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Original article

Small hive beetle, *Aethina tumida* (Coleoptera: Nitidulidae): chemical profile of the cuticle and possible chemical mimicry in a honeybee (*Apis mellifera*) pest

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Abstract – The small hive beetle, *Aethina tumida* (Coleoptera: Nitidulidae), is an economically important pest of the Western honeybee, *Apis mellifera* (Hymenoptera: Apidae). We investigated the effect of rearing environment on the cuticular chemical profile of adult *A. tumida*, using hexane to extract the hydrocarbons and other compounds from the cuticles of beetles. Beetles were collected from *A. mellifera* colonies in Australia as well as reared in single sex laboratory cultures on different diets. We investigated whether rearing environment (laboratory vs. field, different apiaries, access to mating partners, diet) had any effect on cuticular hydrocarbons. Coupled gas chromatography–mass spectrometry analyses of the extracts showed that rearing environment had significant qualitative and quantitative effects on the hydrocarbons detected. The data support the hypothesis that cuticular profiles of *A. tumida* are contingent on environment, partitioning on the basis of rearing diet and source hives. The finding has implications for the regulation of interactions between *A. tumida* and honeybees and improvements in targeting of management strategies.

Cuticular hydrocarbons / Chemical mimicry / Gas chromatography-mass spectrometry / Honeybee pest

1. INTRODUCTION

Insect cuticular hydrocarbons (CHCs) are long-chain hydrocarbons that function to prevent desiccation, can act as mating cues, and can be important determinants of species recognition; they are mainly alkanes, alkenes, and methyl-branched alkanes (Blomquist and Bagnères 2010; Carlson et al. 1971; Chung and Carroll 2015). The cuticle may also hold other compounds of ecological and biological significance, such as lipids including wax esters, sterol esters, ketones, alcohols, aldehydes, and acids (Blomquist and Bagnères 2010). For example, aldehydes account for 2% of the cuticular lipids of the cabbage seed weevil *Ceutorhynchus assimilis* (Paykull) (Coleoptera: Curculionidae) (Richter and Krain 1980), and in the pecan weevil *Curculio caryae* (Horn) (Coleoptera: Curculionidae), odd-chain methylketones represent 1%

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and 3% of adult male and female cuticular lipids, respectively (Espelie and Payne, 1991).

CHC composition between conspecifics can vary for several reasons. First, the profiles may differ between field-collected and laboratory-reared insects; for example wild *Drosophila mojavensis* Patterson (Diptera: Drosophilidae) have fewer hydrocarbons than laboratory-reared flies overall and differ in the relative amounts of several components (Toolson et al. 1990). Moreover, significant behavioural and physiological differences between laboratory and field-collected individuals have been reported in several insect species (e.g. van Zweden et al 2009); these findings are especially relevant to biological control programs as cuticular compounds can be involved in host recognition by natural enemies (Huettel 1976; Huho et al. 2007; Sivinski et al. 1989).

Second, CHC composition can be influenced by diet and environment. In the Argentine ant *Linepithema humile* (Mayr) (Hymenoptera: Formicidae), hydrocarbons used in nest mate recognition can be acquired from insect prey (Liang and Silverman 2000). In *Apis mellifera* L. (Hymenoptera: Apidae), a dietary change from sucrose to pollen or vice versa produced significant changes in cuticular hydrocarbons (Francis et al. 1989). Also in *A. mellifera*, wax combs mediate nest mate recognition, as do floral scents (Breed et al. 1988; D'ettorre et al. 2006). Breed et al. (1995) found that cues from wax comb can be acquired by bees in 5 min or less, and Breed et al. (1988) suggest that wax comb in the colony and the bee's outer layer of cuticle are a continuous medium for any carbon-soluble compounds used as nest mate recognition.

Third, there can be sex-dependent differences in CHC profiles. This may be due to chemical communication in mate finding and mate choice, copulatory stimulants, and short-range attractants. This occurs in the Colorado beetle *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) (Dubis et al. 1987) as well as in two *Chrysochus* spp. Gebler (Peterson et al. 2007), mediating male mate choice and reproductive isolation. CHC composition also can differ depending on the mated status of an insect. In *Drosophila melanogaster* Meigen (Diptera: Drosophilidae), mating produces dramatic changes in CHC profiles of both male and female flies (Everaerts et al. 2010).

Another important role of cuticular odours sometimes seen is that of chemical mimicry: one species taking on an odour profile of another species. The queen Cuckoo ant *Leptothorax kutteri* Buschinger (Hymenoptera: Formicidae) grooms the host *Leptothorax acervorum* (F.) (Hymenoptera: Formicidae), thereby acquiring the cuticular profile of its host, in particular the cuticular fatty acids, and in so doing is accepted as a nest mate (Franks et al. 1990). The paper wasp parasite *Polistes sulcifer* (Zimmermann) (Hymenoptera: Vespidae) usurps the host *Polistes dominula* (Christ) queen and adopts a colony-specific odour facilitating their acceptance by host females (Sledge et al. 2001). Of course, it is important to note that both of these examples are species of the same genus. In the bee louse *Braula coeca* Nitzsch (Diptera: Braulidae), the CHC profile mirrors that of the host honeybees, even down to small colony differences in their alkene isomer patterns (Martin and Bayfield 2014), and *Varroa destructor* Anderson & Trueman (Mesostigmata: Varroidae) demonstrates passive chemical mimicry of its host's odour (Kather et al. 2015).

