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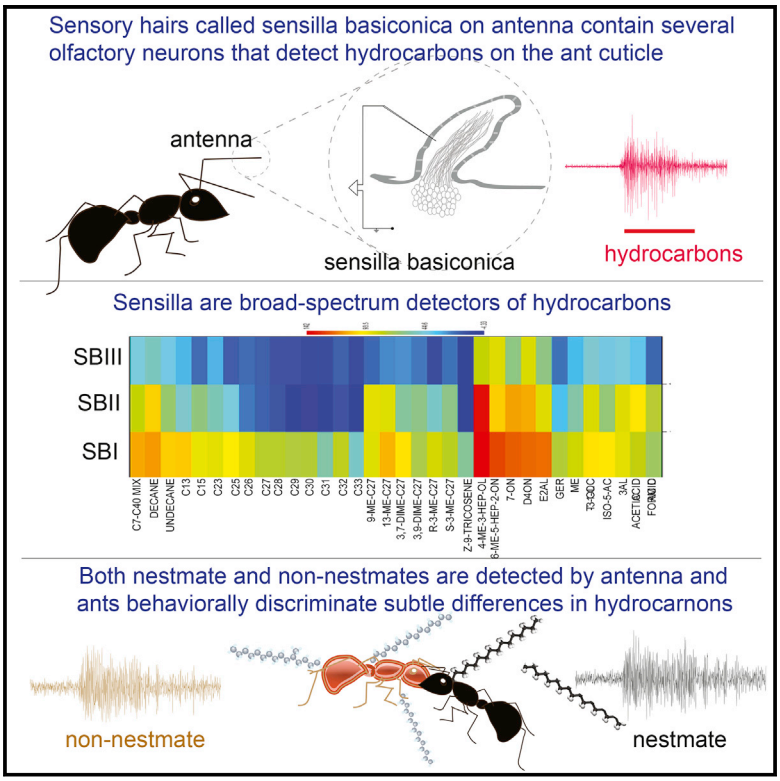
Cuticular Hydrocarbon Pheromones for Social Behavior and Their Coding in the Ant Antenna

Graphical Abstract



Highlights

- Ant antennae are broad-spectrum sensors for cuticular hydrocarbons (CHCs)
- CHCs from various castes and colonies are detected by the antenna
- CHCs that activate the antenna are also sensed behaviorally in discrimination assays
- Ants detect and discriminate *R* and *S* enantiomers of a queen pheromone

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In Brief

Sharma et al. show that ants can detect a number of hydrocarbons present on the cuticle, therefore recognizing different castes such as workers and queens from their own colony as well as different colonies. They also show that ants are able to smell and discriminate minor differences among hydrocarbons.

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Cuticular Hydrocarbon Pheromones for Social Behavior and Their Coding in the Ant Antenna

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SUMMARY

The sophisticated organization of eusocial insect societies is largely based on the regulation of complex behaviors by hydrocarbon pheromones present on the cuticle. We used electrophysiology to investigate the detection of cuticular hydrocarbons (CHCs) by female-specific olfactory sensilla basiconica on the antenna of *Camponotus floridanus* ants through the utilization of one of the largest family of odorant receptors characterized so far in insects. These sensilla, each of which contains multiple olfactory receptor neurons, are differentially sensitive to CHCs and allow them to be classified into three broad groups that collectively detect every hydrocarbon tested, including queen and worker-enriched CHCs. This broad-spectrum sensitivity is conserved in a related species, *Camponotus laevigatus*, allowing these ants to detect CHCs from both nestmates and non-nestmates. Behavioral assays demonstrate that these ants are excellent at discriminating CHCs detected by the antenna, including enantiomers of a candidate queen pheromone that regulates the reproductive division of labor.

INTRODUCTION

Colonies of eusocial insects are characterized by elaborate cooperation among nestmates that is largely mediated by chemical communication. Division of labor between the reproductively specialized morph, the queen, and her helpers, the workers, is mediated by queen pheromones, whereas the discrimination between members of the nest and foreigners is based on cues emitted by workers (Hölldobler and Wilson, 2009). In ants, many of these pheromones and cues are cuticular hydrocarbons (CHCs), several of which are differentially produced by the queen and her worker offspring, who are also females (Holman et al., 2010, 2013; Liebig, 2010; Van Oystaeyen et al., 2014; van Zweden and D'Ettorre, 2010). In the formicine ant *Camponotus floridanus*, a single mated queen produces a pheromone in the form of a characteristic suite of CHCs that signals the queen's fertility to the workers in the nest and prevents them from being reproductively active. Although reproductive division of labor is central to eusociality, and despite progress in sequencing their genomes and understanding epigenetic differences underlying ant castes, less is understood about the precise composition of cuticular pheromones or the cellular and molecular pathways that underlie their recognition (Bonasio et al., 2010, 2012; Simola et al., 2013; Smith et al., 2011a, 2011b; Suen et al., 2011; Wurm et al., 2011).

