Mate and nestmate recognition in the primitively eusocial wasp *Ropalidia marginata*

Thesis submitted in partial fulfillment of the requirements of

Five Year BS-MS Dual Degree Program

by

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Certificate

This is to certify that this dissertation entitled "**Mate and nestmate recognition in the primitively eusocial wasp *Ropalidia marginata***" towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents original research carried out by **Ravindra P N** at the Indian Institute of Science, Bangalore (IISc) under the supervision of **Prof. Raghavendra Gadagkar, Professor, Centre for Ecological Sciences (CES)** during the academic year 2014-2015.

Prof. Raghavendra Gadagkar

Centre for Ecological Sciences

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Declaration

I hereby declare that the matter embodied in the report entitled "**Mate and nestmate recognition in the primitively eusocial wasp *Ropalidia marginata***" are the results of the investigations carried out by me at the Centre for Ecological Sciences, Indian Institute of Science, under the supervision of Prof. Raghavendra Gadagkar and the same has not been submitted elsewhere for any other degree.

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Abstract

Chemical communication has a vital role in social insects. Chemicals like cuticular hydrocarbons (CHCs) are involved in eliciting behaviors such as nestmate and mate recognition. For CHCs to act as mate recognition chemicals, they should be sexually dimorphic. It is known that there is no sexual dimorphism of non-volatile CHCs. So, we checked if males and females of the primitively eusocial wasp*, Ropalidia marginata*, vary with respect to volatile CHCs (if any). Our results, from gas chromatography suggest no volatile CHCs and confirm no sexual dimorphism with respect to CHCs, and a bioassay fails to find any evidence for long distance mate attraction, raising more questions about mate recognition in this species.

Furthermore, we investigated nestmate recognition in the context of nest when an anesthetized wasp is introduced onto a natal and a non-natal nest. It is known that less than six to eight days old non-nestmate wasps are accepted into nests while older non-nestmate wasps are not. In addition, they both differ in their CHC profile which could be environmentally determined; exposure to nestmates/nest is known to change CHC composition. So, we asked if 13-18 day old wasps, isolated from their nest on their eclosion day, are treated differently than similar age wasps that were allowed to stay on their nest (exposed wasps). Using this, we speculate implications of isolation (after eclosion) on CHC dynamics. Our results show that isolated and exposed wasps are not treated differently, and non-nestmates in both the cases are less tolerated than nestmates. This could mean that when isolated for around 15 days, the wasps have a CHC profile that could be closer to their natal nest profile even after the long isolation from their nest and nestmates, and thus similar (since they may acquire colony labels before isolation from nest) to exposed wasps. However, if a wasp is exposed to a foreign nest and its wasps, its own profile could be overridden by the common colony odor. Our study, done in natural context (of nest), confirms earlier study done in artificial conditions.

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Chapter 1

Introduction

Smells can convey message(s). In fact, just think if you smell something while you are reading this—your own sweat, or some pheromones from your neighbor, perhaps indicating the person's gender. If you were a social insect, the smells could deliver messages and elicit different behaviors like recruitment, recognition, territorial behavior etc. (Jackson and Morgan, 1993). Many insects have the ability to deliver messages using chemicals (collectively termed 'pheromones', as they convey messages to other members of their species) such as cuticular hydrocarbons, proteins and peptides. For example, Silk moth females release pheromones that attract males, social insects pheromones can convey their colony membership, sex, trail signal, alarm signal, smell of royalty etc.

Among all the chemicals, cuticular hydrocarbons (CHCs), which form the greasy layer on the exoskeleton of the insects, are widely studied. Social insects like wasps, for example, have complex CHC profiles (Howard and Blomquist, 2004), that differ in composition and relative quantity, forming a cocktail of chemicals, and thus can act as recognition labels (Mitra et al., 2014; Howard and Blomquist, 2004; Richard and Hunt, 2013). The composition could be both genetic and environmentally determined (Gamboa et al., 1986; Gadagkar, 2009). Social interactions like allogrooming, trophallaxis and even the nest material and diet may change the cocktail leading to a uniform but adaptively changing colony specific CHC composition. In social insects, CHCs are involved in wide range of functions. They include protection from desiccation, as task specific cues, dominance and fertility cues, as species, nestmate and mate recognition cues (Howard and Blomquist, 2004; Richard and Hunt, 2013). This thesis deals with two of the important behaviors associated with CHCs: mate and nestmate recognition in *Ropalidia marginata.*

Ropalidia marginata is a primitively eusocial, common tropical paper wasp (Figure 1). Their nests are without any envelope and thus observing and collecting the wasps is easy. Each colony has one reproductive individual (which is morphologically identical to

other workers) and a few workers. For these wasps, and many other social insects, nest is the centre of social interactions and brood rearing. So nest location and nest are important resources; they need to prevent social parasitism and defend the colony. However, there is also drifting of individuals from one colony to another, and sometimes there is usurpation (Gadagkar, 2009), making the ability to recognize and accept nestmates and reject aliens/social parasites a vital attribute .

Figure 1. *Ropalidia marginata* nest (Photo by Tresiamma).

Males of *R. marginata* leave their nest after a period of up to 12 days after their eclosion (Sen and Gadagkar, 2010) and females go out of their nest for mating (Sen et al., 2010). So, recognizing the opposite sex is important for successful mating. But, how do they recognize their opposite sex? Since their courtship behavior involves antennation (Sen and Gadagkar, 2010), do they use chemicals like CHCs to communicate?

The scent of a wasp; is it in the air?

For many insects, CHCs are shown to act as sex pheromones (reviewed in Howard and Blomquist, 2004). They can be perceived by direct contact, if they are non-volatile, or over short distance, if they are volatile (Shorey, 1973). But for CHCs to act as recognition cues, individuals must be sexually dimorphic with respect to CHCs (quantitatively and/or qualitatively). Sexual dimorphism in CHC composition (quantitative and/or qualitative) is common in insects like a few ants, flies, crickets etc., and this is known to help in mate recognition (Cuvillier-Hot et al., 2001; Ferveur, 2005; Howard and Blomquist, 2004; Thomas and Simmons, 2008). In *R. marginata*, it is known that the non-volatile CHCs on males and female are not significantly different (Mitra et al., unpublished data). However, owing to an evaporation step in the extraction of the CHCs—which was required to get sufficient concentration to analyze the sample using Gas Chromatography (GC)—previous experiments could not consider volatile CHCs in the comparison. So, here we asked if volatile CHCs are involved in mate

recognition in this species. Is there sexual dimorphism with respect to volatile CHCs in *R. marginata*? To answer these questions, we used a modified method to extract CHCs, chemical analysis using GC and a bio-assay. Furthermore, we investigated another important behavior that involves CHCs—nestmate recognition.

Us vs. them; who is nestmate and who is not?

For successful nestmate recognition, every wasp should carry a label on its body, which indicates its nestmateship, and a template in its brain, which can be used to compare other wasp's label with its own (Gadagkar, 2009). Because of the possibility of various combinations, CHCs are thought to be acting as the main labels for nestmate recognition (D'Ettorre and Lenoir, 2010; Mitra et al., 2014, for *R. marginata*). It is known that female *R. marginata* wasps discriminate between nestmates and non-nestmates, presumably based on the perception of reproductive threat, outside the context of nest (Venkataraman and Gadagkar, 1992; Venkataraman et al., 1988). What happens in the context of nest is unknown. Further, isolated older non-nestmates (>6 to 8-day-old) are not accepted onto unrelated colonies but newly eclosed (< 6 to 8-day-old) wasps are (Arathi et al., 1997). This is explained by the fact that the newly eclosed females (<5 day post-eclosion age) have different CHC profile and lesser in quantity than the older females (Mitra et al., 2014). In addition, we know that nestmate recognition labels are acquired from sources outside their body, may be nest material or nestmates (Venkataraman et al., 1988). So, what changes happen to their CHC profile when they are older, say around 2 weeks old, and isolated post-eclosion? If there are any changes, it would be reflected in the way the wasps are treated when they are introduced to their natal and a non-natal nest after a few days of isolation. So, we asked if 13-18 day old wasps, isolated from their nest and nestmates on their eclosion day, are treated differently, in the context of nest, than similar age wasps that were allowed to stay on their nest (exposed wasps). We also asked if nestmates and non-nestmates in each of cases are treated differently in the natural context of nest. To answer the questions, we performed introduction of each wasp (after anesthetization on ice) by holding it using a pair of forceps 1-2 cm over its natal nest or a non-natal nest and recorded the response of the resident wasps towards it.

Chapter 2

Mate Recognition

To find out if there are volatile CHCs and sexual dimorphism with respect to volatile CHCs in *R.marginata* we extracted CHCs and analyzed them using gas chromatography (section 2.1). In addition, we performed a bio-assay to confirm the presence/absence of volatiles in mate recognition (section 2.2).

Methods

2.1 Down to the compounds: a chemical analysis

Animal collection and rearing

Newly eclosed male and female wasps were collected from 6 different nests, in the IISc campus, within 24 hours of their eclosion. All the animals were collected during late October and November 2014. Each wasp was isolated and reared in a closed, ventilated plastic box with *ad libitum* food, diluted honey, *Corcyra cephalonica* (rice moth) larvae, building material (soft wood) and tap water (Figure 2) till they are seven days old. We chose 7 days, since 5-20 day old *R. marginata* wasps can mate (Sen et al., 2010), and thus they can be expected to have the sex pheromones. On the eighth day, the wasps were frozen and kept at -20ºC till they were used for CHC extraction. Eight males and eight females were collected in total.

