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RESEARCH ARTICLE

Comparative analysis of fertility signals and sex-specific cuticular chemical profiles of *Odontomachus* trap-jaw ants

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ABSTRACT

The lipid mixture that coats the insect cuticle contains a number of chemical signals. Mate choice in solitary insects is mediated by sexually dimorphic cuticular chemistry, whereas in eusocial insects, these profiles provide information through which colony members are identified and the fertility status of individuals is assessed. Profiles of queens and workers have been described for a number of eusocial species, but there have been few comparisons of fertility signals among closely related species. Additionally, sexual dimorphism in cuticular lipid profiles has only been reported in two species of ants. This study describes the cuticular chemical profiles of queens, workers and males of three species of *Odontomachus* trap-jaw ants: *O. ruginodis*, *O. relictus* and *O. haematodus*. These are compared with fertility signals and sexually dimorphic profiles already described from *O. brunneus*. We report that fertility signals are not conserved within this genus: chemical compounds that distinguish queens from workers vary in number and type among the species. Furthermore, the compounds that were most abundant in cuticular extracts of *O. ruginodis* queens relative to workers were novel 2,5-dialkyltetrahydrofurans. Bioassays of extracts of *O. ruginodis* queens indicate that the dialkyltetrahydrofuran and hydrocarbon fractions of the profile are likely to work synergistically in eliciting behavioral responses from workers. In contrast, cuticular lipids that distinguish males from females are more conserved across species, with isomeric and relative abundance variations comprising the main differences among species. Our results provide new insights into how these contact chemical signals may have arisen and evolved within eusocial insects.

KEY WORDS: Lipid, Cuticular hydrocarbons, Division of labor, Pheromones, Sex pheromone, Social insects

INTRODUCTION

The cuticular chemical profiles of social insects encode information necessary for maintaining colony identity and social cohesion, providing the foundation through which eusociality is maintained (Blomquist and Bagnères, 2010). Signals of colony membership result from the blending of individual profiles through constant social interactions and assessments of nestmate profiles (van Zweden and d'Ettorre, 2010). Simultaneously, reproductive division of labor is maintained by a specific subset of cuticular chemicals expressed by queens and reproductive workers that signal their fertility (Liebig, 2010). Additionally, some species display sex-specific chemical profiles that distinguish males from female castes in the colony (Smith et al., 2014). Although these communicative chemical profiles have been generally described for a number of species (e.g. Van Oystaeyen et al., 2014), there have been few detailed comparisons among closely related species.

The cuticular chemical profiles of insects usually are composed primarily of straight-chain and branched-chain alkanes and alkenes of 19–35 carbons (Martin and Drijfhout, 2009; Blomquist and Bagnères, 2010). Lesser amounts of other classes of chemicals also have been documented on the cuticle including very long-chain hydrocarbons, triacylglycerides and oxygenated compounds (Cvacka et al., 2006; Buckner, 2010; Yew et al., 2011). Some of these non-hydrocarbon cuticular compounds (e.g. triacylglycerides) are important components of pheromone blends (Kuhbandner et al., 2012), interacting synergistically with cuticular hydrocarbons.

Cuticular chemical profiles of queens and workers have been compared for more than 60 species of social insects, and bioassays have resulted in the experimental identification of specific cuticular compounds responsible for fertility signaling in seven of these species (Van Oystaeyen et al., 2014). Phylogenetic comparisons indicate that particular cuticular compounds or classes of compounds (saturated alkanes) are highly conserved across genera as queen fertility signals (Van Oystaeyen et al., 2014; van Zweden et al., 2014). Several recent reviews highlight the theoretical implications that the observed high level of conservation of queen signals among species has for our understanding of signal evolution (Oi et al., 2015; Peso et al., 2015). Rapidly evolving queen signals are predicted to be a product of conflict between workers and queens when queen signals manipulate worker physiology against the interests of workers. In contrast, conserved queen signals are indicative of honest advertisement of queen fecundity rather than exploitative manipulation of worker reproductive physiology (Keller and Nonacs, 1993). However, comparative chemical data on more than one or two species within a genus are not available for the majority of social insect genera, and current phylogenetic comparisons and theories of fertility signal evolution rely heavily on equivocal comparisons across lineages rather than comparisons among closely related species (Van Oystaeyen et al., 2014). More finely focused, within-genera comparisons of queen and worker cuticular profiles are necessary to fully understand how these signals evolved.

Ants are the most well studied social insects in terms of the chemical profiles of queens (described for over 40 species), yet only two studies have directly compared the cuticular profiles of queens and workers of congeneric species. Brunner et al. (2011) found that the profiles of queen and worker ants of six *Temnothorax* species were dominated by linear and branched alkanes, but there was no clear trend in compounds that characterized queens across species. In contrast, Holman et al. (2013) found that queens of 11 *Lasius* species had greater relative amounts of 3-methylalkanes than did

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workers. For two of these species, *L. niger* and *L. flavus*, 3-methylhentriacontane was experimentally identified as the compound used for signaling queen fertility (Holman et al., 2010, 2013). Beyond these directly comparative studies, queens of three species of *Neoponera* (formerly *Pachycondyla*) species were distinguished by relative amounts of particular alkenes, methylalkanes or dimethylalkanes (Heinze et al., 2002; Kellner, 2005; Evison et al., 2012). Species-specific blends of straight- and branched-chain alkanes also distinguished queens from workers in three *Camponotus* species (Bonavita-Cougourdan and Clement, 1994; Endler et al., 2006; Campos et al., 2012). These comparative studies of four genera from three ant subfamilies suggest that there is little evidence for conservation of ant fertility signaling compounds within genera, with the exception of the genus *Lasius*.

Cuticular chemical profiles of males have been reported for eight species of ants, only two of which, *Diacamma ceylonense* and *Odontomachus brunneus* (both in the subfamily *Ponerinae*), exhibit male-specific profiles (Cuvillier-Hot et al., 2001; Smith et al., 2014). Sexually dimorphic cuticular chemical profiles are thought to be the ancestral condition from which eusocial queen signals evolved, because of their widespread use as sex-specific mate recognition signals in solitary insects, including solitary and primitively eusocial Hymenoptera (Paulmier et al., 1999; Ayasse et al., 2001; Liebig et al., 2009; Kocher and Grozinger, 2011; Van Oystaeyen et al., 2014; Oi et al., 2015). Further investigation of the hydrocarbon patterns of males relative to those of female castes may prove essential in understanding the evolution of chemical signaling in social insects.

Here, we compared the cuticular chemical profiles of queen, worker and male ants of three species of *Odontomachus* ants that occur in the southeastern USA, and contrast these findings with earlier research on *O. brunneus*. The fertility signal of *O. brunneus* queens and workers was identified experimentally as (*Z*)-9-nonacosene (Smith et al., 2012, 2013, 2015). Cuticular profiles of male *O. brunneus* were dominated by pentacosenes and pentacosadienes, which were present in low amounts on workers (average combined relative abundance of 36% and 0.5%, respectively; Smith et al., 2014). Additionally, we report the identification of several novel 2,5-dialkyltetrahydrofurans that are major components of the cuticular lipids of *O. ruginodis* queens. We also present results of bioassays that tested the responses of workers to crude extracts of *O. ruginodis* queens, and to fractions of crude extracts that contained different classes of cuticular chemicals.

MATERIALS AND METHODS

Study organisms, collections and culture

We compared the cuticular hydrocarbon profiles of four species of *Odontomachus* that co-occur in the state of Florida in the southeastern USA (see MacGown et al., 2014): (1) *O. brunneus* (Patton), a widespread native species, (2) *O. relictus* Deyrup and Cover, a native species restricted to two Florida populations, (3) *O. ruginodis* M. R. Smith, widespread throughout Florida and probably introduced, and (4) *O. haematodus* (Linnaeus), an introduced South American species that now occurs from Alabama to Florida. Phylogenetic research has revealed that *O. relictus* is closely related to *O. brunneus*, whereas *O. ruginodis* and *O. haematodus* are more distantly related but in the same New World clade (Spagna et al., 2008; Schmidt, 2009; Larabee, 2015). The cuticular chemical profiles of workers, queens and males of *O. brunneus* have been described previously (Smith et al., 2012, 2013, 2014, 2015).

Colonies and foundresses of *O. ruginodis* were collected in August 2012 from the MacArthur Agro-ecology Research Center in Lake Placid, Florida. Colonies of *O. relictus* were collected in August 2012 from the Archbold Biological Station in Venus, Florida, and colonies of *O. haematodus* were collected in March 2014 from the Weeks Bay Preserve in Fairhope, Alabama.

In the laboratory, multiple colonies of each species were separately housed in two to three interconnected 60×15 mm Petri dishes with plaster-lined bottoms that were moistened twice per week. Colonies received a constant supply of water and 20% sugar–water solution, and were fed twice a week on termites and freeze-killed crickets. All colonies were kept at an average temperature of 27°C. Colonies were used for chemical analysis after they had been kept under laboratory conditions for at least 4 weeks. Queens were sampled only if their colony had produced only female-destined brood and an egg pile was consistently present in the colony. For *O. ruginodis* foundresses, chemical samples were taken after the first clutch of 1–5 eggs was laid and before any of those eggs developed into larvae. Samples were taken from males present in the colonies when they were collected, and from males reared in the laboratory by queenless colonies containing reproductive workers.

Chemical sampling and analysis

The cuticular chemical profiles of queens, workers and foundresses were sampled from live ants by solid-phase microextraction (SPME). A SPME fiber (100 µm polydimethylsiloxane; Supelco Inc., Bellefonte, PA, USA) was lightly rubbed on the abdomen of a restrained ant (see bioassays section, below, for a description of the restraint) for 5 min, then thermally desorbed for 5 min in the injection port of a Hewlett-Packard 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA), equipped with a DB-5MS column (30 m×0.25 mm×0.25 µm film; J&W Scientific, Folsom, CA, USA), connected to a Hewlett-Packard 5973 series mass-selective detector. The GC injection port and transfer line were set to 280°C. The column temperature was held at 60°C for 2 min, increased to 220°C at 40°C min^{-1}, and then to 315°C at 4° C min^{-1}. Helium was the carrier gas at 1 ml min^{-1}, and samples were injected in splitless mode with a purge time of 2 min. Electron impact ionization mass spectra were obtained using 70 eV ionizing voltage, with a source temperature of 230°C.

The cuticular profiles of males and workers were compared by GC-MS analysis of solvent extracts of each sex. Ants were freeze-killed and submerged, individually, in 125 µl of hexane for 5 min. The extract was concentrated under a stream of nitrogen to a volume of ∼10 µl, and 1 µl was analyzed by GC-MS as described above, injecting in splitless mode.

Linear hydrocarbons were identified from their mass spectra, including the molecular ion if present, and by matching retention times with authentic standards. Branched chain compounds were identified from a combination of their mass spectra, looking specifically for enhanced ions due to fragmentation on either side of methyl branch points, and by comparison of their retention indices with those of straight-chain hydrocarbons (Carlson et al., 1998).

To identify the novel tetrahydrofuran compounds, a hexane extract of eight *O. ruginodis* queens was fractionated by liquid chromatography on a column of oven-dried silica gel (200 mg). The column was first wetted with hexane, then loaded with the extract, which had been concentrated to ∼100 µl under a stream of nitrogen, and the extract was flushed into the bed with a few drops of hexane. The column was then eluted sequentially with 1×1 ml hexane, 2×0.5 ml 10% ether in hexane, and 1×0.5 ml ether. The saturated and unsaturated tetrahydrofurans eluted cleanly in the second fraction.

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High resolution mass spectra of the tetrahydrofurans were obtained on a Waters GCT coupled gas chromatograph–high resolution mass spectrometer, run in EI mode (70 eV). The GC was equipped with a DB-5 column (30×0.25 mm i.d., J&W Scientific), programmed from 100 to 280°C at 10°C min^{-1}, with a final time of 20 min.

An aliquot of the fraction containing the tetrahydrofurans was diluted to ∼0.5 ml with hexane, and stirred for 2 h with a few milligrams of 5% palladium on carbon under a hydrogen atmosphere. The mixture was then filtered through a small pad of Celite to remove the catalyst, concentrated under a stream of nitrogen, and analyzed by GC-MS.

To determine the double bond positions in the unsaturated tetrahydrofuran compounds, a second aliquot of this fraction in a screw-cap vial was treated with 5 µl dimethyldisulfide and 10 µl of a 2% solution of iodine in tetrahydrofuran, warming at 50°C overnight with stirring. After cooling, the mixture was diluted with 0.5 ml each of hexane and 1 mol l^{-1} aqueous sodium thiosulfate, and vortexed until the organic layer was colorless. The organic layer was pipetted off and passed through a plug of anhydrous Na_2SO_4 to remove traces of water, then concentrated under a stream of nitrogen, and analyzed by GC-MS using a medium polarity DB-17MS capillary column (25 m×0.20 mm, 0.33 µm film thickness; J&W Scientific, Inc.). Injections were made in splitless mode, and the oven was programmed from 100 to 280°C at 10° min^{-1}, with a final time of 20 min.

