed this ball to avoid disturbing the heat-balling bees to a distance of at least 10 m from the nest entrance and placed it on a clean glass petri dish (10 cm diameter) to facilitate our measurements. We carefully inserted a clean PFTE tube (1.5 mm diameter) into the ball. The other end of the tube was connected to a pump drawing air out at 1 ml/s. We penetrated the wall of the PTFE tube with a 65 μm PDMS/DVB solid-phase microextraction (SPME) fibre (Supelco, Bellefonte, PA, U.S.A.) to collect volatiles from the ball without contacting bees (Fig. 2a) and performed chemical analyses. We collected only one sample per day. We used three Ac and three Am colonies (six total analyses). We observed that the initial approach of guard bees often caused the hornet to produce a droplet of venom, whose volatiles are the source of hornet alarm pheromone, on the tip of its stinger. However, once the heat ball had formed, only bee sting alarm pheromones could be detected from the ball, likely because of the large number of attacking bees. We therefore also quantified the volatiles found in Ac and Am sting glands, using hexane extractions of freshly dissected individual sting glands (3 bees from 3 colonies from each species).

Chemical analyses

To analyse the volatiles that we collected with SPME, we used gas chromatography-flame ionization detector (GC-FID) analysis with a HP 7890B (Agilent, Santa Clara, CA, U.S.A.) gas chromatograph, a HP-5 column (30 m × 320 μm × 0.25 μm; Agilent) through which helium carrier gas flowed at 37 cm/s. The oven ramp was 50 °C for 2 min, then increased by 10 °C/min to 280 °C. The transfer line was heated to 250 °C. Each SPME fibre was desorbed into the injector port for 1 min. During desorption, the split vent was closed.

To identify the volatile compounds, we used an HP 7890A-5975A gas chromatography-mass spectrometer (GC-MS), with a HP-5ms capillary column (30 m × 250 μm × 0.25 μm; Agilent) and the conditions described above. In the quadrupole mass spectrometry, a 70 eV EI ion source was used and heated to 230 °C. The mass range scanned was m/z 28.5–380 at a rate of two scans/s (MS detector), with the A/D sampler collecting data at four scans/s. The

