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Olfactory cues and nest recognition in the solitary bee *Osmia lignaria*

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Abstract. The use of olfactory cues for nest recognition by the solitary bee *Osmia lignaria* is studied in a greenhouse environment. Glass tubes are provided as nesting cavities to allow the in-nest behaviour of bees to be observed. In addition, each glass tube is cut into three sections for experimental manipulation and for subsequent chemical analysis. Nesting females drag their abdomen along the tube before exiting, spiral inside the tube, and sometimes deposit fluid droplets from the tip of the abdomen. For the manipulation, the outer section, the middle section, or both sections are removed and replaced with similar clean glass tube sections, and the behaviour exhibited by test females is recorded upon arrival in front of the nesting site and inside the nesting tubes. The resulting hesitation behaviour displayed by females after treatments appears to indicate the loss of some olfactory cues used for nest recognition inside the entire nest. Chemical analysis of the depositions inside the nesting tube, as well as analysis of the cuticular lipids of the nesting bees, reveals the presence of free fatty acids, hydrocarbons and wax esters.

Key words. Lipid identification, nest marking, nest recognition, olfactory cues, *Osmia lignaria*.

Introduction

Studies concerning olfactory cues have addressed nest and nestmate recognition in ants, social and solitary bees, and social wasps (Michener, 1982; Breed & Julian, 1992; Singer & Espelie, 1992, 1996; Breed, 1998; Singer *et al*., 1998; Vander Meer & Morel, 1998); alarm signals or recruitment trail pheromone in stingless bees and ants (Michener, 1974; Hölldobler & Wilson, 1990; Vander Meer & Alonso, 1998); and nest entrance location with social bees (Butler *et al*., 1969; Cederberg, 1977). For solitary bees that nest in aggregations, short-range orientation may involve both visual and olfactory cues, but the decision to enter a nesting cavity, or nest recognition, appears to be dictated by olfactory cues (Steinmann, 1985, 1990; Anzenberger, 1986; Raw, 1992; Fauria, 1998). The present study examines nest recognition in *Osmia lignaria* Say (Hymenoptera: Megachilidae), an economically important bee whose management could benefit from a better understanding of its nesting behaviour.

Osmia lignaria is a cavity-nesting solitary bee, with each female bee being fertile, and thus building and provisioning her own nest. Although solitary, *O. lignaria* is gregarious (Torchio, 1991) and can be managed at artificial nesting sites for commercial or experimental purposes. Commonly called the blue orchard bee, *O. lignaria* is active during the spring and is an important pollinator of orchard crops, such as apples, cherries and almonds (Torchio, 1991; Bosch & Kemp, 2001). *Osmia lignaria* females build linear series of cells, with each cell containing a mixture of nectar and pollen on which an egg is laid (Torchio, 1989). Foraging for cell provisions requires many departures and returns to the nest that can be used in observations, particularly in short-range orientation studies.

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Because they are gregarious, individual nesting females must locate their nests among many closely spaced nesting cavities. Normally, these bees show little or no hesitation when entering their nest, suggesting the use of some visual and/or olfactory cues. However, there are times when a female returning from a foraging trip enters the wrong nesting cavity. Such a female quickly appears to recognize the mistake, exits the cavity and scans over other cavities before entering the correct nesting hole (personal observations). Additionally, when visual cues are altered at the nesting site, *O. lignaria* females hover and inspect several nest entrances, only inserting the head and then withdrawing immediately from alien cavities (Guédot, 2004). These observations suggest that olfactory cues may be used by *O. lignaria* for nest recognition.

In studies involving different solitary bee genera, females have been observed smearing the tip of their abdomen at the nest entrance, presumably depositing chemicals used in nest marking (Kapil & Dhaliwal, 1968; Gerber & Klostermeyer, 1972; Hefetz, 1992, 1998; Strohm *et al.*, 2002). Some reports propose the use of mandibular secretions for nest marking in other solitary bees (Shinn, 1967; Steinmann, 1976; Anzenberger, 1986). At least one study suggests that bees mark the nest entrance with both the abdomen and the glossae (Hefetz *et al.*, 1986). However, no definitive information concerning nest marking is available for *O. lignaria*.

Different methodologies have been employed to investigate the use of olfactory cues for nest recognition. Such methods include replacing the entire nest by another active or inactive nest (Tirgari, 1963; Tepedino *et al.*, 1979; Hefetz, 1992; Raw, 1992); replacing the nest entrance by a clean entrance or by the nest entrance of another active nest (Steinmann, 1976; Foster & Gamboa, 1989; Hefetz *et al.*, 1990; Hefetz, 1992); or washing the inside of the nest entrance with a solvent (Steinmann, 1976; Wcislo, 1990, 1992). All these manipulations result in a delay at the nest entrance by returning females, indicating the importance of olfactory cues for nest recognition. However, in none of the studies are detailed observations of nest-marking behaviour inside the entire nest possible. Furthermore, to identify the secretions deposited inside the nest, researchers only extracted the chemicals deposited at the nest entrance (Brooks & Cane, 1984; Kronenberg & Hefetz, 1984; Hefetz *et al.*, 1986).

