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RESEARCH ARTICLE

Characterization of cuticular hydrocarbons in a subsocial bee, *Ceratina calcarata*

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Abstract
Research on cuticular hydrocarbons of solitary and eusocial bees has greatly contributed to our understanding of the evolution of eusociality. However, to understand the transition from simple to complex societies, understanding subsocial species is necessary. Subsociality, defined as prolonged parental care, is a pre-requisite for eusociality yet cuticular hydrocarbons of subsocial bees are not well characterized. Here we present the first characterization and analysis of cuticular hydrocarbons in a subsocial bee, *Ceratina calcarata* Robertson. We found that cuticular hydrocarbons signal both age and reproductive status in this species. Pre-reproductive, virgin females overexpress three compounds, heneicosane, farnesol, and nonadecane. We show that pentacosane, a known fertility signal, is likely used to signal reproduction in *C. calcarata*. Since we found a possible reproductive signal in a subsocial bee this suggests that queen pheromones likely evolved from pre-existing fertility signals.

Keywords Cuticular hydrocarbons · Social evolution · Subsocial · Eusocial · Pentacosane · Queen pheromones · Fertility signals

Introduction

Cuticular hydrocarbons (CHCs) are long chain hydrocarbons, primarily alkanes and alkenes, and are present on the cuticle of insects. CHCs play an essential role in water balance in insects (Howard and Blomquist 1982). While CHCs originally evolved for their anti-desiccation properties, they have been co-opted for use in communication in many taxa (Howard and Blomquist 2005; Howard 1993). CHCs have evolved to serve a variety of derived functions such as signaling age (Jackson and Bartelt 1986; Cuvillier-Hot et al. 2001; Nunes et al. 2009), sex (Thomas and Simmons 2008; dos Santos and Nascimento 2015; Weiss et al. 2015), reproductive, social and sexual experience (Oppelt and Heinze 2009; Oliveira et al. 2015; Gershman and Rundle 2016; Pascoal et al. 2016; Holman 2018).

Since cuticular hydrocarbons mediate behavioral interactions, understanding their functions is therefore essential to our understanding of the evolution of eusociality (Van Oystaeyen et al. 2014). Eusociality is a highly derived, extremely successful reproductive strategy defined by overlapping generations, cooperative brood care, and reproductive division of labor (Batra 1966). In eusocial insects, one individual (queen) monopolizes reproduction while workers carry out tasks to maintain a functional colony (Wilson 1971; Michener 1974).

Chemical communication of physiological and behavioral status is important in eusocial species (Hölldobler and Wilson 1990; Liang and Silverman 2000; Lorenzi et al. 2005; Pradella et al. 2015; Esponda and Gordon 2015). CHCs are used to distinguish queens from workers, and young versus old individuals in stingless bees (Nunes et al. 2009). Additionally, there are different types of workers, each with a specialized task, and CHCs are used to communicate between worker sub-castes in ants (Grüter and Keller 2016). However, CHCs are used for recognition in solitary bee species suggesting that social traits may have adaptive significance in nest protection for solitary species (Flores-Prado et al. 2008; Smith and Breed 2012).

Research on eusocial Hymenoptera (ants, bees, and wasps) has shown that CHCs signal fertility (Bonavita-Cougardan et al. 1991; Ayasse et al. 1995; Peeters et al. 1999; Liebig et al. 2000; Heinze et al. 2002) and direct evidence

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has identified several cuticular compounds that are fertility signals regulating worker reproduction (Smith et al. 2009; Holman et al. 2010, 2013; Holman 2014; Van Oystaeyen et al. 2014). While fertility signals have been identified, their broad role in the evolution of eusociality has not been addressed until recently. Van Oystaeyen et al. (2014) compared CHCs across Hymenoptera and found that the overexpression of saturated hydrocarbons in queens relative to workers was common across 64 eusocial species. Thus, the current hypothesis is that fertility signals were present in solitary ancestors and have been subsequently co-opted as to regulated worked reproduction in eusocial species (Oliveira et al. 2015; Van Oystaeyen et al. 2014; Oi et al. 2015).