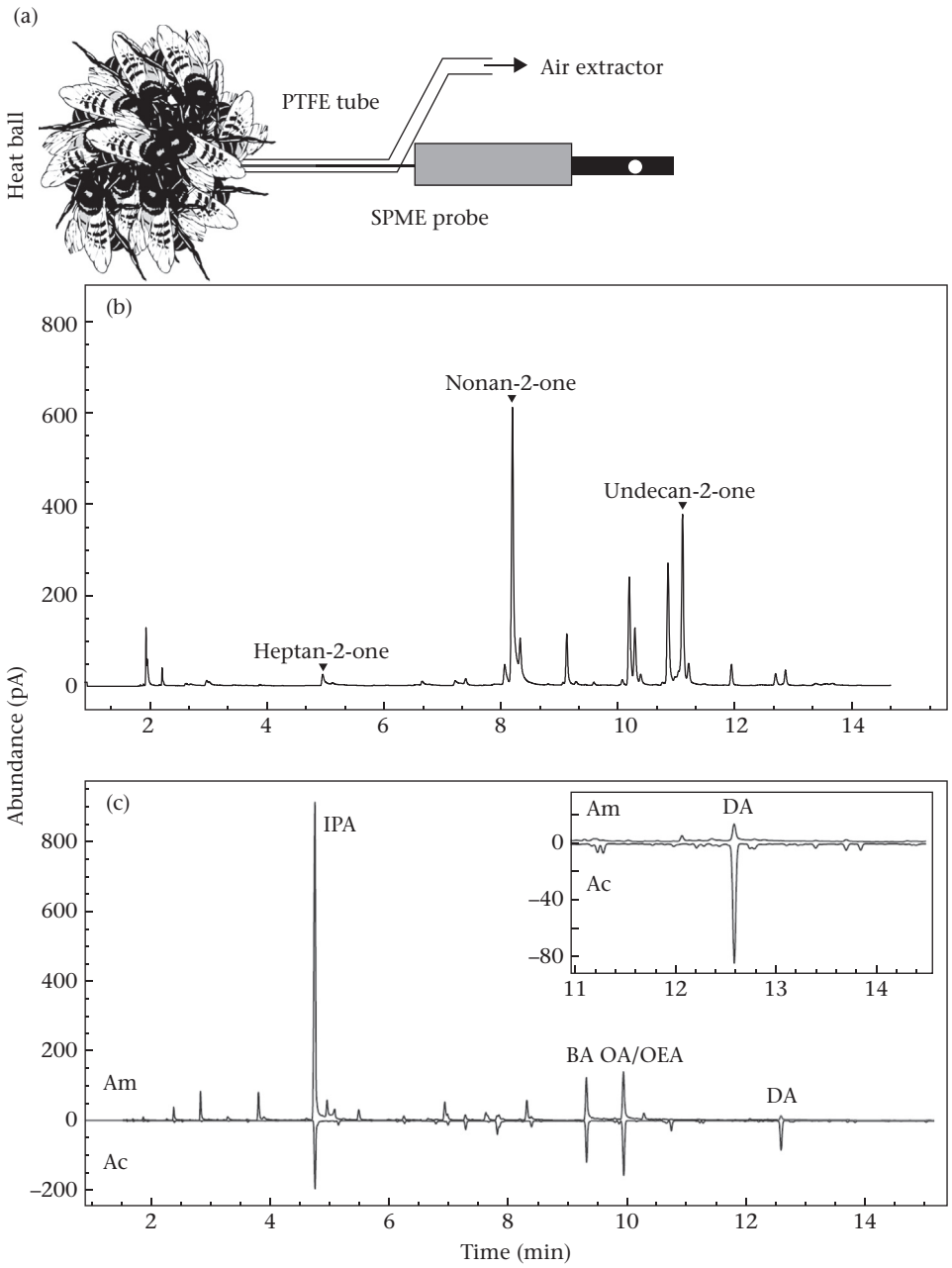

Figure 2. Chemical analyses of volatile compounds released by heat balls forming around a live hornet. (a) Diagram showing how odours were extracted by pumping air through a clean tube into which the SPME probe was inserted (the image illustrates a later phase when a heat ball had formed). (b) SPME analysis of dissected venom glands of hornets. (c) Once heat balls had formed, SPME analyses (three colonies of each species) revealed the presence of honey bee sting alarm pheromone, but not hornet alarm pheromone, perhaps because of the large number of alarmed bees relative to the single hornet. Analyses were performed with three colonies of each species: *A. mellifera* (Am) and *A. cerana* (Ac). Since there were no differences based upon bee species, we show a single representative chromatogram. Typical chromatograms are shown, with all major peaks corresponding to known components of sting alarm pheromone in Am and Ac: IPA (isopentyl acetate), BA (benzyl acetate), OA (octyl acetate)/OEA ((E)-oct-2-en-1-yl acetate) (Wen et al., 2017) and DA ((E)-2-decen-1-yl acetate). The magnified inset shows that Am also produced DA, a known component of Am sting alarm pheromone, when heat balling hornets. The relative amounts of each compound were variable, depending upon the number of bees in a ball and the exact placement of the probe within the ball. These chromatograms therefore enable compound identification but not quantification.

abundance threshold for detection was set to 10 ions per scan. Data were analysed using Chemstation software (Agilent). We confirmed our preliminary compound identifications with authentic standards by comparing their retention times and mass spectra when run on the same equipment. Commercially available isopentyl acetate (IPA), benzyl acetate (BA) and octyl acetate (OA) were obtained from TCI Co. Ltd (Tokyo, Japan). (*E*)-Dec-2-en-1-yl acetate (DA) was synthesized by acetylation of (*E*)-dec-2-en-1-ol (Wen et al., 2017) using acetyl chloride in hexane with triethylamine (Hinkens, McElfresh, & Millar, 2001), and then purified with silica chromatography. Hornet alarm pheromone compounds (heptan-2-one, nonan-2-one and undecan-2-one) were also purchased from TCI Co. Ltd.

We also used the GC-FID set-up to quantify the volatiles found in average Am and Ac worker sting glands. We used hexane solvent extracts for better quantification accuracy and injected the extracts directly into the GC-MS (18 separate analyses). Quantification followed the procedure described in Wen el al. (2017).

Experiment 4: Effects of Individual Sting Alarm Pheromone Components on Heat Balling

We identified major Ac and Am sting pheromone volatiles (IPA, BA, OA/OEA, DA; Fig. 2b) (Wang et al., 2016) released by heat balling and therefore sought to determine whether one of these individual compounds could elicit heat balling. All of these compounds, particularly IPA, are associated with colony alarm responses (Wen et al., 2017). BA increases the number of fanning Am hive workers, which may play a role in colony defence (Collins & Blum, 1982; Free, 1987; Wager & Breed, 2000) and provides a warning signal that inhibits Ac foraging at a marked food source (Wen et al., 2017). OA assists in orienting Am workers towards a moving target (Wager & Breed, 2000), and DA may provide orientation cues to *Apis dorsata* and *Apis florea* attackers (Koeniger, Weiss, & Maschwitz, 1979; Veith, Weiss, & Koeniger, 1978).

We diluted these components in dichloromethane and presented quantities in ascending order: 0 μg (solvent only), 1 μg, 10 μg and 100 μg. Each sample was applied to a new filter paper strip (15 × 4 mm) attached with an insect pin to the tergum of a de-scented dead hornet. As in our other experiments, we presented the hornet 3 cm from the colony entrance and counted the number of heat-balling bees over a 3 min trial. Between tests of each quantity, we waited 30 min (see experiment 2). Each day, we tested multiple colonies but only tested one randomly selected compound (all four quantities) per colony. We used six Ac and six Am colonies.

Experiment 5: Effects of Hornet Alarm Pheromone Components on Heat Balling

Cheng et al. (2017) showed that alarmed *V. velutina* workers release sting gland volatiles that act as an alarm pheromone: they are readily detected by hornet antennae, are strongly attractive to hornets and elicit attacks. They identified three major volatile components that triggered hornet attacks: heptan-2-one (mean ± SE = 31.3 ± 4.1 ng/hornet), nonan-2-one (852 ± 141.7 ng/hornet) and undecan-2-one (178.4 ± 30.9 ng/hornet). Heptan-2-one is produced in the mandibular glands of Am (Papachristoforou et al., 2012) but not in Ac (Morse, Shearer, Boch, & Benton, 1967). However, the amount of heptan-2-one in Am is 30 880 ng/guard bee (Papachristoforou et al., 2012) and is therefore 987-fold greater than the amount per hornet sting.

We therefore repeated experiment 4 but tested the following treatments per trial: control (solvent only), heptan-2-one, nonan-2-one, undecan-2-one and a mixture of these three compounds, all at one hornet-equivalents. Each day, we tested multiple colonies but only conducted one trial per day with any given colony. We randomized compound presentation order, and we waited 30 min between treatment presentations. We used 12 Ac and 12 Am colonies.