To identify the double bond positions in alkenes and dienes present in crude hexane extracts of queens, the extracts were first fractionated on $AgNO_3$-impregnated silica gel. Thus, crude extracts from 2–5 *O. ruginodis*, *O. relictus* and *O. haematodus* males were concentrated to ∼100 µl, and fractionated on a column of 10% $AgNO_3$ on silica gel (400 mg) contained in a Pasteur pipette with a glass wool plug. The column was dried immediately before use in an oven at 120°C for 2 h. After cooling to room temperature, the column was wetted with hexane, then loaded with the concentrated extract which was rinsed into the bed with a few drops of hexane. The column was then eluted sequentially with 2 ml hexane, 2 ml 10% cyclohexene in hexane, and ether to provide fractions containing saturated hydrocarbons, unsaturated hydrocarbons and more polar compounds, respectively. The unsaturated hydrocarbons fraction was then derivatized with dimethyl disulfide (DMDS) and the resulting adducts were analyzed by GC-MS using a medium polarity DB-17MS capillary column, as described above.

Compounds were included in data analyses if they appeared in ≥70% of one of the castes of individuals (workers, queens, foundresses or males) sampled. Statistical comparisons of the cumulative abundances of compound classes across caste and species were carried out with Mann–Whitney *U*-tests performed used JMP statistical software (SAS Institute Inc., Cary, NC, USA). For comparisons between workers and males, diagnostic power (DP) of individual compounds was calculated (van Zweden and d'Ettorre, 2010). DP is the standard deviation of the standardized peak area of all sampled individuals divided by the pooled standard deviation within the grouping of interest. Groupings of interest were population and sex and species; therefore, two measures of DP for each compound were calculated. Compounds with the highest DP are the most consistent within a group while being variable between groups; therefore, they are compounds most likely to be 'diagnostic' of the grouping (van Zweden and d'Ettorre, 2010).

Bioassays of cuticular extracts and fractions thereof of *O. ruginodis* queens

Three workers from nine colonies (27 workers in total) were harnessed in a restraint fashioned from a 5×5 cm square of paper. A radial slit was made through the paper square, ending in a small hole at the center. The petiole (constriction between the thorax and the second abdominal segment) of the test ant was placed in the hole, so that the legs, thorax and head protruded from one side of the paper, and the abdomen from the other. Responses to stimuli were assessed by contacting the antennae with the stimulus and measuring antennal retraction, a stereotypical submissive posture displayed by workers of *Odontomachus* species when in proximity to a reproductive queen (Medeiros et al., 1992; Powell and Tschinkel, 1999). The worker pulls its antennae away from the queen such that the antennal scape is perpendicular to the long axis of the head. This assay has been used previously in studies of fertility signal perception in *O. brunneus* (Smith et al., 2015).

The first experiment presented restrained workers with cold-anesthetized non-nestmate queens or non-nestmate workers from the same colony. For the second experiment, three reproductive queens and three corresponding nest workers were freeze killed and extracted in 125 µl of hexane. The whole extract was then transferred in 10 µl increments to the tip of a glass rod (6 cm diameter), allowing the solvent to evaporate to coat the tip with the extract. The glass rod was then presented to non-nestmate ants as stimuli. For the third experiment, three additional queens and corresponding nest workers were freeze killed and extracted in 125 µl of hexane. Extracts of queens and workers were fractionated separately using a small column of 500 mg oven-dried 10% silver nitrate-impregnated silica gel in a Pasteur pipette with a glass wool plug. Columns were first wetted with 2×1 ml of hexane, then loaded with extract, and extract was eluted with 2.5 ml 20% cyclohexene in hexane to yield a complete hydrocarbon fraction followed by 2.5 ml of 20% ether in hexane to yield a fraction containing more polar constituents (including the 2,5-dialkyltetrahydrofurans). The worker extracts were run through an identical column, eluting with 20% cyclohexene in hexane. Fractions were concentrated under a stream of nitrogen, then resuspended in 125 µl of hexane, transferred to a glass rod, and presented to test ants as described above.

A different set of test workers was used for each of the three experiments. The stimulus queens, workers and extracts were presented in random order, and presented three times per trial. All trials were video recorded and given a coded title which ensured that the data transcriber was blind to the treatments and identity of test ants and stimuli. The consensus response (≥2 of 3 submissive or non-submissive responses) was recorded for each test ant. Data from experiments 1 and 2 were analyzed using McNemar's test, and data from experiment 3 were analyzed by Cochran's *Q*-test. All analyses were performed used JMP statistical software (SAS Institute Inc.).

RESULTS

Identification of tetrahydrofuran compounds

GC-MS analysis on a medium polarity DB-17 column of the 10% ether in hexane fraction from silica gel chromatography of the crude cuticular extract of *O. ruginodis* queens showed two pairs of peaks (compounds 1–4, compound numbers in this section do not correspond to compound numbers in the figures or tables), each in approximately a 1:10 ratio. The EI mass spectrum of the first peak (compound 1) in the earlier eluting pair did not show a molecular ion, but had a base peak at *m/z* 183 and a large fragment at *m/z* 281. The larger, second peak in the pair (compound 2) had a base peak at *m/z* 181 and a large fragment at *m/z* 281, suggesting that 1 and 2 were analogs differing only by the presence or absence of a double bond. Compound 2 had a clearly visible molecular ion at *m/z* 392, with a small fragment at *m/z* 374 from loss of water, indicating that

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it contained at least one oxygen, giving a possible molecular formula for compound 2 of $C_{27}H_{52}O$, a formula requiring two rings or double bonds. This molecular formula was confirmed by high resolution mass spectrometry (calculated for $C_{27}H_{52}O$: 392.4018 amu; found 392.4027 amu). Given the evidence for a C=C double bond in compound 2 due to its differing in mass from compound 1 by 2 amu, and the fact that compounds 1 and 2 had the same ion at *m/z* 281, this suggested that the remaining site of unsaturation was a ring. Furthermore, the base peak of compound 2 at *m/z* 181 and the large fragment at *m/z* 281 suggested losses of $C_{15}H_{31}$ and C_8H_{15}, respectively, from the molecular ion at *m/z* 392, leaving C_4H_6O unaccounted for from the possible molecular formula, suggesting that the structure was a 2,5-disubstituted tetrahydrofuran ring, i.e. 2-octenyl-5-pentadecyltetrahydrofuran. This structure would be expected to provide the large fragments seen, by loss of the alkyl chains from the carbons attached to the oxygen, with concomitant stabilization of the resulting cations by the oxygen. The smaller, earlier eluting compound, lacking the double bond and with a base peak at *m/z* 183 and a prominent ion at *m/z* 281, would then correspond to the saturated analog, 2-octyl-5-pentadecyltetrahydrofuran.

The mass spectra of the later-eluting pair of peaks indicated that they were simply analogs of the first two compounds. Thus, the smaller, earlier eluting peak 3 had a base peak at *m/z* 183 and a large fragment at *m/z* 309, with no visible molecular ion at *m/z* 422, whereas peak 4 had a base peak at *m/z* 181, a large fragment at *m/z* 309, a clearly visible molecular ion at *m/z* 420, and a smaller peak at *m/z* 402 from loss of water from the molecular ion. The high resolution mass spectrum of compound 4 provided corroborating evidence for a molecular formula of $C_{29}H_{56}O$ (calculated for $C_{29}H_{56}O$: 420.4312 amu; found 420.4326 amu). These data suggested that compounds 3 and 4 were 2-octyl-5-heptadecyltetrahydrofuran and 2-octenyl-5-heptadecyltetrahydrofuran, respectively.

Catalytic reduction of an aliquot of the fraction containing the tetrahydrofuran compounds with a catalyst of 5% palladium on carbon under a hydrogen atmosphere resulted in the disappearance of peaks 2 and 4, leaving only two peaks from compounds 1 and 3, confirming that 2 and 4 were analogs of the former compounds, with one double bond. To locate the positions of the double bonds in peaks 2 and 4, an aliquot of the fraction containing the tetrahydrofurans was derivatized with dimethyldisulfide. The resulting adduct from compound 2 gave a small molecular ion at *m/z* 486 (3% of base peak), a base peak at *m/z*

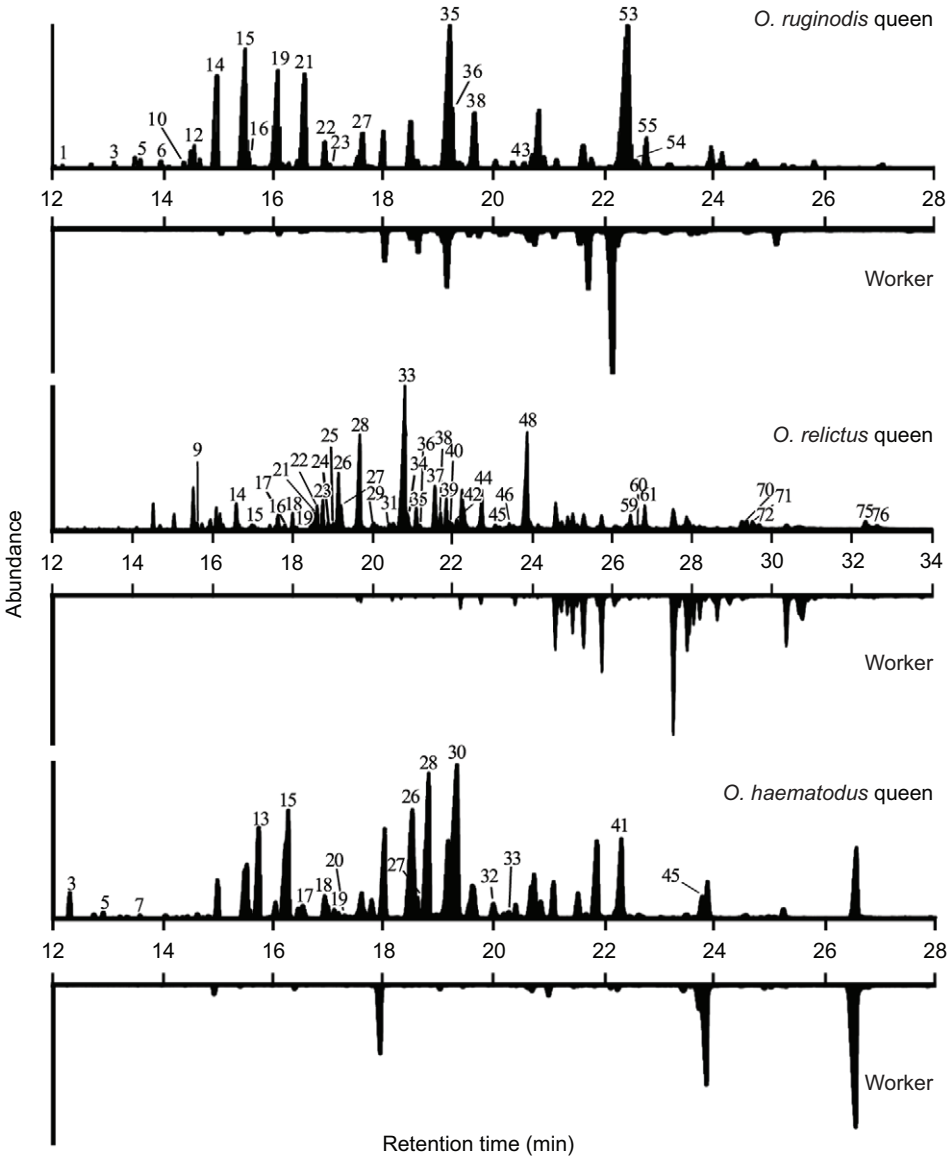

Fig. 1. Representative chromatograms of the cuticular extracts of queens and workers of three *Odontomachus* species. Labeled peaks correspond to the identified compounds (Table 1; Tables S1–3) that are more abundant on queen profiles relative to worker profiles. Tables S1–3 and Figs S1–3 contain a complete summary of compounds identified for all species.

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425 from loss of C_2H_5S, with a corresponding ion at *m/z* 61 for $C_2H_5S^+$, indicating that the double bond was in the terminal position. Thus, compound 2 was conclusively identified as 2-(oct-7-enyl)-5-pentadecyltetrahydrofuran. There are four possible stereoisomers for this structure (two diastereomeric pairs of enantiomers), and it has not yet been possible to determine which isomer is produced by the ants.

Similarly, the DMDS adduct of compound 4 displayed a small molecular ion at *m/z* 514 (3% of base peak), and diagnostic ions at *m/z* 453 (base peak) from loss of C_2H_5S and *m/z* 61 for $C_2H_5S^+$, again showing that the double bond was in the terminal position, proving the structure of compound 4 to be 2-(oct-7-enyl)-5-heptadecyltetrahydrofuran.

Queen and worker chemical profiles across species

Profiles of *O. ruginodis* queens were distinguishable from those of workers and foundresses by increased relative abundances of several methylalkanes and dimethylalkanes as well as the heretofore undiscovered 2,5-dialkyltetrahydrofurans (Fig. 1, Table 1; see Fig. S1 for *O. ruginodis* foundress data, and Tables S1–3 for complete compound identification and abundance data for all species). The 2,5-dialkyltetrahydrofurans were only found in extracts from queens, and one of them [2-(oct-7-enyl)-heptadecyltetrahydrofuran, compound 53; Table 1] was the most abundant queen compound in this species.