The present study aims to determine whether *O. lignaria* females rely on olfactory cues for nest recognition. For this purpose, five aspects are defined: (i) describe any behaviours exhibited by *O. lignaria* females that are consistent with nest marking; (ii) locate where the marking occurs within the nest; (iii) determine if this marking is used for nest recognition; (iv) confirm the presence of nest-marking components through chemical analysis; and (v) identify the chemical compounds used in nest marking as well as the cuticular compounds of the bee that left those nest markings.

Materials and methods

Bee population

A population of field-trapped *O. lignaria* brood maintained within paper straw nests was collected from an orchard located in North Logan, Utah, and brought to the laboratory in June 2002. The brood was placed in a 22 °C incubator and allowed to complete development to adulthood (confirmed using X-radiography in mid-September 2002). The nests containing adults within cocoons were then cooled in a 14 °C incubator for 2–4 weeks (Bosch & Kemp, 2001), and finally transferred to a 4 °C cooling unit where they remained from October 2002 through April 2003. Five groups of *O. lignaria* cocoons containing adult bees were transferred at 7-day intervals to a 26 °C incubator until emergence (1–3 days for males and 4–7 days for females). As females emerged, they were cooled (4 °C) temporarily and marked for individual identification with enamel or acrylic paint (Testors, Rockford, Illinois) on the thorax. Females were released in groups, so that 25, 35 and 20 females were released in April, and 24 and 20 females in May 2003. For each female group, twice as many males were released. Data were collected no sooner than 1 week after release, allowing time for the bees to mate and for females to select a nesting cavity and initiate nest-provisioning.

Study sites and nesting materials

The greenhouse study was conducted at the USDA-ARS Bee Biology & Systematics Laboratory on the Utah State University Campus in North Logan, Utah, from April to June 2003. Two greenhouse sections (greenhouse body $8.7 \times 9.8 \times 2.4$ m; apex of triangular roof = 4.5 m) were planted with *Phacelia tanacetifolia* Bentham (Hydrophyllaceae), which provides a good pollen and nectar resource for *O. lignaria* (Williams & Christian, 1991; Carreck & Williams, 1997). An observation room (2.44 m^3) was located at the centre of the north wall of each greenhouse section. A brown plywood board (1.22 m^2) was attached 64 cm from the ground to the centre of the outer south face of each observation room. Two hundred and twenty-five holes (diameter = 8 mm), arranged in 15 rows of 15 holes (interhole distance = 2 cm), with every other row offset by 1 cm, were drilled into the centre of the board, and clustered in a 28 cm^2 area. From within the observation room, the tips of glass tubes (total length = 14 cm; inner diameter = 7.5 mm) were inserted into 25 of these holes for the bees to use as nesting cavities. The tubes fit tightly inside the holes, and were thus suspended perpendicularly from the inner central wall of each observation room. Glass was the preferred material for in-nest behaviour observations and subsequent chemical extraction. Each glass tube consisted of three linear sections: an outer section (2 cm) opening to the greenhouse, a middle section (4 cm) and an inner section (8 cm) plugged

at the rear with cigarette filter material. The three glass sections comprising each tube were held together with short sections (c. 1 cm) of clear Tygon® (Saint-Gobain Performance Plastics, Akron, Ohio) plastic tubing (inner diameter = 1 cm). Within the observation room, the glass tubes were covered with loose-fitting black paper sleeves to exclude sunlight from filtering into the observation room from the greenhouse. Sleeves were removed during in-nest observations. The remaining 200 holes in the board were blind (covered with black paper at the rear) to discourage the bees from relying on only visual cues to locate their nest.

Experimental design

To observe and describe any behaviours consistent with nest marking, 12 nesting *O. lignaria* females were watched inside their nesting tubes intermittently from 21 April to 1 May 2003 between 09.00 and 18.00 h (MST).

To locate where the marking behaviour occurs within the nest, manipulations of the glass tube sections were made during the period of peak bee activity between 11.00 and 17.00 h (MST). Temperatures inside the greenhouses during observation times averaged 26.8 ± 0.27 °C. Two observers were present for observations during manipulations, one inside the greenhouse facing the nesting holes, the other inside the observation room observing in-nest behaviour of the test bee.

The test procedure for each nesting female consisted of three steps. First, a female whose nest contained at least one, but no more than three cells, was observed leaving and returning to her nest. If the female entered her nest without hesitation and deposited her pollen-nectar load, she was selected for testing. After the test female departed for a new foraging trip, the manipulation of the nesting tube (described below) was performed. Upon return of the test female to the nesting site, an audiotape was used to record the behaviour exhibited by the female at the nest entrance as well as inside her nesting tube (from inside the observation room). Each female tested was then removed from the experiment along with her glass nest, so that each female was exposed to only one manipulation. Manipulations inside the observation room were conducted under red light to avoid disturbing the bees (Gould & Gould, 1988) and wearing latex gloves to avoid contamination of the nesting tube with extraneous chemicals.

