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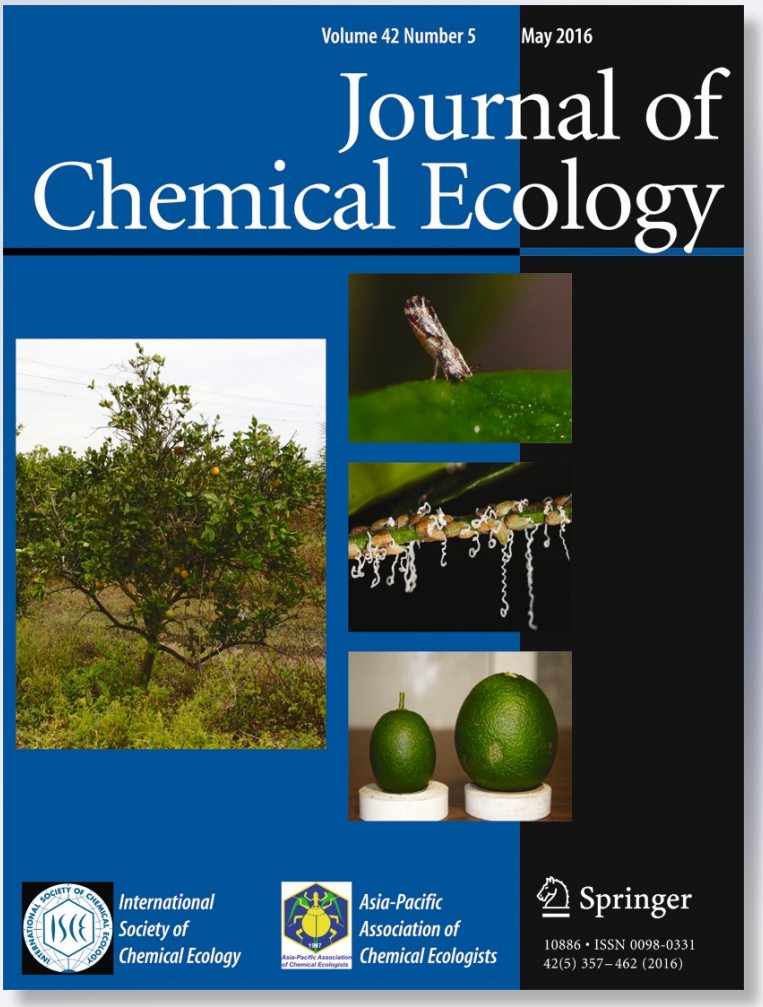
Journal of Chemical Ecology

ISSN 0098-0331
Volume 42
Number 5

J Chem Ecol (2016) 42:444-451
DOI 10.1007/s10886-016-0700-4



Springer

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Cuticular Hydrocarbon Compounds in Worker Castes and Their Role in Nestmate Recognition in *Apis cerana indica*

Seydur Rahman[1] · **Sudhanya Ray Hajong**[1] · **Jérémy Gévar**[2] · **Alain Lenoir**[2] · **Eric Darrouzet**[2]

Received: 13 October 2015 / Revised: 3 April 2016 / Accepted: 20 April 2016 / Published online: 7 May 2016

Abstract Differences in cuticular hydrocarbons (CHCs) among worker castes and colonies were examined in *Apis cerana indica*. The roles of tetracosanoic acid, hexadecanoic acid, pentacosane, and (*Z*)-9-tricosene in nestmate recognition were studied. The CHC profiles of different castes, i.e., newly emerged bees, nurse bees, and forager bees, were found to differ among colonies. The CHC profiles of nurse bees were similar across different colonies, but forager bees in all colonies had significantly greater amounts of alkanes. In nestmate recognition experiments, guard bees reacted significantly more aggressively to foragers treated with tetracosanoic acid, hexadecanoic acid, and (*Z*)-9-tricosene. Pentacosane provoked no such effect.

Keywords Tasks · Cuticular hydrocarbons · *Apis cerana indica* · Alkanes · Fatty acids

Introduction

In insects, the cuticular surface is covered by a thin layer of lipids whose purported main role is to prevent desiccation (Lockey 1988). Most of these lipids are hydrocarbons, of which there are a vast number in nature. Cuticular hydrocarbons (CHCs) in insects can be complex and may consist of a mixture of compounds, including linear alkanes and alkenes, terminally branched monomethyl alkanes, and internally branched mono-, di-, tri-, and tetramethyl alkanes. For example, more than 1000 different CHCs have been found just in ants (Martin and Drijfhout 2009).

