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Original article

A coat of many scents: Cuticular hydrocarbons in multitrophic interactions of fig wasps with ants

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ABSTRACT

The fig–fig wasp system of *Ficus racemosa* constitutes an assemblage of galler and parasitoid wasps in which tritrophic interactions occur. Since predatory ants (*Oecophylla smaragdina* and *Technomyrmex albipes*) or mostly trophobiont-tending ants (*Myrmicaria brunnea*) were previously shown to differentially use volatile organic compounds (VOCs) from figs as proximal cues for predation on fig wasps, we examined the response of these ants to the cuticular hydrocarbons (CHCs) of the wasps. CHC signatures of gallers were distinguished from those of parasitoids by the methyl-branched alkanes 5-methylpentacosane and 13-methylnonacosane which characterised trophic group membership. CHC profiles of wasp predator and wasp prey were congruent suggesting that parasitoids acquire CHCs from their prey; the CHC composition of the parasitoid *Apocrypta* sp 2 clustered with that of its galler host *Apocryptophagus fusca*, while the CHC profile of the parasitoid *Apocryptophagus agraensis* clustered with its galler prey, the fig pollinator *Ceratosolen fusciceps*. In behavioural assays with ants, parasitoid CHC extracts evoked greater response in all ant species compared to galler extracts, suggesting that parasitoid CHC extracts contain more elicitors of ant behaviour than those of plant feeders. CHCs of some wasp species did not elicit significant responses even in predatory ants, suggesting chemical camouflage. Contrary to earlier studies which demonstrated that predatory ants learned to associate wasp prey with specific fig VOCs, prior exposure to fig wasp CHCs did not affect the reaction of any ant species to these CHCs.

1. Introduction

Ants have varied foraging strategies and food sources (Carroll and Janzen, 1973; Traniello, 1989) depending on which they employ a variety of food recognition modalities that are primarily chemical but may also be assisted by visual cues (Eriksson, 1985). Ants associated with plants as herbivores, seed predators or seed dispersers use plant secondary compounds (Cherrett and Seaforth, 1970; Saverschek et al., 2010), plant nutrients (Marshall et al., 1979; Skidmore and Heithaus, 1988) or other chemical features of their mutualistic partners (Ghazoul, 2001; Pierce et al., 2002; Stadler and Dixon, 2005; Choe and Rust, 2006; Youngsteadt et al., 2008; Willmer et al., 2009; Hojo et al., 2014) as recognition, feedant or anti-feedant cues. Predatory ants that feed on plant-associated insect prey may use volatile, plant-derived compounds to obtain information about the location and type of insect prey available on plant resources (Ranganathan and Borges, 2009; Schatz and Hossaert-McKey, 2010). However, such predatory ants feeding on insects associated with plants may also use less volatile chemicals such as insect cuticular hydrocarbons (CHCs) as feedant cues since CHCs often play an important role in insect predator–prey relationships (Espelie et al., 1991).

While insect CHCs are involved in several important discriminatory functions in ants such as mate recognition, nestmate recognition, colony regulation, chemical mimicry and camouflage (Howard and Blomquist, 2005; Blomquist and Bagnères, 2010; Tsutsui, 2013; Guillem et al., 2014; Menzel et al., 2014), their role in predator–prey interactions by providing feedant or anti-feedant cues to ants regarding prey has received less attention. Since qualitative or quantitative differences in CHC profiles can elicit aggressive, appeasement, or indifferent behaviour by ants towards conspecific or heterospecific ants (Endo and Itino, 2012, 2013; Menzel et al., 2013; Lenoir et al., 2013), it is possible that CHCs of insect prey may evoke differential responses in ants for varied prey

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types and across predatory or non-predatory ants. Indeed, non-predatory, trophobiont-tending ant species were indifferent to plant volatile cues that were used by predatory arboreal ants to locate plant-associated insect prey (Ranganathan and Borges, 2009). Furthermore, the behavioural response of the predatory arboreal ants to plant volatiles was a learned association between the presence of the volatiles and the presence of insect prey (Ranganathan and Borges, 2009); consequently, the response to prey-associated plant volatiles was not innate in these ants.

CHCs of both plant and insect cuticles have been implicated in mediating multitrophic interactions between plants, insect herbivores and their predators and parasitoids (Espelie and Hermann, 1988; Espelie and Brown, 1990; Espelie et al., 1991). Insects may acquire CHCs from their diets (Liang and Silverman, 2000; Richard et al., 2004), via contact (von Beeren et al., 2011), or synthesise them *de novo* (Fan et al., 2003). Since ant feeding behaviour on plant products such as seeds or extrafloral nectar is elicited by resource chemistry (Skidmore and Heithaus, 1988; Shenoy et al., 2012), ants that feed on plant-feeding insects or their parasitoids may exhibit differential responses to CHCs of plant-feeding insect gallers versus carnivorous parasitoids based on differences in their CHC profiles, if any. Also, as in the case of plant volatiles (Ranganathan and Borges, 2009), such differential responses to CHCs by ants may be acquired and may not be innate.

Infochemical use by carnivorous insects in complex tritrophic interactions has scarcely been examined (Steidle and van Loon, 2003). Whether ants with different lifestyles or experience can show similar learning with regard to CHCs has not been examined in these complex multitrophic systems. In order to investigate the differential responses of ants to CHC profiles of a multitrophic prey community, we chose the co-evolved system of figs associated with fig wasps, since galler and parasitoid fig wasps form an important prey resource for arboreal ants (Schatz et al., 2006, 2008; Ranganathan et al., 2010; Zachariades et al., 2010; Bain et al., 2014) and ants are predictably available as dominant predators on fig trees. Stable and predictable plant-based prey sources such as figs can therefore serve as important model systems to understand ant foraging behaviour (Heil and McKey, 2003; Debout et al., 2005; Ranganathan and Borges, 2009), particularly the response of ants to cuticular compounds of their prey.