Experiment 6: Comparing the Effects of Hornet and Honey Bee Sting Alarm Pheromone on Heat Balling

Based upon the results of experiment 5, we next tested the effects of synthetic hornet alarm pheromone, natural honey bee alarm pheromone ($Am_{sting\ pheromone}$ for Am and $Ac_{sting\ pheromone}$ for Ac), and the combination of these two pheromones upon heat-balling behaviour. We used natural sting pheromone because this elicited the most consistent heat-balling responses and we tested the combination of bee and hornet alarm pheromones to determine whether there was a potential synergistic effect. We followed the same protocol as in experiment 2, used a clean de-scented hornet as a control treatment, obtained one bee-equivalent of freshly alarm pheromone (<10 s before testing) and a synthetic equivalent of hornet sting pheromone (one hornet-equivalent, same blend and amounts as in experiment 5). All test compounds were added directly to a de-scented hornet. We counted the number of attackers each min over 3 min to determine whether hornet or bee alarm pheromones were differentially effective at earlier or later stages of heat balling. Each day, we tested multiple colonies but only conducted one trial per day with any given colony. We randomized compound presentation order, and we waited 30 min between treatment presentations. We used 10 Ac and 10 Am colonies.

Experiment 7: GC-EAD Analysis

We compared Ac and Am antennal responses to natural hornet alarm pheromone, using gas chromatography-electroantennographic detection (GC-EAD). We carefully captured guard bees in individual glass vials at colony entrances and followed previously published procedures for EAD recording (Wen et al., 2017). We filled a glass micropipette with honey bee Ringer's solution, connected it to the EAG amplifier input and grounded it with platinum wires. We randomly severed one antenna (right or left) at its base with microscissors. We also cut the distal end of the antenna to facilitate recording, and then mounted the antennae between the tips of the recording and grounding pipettes.

We used a highly sensitive, custom-built EAD system (Wen et al., 2017) with a HP7890B GC running with the same conditions described above for our GC-FID analyses. To obtain hornet sting alarm pheromone, we followed the procedures described in experiment 2. We used venom extruded from one hornet sting gland per EAD trial. To record the antennal responses, we built a custom amplifier and used a HP34465A digital multimeter (Keysight, Santa Rosa, CA, U.S.A.) controlled by BenchVue software (Keysight) run on a PC. The GC trigger signal triggered the EAG recording.

Statistics

Heat balling is a colony response to hornet attack. To analyse the attack distance data (experiment 1), we therefore used a mixed model repeated measures analysis of covariance (ANCOVA) with colony as the replicated individual unit (random effect) and attack distance as a continuous fixed effect. Based upon residuals analysis, we log transformed the number of bees that attacked the target.

To analyse the effects of different visual and olfactory treatments on the number of attacking bees (experiment 2) and the effects of hornet alarm pheromone compounds (experiment 5), we used a repeated measures ANOVA (REML algorithm) with colony as the replicated unit (random effect). Bee species and treatment were fixed effects. Based upon inspection of the residuals, we log transformed the number of attacking bees for experiment 2 (but not for experiment 5) and used Tukey's honest significant difference (HSD) tests to make all pairwise comparisons, corrected for type I error.

To examine differences in heat-balling responses to the different compounds within each bee species (experiment 4), we first applied a repeated measures ANOVA (REML algorithm) with colony as the replicated unit (random effect) and species and treatment (where each dose of each compound was a separate nominal response) as fixed effects. We used Tukey's HSD tests to make all pairwise comparisons. Because we were interested in the levels at which different compounds would elicit a significantly elevated heat-balling response, we next examined each species separately. We applied a univariate repeated measures ANOVA with colony as the replicated unit and used Dunnett's test to make all pairwise

comparisons (corrected for type I error) between the different compound treatments and the control.

For experiment 5, we applied a repeated measures ANOVA (REML algorithm) with colony as the replicated unit (random effect) and species and treatment as fixed effects. For experiment 6, we log transformed the number of attackers and also used a repeated measures ANOVA (REML algorithm) with colony as the replicated unit (random effect) and species, treatment and time (with all interactions) as fixed effects. We used Tukey's HSD tests to make all pairwise comparisons for these experiments.

For all analyses, we used JMP Pro V13 software (SAS Institute Inc., Cary, NC, U.S.A.). We report means ± 1 SE.

RESULTS

Ac Had a Stronger Attack Response Than Am As the Hornet Approached

Guard bees began attacking hornets when they were within 4 cm of the colony entrance. However, 10.6-fold more Ac than Am workers attacked the hornet at this distance, and the number of Am workers was never sufficient to form a complete ball around the hornet (Fig. 1a). There was a significant effect of species ($F_{1,20} = 20.07$, $P = 0.0002$) and hornet distance ($F_{1,141} = 55.84$, $P < 0.0001$). The number of attacking Ac bees also increased more rapidly than the number of Am bees as the hornet approached the nest entrance (significant interaction of species∗hornet distance: $F_{1,141} = 4.60$, $P = 0.028$; Fig. 1a). Our model accounted for 24% of variance in the number of attacking bees.

Ac but Not Am Eavesdropped Upon Hornet Alarm Pheromone

We observed attacks during our trials and also reviewed attack videos. Once the first bee landed on a hornet, the hornet would try to attack it, biting and stinging. A droplet of hornet sting venom was often visible at the tip of the hornet's stinger. Bees would eventually also try to sting the hornet. We therefore compared the effects of natural stimuli. The honey bee species responded differently ($F_{1,18} = 60.64$, $P < 0.0001$), and there was a significant effect of treatment ($F_{3,54} = 31.12$, $P < 0.0001$) and a significant species∗treatment interaction ($F_{3,54} = 7.97$, $P = 0.0002$).

Between species, significantly more bees from Ac colonies than from Am colonies heat balled in the following treatments: hornet alarm pheromone, bee sting alarm pheromone and live hornet (Tukey's HSD test: $P < 0.05$; Fig. 1b). As expected, there was no significant difference between Ac and Am responses to the control (Tukey's HSD test: $P > 0.05$).