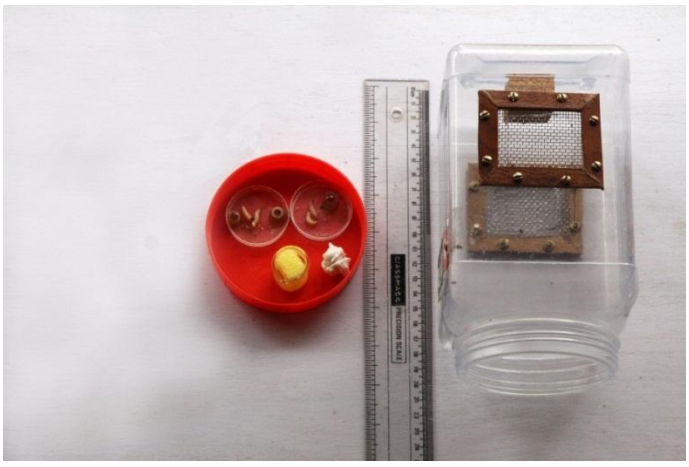


Figure 2: Ventilated box and food to maintain isolated wasps in vespiary (photo:Souvik).

CHC extraction and gas chromatography (GC)

CHC was extracted by first pouring 100microlitres of chilled acetone on the wasp kept in chilled GC vial and gently shaking the vial in ice for about 30 seconds. Previously a different method, which included an evaporation step to increase the concentration of extracted CHCs, was used to extract CHCs from the wasps. But our experiment was done on ice so that no volatiles can evaporate. Eight microliters were drawn from the 100 microliter and injected immediately into the GC machine. GC conditions were identical to those mentioned in (Mitra et al., 2014). After acetone run, the wasp was put in a fresh chilled GC vial and the same procedure was repeated with pentane as a solvent. We chose two solvents because pentane can dissolve non-polar CHCs (that could be insoluble in acetone), while acetone can dissolve both polar CHCs with non-polar tails and also some fully non-polar CHCs.

Mass spectrometry

Identification of compounds for *Ropalidia marginata* was already done in (Mitra et al., 2014) by interpretation of their mass fragmentation patterns produced by electron impact ionization at 70 eV. This was used to match the retention times of the current data and all the peaks in our data could be identified.

Data analysis

Univariate analysis

First, all the solvent peaks were deleted from the raw data. Percentage area under each peak was then calculated. All the peaks that were there in at least 6 out of 8 animals were retained. Only those peaks from both male and female (taken separately) that are more than one percent peak area in at least six out of eight animals were retained. This is to remove small peaks which could potentially be contaminants/noise as they are close to baseline. The remaining peaks from male and female are then compared using Mann-Whitney U test with bonferroni correction. Analysis was done using R Studio (Version 0.98.1091)

Multivariate Analysis

Area under all the peaks considered for univariate analysis was added with 0.001 to get rid of zeros. The data were then log-geo transformed using the formula: $Z_{p,j}=\ln[A_{p,j}/g(A_j)]$, where $A_{p,j}$ is area of peak p for individual j, $g(A_j)$ is geometric mean of all peaks considered for analysis in individual j, ln is natural logarithm, $Z_{p,j}$ is transformed peak area of peak p of individual j (Reyment, 1989). The transformed areas were then subject to Principal Component Analysis. Those peaks with communality <0.8 were considered for Discriminant Analysis. Percentage of correct classification and Wilk's lambda was examined to evaluate the validity of the discriminant function. This analysis was done using StatistiXL.

Random forest analysis

Percent peaks of all the peaks used for univariate analysis were analyzed by random forest using the package randomForest 4.6-6 in R Studio (Mitra and Gadagkar, 2012, 2014; Mitra et al., 2014). The number of variables used randomly at each decision branch was set to 4 and 100,000 trees were generated. Euclidean distance between the individuals in random forest was measured by one minus proximity (The proximity between any two points is the proportion of times the two points occur at the same terminal node of the trees), and that distance matrix was scaled to two dimensions to visualize their relative positions. Further, Non-metric Multidimensional Scaling was done on the percent peaks to visualize their relative positions on the scaled dimension (Mitra and Gadagkar, 2012).

2.2 Smelling the air: a bio-assay

Animal collection and rearing

Newly eclosed male (n=9) and female (n=9) wasps were collected from their nests (total of 5), in the IISc campus, within 24 hours of their eclosion. All the animals were collected during late October and November 2014. Each wasp was isolated and reared in a closed, ventilated plastic box with *ad libitum* food, diluted honey, *Corcyra cephalonica* (rice moth) larvae, building material (soft wood) and tap water (Figure 2,

Gadagkar, 2009). For each set of experiment, two males and two females with a maximum age difference of three days were used. These wasps were kept for a maximum of 8 days of age and a minimum of 5 day old before they were used for the experiment.

Setup

A T-tube olfactometer made of glass was used for the assay (Figure 3).

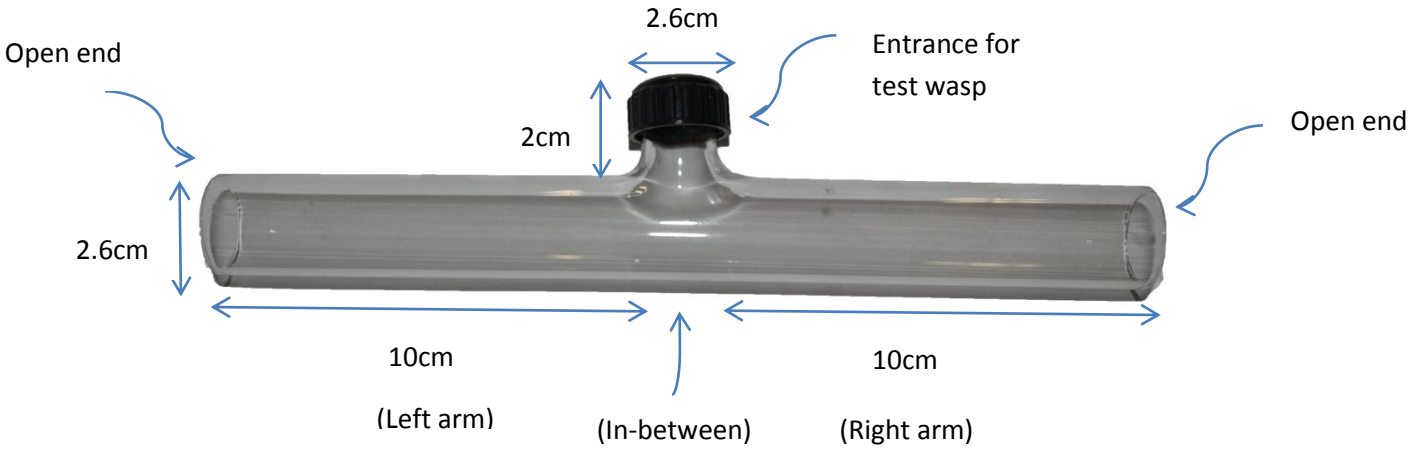


Figure 3: T-tube olfactometer.

On either of the open ends of T-tube, 5cm vial with a male or female wasp can be inserted (Figure 4) snugly (such that there is still air circulation in the tube). Mouth of this vial was covered with a plastic mesh (mesh 1) that does not allow wasps to enter the T-tube. In addition to this mesh, another mesh (mesh 2) which the wasp in the vial cannot touch, so as to avoid any chemical trace of the wasp on the mesh, was also put before inserting the 5cm vial (Figure 4, zoomed out).

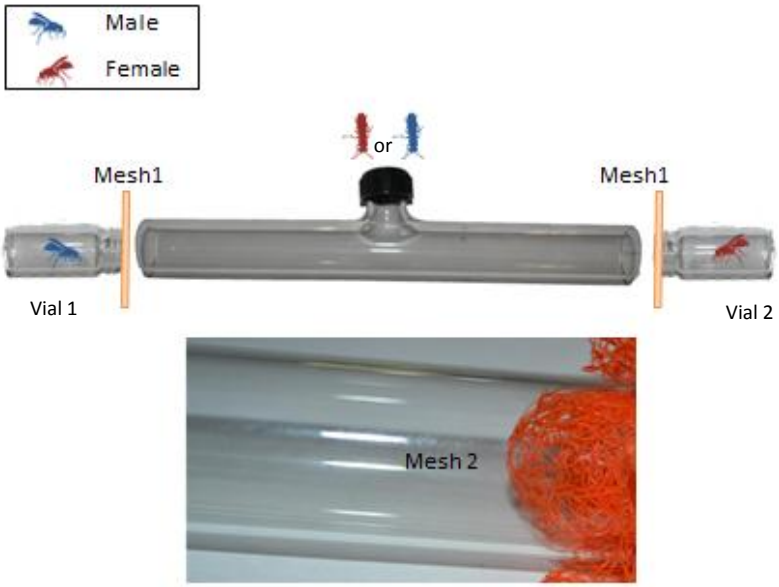


Figure 4: T-tube apparatus with choice vials and meshes.

Experiment

Two males (M1, M2) and two females (F1, F2) of 5 to 8 day old, with a maximum age difference of 3 days were used for the experiment. The test wasp was given two choice tests (a, b) whose sequence was random.

1. Choice test (a): test wasp (say M2 or F2) was introduced into the T-tube with empty vials on either side. Time spent by the wasp in left arm, right arm and in-between was recorded for 10 minutes before the wasp is taken out and kept aside in a glass vial for next choice test.
2. Choice test (b): a male and female wasps (M1, F1) were introduced in vial 1 or 2 randomly, decided with a coin toss, and the vials were keep attached to the either sides of a fresh T-tube, with mesh1 and mesh 2, for five minutes (so that volatiles CHCs from the wasps, if any, can start spreading).
   After five minutes, test wasp, either M2 or F2 (decided with a coin toss), was introduced from T-end (Figure 4). Time spent by the wasp in left arm, right arm and in-between was noted down for 10 minutes.