Three treatments were used to manipulate the glass tube being used by an actively nesting female. For one treatment, the outer 2-cm section of the glass tube was removed and replaced by a similar clean section. For another treatment, the middle 4-cm section of the glass tube was removed and replaced by a similar clean section. For the last treatment, both outer and middle sections of the glass tube were removed and replaced by similar clean sections. As a control, the glass tube was disassembled and reassembled to account for the effect of any manipulation during treatments.

Once a treatment was applied, the behaviours of each test bee were recorded at her own nest hole or at other holes. Behaviours at her own nest hole included touching the entrance of the nest with the antennae, inserting the head, entering the outer section, entering the middle section, and touching the provision. At other holes, behaviours included touching the entrance of the hole with the antennae or entering the hole. A 'nest recognition attempt' was defined as a female touching or entering her nest or another hole without depositing the pollen-nectar load, and then exiting the cavity. Each 'attempt' was assigned a score of 1. The frequency of a bee's nest recognition attempt at her own nest hole and at other holes was averaged. Whether or not the female eventually deposited the pollen-nectar load in her nest, and whether these females marked their nest as they first exited after manipulations, were also recorded.

Statistical analysis

StatXact3 for Windows (Cytel Software Corp., Cambridge, Massachusetts) was used to perform a two-sided nonparametric median test with a Monte Carlo estimate for the mean nest recognition attempts at a bee's own nesting cavity and for the mean nest recognition attempts at other holes (*t*-test). Chi-square analysis was performed using SAS Version 8 (SAS Institute Inc., 1999–2001) for the deposition scores, as well as the number of females that marked the nest after treatment. Because multiple comparisons were made between treatments, a sequential Bonferroni correction was applied with all tests to adjust the significance level (Rice, 1989).

Chemical analysis

Ten *O. lignaria* females tested in the behavioural study were collected along with each bee's outer and middle sections of the glass nesting tube; the inner section, containing nest cells, was not used for chemical extraction. Each of the 10 females was placed in an individual glass vial and freezer-killed at −16 °C. The glass tube sections were individually wrapped in aluminium foil and kept at −16 °C. Both the outer and middle sections of a clean glass tube also were maintained as controls. All samples were then shipped to the USDA-ARS Biosciences Research Laboratory in Fargo, North Dakota, and held at 0 °C until processing for chemical analysis.

For chemical analyses, separate solvent extractions were made of the glass tube sections, the bee cuticles and the pollen from the greenhouse. Lipids were extracted from glass tube sections by slowly passing three 500 μL aliquots of solvent down the inner walls at the same time as rotating each tube, and then collecting the solvent in a test tube. For the first six glass tube samples, hexane ($n = 5$) or chloroform ($n = 1$) was used first as the solvent, and the samples were analysed as described below. The same tubes were extracted again with the same procedure except using 2 : 1

chloroform : methanol as the solvent (Buckner *et al*., 2004), and the samples analysed again. Because additional lipids were obtained in the second extraction, extracts from both solvent rinses were pooled for the final analysis, which is reported. Thus, only 2 : 1 chloroform : methanol was used as the solvent for lipid extraction of five additional glass tube samples.

Cuticular lipids were removed from each bee either by submersion in 10 mL hexane for 1 min (Espelie & Hermann, 1990; Page *et al*., 1991) followed by a 5 mL hexane rinse for 20 s ($n = 5$), or in 10 mL hexane for 1 min followed by submersion for 30 s in 10 mL chloroform, then a 15 s rinse in 5 mL chloroform ($n = 5$) (Buckner *et al*., 1984; Nelson & Fatland, 1992; Buckner *et al*., 2004). Extraction solvents were filtered and, for each sample, all solvent rinses were pooled and the volume reduced under vacuum and/or a stream of nitrogen gas. Pollen was recovered from the anthers from *P. tanacetifolia* flowers and the pollen lipid removed by sonicating in 2 : 1 chloroform : methanol for 40 min. Pollen extracts were filtered and reduced in the same manner as the bee cuticular extracts.

Individual lipid components were separated by capillary gas chromatography (GC), quantified by their flame ionization detector (FID) response and identified by GC-mass spectrometry (GC-MS). GC analyses were performed using an Agilent 6890 gas chromatograph (Agilent Technologies, Palo Alto, California) equipped with a temperature- and pressure-programmable on-column injector, an Alltech AT^{TM}-1HT capillary column (Alltech Associates, State College, Pensylvania) (0.25 mm × 15 m) and an FID. The column oven temperature was held at 75 °C for 30 s, increased to 225 °C at 25 °C min^{-1}, next increased at 10 °C min^{-1} to 300 °C, and then increased at 25 °C min^{-1} to 320 °C and held for 45 min. Samples were introduced onto the 0.1-μm phase thickness column via a 2-m retention gap of uncoated, deactivated fused silica with the hydrogen carrier at 20 psig. After 30 s, the pressure was reduced to 7 psig, and then increased at 1 psi min^{-1} to 30 psig where it was held until the end of the run.