Ant cuticular extracts have been tested in relatively few studies, where neuronal activity was reported in the antenna and antennal lobes, suggesting that at least one or more chemicals are detected by olfactory receptor neurons (ORNs) (Brandstaetter and Kleineidam, 2011; Brandstaetter et al., 2011). The extracts are complex mixtures of dozens of hydrocarbons of which only three (3,11-dimethyl C_{27}, 3-methyl C_{31}, and cis-9-tricosene) have been tested for electrophysiological responses (D'Ettorre et al., 2004; Holman et al., 2010; Brandstaetter et al., 2010). The methods to study the responses of these low-volatility compounds are challenging, and the precise identities of the cuticular CHCs that are detected by ant ORNs remain open questions.

ORNs are housed in specialized olfactory sensilla on the antenna, representing the primary non-contact detectors of pheromones that convert chemical signals into neuronal activity and convey that information to glomeruli in the antennal lobe (Kelber et al., 2009, 2010; Mysore et al., 2010; Nakanishi et al., 2009). *Camponotus* ants display two types of multiporous sensilla, sensilla basiconica (SBs), which are present only in

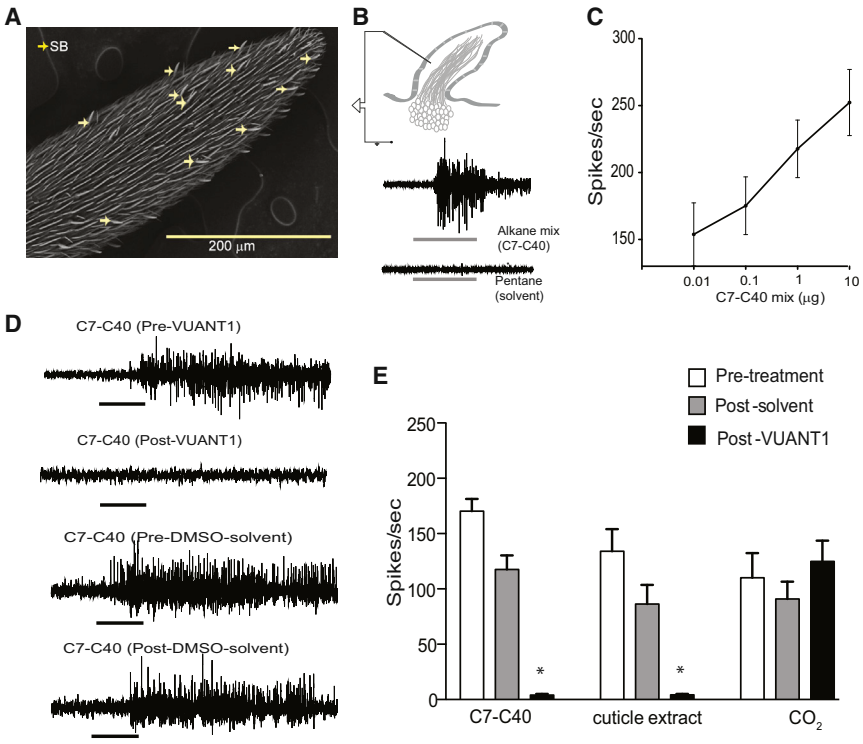

Figure 1. The Antennal Basiconic Sensilla Respond to Hydrocarbon Odorants

(A) Scanning electron micrograph of the last flagellar segment of a *C. floridanus* worker antenna showing olfactory sensilla. Arrows indicate a few representative basiconic sensilla.

(B) Schematic illustrating the SB innervated by numerous ORN dendrites and the typical electrode placement for single-sensillum recordings shown underneath.

(C) Dose responses of SBI sensilla to C7-C40 mix (n = 10 each). Error bars show SEM.

(D) Representative action potential traces of SBs showing activity to a 1-s stimulus of C7-C40 after a 1-s exposure to VUANT1 (Orco antagonist) or solvent (DMSO).

(E) Mean response of a worker SB to C7-C40 mix, *C. floridanus* colony D601 worker cuticular extract, and CO_2 before and after exposure to solvent or VUANT1 (n = 7–13).

females (worker and queen), and sensilla trichodea curvata (STrCs), which are present in both males and females (Figure 1A; Mysore et al., 2010; Nakanishi et al., 2009). The female-specific basiconic sensilla are innervated by the dendrites of a large number (>100) of ORNs (Kelber et al., 2006, 2010; Nakanishi et al., 2009). A comparable number of Odorant receptor (*Or*) gene family members are found to be worker antenna-enriched (Koch et al., 2013; Zhou et al., 2012). Insect antennae express several Ors, each ORN usually expressing a single Or along with an obligate Orco coreceptor, which, together, form a functional receptor. These ORNs and their associated molecular receptors have been proposed to play a central role in detecting the queen CHCs that act as signature cues and are involved in regulating complex worker-specific behaviors and physiological effects such as suppressing ovarian function (Kidokoro-Kobayashi et al., 2012; Nakanishi et al., 2010; Nishikawa et al., 2012; Ozaki et al., 2005; Smith et al., 2013). In this study, we characterize the electrophysiological responses of a panel of cuticular hydrocarbons and non-cuticular hydrocarbons on the worker-specific basiconic sensilla of the antenna and find that they are broad-spectrum detectors of hydrocarbons. Using a training assay, we confirm that hydrocarbons activating the antenna are behaviorally perceived. Finally, we show that a proposed queen pheromone can be detected in both *R* and *S* forms and be discriminated behaviorally.