The live hornet elicited the strongest responses in both bee species. Ac showed a significantly stronger heat-balling response (21-fold more bees) to a live hornet as compared to the dead, de-scented control hornet (Tukey's HSD test: $P < 0.05$; Fig. 1b). Ac also responded significantly more strongly to hornet alarm pheromone and Ac alarm pheromone than to the control (Tukey's HSD test: $P < 0.05$). In Am, the only significant difference was a stronger response to a live hornet than to the control treatment (Tukey's HSD test: $P < 0.05$). Although 1.2-fold more bees heat balled in response to Am sting pheromone (one bee-equivalent) than the control, this was not significantly different (Tukey's HSD test: $P < 0.05$; but see results of experiment 6, Fig. 5). Our model accounted for 74% of variance.

The bee species had different responses to hornet alarm pheromone. The sympatric Ac had a significant five-fold higher response to hornet alarm pheromone than to the control (Tukey's HSD test: $P < 0.05$). The allopatric Am did not respond significantly to hornet alarm pheromone (Tukey's HSD test: $P > 0.05$). Ac had a six-fold higher response to hornet alarm pheromone than did Am (Tukey's HSD test: $P > 0.05$). There was no significant difference between the response of Ac to its own sting alarm pheromone or to hornet alarm pheromone (Tukey's HSD test: $P > 0.05$; Fig. 1b).

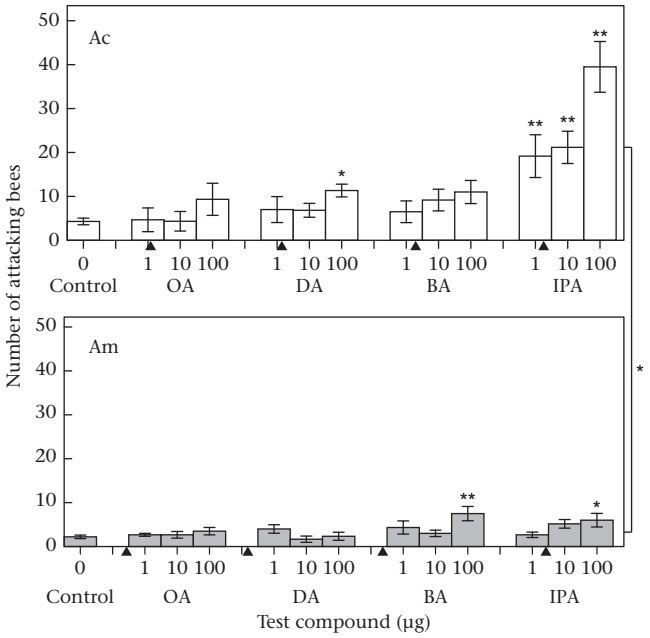

Figure 3. Heat-balling responses of *A. cerana* (Ac) and *A. mellifera* (Am) to abundant and active compounds found in the sting alarm pheromones of both species: OA (octyl acetate), DA ((E)-2-decen-1-yl acetate), BA (benzyl acetate) and IPA (isopentyl acetate). All treatments were presented on a dead, de-scented hornet. Means and standard errors are shown. Within each species, all comparisons are with the blank control (0 μg compound): Dunnett's test: $^{*}P < 0.05$; $^{**}P < 0.006$. Arrowheads indicate one bee-equivalent of each compound (Ac, Am): IPA (1.2, 2.3 μg), DA (2.3, 0.2 μg), BA (2.1, 0.3 μg) and OA (1.4, 0.4 μg). Samples sizes are as follows: $N_{colonies} = 6$ Ac and 6 Am, $N_{trials} = 1$ trial per colony with each of the 16 treatments (0 μg control was replicated for each compound test but is shown pooled in this plot), $N_{attackers\ from\ all\ trials} = 1003$ Ac and 326 Am. For the compound quantifications: $N_{colonies} = 3$ Ac and 3 Am and $N_{bees} = 9$ Ac and 9 Am. The only significant difference between species was the elevated response of Ac, as compared to Am, to 100 μg of IPA (Tukey's HSD test of all pairwise comparisons: $^{*}P < 0.05$).

Volatiles Released During Heat Balling: Hornet and Bee Alarm Pheromones

We used SPME to sample heat-balling volatiles (Fig. 2a). After the heat ball had formed (3 min after hornet introduction), we detected only honey bee sting alarm pheromone volatiles: isopentyl acetate (IPA), (E)-2-decen-1-yl acetate (DA), benzyl acetate (BA) and octyl acetate (OA) (Fig. 2c). These SPME analyses, per attack phase, consisted of six trials (1 per colony, with 3 Ac colonies and 3 Am colonies), and all identified the same compounds.

IPA Elicited the Strongest Heat-balling Response in Both Species

Given the strong response of Ac to its own sting pheromone and prior research demonstrating that Ac sting pheromone can elicit heat balling (Tan et al., 2016), we tested the effects of individual sting pheromone compounds (IPA, DA, BA, OA) at different levels. To determine which levels corresponded to a biologically relevant level of one bee sting-equivalent, we used hexane extraction for quantification, employing the same equipment and techniques for consistency. These volatiles occurred at the following levels per bee: IPA (Ac: 1.2 ± 0.3 μg; Am: 2.3 ± 0.3 μg), DA (Ac: 2.3 ± 0.4 μg;