Over the course of evolution, CHCs have evolved as communication cues that play a significant role in the life of social insects. They are used as attractants, involved in recognition by nestmates, members of the opposite sex, and different species, and they elicit defense behavior (Breed et al. 2004a, b; Chung and Carroll 2015; Ferreira-Caliman et al. 2010; Murakami et al. 2015; Valadares et al. 2015; Weiss et al. 2015). For instance, insect gregariousness is maintained by these hydrocarbons (Deneubourg et al. 2002; Gordon 1996; O'Donnell and Bulova 2007).

In social insect colonies, labor is divided between a reproductive queen and sterile workers (Wilson 1971). The distribution of tasks among individuals requires interactions among colony members, which involves the recognition of different castes (e.g., queens vs. workers), eggs, and larvae. This process is based on cues encoded in CHCs. For example, in bees and termites, castes can be distinguished because their CHC profiles differ (Bagneres et al. 2011; Darrouzet et al. 2014; Haverty et al. 1996; Howard et al. 1978, 1982; Kaib et al. 2002; Nunes et al. 2009).

During different periods of their lives, honey bee workers perform different specialized tasks, such as queen care or brood tending, comb building and maintenance, protection of nest areas, and foraging for pollen and nectar, which all depend on the colony's needs (Gordon 1996, 2002). These activities are not regulated by any one specific caste, and even the queen cannot control the individual behavior of colony members (Gordon 1996). Each worker makes behavioral choices based on her own cues, as well as those received from fellow honey bees (Detrain and Deneubourg 2006; Greene and Gordon 2003, 2007; Pratt 2005). In stingless bees, individual workers belonging to different castes show qualitative

✉ Seydur Rahman
seydurr@gmail.com

[1] North-Eastern Hill University, Shillong-22, India

[2] IRBI UMR CNRS 7261 Université François Rabelais, Faculté des Sciences, Parc de Grandmont, 37200 Tours, France

and quantitative differences in their CHC profiles (Ferreira-Caliman et al. 2010). In the European honey bee, *Apis mellifera*, young workers have a higher proportion of alkenes than do nurses and foragers, while foragers have more diverse cuticular profiles than do young workers. Kather et al. (2011) demonstrated that worker bees may use different proportions of alkenes to distinguish among different task -groups.

Almost all insects have the ability to recognize and respond to signals signifying self vs. non-self, and this ability is particularly important in social insects (Holldobler and Wilson 2008). Chemical cues are used to identify insects belonging to other colonies; based on this information, individuals are able to discriminate among nestmates and non-nestmates. These cues are encoded in the CHC profile of a species, which may comprise a few to more than a hundred compounds that differ in proportion, chemical properties, and quality. The compounds involved in nestmate recognition have been extensively studied in honey bees (Breed et al. 1988a, b; Châline et al. 2005; Couvillon et al. 2007; Dani et al. 2005; d'Ettorre et al. 2006), stingless bees (Nunes et al. 2009), ants (Larsen et al. 2014), and wasps (Dani et al. 2001; Murakami et al. 2015).

The Indian honey bee *A. cerana indica* is one of the world's most economically important honey bees, especially in the hilly regions of the Himalayan belt, which includes the state of Meghalaya, where the species is a significant source of income for marginalized groups in rural and urban areas. *A. c. indica* is approximately two-thirds the size of *A. mellifera* (Dyer and Seeley 1987). Compared to *A. mellifera*, *A. c. indica* has several distinguishing behavioral traits. First, it has a high propensity to swarm, forming around 6–10 swarms a year (Engel 2001). It can adapt to diverse climatic conditions and survive extreme temperature fluctuations (Xu et al. 2009). Foraging distances range between 1 and 2 km (Oldroyd and Wongsiri 2006), and colony defense behavior is well coordinated (Ono et al. 1987). *Apis cerana* guard bees often cluster together to defend their colony when it is attacked by predators or intruders, and they release alarm pheromones that help organize the defense response (Morse et al. 1967; Ono et al. 1987, 1995). Comparative studies between *A. mellifera* and *A. cerana* have revealed that *A. cerana* workers were less sensitive to nestmate recognition than *A. mellifera* when sealed queen cells were introduced heterospecifically between queenless colonies (Tan et al. 2010).