Profiles of *O. relictus* queens were distinguishable from those of workers by increased relative abundances of several methylalkanes and dimethylalkanes, a small subset of linear alkanes, and some alkenes and dienes (Fig. 1, Table 1). The largest increase in the relative abundance of a single compound on queens was for (*Z*)-9-nonacosene. Queens of the closely related species *O. brunneus* were distinguished from workers by an increase in this same compound (Smith et al., 2012, 2013).

Profiles of *O. haematodus* queens were distinguishable from those of workers by increased relative abundances of several methylalkanes and dimethylalkanes, along with a subset of linear alkanes and a diene (Fig. 1, Table 1). The compounds with the largest proportional increases in queens were dimethylalkanes.

Comparisons of the collective total percentages of compound classes that were more abundant on the cuticles of queens of the four *Odontomachus* species revealed that queens have species-specific chemical profiles (Fig. 2). Worker profiles were also species specific, and whereas queens shared some compounds with their respective workers, they were differentiated from workers by diverse and species-specific sets of compounds.

Bioassays of cuticular extracts and fractions thereof of *O. ruginodis* queens

Odontomachus ruginodis workers responded to anesthetized non-nestmate queens with antennal retraction significantly more frequently than they did when presented with non-nestmate workers (Fig. 3). Crude cuticular extracts coated onto the tip of a glass rod elicited analogous queen-specific antennal retractions (Fig. 3). However, when the crude extract of a queen was divided into hydrocarbon and dialkyltetrahydrofuran fractions, neither fraction alone elicited a queen-specific response when presented on a glass rod to conspecific workers (Fig. 3), indicating possible synergism between components in the two fractions.

Cuticular lipid profiles of males across species

Cuticular profiles of *O. ruginodis*, *O. relictus* and *O. haematodus* males were distinguished from those of all females by increases in relative abundances of several alkanes, alkenes and dienes (Fig. 4, Table 2). The most pronounced differences were in pentacosadienes, pentacosenes and pentacosane, which together constituted 39–58% of the profiles of males, across species. In comparison, that set of compounds constituted 1–3% of the profiles of workers of the same species. Compounds with the highest relative abundances on males (e.g. pentacosadienes, pentacosenes, tricosane) were the most diagnostic of sex, and among the compounds that were least diagnostic of species (Table 2), suggesting that the patterns of relative compound abundances that distinguish male profiles from females may be conserved across species. However, when examined more closely, species specificity was apparent in the patterns of alkene and diene isomer variation. For example, 5,9-alkadienes were present only in the cuticular extracts of *O. haematodus*, whereas 1,9-pentacosadiene was present only in extracts of *O. ruginodis* (Table 2). The remainder of the profiles of males consisted of relatively small amounts of the longer-chain hydrocarbons that make up the species-specific profiles of the respective workers (Fig. 4).

DISCUSSION

For social insects, the cuticular hydrocarbon profile encodes information permitting the identification of the sex, fertility status and colony identity of individuals (Blomquist and Bagnères, 2010). Previous work on *O. brunneus* found queens to be differentiated from workers primarily by a single fertility-signaling compound, (*Z*)-9-nonacosene (Smith et al., 2012, 2013). In contrast, queens of the three additional species surveyed here were distinguished from workers by a number of cuticular chemicals (Fig. 1, Table 1). Furthermore, profiles of queens of each of these species were characterized by an increased abundance of different classes of compounds (Fig. 2). The profile of queens of the species most closely related to *O. brunneus*, *O. relictus*, was somewhat similar in that the compound with the largest increase in relative abundance in comparison to workers was (*Z*)-9-nonacosene (relative abundance on workers: 1%, queens: 18%; Table 1). However, (*Z*)-9-nonacosene was not the only queen-distinguishing compound for *O. relictus*, nor did it differentiate queens from workers in *O. ruginodis* or *O. haematodus*.

Several dialkyltetrahydrofurans were found in cuticular extracts of *O. ruginodis* queens but not the corresponding extracts of workers or foundress queens (Table 1; see Table S1 and Fig. S1 for foundress data). To our knowledge, these compounds have not been found in nature before, and analogous 2,5-dialkyltetrahydrofurans have only been found in one other insect order, the Lepidoptera (Takabayashi and Takahashi, 1986a; Schulz et al., 1998). Braconid parasitoid wasps have been shown to exploit these compounds as kairomones for location and recognition of their lepidopteran hosts (Takabayashi and Takahashi, 1986b). 2-Octyl-5-pentadecyltetrahydrofuran was also found in low abundance co-eluting with 3-methylheptacosane in the profile of *O. haematodus* queens (see Fig. S3 and Table S3). Whereas all previously identified cuticular fertility signals in ants have been cuticular hydrocarbons (Van Oystaeyen et al., 2014), other social insects are known to signal their fertility with non-hydrocarbon compounds. For example, reproductive workers and breeding queens of the bumblebee *Bombus terrestris* produce long-chain esters and aldehydes (Sramkova et al., 2008). However, a recent bioassay demonstrated that queen-specific hydrocarbons were more effective than queen-specific esters at eliciting the typical physiological response of workers to a bumblebee queen (Van Oystaeyen et al., 2014). In a more concrete example, the queen pheromone of the honeybee, *Apis mellifera*, is a product of the mandibular gland and

Table 1. Compounds on cuticles of queens and workers of *Odontomachus* species

Compound	*O. ruginodis* (*N*=8)			*O. relictus* (*N*=6)			*O. haematodus* (*N*=12)		
	No.	Queen	Worker	No.	Queen	Worker	No.	Queen	Worker
Tricosane	1	0.18 (0.07, 0.43)	0				3	1.16 (0.4, 1.93)	0.01 (0, 0.17)
5-Methyltricosane							5	0.26 (0.07, 0.4)	0
3-Methyltricosane	3	0.25 (0.12, 0.44)	0						
Tetracosane							7	0.13 (0.06, 0.26)	0
3,7-Dimethyltricosane	5	0.4 (0.22, 0.61)	0						
11-Methyltetracosane	6	0.31 (0.08, 0.61)	0						
2-Methyltetracosane	10	1 (0.14, 3.06)	0						
x-Pentacosene	12	0.82 (0.44, 2.02)	0.02 (0, 0.16)						
Pentacosane	14	7.01 (5.5, 9.15)	0.49 (0.2, 0.72)						
11-Methylpentacosane	15	7.5 (4.29, 11.01)	0.26 (0, 0.5)						
7-Methylpentacosane	16	0.58 (0.41, 0.8)	0	9	0.43 (0.24, 0.65)	0			
5-Methylpentacosane							13	4.96 (2.52, 8.02)	0.51 (0, 1.79)
3-Methylpentacosane	19	6.74 (5.62, 8.18)	0.26 (0, 0.71)						
x,*y*-Dimethylpentacosane							15	8.65 (4.41, 12.2)	0.89 (0, 3.18)
3,15-Dimethylpentacosane	21	5.34 (3.42, 7.07)	0						
3,9-; 3,7-Dimethylpentacosane							17	0.7 (0.53, 1)	0
3,7-Dimethylpentacosane				14	2.16 (1.3, 3.58)	0.18 (0, 0.58)			
13-, 12-, 11-; 10-, 9-Methylhexacosane				15	0.39 (0.28, 0.57)	0			
13-; 11-Methylhexacosane							18	1.45 (1.05, 1.95)	0
11-Methylhexacosane	22	1.06 (0.6, 1.47)	0						
10-, 9-; 8-Methylhexacosane	23	0.22 (0.14, 0.31)	0						
6-Methylhexacosane							19	0.34 (0.18, 0.53)	0
5-Methylhexacosane							20	0.18 (0.14, 0.21)	0
x-Heptacosene	27	1.67 (0.59, 2.88)	0.04 (0, 0.33)	16	1.16 (0.65, 1.83)	0			
4,8-Dimethylhexacosane				17	0.43 (0.27, 0.61)	0			
Heptacosane				18	0.68 (0.17, 0.99)	0			
3,7-; 3,9-Dimethylhexacosane				19	0.37 (0.16, 1.09)	0			
13-; 11-Methylheptacosane							26	8 (4.75, 12.13)	1.21 (0.18, 2.82)
9-Methylheptacosane				21	1.02 (0.76, 1.4)	0			
7-Methylheptacosane				22	1.54 (1.13, 2.05)	0	27	0.65 (0, 0.99)	0.06 (0, 0.3)
5-Methylheptacosane				23	1.99 (1.3, 2.55)	0	28	7.83 (5.41, 9.45)	0.96 (0, 2.68)
11,15-Dimethylheptacosane				24	0.55 (0.44, 0.67)	0			
7,11-Dimethylheptacosane				25	0.5 (0.39, 0.58)	0			
3-Methylheptacosane				26	3.3 (2.47, 4.03)	0.53 (0, 1.69)			
2-(Oct-7-enyl)-5-pentadecyltetrahydrofuran	35	14.72 (7.64, 20.52)	0						
5,19-Dimethylheptacosane							30	12.58 (10.08, 16.29)	1.42 (0, 4.19)
5,9-Dimethylheptacosane				27	1.95 (1.45, 2.41)	0			
2-Octyl-5-pentadecyltetrahydrofuran	36	1.48 (0.38, 3.47)	0						
3,15-Dimethylheptacosane	38	3.44 (2.73, 4.23)	0.48 (0, 1.75)						
3,9-; 3,7-Dimethylheptacosane				28	10.02 (7.65, 12.79)	0.78 (0, 1.36)			
14-; 13-; 12-; 11-; 10-Methyloctacosane				29	0.72 (0.54, 0.89)	0	32	0.76 (0.42, 1.04)	0
5-Methyloctacosane							33	0.34 (0.16, 0.72)	0
4-Methyloctacosane				31	0.44 (0.28, 0.57)	0			
2-Octenyl-5-hexadecyltetrahydrofuran	43	1.13 (0.82, 1.4)	0						
(*Z*)-9-Nonacosene				33	18.34 (11.77, 23.78)	1.05 (0.4, 2.27)			
x-Nonacosene				34	1.14 (0.79, 1.29)	0			
Nonacosane				35	1.37 (1, 1.66)	0.09 (0, 0.53)			
3,7-Dimethyloctacosane				36	0.3 (0.25, 0.38)	0			

Continued

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Table 1. Continued

Compound	*O. ruginodis* (*N*=8)			*O. relictus* (*N*=6)			*O. haematodus* (*N*=12)		
	No.	Queen	Worker	No.	Queen	Worker	No.	Queen	Worker
15-; 13-Methylnonacosane				37	2.99 (1.92, 4.06)	0.22 (0, 0.68)			
7-Methylnonacosane				38	0.87 (0.72, 1.09)	0.07 (0, 0.45)			
5-Methylnonacosane				39	2.25 (1.65, 2.66)	0			
13,17-; 11,15-Dimethylnonacosane				40	0.32 (0.19, 0.49)	0			
7,11-; 7,13-Dimethylnonacosane				42	0.41 (0.23, 0.76)	0			
x,*y*-Dimethylnonacosane							41	4.94 (3.52, 6.28)	0
2-(Oct-7-enyl)-; 2-Octyl-5-heptadecyltetrahydrofuran	53	21.39 (15.88, 27.79)	0						
x-Triacontene	54	0.59 (0.37, 1.01)	0						
3,15-Dimethylnonacosane	55	2.04 (0.91, 2.96)	0.04 (0, 0.3)						
3,7-; 3,9-Dimethylnonacosane				44	2.99 (2.09, 4.05)	0.69 (0, 1.34)			
15-; 14-; 13-; 12-; 11-Methyltriacontane				45	0.45 (0.24, 0.72)	0			
x,*y*-Hentriacontadiene				46	0.93 (0.55, 1.3)	0	44	0.32 (0.1, 0.77)	2.66 (0.99, 8.59)
x,*y*-Hentriacontadiene							45	1.72 (0.58, 3.33)	0
x-Hentriacontene				48	9.79 (7.41, 12.19)	0.44 (0, 1.41)			
x-,*y*-Tritriacontadiene				59	1.52 (1.17, 2.38)	0.1 (0, 0.59)			
x-Tritriacontene				60	0.42 (0.28, 0.61)	0			
x,*y*-Tritriacontadiene; *x*-Tritriacontene				61	2.21 (1.34, 2.91)	0			
x,*y*-Pentatriacontadiene; *x*-Pentatriacontene				70	0.8 (0.6, 1.19)	0			
x-Pentatriacontene				71	1.05 (0.92, 1.17)	0			
x-Pentatriacontene; *x*,*y*-Pentatriacontadiene				72	0.59 (0.32, 0.83)	0			
x,*y*-Heptatriacontadiene				75	1.46 (1.18, 1.78)	0			
x-Heptatriacontene				76	0.83 (0.53, 1.63)	0			

Compounds listed are those that were relatively more abundant in profiles of queens and with relative abundance ranges non-overlapping with that of workers. Compound number and identification correspond to compounds numbered according to species in Fig. 1, and FigsS 1–3 and Tables S1–3 (where complete lists of compounds identified for all species are provided). Relative compound abundances are given as averages, with minimum and maximum values in parentheses.

'*x*-', '*y*-' denotes uncertain double bond position.