The fig (*Ficus*: Moraceae) syconium is a specialised globular inflorescence within which fig wasps breed. These wasps could be gallers, kleptoparasites feeding on galled plant tissue, parasitoids or hyperparasitoids and develop within the syconium (Cook and Rasplus, 2003; Herre et al., 2008; Borges, 2015). All wasp species are usually highly specific to their natal fig species (Herre et al., 2008; Jousselin et al., 2008); however, a single parasitoid wasp species may parasitise several wasp species developing within the same syconium or in the same fig species (Ghara and Borges, 2010; Ghara et al., 2014; Borges, 2015). Therefore, in this tritrophic interaction, the predatory parasitoids could be generalists at the prey level but are specialists at the host plant level (*sensu* Vet and Dicke, 1992). Owing to host-plant specificity of fig wasps, and assuming that some CHCs could be acquired from the diet, all gallers could acquire elements of their CHC profiles from the fig species they feed upon, and in turn all parasitoids could acquire components of their CHC signature from the various gallers or parasitoids (in the case of hyperparasitoids) they prey upon within the same fig species (Fig. 1). All else being equal, we expected close correspondence between CHC profiles of predator–prey species pairs.

We selected a reasonably speciose community of fig wasps and ant predators in a common fig species *Ficus racemosa* L. (Moraceae) in India since we had knowledge of the trophic level of the fig wasps (Ghara and Borges, 2010; Ghara et al., 2011, 2014), had established that predatory ants but not trophobiont-tending ants in this system learn to associate insect prey with plant volatiles (Ranganathan and Borges, 2009), and we also knew the predation levels of the ants on the different species of fig wasps (Ranganathan et al., 2010). We therefore asked the following questions: 1) What are the CHC profiles of galler and parasitoid fig wasps developing within *F. racemosa* syconia, and how do they differ? 2) Are the CHC profiles of parasitoid fig wasps congruent with those of their prey? 3) What is the response of ants to CHC extracts of the different fig wasps? 4) Is there a difference between predatory and trophobiont-tending ants in this response? 5) Are these responses learned? To the best of our knowledge, this is the first study to address such questions in a highly specific and complex multitrophic interaction such as that of figs and fig wasps interacting with generalist ants.

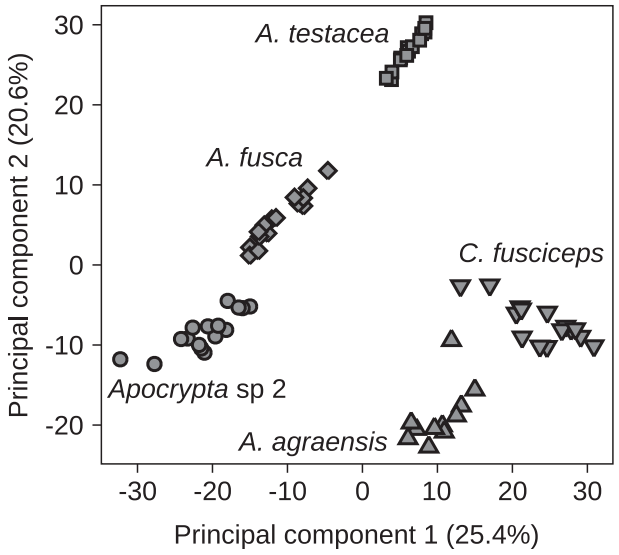


Fig. 1. Principal Component Analysis (PCA) plot based on proportional abundance of fig wasp CHCs.

2. Materials and methods

2.1. Natural history of the fig wasp community

F. racemosa (Moraceae: Subgenus *Sycomorus*) bears globular enclosed inflorescences termed syconia (figs) and produces syconia 5–6 times per year. This fig species is pollinated by an agaonid wasp *Ceratosolen fusciceps* which enters the syconia and breeds by galling some pollinated flowers into which eggs are deposited. The development of syconia goes through distinct phases. In the pre-pollination phase, syconia are small with floral buds; in the pollen receptive phase, syconia contain female flowers that are ready to be pollinated by *C. fusciceps*. Ovipositing pollinators die within a few hours of entering syconia. Pollinator larvae and seeds develop during an inter-floral phase. The wasp-dispersal phase follows when male flowers produce pollen; wingless male fig wasps eclose from galled flowers, and mate with eclosed female pollinating fig wasps while they are still inside their galls. The females collect pollen and exit the syconium via the exit hole chewed out cooperatively by the pollinator males which later die within the syconium.

This obligate brood-site pollination mutualism between *F. racemosa* and its pollinating wasp is subject to parasitism by several species of galling and parasitoid chalcid wasps that do not enter the syconium but oviposit into the fig syconia from the outside using long ovipositors during the various development phases of the

syconium described earlier (Ghara and Borges, 2010; Ranganathan et al., 2010). The gallers aggregate for oviposition in dense concentrations on the syconium surface either early or late in syconium development as do the parasitoids (Krishnan and Borges, 2014). These non-pollinating fig wasps (NPFWs) include the gallers *Apocryptophagus stratheni*, *Apocrytophagus testacea and Apocryptophagus fusca*, as well as the parasitoids *Apocryptophagus agraensis*, *Apocrypta* sp 2 and *Apocrypta westwoodi*. Based on published experiments and ongoing investigations (Ghara et al., 2014; Ghara, Yadav, Krishnan and Borges, unpublished data), we have established the following putative galler–parasitoid (i.e. prey–predator) associations: 1) *C. fusciceps*–A. *agraensis* (major predator) + *Apocrypta* sp 2; 2) *A. stratheni*–*Apocrypta* sp 2 + *Apocrypta westwoodi*; 3) *A. testacea*-*Apocrypta* sp 2 + *Apocrypta westwoodi*; 4) *A. fusca*–*Apocrypta* sp 2. All these NPFWs are highly host-plant species specific and parasitise *F. racemosa* throughout its range that extends from India through Australia; *A. stratheni* may, however, be restricted to the Indian subcontinent (J-Y Rasplus, pers. comm.). It may be noted that the genus *Apocryptophagus* is equated with *Sycophaga* which is the older, and more preferred name according to some authorities (Cruaud et al., 2011).

The NPFWs eclose and leave the syconium synchronously along with female pollinating wasps using the exit hole made by male pollinators; hundreds of fig wasps exit each syconium at approximately the same time. Owing to this specialized ontogeny and regular patterns of arrival at or departure from syconia, pollinators and parasites are available at fig syconia in predictable temporal pulses across the development of the syconium and provide dense concentrations of prey available to ants on the surface of each syconium (Ghara and Borges, 2010; Ranganathan et al., 2010). Pollinators and parasites are available to ants only on the syconium surface since ants cannot enter the syconia.