RESULTS AND DISCUSSION

To examine the detection of CHCs by the basiconic sensilla on the antennae, we developed a single-sensillum extracellular recording (SSR) method in the larger form of the two worker castes ("majors") that is similar to methods used in *Drosophila* and other insects (Figures 1A and 1B). In this paradigm, the sensilla showed robust and reproducible responses to a mixture of hydrocarbons but little response to the solvent (Figure 1B). Multiple action potential amplitudes were observed in each sensillum, representing the activation of a large number of ORNs. Each sensillum houses the dendrites of a very large number of ORNs, (>100) in a related *Camponotus* species (Nakanishi et al., 2010), and, therefore, individual ORN responses could not be clearly assigned to distinguishable spike amplitudes despite utilizing software sorting methods and manual inspection. The total increase in action potential frequency was therefore used to reliably quantify the overall strength of response, as has been done previously with other sensilla (Yao et al., 2005). To examine overall electrophysiological responses, we performed additional assays with varying concentrations of the hydrocarbon mixture to reveal a dose-dependent relationship between stimulus and response (Figure 1C).

We utilized a pharmacological approach to test whether members of the large *Or* gene family act as hydrocarbon chemoreceptors in the antennal ORNs. To accomplish this, we took advantage of VUANT1 (also called VU0183254), which has recently been identified as an antagonist of the conserved Orco co-receptor and is therefore expected to selectively block activity from Or family receptors (Jones et al., 2012). Because the solvent DMSO alone also activates spikes from the basiconic sensilla, we designed an experimental paradigm where VUANT1 vapor would be applied first, followed by subsequent application within the next 30 s of a hydrocarbon mixture (C_7-C_{40}) or various CHC extracts from worker ants. As expected, the responses to the alkane mixture and the CHC extract were lost completely after a brief pre-exposure to VUANT1, consistent with the hypothesis that CHC stimuli are detected by members of the Or family (Figures 1D and 1E). The hydrocarbon responses were

unaffected by pre-exposure to the solvent DMSO, and the response to carbon dioxide, a potentially non-Or ligand, was also unaffected by the antagonists (Figure 1E).

Using an electrophysiology assay, we next tested single compounds from a panel of and found that most odorants and cuticular hydrocarbons elicited an increase in action potential frequency (>25 spikes/s) (Figure 2A) when averaged across a total of 30 sensilla from which the responses were recorded (light gray bars on the left of Figures 2B–2D and grayscale heatmap in Figure 2E). The responses to individual compounds varied across different sensilla, and, to classify them into groups, a cluster analysis was performed based on the responses of the 30 sensilla to 34 stimuli. The responses from the sensilla clustered into three response groups, denoted SBI, SBII, and SBIII (Figure S1; Table S1), which could represent subsets that differ in responsiveness or differences in expressed receptor repertoires.

In *C. floridanus,* the cuticle of the queen is enriched in a collection of specific CHCs, many of which may be sensed by the workers and act as pheromones (Endler et al., 2004; Table S2). We tested the antennal basiconic SSR responses in major workers to nine queen-enriched CHCs and found that the SBI and SBII classes of basiconic sensilla responded to them, whereas the SBIII class showed very little response (Figures 2B and 2E). Workers may therefore be able to detect the queen using multiple CHCs from her cuticle, including saturated hydrocarbons, which have been proposed to regulate reproduction in workers across several social insect species (D'Ettorre et al., 2004; Holman et al., 2010; Van Oystaeyen et al., 2014).