In this study, we analyzed caste-related differences in CHC profiles in *A. c. indica*. We also experimentally tested four compounds (hydrocarbons and fatty acids) to determine their role in nestmate recognition for different bee groups (newly emerged bees, nurse bees, and forager bees). Although newly emerged bees do not technically represent a task group, we nonetheless defined them as such for two reasons. First, they often are observed inspecting cells in the hive (personal observation). Second, we can easily characterize their CHC profiles, which can serve as a type of baseline. Then, we experimentally exposed workers to tetracosanoic acid, hexadecanoic acid, pentacosane, and (*Z*)-9-tricosene to analyze responses from guard bees. We chose these hydrocarbons as they are commonly found in *Apis* and reported to affect nestmate recognition in *A. mellifera* (Breed 1998; Breed and Stiller 1992; Getz et al. 1989; Getz and Page 1991; Page et al. 1991). On the other hand, honeybees may not use hydrocarbons as the only source of nestmate recognition cues. Wax secreted by bees for nest construction, also is involved in nestmate recognition cues among individuals in honeybee colonies (Breed et al. 1988a, b; Couvillon et al. 2007; d'Ettorre et al. 2006). The major compounds found in this wax are free fatty acids (12 % of the total wax content), and hydrocarbons (14 %) (Breed 1998). The wax of *A. mellifera* contains 16 % hydrocarbons, 35 % esters, and 14 % fatty acids (Tulloch 1980; Hepburn et al. 1991).

Methods and Materials

Insect Material The three *A. c. indica* colonies (A, B, and C) were housed in eight-framed wooden boxes; the dimensions of the super chamber, brood chamber, and floor bed were 10 × 35.5 in. 20 × 35.5 in. and 35 × 45 in. respectively. Each colony contained approximately 6000 bees. All colonies were kept in the botanical garden of North-Eastern Hill University, Shillong, India. (25°34′32″N/91°52′23″E). The university is situated 1432 m above sea level, and the temperature varies from 4 to 25 °C. The campus is situated in a pine tree forest.

Sample Collection Newly emerged bees were sampled from each colony by placing combs containing mature pupal cells overnight in an incubator at 34.5 °C and 60 % relative humidity. Nurse bees were collected when they inserted their heads into cells containing larvae. Forager bees were collected by blocking the entrance to the hive as bees returned to their respective colonies after foraging trips, i.e., they had pollen attached to their hind legs.

Chemical analyses were conducted on the bees collected. The objective was two-fold: first, to explore task-group-specific differences in CHCs within colonies, and second, to uncover any significant differences in CHCs among colonies. Bees were sacrificed by freezing (−10 °C for 10 min). They were placed in individual 2-ml glass vials containing 1 ml hexane, in which they were submerged for 15 min to extract their CHCs. Subsequently, the extract was dried by evaporation under a stream of nitrogen and stored at −20 °C until chemical analyses could be performed.

Analyses by Gas Chromatography-Flame Ionization Detection (GC-FID) and Gas Chromatography–Mass Spectrometry (GC-MS) Pentane (500 μl) was added to each dried sample. Immediately prior to the analyses, 10 μl of *n*-eicosane solution in hexane (C_{20}; 10^{-3}g/ml) was added to each tube as an internal standard.

GC-FID analyses were carried out on an Agilent Technologies 7820A System (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with flame-ionization detector (FID) and a capillary column (HP5, 30 m × 0.32 mm × 0.25 μm; Agilent) using helium as a carrier gas (1.7 ml/min). The oven temperature was increased from 50 to 200 °C at 8 °C/min, and then from 200 to 315 °C at 5 °C/min, with a 5-min hold at 315 °C. Injection was splitless (250 °C), and sample size was 2 μl. To characterize the CHC profiles, the areas of the main peaks were determined using ChemStation software (Agilent Technologies), and the relative proportions of each peak were calculated as described in Bagneres et al. (1990).

Compounds were identified using an Agilent Technologies 7000C Triple Quad GC-MS with a 7890B GC System equipped with the same column as above, and operated with the same temperature program as above. Compounds were identified using standard alkanes, mass spectral library data, and Kovats retention indices.