The fig trees were patrolled by three ant species *Oecophylla smaragdina* (Fabricius) (Formicinae), *Technomyrmex albipes* (Smith) (Dolichoderinae) and *Myrmicaria brunnea* Saunders (Myrmicinae) which are the dominant ants in this community (Ranganathan and Borges, 2009; Ranganathan et al., 2010) and were studied within the campus of the Indian Institute of Science, Bangalore, India (12°58′N, 77°35′E). *Oecophylla smaragdina* is a largely predatory ant species and constructs polydomous or multiple nests spanning several trees to harbour its huge colony size. *Technomyrmex albipes* is a small predatory ant species with a much smaller colony size, and also exhibits nesting polydomy. *Myrmicaria brunnea* is a largely trophobiont-tending ant and is also a scavenger constructing terrestrial nests.

2.2. Fig wasp cuticular hydrocarbon signatures

Since *Apocryptophagus stratheni* is an extremely rare species, and it was also impossible to obtain sufficient numbers of fig wasps of *Apocrypta westwoodi* for this study, we investigated the CHC profiles of the following members of the fig wasp community – the gallers: *C. fusciceps* (n = 15 samples, each of 100 wasp extracts), *A. fusca* (n = 16), and *A. testacea* (n = 14), and the parasitoids: *A. agraensis* (n = 11), and *Apocrypta* sp 2 (n = 18). For each CHC extract, we immersed 100 freshly freeze-killed females of each species in 5 ml of pentane for 10 min (Van der Meer et al., 1989; Kather and Martin, 2012); freshly eclosed wasps were collected from D-phase figs in the laboratory. These numbers of wasps were required for each sample since the wasps are very small (0.22–0.35 mg per wasp; data from Ghara and Borges, 2010). Each extract was vortexed for 1 min and allowed to evaporate completely. To this we added 100 μl of pentane with 1 μl of methyl stearate (at 200 ng μl^{-1} as internal standard). We injected 1 μl of this sample into a gas chromatograph and mass spectrometer (Agilent-HP GC model 6890N, MS model 5973N) operating in the split mode (ratio 10:1), and fitted with an HP1 column of 30 m length, 0.25 mm internal diameter and 0.25 μm thickness using helium as a carrier gas (total flow rate of 2 ml min^{-1}). We optimized separation of the extract by using a temperature profile in which the analysis began at 100 °C for 2 min, and rose to 280 °C at 7 °C min^{-1} after which it was held for 25 min. The transfer line from the GC to the mass spectrometer was set at 280 °C. Electron impact ionization was 70 eV. Hydrocarbons were identified by their mass spectra (e.g. Scammells and Hickmott, 1976; Blomquist et al., 1987) and corroborated by their retention indices (Kováts, 1965; Genin et al., 1986; Carlson et al., 1998). Peak areas relative to those of the internal standard methyl stearate were used to quantify CHCs. Methyl alkanes were identified by their diagnostic ions (e.g. 140/141 for 9-methyl alkanes, or 168/169 for 11-methyl alkanes), taking the possibility of co-elution of these alkanes into account. Determination of double bond positions in alkenes by derivatisation using dimethyl disulphide (DMDS) (Carlson et al., 1989) was attempted but was unsuccessful. Double bond locations in alkenes were therefore obtained by preparing epoxy-derivatives using m-chloroperbenzoic acid (MCPBA) and then examining their mass fragmentation pattern (Krokos et al., 2001). This involved addition of ~500 μg of MCPBA to a 100-wasp equivalent extract in dichloromethane. The double bonds were converted to their corresponding epoxy-derivatives after incubation at room temperature for 15–30 min. These epoxy-derivatives were analysed via GC–MS using the same temperature ramping mentioned above. The positions of double bonds for alkadienes were not identified. The identity of alkatrienes was confirmed by comparison with published mass spectra (e.g. Witte et al., 2009). No attempt was made to identify the stereo-geometry (*E* or *Z*) of these double bonds. However, the (*Z*) form appears to be the most common biologically relevant stereoisomer (Blomquist et al., 1987). All analytical grade chemicals were obtained from Sigma–Aldrich, India.

2.3. Statistical analyses of fig wasp CHCs

We used principal component analysis (PCA) on the relative proportions of the fig wasp CHCs using the *prcomp* function of the software package R to visualise the clustering of the CHCs of the different fig wasp species. To arrive at a quantitative measure of the similarities between the fig wasp species based on their CHC profiles, we used the *pvclust* function of the R package *pvclust*. For clustering, we used 10,000 bootstrapping iterations with Euclidean distance as the distance measure and Ward's minimum variance as the agglomerative method. Clusters with approximately unbiased (AU) values of ≥95 were considered stable. We used the Random Forests algorithm to identify CHCs that were unique to a particular fig wasp species (see validation of this method to determine unique volatile compound signatures of fig species in Ranganathan and Borges, 2010, 2011). This tree-based algorithm performs hierarchical clustering via multi-scale and combinatorial bootstrap resampling and is most appropriate for data where the variables (i.e. CHCs in this case) are many more than the number of samples, and where there may also be autocorrelation between CHCs (van Wilgenburg et al., 2010) which is the problem faced by conventional multivariate analysis (Breiman, 2001; Martin and Drijfhout, 2009). Because of its versatility, Random Forests is being regularly used in chemical ecology to find discriminator compounds between different samples (Junker et al., 2011; Parachnowitsch et al., 2012; Spaëthe et al., 2013; Bischoff et al., 2014). We used a *one* versus *the rest* classification where *one* is the group of interest and *the rest* is the universe consisting of all other samples, as well as an *all* versus *all* classification to investigate species-specific signatures.

The former classification will find CHCs unique to each species against a background of all other wasp CHCs while the latter will identify CHC signatures which distinguish each species from the other. We also used the Random Forests algorithm to find galler- and parasitoid-specific CHC signatures, if any. We used the *varSelRF* package for the Random Forests algorithm which, in addition to finding a minimum set of predictor variables, also gives prediction error estimates for the classification using the .632+ bootstrap method (see Ranganathan and Borges, 2010, 2011). Such analyses can generate sets of compounds that can be used as predictor variables in subsequent assays with ants to determine proximate mechanisms for recognition of insect prey by ants. All statistical analyses were performed using R software version 2.11.1 (R Development Core Team, 2009).

2.4. Behavioural assays with ants: response to fig wasp CHCs

We chose antennations by ants as the measure of behavioural response to filter paper discs soaked in fig wasp CHCs since antennation is an important part of the early stages of predatory behaviour in ants (Déjean et al., 1993; Pekár and Křál, 2002). In some cases, antennations were followed by disc removal; however, since this was not a consistent and replicated behaviour, we used only antennations by ants of CHC-soaked discs as an indication of their interest in the CHCs, and thereby of response to prey.