When we tested CHCs present in both the worker and the queen cuticles, the SBIs responded robustly, whereas SBII ORNs showed a weaker response (Figures 2B and 2D). These results indicate that workers are able to detect both the queen and other workers using the ORNs housed in these SBs. The SBIIs show differential responses to queen and worker-derived CHCs and could contribute to differences in detection of these two castes. Interestingly, the SBI and SBII sensilla were also able to detect a variety of hydrocarbons that are not present on the *C. floridanus* cuticle (Figure 2C), suggesting that they are broad-spectrum detectors of hydrocarbons that not only detect complex intra-colony pheromones but also diverse chemosensory cues derived from non-nestmate conspecifics as well as other insect species that might comprise both prey and predators. The sensilla also contain ORNs that responded to several odorants associated with plants and other sources (Figure S2). From previous reports regarding ants and honeybees, it is also evident that they can detect and discriminate both pheromonal and non-pheromonal odors (Brill et al., 2013; Dupuy et al., 2010; Nishikawa et al., 2012; Rössler and Zube, 2011; Zube and Rössler, 2008). The characterization of responses to several hydrocarbons provided an opportunity to study the differential detection of the low-volatility compounds by a subset of the ant olfactory system. Individual chemicals arranged by structure were sorted by the response of SB sub-types in a heatmap of activity (Figure 2E). In this analysis, the SBI class is the most broadly responsive and differs from the SBIIs in its ability to better detect long-chain CHCs (C27–C33). The SBIIIs, on the other hand, show weak responses to most hydrocarbons.

To test whether the ability to detect hydrocarbons broadly is conserved, we collected another related ant species from southern California, *Camponotus laevigatus*. Using a smaller diagnostic panel of hydrocarbon and odorant stimuli, we performed parallel electrophysiology analyses of the basiconic sensilla of both *C. laevigatus* (n = 38) and *C. floridanus* (n = 72) spread across the nine flagellar sections of the antenna. When comparing these sensillar responses, a number of similarities in responsiveness to CHCs and odorants were observed across the two species that, taken together, reveal some conservation of the general organization of sensilla (Figures 3A and 3B). That said, the relative distribution of the three response classes of basiconic sensilla differed, with the broadly responsive SBI class being most abundant (~55% and 42%) in both (Figures 3C and 3D). The breadth of tuning of the three sensillum classes was visualized by plotting tuning curves, as done for other species (Hallem and Carlson, 2006), and computing the kurtosis (K) values (Figures 3E and 3F). Taken together, these results indicate that the antennae of *Camponotus* workers have the capability to detect a wide variety of cuticular hydrocarbons from the queen as well as workers.

Worker ants from one nest are usually highly aggressive toward non-nestmate workers, and, in this context, the accurate recognition of non-nestmates is a critical function of the ant's olfactory system. It has been proposed that the worker basoconic sensilla are the discriminators of this response by responding only to non-nestmate CHC extracts to the exclusion of nestmate CHC extracts, which differ only by subtle changes in the levels of individual CHCs in the mixtures (Kidokoro-Kobayashi et al., 2012; Ozaki et al., 2005). Given our observation that the basiconic sensilla are broadly tuned to numerous CHCs, it was puzzling how ants respond to one and not the other. To directly examine this, we utilized our SSR method to assess the response of *C. floridanus* worker antennal SBs to cuticular extracts from nestmates (D229) and non-nestmates. In these studies, one set of non-nestmates was collected from the same key in Florida (D260, Long Key), whereas another was from a distant one (D601, Sugarloaf Key). Although it is difficult to precisely quantify the differences in specific hydrocarbons within the scope of this study, gas chromatograms of each cuticular extract were examined for hydrocarbon constituents to demonstrate that they qualitatively showed general patterns of CHCs typical for these populations, indicating that the extracts could be tested further with electrophysiology (Moore and Liebig, 2010; Figure 4A). Interestingly, we found that all three classes of D229 SBs responded to nestmate CHC extracts at comparable levels as non-nestmates (Figures 4B and 4E). This ability was conserved in *C. laevigatus*, which also showed responses from basiconic sensilla to nestmates and non-nestmate colonies collected in San Bernardino, California (Figures 4C, 4D, and 4F). Although the summed spike frequencies in the sensilla are comparable, differences, which almost certainly exist at the level of individual ORNs, are not distinguishable by this technique.

Because the widespread responses to various hydrocarbons found using electrophysiology were somewhat unexpected, we wanted to test it using another method, such as behavior. To test whether hydrocarbons eliciting an electrophysiology