Much of the Hymenoptera CHC literature in the past thirty years has focused on eusocial and solitary species. While these studies have greatly contributed to our understanding of the evolution of eusociality, there is a severe lack of research on non-eusocial hymenopteran species (Andrade-Silva and Nascimento 2015; Steitz et al. 2018, 2019, Steitz and Ayasse 2020). To understand how eusociality evolved, simple societies must be well studied (Rehan and Toth 2015). Subsociality, defined as prolonged parental care, is the simplest form of social behavior and is a prerequisite for eusociality (Wilson 1971; Michener 1974). Therefore, elucidating the function of CHCs in subsocial species is necessary to understand the transition from solitary to complex insect societies.

To fully support the hypothesis that regulation of worker reproduction evolved from pre-existing fertility signals in solitary ancestors, confirmation that CHCs signal fertility in subsocial species is necessary. Here we provide characterization of cuticular hydrocarbons in a subsocial small carpenter bee, *Ceratina calcarata* Robertson (Hymenoptera: Apidae: Xylocopinae). The aims of this study were two-fold: first, to characterize the cuticular hydrocarbon profiles of *C. calcarata*; and second, to examine the relationship between reproductive status and cuticular hydrocarbon profiles in this species.

Methods

Nest collections and dissections

Twenty five *Ceratina calcarata* nests were collected from May through August 2014 from sumac trees, *Rhus typhina*, in Durham, New Hampshire (43°08′02″N 70°55′35″W). Nests were collected between 6:00 and 8:00 AM to ensure the bees were still in the nest. To avoid damage to the contents of the nests and any offspring present, the dead broken stem was cut with garden shears at the junction with another branch. Masking tape was then applied to the nest entrance to contain the bees living inside. Collected nests were then brought back to the lab and stored in a 4 °C cold room until dissection the same morning of nest collection.

Nests were dissected with extreme care to avoid damaging the contents of the nest. The dead broken stems (nests) were split long-ways using a sharp non-serrated knife. Nest classification (described below) was determined and recorded. Nest occupants were captured and stored in chemically neutral tubes. Cuticular washes occurred immediately upon nest dissection to avoid any changes due to prolonged captivity.

Since we were primarily concerned with how CHCs vary as a function of reproductive status we collected newly eclosed (late summer), pre-reproductive (next spring), actively reproductive (early summer), and post-reproductive (mid-summer) *Ceratina calcarata* females using previously defined female classifications for this and related species (Rehan and Richards 2010a; Rehan et al. 2015). In spring, overwintering females disperse from their natal nest and establish their own nest solitarily and their nests do not contain any feces or pollen and have clean nest walls. The females collected from these nests were classified as *founding nest* (FN). Therefore, FN females have developed ovaries, but are not yet laying eggs. In early-mid-summer, females are fully reproductive and begin egg laying. Females collected from nests containing at least one pollen ball and egg were classified as *active brood* (AB). In mid-late summer when the brood cell closest to the nest entrance contains a larva or pupa, and the mother's reproductive effort for the season is complete and she will not be laying any more eggs. Females collected from these nests were classified as *full brood* (FB). Finally, in late summer all offspring in the nest have finished developing into adults. Adult offspring remain in the nest until they disperse the following spring, in which the cycle starts over. We collected these callow *pre-dispersal* (PD) females from late summer nests.

Morphological measurements

All morphological measurements were recorded after cuticular washes using a NIKON H550S dissecting scope. Head width (i.e., greatest distance of head width, including eyes) was measured as an accurate metric of body size (Rehan and Richards 2010a). Bees were dissected to score ovarian development, recorded as the sum of the length (mm) of the three largest oocytes.

Cuticular hydrocarbon characterization

Adult bees were washed in 500µL of pentane for 45 min in chemically neutral glass vials (Flores-Prado et al. 2008). Cuticular hydrocarbon profiles were analyzed using Gas Chromatography coupled with Mass Spectrometry (GC/MS), using Sigma Aldrich Retention Index Standards for all analyses. A Micromass AutoSpec with a Hewlett-Packard 5890 series II

gas chromatograph with helium (75 kPa) as a carrier gas was used to carry out the GC/MS analysis. The injector temperature was 250 °C and the transfer line temperature was 300 °C (Nunes et al. 2009). The initial temperature of the oven was 50 °C and held for 1 min. Then, the temperature was raised 5 °C/min to a final temperature of 300 °C, which was held for four minutes. The total oven program was 55 min. Compounds were identified using three commercial libraries (Wiley 275, NIST 98, and Adams EO library 2205). The mass spectrometer was operated at 70 eV with an ion source temperature of 250 °C and MS scan range of 45–650, at a data acquisition rate once per second (0.9 + 0.1 = 1 s/scan). The detector voltage was 2600 V with a trap current of 164 Ua and solvent delay of 1.3 min. A 1 µL sample of each pentane wash was injected. The washes of five PD females, seven FN females, five AB females, and eight FB females were used for analysis.