To obtain fig wasp CHC extracts for these assays, one hundred fig wasps of each of the following species: *C. fusciceps*, *A. testacea*, *A. fusca*, *A. agraensis*, and *Apocrypta* sp 2 were separately treated with 1 ml pentane for 10 min in a glass vial. Pentane was chosen since pentane extracts elicited good antennation responses from ants in pre-trial experiments compared to other solvents (results not shown). Each species-specific wasp extract was allowed to evaporate completely to remove any volatile components, after which it was reconstituted with 100 μl of pentane. One drop (6.25 μl; equal to 6 wasp equivalents) of this extract was placed on a Whatman filter paper disc, allowed to dry and used for the choice experiments. For *O. smaragdina* and *M. brunnea* we used 5 mm diameter filter paper discs, whereas for *T. albipes* we employed 3 mm diameter discs since these ants are smaller in size. Discs immersed in pentane were used as control discs.

We performed the ant assays first with experienced ant colonies of each species. We defined experienced ants as those belonging to colonies that had nests on (*O. smaragdina* and *T. albipes*) or near the base of (*M. brunnea*) fig trees (Ranganathan and Borges, 2009). Each block of the choice assay included 10 discs, 5 of which were fig wasp extracts (1 disc for each of the 5 wasp species tested) and 5 solvent discs. All experiments were conducted on natural ant trails outside the laboratory near fig trees. All observations were conducted from 10:00–13:00 h as this was previously noted as a peak activity period for the ants (Ranganathan et al., 2010). In order to control for the fluctuating numbers of ants on trails during the assays, we randomized the presentation sequence of the different discs. Each assay involved placing a disc on an active ant trail and the number of antennations the disc received was recorded for a 3 min period. Each observation period for every disc was preceded and followed by a gap of 1 min to allow for any disturbances in the ant trail caused by disc placement to subside. The next disc in the random sequence was then presented on the ant trail, and so on. For each ant species, 20 such blocks of choice assays were performed; thus $n = 20$ per species. We used antennation as an indication of attractiveness or interest shown by the ants to CHC extracts. Antennations made singly by an individual ant, repeatedly by the same ant, as well as antennations by multiple ants on a single disc were pooled together as the total number of antennations. The number of antennations received by discs in each wasp species category was tested against the median value of the number of antennations received by the control solvent-only discs using a Wilcoxon signed rank test. This examination of ant reaction to solvent-only discs was done to control for intrinsic differences between ant species in antennation behaviour and thus to allow for comparisons between species.

We repeated the same set of experiments with naïve ants of each species to determine whether the response towards fig wasp CHC extracts was a learned response. We defined naïve ants as in Ranganathan and Borges (2009) as those ants which patrol and nest in non-*Ficus* trees at least 300 m away from the nearest *F. racemosa* tree, and hence were unlikely to have encountered fig wasps as prey, especially since the *Ficus* and non-*Ficus* trees used in these experiments were also separated by massive buildings and concrete structures on the campus. Therefore for naïve ants, the assays were conducted on ant trails around such non-*Ficus* trees in the same way as they were done for ant trails around *Ficus* trees and the data were analysed separately.

3. Results

3.1. Fig wasp CHCs

The CHC profile of each fig wasp species was unique (Fig. 1). A total of 65 CHC compounds were identified in all examined fig wasp species; these included *n*-alkanes, monomethyl- and dimethyl-branched alkanes, and alkenes (Supplementary material 1, Supplementary material 2). The CHC profiles were dominated by methyl-branched alkanes and alkenes rather than alkanes (Table 1). Across all fig wasp species, alkanes were far fewer in types of compounds (8) compared to methyl-branched alkanes (36) and alkenes (18) ($\chi^2 = 19.48$, $df = 2$, $P << 0.001$). Yet, the most abundant compound in each species was an alkatriene except in *A. testacea* where it was a methyl-branched alkane (Table 1). *Apocrypta* sp 2 and *Apocryptophagus fusca* (predator–prey pair; see later) shared the most abundant compound in their species-specific CHC profiles, i.e. 3,6,9-nonacosatriene (Table 1). Homologous hydrocarbons, i.e. those differing only in chain length, within a species were found mostly in the alkanes and alkatrienes, e.g. 3,6,9-pentacosatriene and its homologs (Supplementary material 1). There was variation between wasp species in total extractable CHCs (ng/100 wasps) (Kruskal–Wallis $\chi^2 = 15.66$, $df = 4$, $P = 0.003$); however, pair-wise tests with Bonferroni corrections showed that only *A. testacea* and *Apocrypta* sp 2 were significantly different in total extracted CHCs with *A. testacea* having the highest amounts of extractable CHCs (Table 1).

The results of the *one* versus the *rest* Random Forests algorithm (where each species was distinguished from all other samples when the identities of the other species were masked) indicated that the set of compounds which could be used to uniquely identify each fig wasp species (Table 2) from a background of all others had lower coefficients of variation (CV) than the most abundant compound in each species (Table 1), indicating that the most abundant compound may not be the most unique and invariant compound in the species-specific CHC signature. The only exception was 11-methylhentriacontane in *A. testacea* which was the most abundant compound and also had a low CV. The Random Forests results indicated that the model frequency for the predictor compounds (i.e. their predictability) was considerably lower for most gallers (10% in *A. testacea*, and 16% in *A. fusca*; Table 2) compared to the parasitoids (100% in *A. agraensis*, 34% in *Apocrypta* sp 2), with the exception of a model frequency of 41% in *C. fusciceps*, suggesting that prediction of trophic group membership using CHCs was better for parasitoids than for gallers (lower model frequency

Table 1
Summary of cuticular hydrocarbons (CHCs) in the fig wasps of *Ficus racemosa*.

Fig wasp species	Number of CHC compounds	Most abundant CHC	Mean percent (%), CV[a]	Mean quantity (ng/100 wasps), CV[a]
C. fusciceps	33	x-pentacosene	22.34, 0.47	40.10, 0.44
A. testacea	25	11-methylhentriacontane	26.12, 0.07	50.33, 0.25
A. fusca	26	3,6,9-nonacosatriene	15.75, 0.29	31.84, 0.49
A. agraensis	31	3,6,9-heptacosatriene	26.85, 0.36	48.66, 0.73
Apocrypta sp 2	22	3,6,9-nonacosatriene	35.29, 0.20	33.66, 0.79

[a] Coefficient of variation of most abundant CHC.