The small hive beetle, *Aethina tumida* Murray (Coleoptera: Nitidulidae), is a pest of Western honeybees (*Apis mellifera* L.) (Hymenoptera: Apidae) and is economically significant in the USA and Australia (Neumann et al. 2016). The beetle has also been reported to invade bumblebee, stingless bee, and solitary bee nests (Gonthier et al. 2019; Hoffmann et al. 2008; Neumann et al. 2016). This invasive insect, which originates in sub-Saharan Africa (Lundie 1940), is now recorded on every continent except Antarctica (e.g. Al Toufailia et al. 2017; Cervancia et al. 2016; Hassan and Neumann 2008; Lee et al. 2017; Mostafa and Williams 2002; Muli et al. 2018; Ritter 2004). *Aethina tumida* beetles have been shown to be associated with a yeast, *Kodamaea ohmeri* (Etchells & Bell) Yamada et al. (Saccharomycetales: Saccharomycetaceae), at every life stage (Amos et al, 2018). Adult *A.*

tumida are attracted to *A. mellifera* hive odours including the honeybee alarm pheromone isopentyl acetate (Torto et al. 2007), and, as active fliers, they can travel up to 10 km (Neumann and Elzen 2004), providing an ability to source new hives for invasion. However, host honeybee colony choice is not well understood, and *A. tumida* have been recorded to undertake long-range flights rather than enter nearby host colonies (Neumann et al. 2012). Aggregation of small hive beetle is known to be important for mating success (Mustafa et al 2015).

Once inside the hive, female *A. tumida* lay eggs in brood cells and in crevices in the brood and pollen comb. Eggs hatch after 3 days and larvae feed on brood, honeycomb, and pollen stores; final instar larvae leave the hive to pupate in the soil (Lundie 1940; Neumann et al. 2013).

Host honeybee behaviour towards *A. tumida* has been studied extensively (Atkinson and Ellis 2011; Ellis et al. 2003a, 2002a, 2003b; Hepburn and Radloff 1998; Neumann et al. 2015). Trophallaxis involving *A. tumida* has been recorded in *A. mellifera* colonies when host bees socially encapsulate *A. tumida* adults by corralling them and trapping them in resin 'prisons' (Ellis et al. 2003a, 2002a). *Aethina tumida* trophallactic solicitation ('begging', i.e. obtaining a drop of food from a donor bee) has an overall high degree of success (~ 50%), and it has been reported to be an innate behaviour that can be influenced by both sex and experience (Neumann et al. 2015). However, it remains unclear whether there is a chemical basis to *A. tumida* trophallactic solicitation and other interactions.

The CHC profile of *A. tumida* has not been studied previously, and as such, any role that these compounds might have in communication is unexplored. Kollmann et al. (2016) report that the antennal lobes of *A. tumida* are large in relation to other brain areas; this and the specialised lifestyle of *A. tumida* suggest that olfaction plays a crucial role in the species. Neumann et al. (2015) note that the fact that *A. tumida* can be attacked by host honeybees does not preclude olfactory mimicry that might occur in parallel and recommend further investigation. The current work describes the cuticular profile of *A. tumida* to answer a range of questions:

1. Does the CHC profile of the beetles differ between laboratory-reared and field collected beetles?
2. Does the apiary of collection affect the CHC profile of field-collected beetles?
3. Are there any effects of rearing diet or mated status on small hive beetle CHCs?
4. If CHC profiles differ, does this affect the behaviour of beetles, especially with respect to aggregation?

Understanding chemical communication in *A. tumida* can inform pest management of this species. It also has relevance to research of this species using laboratory culture, when compared with field populations, and more broadly, this study contributes to our understanding of social insect pests and adaptive chemical mimicry.

2. MATERIALS AND METHODS

2.1. *Aethina tumida* laboratory rearing

Laboratory-reared *A. tumida* were maintained as described by Cribb et al. (2013). All insects were maintained at 27 °C and R H 65%; adult insects were maintained at light/dark, 12:12 h, while larvae and pupae were reared in continuous darkness. To obtain individual, unmated beetles, final instar larvae were retrieved from the culture and placed individually in vials (5 mL) filled with sandy soil (7 g) (moistened to 5% w/w) and then incubated in darkness. Freshly emerged adults were collected at the soil surface and sexed following Neumann et al. (2013).

2.2. Hexane wash extraction of adult *A. tumida* sourced from laboratory culture and apiaries

The chemicals on the cuticle of individual insects (total 40 female, 40 male from laboratory, 66 female, 59 male, and 22 unsexed individuals from six different apiary sites—see Table I for origins, rearing conditions, mating access, and sex) were extracted by submerging

Table I
Sources and details of *Aethina tumida* adults used for hexane wash extracts

Source of *A. tumida* adults		Rearing material	Mating status	Treatment	No./sex sampled
Laboratory culture		Hive products[a]	Unmated	Untreated (≤ 15 d since emergence)	20 ♂ 20 ♀
		Hive products[a]	Possibly mated	Untreated (≤ 15 d since emergence)	10 ♂ 10 ♀
		Pollen dough[b]	Unmated	Untreated (≤ 15 d since emergence)	10 ♂ 10 ♀
Collected from *A. mellifera* hives	Esk 27.23° S 152.42° E	Hive products	Possibly mated	Untreated	11 ♂ 12 ♀
	Petrie 27.26° S 152.98° E	Hive products	Possibly mated	Untreated	12 ♂ 11♀
	Bellbowrie 27.55° S 152.89° E	Hive products	Possibly mated	Untreated	10 ♂ 7 ♀
				From 'slime-out'[c] hive	10 ♂ 10 ♀
	Gumdale 27.49° S 153.16° E	Hive products	Possibly mated	Untreated	10 ♂ 10 ♀
	Taree 31.89° S 152.44° E	Hive products	Possibly mated	Untreated	22 unsexed
	Richmond 33.60° S 150.75° E	Hive products	Possibly mated	Untreated	6 ♂ 16 ♀