Statistical analyses

All statistical analyses were calculated using R Studio 3.2.2 (R Core Team 2014). Shapiro–Wilk tests were used to test for normal distribution of ovarian development, head width, and wing wear. All three physiological measurements were not normally distributed and non-parametric statistics were used. Spearman Rank Correlations of head width and ovarian development versus relative abundance were employed to determine if cuticular hydrocarbons were correlated with body size and reproductive status, respectively.

Kruskal–Wallis tests were used to determine if there were significant differences in physiological measurements between PD, FN, AB, and FB females (Mant et al. 2005). Additionally, Kruskal–Wallis tests were used to determine if there were significant differences in relative abundance of CHCs between PD, FN, AB, and FB females. Post-hoc HSD and LSD tests (R package "agricolae"; de Mendeburu 2015), and Dunn's tests (R package "CRAN") were run after Kruskal–Wallis tests to determine the source of significant differences between groups. Bonferroni corrections were calculated to control for multiple comparisons. There were fourteen chemical compounds detected and thus alpha was adjusted for multiple comparisons (Bonferroni corrected $p = 0.05/14 = 0.00357$). Principal Component Analysis (PCA) using centered log ratio (clr) transformation was used to determine if PD, FN, AB, and FB females differed in their cuticular hydrocarbons profiles.

Results

Cuticular hydrocarbon profiles

A total of fourteen different hydrocarbons from four major classes were identified from cuticular extracts of *Ceratina calcarata* females. Alkanes and alkenes were the most abundant class of hydrocarbons, although three ethyl esters and one alcohol was identified (Table 1). The hydrocarbons ranged from 15 to 27 carbons in length with pentadecane as the shortest length and heptacosane was the longest length identified.

Nonadecane, heneicosane, pentacosane, ethyl hexadecenoate, and farnesol relative abundances were significantly different between females of different nest class stages (Table 1). PD females had significantly higher relative abundance of farnesol, nonadecane, and heneicosane than FN, AB, and FB females (Fig. 1). AB females had significantly higher levels of pentacosane than FB and PD females but not significantly higher than FN females, and significantly greater amounts of ethyl hexadecenoate than FN and FB females, but not significantly higher than PD females (Fig. 2). Principal Component Analysis (PCA) revealed that two principal components explain 74.7% of the variance in CHC profiles, and shows three unique clusters (Fig. 3). PD females were clustered together, AB females were clustered together, and FN and FB females were clustered together.

Morphological measurements

Active brood females had the largest ovarian development, and were significantly more developed than full brood and founding nest bees, but did not have significantly larger ovaries than PD females. Full brood females had the smallest ovaries and founding nest females had the second smallest ovaries (Kruskal–Wallis test: Chi-squared = 16.30, df = 3, $p = 0.001$; Fig. 4A). There was a significant correlation between ovarian development and relative abundance of nonadecane (Table S1). There were no other significant correlations between ovarian development and relative abundance of the compounds identified in *Ceratina calcarata* (Table S1). There were no significant differences in head width between females of different nest class stages (Kruskal–Wallis test: Chi-squared = 2.86, df = 3, $p = 0.41$; Fig. 4B). There was a significant positive correlation between head width and relative amounts of heneicosane (Table S2). There were no other correlations between head width and relative abundance of the compounds identified in *C. calcarata* females (Table S2).

Discussion

Here we provide the first characterization of cuticular hydrocarbon profiles in a subsocial bee. There are three main findings from this study. First, we show that pentacosane is most abundant in reproductive females, indicating that it may signal reproductive status in this species (Figs. 2, 3). Second, we show that cuticular hydrocarbons may signal

Table 1 The relative proportions of fourteen hydrocarbons found on the cuticles of *Ceratina calcarata* females at different stages of reproduction and life cycle