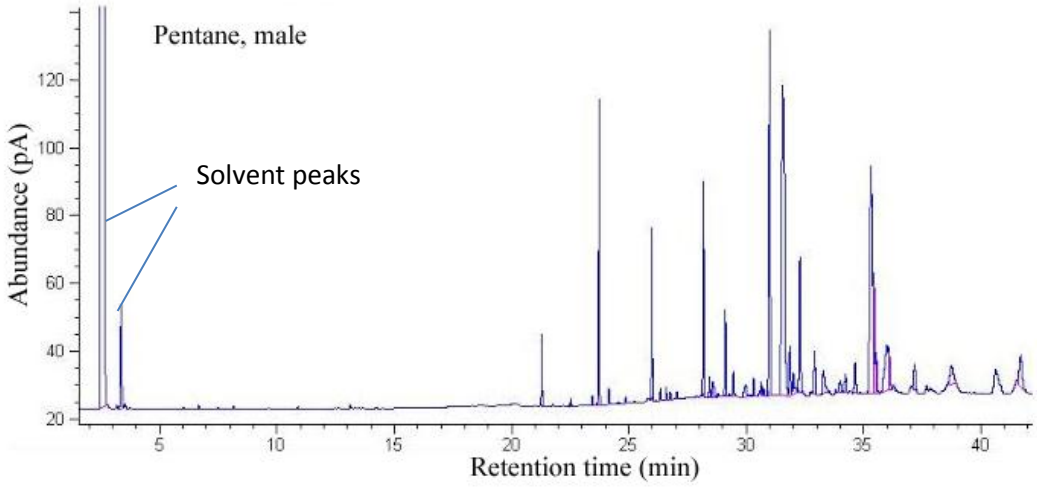
Data Analysis

Proportion of time spent in each side was calculated (Table 3 and 4, for male and female side). Then the proportion of time spent in between was excluded and the proportion for male and female side was re-calculated. The new proportions were log-geo transformed (Reyment, 1989) after adding 0.001 to all the values to remove zeros. We did the transformation to remove simplex structure and constant sum constraint of compositional data (values always add up to a number less than one, in this case). The resultant numbers were compared using Wilcoxon Paired-Sample test. Same was done with the choice test with both blanks on either side.

Results

Chemical analysis

CHC profile for pentane was qualitatively similar to what was found earlier in *R. marginata* (Mitra et al., 2014). From gas chromatography, we found that there are no volatiles (those CHCs that evaporate in room temperature) with both pentane and acetone as solvents (Figure 5-8). Notice that there are no peaks after the solvent peaks (between 2-4 minutes) and before the first at 21 min (first non-volatile peak seen in Mitra et. al unpublished). We also did not observe any consistent qualitative difference between male and female CHC profiles.



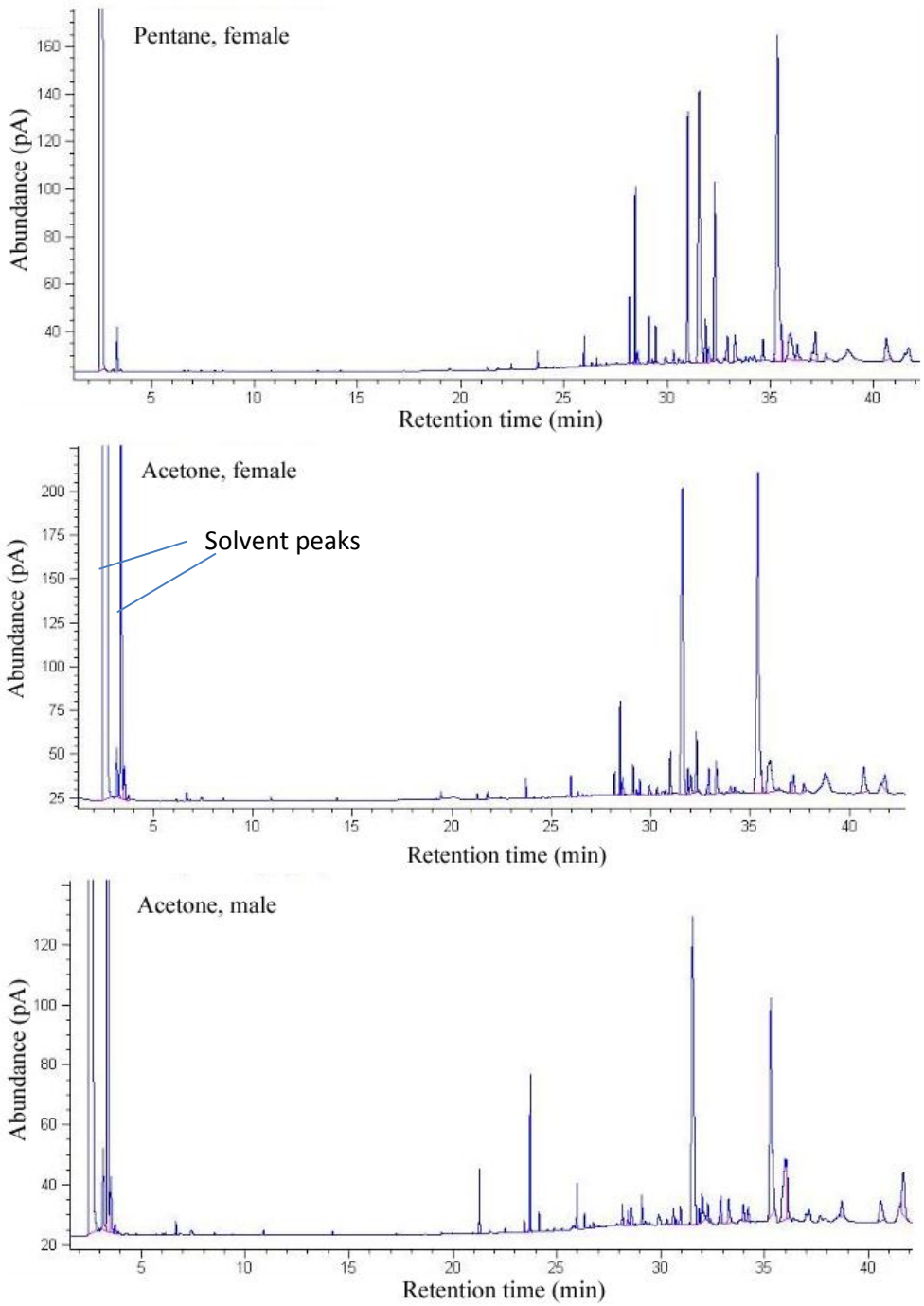


Figure 5-8: Flame ionization detection gas chromatograms of a male (Male7) and a female (Female7) using pentane and acetone as solvents. Observe that there are no significant peaks after the solvent peaks (between 2-4 minutes) and before 21 minutes (first non-volatile peak from Mitra et. al. unpublished). On y-axis is current in pA; it shows quantity of the chemical component.

Univariate analysis

After the cut-offs as mentioned in the methods section, From an average of 35 peaks for pentane and 30 peaks for acetone, 17 peaks remained for pentane and 15 for acetone. When each of the individual peak for a solvent was compared using Mann-Whitney U test, we found that all the peaks do not differ significantly except one peak in each case (peak 13 in pentane, peak 14 in acetone, see Table 1 and 2). Without bonferroni, 4 peaks were significant for pentane, 6 (+3 marginally) peaks significant for acetone.

Table 1: Peak wise comparison of male and female CHCs using pentane as solvent.
* indicates significant after bonferroni correction, $p_{critical}$= 0.0029

Typical retention time	**Peak number**	**Compound identity**	**U**	**p-value**
23.74477	peak 1	Tricosane	21	0.2701
26.00041	peak 2	Pentacosane	14	0.06608
28.19262	peak 3	Heptacosane	25	0.4948
29.12621	peak 4	3-methyl heptacosane	25	0.4948
29.47032	peak 5	octacosane	50	0.06608
31.01362	peak 6	Nonacosane	45	0.1893
31.56927	peak 7	Mixture of 11, 13, 15 methylnonacosane	20	0.2271
31.87729	peak 8	5-methylnonacosane	40	0.4309
32.32422	peak 9	3-methylnonacosane	49	0.08312
32.93092	peak 10	8-methyltriacontane	12	0.04057
33.30376	peak 11	14, 16-dimethyltriacontane	39	0.4948
35.34896	peak 12	Mixture of 11, 13, 15- methylhentriacontane	53	0.03132
35.45755	peak 13	Mixture of 11, 13, 15- methylhentriacontane	4	**0.00146***
35.56266	peak 14	Mixture of 7, 9-methylhentriacontane	49	0.08312
35.99532	peak 15	Mixture of 11, 17 and 13, 17-methylhentriacontane	8	0.01352
37.18245	peak 16	8-methyldotriacontane	41	0.372
40.61946	peak 17	Mixture of 13, 15, 17-methyltritriacontane	33	0.958

Table 2: Peak wise comparison of male and female CHCs using Acetone as solvent. The peaks in acetone were not always the same peaks as in pentane.