Table 2
Model frequency and predictor compounds of fig wasp cuticular hydrocarbons (CHCs) according to the Random Forests algorithm based on percent abundance of compounds (*one* versus *the rest*).

Species	Model frequency	.632+ Prediction error	Predictor CHCs	Percentage (%) mean, CV[a]
C. fusciceps	41%	0.0069	*n*-nonacosane	–
			2-methyltriacontane	10.30, 0.39
A. testacea	10%	0.0017	11-methylhentriacontane	26.12, 0.07
			3-methylnonacosane	13.40, 0.16
A. fusca	16%	0.0009	x,y-dimethylhexacosane	2.70, 0.13
			5,15-dimethyltriacontane	2.46, 0.20
A. agraensis	100%	0.0057	x-tritriacontene	9.33, 1.68
			5,x-dimethyloctacosane	–
Apocrypta sp 2	34%	0.0074	3,6,9-nonacosatriene	35.29, 0.20
			5,15-dimethyltriacontane	5.09, 0.21

[a] Coefficient of variation.

would mean "more than one unique" way to differentiate two groups).

We also performed an *all* versus *all* classification (i.e. with the identities of each species being retained) to compare species uniqueness based on CHC proportions. In such a classification, only three compounds, 3,6,9-nonacosatriene, 9-hentriacontene and 5-methylhentriacontane were sufficient to be used as predictors to distinguish all species with 100% model frequency (Fig. 2). The proportional abundance of these compounds in the CHC signatures ranged from as high as 35.3% of 3,6,9-nonacosatriene in *Apocrypta* sp 2 to as low as 1.6% in *C. fusciceps* (Supplementary material 1; Fig. 2). The Random Forests algorithm predicted that, with 100% model frequency (prediction error of 0.0012), gallers (*C. fusciceps*, *A. fusca*, *A. testacea*) could be distinguished from the parasitoids (*A. agraensis*, *Apocrypta* sp 2) using the proportions of two compounds, 5-methylpentacosane and 13-methylnonacosane, of which the latter was also absent in parasitoids (Fig. 3).

The degree of similarity between the CHC profiles of species indicating either trophic level or predator–prey relationships was apparent from the dendrogram generated by hierarchical clustering (Fig. 4). The galler *C. fusciceps* and its parasitoid *A. agraensis* formed a stable cluster with a very high (99%) approximately unbiased (AU) value while the parasitoid *Apocrypta* sp 2 and its prey, i.e. the galler *A. fusca*, constituted another cluster though with a slightly lower (92%) approximately unbiased (AU) value (Fig. 4). The galler *A. testacea* clustered along with the pair of the galler *C. fusciceps* and its major parasitoid *A. agraensis* (Fig. 4).

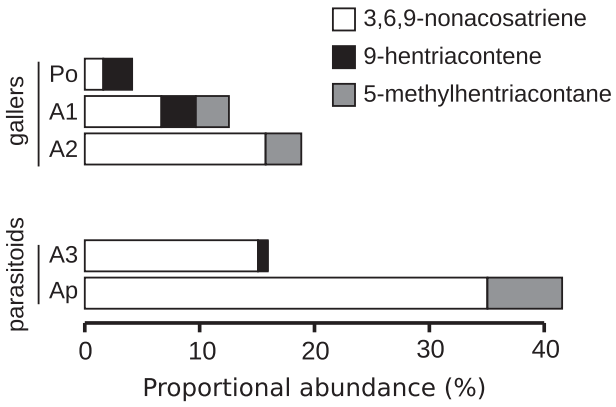


Fig. 2. Random Forests *all* versus *all* classification using proportional abundance. For sake of clarity, only median values are plotted. Po = *C. fusciceps*, A1 = *A. testacea*, A2 = *A. fusca*, A3 = *A. agraensis* and Ap = *Apocrypta* sp 2.

3.2. Behavioural assays with ants: response to fig wasp CHCs

Each ant species displayed a different degree of attraction towards the fig wasp CHC extracts (Fig. 5); however, general patterns also emerged. The antennations received by the solvent (control)

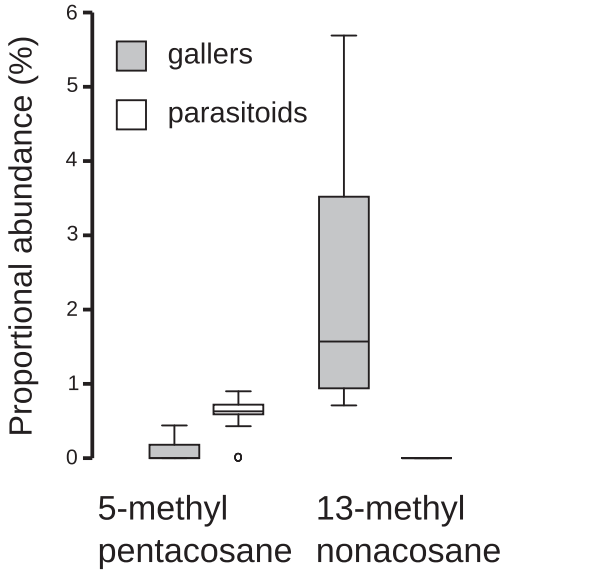
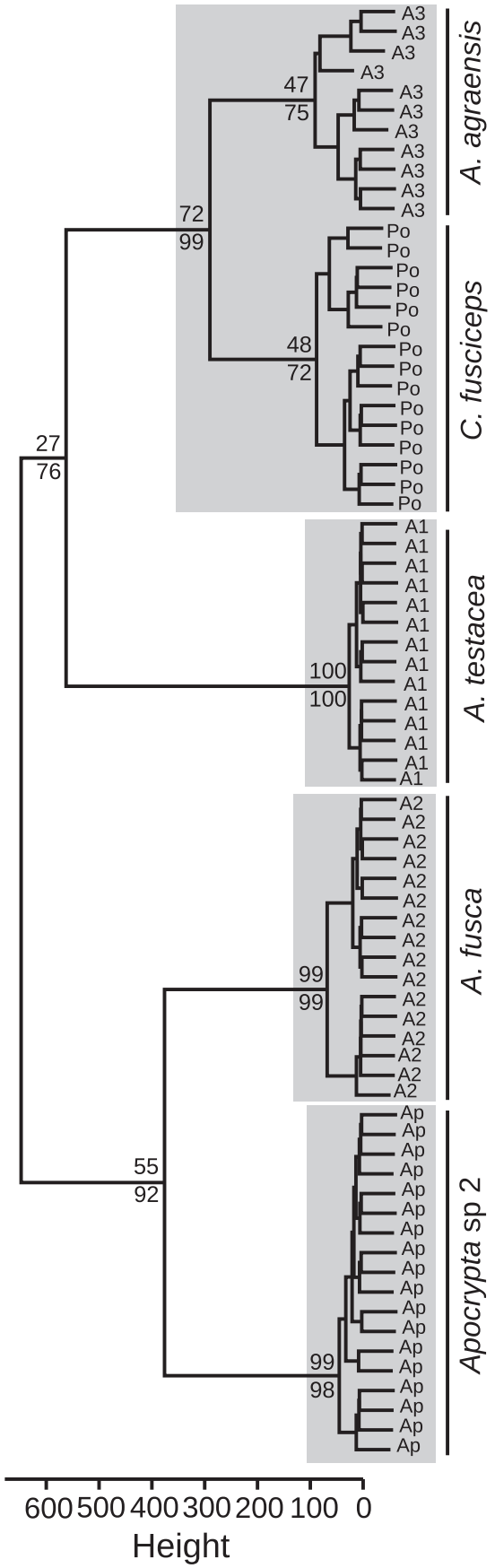