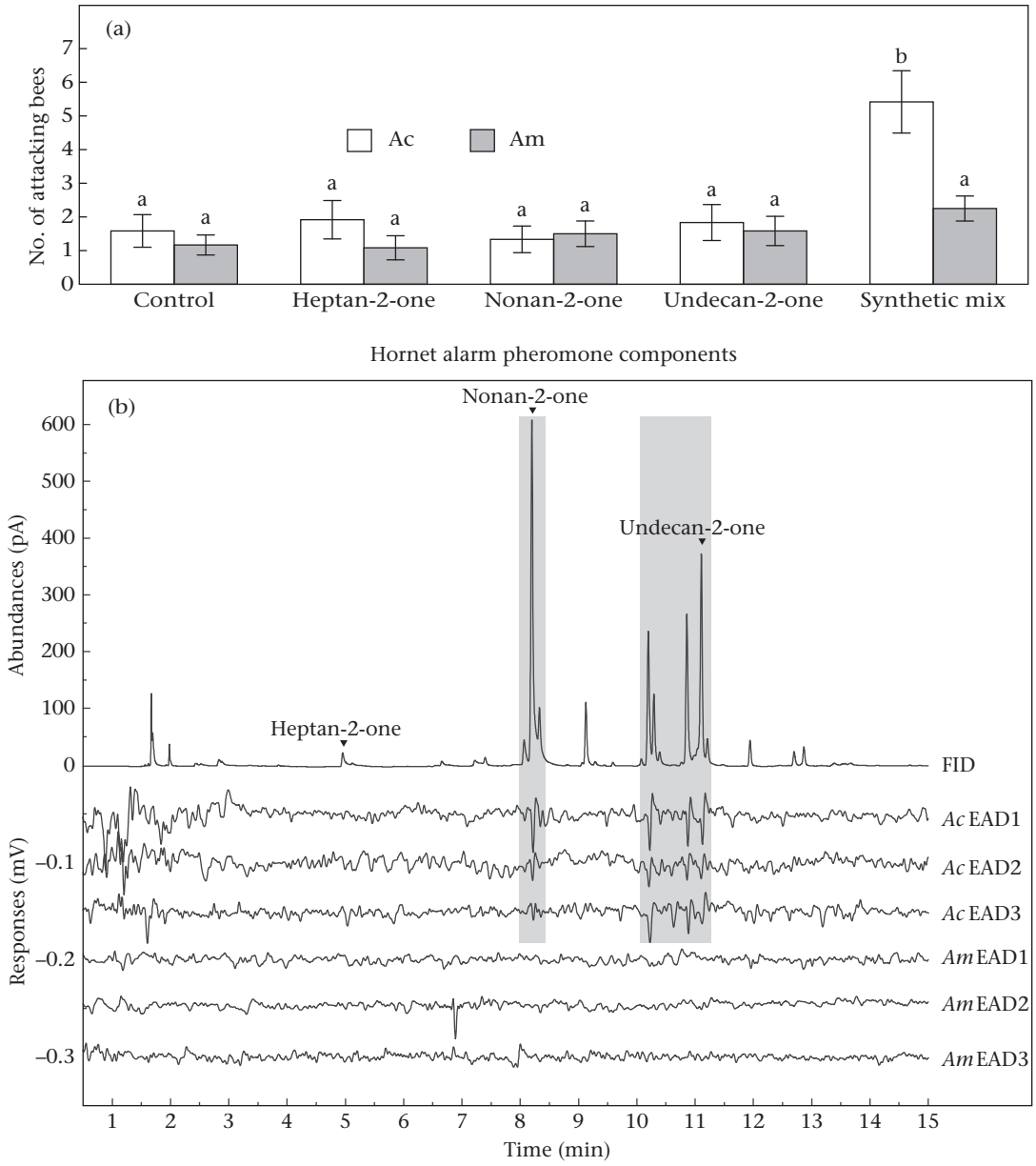

Figure 4. Heat-balling responses and antennal detection by *A. cerana* (Ac) and *A. mellifera* (Am) of abundant and active compounds found in the alarm pheromone of *V. velutina*. (a) Heat-balling responses of both bee species to heptan-2-one (31.3 ng/hornet), nonan-2-one (852 ng/hornet) and undecan-2-one (178.4/hornet). All treatments were presented on a dead, de-scented hornet. We provided all compounds, including the mixture of three compounds, at the level of one hornet-equivalent. Means and standard errors are shown. Different letters indicate significant differences (Tukey's HSD test: $P < 0.05$). Samples sizes are as follows: $N_{colonies} = 12$ Ac and 12 Am, $N_{trials} = 1$ trial per colony with each of the five treatments, and $N_{attackers\ from\ all\ trials} = 145$ Ac and 91 Am. (b) Antennal responses (GC-EAD) of Ac and Am guard bees to compounds in natural *V. velutina* sting alarm pheromone (three analyses per species, each with a bee from a different colony). Grey areas show where Ac, but not Am, responded to major hornet venom volatiles. Strong and consistent antennal responses were only detected by Ac antennae for nonan-2-one and undecan-2-one. Heptan-2-one is produced in the mandibular glands of Am, but at nearly 1000-fold higher level per bee than in one hornet-equivalent. The lack of an Am antennal response to heptan-2-one may therefore have arisen from the small quantity per hornet sting gland.

Am: 0.2 ± 0.1 μg), BA (Ac: 2.1 ± 0.8 μg; Am: 0.3 ± 0.1 μg) and OA (Ac: 1.4 ± 0.3 μg; Am: 0.4 ± 0.1 μg).

The species responded differently ($F_{1,11} = 21.31$, $P = 0.0007$). Different treatments elicited significantly different responses ($F_{12,156} = 7.10$, $P < 0.0001$), and there was a significant interaction of species * treatment because Ac had a stronger heat-balling response than Am ($F_{12,156} = 2.65$, $P = 0.003$; Fig. 3). Our model accounted for 53% of variance.

IPA elicited the strongest responses, and Ac responded significantly more strongly than Am to 100 μg of IPA (Tukey's HSD test: $P < 0.05$). There were no other pairwise species differences between compounds tested at the same level (Tukey's HSD test: $P < 0.05$; Fig. 3).

