Title

Effects of natural and synthetic alarm pheromone and individual pheromone components on foraging behavior of the giant Asian honey bee, Apis dorsata

Journal

Journal of Experimental Biology, 217(19)

ISSN

0022-0949

Authors

Li, Jianjun
Wang, Zhengwei
Tan, Ken
et al.

Publication Date

2014-10-01

DOI

10.1242/jeb.110171

Peer reviewed

© 2014. Published by The Company of Biologists Ltd | The Journal of Experimental Biology (2014) 217, 3512-3518 doi:10.1242/jeb.110171

RESEARCH ARTICLE

Effects of natural and synthetic alarm pheromone and individual pheromone components on foraging behavior of the giant Asian honey bee, *Apis dorsata*

Jianjun Li[1], Zhengwei Wang[1], Ken Tan[1,2,*], Yufeng Qu[1] and James C. Nieh[3]

ABSTRACT

Social pollinators such as honey bees face attacks from predators not only at the nest, but also during foraging. Pollinating honey bees can therefore release alarm pheromones that deter conspecifics from visiting dangerous inflorescences. However, the effect of alarm pheromone and its chemical components upon bee avoidance of dangerous food sources remains unclear. We tested the responses of giant honey bee foragers, *Apis dorsata*, presented with alarm pheromone at a floral array. Foragers investigated the inflorescence with natural alarm pheromone, but 3.3-fold more foragers preferred to land on the 'safe' inflorescence without alarm pheromone. Using gas chromatography–mass spectrometry analysis, we identified eight chemical components in the alarm pheromone, of which three components (1-octanol, decanal and gamma-octanoic lactone) have not previously been reported in this species. We bioassayed six major compounds and found that a synthetic mixture of these compounds elicited behaviors statistically indistinguishable from responses to natural alarm pheromone. By testing each compound separately, we show that gamma-octanoic lactone, isopentyl acetate and (E)-2-decen-1-yl acetate are active compounds that elicit significant alarm responses. Gamma-octanoic lactone elicited the strongest response to a single compound and has not been previously reported in honey bee alarm pheromone. Isopentyl acetate is widely found in the alarm pheromones of sympatric Asian honey bee species, and thus alarmed *A. dorsata* foragers may produce information useful for conspecifics and heterospecifics, thereby broadening the effects of alarm information on plant pollination.

KEY WORDS: Alarm pheromone, Foraging, Honey bee, Information flow, Predator attack

INTRODUCTION

Alarm pheromones play an important role in social insects and enhance collective fitness by providing information about dangers such as predators (Billen and Morgan, 1998). For example, honey bees can use alarm pheromones to co-ordinate colony defence (Free, 1987; Pirk et al., 2011). However, the role of alarm pheromones in communicating danger at food sources is less well understood (Nieh, 2010; Goodale and Nieh, 2012). Honey bees (*Apis mellifera*, *A. dorsata* and *A. florea*) avoided flowers upon which a conspecific had been attacked by predators and therefore potentially released alarm-based odors (Llandres and Rodriguez-Gironés, 2011; Llandres et al., 2013). Although it is highly likely that these bees released alarm pheromone compounds that elicit conspecific avoidance of these inflorescences, it was not clear whether they were avoiding alarm pheromone or other olfactory cues. Therefore identifying and testing different chemical components of alarm pheromone in honey bees is important for understanding what elicits avoidance following attacks at natural food. In fact, with the exception of the European honey bee, *A. mellifera*, little is known about which alarm pheromone components elicit alarm. Understanding the role of different alarm pheromone components is relevant because this tells us which components elicit alarm, provides information on shared and thus potentially ancestral components in different honey bee species, and opens the possibility of heterospecific responses to shared pheromone components.

Most social insects use alarm pheromones, which have multiple evolutionary origins, in some cases evolving from compounds with toxic properties (Blum, 1969). In ants, alarm pheromones occur in nearly all tested species and are typically produced by glands associated with defence or offence, such as sting poison glands (Holldobler and Wilson, 1990). Alarm pheromones are similarly widespread in social wasps and are found in the *Vespa*, *Vespula*, *Polistes*, *Ropalidia*, *Polybia*, *Dolichovespula*, *Provespa* and *Polybioides*, where they are generally released from sting venom glands (Fortunato et al., 2004). However, one species, *Vespula squamosa*, evidently releases alarm pheromone from a gland in the head (cephalic gland) (Landolt et al., 1999). This strategy is shared by all studied species of stingless bees (Hymenoptera, Apidae, Meliponini), which lack stings and therefore attack by biting (Roubik, 1989). Stingless bees use cephalic secretions as alarm pheromones, in all examined cases from mandibular glands (Nieh, 2004; Schorkopf et al., 2009). Surprisingly, bumble bees (Hymenoptera, Apidae, Bombini) generally do not seem to use alarm pheromones (Maschwitz, 1964), although Llandres et al. (Llandres et al., 2013) recently demonstrated that *Bombus terrestris* would avoid flowers upon which a conspecific had been attacked and potentially deposited an alarming odor. The source of this odor remains unknown.

Honey bees (Hymenoptera: Apidae, Apini) produce alarm pheromone in the sting gland and a pheromone that has sometimes been described as an alarm pheromone in their mandibular glands, but which does not galvanize colony aggression (Vallet et al., 1991; Couvillon et al., 2010). In fact, recent evidence suggests that *A. mellifera* mandibular gland pheromone is a defensive compound for paralysing hive intruders (Papachristoforou et al., 2012). Suwannapong et al. (Suwannapong et al., 2011) tested the response of *A. andreniformis* and *A. florea* foragers to synthetic compounds found in bee mandibular glands. They applied these compounds to filter papers placed beneath flowers and found that some compounds, depending upon their concentration, repelled or

[1]Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Kunming, Yunnan Province 650223, China. [2]Eastern Bee Research Institute, Yunnan Agricultural University, Kunming 650201, China. [3]Division of Biological Sciences, Section of Ecology, Behavior and Evolution, University of California, San Diego, La Jolla, CA 92093, USA.

*Author for correspondence (kentan@xtbg.ac.cn)

Received 26 June 2014; Accepted 5 August 2014

The Journal of Experimental Biology

The Journal of Experimental Biology (2014) doi:10.1242/jeb.110171

attracted foragers of *A. andreniformis*. The tested compounds only repelled *A. florea*. It would therefore be useful to determine the responses of honey bees to alarm compounds, not only from mandibular glands, but from the entire bee.