Nestmate Recognition Bioassay In the nestmate recognition experiment, the CHC-treated bees came from the same colony. The three colonies were used in this bioassay. Forager bees returning to the hive with pollen baskets were collected at the hive entrance ($N=20$ individuals per colony) using a clean pair of forceps, and were placed in resealable plastic bags. Individual foragers were chilled such that they could walk but not fly, and then were deposited in 15-ml glass vials containing 80 mg of either tetracosanoic acid, pentacosane, or hexadecanoic acid, which are all solids at room temperature; the bees were left in the vials for 5 min. Since (*Z*)-9-tricosene is a liquid at room temperature, 0.5 μl was applied directly to the thoraces of the bees by using fine capillary tubes. We treated insects with the same concentrations as used by Breed et al. (2004a, b) and Buchwald and Breed (2005) when adjusted for the different sizes of the bees and the bioassay containers. Given the brief period of exposure to the test compounds, it was considered unlikely that the natural blend of CHC's of treated bees would be significantly changed other than by the addition of the test compound. Tetracosanoic acid, pentacosane, and (*Z*)-9-tricosene standards were obtained from Sigma-Aldrich (St. Louis, MO, USA). Hexadecanoic acid was obtained from Sisco Research Laboratories (India Limited, Mumbai). All standards were 99 % pure, except for the (*Z*)-9-tricosene, which was 97 % pure.

Captured control workers were placed in individual, loosely capped 15-ml glass vials and chilled on dry ice for 50–55 sec until they could walk but not fly. To avoid changing the smell of these bees, they were not labelled.

CHC-treated bees were placed at the entrance to the hive when guard bees were present (between 09:00 and 14:00 h). The acceptance and rejection of the treated bees by the guards was defined according to Downs and Ratnieks (1999). They were considered to be rejected if any of the following occurred: they were grappled, pulled, or bitten on the legs, head, or wings; they were subject to immobilization attempts; the guards deposited resin on them; or they tried to enter the hive by force. They were considered to be accepted if they were left unmolested; allowed to enter the hive; and none of the above hostile behavior was displayed. Only one compound per colony could be tested per day.

Statistical Analysis Task-group-related differences in total compound quantities were examined using a one-way ANOVA and Tukey *post-hoc* tests. A two-way MANOVA using Pillai's trace test was carried out to explore task-group related differences in CHC profiles among colonies.

A stepwise discriminant analysis was used to determine if the task groups and colonies could be distinguished based on their profiles and, if so, which compounds were playing a role there. The relative areas of the peaks associated with the individual compounds found in the CHCs profile of each bee were standardized to 100 %. The relative quantity of each compound was calculated by dividing the area of the corresponding peak by the total area of all the hydrocarbon peaks in the entire chromatogram. The standardized peak areas then were transformed using the following formula:

$$Z = \ln[\mathrm{Ap}/g(\mathrm{Ap})]$$

where Ap is the area under the peak, g (Ap) is the geometric mean of all compounds, and Z is the transformed peak area (Atchinson 1986). The transformed peak areas were used in a discriminant analysis to determine if workers' different task groups and colonies could be distinguished based on their CHC profiles.

In the nestmate recognition bioassay, Fisher's exact test was used to compare the rates of acceptance and rejection of nestmates and non-nestmates. Statistical analyses were performed using SPSS v. 16.

Results

Caste-Related CHC Differences Of the 106 compounds detected, only the 8 main compounds that were present (i.e., relative abundance of at least 0.2 %) in 75 % of the individuals in each group were included in the statistical analyses. These were heneicosane (C_{21}), (*Z*)-9-tricosene ($C_{23:1}$), tricosane (C_{23}), pentacosane (C_{25}), hexacosane (C_{26}), heptacosane

(C_{27}), octacosane (C_{28}), and 15,17-dimethylnonacosene (15, 17-Dime$C_{29:1}$).

The worker castes (i.e., newly emerged bees, nurse bees, and forager bees) differed significantly in C_{21}, C_{23}, and C_{27} in all three colonies ($P<0.001$; Fig. 1). C_{25} differed among castes in colony A and colony C ($P<0.01$), but $C_{23:}$ only differed among castes in colony B ($F_{2,29}=6.73$, $P<0.001$). There were no significant caste-related differences in C_{26}, C_{28}, or 15,17-Dime-$C_{29:1}$ for any of the colonies ($P>0.05$).

Percentage of *n*-Alkanes Castes differed significantly in their relative levels of *n*-alkanes in all three colonies (Colony A: $P<0.001$; Colony B: $P<0.001$; Colony C: $P<0.01$; $df=2$). Forager bees had the highest levels, and newly emerged bees had the lowest levels (Fig. 2).

Colony Differences in CHCs Both newly emerged bees (MANOVA: $F_{2,29}=2.42$, $P<0.001$) and forager bees ($F_{2,29}=3.83$, $P<0.001$) displayed different CHC profiles in different colonies. Nurse bees did not differ in their profiles across colonies ($F_{2,29}=1.19$, $P=0.30$). Discriminant analysis revealed that CHC profiles were task-group specific (Fig. 3). All the individuals were correctly assigned to their castes.