[a]Honeycomb, brood comb, and pollen stores sourced from apiary at Bellbowrie

[b]Bee Build ™ produced by L. & P. Dewar, Kalbar, Queensland, Australia; sterilised by irradiation (8 kGy, 24 h) and stored at 4 °C

[c]Refers to the destruction of a hive by *A. tumida* with fermentation of hive materials and the presence of final instar larvae

single adult beetles in 99% n-hexane (RCI Labscan Ltd.) (200 µL; 14 min) and then vortexing them for a further 90 s. Pilot trials (data not presented) determined that using our analytical system, this was the minimum amount of time to wash insects in order to extract sufficient compound for detection and identification in GC–MS analysis of extracts. Despite this relatively long wash time, all compounds we detected appear to be cuticular compounds, based on previously reported CHC components (e.g. Blomquist and Bagnères 2010). We did not detect any compounds that are characteristic of glandular secretions or haemolymph.

Extracts were dried to ≈ 30 µL under nitrogen and samples (1 µL) analysed in a gas chromatograph (6890 Series: Agilent, Santa Clara, CA, USA) coupled to a mass spectrometer (Agilent 5975) (GC–MS) fitted with a silica capillary column (Agilent, model HP5-MS, 30 m × 250 µm ID × 0.25 µm film thickness). GC conditions for acquiring data were inlet temperature, 250 °C; carrier gas, helium at 51 cm/s; split ratio, 13:1; transfer-line temperature, 280 °C; initial temperature, 80 °C; rate, 20 °C/min to 180 °C then 8 °C/min; final temperature, 280 °C; and final time, 6 min. The MS was held at 280 °C in the ion source, scanned between 35 and 550 amu,

and the scan rate was 4.45 scans/s. A standard Retention Index Mixture (Sigma) was injected under the same conditions to allow calculation of Kovats Retention Index. Compounds were tentatively identified based on comparison to the NIST mass spectral library (version 2.0), Kovats Retention Indices (after van den Dool and Kratz 1963), and synthetic standards (Sigma). Mass spectra of peaks from different samples with the same retention time were compared to ensure that the compounds were indeed the same.

2.3. Hexane wash analysis

To ascertain whether the hexane washes extracted different cuticular compounds from beetles from different sources (e.g. field vs laboratory, different apiaries, diet, mating status), the presence of peaks in the chromatograms and their relative areas were fourth root transformed and analysed by non-parametric methods (Bray–Curtis cluster analysis). They were then visualised by a two-dimensional representation of non-metric multi-dimensional scaling (nMDS) ordination (Clarke 1993) to determine whether any differences could be detected among the samples. To determine if clusters of individual insect washes were significantly different from each other, an analysis of similarity (ANOSIM) was used. The ANOSIM tests are a range of Mantel-type permutations of randomisation procedures, which make no distributional assumptions. These tests depend only on rank similarities and thus are appropriate for these types of data. A similarity percentage (SIMPER) analysis was employed to investigate the relative contribution of each of the components to assign the individual insects to a priori determined groups to determine differences among groups and to assess similarity among individuals within each group. The software used for the multivariate analysis was PRIMER-E for Windows (V 7) (Clarke and Gorley 2015).

2.4. Behavioural assay

Bioassays were conducted to determine whether *A. tumida* origin influenced conspecific aggregation behaviour, do laboratory-reared and field collected beetles behave differently to beetles from the other environment? Aggregation is important in mating success in small

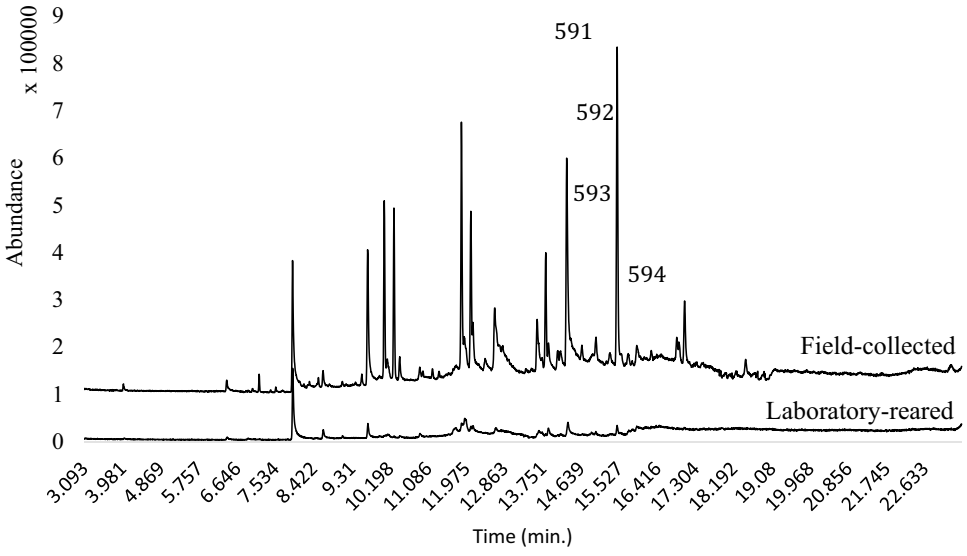


Figure 1. Total ion chromatograms contrasting hexane wash extracts from one exemplar laboratory-reared against one exemplar field-collected (Bellbowrie: 27.55° S, 152.89° E) *Aethina tumida* adult.