Upon attack, honey bees produce an alarm pheromone that elicits different responses depending upon the setting. For example, exposure to *A. mellifera* sting alarm pheromone results in attacks from defenders near the nest (Wager and Breed, 2000) but causes aversion at food (Goodale and Nieh, 2012) and reduces recruitment to this food location (Nieh, 2010). Such context dependency is also exhibited by honey bee queen pheromone, which elicits a wide variety of behaviors (the queen retinue, division of labor, swarm clustering, drone attraction, etc.) depending upon the receiver and the environment (Free, 1987).

In the highly social bees (honey bees and stingless bees), alarm pheromones can increase aggression and flight activity at the nest entrance, a defensive response (Boch and Shearer, 1971; Roubik, 1989). However, at a food source, alarm pheromones ultimately cause avoidance. Stingless bee foragers (*Melipona panamica*) hover around a feeder with mandibular gland alarm pheromone extract, but then preferentially land on a control feeder without the pheromone (Nieh, 1998). Other stingless bee species, *Scaptotrigona depilis* and *Trigona spinipes*, avoid a nectar feeder with conspecific mandibular gland extracts (Schorkopf et al., 2009). Similarly, honey bee (*A. mellifera*) foragers avoid sting gland alarm pheromone placed on a sucrose feeder (Goodale and Nieh, 2012). A major component of *A. mellifera* sting gland pheromone, isopentyl acetate, will repel bees from visiting when added to a sugar solution feeder (Ferguson and Free, 1979) or to oil-seed rape, field beans and sunflowers (Free et al., 1985). However, it is not clear whether other compounds in honey bee alarm pheromone can also elicit such avoidance.

Predation at food sources can elicit alarm, and our study was inspired by predation observations. Hornets (*Vespa tropica*) caught *A. dorsata* foragers on flowers of *Syzygium jambos* L. near our field site in Menglun, China. We also observed weaver ants (*Oecophylla smaragdina*) attacking *A. dorsata* foraging on inflorescences (*Calliandra haematocephala*). Ants attacked 3% of visiting bees (980 bee visits observed) and 31% of these attacks successfully killed the bee forager (Li et al., 2014). Ant presence alone significantly reduced bee floral visitation by 40% (Li et al., 2014). During these attacks, we smelled *A. dorsata* alarm phermone released by attacked bees, and we wished to determine whether this alarm pheromone would repel foragers. *Apis dorsata* is a good species to study because little is known about its behavioral responses to alarm pheromone. Comparative information about alarm pheromone responses from multiple bee species is necessary to understand alarm pheromone evolution. In addition, *A. dorsata* is widespread throughout Asia (Oldroyd and Wongsiri, 2006; Hepburn and Radloff, 2011), where it is an important pollinator of crops (Wongsiri et al., 2001; Partap, 2011) and native plants (Corlett, 2011). Finally, *A. dorsata* is sympatric with two other native *Apis* species with which it shares alarm pheromone components (Boch et al., 1962; Morse et al., 1967; Koeniger et al., 1979) and, potentially, alarm information.

We therefore tested the effects of natural alarm pheromone, individual pheromone components that we chemically identified, and a synthetic mixture of these components on *A. dorsata* foraging at *C. haematocephala* inflorescences. Our goal was to understand how *A. dorsata* alarm pheromone would affect conspecific foraging behaviour, and to determine which chemical components have the strongest effect on conspecific foragers.

RESULTS

Gas chromatography–mass spectrometry analysis

We used solid phase micro-extraction (SPME) fibers to collect alarm pheromones from the airspace around bees captured in vials. The alarm pheromone components that we identified (Fig. 1, Table 1) largely match those reported by previous investigators (Table 2). The two most abundant compounds are gamma-octanoic lactone and isopentyl acetate. Gamma-octanoic lactone has not been previously identified in any honey bee alarm pheromone, although it is found in the cephalic extract of *A. laboriosa* (Table 2). In addition, we provide the first identification of the following minor *A. dorsata* alarm pheromone compounds: 1-octanol (found in *A. mellifera* sting gland pheromone), and decanal (not previously reported in *Apis* alarm pheromone; Tables 1, 2).

Floral choice test

We gave foragers a choice between two inflorescences, one with the treatment odor and the other with a control. We assayed the choices of 1561 bees, counting each choice only once. For compounds that elicited a strong aversion, such as natural alarm pheromone (Table 3, Fig. 2), foragers exhibited a classic behavior. They slowly hovered over the inflorescence with the alarm odor, and then landed on the control inflorescence or flew away from the array. Foragers significantly avoided landing on inflorescences with natural alarm pheromone, the synthetic mixture, and all alarm pheromone components (χ^2 tests, $P \leq 0.006$), with the exception of isopentyl propionate, octyl acetate and 3-methyl-1-butanol ($P \geq 0.42$; Table 3).

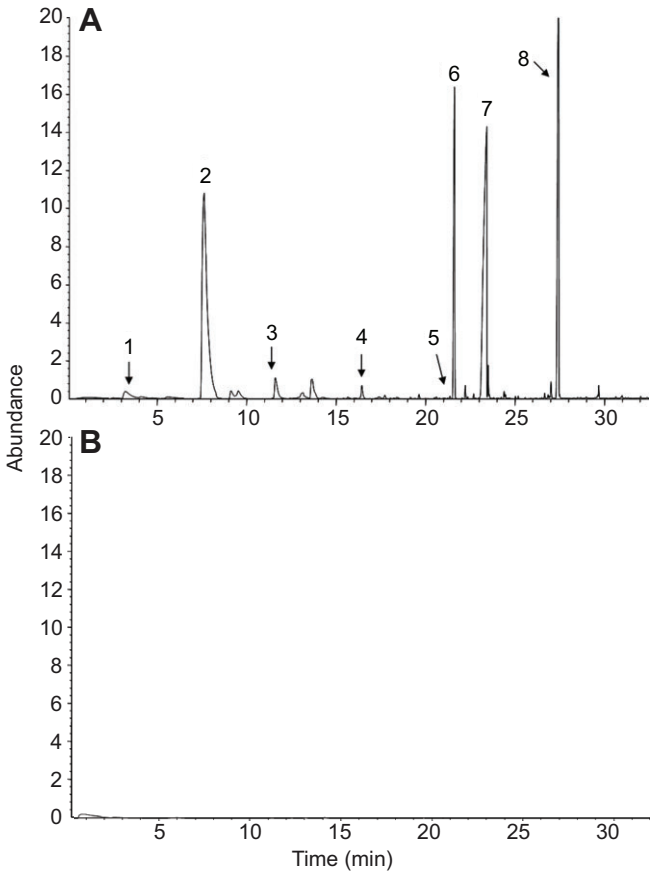

Fig. 1. Results of GC–MS analyses. Typical GC–MS chromatograms of compounds produced by (A) alarmed and (B) non-alarmed *A. dorsata* foragers and adsorbed onto SPME fibers. Numbers correspond to compounds listed in Table 1.