Discriminant analysis showed that the CHC profiles of worker bees are colony specific and that the bees clustered according to their colony of origin; about 96.5 % of the bees were correctly classified. The first two discriminant functions explained 100 % of the variance. Function 1 explained 66.5 % (canonical correlation = 0.908, *Wilks' lambda* = 0.052, $X^2=221.95$, $P<0.001$), and function 2 explained 33.5 % (canonical correlation = 0.839, *Wilks' lambda* = 0.296, $X^2=91.24$, $P<0.001$). The plot of the first vs. the second roots of the discriminant analysis (Fig. 4) clearly shows that worker bees from different colonies could be distinguished based on their CHC profiles.

Nestmate Recognition The levels of aggression displayed by guard bees towards nestmates treated with different CHCs are summarized in Fig. 5. Significantly more aggression was directed towards bees treated with tetracosanoic acid (Colony A: 60 % of individuals; $P=0.008$; Colony B: 65 % of individuals; $P=0.001$; Colony C: 65 % of individuals;: $P=0.003$), hexadecanoic acid (Colony A: 70 % of individuals; $P=0.001$; Colony B: 80 % of individuals; $P=0.001$; Colony C: 90 % of individuals; $P=0.001$) and (*Z*)-9-tricosene (Colony A: 70 % of individuals; $P=0.001$; Colony B: 45 % of individuals; $P=0.008$; Colony C: 85 % of individuals; $P=0.001$). In contrast, bees treated with pentacosane did not experience increased aggression as compared to the control (Colony A: 30 % of individuals; $P=0.451$; Colony B: 25 % of individuals; $P=0.182$; Colony C: 5 % of individuals; $P=0.605$).

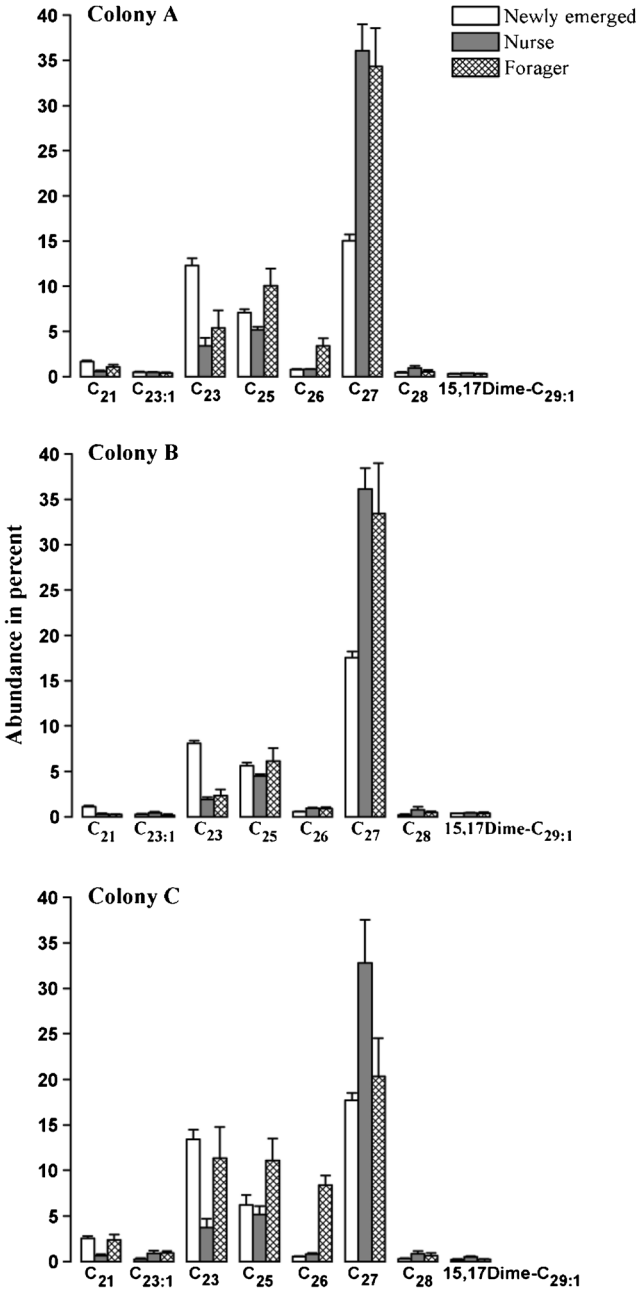

Fig. 1 Differences in major CHCs produced by newly emerged bees, nurse bees, and forager bees in each colony ($N=10$ for each group; *error bars* represent standard errors)