hive beetle (Mustafa et al 2015), and thus any differences in behaviour to field and laboratory beetles is important. In each trial, sixteen adult *A. tumida* were sedated by exposure to CO_2 for 15 s and placed in a glass Petri dish (15 cm diameter), and each beetle was only ever used once. The Petri dish was then positioned on a marked template that divided the base of the dish into eight equal-sized segments; two beetles were placed in each segment. The field-collected *A. tumida* adults used were collected from *A. mellifera* hives (Bellbowrie, Queensland, Australia) and used in assays within 2 h of collection. To attempt to control for the unknown age of field-collected beetles, they were size-matched with laboratory beetles. Preliminary tests showed that marking beetle elytra with nail varnish (approximately 50 µL, allowed to dry for 10 min after application) did not impede movement or have any observable impact on behaviour. Laboratory-reared beetles (≤ 15 days post emergence from soil) were marked to distinguish them from field-collected beetles, which were not marked. In each assay, eight field-collected beetles of one sex were placed with eight colony-reared beetles of the same sex; one beetle from each source was placed in each segment. Pilot studies showed that beetles would readily cluster with individuals of the same sex, so sexes were tested separately. The arena was evenly illuminated by two overhead lamps and a video camera (Canon© Legria HFM52 HD camcorder) used to record the movement of beetles for 15 min; the position of beetles was then monitored every 30 s after release by playing back the recording. In experiments, a 'cluster' was defined as at least five beetles within one beetle length (approx. 6 mm) of each other. The number of marked (laboratory) and unmarked (field-collected) beetles in clusters in the first 5 min was recorded. Petri dishes were washed, wiped with ethanol (100%, Sigma), and air-dried between each assay. The assay was repeated seven times using female beetles and six times with male beetles. In addition to this, assays using laboratory-reared beetles only were conducted to act as a control; this assay was repeated eight times using female beetles and eight times using male beetles. The mean time to form a cluster and the maximum cluster size over the 15 min period were analysed between the 50:50 laboratory/field treatment and the control using Mann–Whitney U tests using GraphPad Prism for Mac OS X (v 7.0c) (Ivanshchenko 2017).

Figure 2. a Non-metric multi-dimensional scaling ordination (nMDS) of hexane wash extracts of *A. tumida* sourced from laboratory culture or field-collected (six apiaries in Queensland and NSW, see Table I for locations) from honeybee (*Apis mellifera*) hives. **b** Non-metric MDS of hexane wash extracts of *A. tumida* sourced from six apiaries in Queensland and NSW, Australia (see Table I for locations) (ellipses diagrammatic and added as visual aid).