Table 1. Components of *Apis dorsata* alarm pheromone

ID no.	Time (min)	Compound	Relative amount per bee (%)		
			Mean	s.d.	Estimated amount (μg) per bee
1	3.238	*3-Methyl-1-butanol*	5.18	6.00	1.80
2	7.734	*Isopentyl acetate*	57.62	21.58	20.00
3	11.614	*Isopentyl propionate*	1.40	1.47	0.49
4	16.449	1-Octanol	3.70	1.24	
5	21.364	Decanal	0.82	0.48	
6	21.616	*Octyl acetate*	5.53	5.60	1.92
7	23.424	*Gamma-octanoic lactone*	18.59	19.36	6.46
8	27.44	*(E)-2-decen-1-yl acetate*	7.18	7.99	2.49

Compounds in italics were chosen for testing because they were among the most abundant. Measurements were obtained from GC–MS analyses of 36 different bees (18 alarmed and 18 non-alarmed foragers). Analyses of the 18 non-alarmed controlled bees resulted in no detectable levels of these eight compounds (Fig. 1B). The estimated amount of each identified compound per bee is shown, based upon a known average of 20 μg of isopentyl acetate per *A. dorsata* forager (Morse et al., 1967; Koeniger et al., 1979).

The overall repellency of our floral array matches these landing choices: 33–49% of approaching foragers left without making a choice when the array presented natural alarm pheromone, the synthetic mixture, gamma-octanoic lactone, isopentyl acetate or (E)-2-decenyl acetate. However, only 20–24% of approaching bees left without landing when the array presented isopentyl propionate, octyl acetate or 3-methyl-1-butanol (Table 3).

There is a significant effect of compound type on the number of bees that landed (ANOVA, $F_{9,288}$=21.68, P<0.0001; Fig. 2). There is no effect of trial date ($F_{1,288}$=0.01, P=0.91). The numbers of bees that landed on the inflorescence-only control (no added odor) and on the hexane-only treatment are, respectively, 5.6±1.3 and 6.0±1.9 bees per 10 min trial [no significant difference, Tukey's honestly significant difference (HSD) test, P>0.05]. Releasing natural alarm pheromone at an inflorescence significantly decreased the number of landing bees to 1.7±1.5 bees (significantly lower than either control treatment, Tukey's HSD test, P<0.05; Fig. 2).

There is no significant difference between aversion to natural alarm pheromone and the synthetic mixture (Tukey's HSD test, P<0.05). The two control treatments and three of the test treatments (isopentyl propionate, octyl acetate and 3-methyl-1-butanol) elicited the least aversion and were visited by approximately equal numbers of bees (no significant differences, Tukey's HSD test, P<0.05). All other tested compounds elicited intermediate aversion levels (Tukey's HSD test, P<0.05; Fig. 2).

DISCUSSION

Apis dorsata foragers avoided their alarm pheromone when this was presented at a food source, a situation that occurs when foraging bees are attacked by predators. This avoidance lasted for at least

Table 2. Summary of chemical compounds detected in the sting glands of different honey bee species (genus *Apis*)

Compound	Species					References
	A. mellifera	*A. cerana*	*A. dorsata*	*A. florea*	*A. laboriosa*	
Isopentyl acetate[1]	+s, r	+	+#	+		Boch et al., 1962; Morse et al., 1967; Koeniger et al., 1979
1-Hexanol	+r					Collins and Blum, 1982
Octyl acetate	+a	+	+#			Schmidt et al., 1997; Wager and Breed, 2000
Butyl acetate	+r					Hepburn et al., 1994
1-Butanol	+r					Collins and Blum, 1982
1-Octanol	+r		#			Collins and Blum, 1982
2-Methyl-1-butanol	+					Wager and Breed, 2000
3-Methyl-1-butanol	+		#			Wager and Breed, 2000
Hexyl acetate	+r		+			Boch et al., 1962
(E)-2-decen-1-yl acetate[2]			+#	+e		Veith et al., 1978; Koeniger et al., 1979
2-Nonanol	+r	+				Collins and Blum, 1982; Schmidt et al., 1997
(Z)-11-eicosen-1-ol	+s,a	+				Pickett et al., 1982; Schmidt et al., 1997
Eicosenol	+c	+				Schmidt et al., 1997
Farnesol			+			Blum et al., 2000
Benzyl acetate	+					Hepburn et al., 1994
Decyl acetate			+			Blum et al., 2000
Undecyl acetate			+			Blum et al., 2000
Farnesyl acetate			+			Blum et al., 2000
Decane			+			Blum et al., 2000
Isopentyl propionate			+#			Blum et al., 2000
Palmitic acid			+			Blum et al., 2000
Stearic acid			+			Blum et al., 2000
Gamma-octanoic lactone			#		+	Blum et al., 2000
Napthalene			+			Blum et al., 2000
Decanal			#			

[1]Synonymous with isoamyl acetate and iso-amyl acetate.
[2]Synonymous with 2-decen-1-yl-acetate (Koeniger et al., 1979; Veith et al., 1978) and 1-acetoxy-2-decene (Blum et al., 2000).
+, compound reported in a previous study; #, compound reported in this study (includes all pheromones produced during alarm); s, elicits stinging; a, elicits attraction; e, extended the duration of other alarm components; c, carrier of other active alarm pheromones; r, attracts recruits.

The Journal of Experimental Biology (2014) doi:10.1242/jeb.110171

Table 3. Forager landing preferences for different olfactory compounds

	Aversion to array			Forager landing choices		
Test substance	Percent of approaching bees that departed without landing	*N* (total no. approaching bees)	Percent of landing bees that chose the test inflorescence	χ_1^2	*P*	*N* (total no. landing bees)
Natural alarm pheromone	49.3	284	22.9	42.25	<<0.00001	144
Synthetic mixture	46.4	267	25.9	33.29	<<0.00001	143
Gamma-octanoic lactone	35.5	287	31.4	25.74	<<0.00001	185
Isopentyl acetate	33.2	301	36.8	13.98	0.0002	201
(E)-2-decenyl acetate	33.3	288	40.1	7.52	0.006	192
Isopentyl propionate	23.8	294	47.3	0.64	0.42	224
Octyl acetate	24.1	291	48.9	0.11	0.74	221
3-Methyl-1-butanol	20.3	315	48.6	0.20	0.66	251

For each test substance, bees chose between two inflorescences, one with the test substance in hexane and a control inflorescence with an equal volume of hexane only. The choice of each bee was recorded in the absence of other bees near or on the inflorescences and every bee (including ones that approached but did not land) was captured so that each bee was counted only once.