GC-MS was performed on an Hewlett Packard Model 5890A gas chromatograph (Agilent Technologies, Palo Alto, California) equipped with a temperature- and pressure-programmable on-column injector and a 1-m retention gap, connected to a J&W Scientific DB-1MS capillary column (Folson, California) (0.2 mm × 12.5 m, 0.33 μm phase thickness) coupled to an HP 5970B quadrupole mass selective detector. The carrier gas was 0.75 mL min^{-1} helium, programmed for constant flow. The column temperature was initially held at 150 °C for 4 min, then programmed to 320 °C at 4 °C min^{-1} where it was held until all peaks eluted.

Quantities of hydrocarbons and wax esters were determined using the integrated peak area data from the FID response to increasing quantities (0.39–200 ng) of the authentic standards, *n*-octacosane and tricosanyl heptadecanoate, respectively. When GC-MS analysis revealed a phthalate contaminant that coeluted with the 25:0 hydrocarbon, the hydrocarbon was quantified using the integrated peak area after GC-MS selected ion monitor (SIM) analysis of its molecular ion (352 amu). Non-linear slope data of the integrated peak area from SIM analysis of the molecular ion (394 amu) of increasing amounts (1.56–200 ng) of the authentic standard, *n*-octacosane, were used to convert peak areas to quantities of 25:0 hydrocarbon.

Results

Behavioural study

The in-nest behaviour of 12 *O. lignaria* females was observed inside glass tubes. After mating, females inspected cavities and, when a cavity was accepted, females collected mud and deposited it at the far end of the nest. When the first mud partition was completed, females began collecting nectar and pollen to provision the first cell.

When returning from a foraging trip, females first deposited the nectar, turned around inside or outside the nest and deposited the pollen against the mud partition. Females thus exited the nest facing the entrance. When exiting the nest, females dragged the tip of the abdomen along the entire tube, spiralling and apparently marking also the upper part of the nesting tube. Females regularly stopped and brushed the ventral part of the abdomen with the hind legs, bringing the tarsi together in contact with the abdomen, and lowering the abdomen so the tip came into contact with the glass tube. It was not determined whether the brushing of the abdomen is associated with marking of the nest or just involved in cleaning the abdomen to remove any remaining pollen. Females were often observed depositing a tiny fluid droplet from the abdomen. The fluid droplet appeared clear most of the time; however, sometimes it was purplish, suggesting the presence of the purple pollen and/or nectar of *P. tanacetifolia* in the secretions. When secreting the fluid droplet, females usually continued walking, but occasionally stopped and smeared the droplet with the legs and tip of the abdomen. No females exhibited any behaviour using the mandibles or any other part of the body that could be interpreted as nest marking.

Osmia lignaria females were very sensitive to the removal of the nest markings due to the replacement of a nest tube section (Table 1). In all treatments, when confronted with the new clean sections, the returning females hesitated in front of or inside their nests and exhibited significantly more recognition attempts than in the control (Table 1). When tested with the control, the 15 females entered their nest without hesitation and all deposited the pollen-nectar load in their nest.

With the replacement of the outer section by a similar clean section, the females did not enter the nest but rather touched the entrance with the antennae or inserted the head inside the outer section (Table 1), suggesting that the bees failed to detect an expected recognition cue as soon as their antennae contacted the nest entrance. These females

Table 1. Mean nest recognition attempts at own nest hole and at other holes, number of *Osmia lignaria* females that deposited pollen load, and number of these females that marked own nest when exiting after manipulations of the nest sections.

Treatment sections replaced (n = 15)	Mean attempts ± SE		Number of bees deposited pollen load	Number of bees marked own nest
	Own nest hole	Other holes		
Control (none)	0^{a}	0.3 ± 0.2^{a}	15^{a}	3^{a}
Outer section	4.7 ± 1.3^{b}	$5.5 \pm 2.6^{a,b}$	14^{a}	11^{b}
Middle section	14.6 ± 1.8^{c}	$11.5 \pm 3.7^{b,c}$	12^{a}	$7^{a,b}$
Both sections	$14.3 \pm 3.3^{b,c}$	15.9 ± 4.3^{c}	5^{b}	5^{b}

Superscript letters indicate, within columns, the values that are significantly different from those values not sharing that letter ($P < 0.05$). *n*, Sample size per treatment.

checked several other holes, coming back to their own nest several times without entering. When the females finally entered their nest, they walked to the provision without hesitation and deposited the pollen-nectar load. Fourteen out of the 15 females eventually deposited the pollen-nectar load in their own nest. The fifteenth female abandoned her nest, subsequently usurped another active nest, and marked it repeatedly.