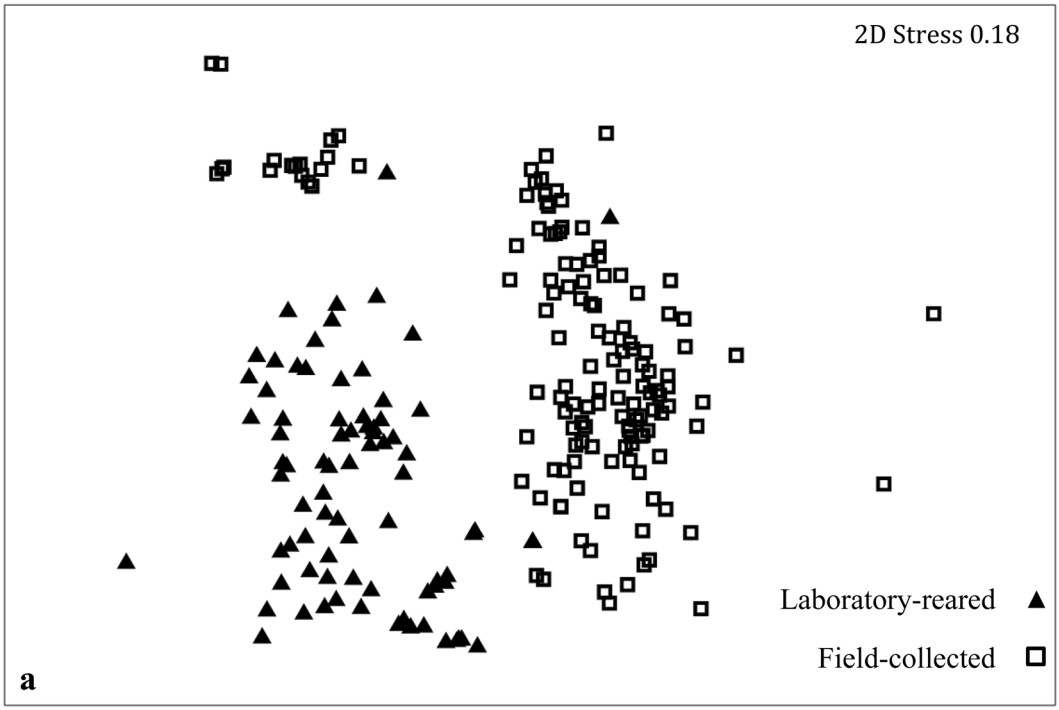
3. RESULTS

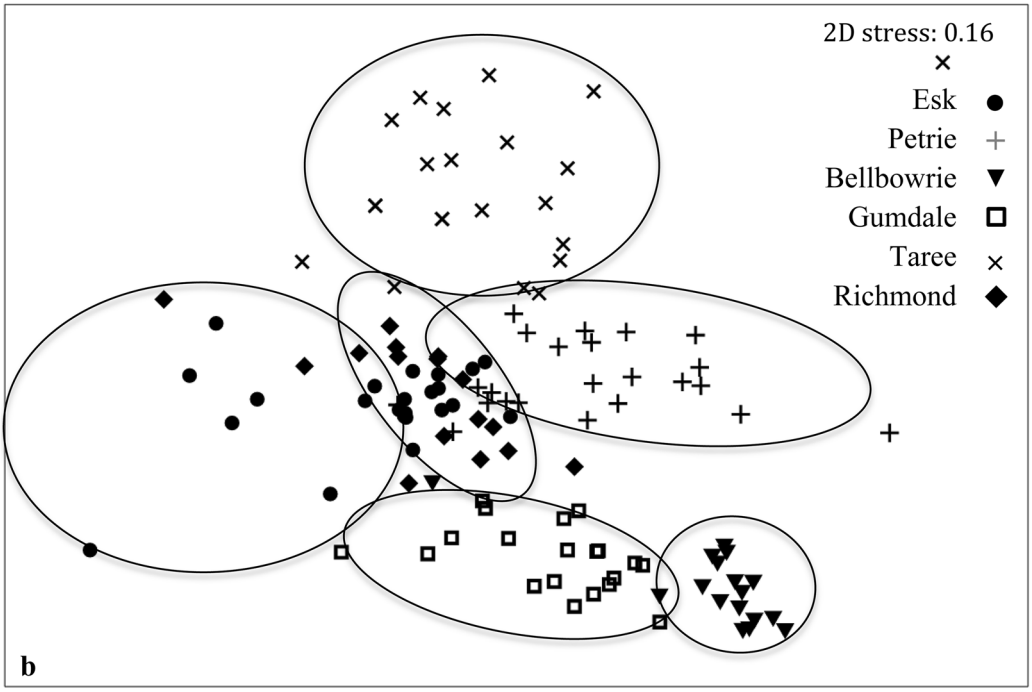
Irrespective of *A. tumida* origin or rearing conditions, the compounds identified in extracts from hexane washes of adult insects were primarily straight chain or methyl-branched saturated

Table II
Results and analysis of behavioural arena assays with laboratory-reared and field-collected (Bellbowrie: 27.55° S, 152.89° E) *Aethina tumida*

	Laboratory-reared[a] *A. tumida* ($n = 16$)	Laboratory-reared[a] + field-collected *A. tumida* ($n = 13$)	Analysis
Time (min/sec) to form cluster: mean (± SEM)	1:50 ± 0:16	2:30 ± 0:17	U = 66 P = 0.1
Largest cluster size: mean (± SEM)	12.06 ± 0.54	10.31 ± 0.58	U = 59.5 P = 0.05

[a]Laboratory-reared beetles fed on a diet of honeycomb, brood comb, and pollen stores sourced from apiary at Bellbowrie


2D Stress 0.18
Laboratory-reared
Field-collected
a


2D stress: 0.16
Esk
Petrie
Bellbowrie
Gumdale
Taree
Richmond
b

or mono-unsaturated long-chain hydrocarbons or oxygenated compounds, including aldehydes, esters, and alcohols (see Online Resource 1).

There was a significant difference between the components of the hexane wash extracts from insects from the laboratory culture and from the field (ANOSIM: $R=0.707$, $P=0.001$) (Figs. 1 and 2a; Online Resource 2). There was a significant difference between the components of the hexane wash extracts from field-collected insects sourced from different honeybee apiaries in Queensland and New South Wales, Australia (ANOSIM: $R=0.678$, $P=0.001$) (Fig. 2b; Online Resource 2).

There was a significant difference between the components of the hexane wash extracts from insects sourced from a 'slimed-out' hive overrun by *A. tumida* at Bellbowrie (QLD, Australia) and healthy hives at Bellbowrie (ANOSIM: $R=0.939$, $P=0.001$) (Fig. 3; Online Resource 2).

The components of the hexane washes of laboratory-reared beetles varied with rearing diet and mate-access status. Among laboratory-reared beetles reared on hive products (Table I), there was a significant difference for the hexane washes of beetles that had been reared individually (and were unmated) and those reared with access to the opposite sex (potentially mated) (ANOSIM: $R=0.491$, $P=0.001$) (Fig. 4a; Online Resource 2). There was also a significant difference in the components of the hexane washes of unmated beetles that had been reared on pollen dough and those reared on hive products (sourced

Figure 4. a Non-metric multi-dimensional scaling ordination (MDS) of hexane wash extracts of *A. tumida* sourced from the laboratory culture, reared on hive products, individually reared (unmated), and reared with access to the opposite sex. **b** Non-metric MDS of hexane wash extracts of *A. tumida* sourced from the laboratory culture, unmated, and reared on pollen dough (Bee Build™) and on hive products (from apiary at Bellbowrie: 27.55° S, 152.89° E).