10 min, the duration of our trials. Using gas chromatography–mass spectrometry (GC–MS) analysis, we identified eight chemical components in the alarm pheromone, of which three components (1-octanol, decanal and gamma-octanoic lactone) have not previously been identified in this species. GC–MS analyses of hexane extracts of sting glands would probably have yielded more compounds, but we used SPME to focus on airborne volatiles. We then tested six major compounds [3-methyl-1-butanol, isopentyl acetate, isopentyl propionate, octyl acetate, gamma-octanoic lactone and (E)-2-decen-1-yl acetate] and found that a synthetic mixture of these compounds (in the average ratio identified in our GC–MS analyses) elicited behaviors statistically indistinguishable from forager responses to an alarmed conspecific.

We show that gamma-octanoic lactone, isopentyl acetate and (E)-2-decen-1-yl acetate are active compounds and act like natural alarm pheromone (Table 3). It is possible that foragers also respond to other alarm pheromone compounds, but these three compounds are sufficient to elicit forager aversion. Of all the compounds tested, gamma-octanoic lactone appears to be the most effective. It elicited a landing aversion response that was not significantly different from the response to natural alarm pheromone (Fig. 2). Isopentyl propionate, octyl acetate and 3-methyl-1-butanol did not repel bees from landing (Table 3). Responses to these compounds were not significantly different from responses to the hexane-only control (Fig. 2). These reactions were probably not nestmate-specific because bees visiting our inflorescences probably came from multiple nearby colonies. Moreover, in the context of defence, honey bee alarm pheromone can elicit a general defensive response from nearby colonies, not just from the colony of the attacked bee (Graham, 1992).

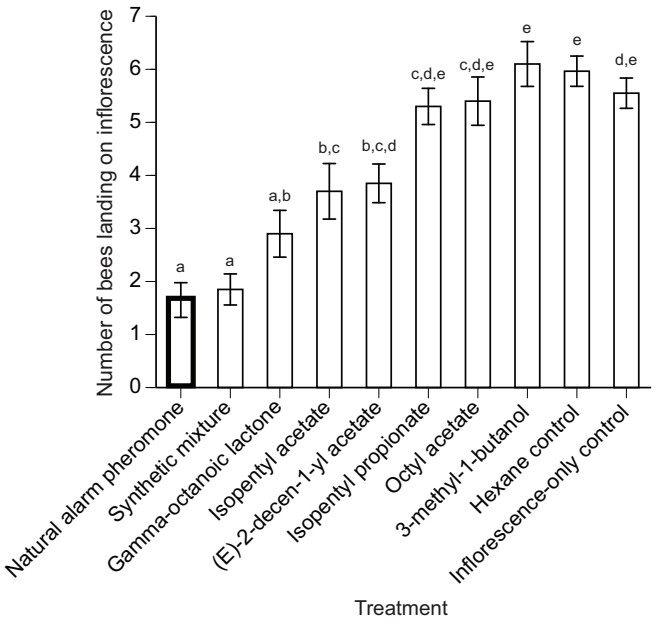

Fig. 2. The number of landing foragers on control and treatment inflorescences (mean values with standard error bars). Responses to natural alarm pheromone are highlighted with a thicker rectangle. In each test, foragers chose between two inflorescences, one with the test substance and one control. Table 1 shows the proportion choosing the test inflorescence. This figure shows the number of bees that landed on each inflorescence, which allows comparisons between the different olfactory treatments. Different letters indicate significant differences (Tukey's HSD test, $P<0.05$).

We identified three new compounds not previously reported in *A. dorsata* alarm pheromone (Table 2). It is possible that some of these compounds, particularly those with low relative abundance, were not part of the alarm pheromone but were volatiles produced by other bee body parts. However, gamma-octanoic lactone was produced in high relative abundance (Table 1) and therefore may be part of the alarm pheromone. With respect to the two *A. dorsata* alarm compounds, 1-octanol is found in the sting alarm pheromone of *A. mellifera* (Collins and Blum, 1982; Wager and Breed, 2000), and decanal has been detected in adult *A. mellifera* workers (Torto et al., 2005).

Changes in inflorescence visual appearance are unlikely to account for our results. When we applied the synthetic alarm pheromone, we were careful to not bruise the inflorescence and did not touch it with the tip of our pipette. When we applied natural alarm pheromone, we took care to prevent the struggling bee from bruising the inflorescence during alarm pheromone deposition pheromone. It is possible that the appearance or natural odor of the test inflorescence was slightly altered by contact with the struggling bee, not by the bee's alarm pheromone. Nonetheless, bees exhibited the same statistically indistinguishable level of landing avoidance to natural and synthetic alarm pheromone (Fig. 2), even though no bee made physical contact with the inflorescence in the synthetic alarm pheromone tests.

Other bee-deposited odor marks probably did not affect our overall results. Bees can leave odor marks that are repellant or attractive (Saleh et al., 2007; Yokoi and Fujisaki, 2009; Llandres et al., 2013). To exclude prior odor-marking, we enclosed all inflorescences in bags while they were still buds, before they produced nectar and would have attracted bees. We only removed the bags right before a test. During a trial, we immediately removed bees as soon as they landed, but it possible that they released odors when they landed. Although landing bees or the process of capturing bees may have produced odors that influenced the choices of subsequent bees, there are

nonetheless strong, significant differences between the number of bees choosing the different treatments, even though all trials were conducted under similar, standardized conditions.

Finally, bee avoidance of our test odors probably does not arise from avoidance of inflorescences that smelled differently because of our treatments. Bees rejected specific olfactory compounds that we added, but did not reject isopentyl propionate, octyl acetate or 3-methyl-1-butanol (Table 3, Fig. 2). Honey bees do not necessarily reject food sources when novel odors are subtracted or added. Heiling et al. (Heiling et al., 2003) showed that honey bees continued to visit flowers at the same rate even when floral odors were removed. Suwannapong et al. (Suwannapong et al., 2011) added different compounds (identified from honey bee mandibular glands) to flowers and reported attraction, repulsion or no significant behavioral changes depending upon the compound and its concentration. Likewise, Goodale and Nieh (Goodale and Nieh, 2012) showed that honey bees could smell and avoid the odor of conspecific haemolymph but did not avoid the odor of bumble bee hemolymph added to a food source.