* indicates significant after bonferroni correction $p_{critical}$= 0.0033

Typical retention time	Peak number	Compound identity	U	p-value
23.75376	peak 1	Tricosane	19	0.1893
26.00677	peak 2	Pentacosane	12	0.04057
28.60368	peak 3	Unidentified contaminant	12.5	0.04584
29.13605	peak 4	3-methyl heptacosane	25	0.5054
30.99932	peak 5	Nonacosane	47	0.1304
31.56573	peak 6	Mixture of 11, 13, 15 methylnonacosane	11	0.02813
31.8841	peak 7	5-methylnonacosane	47	0.1304
32.03382	peak 8	11, 15-dimethylnonacosane	15	0.08298
32.31044	peak 9	3-methylnonacosane	54	0.02067
32.93996	peak 10	8-methyltraicontane	7	0.006993
33.31143	peak 11	14, 16-dimethyltriacontane	51	0.04988
35.35132	peak 12	Mixture of 11, 13, 15- methylhentriacontane	54	0.02067
36.0195	peak 13	Mixture of 11, 17 and 13, 17-methylhentriacontane	30	0.8785
40.65024	peak 14	Mixture of 13, 15, 17-methyltritriacontane	60	**0.00146***
41.70305	peak 15	13, 19-dimethyltritriacontane	8	0.004569

Multivariate analysis

Males and females could be differentiated completely, based on their CHC profile, by discriminant analysis (Wilks' Lambda=0.036, DF=12, p=0.01, n=8 each, for pentane; Wilks' lambda=0.068, DF=6, p=0.00005, n=8 each, for acetone; classification analysis: 100% correct classification) implying sexual dimorphism with respect to CHCs. However these results are not stringent and are based on many assumptions (see discussion for details).

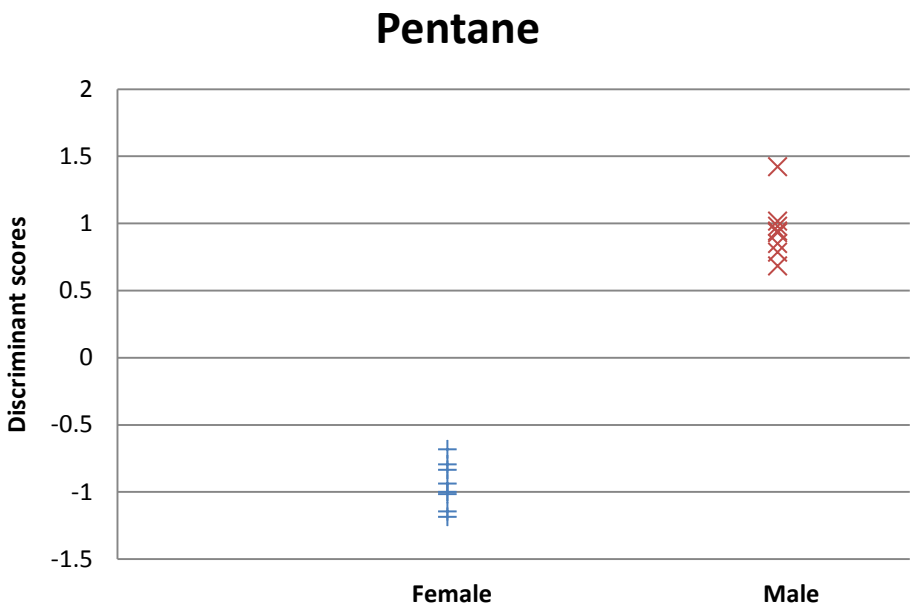


Figure 9: Scores in discriminant functions for males and females using pentane solvent for CHC extraction (Wilks' Lambda=0.036, DF=12, p=0.01, n=8 each, classification analysis: 100% correct classification).

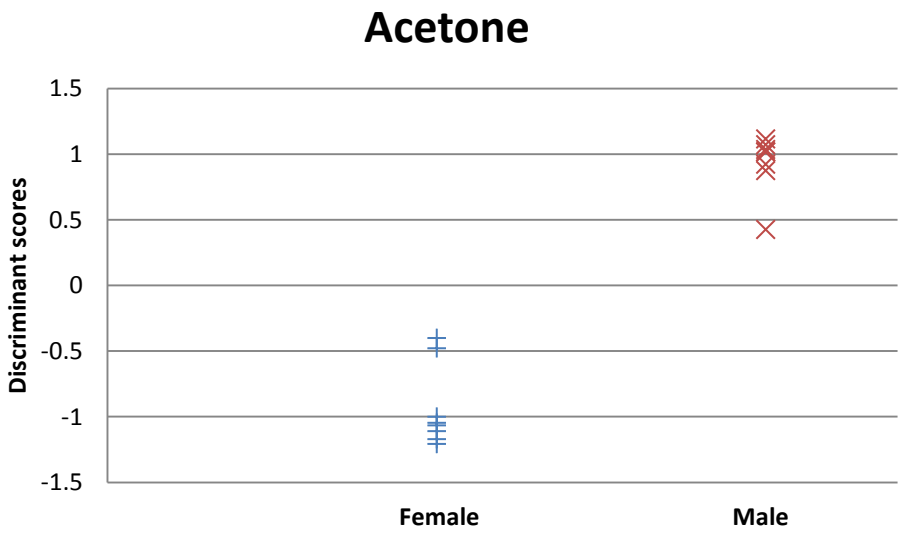


Figure 10: Scores in discriminant functions for males and females using acetone solvent for CHC extraction (Wilks' lambda=0.068, DF=6, p=0.00005, n=8 each, classification analysis: 100% correct classification).

Random forest

When males and females were mingled and run on the random forest, they did not show any clear separation as shown in the multidimensional scaling (Figure 11-12). Based on the value of mean decrease gini, of all the 17 peaks on which random forest was run, peaks 11, 9, 5,7,14 for pentane and of the 15 peaks, peaks 6, 10,7,11,2,9,1 for acetone had higher relative importance.

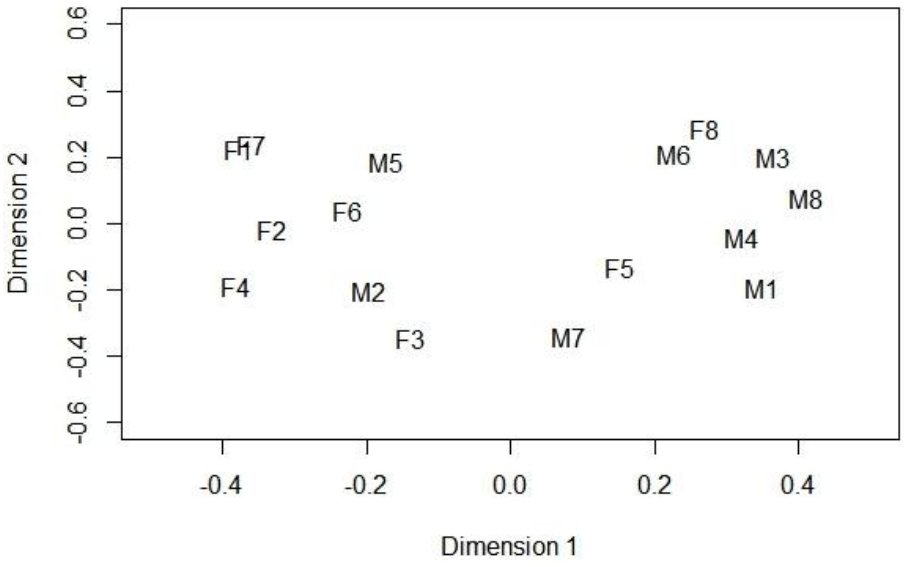


Figure 11: Two dimensional non-metric multidimensional scaling done on percent peaks for all individuals on the random forest,data from pentane as solvent, (100,000 tress, number of randomly selected variables used at each decision branch=4, n=8 each). Notice no clear separation of males and females.

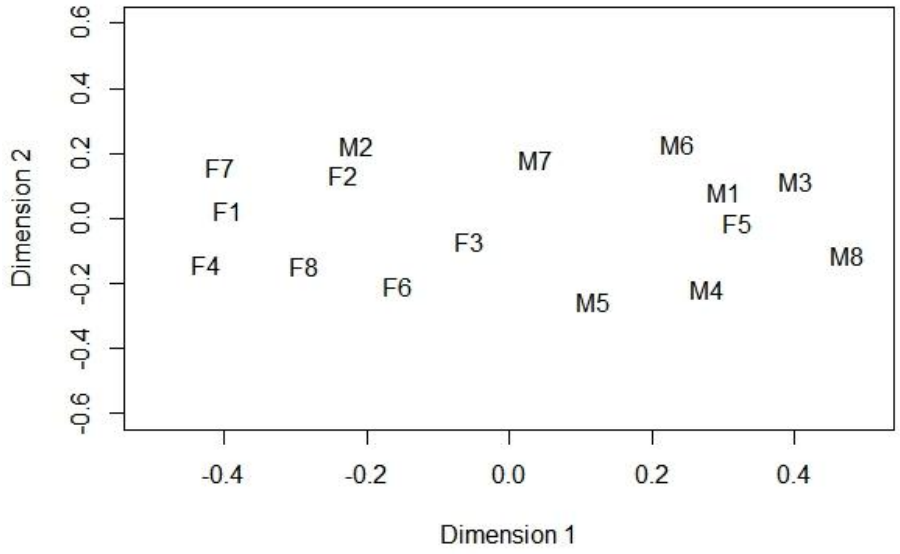


Figure 12: Two dimensional non-metric multidimensional scaling done percent peaks for all individuals on the random forest, data from acetone as solvent, (100,000 tress, number of randomly selected variables used at each decision branch=4, n=8 each). Notice no clear separation of males and females.