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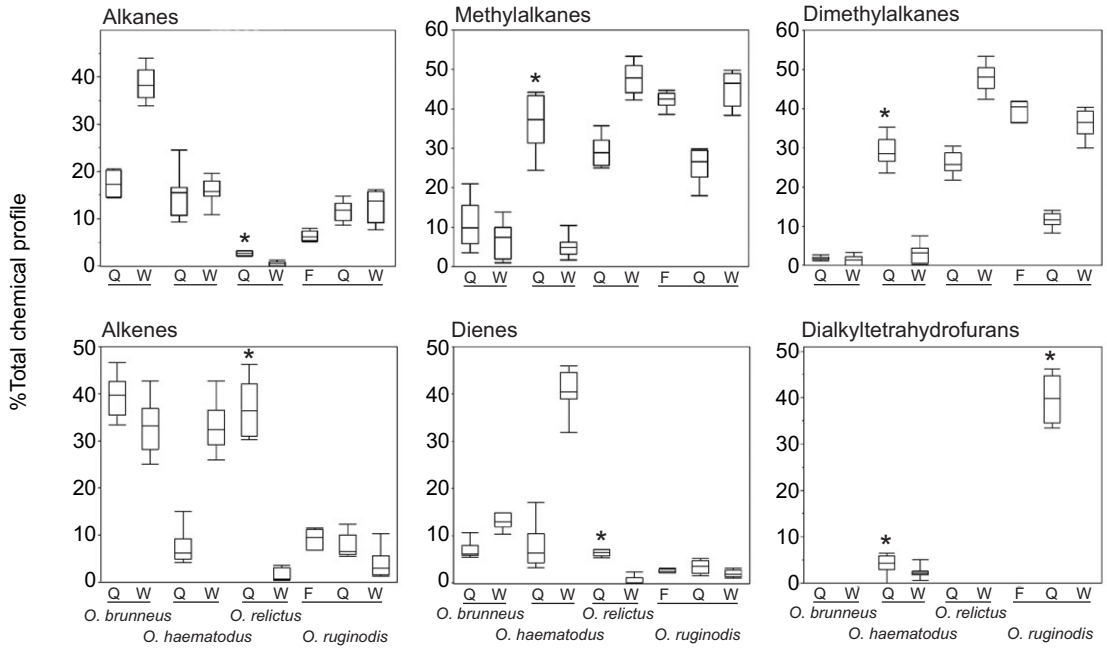

Fig. 2. Percentage of particular classes of compounds in the total cuticular chemical profiles of four *Odontomachus* species. Data are medians, quartiles and ranges. Q, queen; W, worker; F, foundress. Sample sizes: *O. brunneus* *N*=6, *O. haematodus* *N*=12, *O. relictus* *N*=6, *O. ruginodis* *N*=8 (workers and queens), *N*=6 (foundresses). Asterisks represent compound classes that are significantly (Mann–Whitney *U*-test, $P<0.01$) more abundant on queen than on worker cuticles. Statistical comparisons were not made for compound classes more abundant on workers relative to queens. *Odontomachus brunneus* data are from an Archbold Biological Station population as previously reported in Smith et al. (2013).

consists of (*E*)-9-oxodec-2-enoic acid and a blend of other non-hydrocarbon components (Slessor et al., 1988; Hoover et al., 2003). Furthermore, queens of the termite *Reticulitermes speratus* are reported to signal their fertility by emitting *n*-butyl *n*-butyrate and 2-methyl-1-butanol, and these compounds also have been found on their eggs (Matsuura et al., 2010; Yamamoto and Matsuura, 2011). However, neither the honeybee nor the termite pheromones are cuticular-based contact pheromones.

Our comparative data clearly show that the cuticular hydrocarbons that distinguish queens from workers are not conserved within the genus *Odontomachus*. Although recent phylogenetic comparisons suggested that queen fertility signals may be highly conserved throughout social hymenoptera (Van Oystaeyen et al., 2014), these comparisons may be misleading because they were usually based on data from single species from distantly related genera rather than from congeners or other closely related species. As an extreme example of a single chemical being used as a signal in distantly related insect groups, (*Z*)-9-nonacosene is a contact pheromone for females of the longhorned beetle *Megacyllene caryae* (Ginzel et al., 2006), but also the fertility signal of *O. brunneus* queens and workers (Smith et al., 2012). Nevertheless, this compound is not conserved as a fertility signal within the genus *Odontomachus* (Fig. 2, Table 1), or as a contact pheromone within the genus *Megacyllene* (Ginzel et al., 2003).

The apparent diversification of queen fertility signals in the genus *Odontomachus* suggests that these compounds manipulate worker reproductive physiology, and diversified as a result of conflicting interests of queens who chemically manipulate workers, and workers evolving resistance to those signals (Keller and Nonacs, 1993). However, our bioassays with *O. ruginodis*, and previously with *O. brunneus*, suggested that fertility signals are interpreted as such by workers only in the context of the other cuticular chemicals, rather than being chemicals that trigger responses in every worker that encounters the signal compound(s). Thus, cuticular extracts from *O. ruginodis* queens, when presented to workers on a neutral substrate, were as effective at eliciting a queen-specific releaser effect from workers as was a live queen (Fig. 3). However, neither the dialkyltetrahydrofuran nor the hydrocarbon fractions of extracts from queens elicited responses from workers (Fig. 3). (It should be noted that more polar

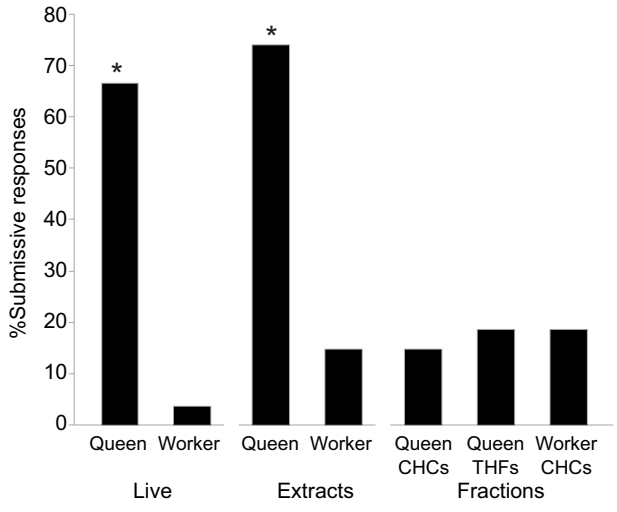

Fig. 3. Percentage of total submissive responses by restrained *O. ruginodis* workers following stimulation. Worker ants were stimulated by antennal contact with live ants, crude cuticular extracts, or fractions of cuticular extracts of *O. ruginodis* queens and workers. CHC, cuticular hydrocarbon; THF, tetrahydrofurans. Asterisks represent significant differences (McNemar's test, $P<0.0001$). Reactions to the fractions were not significantly different (Cochran's *Q*-test, $P=0.36$). $N=27$ for all groups.

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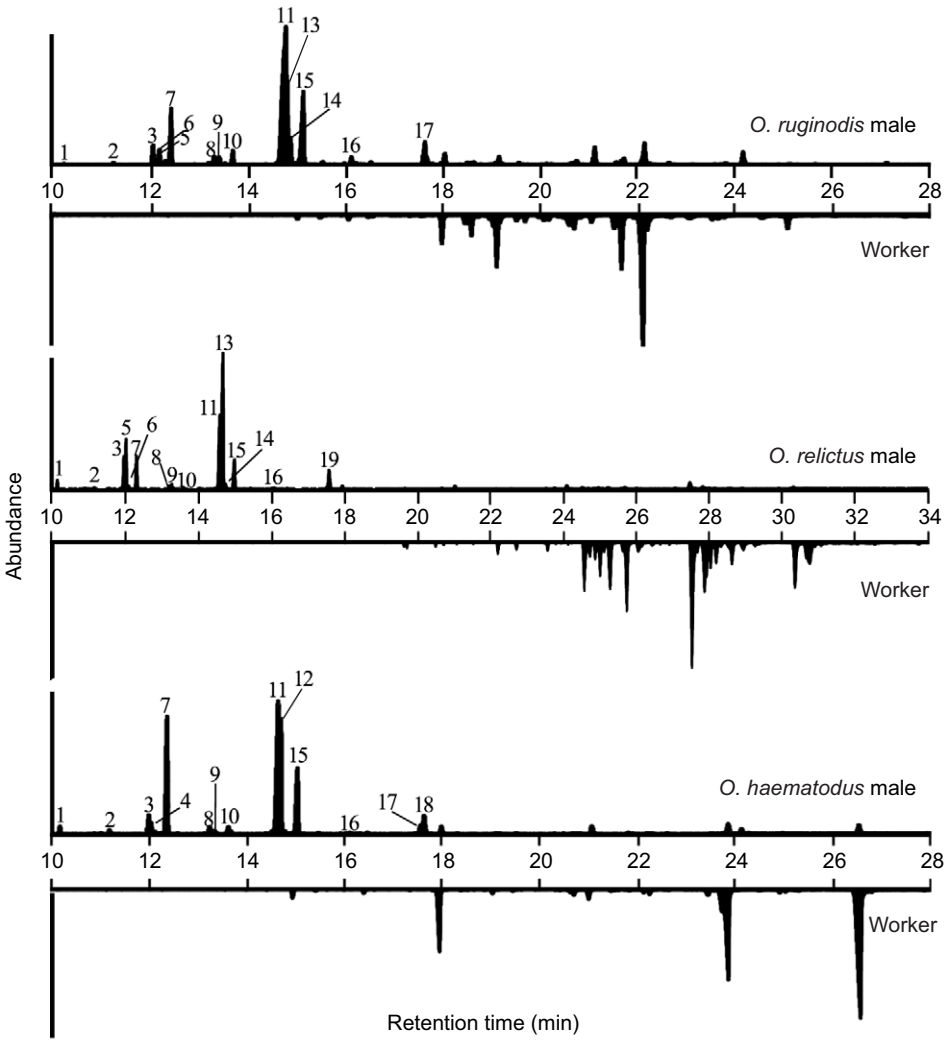

Fig. 4. Representative chromatograms of cuticular extracts of male *O. ruginodis*, *O. relictus* and *O. haematodus* contrasted with worker profiles. Labeled peaks correspond to compounds identified in Table 2.

compounds present in crude cuticular extracts may have been excluded from the fractions.) Furthermore, a recent series of experiments with *O. brunneus* revealed that its fertility signaling compound, (*Z*)-9-nonacosene, is only perceived by workers as a fertility signal when presented as a component in a nestmate or near-nestmate background chemical profile (Smith et al., 2015). Our current results suggest that, as with *O. brunneus*, *O. ruginodis* workers require a chemical context (some or all of the remainder of the cuticular chemical profile) within which to interpret queen signals, and do not respond to fertility signaling compounds that are presented out of chemical context. These results indicate the need for more systematic studies involving fractionating and recombining cuticular extracts to determine in bioassays how individual signal components are perceived, and how they might act synergistically with the remainder of the chemical profile.

To date, cuticular chemical fertility signals have been identified by manipulating concentrations of individual compounds, usually by applying them to the cuticle of workers (Smith et al., 2009) or to a neutral substrate (Holman et al., 2010). However, a single compound is unlikely to be solely responsible for the queen fertility signal of any of the three ant species studied here, as opposed to the single component fertility signal of *O. brunneus*. Queens of *O. brunneus* are distinguished from non-reproductive workers largely by a single compound (Smith et al., 2013), and applying the compound to the cuticle of a worker resulted in nestmates responding to the treated worker as though it were a queen (Smith et al., 2012, 2015). However, profiles of queens of the three species described herein were distinguished from those of workers by a number of compounds, suggesting that the queen signal in these species is likely to consist of multiple components. Work in other ant species, such as the carpenter ant *Camponotus floridanus* and the Argentine ant *Linepithema humile*, has shown that the hydrocarbon profiles of queens differ from those of workers in the relative abundances of the majority of their components (de Biseau et al., 2004; Endler et al., 2006). This suggests that the queen signals in these species might also consist of multiple components. Bioassays that start with tests of complete profiles followed by bioassays with fractions of the profiles may reveal that the context of a (more) complete chemical profile is necessary for perception of a fertility signal.