Upon replacement of the middle section, the returning females did not hesitate at the nest entrance and entered the outer section of the nest (Table 1). However, they abruptly stopped at the beginning of the middle section, touching it with their antennae. Females stayed in the outer section of the nest, inspecting it with the antennae and spiralling inside the tube. Females then exited the nest, checking several other holes, returning to and exiting the outer section of the correct nest several times. These females attempted their own nests significantly more often that when the outer section had been replaced (Table 1). Twelve out of the 15 females ultimately crossed the middle section very slowly, resuming a normal pace when contacting the unchanged inner section, and subsequently deposited the pollen-nectar load. The remaining three females abandoned their nest, either usurping the nest of another female (depositing the pollen-nectar load and/or marking) or initiating a new nest in a clean nesting tube (depositing mud).

After replacing both outer and middle sections, returning females hesitated at the nest entrance. They did not enter the nest, but rather touched or inserted the head in the outer section (Table 1), exhibiting similar patterns of behaviour as described with the outer section treatment. The females examined several other holes significantly more often than bees in the outer section treatment, returning to their nest less and less frequently. Females were observed checking other active nests rather than blank nesting tubes, and only five out of the 15 females ultimately entered their nest and deposited pollen-nectar. The remaining 10 females abandoned their nest, either usurping an active nest or nesting anew in a clean nesting tube. Compared with the middle section treatment, replacing both outer and middle sections did not result in an increase in the mean attempts at the bees' own nest (median test: $t = 0.1$, $n = 15$, $P > 0.9$) or at other holes (median test: $t = 2.1$, $n = 15$, $P > 0.2$). However, fewer females deposited pollen-nectar in their nest with the both sections treatment compared with the middle section treatment ($\chi^2 = 6.7$, d.f. = 1, $P < 0.01$; Bonferroni correction, $\alpha < 0.013$).

Once females accepted their nest after manipulation and deposited the pollen-nectar load, they immediately began to mark the nest cavity intensely before exiting on a foraging trip. Indeed, fewer females marked their nest after the control manipulation compared with the outer section treatment ($\chi^2 = 9.95$, d.f. = 1, $P < 0.002$; Bonferroni correction, $\alpha < 0.01$), the middle section treatment (although not significant: $\chi^2 = 4.2$, d.f. = 1, $P > 0.04$; Bonferroni correction, $\alpha < 0.013$), or the both sections treatment ($\chi^2 = 10.0$, d.f. = 1, $P < 0.002$; Bonferroni correction, $\alpha < 0.008$).

To confirm that the absence of olfactory cues was the cause for nest abandonment in the both sections treatment, the original sections of their nests were returned approximately 15 min after manipulations for seven of the total females. Six of those seven females identified their nest within a few minutes and resumed nesting after intensive marking.

Chemical analysis

The analyses performed on solvent extracts from both outer and middle nest tube sections and the bee cuticles revealed the presence of free fatty acids, long chain hydrocarbons and wax esters (Fig. 1; Table 2). The major compounds found in all samples were hexadecanoic, octadecadienoic, octadecenoic and octadecatrienoic acids, pentacosene, pentacosane, heptacosene, heptacosane and nonacosene. The free fatty acids (peaks 1–5) were also found in the pollen samples, and were thus suspected to be contaminants in the tubes and on the cuticular surface of the nesting bees. This suspicion was confirmed by comparative analysis of the cuticular lipids of laboratory-emerged, pollen-free *O. lignara* females (Buckner & Pitts-Singer, unpublished results). Some of the unidentified components also originated from pollen (peaks 26, 28 and 30) (Table 2).

As for all samples of glass tubes, the results of GC-FID analyses of the outer and middle tube sections of a representative *O. lignaria* female revealed very similar patterns. Noticeable differences were found in the relative proportions of hydrocarbons peaks 8 and 9. There were also