Bio-assay

When the transformed proportion of time spent on left and right sides in blank choice test was compared for males and females separately, using two-tailed Wilcoxon Paired-Sample Test, we found that they do not significantly differ for both males as well as females (T=21, p=0.91, n=9, for males; T=8, p=0.10, n=9, for females). Similarly, for choice test with a male and female on either side of the tube, both test males and test females showed no significant difference in proportion of time spent (One-tailed Wilcoxon Paired-Sample test, T=22, p=0.48, n=9, for males, alternate hypothesis: proportion of time spent on female side>male side; T=27, p=0.71 , n=9, for females, alternate hypothesis: proportion of time spent on male side>female side). To check if an animal behaves (time spent in different arms) the same way when animals are present as a choice and when no animals are present (both blank test), we compared each side in blank test with the same side in choice test (b) using two-tailed Wilcoxon Paired-Sample test and found no significant difference (for males: T=20, p=0.82, n=9, for both

comparisons—male side and female side with same side on blank; for females: T=21, p=0.95, n=9, for both comparisons).

Table 3: Proportion of time spent (measured in seconds) by male wasps in different sides of the T-tube.

Male side	Female side
0.27	0
0.58	0.35
0.29	0.47
0.46	0.45
0.02	0.14
0.07	0
0.30	0.50
0.47	0.51
0.23	0.52

Table 4: Proportion of time spent (measured in seconds) by female wasps in different sides of the T-tube.

Male side	Female side
0.07	0.77
0.42	0.44
0	1
0.16	0
0.44	0.47
0	1
0	1
1	0
0.94	0.05

Discussion

Using gas chromatography, we failed to find any volatile CHCs and also re-confirmed that there is no sexual dimorphism of CHCs, and from a bio-assay we found no evidence for long distance mate attraction in *R.marginata,* thus implying that they may not be acting as sex pheromones in this wasp.

Contact pheromones like CHCs are known to have a role in mate recognition in beetles (Ginzel et al., 2003), *Drosophila melanogaster* (Antony and Jallon, 1982) and a few other insects (reviewed in Howard and Blomquist, 2004). Hymenopterans like halictine bees use volatile pheromones to locate mate, and once they approach, they may use contact pheromones or CHCs for courtship responses (Ayasse et al., 1999). For CHCs to act as sex pheromones, they are expected to be sexually dimorphic. In *Ropalidia marginata*, we know that the non-volatile CHCs are not sexually dimorphic (Mitra et al. unpublished). Since there is a possibility of volatiles acting as sex pheromones, we checked for sexual dimorphism with respect to volatiles (if any) using gas chromatography and found no significant peaks in the volatile range, presumably having lower retention time (Figure 5-8). We re-confirmed that there is no sexual dimorphism even with our changed protocol for CHC extraction. In addition, we used both acetone and pentane (separately) as solvents to boost our conclusion.

From univariate comparisons, although we found that one peak significantly differs between males and females (Table 1 and 2), the difference is not consistent as there is overlap of peaks when a male and female are compared, thus suggesting it may not have biological significance. Further, the difference in not consistent with acetone and pentane. Multivariate analysis using PCA and LDA, shows that males and females differ in CHC profile (Figure 9-10, significant Wilks' lambda indicates that between group variance is higher than within group variance). However, compositional data analysis using these statistical methods has many drawbacks (Martin and Drijfhout, 2009). First of all, LDA assumes normality and homoscedasticity and higher sample size (at least, twice the number of variables); all of which do not hold true for most cases of CHC data due to the nature of it. Furthermore, since it is a supervised learning method (identity of male and female group is used during analysis), and as it tries to separate the centroids

of the 2 groups maximally, there are high chances of over fitting the data. So, we decided to check our data using a more stringent method called random forest.

Random forest is an unsupervised machine learning process (gender identity is not considered to make decision trees, thus it is unsupervised) that takes random subset of variables out of the raw data (without any transformations), classifies all individuals based on the variables by making a decision tree. The tree partitions the data into homogenous (as much possible) regions with respect to the variables. The process is iterated many times for all the individuals to generate many trees forming a 'random forest'. Since the forest has many trees, it is not possible to directly visualize the percent peaks for all individuals in the forest and so they are scaled on two dimensions using non-metric multidimensional scaling. Thus, using this stringent method, we showed that males and females do not separate in multidimensional scaling plot (Figure 11 and 12) indicating that males and females cannot be differentiated in a multivariate space.

Differences in volatile CHC profiles, if found, need to have biological significance. So, we performed a bio-assay to confirm our chemical analysis results (that showed no volatiles), and to check if there is any long distance mate attraction (because of volatiles apart from CHCs, in addition to them). In this assay, we had two choice tests and the test wasp could not contact with the choice wasps as they were separated by two different meshes, and thus if any choice is made it should be based on sensing the volatiles (CHCs or otherwise) from either side (Figure 8). We chose time spent on each side of the T-tube as a proxy for choice as we know that in artificial conditions (in plastic box), the wasps mate almost within a few minutes once they are near to another wasp.. Choice test (a), with both blanks on either sides, rules out the possibility that if there were volatiles and wasp sensed it but did not have a choice to make (meaning no choice for either of the smells). Choice test (b) gives the actual time spent on male and female side thus showing differences in time spent, if there is any choice made based on volatiles. This assay showed no evidence for long distance mate attraction cues that may originate from sources other than and in addition to CHCs. Results from the bio-assay corroborate our chemical analysis confirming that no volatiles are involved in mate recognition in *R. marginata*.

Conclusion

Our study shows that males and females of *R. marginata* do not differ in their CHC profiles (qualitatively and quantitatively) and no volatiles CHCs on these wasps, and absence of long distance mate attraction, thus ruling out CHC's role as sex pheromones. Our results are similar to another study on *Formica exsecta* ants (Martin SJ, Shemilt S, 2014). Although our study does not answer the question of sex recognition in this species, it opens up many questions about this phenomenon. In *R. marginata,* males have a yellow clypeus which is brownish in females. So, vision and olfaction could be integrated and used at different priorities (Baracchi et al., 2015) or recognition solely by vision cannot be ruled out as well. They may also be using tactile cues, conditional pheromone release from exocrine glands (ex: releasing at the sight of opposite sex) or less likely option of chance encounter. Another possibility is that they may use proteins and peptides on their body to recognize opposite sex. Further detailed observation of the actual mating process might give some clues as to how they recognize their opposite sex.

Chapter 3

Nestmate recognition

Return of the nestmate and non-nestmate

We studied nestmate recognition in *R. marginata* in the context of nest and compared tolerance towards isolated and exposed wasps when each was anesthetized and introduced onto its natal nest and a non-natal nest.

Methods

Animal collection and rearing (for isolated wasps)

Newly eclosed female wasps (n=30) were collected from 11 different nests in the IISc campus, within 24 hours of their eclosion. Each wasp was isolated and reared in a closed, ventilated plastic box with *ad libitum* food, diluted honey, *Corcyra cephalonica* (rice moth) larvae, building material (soft wood) and tap water (Figure 2) till they are 13 to18 days old (on an average, 15 days). All the collection was done either in November 2014 or January 2015.

Introduction of isolated individuals

First, isolated wasp of age between 13 to 18 days was anesthetized after putting the wasp in a vial kept in ice, for 3 minutes. It was then held by its legs using clean forceps and was introduced back to its natal nest or a non-nest nest. The sequence of this introduction was chosen randomly (with a coin toss). The wasp was held 1-2cm above the centre (approximately) of the nest for one minute. If no wasp responded to the introduced wasp after 30 seconds, the wasp was presented in front of the nearest wasp and so on. All the responses of the wasps in the nest were video recorded for further analysis. The same procedure was repeated for non-natal nest or natal nest (depending on the coin toss). At least 15 minutes of gap was given between each introduction to a nest, and similarly each test wasp was given at least half an hour gap before its next introduction.

Introduction of exposed individuals

Grey eyed (meaning >6 day old) female wasps (n=30) were collected from their nest (6 in total) and isolated in a glass vial for about half an hour before introducing them back to their natal nest/non-natal nest as described in the above section for isolated wasps.

Data analysis

Recorded videos were observed for different behaviors shown to the introduced wasp. While doing this, the observer was blind to whether the wasp is a nestmate or non-nestmate to avoid any confirmation bias (Van Wilgenburg and Elgar, 2013). Number of wasps that interacted with the introduced wasp, their time of interaction, number of different behaviors was tallied from the videos and nestmate vs. non-nestmate and isolated vs. exposed wasps were compared using Wilcoxon signed rank test. From the recordings, a total of five behaviors were observed: Antennation, pecking, nibbling, aggressive biting and attack. Only one out of 120 introductions led to killing of the introduced wasp (the wasp died after the observation time).

All the observed behaviors were ranked in order, similar to (Venkataraman et al., 1988), and a tolerance index was calculated and compared using Wilcoxon signed rank test. $T=\Sigma_{i=1-5}(P_iR_i)$, Where T=tolerance index, P is proportion of the *i*th behavior, and R is the rank of the *i*th behavior.

Rank	**Behavior**
1	Aggressive bite
2	Attack
3	Peck
4	Nibble
5	Antennation

Results

Isolated individuals

Number of wasps that interacted with test animal was not different when compared between nestmates and non-nestmates (Wilcoxon signed rank test, V=105, p=0.47,

n=30 each), but time spent interacting (out of 60 seconds of introduction) was significantly different (Wilcoxon signed rank test, V=113, p=0.01, n=30 each Figure 13). Sum of number of acts of the five behaviors shown towards the introduced wasps was significantly different (Wilcoxon signed rank test , V=63.5, p=0.002, n=30 each, Figure 14). Whether nestmate introduction was done before non-nestmate introduction or the other way, did not significantly differ (nestmates (Wilcoxon signed rank test , V=8.5, p=0.21, n=30 each, Figure 15), non-nestmates (Wilcoxon signed rank test , V=52, p=0.1, n=30 each, Figure 15). Tolerance index for nestmate vs. non-nestmate introductions was significantly different (Wilcoxon signed rank test, V=181.5, p=0.02, n=30 each, Figure 16).