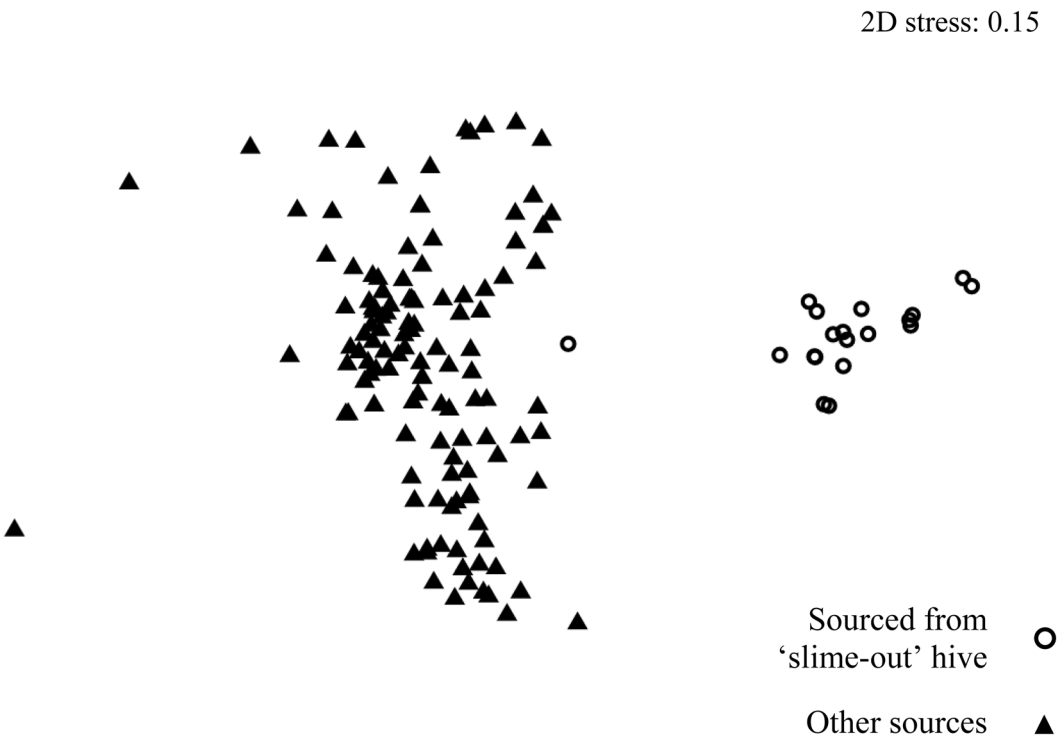
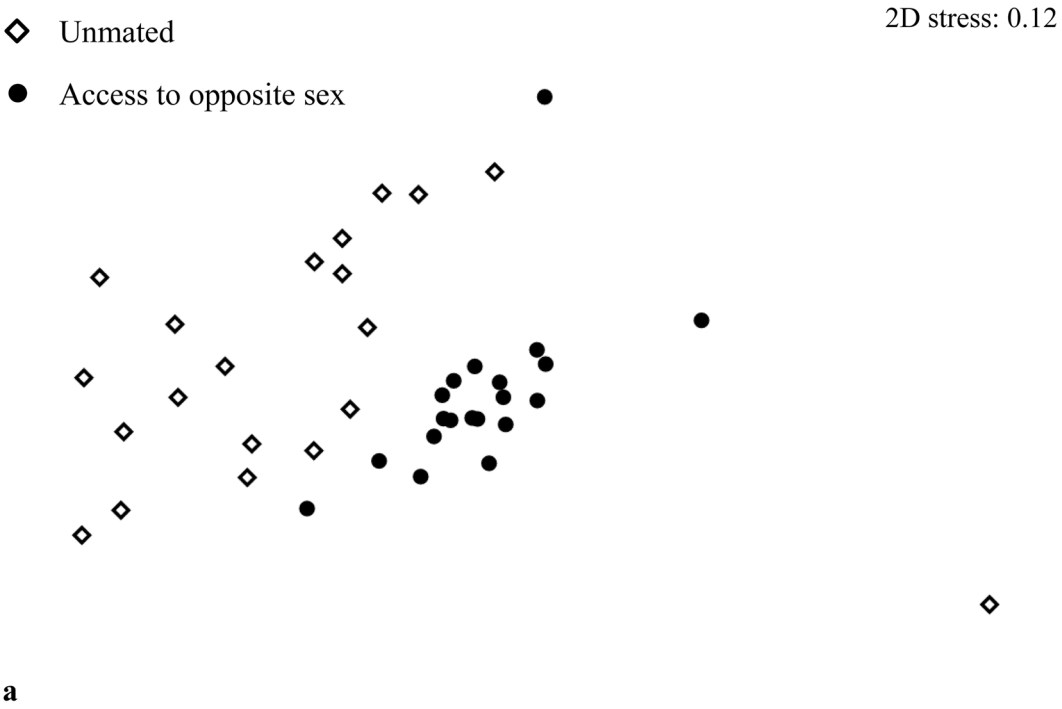
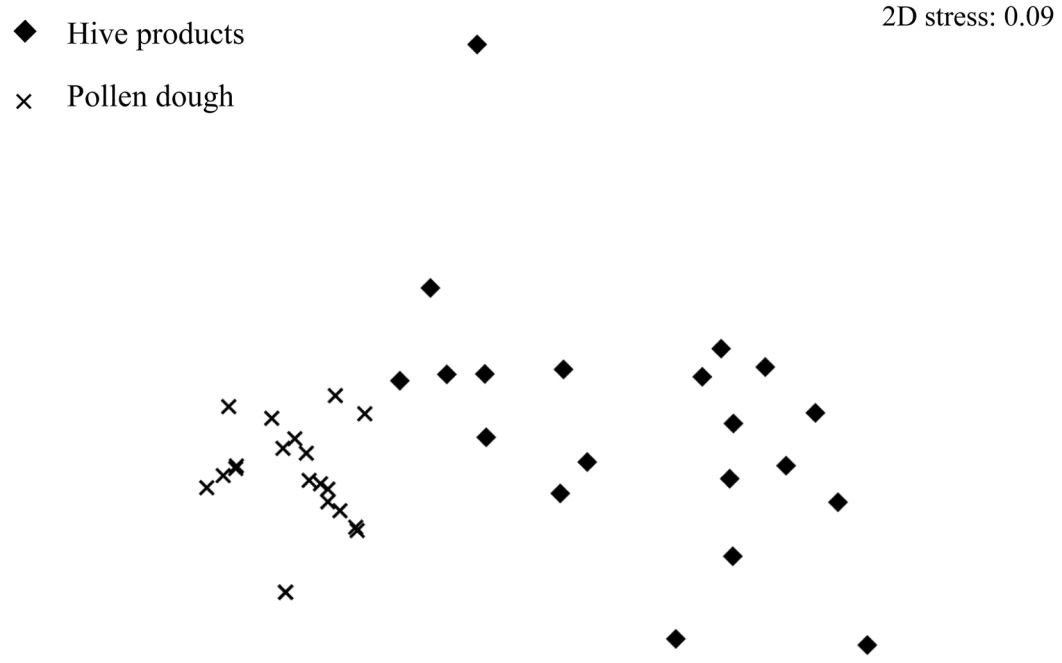