		Retention time	Relative abundance (%)				X^2
			PD ($n=5$)	FN ($n=7$)	AB ($n=5$)	FB ($n=8$)	
Alkanes							
C15	Pentadecane	25.3	11.91 ± 5.8	0.27 ± 0.27	3.00 ± 2.30	8.30 ± 4.00	5.63
C17	Heptadecane	30.3	29.00 ± 10.93	34.59 ± 12.84	16.36 ± 11.7	38.14 ± 10.80	1.99
C19	Nonadecane	34.6	1.28 ± 0.89	0	0	0	8.33*
C21	Heneicosane	38.9	8.14 ± 5.12	0.19 ± 0.50	1.69 ± 2.33	1.50 ± 3.4	13.10**
C23	Tricosane	42.6	3.45 ± 2.43	3.55 ± 3.38	2.50 ± 1.41	1.11 ± 1.11	4.68
C25	Pentacosane	46.0	6.03 ± 3.13	16.63 ± 11.63	19.66 ± 13.70	1.12 ± 2.96	12.63**
C27	Heptacosane	49.2	1.51 ± 0.74	14.26 ± 6.36	3.89 ± 2.16	2.53 ± 2.53	5.65
Alkenes							
C17:1	Heptadecene	29.8	4.15 ± 3.74	1.63 ± 1.47	6.32 ± 5.30	28.79 ± 12.10	4.16
C19:1	Nonadecene	34.3	0.44 ± 0.44	0.157 ± 0.157	0.59 ± 0.59	0	1.76
C23:1	Tricosene	42.2	24.28 ± 14.92	19.43 ± 5.57	21.43 ± 12.30	14.28 ± 7.92	1.52
Ethyl Esters							
Et-C16	Ethyl Hexadecanoate	37.2	1.20 ± 0.08	0	2.46 ± 2.07	0	6.79
Et-C16:1	Ethyl Hexadecenoate	36.3	3.10 ± 2.11	0	14.29 ± 9.82	0.17 ± 0.17	7.85*
Et-C18:1	Ethyl Octadecenoate	40.1	2.10 ± 1.76	1.49 ± 1.34	1.47 ± 0.911	1.02 ± 1.02	2.35
Alcohols							
C15-OH	Farnesol	30.8	3.41 ± 3.58	0	0.10 ± 0.226	0	10.34*

Retention times for each compound are reported in minutes

The number of bees washed is provided in parentheses. Chi-Squared values from Kruskal–Wallis non-parametric ANOVA. *$p<0.05$, **$p<0.01$

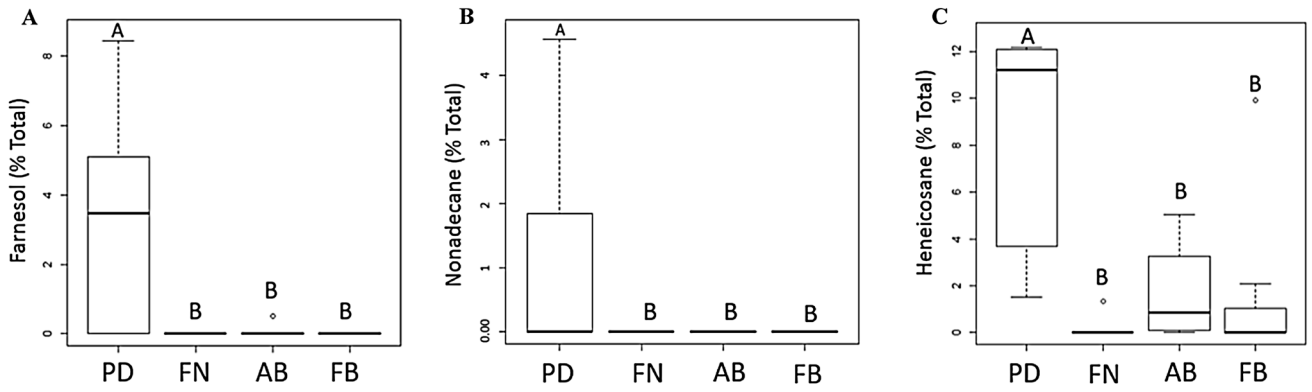

Fig. 1 **A** Pre-dispersal (PD) *Ceratina calcarata* daughters contained significantly more farnesol than founding nest (FN), active brood (AB), and full brood (FB) females (Kruskal–Wallis test: Chi-squared = 10.34, df = 3, $p=0.016$). **B** PD daughters had significantly more nonadecane than FN, AB, and FB females (Kruskal–Wallis Chi-squared = 8.33, df = 3, $p=0.04$). **C** PD daughters had significantly more heneicosane than FN, AB, and FB females

age, as young pre-dispersal females had higher concentrations of farnesol, nonadecane, and heneicosane (Fig. 1) than older females.