The hydrocarbon profiles of males of the three *Odontomachus* species studied here were broadly similar to those of male *O. brunneus*, with pentacosane, pentacosenes, pentacosadienes and tricosane representing more than half of the total hydrocarbons, whereas these compounds only constitute a small percentage of the hydrocarbons of females (Smith et al., 2014). These compounds were highly diagnostic of sex, rather than species (Table 2). Broadly, the classes of compounds that distinguished males from females in

Table 2. Chemical composition of cuticular extracts from males and workers of *Odontomachus* species

			O. ruginodis (*N*=9)		*O. haematodus* (*N*=5)		*O. relictus* (*N*=3)		Species		Sex	
No.	Compounds	RI	Workers	Males	Workers	Males	Workers	Males	DP	Rank	DP	Rank
1	Heneicosane	2100	0	0.17 (0, 0.38)	0	0.55 (0, 1.13)	0	1.22 (1, 1.49)	1.24	43	1.21	14
2	Docosane	2200	0	1.06 (0, 8.78)	0	0.29 (0, 0.49)	0	0.22 (0.19, 0.26)	1.1	61	1.01	58
3	*x*,*y*-*; 6,9-Tricosadiene	2269	0.03 (0, 0.27)	3.39 (0.31, 10.03)	0	1.58 (0.41, 2.65)	0	6.27 (4.72, 8.23)	1.14	52	1.3	9
4	5,9-Tricosadiene	2271	0	0	0	0.85 (0.28, 1.51)	0	0	1.33	31	1.06	36
5	(*Z*)-9-Tricosene	2273	0	0.98 (0, 1.81)	0	0.07 (0, 0.33)	0.4 (0, 1.21)	8.73 (7.47, 9.65)	1.45	21	1.08	27
6	*x*-Tricosene	2278	0.03 (0, 0.28)	0.86 (0, 1.55)	0	0	0	0.73 (0.5, 1.16)	1.2	46	1.23	12
7	Tricosane	2300	0.44 (0, 3.16)	10.49 (3.52, 24.13)	0.21 (0, 0.5)	14.64 (8.07, 21.01)	0.34 (0, 1.02)	6.07 (5.99, 6.18)	1.11	56	1.53	1
8	*x*,*y*-Tetracosadiene	2370	0	1.1 (0, 2.35)	0	0.69 (0.29, 1.09)	0	0.6 (0.47, 0.79)	1.11	57	1.46	4
9	(*Z*)-9-Tetracosene	2377	0	0.21 (0, 1.16)	0	0.18 (0, 0.49)	0	1.18 (0.99, 1.28)	1.24	40	1.13	20
10	Tetracosane	2400	0.12 (0, 1.04)	1.2 (0.47, 2.37)	0	0.84 (0.57, 1.09)	0	0.3 (0.24, 0.34)	1.15	51	1.42	7
11	1,9-*; 6,9-Pentacosadiene	2475	1.68 (0, 10.74)	21.28 (1.15, 48.75)	0.51 (0, 0.98)	31.27 (29.62, 33.07)	0.77 (0, 2.31)	20.08 (15.61, 25.14)	1.1	59	1.51	2
12	5,9-Pentacosadiene	2476	0	0	0.34 (0, 0.63)	14.91 (10.77, 21.77)	0	0	1.41	24	1.07	30
13	(*Z*)-9-Pentacosene	2478	0	2.43 (0, 6.68)	0	0.17 (0, 0.83)	2.25 (0, 6.75)	31.51 (26.09, 35.04)	1.49	17	1.06	40
14	(*Z*)-7-Pentacosene	2482	0.27 (0, 2.24)	0.94 (0, 3.15)	0	0	0	1.51 (0.76, 2.76)	1.16	50	1.06	39
15	Pentacosane	2500	1.48 (0.2, 8.39)	15.27 (6.68, 37.38)	0.92 (0.6, 1.81)	12.92 (10.5, 16.28)	0.44 (0, 1.33)	5.84 (4.88, 6.66)	1.12	54	1.43	5
16	*x*-Hexacosene	2571	0.2 (0, 0.61)	0.59 (0, 1.08)	0	0.15 (0, 0.35)	0	0.39 (0.3, 0.5)	1.24	42	1.17	16
17	6,9-Heptacosadiene	2673	0.2 (0, 0.83)	2.72 (0.93, 5.9)	0	2.45 (1.67, 3.55)	0	1.36 (0, 4.07)	1.1	58	1.42	6
18	5,9-Heptacosadiene	2677	0	0	0.06 (0, 0.29)	6.03 (3.49, 9.96)	0	0	1.33	30	1.06	38
19	(*Z*)-9-Heptacosene	2679	0	0	0	0	0	3.41 (0.23, 5.04)	1.3	33	1.02	56

Compounds listed are those that were relatively more abundant in profiles of males compared with those of workers. Relative compound abundances are given as averages, with minimum and maximum values in parentheses.

Peak numbers correspond to those in Fig. 4. '*x*-', '*y*-' denotes uncertain double bond position. RI, Kovat's retention index. Measures of diagnostic power (DP) of individual compounds for species and sex are given and ranked according to the diagnostic power of all cuticular compounds.

*Dienes specific to *O. ruginodis*.

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Odontomachus were conserved to a much greater degree than the classes of compounds that distinguished queens from workers. However, when examined in greater detail, the patterns of isomeric variation of these male-distinguishing compounds as well as the low-abundance portion of the profile that is shared with workers render the male profiles species specific (Fig. 4, Table 2; Smith et al., 2014).

Many studies of social insect fertility signals have noted potential homologies with the contact sex pheromones of females of solitary insect species (Liebig et al., 2009; Kocher and Grozinger, 2011; Van Oystaeyen et al., 2014; Oi et al., 2015). Indeed, sex-based differences in the cuticular chemical profiles of males and females are the basis for short-range mate recognition for many solitary insects (Tregenza and Wedell, 1997; Ferveur and Cobb, 2010; Ginzel, 2010). In contrast, sex-based differences in cuticular profiles have rarely been investigated in ants, and to date have only been reported for one *Odontomachus* and one *Diacamma* species, both of which belong to the basally derived ant subfamily Ponerinae (Cuvillier-Hot et al., 2001; Smith et al., 2014). Our results suggest that these sex-based differences are the most deeply conserved cuticular chemical patterns within *Odontomachus*. If these patterns are derived from signals from a solitary insect ancestral condition, in the evolutionary history of eusocial Hymenoptera, selection on females may have driven the divergence in hydrocarbon profiles among species that would be required for species-level mate recognition. Males might have been the first receivers of signals differentiating fertile from non-fertile females, which in turn may have driven the diversification of such signals. However, testing this hypothesis will require further investigation into male-specific signals in other social Hymenoptera, phylogenetic analysis of such signals, and a detailed knowledge of the reproductive biology of senders and receivers within each species.

In conclusion, our comparison of queen fertility signals among four *Odontomachus* species suggests that even within genera, these signals can be widely divergent. In contrast, male-specific hydrocarbon patterns appear to be more conserved within the genus, with species being differentiated by different patterns of isomers of the same chain lengths. Further comparative studies within genera will help to elucidate how these important eusocial signals arose and evolved, and investigations into sex-based cuticular chemical differences may provide insights into the evolutionary history of chemical signaling in social insects in general.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
All authors conceived of the study and drafted and revised the manuscript. A.A.S. collected and analyzed the data. J.G.M. collected and analyzed chemical data.

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Supplementary information
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References
Ayasse, M., Paxton, R. J. and Tengö, J. (2001). Mating behavior and chemical communication in the order Hymenoptera. *Annu. Rev. Entomol.* **46**, 31-78.
Blomquist, G. J. and Bagnères, A. G. (2010). *Insect Hydrocarbons: Biology, Biochemistry, and Chemical Ecology*. Cambridge: Cambridge University Press.
Bonavita-Cougourdan, A. and Clement, J. (1994). Complexité du message chimique cuticulaire chez les fourmis: Le modéle *Camponotus vagus* (Scop.) (Hymenoptera, Formicidae). *Memorbilia Zool.* **48**, 23-37.
Brunner, E., Kroiss, J., Trindl, A. and Heinze, J. (2011). Queen pheromones in *Temnothorax* ants: control or honest signal? *BMC Evol. Biol.* **11**, 55.
Buckner, J. S. (2010). Oxygenated derivatives of hydrocarbons. In *Insect Hydrocarbons: Biology, Biochemistry, and Chemical Ecology* (ed. G. J. Blomquist and A. G. Bagnères), pp. 187-203. Cambridge: Cambridge University Press.
Campos, M. C. G., Campos, M. L. G., Turatti, I. C. and Nascimento, F. S. (2012). Cuticular hydrocarbon variation of castes and sex in the weaver ant *Camponotus textor* (Hymenoptera: Formicidae). *Sociobiology* **59**, 1025-1036.
Carlson, D. A., Bernier, U. R. and Sutton, B. D. (1998). Elution patterns from capillary GC for methyl-branched alkanes. *J. Chem. Ecol.* **24**, 1845-1865.
Cuvillier-Hot, V., Cobb, M., Malosse, C. and Peeters, C. (2001). Sex, age and ovarian activity affect cuticular hydrocarbons in *Diacamma ceylonense*, a queenless ant. *J. Insect Physiol.* **47**, 485-493.
Cvacka, J., Jiros, P., Sobotnik, J., Hanus, R. and Svatos, A. (2006). Analysis of insect cuticular hydrocarbons using matrix-assisted laser desorption/ionization mass spectrometry. *J. Chem. Ecol.* **32**, 409-434.
de Biseau, J.-C., Passera, L., Daloze, D. and Aron, S. (2004). Ovarian activity correlates with extreme changes in cuticular hydrocarbon profile in the highly polygynous ant, *Linepithema humile*. *J. Insect Physiol.* **50**, 585-593.
Endler, A., Liebig, J. and Hölldobler, B. (2006). Queen fertility, egg marking and colony size in the ant *Camponotus floridanus*. *Behav. Ecol. Sociobiol.* **59**, 490-499.
Evison, S. E. F., Ferreira, R. S., D'Ettorre, P., Fresneau, D. and Poteaux, C. (2012). Chemical signature and reproductive status in the facultatively polygynous ant *Pachycondyla verenae*. *J. Chem. Ecol.* **38**, 1441-1449.
Ferveur, J. F. and Cobb, M. (2010). Behavioral and evolutionary roles of cuticular hydrocarbons in Diptera. In *Insect Hydrocarbons: Biology, Biochemistry, and Chemical Ecology* (ed. G. J. Blomquist and A. G. Bagnères), pp. 325-343. Cambridge: Cambridge University Press.
Ginzel, M. D. (2010). Hydrocarbons as contact pheromones of longhorned beetles (Coleoptera: Cerambycidae). In *Insect Hydrocarbons: Biology, Biochemistry, and Chemical Ecology* (ed. G. J. Blomquist and A. G. Bagnères), pp. 375-389. Cambridge: Cambridge University Press.
Ginzel, M. D., Millar, J. G. and Hanks, L. M. (2003). (*Z*)-9-Pentacosene – contact sex pheromone of the locust borer, *Megacyllene robiniae*. *Chemoecology* **13**, 135-141.
Ginzel, M. D., Moreira, J. A., Ray, A. M., Millar, J. G. and Hanks, L. M. (2006). (*Z*)-9-Nonacosene – major component of the contact sex pheromone of the beetle, *Megacyllene caryae*. *J. Chem. Ecol.* **32**, 435-451.
Heinze, J., Stengl, B. and Sledge, M. F. (2002). Worker rank, reproductive status and cuticular hydrocarbon signature in the ant, *Pachycondyla cf.* inversa. *Behav. Ecol. Sociobiol.* **52**, 59-65.
Holman, L., Jorgensen, C. G., Nielsen, J. and d'Ettorre, P. (2010). Identification of an ant queen pheromone regulating worker sterility. *Proc. R. Soc. B Biol. Sci.* **277**, 3793-3800.
Holman, L., Lanfear, R. and d'Ettorre, P. (2013). The evolution of queen pheromones in the ant genus *Lasius*. *J. Evol. Biol.* **26**, 1549-1558.
Hoover, S. E. R., Keeling, C. I., Winston, M. L. and Slessor, K. N. (2003). The effect of queen pheromones on worker honey bee ovary development. *Naturwissenschaften* **90**, 477-480.
Keller, L. and Nonacs, P. (1993). The role of queen pheromones in social insects: queen control or queen signal. *Anim. Behav.* **45**, 787-794.
Kellner, K. (2005). Reproductive conflicts and within colony recognition in the ant genus Pachycondyla. *MSc Thesis*, Universität Regensburg.
Kocher, S. D. and Grozinger, C. M. (2011). Cooperation, conflict, and the evolution of queen pheromones. *J. Chem. Ecol.* **37**, 1263-1275.
Kuhbandner, S., Sperling, S., Mori, K. and Ruther, J. (2012). Deciphering the signature of cuticular lipids with contact sex pheromone function in a parasitic wasp. *J. Exp. Biol.* **215**, 2471-2478.
Larabee, F. J. (2015). The evolution and functional morphology of trap-jaw ants. *PhD dissertation*, University of Illinois at Urbana-Champaign.
Liebig, J. (2010). Hydrocarbon profiles indicate fertility and dominance status in ant, bee, and wasp colonies. In *Insect Hydrocarbons: Biology, Biochemistry, and Chemical Ecology* (ed. G. J. Blomquist and A. G. Bagnères), pp. 254-281. Cambridge: Cambridge University Press.
Liebig, J., Eliyahu, D. and Brent, C. S. (2009). Cuticular hydrocarbon profiles indicate reproductive status in the termite *Zootermopsis nevadensis*. *Behav. Ecol. Sociobiol.* **63**, 1799-1807.
MacGown, J. A., Boudinot, B., Deyrup, M. and Sorger, D. M. (2014). A review of the nearctic *Odontomachus* (Hymenoptera: Formicidae: Ponerinae) with a treatment of the males. *Zootaxa* **3802**, 515-552.