Discussion

The findings demonstrate that *n*-alkanes were present in high amounts in all three colonies of *A. c. indica*. We also showed that CHC profiles differed among task groups. Foragers possessed relatively higher levels of *n*-alkanes. Similarly, *A. mellifera* foragers have been found to have relatively more *n*-alkanes in their CHC profiles (Kather et al. 2011). Differences in CHC profiles and proportions across different age groups and subcastes may serve as the basis for task

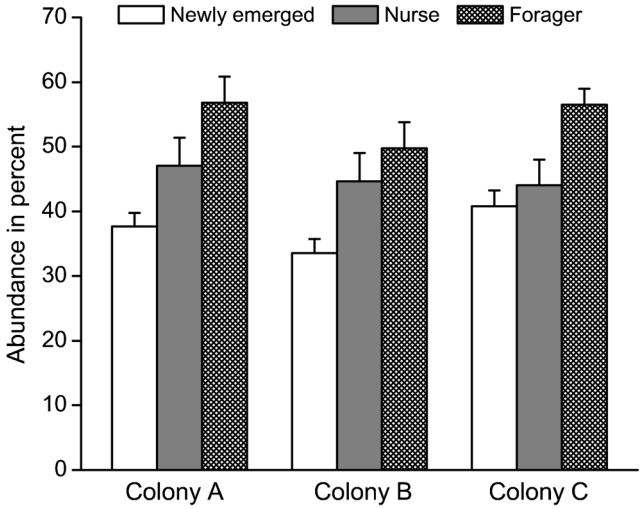

Fig. 2 Total amount of *n*-alkanes produced by newly emerged bees, nurse bees, and forager bees in the three colonies ($N = 10$ for each group; error bars represent standard errors)

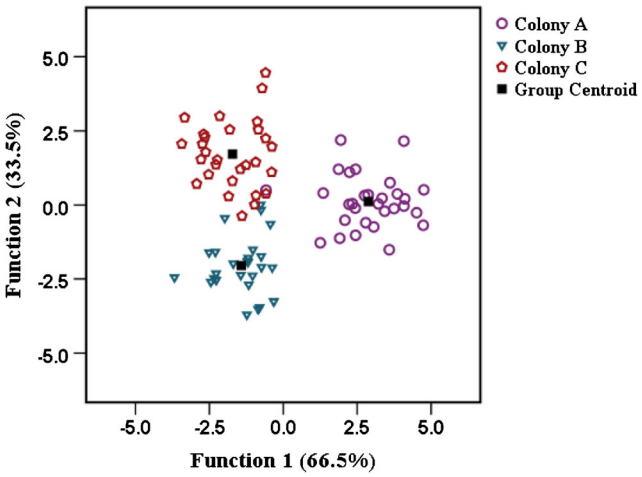

Fig. 4 Colony clustering in *A. c. indica* based on the CHC discriminant analysis; 96.5 % of workers were correctly classified ($N = 30$ for each colony)

allocation and nestmate recognition (Bonavita-Cougourdan et al. 1993; Scholl and Naug 2011). Foragers are more often exposed to unfavorable environmental conditions, such as hot temperatures, high humidity, and harmful chemicals. Consequently, the increased levels of *n*-alkanes in foragers (relative to those of bees remaining inside the hive) suggest that this class of hydrocarbons is involved in regulating water impermeability as opposed to communication. *n*-Alkanes are considered to be among the best hydrocarbons for use in preventing desiccation (because of their high melting point), and they may provide better waterproofing in the face of changing environmental conditions (Lockey 1988). High concentrations of the *n*-alkanes C_{23}, C_{25}, and C_{27} have also been observed in ants and bees (Bonavita-Cougourdan et al. 1993; Kather et al. 2011; Martin and Drijfhout 2009; Wagner et al. 1998). The *n*-alkanes C_{23}, C_{25}, and C_{27} may help in distinguishing task groups in *A. c. indica*; further studies are needed to elucidate the roles of these hydrocarbons.

We found that the CHC profiles of newly emerged bees and forager bees varied within colonies, whereas those of nurse bees did not. Furthermore, nurse bees showed no significant differences among colonies. This pattern may be due to the fact that all nurse bees experience the same conditions inside the hive. In contrast, each forager experiences different abiotic conditions (Martin et al. 2008). Consequently, it makes sense that the CHC profiles of foragers would differ, while the CHC profiles of nurse bees would remain consistent.