Gamma-octanoic lactone

We will now consider each of the major alarm compounds in order of response strength. Gamma-octanoic lactone elicited the greatest response of any single compound (Fig. 2). The landing aversion response elicited by this compound was indistinguishable from forager responses to natural alarm pheromone (Fig. 2). It is also one of the most abundant compounds that we identified in *A. dorsata* alarm pheromone (Table 1). This compound was first reported in the cephalic extracts of *A. laboriosa*, but was not previously found in the alarm pheromone of *A. dorsata* (Blum et al., 2000). It has not been reported in any other honey bee species.

It is possible that Blum et al. (Blum et al., 2000) did not previously identify gamma-octanoic lactone in *A. dorsata* alarm pheromone because of methodological differences. We used SPME analysis of the total volatile odors produced by alarmed *A. dorsata* foragers. The advantage of this approach is that it captures all of the volatile odors that bees release when they are alarmed, not just the components of a single gland. Our analysis could therefore include other components produced during alarm such as compounds produced by cephalic glands. Blum et al. (Blum et al., 2000) dissected out sting shafts of *A. dorsata* workers into a solvent and then analysed the resulting mixture. Interestingly, Blum et al. (Blum et al., 2000) found gamma-octanoic lactone in *A. laboriosa* cephalic extracts, although they did not find this compound in *A. dorsata* cephalic extracts. There may also be differences in the alarm pheromone components of different *A. dorsata* populations. We used *A. dorsata* foragers collected in Xishuangbanna, China, and Blum et al. (Blum et al., 2000) used *A. dorsata* workers from Nepal.

Isopentyl acetate

This compound has now been identified in the alarm pheromone of every *Apis* species whose alarm pheromone has been analysed, with the exception of *A. laboriosa*, and is therefore the most commonly found sting pheromone compound in *Apis*. Isopentyl acetate also occurs in the sting gland pheromone of *A. mellifera*, *A. cerana*, *A. dorsata* and *A. florea* (Boch et al., 1962; Morse et al., 1967). Of these four species, *A. dorsata* produces the highest amount of isopentyl acetate per bee (Koeniger et al., 1979). In *A. mellifera*, *A. florea* and *A. cerana*, this compound can elicit attacks when provided within 60 cm of the nest (Morse et al., 1967). Koeniger et al. (Koeniger et al., 1979) reported that *Apis dorsata* foragers will attack a moving ball placed near the nest and baited with isopentyl acetate. At a floral resource, isopentyl acetate strongly repulsed *A. dorsata* foragers from landing (Table 3, Fig. 2).

(E)-2-decen-1-yl acetate

Blum et al. (Blum et al., 2000) reported that the major component of sting shaft extracts of *A. dorsata* workers was 1-acetox-2-decene [synonymous with the 2-decen-1-yl acetate reported by Koeniger et al. (Koeniger et al., 1979) and Veith et al. (Veith et al., 1978)]. The correct name for this compound is (E)-2-decen-1-yl acetate (J. Millar, personal communication). Veith et al. (Veith et al., 1978) first discovered this compound in *A. dorsata* and *A. florea* workers, and in both species it elicits aggression when placed on a bait near the nest (Koeniger et al., 1979). Our study tested forager responses, and (E)-2-decen-1-yl acetate repelled foragers from landing on an inflorescence (Table 3, Fig. 2).

Isopentyl propionate

Finally, our study and Blum et al. (Blum et al., 2000) also identified a minor component, isopentyl propionate, in *A. dorsata* sting alarm pheromone. However, this compound did not elicit aversion. In our paired choice test, 47% of foragers landed on the inflorescence with this compound, a pattern not significantly different from random (P=0.42; Table 3).

Conclusions

We suggest that alarm pheromones evolved primarily in the context of colony defence, not for warning foragers at a food source, because the colony is the unit of selection and the likelihood of encountering a nestmate, even in species that communicate food location, is lower at an inflorescence than near a nest. However, even if the primary benefit of alarm pheromone signal production is colony defence, foragers from the same colony, other colonies of the same species, and even different species can benefit from sensing and using this information. Thus the simple, reflexive and reliable response of an attacked bee producing alarm pheromone, regardless of context, has broader implications for information flow. For example, *A. cerana*, *A. dorsata* and *A. florea* have overlapping distributions and share a common alarm pheromone component, isopentyl acetate (Table 2). Predation upon one species could therefore alert other species of danger and amplify the effects of a predator on pollination, a key ecosystem service provided by bees (Brittain et al., 2013). It would be interesting to test for such alarm pheromone eavesdropping. In the future, it would also be valuable to determine the alarm pheromone components of all *Apis* species for a detailed evolutionary analysis that considers biosynthetic pathways, behavior and phylogeny. Such knowledge can enhance our understanding of the ecology of information use (Schmidt et al., 2010) and how these alarm signals evolved.

MATERIALS AND METHODS

This research was conducted in full compliance with the laws of the People's Republic of China. No permits were required for our field studies. We used *A. dorsata*, an abundant, non-protected species.

Study species and site

The giant Asian honey bee *A. dorsata* ranges from western India throughout continental and oceanic Asia, including Sulawesi, Indonesia and the Philippines (Oldroyd and Wongsiri, 2006; Hepburn and Radloff, 2011). We conducted our study (January to April 2013 and December 2013 to January 2014) at the Xishuangbanna Tropical Botanical Garden in Menglun, China (21.921°N, 101.231°E), during the dry season, when *C. haematocephala* blooms. At this site, there was a 300 m^2 patch of *C. haematocephala* (Nevling and Elias, 1971) trees in bloom. We observed wild colonies of *A.*

dorsata foraging at this patch. The foragers probably came from ~40 colonies of *A. dorsata* located about 1 km away from our study site. Because we used naturally foraging bees, we could not determine how many different colonies came to our inflorescences. However, we conducted our studies for more than 1 year and used 20 different trees, leading to a high likelihood that we used bees from multiple colonies. We also captured all bees that approached our test array, and therefore recorded each bee's choice only once. Captured bees were subsequently frozen to eliminate potential pseudoreplication.

Alarm pheromone analysis

We separately analysed the alarm pheromone volatiles of 18 different bees. We used a small, clean glass bottle to capture each bee while it was foraging on an inflorescence. To elicit alarm, we clipped the bee's legs together with a clean metal binder clip. The bees soon began producing alarm pheromone. The advantage of this approach is that it captures all of the volatile odors that bees release when they are alarmed, not just the components of a single gland. For controls, we analysed the volatiles produced by 18 different non-alarmed bees. We also obtained these bees by using a small glass bottle to gently capture each bee as it foraged on an inflorescence. However, we did not clip the bee's legs together with a binder clip and took care to avoid agitating or disturbing the bee.