Figure 3. Non-metric multi-dimensional scaling ordination of hexane wash extracts of *Aethina tumida* sourced from a 'slimed-out' hive and all other field sources (see Table I for locations). Each point in the ordination represents a single insect hexane wash.


2D stress: 0.12
Unmated
Access to opposite sex

a


2D stress: 0.09
Hive products
Pollen dough

b

from Bellbowrie, QLD) (ANOSIM: $R = 0.737$, $P = 0.001$) (Fig. 4b; Online Resource 2).

There was no difference in the components extracted from the hexane wash extracts of male and female insects, regardless of their source, or the rearing material (Table I; Online Resource 2) (ANOSIM: $R = 0.002$, $P = 0.28$).

3.1. Behavioural assay

The behaviour of laboratory-reared and field-collected *A. tumida* was analysed. There was no difference in the mean time to form a cluster (five or more beetles) in laboratory-reared and laboratory-reared plus field-collected beetle treatments (Table II). Neither was there a difference in the mean maximum cluster size of each treatment over the 15-min assay time (Table II). In clusters of five or more beetles that formed in the first 5 min of the assay, the mean (± SE) proportion of field-collected beetles in each cluster was 0.49 (± 0.04). Please note that field-collected beetles were only sampled from one apiary, so some caution must be used in generalising these results to all beetles.

4. DISCUSSION/CONCLUSION

This study supports the hypotheses that the CHC profiles of *A. tumida* are contingent on the physical environment, especially the source hive, diet, and access to potential mating. The findings have implications for the regulation of interactions between *A. tumida* and honeybees, especially if this allows the beetles to persist undetected in a honeybee hive or at least have a lower chance of being detected.

We found differences when comparing hexane wash extracts of adult *A. tumida* from managed *A. mellifera* colonies in six different apiaries and a laboratory culture, reared in the presence of the associated yeast *K. ohmeri*. Importantly though, adult *A. tumida* from different sources will aggregate together.

Previous work has shown that adult *A. tumida* response to attractant traps in laboratory assays varies depending on whether *A. tumida* are field-collected or laboratory-reared (Amos 2013). In addition to this behavioural distinction, hexane extracts from field-collected beetles are significantly different from those of laboratory-reared individuals, in both the number of compounds detected and in their relative abundances (Fig. 1). Furthermore, we found significant differences in hexane wash extracts between small hive beetles from *A. mellifera* colonies in different apiaries in South East Queensland and New South Wales, Australia (Fig. 2b). The compounds that distinguished between laboratory and field-collected insects were mainly methyl-branched long-chain alkanes and long chain alkenes; overall there was a greater complexity and abundance of these compounds in the field-collected insects (see Online Resource 2 for compounds contributing to 50% of the dissimilarity between groups). These are likely acquired from the hive itself.

Leonhardt et al. (2015) suggest that environmentally derived compounds on the insect cuticle may provide an advantage by protecting against predators and determined that predatory ants prefer stingless bees (*Tetragonula carbonaria* (Smith) and *Austroplebeia australis* (Friese) (Hymenoptera: Apidae: Meliponini)) without resin-derived compounds. In the case of *A. tumida*, perhaps environment (hive)-derived compounds reduce levels of host honeybee hostility and accompany the behavioural mimicry enabling trophallaxis (Ellis et al. 2002a), although levels of hostility are not reduced to zero.