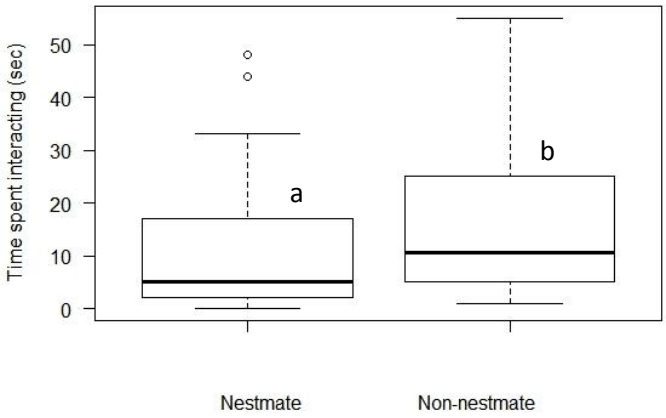


Figure 13: Time spent interacting with the introduced isolated wasps (Wilcoxon signed rank test, V=113, p=0.01, n=30 each). Different alphabets over the bars indicate significant difference.

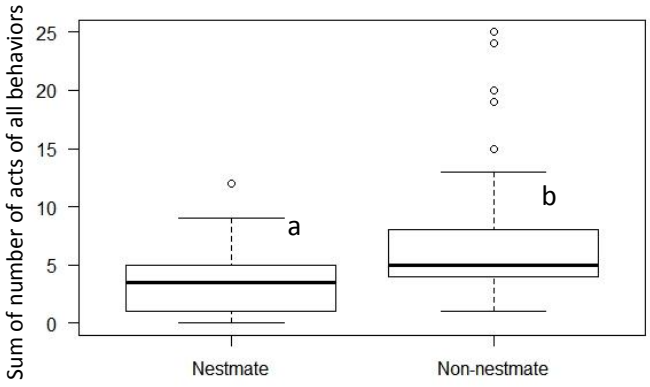


Figure 14: Sum of number of acts of the five behaviors shown to the introduced isolated wasps (Wilcoxon signed rank test, V=63.5, p=0.002, n=30 each). Different alphabets over the bars indicate significant difference.

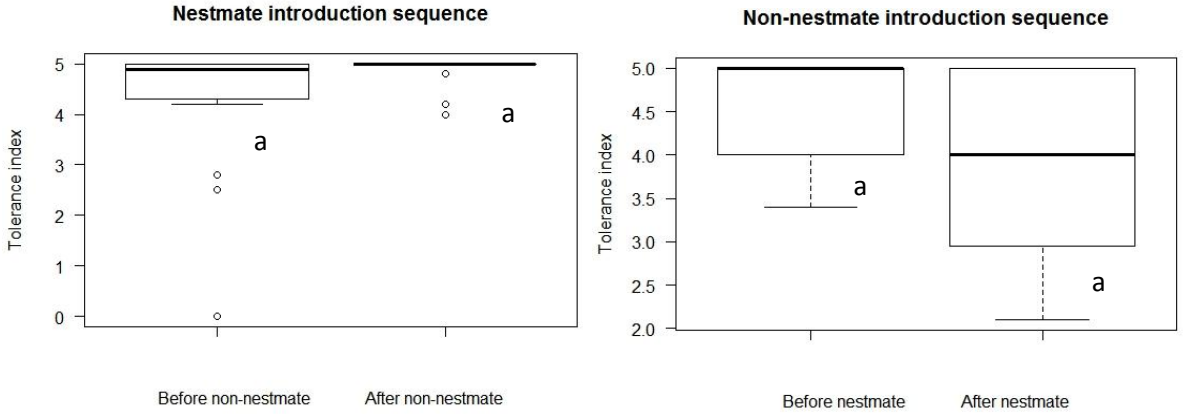


Figure 15: Sequence of introduction for isolated wasps (Wilcoxon signed rank test, Nestmates: V=8.5, p=0.21, n=30 each; Non-nestmates: V=52, p=0.1, n=30 each)

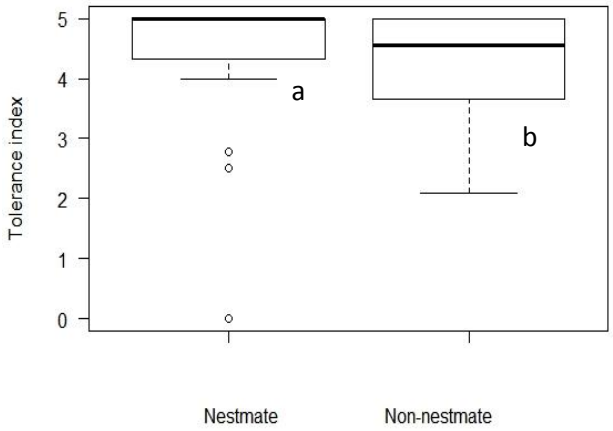


Figure 16: Tolerance index for isolated individuals when nestmate and non-nestmate introductions are compared (Wilcoxon signed rank test, V=181.5, p=0.02, n=30 each)

<u>Exposed individuals</u>

Number of wasps that interacted with test animal was not different when compared between nestmates and non-nestmates (Wilcoxon signed rank test, V=111.5, p=0.27, n=30 each), but time spent interacting (out of 60 seconds of introduction) was significantly different like in the isolated case (Wilcoxon signed rank test,, V=78, p=0.002, n=30 each, Figure 17). Sum of number of acts of the five behaviors shown

towards the introduced wasps was significantly different (Wilcoxon signed rank test,, V=83, p=0.03, n=30 each, Figure 18). Whether nestmate introduction was done before non-nestmate introduction or the other way, did not significantly differ; nestmates (V=1.5, p=0.13, n=30 each), non-nestmates (V=32, p=0.61, n=30 each), Figure 19). Tolerance index for nestmate vs. non-nestmate introductions was significantly different (Wilcoxon signed rank test, V=277, p=0.03, n=30 each; Figure 20).

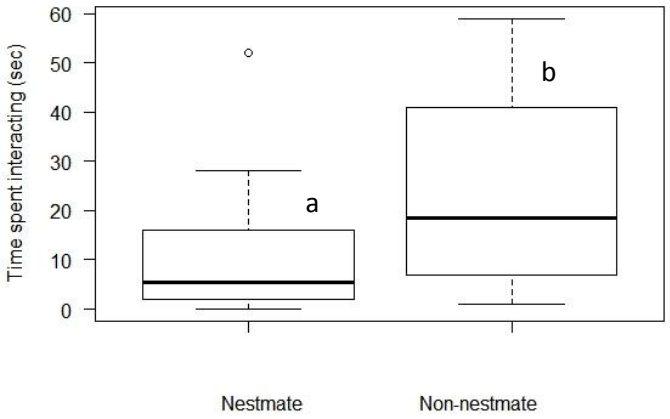


Figure 17: Time spent interacting with the introduced exposed wasps (Wilcoxon signed rank test, V=78, p=0.002, n=30 each).

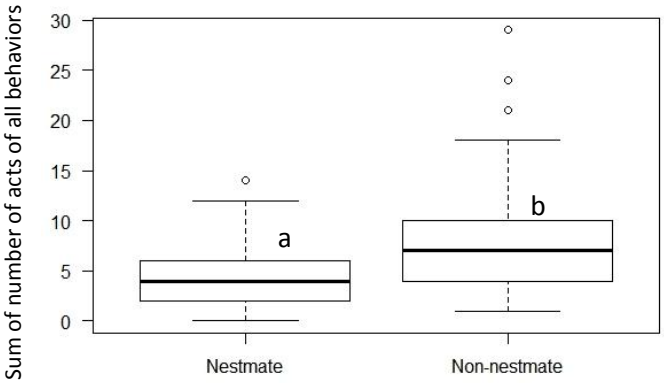


Figure 18: Sum of number of acts of the five behaviors shown to the introduced exposed wasps (Wilcoxon signed rank test, V=83, p=0.03, n=30 each).

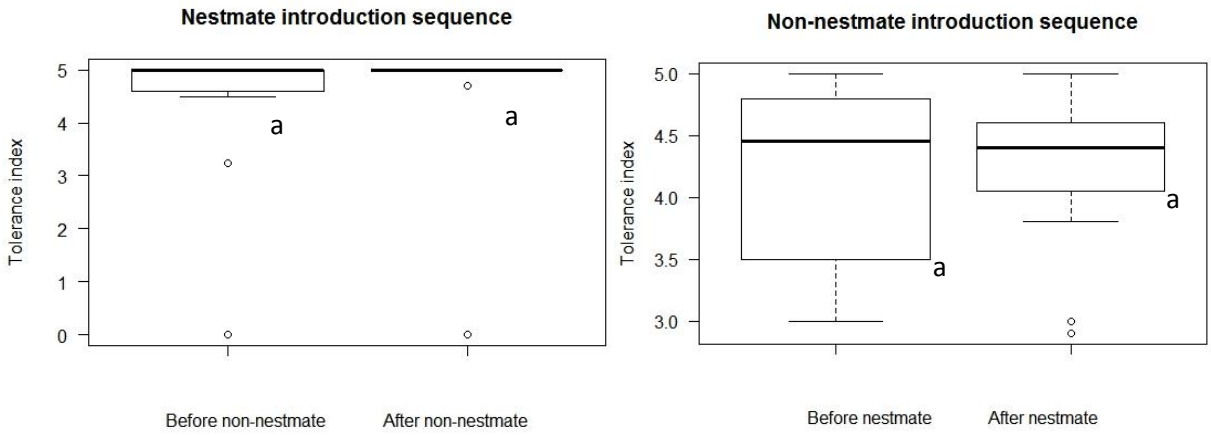


Figure 19: Sequence of introduction for exposed wasps (Wilcoxon signed rank test, Nestmates: V=1.5, p=0.13, n=30 each; Non-nestmates: V=32, p=0.61, n=30 each).