A total of seven alkanes ranging from C15 to C27, three alkenes, three ethyl esters, and one alcohol were identified in this study (Table 1). We found heptadecane was the most abundant compound independent of reproductive status (Table 1). Breed (1998) found that heptadecane did not have an effect on recognition in the honey bee *Apis mellifera* when females were treated with 100 µL of this compound and presented to a sister. Consistent with previous reports that heptadecane likely is not used as a recognition signal we found heptadecane levels are stable across *C. calcarata* females of different reproductive status and therefore it is likely that heptadecane is not a signal compound in this species.

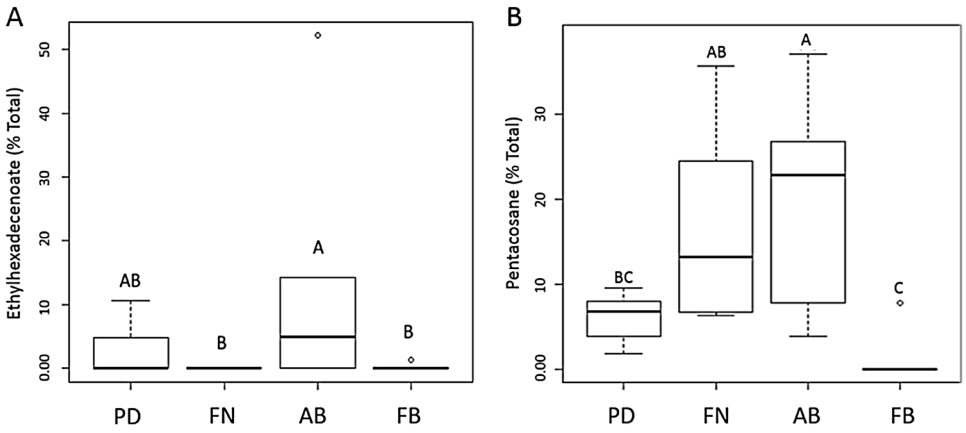

Fig. 2 **A** AB females have significantly higher relative abundances of ethylhexadecenoate than FN and FB females, but not PD females (Kruskal–Wallis chi-squared = 7.85, df = 3, $p = 0.05$). **B** AB females have significantly higher amounts of pentacosane than PD and FB females, but not FN females (Kruskal–Wallis test: Chi-squared = 12.63, df = 3, $p = 0.006$)