We next focused on how each species responded to these different treatments. Significantly more Ac workers heat balled the target for all levels of IPA (1, 10 or 100 μg; Dunnett's test: $P \leq 0.003$) and for the highest level of DA (100 μg; Dunnett's test: $P = 0.047$) as compared to the blank control. No other treatments elicited significantly different responses as compared to the blank control (Dunnett's test: $P \geq 0.13$). Significantly more Am workers heat balled the target when 100 μg of IPA (Dunnett's test: $P = 0.04$) or 100 μg of BA (Dunnett's test: $P = 0.006$) was presented, as

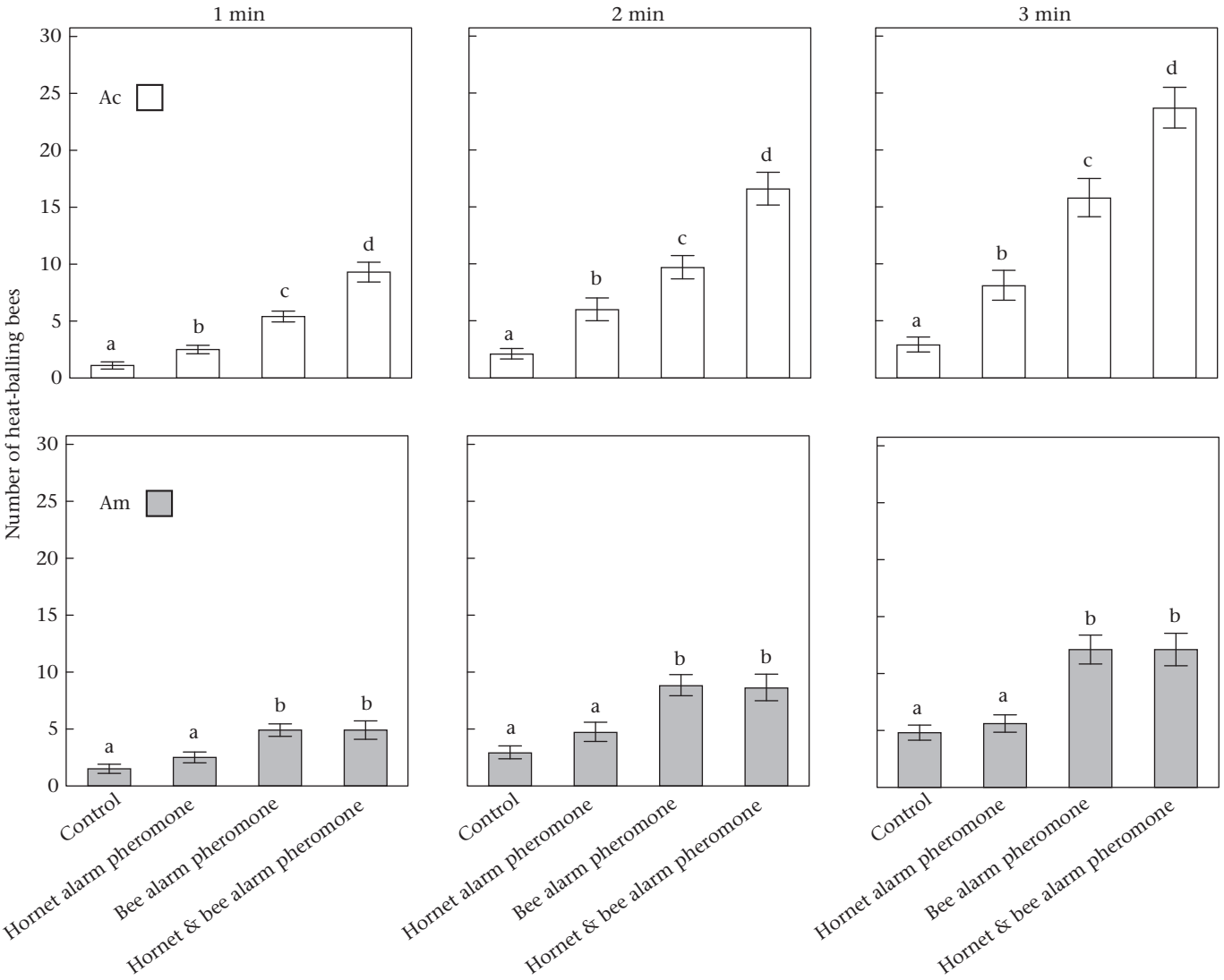

Figure 5. Number of *A. cerana* (Ac) and *A. mellifera* (Am) bees per heat ball 1–3 min following exposure to each treatment: control (dead, de-scented hornet); hornet alarm pheromone (a synthetic blend provided on a dead, de-scented hornet); bee alarm pheromone (each bee species was tested with its natural, species-specific alarm pheromone); and hornet alarm pheromone and species-specific bee alarm pheromones. Different letters indicate significant differences (Tukey's HSD test: $P < 0.05$). Means and standard errors are shown.

compared to the blank control. No other treatments elicited significantly different Am responses compared to the blank control (Dunnett's test: $P \geq 0.10$; Fig. 3).

Ac was therefore more sensitive to the tested bee sting pheromone compounds than Am. In both species, bees exhibited the strongest heat-balling response to IPA, which is the most abundant (Fig. 2c) and volatile active component of sting alarm pheromone in Ac and Am.

Ac but Not Am Eavesdropped Upon Synthetic Hornet Alarm Pheromone

The hornet alarm pheromone treatments elicited significantly different responses ($F_{4,88} = 13.30$, $P < 0.0001$; Fig. 4a). There was no effect of species ($F_{1,22} = 3.33$, $P = 0.08$). However, there was a significant interaction of treatment * species ($F_{4,88} = 5.21$, $P = 0.0008$), because only Ac significantly elevated heat balling (by 3.4-fold) as compared to the control and only for the synthetic mix of all three components (Tukey's HSD test: $P < 0.05$). Am did not significantly elevate heat balling for any treatment (Tukey's HSD test: $P > 0.05$; Fig. 4a).