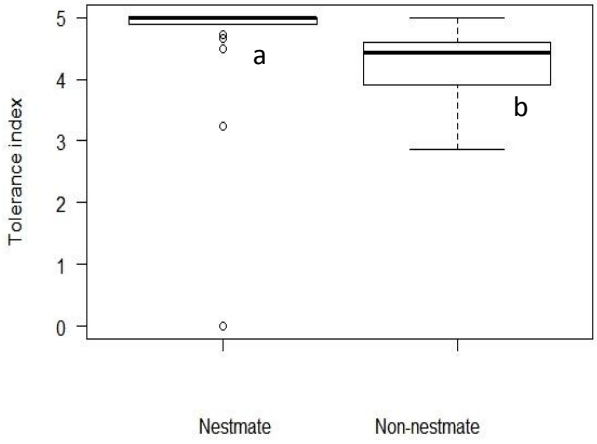


Figure 20: Tolerance index for exposed individuals when nestmate and non-nestmate introductions are compared (Wilcoxon signed rank test, V=277, p=0.03, n=30 each).

<u>Isolated Vs. Exposed individuals</u>

Number of wasps that interacted with test animal was not significantly different (Wilcoxon signed rank test, Nestmate: V=130, p=0.61, n=30 each; Non-nestmate: V=152, p=0.21, n=30 each, Figure 21), and time spent interacting (out of 60 seconds of introduction) was not significantly different (Wilcoxon signed rank test, Nest mate: V=228, p=0.93, n=30 each; Non-nestmate: V=296.5, p=0.19, n=30 each, Figure 22). Similarly, Sum of number of acts of the five behaviors shown towards the introduced wasps was not significantly different (Wilcoxon signed rank test, Nestmates: V=196.5,

p=0.60, n=30 each; Non-nestmates: V=262.5, p=0.54, n=30 each, Figure 23). We found no difference in tolerance index between isolated vs. exposed individuals for both nestmate and non-nestmate introduction (Wilcoxon signed rank test, Nest mate: V=91.5, p=0.81, n=30 each; Non-nestmate: V=180.5, p=0.85, n=30 each, Figure 24).

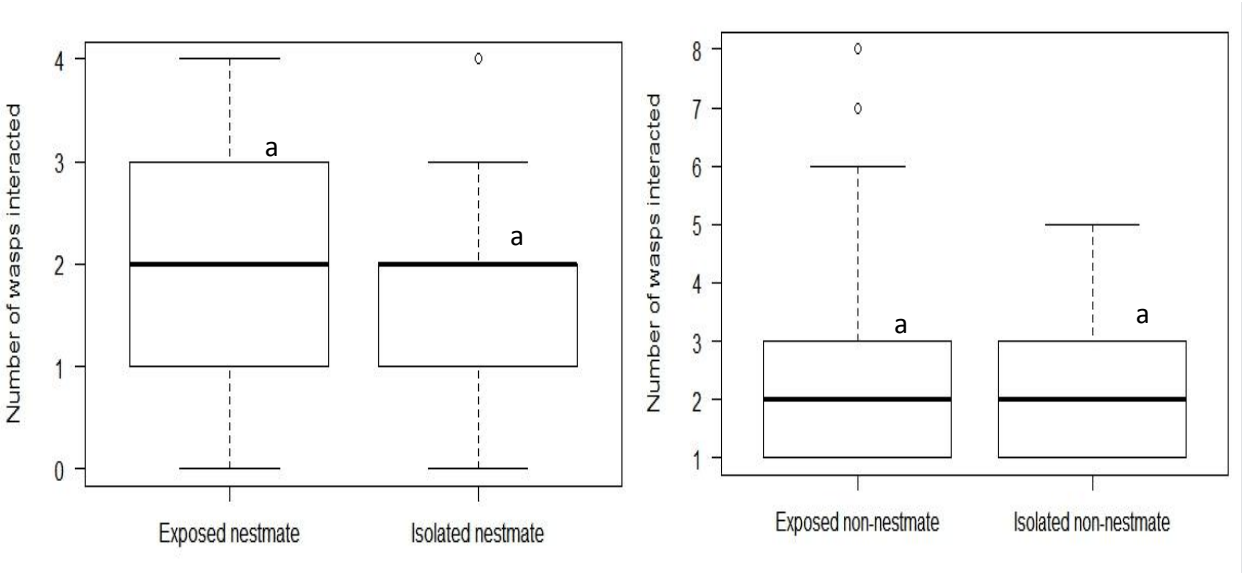


Figure 21: Number of wasps that interacted with the introduced wasp (Wilcoxon signed rank test, Nestmate: V=130, p=0.61, n=30 each; Non-nestmate: V=152, p=0.21, n=30 each). Same alphabets over the bars indicate no significant difference.

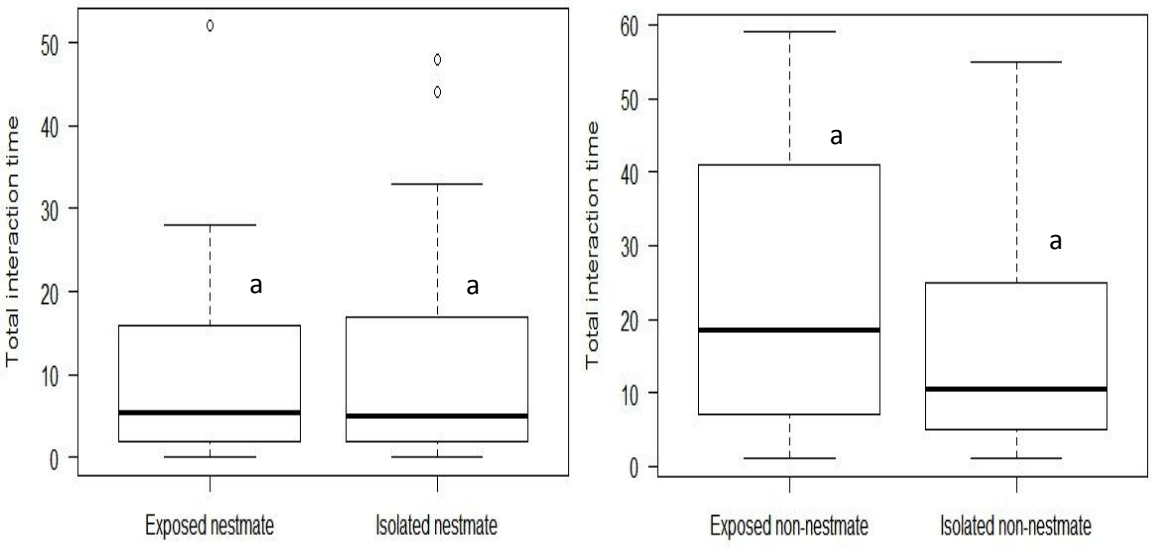


Figure 22: Time spent interacting with the introduced wasps (Wilcoxon signed rank test, Nest mate: V=228, p=0.93, n=30 each; Non-nestmate: V=296.5, p=0.19, n=30 each).

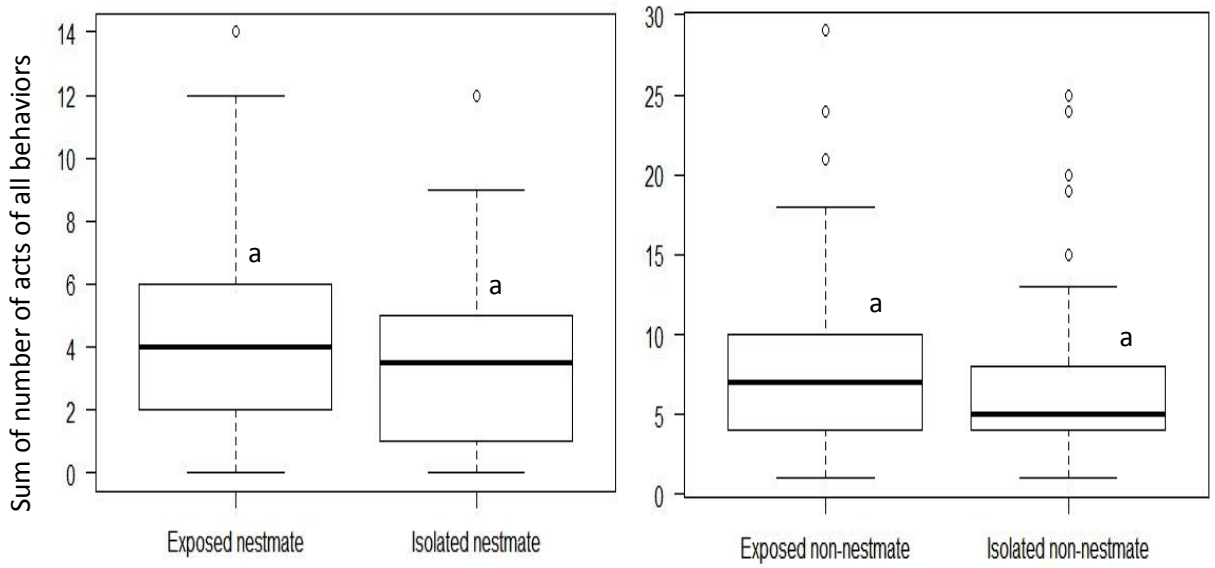


Figure 23: Sum of number of acts of the five behaviors shown to the introduced wasps (Wilcoxon signed rank test, Nestmates: V=196.5, p=0.60, n=30 each; Non-nestmates: V=262.5, p=0.54, n=30 each).