To obtain the odor, we waited 1 min and then quickly removed the bee from the vial to minimize odor loss. We adsorbed odors released into the glass bottle with a SPME fiber (75 µm CAR/PDMS, Supelco, Bellefonte, PA, USA) inserted into the glass bottle for 30 min. For analysis, this fiber was then desorbed in the gas chromatograph inlet at 220°C for 5 min. We used an HP 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA, USA), equipped with a HP-5MS column (30 m×0.25 mm, 0.25 µm film thickness), and linked to an HP 5975C mass spectrometer (Agilent Technologies). Helium was used as a carrier gas at a flow of 1 ml min^{-1}, and the injector temperature was set to 260°C. Column temperature was 40°C and held for 1 min, increased to 80°C at a rate of 3°C min^{-1}, and then finally increased to 220°C at a rate of 5°C min^{-1}. We used the NIST08 MS library to identify possible compounds and then confirmed these compounds by comparing the GC retention times and MS of the alarm pheromone samples with those of authentic compounds (typical chromatogram shown in Fig. 1, average data shown in Table 1).

To estimate the average amount of each compound per forager, we used an average of published values of isopentyl acetate (20 µg) per *A. dorsata* forager (Morse et al., 1967; Koeniger et al., 1979). For all other tested compounds, we then calculated the estimated amount per bee based upon the relative amount (RA) ratios. For example, $RA_{\text{gamma-octanoic lactone}}/RA_{\text{isopentyl acetate}}=0.323$ (Table 1). Thus we estimated the amount of gamma-octanoic lactone to be 0.323×20 µg=6.46 µg per bee. We used these estimates to determine the amount of each compound to use in our floral choice tests.

Floral choice test

From 20 trees, we randomly chose two inflorescences per tree. To obtain inflorescences that contained nectar and no odor marks, we placed mesh bags around *C. haematocephala* inflorescences while they were still buds and waited ~3 days until they became mature inflorescences. To ensure that the trees were ant free, we carefully inspected them before and after we applied ant-excluding rings of sticky Tanglefoot™ resin around the trunks and branches. We used paired choice tests between a treatment and a control inflorescence, following standard designs for testing bee foraging choices (Dukas, 2001; Abbott, 2006). There are two types of control treatments: inflorescence-only control (paired with the natural alarm pheromone treatment) and hexane control (paired with the synthetic compound treatments).

When the inflorescences were fully mature, we used them in a paired choice test by cutting two off and placing one per tripod on two 1.5-m high tripods separated by 40 cm and placed ~1 m away from the tree. We chose this 40 cm distance based upon other studies, which have used paired food sources separated by 6–30 cm and found strong effects on bee orientation when one food source had signals or cues of predation (Dukas, 2001; Abbott, 2006; Goodale and Nieh, 2012; Tan et al., 2013).

Bees then chose between the inflorescences during a test that lasted for 10 min and was conducted between 13:00 and 16:00 h on sunny days with abundant and consistent foraging activity. We chose 10 min because it corresponded to a period in which a fairly constant number of *A. dorsata* visited the *C. haematocephala* inflorescences. We tested eight different treatments: natural alarm pheromone, synthetic alarm pheromone mixture and six synthetic alarm pheromone components. We conducted 20 trials per treatment. Each treatment was replicated with inflorescences from 20 different trees. Inflorescences from the same tree were used for all eight treatments. Each pair of inflorescences was used for only one 10 min trial period and was then discarded.

After setting out the test stimulus, we waited 2 min to allow the alarm pheromone to volatilize and the hexane solvent (in tests of chemical compounds) to fully evaporate. Only a few bees approached the inflorescences during the 2 min pre-trial interval. During this pre-trial interval, we used insect nets to capture any bees that approached and did not count them as part of the trial. We then began the 10 min trial period. To exclude the possibility of bees being influenced by the choices of other bees, we only counted choices made in the absence of other bees foraging near the array. There were abundant natural floral resources nearby. Thus it was rare for one bee to approach the array while another bee was choosing. In all experiments, the treatments consisted of an odor applied to the test inflorescence, and therefore no other bees were present when foragers made choices. The experimenters were blind to which chemical treatment they used during these behavioral tests. During these tests, ants were excluded from the trees, and no other predators or bee species approached the inflorescences.

We counted each bee choice only once. Bees that landed on inflorescences were immediately captured with an aspirator. Bees that investigated the array (approached within ~5 cm of one of the inflorescence) but did not land were captured with insect nets as they began to fly away. Such investigating bees generally flew slowly, and we could therefore capture them with nets. By capturing these approaching bees, we ensured that each approaching bee was a different bee and could therefore calculate the percentage of approaching bees that landed during the trial.

We first tested the effect of natural alarm pheromone on an inflorescence, simulating a predator catching a bee by its legs. We caught an *A. dorsata* forager with a net and used a clean metal binder clip to hold its legs. We then carefully positioned the bee on top of the test inflorescence. The bee immediately began producing alarm pheromone, and we allowed it to mark the inflorescence with alarm pheromone for 1 min. We then removed the bee, sealing it in a glass vial to exclude further alarm pheromone production. We took care to not alter the visual appearance of the inflorescence or to bruise it.

All subsequent experiments focused on forager responses to synthetic compounds. We analysed the chemical components of alarm pheromone (see above) and identified six major compounds that we separately tested: 3-methyl-1-butanol, isopentyl acetate, isopentyl propionate, octyl acetate, gamma-octanoic lactone and (E)-2-decen-1-yl acetate (Fig. 1, Table 1). We also tested a synthetic mixture of these compounds, using the estimated mean quantities per bee (Table 1). To the test inflorescence, we applied one bee-equivalent of the alarm pheromone mixture or one bee-equivalent of the tested individual compound. We obtained all compounds from Aladdin Reagent Database Inc. (Shanghai, China). The (E)-2-decen-1-yl acetate was kindly synthesized for us by J. Millar (University of California Riverside, Riverside, CA, USA).