Adding Synthetic Hornet Alarm Pheromone Increased Heat Balling in Ac but Not in Am

The heat balls grew with time ($F_{2,198} = 176.23$, $P < 0.0001$). There were significant effects of treatment ($F_{3,198} = 87.14$, $P < 0.0001$) and a significant treatment * species interaction ($F_{3,198} = 7.54$, $P < 0.0001$) because Ac eavesdropped upon synthetic hornet alarm pheromone and heat balled but Am did not (Fig. 5). There was no significant effect of species ($F_{1,23} = 0.99$, $P = 0.33$) and no significant interaction of species * time ($F_{2,198} = 0.07$, $P = 0.93$), time * treatment ($F_{6,198} = 0.50$, $P = 0.80$) or species * time * treatment ($F_{6,198} = 1.18$, $P = 0.32$). At all three time points, significantly more Ac (but not Am) heat balled the hornet alarm pheromone treatment compared to the control (Tukey's HSD test: $P < 0.05$), confirming what we found in our first experiment. Honey bee alarm pheromone significantly increased heat balling in both bee

species, but the addition of hornet alarm pheromone only increased heat balling in Ac, as we expected (Tukey's HSD test: $P < 0.05$). The combination of hornet and bee alarm pheromones had a stronger effect than either alarm pheromone alone in Ac (Tukey's HSD test: $P < 0.05$), but this combined effect was not greater than the sum of the individual pheromone effects (Fig. 5).

Ac Antennae Were More Responsive to Hornet Alarm Pheromone Compounds

GC-EAD analyses (one bee per colony, three colonies of each species) revealed that Ac antennae consistently responded more strongly to hornet alarm pheromone components (nonan-2-one and undecan-2-one) than Am antennae (a diminished Am response to nonan-2-one as compared to Ac) when tested with one hornet-equivalent of alarm pheromone (Fig. 4b). Only the most abundant hornet alarm pheromone components elicited EAD responses, including other compounds that remain to be identified due to their relatively complex structures (Fig. 4b). Ac may have slightly responded to heptan-2-one, which is present at relatively low levels in hornet venom (Fig. 4b), but is an alarm-releasing component of hornet alarm pheromone (Cheng et al., 2017). In addition, Ac antennae responded far more strongly to compound peaks with retention times of 10–11 min, unlike Am (Fig. 4b).

DISCUSSION

Am and Ac are now widespread throughout China and overlap over much of their range with *V. velutina* (Akre, 1978; Tan et al., 2005). At our apiary, colonies of both species were frequently attacked by *V. velutina*. Despite this repeated exposure to *V. velutina*, only the sympatric Ac has a highly effective heat-balling defence (Tan et al., 2005) (Fig. 1), and only Ac used olfactory eavesdropping to detect *V. velutina* alarm pheromone (Figs 1, 4 and 5). Although bee alarm pheromone and a live hornet elicited the most heat balling (Fig. 1), eavesdropping significantly increased the number of heat-balling Ac bees, even in the absence of a live hornet (Fig. 5). Ac antennae responded more strongly and consistently to key volatile components of *V. velutina* alarm pheromone than Am antennae. Although Am and *V. velutina* (but not Ac) use heptan-2-one for chemical communication, the lack of an Am response to this compound is perhaps not surprising given that we tested one hornet-equivalent, which is 0.001 Am guard bee-equivalent (Papachristoforou et al., 2012). Our results thus strongly suggest that this ability to use hornet alarm pheromone as a kairomone, olfactory eavesdropping, has evolved as an innate ability in the sympatric Ac and not in the allopatric Am. Likewise, Ac innately possesses the 'I see you' signal that helps to deter hornet attacks, but Am does not (Tan et al., 2012a). This ability of Ac to detect hornet sting pheromone is likely not due to shared ancestry, given that *Vespa* and *Apis* are not closely related (Bank et al., 2017).

Ac exhibited far better hornet detection and defence than Am. Ac had a larger heat-balling response than Am (1) as hornets approached the bee colony, (2) in response to the presence of a live hornet, (3) in reaction to bee alarm pheromone and natural and synthetic hornet sting pheromone and (4) in response to the major pure components of bee sting alarm pheromone. In all experiments, we used treatments that offered the visual stimulus of a hornet, either dead and de-scented or alive. The visual appearance of a hornet and its movements, vibrations and counteraggression likely contribute to heat balling. However, a dead and de-scented hornet alone elicited almost no heat balling by Ac or Am (Figs 1b, 3 and 4). Because heat balling is a cumulative colony response, the visual and olfactory cues and signals added by heat-balling bees likely contributed to heat balling, as they do in the natural context.

Heptan-2-one, nonan-2-one and undecan-2-one are major components of hornet venom volatiles and are therefore released whenever hornets attempt to sting (Cheng et al., 2017). Bees may also respond to other components of hornet alarm pheromone, but the combination of these three components was sufficient to increase the number of heat-balling Ac bees by 3.4-fold in comparison with the control (Fig. 4a). After heat balls had formed, we only detected the major components of Am and Ac alarm pheromone (IPA, BA, OA, DA), as might be expected given the recent finding that Ac significantly increases heat balling in response to Ac sting alarm pheromone (Tan et al., 2016). Once the heat ball had reached it full size, we did not detect hornet sting alarm pheromone (Fig. 2), perhaps because a fully formed heat ball contained many bees and only one hornet, overwhelming our ability to detect compounds released by the hornet (Fig. 1).