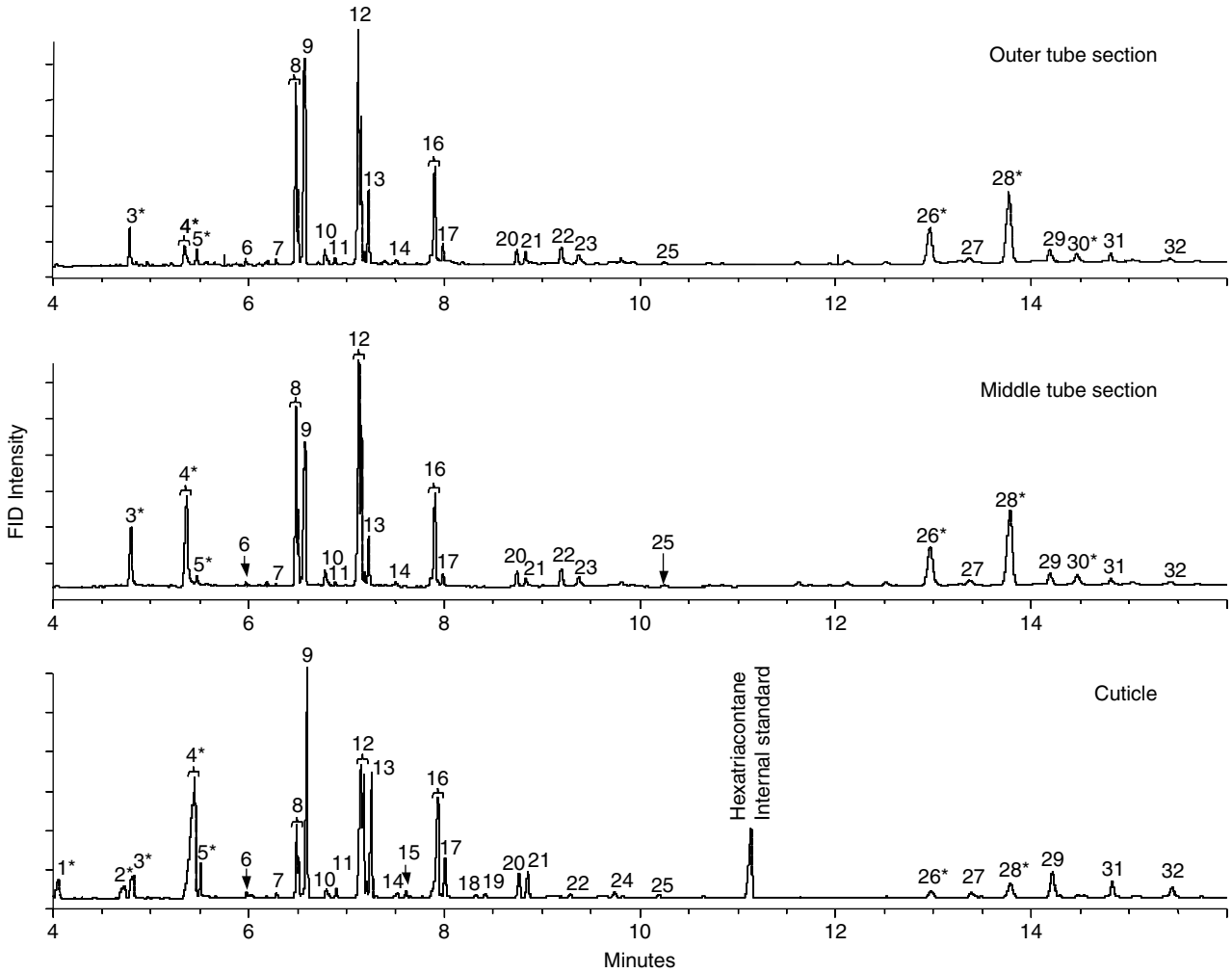

Fig. 1. Gas chromatography-flame ionization detector (FID) results of the outer nest tube section, the middle nest tube section and the cuticle of one representative *Osmia lignaria* female. Identifications of known, numbered peaks are reported in Table 2. Peak numbers followed by an asterisk indicate components shown to originate from *Phacelia tanacetifolia* pollen that contaminated experimental bees and glass tubes.

differences in the presence or proportion of free fatty acid peaks 1–5, which are pollen components and thus are not indicative of differences in nest-marking cues (Fig. 1; Table 2). All the nonpollen lipids that were found on the glass tube sections were also found on the cuticle (Fig. 1; Table 2). However, the relative proportions of peaks 8 and 9 and peaks 12 and 13 differed between the nest tube sections and the cuticle.

Due to the differences in extraction methods for the cuticular lipid compositions, two groups of five insects each are presented in Table 3 (group 1: extracted in hexane only; group 2: extracted in hexane followed by chloroform). On average, 85% of lipid was shown to be removed by hexane alone compared with extraction in hexane followed by chloroform. The only qualitative difference observed by further extraction of hexane-extracted bees with chloroform was the increase in the amounts of fatty acids (Table 3). The average percent composition of the hydrocarbons and wax esters revealed that the same major lipid compounds are present in both outer and middle tube sections, as well as on the cuticle of *O. lignaria* females (Table 3). However, the relative proportions of lipids, particularly for those same major compounds, varied between females as indicated by the standard errors (Table 3).

Furthermore, the total amount of lipid present on the outer section compared with the middle tube section reveals variations between bees. Although several *O. lignaria* females deposited less than half the amount of lipid (mean ± SE) in the outer (5.6 ± 1.6 μg) compared with the middle section (17.2 ± 3.1 μg) ($n = 5$), others deposited equivalent amounts of lipid in both outer (9.7 ± 2.2 μg) and middle (11.2 ± 2.0 μg) sections ($n = 4$). Furthermore, the total amounts of lipid varied greatly between bees in the

Table 2. Compounds identified using gas chromatography-mass spectrometry from outer and middle nest tube sections, and cuticles of *Osmia lignaria* females.