We also found that guard bees reacted aggressively to forager bees treated with tetracosanoic acid, hexadecanoic acid, and (*Z*)-9-tricosene, whereas pentacosane did not elicit such effects. This finding demonstrates that *A. c. indica* largely uses fatty acids and alkenes in nestmate recognition. However, the one *n*-alkane (pentacosane) tested in this experiment failed to

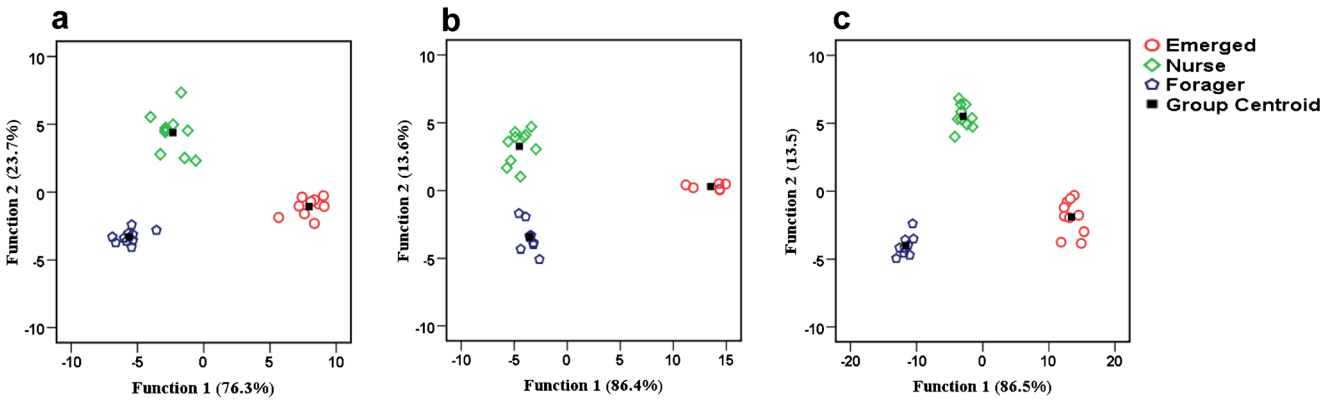

Fig. 3 Task groups clustering based on the discriminant analysis (DA) of CHCs in *Apis c. indica*. In all three colonies, the DA correctly discriminated among bees in different castes (100 %). $N = 10$ for each group. **a** colony A (function 1, *canonical correlation* = 0.987, *Wilks' lambda* = 0.002, $X^2 = 151.19$, $P < 0.001$; function 2, *canonical correlation* = 0.959, *Wilks' lambda* = 0.080, $X^2 = 61.99$, $P < 0.001$); **b** colony B (function 1, *canonical correlation* = 0.992, *Wilks' lambda* = 0.001, $X^2 = 136.95$, $P < 0.001$; function 2, *canonical correlation* = 0.952, *Wilks' lambda* = 0.093, $X^2 = 49.92$, $P < 0.001$) and **c** colony C (function 1, *canonical correlation* = 0.996, *Wilks' lambda* = 0.001, $X^2 = 159.23$, $P < 0.001$; function 2, *canonical correlation* = 0.974, *Wilks' lambda* = 0.051, $X^2 = 61.03$, $P < 0.001$)