Honey bees generally respond more strongly to the complete cocktail of honey bee sting alarm pheromone components than to a single component: Ac (Wen et al., 2017), Am (Nouvian, Reinhard, & Giurfa, 2016) and *A. dorsata* (Li, Wang, Tan, Qu, & Nieh, 2014a). In our study, BA, DA and OA did not elicit strong responses when individually presented at one bee-equivalent. However, Ac formed heat balls of the same size in response to natural Ac alarm pheromone (Fig. 1b) and one bee-equivalent of IPA (Fig. 3). Am did not consistently form significantly larger heat balls in response to one bee-equivalent of natural Am alarm pheromone (Fig. 1b) or one bee-equivalent of synthetic Am alarm pheromone (Fig. 3). However, in experiment 6, we were able to detect slightly but significantly increased heat balling in response to one bee-equivalent of natural Am alarm pheromone (Fig. 5). Multiple factors affect Am colony defensiveness (Hunt, 2007), including season (Pearce, Huang, & Breed, 2001), and experiments 1 and 2 were, respectively, conducted at the beginning and end of our field season. However, the heat-balling responses to the other treatments were consistent for Ac and Am, particularly the ability of Ac (but not Am) to eavesdrop upon hornet alarm pheromone.

The association of sting venom volatiles and alarm pheromone makes sense because the evolution of alarm pheromones from chemical weapons has likely been facilitated by the reliable association between attack and defence. In multiple wasp species, sting venom volatiles are the main source of alarm pheromone: *Polistes dominulus* (Bruschini, Dani, Pieraccini, & Guarna, 2006), *Dolichovespula maculata* (Jimenez et al., 2016), *Vespula squamosa* (Heath & Landolt, 1988; Landolt, Heath, Reed, & Manning, 1995), *Vespa crabro* (Veith, Koeniger, & Maschwitz, 1984), *Vespa mandarinia* (Ono, Terabe, Hori, & Sasaki, 2003), *Vespa simillima xanthoptera* (Ono et al., 2003) and *V. velutina* (Cheng et al., 2017). All honey bee species studied, to date, also use their sting venom volatile as an alarm pheromone (Li et al., 2014a). To avoid Ac eavesdropping, it seems that hornets could eliminate their production of alarm pheromone during Ac nest attacks. However, these hornets are constrained because their sting venom volatiles, produced whenever they attempt to attack or defend themselves by stinging, constitute their alarm pheromone. To turn this signal into a 'whisper', to not sting, would also make them more vulnerable to attack. The hornets are thus in an evolutionary bind that Ac has nicely exploited. A major hornet weapon, sting venom, produces volatiles that Ac can detect. Perhaps hornets could evolve venom that is largely free of volatiles and produce a different alarm pheromone for hornet colony defence. However, these changes would not eliminate the other strategies that Ac uses to detect and fight against hornets.

This hornet signal would only be detectable during the initial stage of attack because Ac attackers also release their own alarm pheromone, which dominates once a heat ball has formed (Fig. 2). Why should Ac therefore pay attention to hornet alarm

pheromone? Hornets do not always persist and attack (Tan et al., 2012a). They can be repelled by guards before a full heat ball forms, with its associated release of high levels of honey bee alarm pheromone. Thus, in the initial engagement between a hornet and a single guard bee, the ability of Ac defenders to detect and rapidly respond to hornet alarm pheromone should be advantageous, facilitating the rapid recruitment or more guards and potentially repulsing the hornet before a heat ball, which can kill a large number of heat-balling bees (Tan et al., 2016), forms. More experiments are necessary to test this hypothesis.

The predator signals used by eavesdroppers may be either learned or innate. For variable signals, learning would be the best strategy. One would not expect innate recognition of highly variable signals. However, learning a predator signal comes at a cost because the learning phase would result in a weaker alarm response and poorer defence. We therefore expect that innate eavesdropping should typically evolve under the following conditions: (1) an advantage accrued via eavesdropping, (2) a relatively high cost imposed by learning and (3) a highly stereotyped sender signal that is under strong selection to remain fixed. Although Ac can detect and respond to their own alarm pheromones, there is evidently an advantage to also detecting olfactory signals produced by the predator. With respect to learning cost, 20–30% of workers in an Ac colony can die from a successful *V. velutina* attack (Tan et al., 2005). Finally, *V. velutina* is presumably under selection to maintain a consistent, highly recognizable alarm pheromone signal for the defence of its own colonies (Cheng et al., 2017). We suggest that Ac innately eavesdrops upon *V. velutina* alarm pheromone, although testing Ac colonies reared in isolation from hornets would help to evaluate this hypothesis. However, the ability of Ac antennae to detect more hornet alarm pheromone components and the higher sensitivity of Ac antennae as compared to Am antennae to hornet venom volatiles (10–11 min; Fig. 4b) supports the innate eavesdropping hypothesis.

One wonders why Am does not learn to recognize *V. velutina* sting alarm pheromone given that Am has excellent olfactory learning (Giurfa, 2007). The lack of sensitivity of Am antennae to the major hornet venom volatiles may help explain this phenomenon. It seems likely that Ac has evolved to have many olfactory sensory neurons with olfactory receptors sensitive to hornet alarm pheromone volatiles. In Ac, the olfactory receptors may also have been tuned to these molecules. Perhaps the larger question is why Am (*A. m. ligustica*) does not show a stronger heat-balling defence. *Apis mellifera cypria* and *Apis mellifera caucasica* have evolved with and form large heat balls around their common predator, *Vespa orientalis* (Kandemir et al., 2012; Papachristoforou, Rortais, Sueur, & Arnold, 2011; Papachristoforou et al., 2007, 2008). Similarly, *A. m. ligustica* forms defensive heat balls against a sympatric hornet predator, *V. crabro* (Baracchi, Cusseau, Pradella, & Turillazzi, 2010). Over evolutionary time, *A. m. ligustica* should likewise evolve this ability in areas where it is subject to hornet predation.

Author Contributions

S.H.D., P.W., K.T. and J.N. conceived of and designed the experiments. S.H.D., P.W., Q.Z., Y.W. and Y.N.C. performed the experiments. S.H.D., P.W. and J.N. analysed the data. P.W. and J.N. contributed reagents, materials or analysis tools. S.H.D., P.W., K.T. and J.N. wrote the paper.

Data Statement

All data will be available, upon publication, via Zenodo (https://doi.org/10.5281/zenodo.1162971).

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