Martin, S. and Drijfhout, F. (2009). A review of ant cuticular hydrocarbons. *J. Chem. Ecol.* **35**, 1151-1161.
Matsuura, K., Himuro, C., Yokoi, T., Yamamoto, Y., Vargo, E. L. and Keller, L. (2010). Identification of a pheromone regulating caste differentiation in termites. *Proc. Natl. Acad. Sci. USA* **107**, 12963-12968.
Medeiros, F. N. S., Lopes, L. E., Moutinho, P. R. S., Oliveira, P. S. and Hölldobler, B. (1992). Functional polygyny, agonistic interactions and reproductive dominance in the neotropical ant *Odontomachus chelifer* (Hymenoptera, Formicidae, Ponerinae). *Ethology* **91**, 134-146.
Oi, C. A., van Zweden, J. S., Oliveira, R. C., Van Oystaeyen, A., Nascimento, F. S. and Wenseleers, T. (2015). The origin and evolution of social insect queen pheromones: novel hypotheses and outstanding problems. *BioEssays* **37**, 808-821.
Paulmier, I., Bagnères, A.-G., Afonso, C. M. M., Dusticier, G., Rivière, G. and Clément, J.-L. (1999). Alkenes as a sexual pheromone in the alfalfa leaf-cutter bee *Megachile rotundata*. *J. Chem. Ecol.* **25**, 471-490.
Peso, M., Elgar, M. A. and Barron, A. B. (2015). Pheromonal control: reconciling physiological mechanism with signalling theory. *Biol. Rev.* **90**, 542-559.
Powell, S. and Tschinkel, W. R. (1999). Ritualized conflict in *Odontomachus brunneus* and the generation of interaction-based task allocation: a new organizational mechanism in ants. *Anim. Behav.* **58**, 965-972.
Schmidt, C. A. (2009). Molecular phylogenetics and taxonomic revision of ponerine ants. *PhD dissertation*, University of Arizona, Tuscon, AZ.
Schulz, S., Beccaloni, G., Nishida, R., Roisin, Y., Vane-Wright, R. I. and McNeil, J. N. (1998). 2,5-Dialkyltetrahydrofurans, common components of the cuticular lipids of Lepidoptera. *Zeit. Naturforsch.* **53**, 107-116.
Slessor, K. N., Kaminski, L.-A., King, G. G. S., Borden, J. H. and Winston, M. L. (1988). Semiochemical basis of the retinue response to queen honey bees. *Nature* **332**, 354-356.
Smith, A. A., Hölldobler, B. and Liebig, J. (2009). Cuticular hydrocarbons reliably identify cheaters and allow enforcement of altruism in a social insect. *Curr. Biol.* **19**, 78-81.
Smith, A. A., Millar, J. G., Hanks, L. M. and Suarez, A. V. (2012). Experimental evidence that workers recognize reproductives through cuticular hydrocarbons in the ant *Odontomachus brunneus*. *Behav. Ecol. Sociobiol.* **66**, 1267-1276.
Smith, A. A., Millar, J. G., Hanks, L. M. and Suarez, A. V. (2013). A conserved fertility signal despite population variation in the cuticular chemical profile of the trap-jaw ant *Odontomachus brunneus*. *J. Exp. Biol.* **216**, 3917-3924.
Smith, A. A., Vanderpool, W., Millar, J. G., Hanks, L. M. and Suarez, A. V. (2014). Conserved male-specific cuticular hydrocarbon patterns in the trap-jaw ant *Odontomachus brunneus*. *Chemoecology* **24**, 29-34.
Smith, A. A., Millar, J. G. and Suarez, A. V. (2015). A social insect fertility signal is dependent on chemical context. *Biol. Lett.* **11**, 20140947.
Spagna, J. C., Vakis, A. I., Schmidt, C. A., Patek, S. N., Zhang, X., Tsutsui, N. D. and Suarez, A. V. (2008). Phylogeny, scaling, and the generation of extreme forces in trap-jaw ants. *J. Exp. Biol.* **211**, 2358-2368.
Sramkova, A., Schulz, C., Twele, R., Francke, W. and Ayasse, M. (2008). Fertility signals in the bumblebee *Bombus terrestris* (Hymenoptera: Apidae). *Naturwissenschaften* **95**, 515-522.
Takabayashi, J. and Takahashi, S. (1986a). Effect of kairomones in the host searching behavior of *Apanteles kariyai* Watanabe (Hymenoptera: Braconidae) and a parasitoid of the common armyworm *Pseudaletia separate* Walker (Lepidoptera: Noctuidae). II: Isolation and identification of arrestants produced by the host larvae. *Appl. Ent. Zool.* **21**, 114-118.
Takabayashi, J. and Takahashi, S. (1986b). Effect of kairomones in the host searching behavior of *Apanteles kariyai* Watanabe (Hymenoptera: Braconidae) and a parasitoid of the common armyworm *Pseudaletia separata* Walker (Lepidoptera: Noctuidae). III: synthesis and bioassay of arrestants and related compounds. *Appl. Ent. Zool.* **21**, 519-524.
Tregenza, T. and Wedell, N. (1997). Definitive evidence for cuticular pheromones in a cricket. *Anim. Behav.* **54**, 979-984.
Van Oystaeyen, A., Oliveira, R. C., Holman, L., van Zweden, J. S., Romero, C., Oi, C. A., d'Ettorre, P., Khalesi, M., Billen, J., Wackers, F. et al. (2014). Conserved class of queen pheromones stops social insect workers from reproducing. *Science* **343**, 287-290.
van Zweden, J. S. and d'Ettorre, P. (2010). Nestmate recognition in social insects and the role of hydrocarbons. In *Insect Hydrocarbons: Biology, Biochemistry, and Chemical Ecology* (ed. G. J. Blomquist and A. G. Bagnères), pp. 222-243. Cambridge: Cambridge University Press.
van Zweden, J. S., Bonckaert, W., Wenseleers, T. and d'Ettorre, P. (2014). Queen signaling in social wasps. *Evolution* **68**, 976-986.
Yamamoto, Y. and Matsuura, K. (2011). Queen pheromone regulates egg production in a termite. *Biol. Lett.* **7**, 727-729.
Yew, J. Y., Dreisewerd, K., de Oliveira, C. C. and Etges, W. J. (2011). Male-specific transfer and fine scale spatial differences of newly identified cuticular hydrocarbons and triacylglycerides in a *Drosophila* species pair. *PLoS ONE* **6**, e16898.

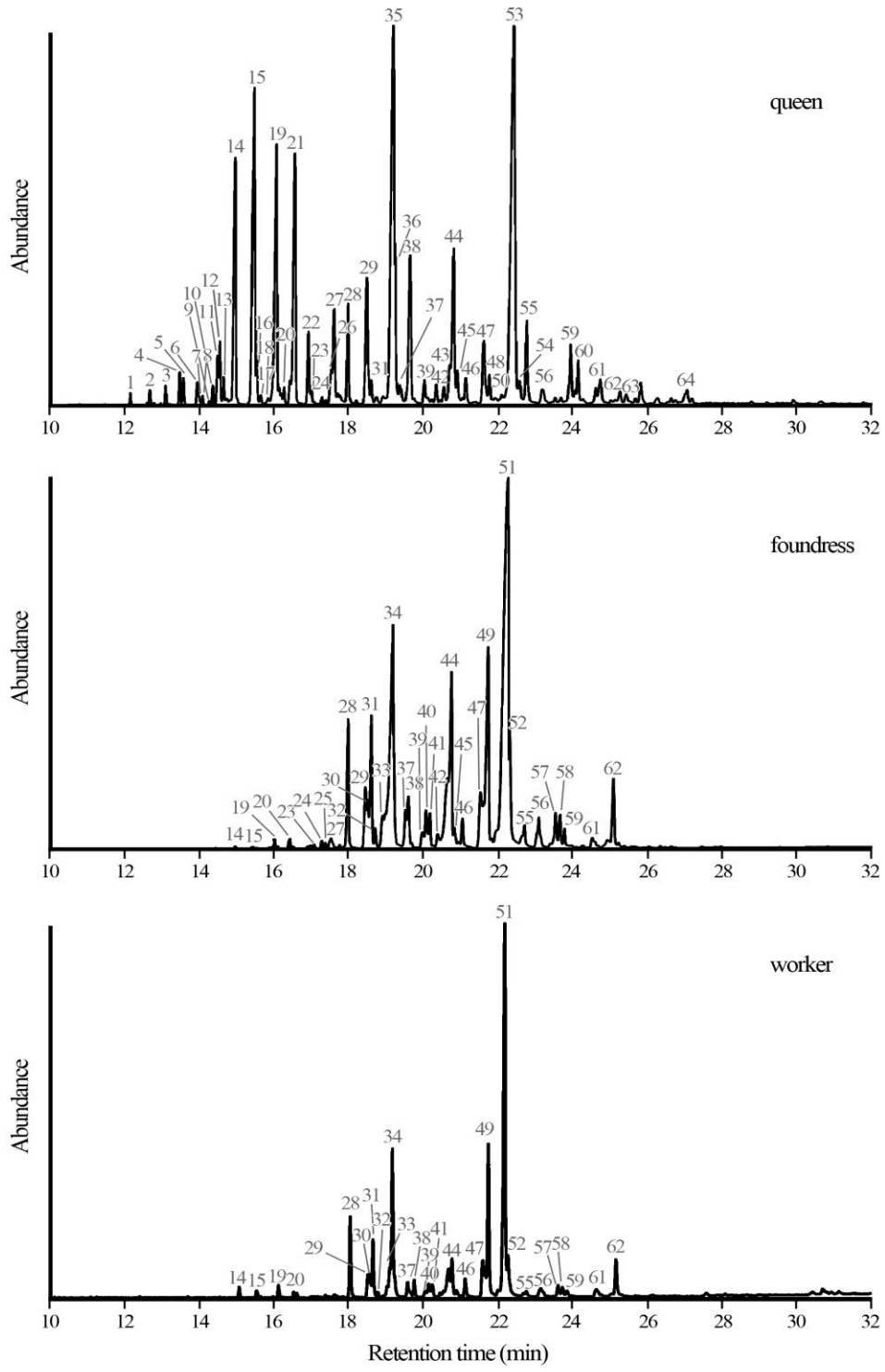

Figure S1: representative chromatograms of cuticular extracts of an *O. ruginodis* queen, foundress, and worker. Labeled peaks correspond to compound identifications in table S1.

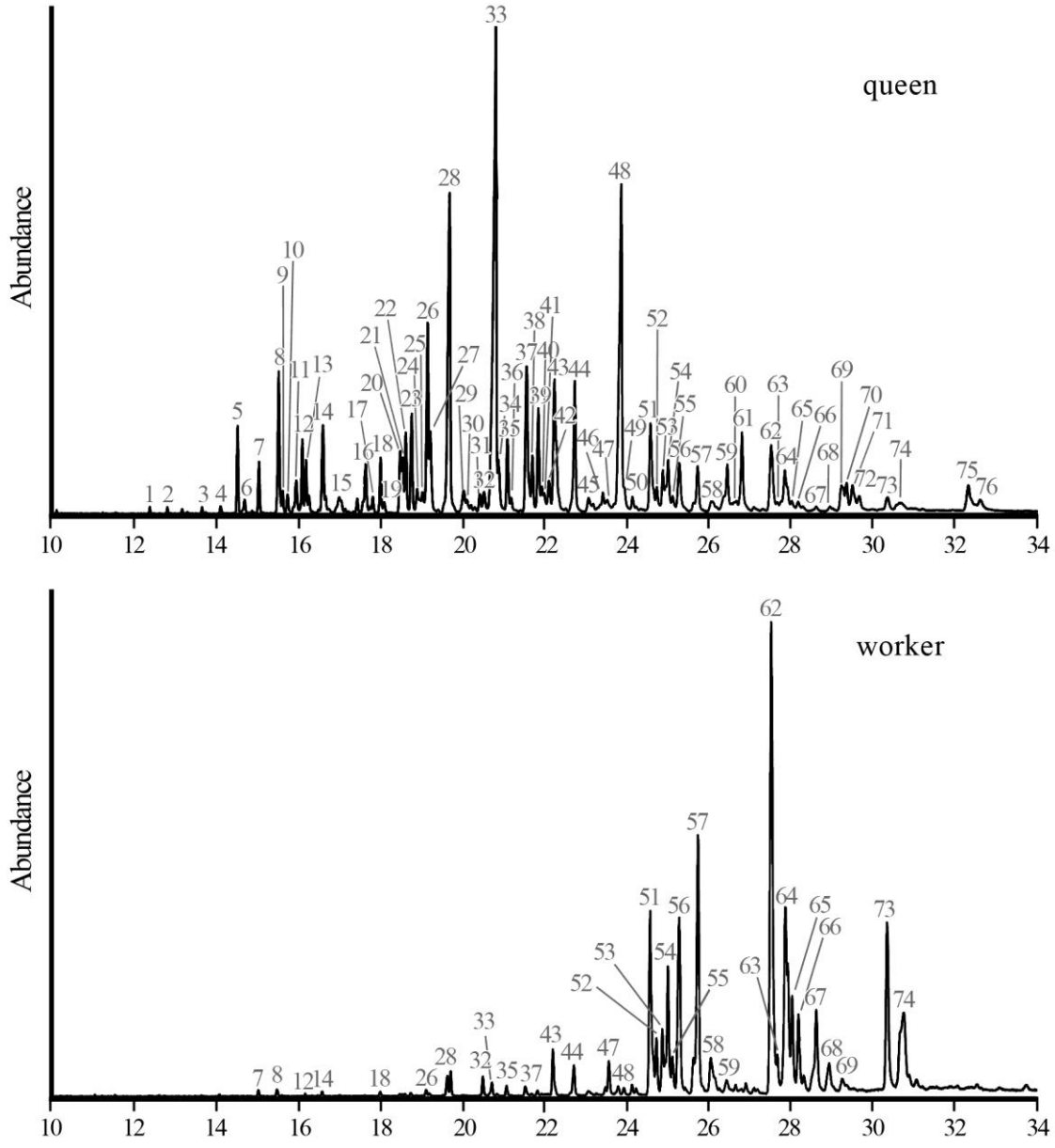

Figure S2: representative chromatograms of cuticular extracts of an *O. relictus* queen and worker. Numbers correspond to table S2.