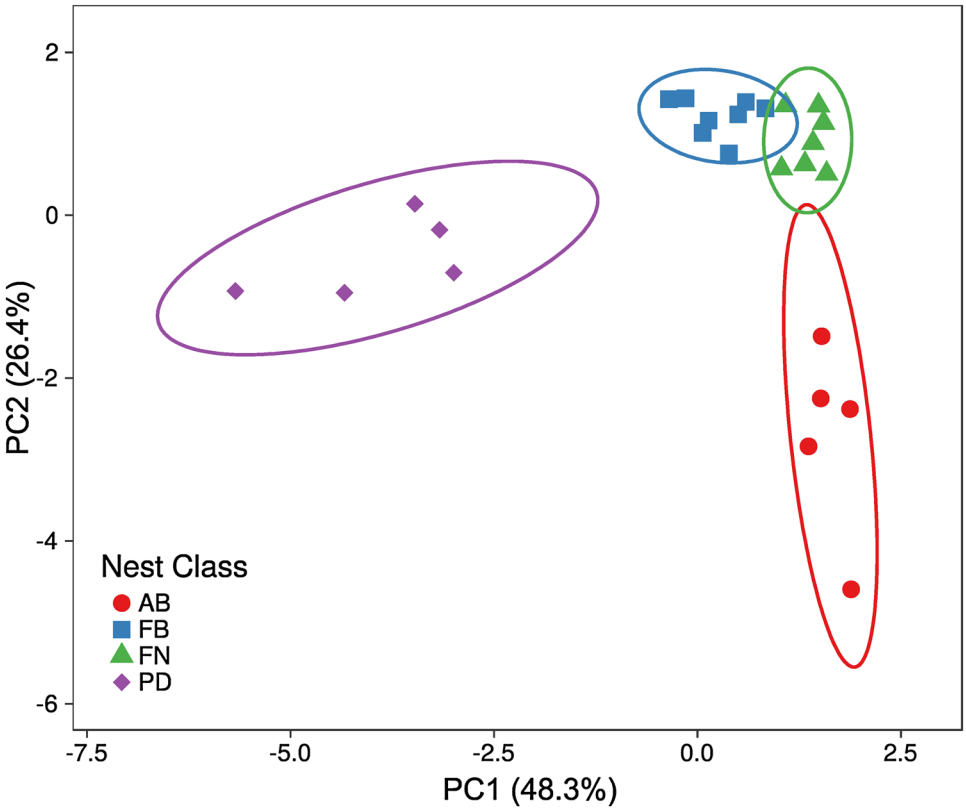

Fig. 3 Principal Component Analysis of cuticular hydrocarbon profiles of *Ceratina calcarata* females at different stages of reproductive development and life cycle. Nest Classes: *PD* pre-dispersal callow females, unmated and still in natal nest, *FN* founding nest females, newly dispersed in spring, *AB* actively reproducing females, *FB* full brood females, which are post-reproductive

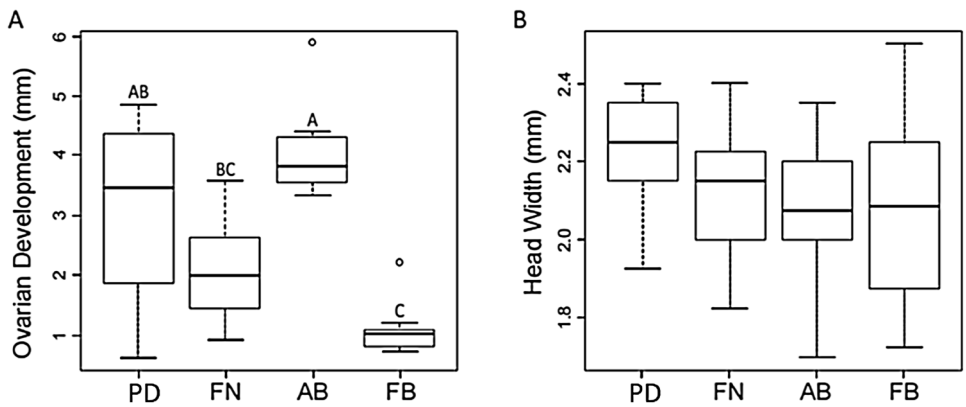

Fig. 4 **A** Active brood (AB) *Ceratina calcarata* females have significantly larger ovaries than founding nest (FN) and full brood females (FB), but not pre-dispersal (PD) females (Kruskal–Wallis test: Chi-squared = 16.30, df = 3, $p = 0.001$). **B** There are no significant differences in head width between nest classes (Kruskal–Wallis test: Chi-squared = 2.86, df = 3, $p = 0.41$)

Reproductive signaling and ovarian development

Previous studies have shown that pentacosane is a fertility signal that influences worker reproduction in a eusocial bumble bee, *Bombus terrestris* (Van Oystaeyen et al. 2014; Holman 2014, 2018). Studies in ants (*Pachycondyla inversa*, Heinze et al. 2002; *Harpegnathos saltator*, Liebig et al. 2000; *Dinoponera quadriceps,* Peeters et al. 1999), bees (*Bombus hypnorum,* Ayasse et al. 1995), and wasps (*Polistes dominulus*, Bonavita-Cougardan et al. 1991) have shown that cuticular hydrocarbon profiles are correlated with ovarian development. Although we did not detect any strong correlations between ovarian development and the compounds identified in *Ceratina calcarata* (Table S1), PCA of compounds show that reproductive females have unique chemical profiles suggesting the use of CHCs to signal reproductive status. Particularly, reproductive females produce the highest levels of the bumble bee fertility signal pentacosane (Fig. 3; Holman 2014). While pentacosane was not statistically greater in AB females than FN females (Fig. 2), these results are still suggestive that *C. calcarata* females may signal fertility and that pentacosane is a putative fertility signal in this species. Moreover, since we found a possible reproductive signal in a subsocial bee, this supports the hypothesis that regulation of worker reproduction likely evolved from pre-existing fertility signals (Van Oystaeyen et al. 2014).