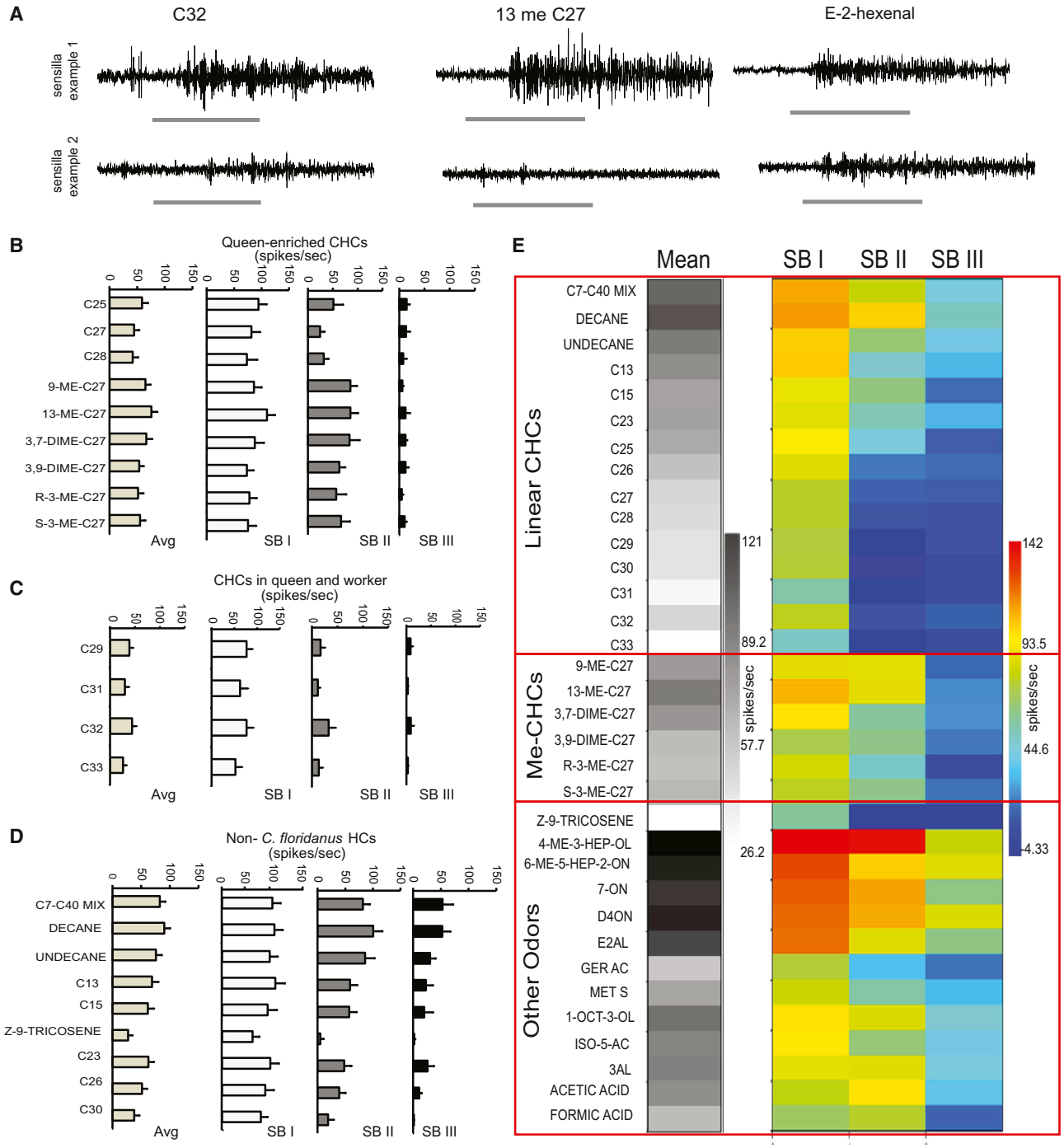

Figure 2. Antennal Basiconic Sensilla Are Broadly Tuned Detectors of Cuticular Hydrocarbon Pheromones

(A) Representative action potential traces from the three antennal SBs to a 0.5-s stimulus of *n*-dotriacontane (C_{32}), 13-methylheptacosane (13-me C_{27}), and *E*-2 hexenal.

(B) Mean response to queen-enriched CHCs. Avg, average.

(C) Mean response to CHCs found in both the queen and worker profile.

(D) Mean response to other non-cuticular hydrocarbons.

(E) Heatmap of mean responses of the three SB subtypes to a 34-odorant panel. Solvent responses are subtracted for responses and presented as bar graphs and heatmaps. Hydrocarbons were tested at 1 μg/μl dilution using a heated delivery system. More volatile hydrocarbons ($<C_{15}$), mix, and other odorants were tested at 10 μg/μl dilution using a regular unheated delivery system. n = 14 (SBI), n = 6 (SBII), n = 14 (SBIII); error bars show SEM.