Using a micropipette, we dispensed 10 µl of alarm pheromone compound (containing one bee-equivalent of the test compound, Table 1) onto the test inflorescence, and 10 µl hexane onto the control. We carefully applied just the fluid onto the inflorescence and did not bruise the inflorescence with the pipette tip. We waited 2 min to allow the hexane to evaporate and then began the 10 min trial. We used hexane because it is an excellent solvent commonly used in olfactory bioassays (Millar and Haynes, 1998). Hexane also rapidly evaporates. At the average air temperature of our trials (>30°C), hexane quickly evaporated because it has a vapor pressure >187.11 mmHg, nearly sixfold greater than the vapor pressure of water under the same conditions (Beyer, 1988). Moreover, honey bees (*A. mellifera*) are not perturbed by hexane and will choose a hexane control feeder over a test feeder with a sting gland extract (Goodale and Nieh, 2012).

Statistics

We used χ^2 tests to determine whether bees were attracted to the different olfactory compounds. Our null hypothesis is that equal numbers of bees will choose the control and treatment inflorescences in our paired choice tests. We then used ANOVA to compare the effect of different olfactory treatments and trial date (day of the year) on the number of bees landing on the different inflorescences. We counted each bee only once. We performed Tukey's HSD test to perform pairwise comparisons of all treatments (Zar, 1984). All data met parametric assumptions as determined through residuals analysis (Zar, 1984). We report means ± 1 s.d.

Acknowledgements

We would like to thank Jocelyn Millar for generously synthesizing (E)-2-decen-1-yl acetate for our experiments, and the Central Laboratory at Xishuangbanna Tropical Botanical Garden for technical support with the GC–MS analysis. We would also like to thank the editor and anonymous reviewers whose comments helped improve our manuscript.

Competing interests

The authors declare no competing financial interests.

Author contributions

J.L., Z.W., K.T. and J.C.N. conceived and designed the experiments. J.L., Z.W. and Y.Q. performed the experiments. K.T. and J.C.N. analyzed the data, and contributed reagents, materials, or analysis tools. J.L., Z.W., K.T. and J.C.N. wrote the paper.

Funding

This work was supported by the Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, and the CAS 135 program (XTBGT01) of Chinese Academy of Sciences, China National Research Fund (31260585) to K.T.