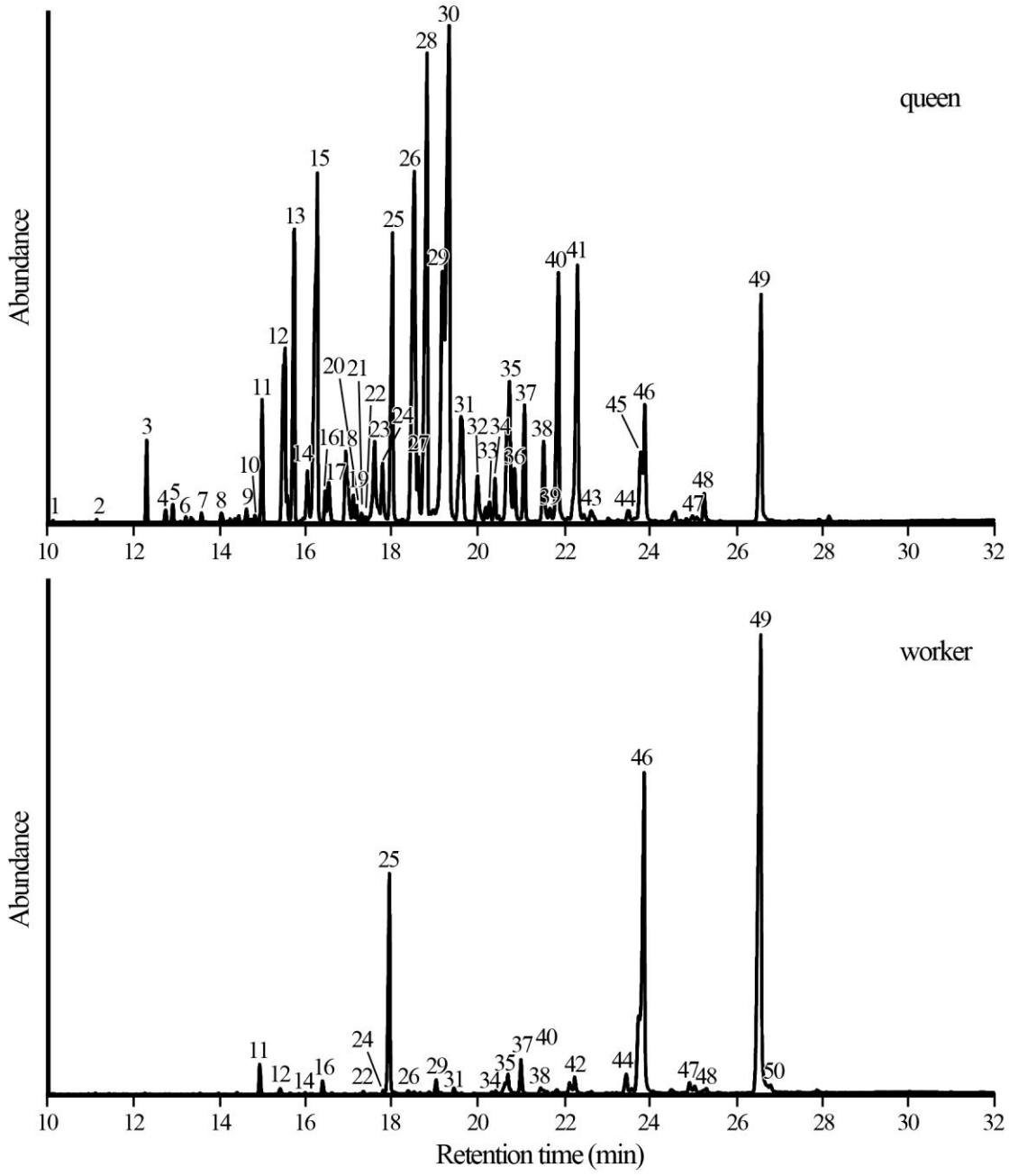

Figure S3: representative chromatograms of cuticular extracts of an *O. haematodus* queen and worker. Numbers correspond to table S3.

Table S1. Compounds in the cuticular extracts of an *O. ruginodis* queens, foundresses, and workers

Number	Identification	RI	Queens	Foundresses	Workers
1	Tricosane	2300	0.18 (0.07, 0.43)	0	0
2	7-Methyltricosane	2339	0.12 (0, 0.36)	0.03 (0, 0.18)	0
3	3-Methyltricosane	2371	0.25 (0.12, 0.44)	0	0
4	Tetracosane	2400	0.45 (0.31, 0.8)	0.1 (0, 0.59)	0
5	3,7-Dimethyltricosane	2406	0.4 (0.22, 0.61)	0	0
6	11-Methyltetracosane	2432	0.31 (0.08, 0.61)	0	0
7	8-Methyltetracosane	2435	0.09 (0, 0.19)	0	0
8	6-Methyltetracosane	2441	0.1 (0, 0.22)	0	0
9	4-Methyltetracosane	2453	0.3 (0.05, 0.67)	0.02 (0, 0.15)	0
10	2-Methyltetracosane	2460	1 (0.14, 3.06)	0	0
11	x,y-Pentacosadiene	2468	1.43 (0.78, 2.71)	0	0.14 (0, 0.85)
12	x-Pentacosene	2472	0.82 (0.44, 2.02)	0	0.02 (0, 0.16)
13	x-Pentacosene	2479	0.27 (0, 1.24)	0	0
14	Pentacosane	2500	7.01 (5.5, 9.15)	0.07 (0, 0.32)	0.49 (0.2, 0.72)
15	11-Methylpentacosane	2535	7.5 (4.29, 11.01)	0.25 (0, 0.73)	0.26 (0, 0.5)
16	7-Methylpentacosane	2539	0.58 (0.41, 0.8)	0	0
17	5-Methylpentacosane	2546	0.15 (0, 0.21)	0	0
18	4-Methylpentacosane	2560	0.18 (0, 0.27)	0.03 (0, 0.18)	0
19	3-Methylpentacosane	2576	6.74 (5.62, 8.18)	0.28 (0.16, 0.56)	0.26 (0, 0.71)
20	Hexacosane	2600	0.53 (0.39, 0.66)	0.14 (0, 0.23)	0.26 (0, 0.56)
21	3,15-Dimethylpentacosane	2608	5.34 (3.42, 7.07)	0.03 (0, 0.16)	0
22	11-Methylhexacosane	2632	1.06 (0.6, 1.47)	0.03 (0, 0.14)	0
23	10-, 9-, 8-Methylhexacosane	2636	0.22 (0.14, 0.31)	0.01 (0, 0.08)	0
24	4-Methylhexacosane	2655	0.11 (0, 0.17)	0.08 (0, 0.19)	0
25	2-Methylhexacosane	2661	0.07 (0, 0.21)	0.19 (0, 0.43)	0
26	x,y-Heptacosadiene	2671	1.3 (0, 2.46)	0	0
27	x-Heptacosene	2676	1.67 (0.59, 2.88)	0.26 (0, 0.51)	0.04 (0, 0.33)
28	Heptacosane	2700	2.55 (1.75, 3.42)	3.97 (2.97, 4.94)	7.89 (4.2, 10.72)
29	11- & 13-Methylheptacosane	2732	2.58 (0.33, 3.38)	1.61 (0.86, 2.66)	1.98 (1.07, 3.77)
30	9-Methylheptacosane	2735	0	1.3 (0.96, 2)	1.49 (0.82, 2.42)
31	7-Methylheptacosane	2739	0.43 (0.25, 0.63)	3.22 (2.33, 3.97)	3 (1.98, 4.44)
32	5-Methylheptacosane	2748	0.15 (0, 0.21)	0.46 (0.38, 0.52)	0.21 (0, 0.39)
33	7,15-Dimethylheptacosane	2769	0	0.37 (0, 2.19)	1.45 (0, 2.31)
34	3-Methylheptacosane	2774	0	12.84 (7.7, 15.65)	12.1 (8.83, 15.68)
35	2-(Oct-7-enyl)-5-pentadecyltetrahydrofuran	2778	14.72 (7.64, 20.52)	0	0
36	2-Octyl-5-pentadecyltetrahydrofuran	2781	1.48 (0.38, 3.47)	0	0
37	Octacosane	2800	0	1.07 (0.72, 1.39)	1.74 (1.17, 2.09)
38	3,15-Dimethylheptacosane	2806	3.44 (2.73, 4.23)	1.16 (0.31, 2.11)	0.48 (0, 1.75)
39	13-Methyloctacosane	2830	0.47 (0.26, 0.62)	0.49 (0.38, 0.66)	0.54 (0.4, 0.74)
40	8-Methyloctacosane	2836	0.04 (0, 0.14)	1.38 (1.17, 1.61)	1.34 (1.12, 1.6)
41	6-Methyloctacosane	2843	0.03 (0, 0.13)	1.05 (0.93, 1.25)	1.02 (0.83, 1.16)
42	4-Methyloctacosane	2863	0.3 (0.09, 0.55)	0.39 (0.31, 0.58)	0.63 (0, 1.79)

43	2-(Oct-7-enyl-5-hexadecyltetrahydrofuran	2873	1.13 (0.82, 1.4)	0	0
44	(*Z*)-9-Nonacosene	2880	2.21 (0.41, 4.71)	4.52 (3.87, 4.98)	3.15 (1.17, 7.5)
45	x-Nonacosene	2886	0.47 (0, 0.84)	4.11 (2.43, 6.4)	0.35 (0, 1.94)
46	Nonacosane	2900	0.74 (0.32, 1.13)	0.97 (0.69, 1.45)	2.25 (1.3, 4.5)
47	11-, 13-, 15-Methylnonacosane	2931	1.38 (0.66, 1.9)	2.69 (2.28, 3.76)	4.42 (2.81, 7.75)
48	7-Methylnonacosane	2940	0.63 (0.13, 1.28)	1.54 (0.63, 4.45)	0
49	5-Methylnonacosane	2950	0.08 (0, 0.26)	9.6 (3.97, 13.55)	12.67 (8.97, 18.15)
50	2-(Oct-7-enyl)-5-heptadecyltetrahydrofuran	2961	0.71 (0, 3.71)	0	0
51	7, 13-Dimethylnonacosane	2969	0	34.85 (31.99, 37.9)	32.04 (24.96, 36.93)
52	3-Methylnonacosane	2974	0	3.4 (2.81, 4.49)	3.5 (2.4, 4.61)
53	2-(Oct-7-enyl)-; 2-Octyl-5-heptadecyltetrahydrofuran	2982	21.39 (15.88, 27.79)	0	0
54	x-Triacontene	2992	0.59 (0.37, 1.01)	0	0
55	3,15-Dimethylnonacosane	3004	2.04 (0.91, 2.96)	0.24 (0, 0.42)	0.04 (0, 0.3)
56	12-, 10-Methyltriacontane	3030	0.34 (0, 0.57)	1.17 (0.84, 1.41)	1.01 (0.04, 2.14)
57	x, y-Dimethyltriacontane	3062	0.14 (0, 0.41)	1.18 (0.99, 1.37)	1.03 (0.5, 1.85)
58	x, y-Dimethyltriacontane	3079	0.58 (0.21, 1.5)	1.33 (0.97, 1.69)	0.78 (0.47, 1.2)
59	x-Hentriacontene	3086	0.22 (0, 0.52)	0.36 (0.18, 0.56)	0.35 (0, 1.94)
60	x-Hentriacontene	3092	1.32 (0.36, 1.86)	0.36 (0.18, 0.56)	0
61	15-, 13-, 11-Methylhentriacontane	3130	0.55 (0, 1.02)	0.24 (0, 0.42)	0.89 (0, 2.23)
62	7,13-Dimethylhentriacontane	3163	0.38 (0, 0.61)	2.97 (1.65, 6.06)	2.19 (0.6, 3.36)
63	2-Octyl-5-nonadecyltetrahydrofuran	3174	0.28 (0, 0.45)	0	0
64	x-Tritriacontene	3278	0.3 (0, 0.57)	0	0

Numbers and identifications correspond to Fig. S1. Relative compound abundances are given as averages, with minimum and maximum values in brackets. RI = Kovat's retention index.