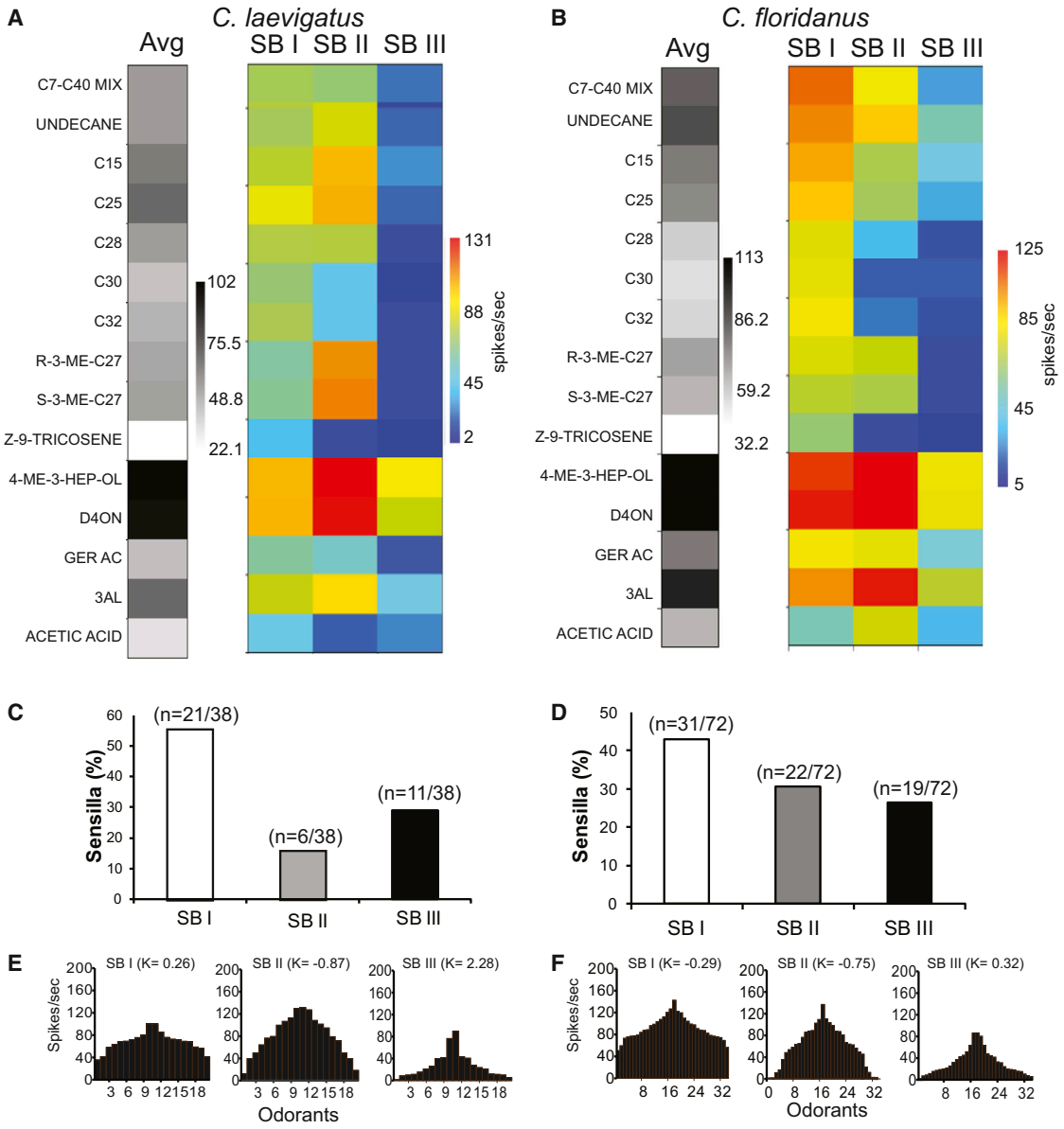

Figure 3. The Broad Detection of Cuticular Hydrocarbons Is Conserved across Ants

(A and B) Heatmap of the mean electrophysiological responses of three SB subtypes to a diagnostic panel of 15 compounds in (A) *C. laevigatus* and (B) *C. floridanus*.

(C and D) The abundance of the three SB subtypes in *C. laevigatus* and *C. floridanus*.

(E and F) Tuning curves of three functional classes of basiconic sensilla in *C. laevigatus* and *C. floridanus*. Individual odorants are arranged on the x axis, with the highest response in the center. Solvent responses are subtracted for responses and presented as bar graphs and heatmaps.

response were indeed being perceived by the worker ants, we performed a series of appetitive learning assays modified from a method established previously (Bos et al., 2012). In this paradigm, each ant was initially offered an individual hydrocarbon associated with a drop of sucrose reward (conditioned stimulus, CS^+) alongside another distinct hydrocarbon in the training arena with no reward (CS^-) (Figure 5A). After five training sessions, the ant was offered a choice between the two hydrocarbons. Preferences were registered manually as a characteristic antennation behavior at the chosen hydrocarbon (Movie S1; Supplemental Experimental Procedures). When an *n*-C_{15} CHC was paired during training with a sucrose reward, 100% of the ants tested chose the *n*-C_{15} stimulus and none opted for the *n*-C_{13} alternative. When the *n*-C_{13} CHC was paired with sucrose in the training runs, 75% of the ants antennated at *n*-C_{13}, whereas, in this instance, the other 25% did not choose either stimulus (Figure 5B). Similar results were obtained with two additional pairs of saturated hydrocarbon compounds (*n*-C_{23} versus *n*-C_{25} and