References

Abbott, K. R. (2006). Bumblebees avoid flowers containing evidence of past predation events. *Can. J. Zool.* **84**, 1240-1247.
Beyer, W. H. (1988). *CRC Handbook of Chemistry and Physics*. Boca Raton, FL: CRC Press.
Billen, J. and Morgan, E. D. (1998). Pheromone communication in social insects: sources and secretions. In *Pheromone Communication in Social Insects* (ed. R. K. Vander Meer, M. D. Breed, K. E. Espelie and M. L. Winston), pp. 3-33. Boulder, CO: Westview Press.
Blum, M. S. (1969). Alarm pheromones. *Annu. Rev. Entomol.* **14**, 57-80.
Blum, M. S., Fales, H. M., Morse, R. A. and Underwood, B. A. (2000). Chemical characters of two related species of giant honeybees (*Apis dorsata* and *A. laboriosa*): possible ecological significance. *J. Chem. Ecol.* **26**, 801-807.
Boch, R. and Shearer, D. A. (1971). Chemical releasers of alarm behaviour in the honey-bee, *Apis mellifera*. *J. Insect Physiol.* **17**, 2277-2285.
Boch, R., Shearer, D. A. and Stone, B. C. (1962). Identification of isoamyl acetate as an active component in the sting pheromone of the honey bee. *Nature* **195**, 1018-1020.
Brittain, C., Williams, N., Kremen, C. and Klein, A.-M. (2013). Synergistic effects of non-*Apis* bees and honey bees for pollination services. *Proc. Biol. Sci.* **280**, 20122767.
Collins, A. M. and Blum, M. S. (1982). Bioassay of compounds derived from the honeybee sting. *J. Chem. Ecol.* **8**, 463-470.
Corlett, R. T. (2011). Honeybees in natural ecosystems. In *Honeybees of Asia* (ed. R. Hepburn and S. Randall), pp. 215-225. Berlin: Springer-Verlag.
Couvillon, M. J., Barton, S. N., Cohen, J. A., Fabricius, O. K., Kärcher, M. H., Cooper, L. S., Silk, M. J., Helanterä, H. and Ratnieks, F. L. W. (2010). Alarm pheromones do not mediate rapid shifts in honey bee guard acceptance threshold. *J. Chem. Ecol.* **36**, 1306-1308.
Dukas, R. (2001). Effects of perceived danger on flower choice by bees. *Ecol. Lett.* **4**, 327-333.
Ferguson, A. W. and Free, J. B. (1979). Production of a forage-marking pheromone by the honeybee. *J. Apic. Res.* **18**, 128-135.
Fortunato, A., Dani, F. R., Sledge, M. F., Fondelli, L. and Turillazzi, S. (2004). Alarm communication in *Ropalidia* social wasps. *Insectes Soc.* **51**, 299-305.
Free, J. B. (1987). *Pheromones of Social Bees*. London: Chapman and Hall.
Free, J. B., Pickett, J. A., Ferguson, A. W., Simpkins, J. R. and Smith, M. C. (1985). Repelling foraging honeybees with alarm pheromones. *J. Agric.Sci.* **105**, 255-260.
Goodale, E. and Nieh, J. C. (2012). Public use of olfactory information associated with predation in two species of social bees. *Anim. Behav.* **84**, 919-924.
Graham, J. M. (1992). *The Hive and the Honey Bee*. Hamilton, IL: Dadant & Sons.
Heiling, A. M., Herberstein, M. E. and Chittka, L. (2003). Pollinator attraction: crab-spiders manipulate flower signals. *Nature* **421**, 334-334.
Hepburn, H. R. and Radloff, S. E. (2011). Biogeography. In *Honeybees of Asia* (ed. R. Hepburn and S. Randall), pp. 51-67. Berlin: Springer-Verlag.
Hepburn, H. R., Jones, G. E. and Kirby, R. (1994). Introgression between *Apis mellifera capensis* Escholtz and *Apis mellifera scutellata* Lepeletier: the sting pheromones. *Apidologie (Celle)* **25**, 557-565.
Holldobler, B. and Wilson, E. O. (1990). *The Ants*. Cambridge, MA: Belknap Press of Harvard University.
Koeniger, N., Weiss, J. and Maschwitz, U. (1979). Alarm pheromones of the sting in the genus *Apis*. *J. Insect Physiol.* **25**, 467-476.
Landolt, P. J., Reed, H. C. and Heath, R. R. (1999). An alarm pheromone from heads of worker *Vespula squamosa* (Hymenoptera: Vespidae). *Fla. Entomol.* **82**, 356-359.
Li, J., Wang, Z., Tan, K., Qu, Y. and Nieh, J. C. (2014). Giant Asian honey bees use olfactory eavesdropping to detect and avoid ant predators. *Anim. Behav.* (in press).
Llandres, A. L., Gonzálvez, F. G. and Rodríguez-Gironés, M. A. (2013). Social but not solitary bees reject dangerous flowers where a conspecific has recently been attacked. *Anim. Behav.* **85**, 97-102.
Llandres, A. L. and Rodríguez-Gironés, M. A. (2011). Spider movement, UV reflectance and size, but not spider crypsis, affect the response of honeybees to Australian crab spiders. *PLoS ONE* **6**, e17136.
Maschwitz, U. (1964). Gefahrenalarmstoffe und gefahrenalarmierung bei sozialen hymenopteren. *Z. Vgl. Physiol.* **47**, 596-655.
Millar, J. G. and Haynes, K. F. (1998). *Methods in Chemical Ecology: Bioassay Methods*. New York, NY: Chapman and Hall.
Morse, R. A., Shearer, D. A., Boch, R. and Benton, A. (1967). Observations on alarm substances in the genus *Apis*. *J. Apic. Res.* **6**, 113-118.
Nevling, L. I. and Elias, T. S. (1971). Calliandra haematocephala: history, morphology, and taxonomy. *J. Arnold Arbor.* **52**, 69-85.
Nieh, J. C. (1998). The role of a scent beacon in the communication of food location by the stingless bee, *Melipona panamica*. *Behav. Ecol. Sociobiol.* **43**, 47-58.
Nieh, J. C. (2004). Recruitment communication in stingless bees (Hymenoptera, Apidae, Meliponini). *Apidologie (Celle)* **35**, 159-182.
Nieh, J. C. (2010). A negative feedback signal that is triggered by peril curbs honey bee recruitment. *Curr. Biol.* **20**, 310-315.
Oldroyd, B. P. and Wongsiri, S. (2006). *Asian Honey Bees: Biology, Conservation, and Human Interactions*. Cambridge, MA: Harvard University Press.
Papachristoforou, A., Kagiava, A., Papaefthimiou, C., Termentzi, A., Fokialakis, N., Skaltsounis, A.-L., Watkins, M., Arnold, G. and Theophilidis, G. (2012). The bite of the honeybee: 2-heptanone secreted from honeybee mandibles during a bite acts as a local anaesthetic in insects and mammals. *PLoS ONE* **7**, e47432.
Partap, U. (2011). The pollination role of honeybees. In *Honeybees of Asia* (ed. R. Hepburn and S. Randall), pp. 227-255. Berlin: Springer Verlag.
Pickett, J. A., Williams, I. H. and Martin, A. P. (1982). (Z)-11-eicosen-1-ol, an important new pheromonal component from the sting of the honey bee, *Apis mellifera* L. (Hymenoptera, Apidae). *J. Chem. Ecol.* **8**, 163-175.
Pirk, C. W. W., Sole, C. L. and Crewe, R. M. (2011). Pheromones. In *Honeybees of Asia* (ed. R. Hepburn and S. Randall), pp. 207-214. Berlin: Springer-Verlag.
Roubik, D. W. (1989). *Ecology and Natural History of Tropical Bees*. New York, NY: Cambridge University Press.
Saleh, N., Scott, A. G., Bryning, G. P. and Chittka, L. (2007). Distinguishing signals and cues: bumblebees use general footprints to generate adaptive behaviour at flowers and nest. *Arthropod-Plant Interact.* **1**, 119-127.
Schmidt, J. O., Morgan, E. D., Oldham, N. J., Do Nascimento, R. R. and Dani, F. R. (1997). (Z)-11-eicosen-1-ol, a major component of *Apis cerana* venom. *J. Chem. Ecol.* **23**, 1929-1939.
Schmidt, K. A., Dall, S. R. X. and van Gils, J. A. (2010). The ecology of information: an overview on the ecological significance of making informed decisions. *Oikos* **119**, 304-316.
Schorkopf, D. L. P., Hrncir, M., Mateus, S., Zucchi, R., Schmidt, V. M. and Barth, F. G. (2009). Mandibular gland secretions of meliponine worker bees: further evidence for their role in interspecific and intraspecific defence and aggression and against their role in food source signalling. *J. Exp. Biol.* **212**, 1153-1162.
Suwannapong, G., Seanbualuang, P. and Benbow, M. E. (2011). Using sensillum potential analysis to quantify pheromone sensing of the antennal sensilla of *Apis florea* Fabricius (1787), foragers and guards. *J. Asia Pac. Entomol.* **14**, 7-10.
Tan, K., Hu, Z., Chen, W., Wang, Z., Wang, Y. and Nieh, J. C. (2013). Fearful foragers: honey bees tune colony and individual foraging to multi-predator presence and food quality. *PLoS ONE* **8**, e75841.
Torto, B., Suazo, A., Alborn, H., Tumlinson, J. H. and Teal, P. E. (2005). Response of the small hive beetle (*Aethina tumida*) to a blend of chemicals identified from honeybee (*Apis mellifera*) volatiles. *Apidologie (Celle)* **36**, 523-532.
Vallet, A., Cassier, P. and Lensky, Y. (1991). Ontogeny of the fine structure of the mandibular glands of the honeybee (*Apis mellifera* L.) workers and the pheromonal activity of 2-heptanone. *J. Insect Physiol.* **37**, 789-804.
Veith, H. J., Weiss, J. and Koeniger, N. (1978). A new alarm pheromone (2-decen-1-yl-acetate) isolated from the stings of *Apis dorsata* and *Apis florea* (Hymenoptera: Apidae). *Experientia* **34**, 423-424.
Wager, B. R. and Breed, M. D. (2000). Does honey bee sting alarm pheromone give orientation information to defensive bees? *Ann. Entomol. Soc. Am.* **93**, 1329-1332.
Wongsiri, S., Chanchao, C., Lekprayoon, C., Wattanasermkit, K., Deowanish, S. and Leepitakrat, S. (2001). Honeybee diversity and management in the new millennium in Thailand. In *Proceedings of the 7th IBRA Conference on Tropical Bees: Management and Diversity & Fifth Asian Apicultural Association Conference,* 19–25 March 2000, Chiangmai, Thailand, pp. 9-14.
Yokoi, T. and Fujisaki, K. (2009). Recognition of scent marks in solitary bees to avoid previously visited flowers. *Ecol. Res.* **24**, 803-809.
Zar, J. H. (1984). *Biostatistical Analysis*. Englewood Cliffs, NJ: Prentice-Hall.