Fig. 3. Galler- and parasitoid-specific CHCs identified by the Random Forests algorithm using proportional abundance.



discs varied among the ant species (median values: 2 for *O. smaragdina*, 4.5 for *T. albipes* and 2 for *M. brunnea*). *Oecophylla smaragdina* exhibited greater interest in parasitoid fig wasp CHCs over control discs (*A. agraensis*: $V = 81$, $P = 0.008$; *Apocrypta* sp 2: $V = 194$, $P < 0.001$); however it did not discriminate galler CHCs from their solvent controls (*C. fusciceps*: $V = 57$, $P = 0.072$, *A. testacea*; $V = 92$, $P = 0.638$, *A. fusca*: $V = 81$, $P = 0.376$). *Technomyrmex albipes* similarly antennated discs impregnated with parasitoid CHCs to a greater extent compared to their controls (*A. agraensis*: $V = 189$, $P = 0.002$; *Apocrypta* sp 2: $V = 198$, $P < 0.001$), and treated those of the gallers *A. testacea* ($V = 115$, $P = 0.722$) and *A. fusca* ($V = 132$, $P = 0.32$) similar to the control discs. However, *T. albipes* antennated discs with CHCs of the galling pollinator *C. fusciceps* to a greater extent compared to the solvent ($V = 191$, $P = 0.001$). The response of *M. brunnea* was similar to that of *O. smaragdina* showing greater interest towards discs impregnated with CHCs of the parasitoids *A. agraensis* ($V = 159$, $P = 0.045$) and *Apocrypta* sp 2 ($V = 177$, $P = 0.007$) but non-significant interest to CHC extracts of the gallers *C. fusciceps* ($V = 130$, $P = 0.359$), *A. testacea* ($V = 155$, $P = 0.064$) and *A. fusca* ($V = 90$, $P = 0.586$) (Fig. 5a).

Therefore, in general, the response of all three ant species was higher compared to the solvent only for the CHC extracts of parasitoids with the sole exception of the interest demonstrated by *T. albipes* towards the CHCs of the galler *C. fusciceps*. The responses of naïve ants were similar to those of the experienced ants (Fig. 5a compared to Fig. 5b; statistical details not shown).

4. Discussion

The CHC profile of gallers in this system could be reliably distinguished from that of the parasitoids by two compounds. The CHC profile of the parasitoid *Apocrypta* sp 2 clustered with one of its prey species, the galler *A. fusca*, while that of the parasitoid *A. agraensis* clustered with that of its major prey, the galler pollinator *C. fusciceps*, suggesting that parastioids acquire CHCs from their prey. The response to fig wasp CHCs was similar in experienced and naïve ants indicating that prior exposure to fig wasp CHCs did not affect the reaction of ants to them. The largely trophobiont-tending ant species exhibited less interest in general to fig wasp CHCs than the predatory ant species.

4.1. The CHC profiles of galler and parasitoid fig wasps

Each examined member of the fig wasp community of *F. racemosa* had a unique CHC profile. This species-specific profile is used by male fig wasps to discriminate between females of the various wasp species in the fig syconia (Krishnan et al., 2014). Species-specific CHC profiles have been found in several insect species even when closely related species have been investigated (Martin et al., 2008; Bagnères and Wicker-Thomas, 2010; Leonhardt et al., 2013). The fig wasp CHC profiles were dominated by methyl-branched alkanes and alkenes rather than alkanes that were of fewer types. N-alkanes are usually minor constituents of several hymenopteran CHC profiles (Brophy et al., 1983; Menzel et al., 2008) as also found in fig wasps. Linear alkanes were thought to be unimportant in species recognition because in several insects they are apparently not perceived (Dani et al., 2001, 2005) or

Fig. 4. Hierarchical cluster dendrogram using proportional abundance of the fig wasp CHC profiles showing affinities between them. Numbers above a split are bootstrap probability (BP) values and those below are approximately unbiased (AU) values. Shaded boxes represent strong clusters supported by AU values 95% or higher. Solid vertical lines indicate clear clustering based on species. Dotted vertical lines indicate clusters where samples of different species cluster together.