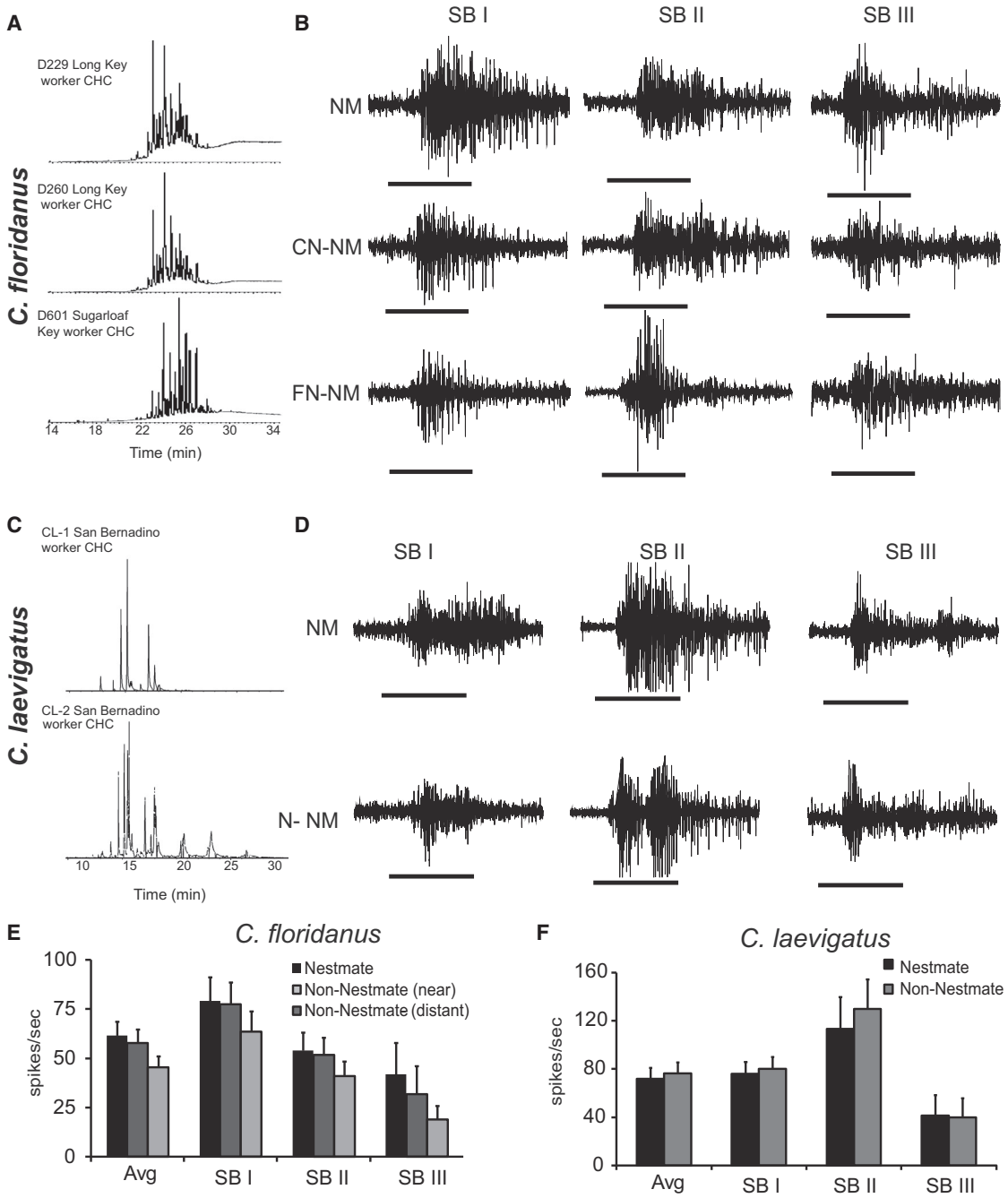

Figure 4. Ants Detect Cuticular Hydrocarbons from Nestmates and Non-nestmates

(A) Representative gas chromatogram spectra of the worker CHC profile of *C. floridanus* D229, D260, and D601 colony CHC extracts.

(B) Representative action potential traces from ORN responses of *C. floridanus* worker antennal SB classes to nestmate (NM), close non-nestmate (CN-NM), and far non-nestmate (FN-NM) cuticular extracts.

(C) Representative GC spectra of the CHC profile of two *C. laevigatus* colonies, CL-1 and CL-4.

(D) Representative action potential traces from *C. floridanus* worker antennal SB classes to NM and non-nestmate (N-NM) cuticular extracts.