Pre-dispersal callow females had developed ovaries (Fig. 4A) despite being unmated non-reproductives. These results are consistent with Sakagami and Maeta (1984), which found that newly emerged *Ceratina japonica* had fully developed ovaries and are capable of laying eggs. Similarly, week old female bumble bees have fully developed ovaries (*B. terrestris,* Duchateau and Velthuis 1989). Several studies have shown that in eusocial insects queens suppress worker (daughter) reproduction via pheromonal influence (e.g., pentacosane in *B. terrestris*; Holman 2014; Van Oystaeyen et al. 2014). In *C. calcarata*, however, pre-dispersal females are about to enter diapause and have no opportunity for reproduction (Rehan and Richards 2010a). Therefore, maternal overexpression of pentacosane for regulation of offspring reproduction is probably not necessary or adaptive in this subsocial bee. The effect of *C. calcarata* mother CHCs on maturing daughter ovarian development should be explored further to directly confirm whether or not mothers in subsocial species influence daughter ovarian development.

CHCs vary as a function of age

We observed differences in cuticular hydrocarbon profiles as a function of age (Figs. 1, 3). Pre-dispersal (PD) females are 8–10 months younger than FN, AB, and FB females (Rehan and Richards 2010a). PD females had a significantly higher relative abundance of heneicosane than FN, AB, and FB females (Fig. 1; Table 1). These results are consistent with data from the mosquito, *Anopheles gambiae*, in which the relative abundance of heneicosane was most abundant in younger individuals, and decreased with age (Polerstock et al. 2002). Although, heneicosane may also signal body size as we found a significant positive correlation between head width and relative abundance of heneicosane (Table S3). In the European hornet, *Vespa crabro*, workers were aggressive towards dead nest mates that were experimentally treated with heneicosane (Ruther et al. 2002). However, PD females are typically not aggressive in *Ceratina calcarata* (Rehan and Richards 2013), suggesting that heneicosane may not elicit the same behavioral responses across all Hymenoptera.

Two additional compounds nonadecane and farnesol were also characteristic of PD females (Fig. 1). PD females had a significantly higher relative abundance of nonadecane than FN, AB, and FB females. Bioassays in the honey bee, *A. mellifera,* found bees experimentally treated with nonadecane received low rates of aggression (Dani et al. 2005). In *C. calcarata* PD females are tolerant of each other in the pre-reproductive assemblages (Rehan and Richards 2013; Rehan et al. 2014). Since PD females have a significantly higher relative abundance of nonadecane than females at other time points in the colony cycle and nonadecane induces low aggression rates in *A. mellifera*, further investigation is warranted to determine if nonadecane helps maintains nest mate tolerance among *C. calcarata* PD females.

Farnesol was found almost exclusively in PD females. In the solitary ground nesting bee *Andrena nigroaenea,* farnesol inhibits copulatory behavior in males, as males use farnesol to distinguish virgin from mated females (Schiestl and Ayasse 2000). Since farnesol was only found in PD *C. calcarata* daughters (virgin), perhaps it functions to prevent young male (brothers) from mating with their sisters in the pre-reproductive assemblages. *Ceratina calcarata* mate in the spring (Rehan and Richards 2010a) and if farnesol inhibited male copulatory behavior in *C. calcarata*, then low levels would be expected in newly dispersed females. Consistent with this idea, we found that spring FN females had significantly lower levels of farnesol than PD females. Furthermore, Rehan and Richards (2010a) reported that males are quiescent and do not interact with females during the summer reproductive phase. This would suggest that females need not produce farnesol during reproduction. Consistent with this notion, we found that AB and FB females had significantly reduced levels of farnesol (Table 1; Fig. 1).

Future directions

Although *Ceratina calcarata* is capable of nest mate recognition (Rehan and Richards 2013), a chemical basis of

this behavior has not yet been verified in this species. Flores-Prado et al. (2008) demonstrated a chemical basis of nest mate recognition in the closely related xylocopine bee, *Manuelia postica*, confirming that eusocial traits have adaptive significance in solitary bees as well. Since subsociality is a necessary pre-requisite for eusociality (Michener 1974), investigating whether *C. calcarata* recognize individuals via chemical profiles is a necessary next step to our understanding of social evolution. Since both solitary (e.g., *M. postica*, Flores-Prado et al. 2008) and social apid bees (e.g., *A. mellifera*, Breed 1998) are known to use pheromones to recognize individuals, we hypothesize that *C. calcarata* can discriminate individuals based on chemical profiles.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00040-021-00833-5.

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