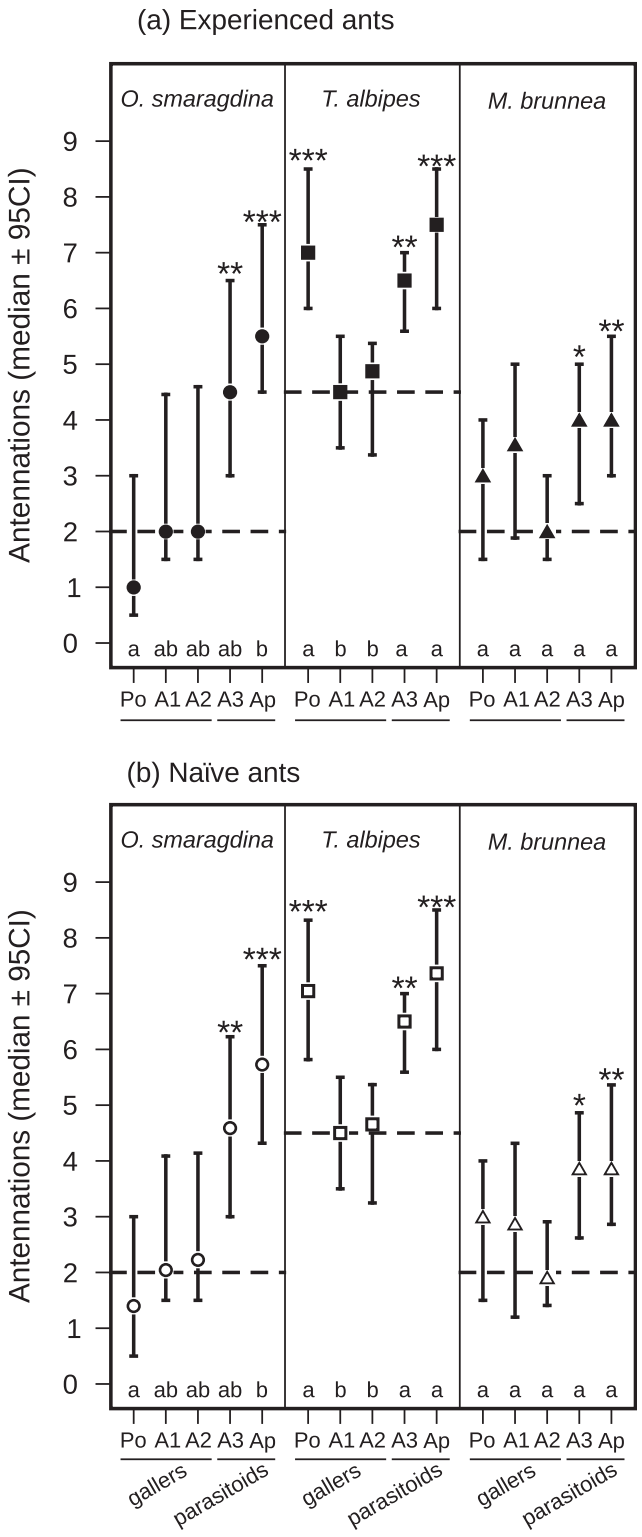


Fig. 5. Response of each ant species to fig wasp CHC extracts (median and 95% CI of the number of antennations each extract received) in (a) experienced ants and (b) naïve ants. For each ant species, the horizontal dashed line indicates the median levels of antennations received by the solvent discs. Asterisks above CIs indicate significant differences compared to solvent discs. Any error bar overlapping the horizontal dashed line is non-significant. ***P < 0.001, **P < 0.01, *P < 0.05 using a Wilcoxon sign rank test. N = 20 for each treatment and for each ant species. For each ant species, pair-wise Mann–Whitney U tests were performed to compare their antennation responses to the CHCs of wasp species pairs. Different alphabets denote significantly different response to CHCs of wasp pairs. Po = *C. fusciceps*, A1 = *A. testacea*, A2 = *A. fusca*, A3 = *A. agraensis* and Ap = *Apocrypta* sp 2.

learned (Châline et al., 2005); however, there is mounting evidence to the contrary (Greene and Gordon, 2007; Bos et al., 2012; Solazzo et al., 2015). The most abundant CHC for each fig wasp species was either a triene or a methyl-branched alkane (Table 1). Methyl-branched alkanes and alkenes appear to be more readily discriminated in Hymenoptera (Dani et al., 2001; Châline et al., 2005). The abundance of methyl-branched alkanes and alkenes in the CHC profiles of fig wasps therefore suggests their role in intra- and inter-species recognition. Alkatrienes have been reported to act as sex pheromones in some insects (Millar, 2000; Ando et al., 2004). Alkatrienes may be part of the CHC components used by male fig wasps for species discrimination and mate recognition within the dark, physically and chemically crowded environments of the fig syconia (Krishnan et al., 2014).

There was great congruency between CHC profiles of fig wasp parasitoids and some of their putative fig wasp prey. The congruency between the CHC profiles of *A. agraensis* and *C. fusciceps* adds confirmatory evidence that this species is a parasitoid, rather than a kleptoparasite, of the pollinator *C. fusciceps*. Earlier studies had doubted whether *A. agraensis* was a kleptoparasite or a parasitoid (Ghara and Borges, 2010), although suggestions that it is a parasitoid of the pollinator *C. fusciceps* were also made by Wang and Zheng (2008). By feeding on the pollinator, *A. agraensis* may acquire several elements of the CHC profile of the pollinator resulting in congruent CHC profiles as has been found in other predator–prey/host–parasitoid systems (Espelie and Brown, 1990; Liang and Silverman, 2000; Richard et al., 2004) or in insect herbivores which acquire hydrocarbons from their host-plant (Espelie and Bernays, 1989). Indeed, *C. fusciceps* is likely to be the only prey of the parasitoid *A. agraensis* (see Materials and methods) which can therefore explain the tight clustering of their CHC profiles (99% AU value of cluster strength; Fig. 4). The co-clustering of the CHC profiles of the parasitoid *Apocrypta* sp 2 and the galler *A. fusca* (92% AU values; Fig. 4) is consistent with the view that *A. fusca* is the prey of *Apocrypta* sp 2. *Apocrypta* sp 2 is likely the sole predator of *A. fusca* in this system (P. Yadav and R. M. Borges, unpublished data), although it may feed on other wasps. In our examined samples, based on the congruency of CHC profiles, it appears that *Apocrypta* sp. 2 parasitizes *A. fusca* more than *A. testacea* or *C. fusciceps*. Whether this pattern changes in other seasons, for other levels of predation by *Apocrypta* sp. 2 on *A. testacea* or *C. fusciceps* is not known, but needs to be investigated. Interestingly, levels of the triene 3,6,9-nonacosatriene in the parasitoid *Apocrypta* sp 2 (constituting 35.29% of its CHC profile) were almost twice that of its prey *A. fusca* (15.75%). Caveats to the interpretation of congruency between CHC profiles as confirmation of host–parasitoid relationships are that such congruencies could also come from shared evolutionary histories or shared *de novo* synthesis of these compounds; however, the fact that the CHC profiles of the three examined *Apocryptophagus* species do not cluster together although they may be considered to be more closely related to each other than to *Ceratosolen* or *Apocrypta* sp 2 suggests the acquisition of CHCs from their diet. The constancy versus the plasticity of these wasp profiles requires much more further investigation which is ongoing.