Table S2. Compounds in the cuticular extracts of an *O. relictus* queens and workers

Number	Identification	RI	Queens	Workers
1	Tricosane	2300	0.02 (0, 0.08)	0
2	11-Methyltricosane	2333	0.02 (0, 0.08)	0
3	Tetracosane	2400	0.02 (0, 0.07)	0
4	12-, 11-Methyltetracosane	2433	0.04 (0, 0.11)	0
5	2-Methyltetracosane	2462	0.38 (0, 1.23)	0
6	x-Pentacosene	2475	0.09 (0, 0.26)	0
7	Pentacosane	2500	0.26 (0, 0.76)	0.33 (0, 1.28)
8	13-, 11-Methylpentacosane	2533	1.06 (0.04, 2.46)	0.32 (0, 1.22)
9	7-Methylpentacosane	2540	0.43 (0.24, 0.65)	0
10	5-Methylpentacosane	2548	0.17 (0, 0.33)	0
11	2-Methylpentacosane	2562	0.24 (0, 0.72)	0
12	3-Methylpentacosane	2572	0.58 (0.11, 1.18)	0.22 (0, 0.85)
13	5,9-Dimethylpentacosane	2578	0.99 (0, 1.73)	0
14	3,7-Dimethylpentacosane	2607	2.16 (1.3, 3.58)	0.18 (0, 0.58)
15	13-, 12-, 11-, 10-, 9-Methylhexacosane	2633	0.39 (0.28, 0.57)	0
16	x-Heptacosene	2676	1.16 (0.65, 1.83)	0
17	4,8-Dimethylhexacosane	2687	0.43 (0.27, 0.61)	0
18	Heptacosane	2700	0.68 (0.17, 0.99)	0
19	3,7-; 3,9-Dimethylhexacosane	2706	0.37 (0.16, 1.09)	0
20	13-, 11-Methylheptacosane	2731	1.19 (0, 1.84)	0
21	9-Methylheptacosane	2735	1.02 (0.76, 1.4)	0
22	7-Methylheptacosane	2740	1.54 (1.13, 2.05)	0
23	5-Methylheptacosane	2749	1.99 (1.3, 2.55)	0
24	11,15-Dimethylheptacosane	2758	0.55 (0.44, 0.67)	0
25	7,11-Dimethylheptacosane	2767	0.5 (0.39, 0.58)	0
26	3-Methylheptacosane	2775	3.3 (2.47, 4.03)	0.53 (0, 1.69)
27	5,9-Dimethylheptacosane	2779	1.95 (1.45, 2.41)	0
28	3,9-; 3,7-Dimethylheptacosane	2809	10.02 (7.65, 12.79)	0.78 (0, 1.36)
29	14-, 13-, 12-, 11-, 10-Methyloctacosane	2831	0.72 (0.54, 0.89)	0
30	8-Methyloctacosane	2836	0.22 (0, 0.31)	0
31	4-Methyloctacosane	2857	0.44 (0.28, 0.57)	0
32	2-Methyloctacosane	2863	0.31 (0.15, 0.53)	0.08 (0, 0.51)
33	(*Z*)-9-Nonacosene	2882	18.34 (11.77, 23.78)	1.05 (0.4, 2.27)
34	x-Nonacosene	2886	1.14 (0.79, 1.29)	0
35	Nonacosane	2900	1.37 (1, 1.66)	0.09 (0, 0.53)
36	3,7-Dimethyloctacosane	2906	0.3 (0.25, 0.38)	0
37	15-, 13-Methylnonacosane	2931	2.99 (1.92, 4.06)	0.22 (0, 0.68)
38	7-Methylnonacosane	2940	0.87 (0.72, 1.09)	0.07 (0, 0.45)
39	5-Methylnonacosane	2949	2.25 (1.65, 2.66)	0
40	13,17-; 11,15-Dimethylnonacosane	2955	0.32 (0.19, 0.49)	0
41	9,13-Dimethylnonacosane	2959	0.3 (0.23, 0.42)	0.58 (0, 1.97)
42	7,11-; 7,13-Dimethylnonacosane	2967	0.41 (0.23, 0.76)	0
43	3-Methylnonacosane	2975	2.94 (1.94, 4.37)	1.5 (0.95, 2.18)

44	3,7-; 3,9-Dimethylnonacosane	3008	2.99 (2.09, 4.05)	0.69 (0, 1.34)
45	15-, 14-, 13-, 12-, 11-Methyltriacontane	3030	0.45 (0.24, 0.72)	0
46	x,y-Hentriacontadiene	3053	0.93 (0.55, 1.3)	0
47	x,y-Hentriacontadiene	3059	0.46 (0.3, 0.54)	0.28 (0, 0.96)
48	x-Hentriacontene	3082	9.79 (7.41, 12.19)	0.44 (0, 1.41)
49	x-Hentriacontene	3088	0.46 (0, 0.81)	0
50	Hentriacontane	3100	0.33 (0.18, 0.43)	0.07 (0, 0.42)
51	15-, 13-Methylhentriacontane	3130	1.9 (0.76, 3.7)	6.79 (5.38, 8.15)
52	7-Methylhentriacontane	3140	0.41 (0.17, 0.92)	1.95 (1.65, 2.19)
53	5-Methylhentriacontane	3150	1 (0.84, 1.19)	2.26 (1.57, 2.89)
54	9,13-Dimethylhentriancontane	3158	0.88 (0.14, 2.52)	4.47 (3.44, 5.24)
55	7,11-; 7,13-Dimethylhentriacontane	3166	0.18 (0, 0.65)	1.16 (0.9, 1.44)
56	5,9-; 5,11-Dimethylhentriacontane	3177	1.24 (0.63, 2.73)	7.12 (5.74, 8.17)
57	3,7-; 3,9-Dimethylhentriacontane	3207	0.91 (0.27, 2.21)	8.72 (6.75, 10.11)
58	16-, 15-, 14-, 13-, 12-, 11-, 10-Methyldotriacontane	3230	0.43 (0.14, 0.98)	2.15 (1.87, 2.37)
59	x-,y-Tritriacontadiene	3256	1.52 (1.17, 2.38)	0.1 (0, 0.59)
60	x-Tritriacontene	3271	0.42 (0.28, 0.61)	0
61	x-Tritriacontene; x-,y-Tritriacontadiene	3280	2.21 (1.34, 2.91)	0
62	17-, 15-, 13-, 11-Methyltritriacontane	3329	1.48 (0.53, 2.87)	22.16 (19.1, 25.99)
63	7-Methyltritriacontane	3339	0.13 (0, 0.25)	1.77 (1.2, 2.4)
64	11,15-Dimethyltritriacontane	3352	1.12 (0.41, 2.56)	9.55 (6.04, 11.91)
65	7,11-Dimethyltritriacontane	3363	0.11 (0, 0.45)	3.16 (2.76, 3.42)
66	5,9-Dimethyltritriacontane	3374	0.16 (0, 0.35)	2.7 (2.18, 3.33)
67	3,7-; 3,9-Dimethyltritriacontane	3405	0.05 (0, 0.22)	3.31 (2.84, 4.2)
68	unknown	3427	0.1 (0, 0.22)	1.83 (1.25, 3.27)
69	x,y-Pentatriacontadiene	3446	0.62 (0, 0.95)	0.12 (0, 0.74)
70	x,y-Pentatriacontadiene; x-Pentatriacontene	3454	0.8 (0.6, 1.19)	0
71	x-Pentatriacontene	3464	1.05 (0.92, 1.17)	0
72	x-Pentatriacontene; x,y-Pentatriacontadiene	3475	0.59 (0.32, 0.83)	0
73	17-, 15-, 13-Methylpentatriacontane	3522	0.31 (0.11, 0.46)	7.69 (6.42, 9.29)
74	11,13-; 11,23-Dimethylpentatriacontane	3545	0.18 (0, 0.46)	5.54 (4.59, 6.88)
75	x,y-Heptatriacontadiene	3657	1.46 (1.18, 1.78)	0
76	x-Heptatriacontene	3676	0.83 (0.53, 1.63)	0

Numbers and identifications correspond to Fig. S2. Relative compound abundances are given as averages, with minimum and maximum values in brackets. RI = Kovat's retention index.

Table S3. Compounds in the cuticular extracts of an *O. haematodus* queens and workers.

Number	Identification	RI	Queens	Workers
1	Heneicosane	2100	0.12 (0, 0.39)	0
2	Docosane	2200	0.05 (0, 0.12)	0
3	Tricosane	2300	1.16 (0.4, 1.93)	0.01 (0, 0.17)
4	11-, 13-Methyltricosane	2334	0.19 (0, 0.34)	0
5	5-Methyltricosane	2348	0.26 (0.07, 0.4)	0
6	3-Methyltricosane	2372	0.1 (0, 0.23)	0
7	Tetracosane	2400	0.13 (0.06, 0.26)	0
8	11-Methyltetracosane	2433	0.15 (0, 0.23)	0.02 (0, 0.19)
9	x-Pentacosene	2475	0.34 (0, 2.04)	0
10	x,y-Dimethyltetracosane	2489	0.12 (0, 0.23)	0
11	Pentacosane	2500	2.09 (0.91, 4.89)	1.37 (0.61, 2.87)
12	13-, 11-, 9-Methylpentacosane	2535	4.18 (1.52, 6.26)	0.82 (0, 1.78)
13	5-Methylpentacosane	2549	4.96 (2.52, 8.02)	0.51 (0, 1.79)
14	3-Methylpentacosane	2572	0.85 (0.06, 1.46)	0.09 (0, 0.52)
15	x,y-Dimethylpentacosane	2585	8.65 (4.41, 12.2)	0.89 (0, 3.18)
16	Hexacosane	2600	0.47 (0.23, 1.03)	0.97 (0, 1.86)
17	3,9-; 3,7-Dimethylpentacosane	2605	0.7 (0.53, 1)	0
18	13-, 11-Methylhexacosane	2631	1.45 (1.05, 1.95)	0
19	6-Methylhexacosane	2643	0.34 (0.18, 0.53)	0
20	5-Methylhexacosane	2648	0.18 (0.14, 0.21)	0
21	2-Methylhexacosane	2655	0.09 (0, 0.14)	0.08 (0, 0.54)
22	x,y-Dimethylhexacosane	2674	0.51 (0, 3.06)	0
23	x-Hexacosene	2674	1.32 (0, 3.58)	0.07 (0, 0.65)
24	x,y-Dimethylhexacosane	2686	0.68 (0, 1.04)	0.28 (0, 0.68)
25	Heptacosane	2700	5.66 (3.03, 11.05)	13.76 (10.96, 19.29)
26	13-, 11-Methylheptacosane	2730	8 (4.75, 12.13)	1.21 (0.18, 2.82)
27	7-Methylheptacosane	2737	0.65 (0, 0.99)	0.06 (0, 0.3)
28	5-Methylheptacosane	2747	7.83 (5.41, 9.45)	0.96 (0, 2.68)
29	3-Methylheptacosane; 2-Octyl-5-pentadecyltetrahydrofuran	2770	4.19 (0, 6.46)	3.04 (0.67, 6.6)
30	5,19-Dimethylheptacosane	2778	12.58 (10.08, 16.29)	1.42 (0, 4.19)
31	Octacosane	2800	2.71 (1.37, 3.24)	0.89 (0.33, 1.56)
32	11-, 12-, 13-, 14-Methyloctacosane	2827	0.76 (0.42, 1.04)	0
33	5-Methyloctacosane	2854	0.34 (0.16, 0.72)	0
34	4-Methyloctacosane	2859	0.4 (0, 0.67)	0.74 (0, 1.95)
35	(*Z*)-9-Nonacosene	2876	3.25 (1.38, 6.51)	2.4 (0, 6.91)
36	x,y-Dimethyloctacosane	2885	0.84 (0.52, 1.23)	1.16 (0, 3.25)
37	Nonacosane	2900	2.3 (1.15, 5.37)	2.74 (1.57, 4.73)
38	15-, 13-, 11-Methylnonacosane	2928	1.22 (0.51, 1.95)	1.01 (0.52, 1.48)
39	7-Methylnonacosane	2938	0.2 (0, 0.31)	0.02 (0, 0.22)
40	5-Methylnonacosane	2947	4.2 (1.98, 6.37)	0.88 (0.24, 2.08)
41	x,y-Dimethylnonacosane	2975	4.94 (3.52, 6.28)	0
42	x-Triacontene	2978	0 (0, 0)	1.93 (0.57, 5.22)
43	Triacontane	3000	0.27 (0, 0.53)	0.04 (0, 0.53)

44	x,y-Hentriacontadiene	3053	0.32 (0.1, 0.77)	2.66 (0.99, 8.59)
45	x,y-Hentriacontadiene	3071	1.72 (0.58, 3.33)	0
46	x-Hentriacontene	3076	2.82 (1.15, 6.52)	28.93 (24.39, 38.5)
47	x,y-Dotriacontadiene	3145	0.19 (0, 0.84)	2.17 (0.42, 5.49)
48	x,y-Dotriacontadiene	3162	0.54 (0.23, 1.34)	0.48 (0, 1.1)
49	x,y-Tritriacontadiene	3244	4.96 (1.67, 13.23)	45.92 (35.87, 61.54)
50	x,y-Tritriacontadiene	3262	0.01 (0, 0.16)	0.96 (0, 1.71)

Numbers and identifications correspond to Fig. S3. Relative compound abundances are given as averages, with minimum and maximum values in brackets. RI = Kovat's retention index.