Apis mellifera guards do not use food-derived odours to recognise non-nest mates (Downs et al. 2001). However, wax combs do mediate nest mate recognition, as do floral scents (Breed et al. 1988; D'ettorre et al. 2006). It is possible that *A. tumida* adults can also acquire these cues from close contact with comb wax, and acquisition of host cues occurs in other host-parasite interactions (e.g. Franks et al., 1990; Sledge et al, 2001). Examining whether beetles have acquired these comb odours would be a sensible next step in understanding this interaction.

Kärcher and Ratnieks (2010) report that honeybee guards did recognise non-nest mates and

intruders through differences in odours, although not necessarily 'harmful intruder' odours since they rejected harmless arthropods and harmful insects to the same degree. The initial aggression shown towards small hive beetle reduces as beetles spend longer in the hive, and the cuticular profile of *A. tumida*, environmentally acquired within a hive, may therefore render beetles classified as 'no different' by both guard honeybees and other castes.

An important consequence of such 'camouflage' might be that with time beetles become invisible to bees within the darkness of a hive. As a result, they would be less likely to be chased by bees into refugia/harbourage traps. Currently the most widely used form of small hive beetle control involves a variety of in-hive trapping systems that rely on recognition of and aggression towards small hive beetles by the bees. The bees chase beetles into the traps, which act as refugia. As a result of adaptation of cuticular chemistry, the effectiveness of these traps may be impacted in a temporal manner following entry by the beetle into the hive, becoming less effective. This concept warrants further investigation.

Intriguingly, the conspecific aggregative behaviour of *A. tumida* appears to be unaffected by the source of the insects involved (Table II). We found no difference in the mean time it took to form a cluster of five or more beetles or in the mean maximum cluster size within 15 min of insects being placed together, and there was no evidence suggesting that laboratory and field-collected individuals failed to cluster together. This suggests that the compounds contributing the differences in the cuticular washes of *A. tumida* do not have a role in such conspecific behaviour. There may though be compounds among those that we found that are species recognition cues, and this warrants further investigation. Such compounds, used for species recognition and probably aggregative behaviour, would likely be highly conserved and not affected by environment and diet.

As for extracts from *A. tumida* sourced from different stages of infested hives, Hayes et al. (2015) found that hive volatiles containing fermenting hive materials and final instar *A. tumida* larvae (called a slime-out) were particularly attractive to the beetles. Unsurprisingly, the data presented here show partitioning between extracts from beetles from infested hives and those from beetles collected from a hive at the slime-out stage (Fig. 3).

Hexane wash extracts from laboratory-reared insects differed between those that had been reared individually and reared en masse and housed with access to the opposite sex (Fig. 4a). These results suggest that mating has an effect either on the production or secretion of cuticular compounds. Notably, all of the compounds contributing to at least 50% of the dissimilarity are lower or absent in the unmated beetles, including hexadecanal and 1-nonadecene which were not detected in unmated beetles. We did not, however, find differences in hexane wash extracts between male and female adult *A. tumida* regardless of source or rearing conditions. This suggests that cuticular compounds including CHCs do not have a role in sex recognition or mate choice in *A. tumida*. However, since Mustafa et al. (2015) report that male and female *A. tumida* can differentiate between sexes and are attracted to the opposite sex at the age of 18–21 days, some signal is likely to be present. The current results suggest that the cues used in this differentiation may be volatile substances perhaps combined with visual and tactile stimuli, as opposed to just chemical cuticle-based cues.

Rearing diet has been shown to influence cuticular compounds in insects, for example Espelie and Bernays (1989) found that in fourth instar *Manduca sexta* L., (Lepidoptera: Sphingidae) larvae, rearing diet significantly influenced the proportions of cuticular lipids. We found differences in hexane wash extracts in laboratory-reared *A. tumida* that had been reared either on pollen dough (Bee Build™) or on hive products from an *A. mellifera* apiary (Fig. 4b). Many laboratories culturing small hive beetles rear them using different rearing materials (Arbogast et al. 2010; Ellis et al. 2002b; Haque and Levot 2005; Neumann et al. 2013). In light of our findings, diet clearly needs to be considered in future examinations of the cuticular profile of this insect, since these

differences could be significant in terms of the responses of host bees to invading *A. tumida* adults. Potentially, host honeybees may react differently to beetles that have different cuticular profiles. If a beetle has gone through its larval stages within a particular honeybee colony (or apiary), this beetle may be better equipped to enter and remain undetected. Although Neumann et al. (2012) report that beetles appear to prefer long-range flights over nearby colonies, they note that beetle dispersal behaviour could differ under less crowded conditions and dispersal is generally dependent on a number of factors (Spiewok et al. 2008). Since cuticular identity for the small hive beetle has now been shown to differ based on source hive, this needs to be considered among such factors in future studies.

SUPPLEMENTARY INFORMATION

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AUTHOR CONTRIBUTION

BAA and RAH conceived the research and designed the experiments; all authors contributed to data interpretation; BAA performed experiments; BAA and RAH performed analysis; and BAA wrote the paper. All authors read, edited, and approved the final manuscript.

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DATA AVAILABILITY

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

CODE AVAILABILITY

Not applicable.

DECLARATIONS

Ethics approval No approval of research ethics committees was required to accomplish the goals of this study because experimental work was conducted with unregulated invertebrate species.

Consent to participate Not applicable.

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Conflict of interest The authors declare no competing interests.

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