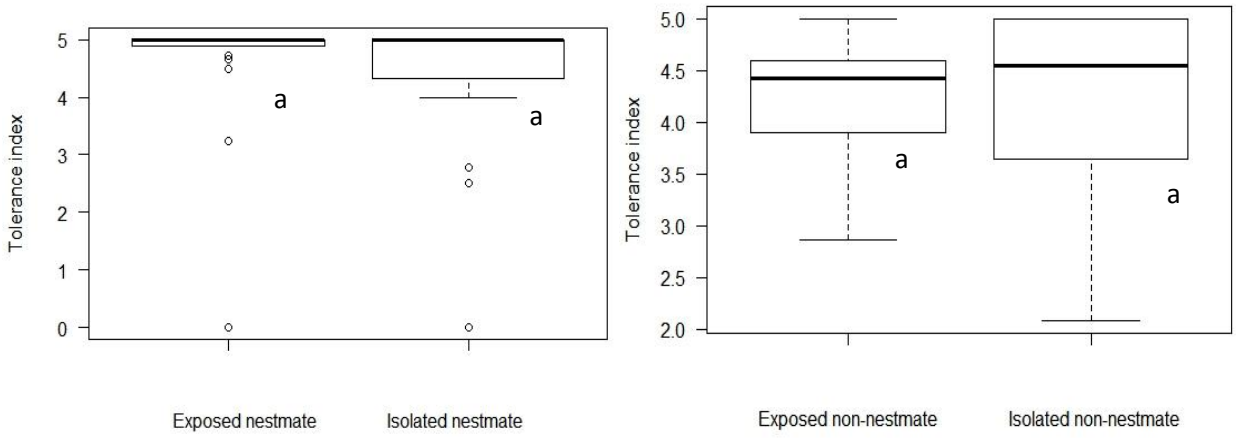


Figure 24: Tolerance index for isolated vs. exposed individuals (Wilcoxon signed rank test, Nest mate: V=91.5, p=0.81, n=30 each; Non-nestmate: V=180.5, p=0.85, n=30 each).

Discussion

Our results from introduction experiments demonstrate that wasps can recognize nestmates in the context of nest, and, isolated and exposed wasps are not treated significantly differently when they are anesthetized and introduced onto their natal nest and a non-natal nest (Figure 21-24). Wasps isolated within 24 hours after eclosion may acquire some colony labels during that time, but those labels seem to remain even after 13-18 days of isolation from nestmates and nest. Non-nestmates, normally expected to be unrelated, are always significantly less tolerated than nestmates (Figure 16, 20), irrespective of whether the introduced wasp was isolated after eclosion for 13-18 days or allowed to stay on the nest (exposed). However, only in one case out of 120 introductions, a wasp (exposed) was killed by non-nestmates (died 3 minutes after the observation time). Further, time spent interacting with the introduced wasp, and sum of number of acts of 5 behaviors shown are also significantly higher for non-nestmates than nestmates indicating that they are recognized (Figure 13, 14, 17, 18), and the sequence of introduction does not matter (Figure 15, 19).

Our results suggest that number of wasps that interact with nestmates is not significantly different from non-nestmates. Since the wasps always need to smell identity of introduced wasp (nestmate or not) before showing any action on them, this result makes sense. In addition, more time is spent interacting with the introduced wasp and higher sum of number of acts of five behaviors is shown to non-nestmate than nestmate wasp. Since, sometimes, non-nestmates could be a threat to the colony; it will be safe if the wasps interact with the non-natal wasp more.

A similar study on *R. marginata* (Venkataraman and Gadagkar, 1992) showed that no foreign animals are accepted into the nest, although nestmates are more tolerated than non-nestmates. In that study, 3 to 4 animals of each category (pre-eclosion-isolated nestmates and non-nestmates, exposed nestmates and non-nestmates) were introduced into a cage containing a nest. Interactions between these wasps and resident wasps were then observed as they occured. But, not all wasps interact in the given condition. Thus, the study did not consider wasps that do not interact or go near the resident wasps. Our experiment involved actually presenting each wasp to its natal

and non-natal nest and observing the responses of the resident wasps. Although the wasps were isolated pre-eclosion in (Venkataraman and Gadagkar, 1992), our results partly agree with it. While isolated nestmates more tolerated than non-nestmates in our experiment, there was no significant difference, from the earlier study. Further, (Venkatraman et. al, 1988) had studied post-eclosion-isolated wasps. But the wasps were tested outside the context of nest, in plastic box, unlike the current study which performed the experiments in the natural context.

In nestmate recognition systems, templates and labels form major components (Gamboa, 1986). They can be acquired and/or genetic (Beye et al., 1997; Gadagkar, 2009; Sorvari et al., 2008; Van Zweden et al., 2009). Wasps can acquire the cues from social interactions like allogrooming, trophallaxis (D'Ettorre and Lenoir, 2010). In such a case, it is suggested that the colony odor is not the sum of labels of all the wasps in the colony but a pattern emerging from the unified whole (*gestalt* model) (D'Ettorre and Lenoir, 2010). Since the chemical cues/labels need to be uniform within a colony, the cocktail of chemicals produce a common colony odor (D'Ettorre and Lenoir, 2010).

In *R. marginata*, cocktail of CHCs can act as cues for nestmate recognition (Mitra et al., 2014). However, the recognition cues can be overlapping, so there cannot be a perfect recognition. It could be also be context dependent (Starks et al., 1998); so costs and benefits of acceptance and rejection should be considered (Reeve, 1989; Venkataraman and Gadagkar, 1992). Considering all this, from our experiments on recognition of isolated and exposed wasps, we speculate a possible mechanism to explain the observed equal tolerance.

We know that the newly eclosed wasps of *R. marginata* have very low amounts of CHCs and as they do not forage out, their chances of attempting to join another colony is very low (Mitra et al., 2014). Thus, the wasps may not discriminate the newly eclosed wasps. However, as they age, they may synthesize more CHCs (if isolated) or get more CHCs from nest and nestmates (if exposed). Also, within the 24 hours after eclosion, the wasps may have attained some colony specific labels. Consequently, the isolated wasps may get some profile intrinsic but not drastically different from its natal nest profile, while the exposed wasps get a common colony specific profile (*Gestalt*)

and its intrinsic profile could get overridden, if it originally came from a different nest. It is remarkable that any labels acquired within 24 hours after eclosion remain on the wasps even after 2 weeks of isolation from nestmates and nest. In other words, isolated wasp may get X proportion of the CHC profile similar to that of its natal nest wasps while the wasp on the nest (exposed) may have >X proportion similar, but both are above a certain threshold required for recognition [Cue dissimilarity model;(Sherman et al., 1997)]. This seems to be an 'all or none' process as described by cue similarity threshold in (Gamboa, 1986). Also, there is evidence for such the presence of quantitative threshold for nestmate recognition in *Polistes dominulus* wasps (Cini et al., 2009).

Our study is perhaps the first of its kind to predict implications to CHC composition after isolation of wasps post-eclosion and to see how they are treated by nestmates and non-nestmates when they are introduced back. Equal tolerance of isolated and exposed wasps may have an implication when wasps re-join their natal nest after leading a solitary life for some time. In this species, although rarely, solitary nests are founded (Gadagkar, 2009). Sometimes, wasps leave their nest to found a new solitary colony. However, if the solitary wasp cannot sustain her colony, she might re-join her old nest. Since these wasps were on their natal nest for a while, even after isolation for some time (at least 13-18 days, from this study), they may have their natal colony specific CHC and thus be accepted back or tolerated.

In our study, both genetic and environmental cues might be playing role but perhaps with different magnitude of influence, similar to another study (Gamboa et al., 1986b). A long term study on carpenter ants showed that with time CHC profile of ants in a colony changes but is slightly converging among colonies and also that the heritable cues of workers play a dominant role in nestmate recognition (van Zweden et al., 2009). If our speculations, which need to be further confirmed with analysis of CHC profile with age, are correct, then our results do not support Fielde's progressive odor theory. This theory says that as the wasps [original theory was proposed for ants] age, they lose their odor and attain a different one.

Conclusion

Our study of introducing anesthetized wasps to natal and a non-natal nest shows that nestmates and non-nestmates are recognized, and isolated (post-eclosion) 13-18 days old and similar age exposed wasps are not treated significantly differently in the context of nest. This could imply that while exposed wasps' own profile is overridden by common colony odor, isolated wasps synthesize their profile which may be not drastically different from its natal nest profile as they may have acquired some cues within the time they were isolated from their nest (<24 hr.). To confirm our speculations, further experiments on the CHC profile differences between isolated and exposed wasps would be fruitful. Future work can throw light on how much of the recognition cues could be intrinsically decided and how much is acquired from the environment (nest and interaction with nestmates). Our study opens up further questions like a.) What changes happen in the CHC profile in isolated and exposed wasps with age?; b.) Are males and females treated differently?; c.) Are foragers (whose CHC is expected to be slightly different as they expose themselves to harsh surroundings) treated differently than non-foragers who stay on the nest? d.) Do proteins and peptides on the cuticle have role in this kind of nestmate recognition? How do they differ between exposed and isolated wasps?

Publications based on this thesis:

1. Aniruddha Mitra, **Ravindra Palavalli Nettimi**, Arathy Ramachandran, Paromita Saha and Raghavendra Gadagkar. (2015). Males and females of the social wasp *Ropalidia marginata* do not differ in their cuticular hydrocarbon profiles and do not use any long distance volatile mate attraction cues. *Insectes Sociaux*. (In press)
2. **Ravindra Palavalli Nettimi** and Raghavendra Gadagkar. (2015). Nestmate recognition in natural context of nest and implications of post-eclosion Isolation of social wasps (In preparation).

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