4.2. Behavioural assays with ants: responses to fig wasp CHCs

Since experienced and naïve ants of all three species exhibited the same responses to all tested fig wasp CHC extracts, these responses were not due to prior exposure to fig wasps, but appear to be general responses to their surface hydrocarbons. However, in all three ant species investigated, only extracts of galler fig wasp species evoked antennation responses that were similar to those of the solvent controls, and these results were the same for naïve as

well as experienced ants. A possible explanation of this pattern could be that galler wasps are more similar in their CHC signatures to plant surface hydrocarbon profiles compared to parasitoids and hence do not evoke strong antennation or recognition responses based on CHCs. There is some evidence which supports this, as the fig syconium also has similar long-chain hydrocarbons on its surface (Ranganathan and Borges, unpublished data) and short-term habituation to background odour is known in insects (Larkin et al., 2010). Alternatively these galler wasps could have lost those CHC compounds of their profile to which ants are responsive. The galler *A. fusca* is the least preyed upon by these ants (Ranganathan et al., 2010), although these wasps occur in large numbers on the surface of the syconia during the receptive phase (Ghara and Borges, 2010). It is possible that these fig wasp species have predation evasive strategies such as oviposition while motionless as well as CHCs which do not elicit predatory behaviour. In fact, *O. smaragdina* walks over motionless ovipositing *A. fusca* individuals on the fig syconium without preying upon them (Y Ranganathan and M Ghara, personal observations). The cuticular profile of the fig syconium could therefore potentially interact with prey detection by providing a similar background for chemical camouflage as seen in other parasitoid–prey (Rostás et al., 2008) or ant–prey systems (Henrique et al., 2005). However, since naive ants (i.e. those unexposed to fig odours) also showed responses to *A. fusca* odour that were indistinguisable from those of the experienced ants, it is unlikely that habituation or similarity to the background is the sole reason for the reduced predation by *O. smaragdina*. Additionally chemical profiles that reduce aggression in ants or that evoke the same response as ant nestmates are also known from studies on myrmecophiles, i.e. species that have parasitic, predatory or mutualistic relationships with ants while living closely with ants and even being accepted into their nests (Liepert and Dettner, 1996; Elgar and Allan, 2004; Choe and Rust, 2006; Silveira et al., 2010). What factors are in operation in the *O. smaragdina–A. fusca* interaction are not yet known but are being investigated.

4.3. Mechanisms underlying ant response to cuticular hydrocarbons in the fig–fig wasp system

In some ant–plant interaction systems, specific chemical elicitors of ant behaviour are known. For example, diglycerides elicit seed-carrying behaviour in seed-dispersing ants (Marshall et al., 1979; Skidmore and Heithaus, 1988), methyl salicylate elicits defensive behaviour in the protective ant mutualist (Schatz et al., 2009), or aliphatic acids ingested by caterpillars from plants evoke predatory behaviour in ants (Weinhold and Baldwin, 2011). In our study, the greater responsiveness of all ant species to the CHC profiles of the parasitoids compared to the galler fig wasp species suggests that there are specific elicitors of ant behaviour in the CHC profile of parasitoids. It is therefore possible that there are elicitors governing prey capture behaviour in the CHC profiles of carnivorous insect prey such as parasitoid wasps as has been found in ant–parasitoid (Liepert and Dettner, 1993; Thomas et al., 2002), plant–herbivore–ant (Henrique et al., 2005) or myrmecochorous seed dispersal systems (Hughes et al., 1994).

The relatively non-predatory and largely trophobiont-tending ant *Myrmicaria brunnea* responded to CHCs of fig wasp species although their predation pressure on fig wasps is minimal (Ranganathan et al., 2010). However, unlike the other two predatory ants (*O. smaragdina* and *T. albipes*), *M. brunnea* did not respond to VOCs produced by figs (Ranganathan and Borges, 2009). Taken together, our results point to the generic nature of the response of ants (predatory or trophobiont-tending) to fig wasp CHCs unlike their differential responses to fig VOCs.

How ants recognise CHCs is still not clearly known (Hefetz et al., 2010; Tsutsui, 2013), although they can detect CHCs at very low concentrations (Ichinose and Lenoir, 2010). Ants may recognise specific CHCs (Menzel et al., 2013), and may also generalise within classes of CHCs such as linear alkanes (Bos et al., 2012). However, they can discriminate methyl-branched alkanes (Martin et al., 2008; Guerrieri et al., 2009; Bos et al., 2012). Ants also recognize CHC profiles different from their own; i.e. they recognize foes and not friends in the process of nestmate identification (Ozaki et al., 2005; Guerrieri et al., 2009). Whether this applies also to prey recognition is not known. Our use of the bootstrapping and combinatorial Random Forests algorithm is an attempt to arrive at a set of possible compounds that may be used by ant chemosensory systems such as those deployed for CHC recognition. Some of these "predictor" compounds had low intraspecific CVs suggesting that they may be important in ant recognition systems (Martin et al., 2013). Multidimensional methods, such as we have employed, that pick out sets of predictor compounds when coupled with innovative metrics of odorants (e.g. Haddad et al., 2008) may therefore provide testable hypotheses for CHC recognition. Although parasitoid shared compounds with their galler prey, ants showed more interest in CHCs of parasitoids than those of gallers indicating that there could be certain CHCs within parasitoid CHC profiles that evoke predatory behaviour in ants. The increased antennation response to CHCs of parasitoids was not due to greater concentrations of CHCs in parasitoids compared to gallers, since *A. testacea* (the galler that was antennated the least and suffered the lowest predation) had significantly higher total CHC levels compared to the parasitoid *Apocrypta* sp 2. This points therefore more specifically to a combination of types of compounds perhaps coupled with their concentration that influences ant response via antennation. Therefore findings that link CHC composition to ant response can point to potential testable hypotheses for CHC recognition. In conclusion, much more needs to be learned about the function of insect CHCs and responses to them in complex multitrophic interactions such as the fig–wasp–ant interaction system including a comparison with such interactions in other fig species.

Author contributions

Yuvaraj Ranganathan (YR) and Renee M Borges (RMB) designed the study, YR collected and analysed the data, Jean-Marie Bessière (JMB) identified the cuticular hydrocarbons, YR and RMB wrote the paper. All authors approved the final draft of the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.actao.2015.05.007.

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