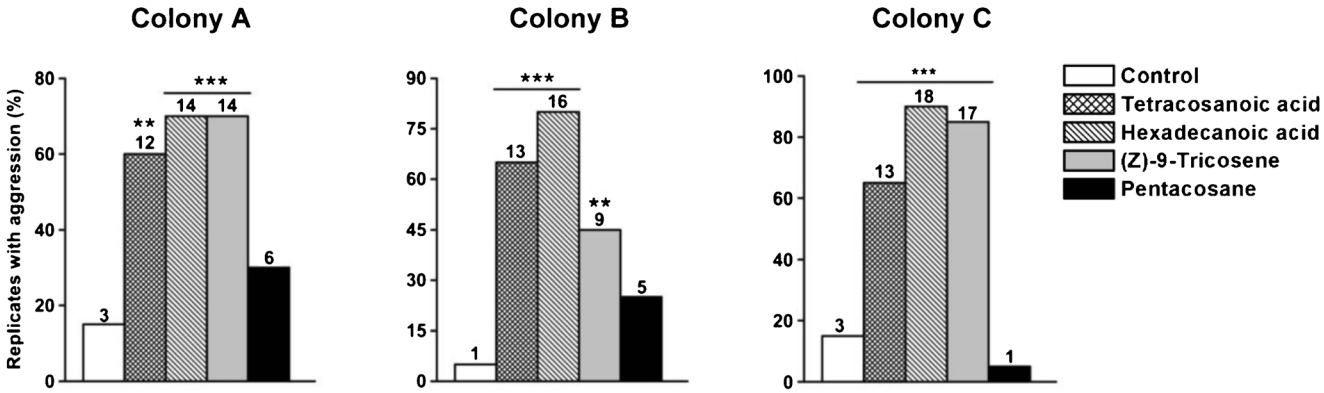

Fig. 5 Effects of cuticular compounds on nestmate recognition in *A. c. indica*. $^{**}P < 0.01$; $^{***}P < 0.001$ (Fisher's exact test). The values above each bar represent the number of forager bees rejected (out of 20) in each trial

change bee behavior. A greater number of *n*-alkanes need to be tested to draw more definite conclusions about their effects. This result is similar to those of Dani et al. (2005) who reported that bees treated with alkenes were attacked more frequently by guard bees than were bees treated with alkanes. Studies in *Formica argentea* have shown that alkenes and methyl-branched alkanes may potentially function in nestmate recognition, whereas *n*-alkanes seem to be relatively unimportant (Krasnec and Breed 2013). It is known that alkenes serve as nestmate recognition cues, and experiments on *A. mellifera* have shown that honey bees are better at learning to recognize and discriminate among alkenes than alkanes (Châline et al. 2005). There is increasing evidence that, within colonies, alkene chain length does not vary; instead, several different colony-specific isomers occur (Kather et al. 2011; Martin et al. 2008; Martin and Drijfhout 2009; Yamaoka 1990). Alkanes differ among colonies and thus do not contribute to nestmate recognition (Kather et al. 2011; Martin and Drijfhout 2009). These results suggest that guards are able to recognize and accept other bees as nestmates if the bees' cuticular compounds match those in the colony's template. Torres et al. (2007) found that, in the Argentine ant (*Linepithema humile*), ants from foreign colonies that were treated with higher CHC concentrations experienced greater aggression than ants treated with lower concentrations. In *A. mellifera* and *Trigona fulviventris*, tetracosanoic acid, hexadecanoic acid, and (*Z*)-9-tricosene have been observed to elicit behavioral reactions (Breed 1998; Breed and Stiller 1992; Buchwald and Breed 2005). Fatty acid and alkene alterations cause bees to attack their own nestmates (Breed 1998). Treatment with 18-carbon fatty acids in *A. mellifera* and *T. fulviventris* resulted in aggression between the treated and untreated nestmate (Breed et al. 2004a, b, 2007; Buchwald and Breed 2005). Although this study and another (Breed and Stiller 1992) found that pentacosane did not affect levels of aggression in *A. c. indica* and *A. mellifera*, respectively, a different result was observed in the ant species *T. fulviventris*, in which the compound appears to serve as a nestmate recognition cue (Buchwald and Breed 2005). CHCs such as C_{17}, C_{20}, C_{23}, C_{25}, C_{29}, and essential oils, along with long-chain fatty acids, do not appear to function in nestmate recognition in *A. mellifera* (Bowden et al. 1998; Breed and Stiller 1992; Downs et al. 2000). Like *A. mellifera*, *A. cerana* guards also have the ability to recognize their nestmates (Chapman et al. 2008). In our study, a modification of the rest of the chemical signature after the treatment was uncertain. Further studies need to verify this point.

Previous workers have reported that caste-related variation in CHCs occurs in social insects (Bagneres et al. 1998; Darrouzet et al. 2014; Haverty et al. 1996; Kaib et al. 2000; Smith and Taylor 1990). Our study suggests that *A. c. indica* also has task-specific chemical signatures. Similarly, *A. mellifera* displays considerable variation in CHC profiles (Kather et al. 2011). Caste-related differences in CHCs may be due to the tasks performed by individuals (Wagner et al. 1998). Thus, CHCs may convey information that helps inform task decision-making.

Acknowledgments The first author was funded by Maulana Azad National Fellowship (University Grant Commission, New Delhi, India). The GC/MS was obtained thanks to a grant from the PRES Centre Val de Loire University (APR-IA 2012) and the CNRS (INEE).

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