Peak no.[a]	Abbreviation	Compound(s)
1	14:0 (free fatty acid)	Tetradecanoic acid[b]
2	16:1 (free fatty acid)	Hexadecenoic acid[b]
3	16:0 (free fatty acid)	Hexadecanoic acid[b]
4	18:2, 18:1, 18:3 (free fatty acids)[c]	Octadecadienoic, octadecenoic and octadecatrienoic acids[b]
5	18:0 (free fatty acid)	Octadecanoic acid[b]
6	23:0	Tricosane
7	24:0	Tetracosane
8	25:1	Pentacosenes
9	25:0	Pentacosane
10	26:1	Hexacosenes
11	26:0	Hexacosane
12	27:1	Heptacosenes
13	27:0	Heptacosane
14	28:1	Octacosenes
15	28:0	Octacosane
16	29:1	Nonacosenes
17	29:0	Nonacosane
18	30:1	Triacontene
19	30:0	Triacontane
20	31:1	Hentriacontenes
21	31:0	Hentriacontane
22		Unknown
23		Unknown
24	33:0	Tritriacontane
25	33B	Dimethyltritriacontane
26		Unknown[b]
27	40:1, 40:0 (wax esters)	Oleoyl docosanoate and palmityl tetracosanoate[d]
28		Unknown[b]
29	42:1, 42:0 (wax esters)	Oleoyl tetracosanoate and palmityl hexacosanoate[d]
30		Unknown[b]
31	44:1, 44:0 (wax esters)	Oleoyl hexacosanoate and palmityl octacosanoate[d]
32	46:1, 46:0 (wax esters)	Oleoyl octacosanoate and palmityl triacontanoate[d]

[a]Peak numbers correspond to those in Figure 1.
[b]Compounds present in extracts of *P. tanacetifolia* pollen grains.
[c]Order of elution.
[d]Components present in highest concentration.

amounts present in the outer section (1.55–33.56 μg), the middle section (5.49–21.67 μg) and even on the cuticle of the females (23.85–89.44 μg) ($n = 10$).

Discussion

Osmia lignaria females mark their nest with olfactory cues that they use for nest recognition in large aggregations of conspecifics. The use of glass tubes allows observers to see that nest marking occurs along the entire length of the cavity, and not only at the nest entrance. This is the first report of nest-marking behaviour exhibited by bees inside the entire nest. Besides behaviours in which the abdomen is used, no other behaviour is consistent with nest marking (e.g. rubbing of the face or mandibles on the nest surface to apply mandibular secretions). These observations contrast with previous reports for other solitary bees, including two other *Osmia* species, *O. cornuta* and *O. bicornis*, which allegedly deposit mandibular secretions inside their nests (Shinn, 1967; Steinmann, 1976; Anzenberger, 1986).

The removal of olfactory cues by manipulating *O. lignaria* nest tube sections elicits temporary or permanent rejection of the bee's nesting cavity, clearly indicating the presence of some olfactory cues used for nest recognition. Although the bioassay was not designed to evaluate the possible use of volatile recognition cues, it appears that the cues in the tubes are persistent. This assumption is supported by the fact that the bees react to manipulations only when they are in close contact with the glass tubes. Furthermore, the cues important for *Osmia lignaria* nest recognition and acceptance are found in the entire nest cavity. Females are affected more by the removal of the internal olfactory cues (middle section) compared with the removal of cues present at the nest entrance (outer section). Because the middle section of the nesting tube is twice as long as the outer section, perhaps the removal of the cues present in this larger nest portion is detected more easily by returning females compared with when the smaller, outer section is removed.

In half of the nesting tubes examined, equivalent amounts of lipid are present in both the short, outer and longer, middle sections. This suggests that females nesting in these tubes mark the outer section of their nest more than the middle section. Abundant marking at the nest entrance could be due to behavioural variability in individual bees because of size, age or experience. For example, some bees increase guarding and intensify nest marking at the entrance in response to aggressive interactions with, or usurpation attempts from, other bees.

Chemical analysis of the deposits inside the nesting tube reveals the presence of free fatty acids (from pollen), hydrocarbons and wax esters. Previous studies of ground-nesting solitary bees, in which the nest cell linings of different species were solvent-extracted, report some or all of the same compounds found in the present study (Brooks & Cane, 1984; Kronenberg & Hefetz, 1984; Shimron *et al*., 1985; Hefetz *et al*., 1986; Williams *et al*., 1986; Espelie *et al*., 1992). The compounds found in the glass nests are similar throughout the entire nest and are similar in composition for nests from different bees (Table 3). However, between bee nests, there are differences in the relative abundance of those compounds. Furthermore, the cuticular lipids from the resident bees match their nest in composition. The differences in relative abundance could provide the variability necessary for individual nest recognition (Barrows *et al*., 1975; Kronenberg & Hefetz, 1984; Shimron *et al*., 1985; Hefetz *et al*., 1986; Hefetz, 1987, 1990). Individual nest recognition could be confirmed by substituting a nest entrance by the entrance of another active nest. However,

Table 3. Average percentage lipid composition present in nest tube sections and cuticles of 10 *Osmia lignaria* females.

		Average ± SD percent composition[a]			
				Cuticle	
Peak no.	ID	Outer section	Middle section	Group 1[b]	Group 2[c]
6	23:0	0.5 ± 0.3	0.4 ± 0.3	0.3 ± 0.1	0.5 ± 0.1
7	24:0	0.6 ± 0.4	0.3 ± 0.2	0.3 ± 0.1	0.5 ± 0.0
8	25:1	19.4 ± 2.3	21.1 ± 3.9	12.5 ± 0.5	9.1 ± 1.3
9	25:0	11.9 ± 5.3	9.0 ± 2.0	15.4 ± 0.8	20.2 ± 1.9
10	26:1	1.7 ± 0.4	2.3 ± 0.3	1.2 ± 0.1	1.3 ± 0.2
11	26:0	0.6 ± 0.4	0.3 ± 0.1	0.6 ± 0.1	1.1 ± 0.4
12	27:1	33.8 ± 2.9	36.2 ± 2.1	30.1 ± 1.9	24.7 ± 1.1
13	27:0	4.8 ± 1.0	3.9 ± 0.7	8.3 ± 1.4	8.1 ± 0.7
14	28:1	0.7 ± 0.3	0.7 ± 0.2	0.5 ± 0.1	0.6 ± 0.1
15	28:0	t[d]	t	0.3 ± 0.1	0.3 ± 0.1
16	29:1	11.5 ± 0.8	12.2 ± 0.7	11.8 ± 0.2	12.1 ± 0.5
17	29:0	2.0 ± 0.8	1.5 ± 0.4	2.7 ± 0.6	2.8 ± 0.2
18	30:1	t	t	0.1 ± 0.1	0.1 ± 0.0
19	30:0	t	t	0.1 ± 0.1	0.2 ± 0.1
20	31:1	1.6 ± 0.4	2.1 ± 0.3	2.5 ± 0.2	2.5 ± 0.2
21	31:0	1.8 ± 1.3	1.6 ± 0.9	1.9 ± 0.4	2.0 ± 0.2
24	33:0	t	t	0.5 ± 0.2	0.4 ± 0.1
25	33B	0.7 ± 0.8	0.3 ± 0.3	t	0.2 ± 0.1
27	40:1, 40:0	1.7 ± 1.3	2.8 ± 2.0	0.9 ± 0.2	1.8 ± 0.4
29	42:1, 42:0	3.4 ± 0.8	2.9 ± 0.7	4.3 ± 0.9	5.1 ± 0.3
31	44:1, 44:0	1.9 ± 0.5	1.2 ± 0.5	3.2 ± 0.4	3.3 ± 0.2
32	46:1, 46:0	1.5 ± 0.5	1.3 ± 0.5	2.7 ± 0.6	3.2 ± 0.6

[a]Percent composition was calculated from the integrated peak area data from the gas chromatography-flame ionization detector response as described in methods and materials.
[b]Bees extracted in hexane only.
[c]Bees extracted in hexane followed by chloroform.
[d]t, Trace amounts (<0.10%).

females that enter an alien nest immediately exit the cavity, strongly supporting the hypothesis that the nest-marking chemistry is unique to the resident bee. Additionally, closely-related individuals could share more similar nest odours, thus exhibiting more similar chemical profiles (Raw, 1992). This nest odour similarity could play a role in nest usurpation, a behaviour known to occur in *O. lignaria* (Tepedino & Torchio, 1994).

The origin of the chemicals used in nest marking in *O. lignaria* is not resolved by the present study. The behavioural evidence supports the idea that odours are deposited inside the nest from the abdominal region of the body. It is quite possible that the chemicals originate from the Dufour's gland (Hefetz *et al.*, 1990; Hefetz, 1992), glands yet to be located, or even the hindgut. In other studies, extractions of the cell lining of several ground-nesting bee species reveal the presence of Dufour's gland secretions, to which several functions have been attributed, including nest entrance marking (Hefetz, 1998; Abdalla & Cruz-Landim, 2001).

The similarity between the compounds extracted from the cuticle of *O. lignaria* females and from their nesting tubes may be explained by the in-nest activity of the bees. The females rub their hind legs on their abdomen, possibly smearing secretions from abdominal glands on their cuticle. Conversely, because females often turn around inside their nest, the cuticle may come into contact with the secretions deposited on the nest surface (Hefetz, 1990), which are picked up by the bee and are revealed in the cuticular extractions. Further investigation into the origin(s) of the chemicals found in *O. lignaria* nests will include chemical analysis of the contents of the Dufour's gland of nesting female bees, as well as a morphological study of this gland and its possible association with the sting apparatus or sternal apertures.

The present study clearly demonstrates the importance of olfactory cues for nest recognition in *O. lignaria*, providing a better understanding of how females identify their own nest among other nearby cavities in large nesting aggregations. The results could have important commercial implications because these olfactory cues might act as an aggregation pheromone, attracting other females to nest in close proximity (Duffield *et al.*, 1984). The compounds present in the depositions inside the nest could be extracted (or synthesized) and applied to commercial nesting boards to induce nesting in commercial situations, improving pollination efficiency (Buttery *et al.*, 1981; Parker *et al.*, 1983).

Alternatively, these compounds could be important attractants for parasitoids (Godfray, 1994) and thus be used in designing traps against specific parasitoids and predators of *O. lignaria*, such as several species of wasps and beetles (Bosch & Kemp, 2001).

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