(E and F) Mean responses of *C. floridanus* (n = 72) and *C. laevigatus* (n = 38) SB classes to the indicated CHC extracts. Statistics: ANOVA, not significant. Error bars show SEM.

n-C_{25} versus *n*-C_{27}) that were tested in individuals from two different colonies (Figure S3). These results indicate that the ants are able to detect all six hydrocarbon compounds presented using their sensory system because they can learn to associate each of them with a sucrose reward. To further test this ability in the ants, we examined two additional hydrocarbon

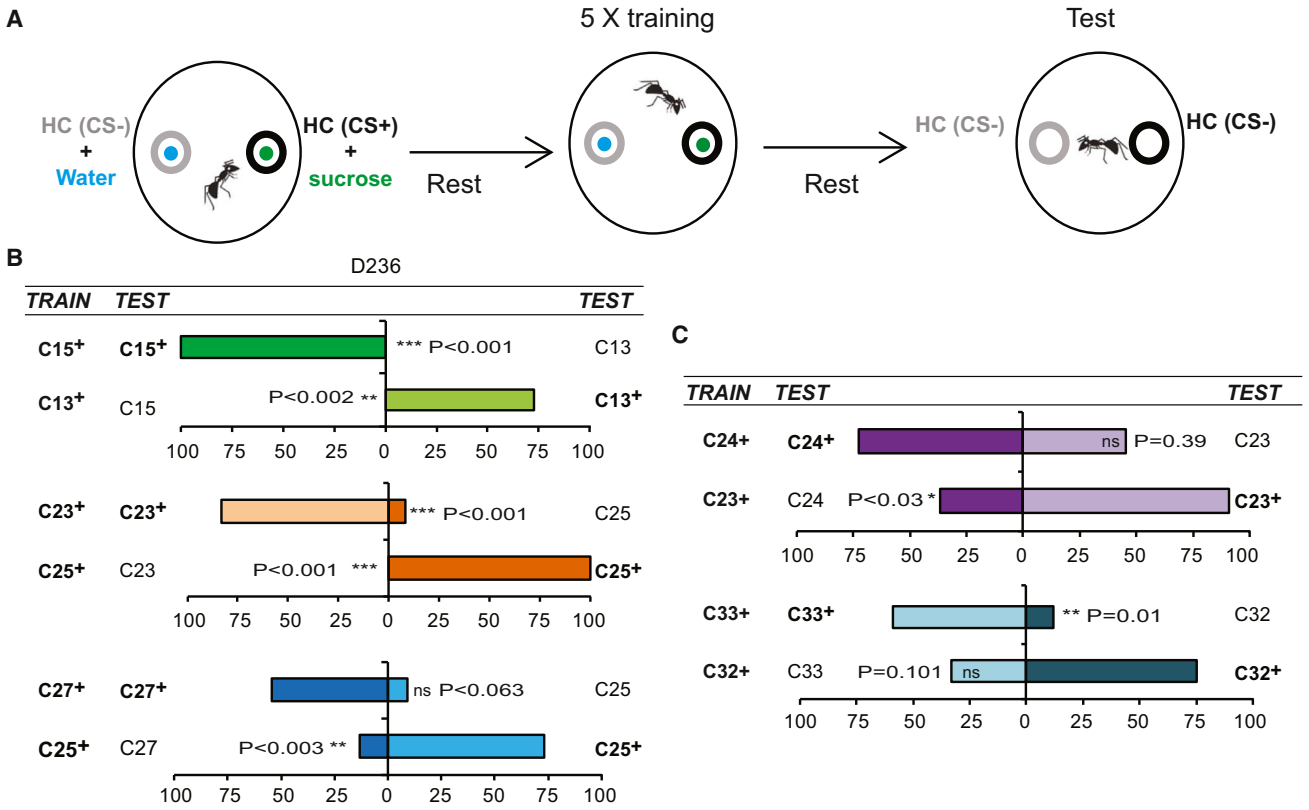

Figure 5. Ants Efficiently Discriminate Closely Related Cuticular Hydrocarbons

(A) Schematic of the hydrocarbon conditioning and discrimination experiment. HC, hydrocarbon. CS, conditioned stimulus.

(B) Post-training discrimination behavior test showing the percentage of ants selecting trained or untrained hydrocarbons in a two-choice assay. The trained odor is indicated with a + sign; n = 10–15 for each test.

(C) A similar discrimination experiment with hydrocarbon pairs separated by one carbon atom using ants from ten colonies. n = 12–17.

pairs that were separated by only one carbon atom (*n*-C_{23} versus *n*-C_{24} and *n*-C_{32} versus *n*-C_{33}) using individuals from ten separate colonies. We found that, for each pair tested, there was an increase in the percentage of ants antennating at the hydrocarbon associated with the reward, with at least one test for each pair being significant (Figure 5C).

The CHC 3-methylheptacosane, which is found in two enantiomeric forms, *R* and *S*, has the highest concentrations in CHC profiles of highly reproductive queens and has been postulated to be part of a fertility signal or, more specifically, of a queen pheromone that inhibits worker ovarian activity (Endler et al., 2004, 2006; Moore and Liebig, 2010). Previous electrophysiology studies with small odorants have shown that heterologously expressed insect odorant receptors can respond differentially to enantiomers (Bohbot and Dickens, 2009; Pask et al., 2011). However, very few studies have been undertaken to examine the ability of insects to behaviorally discriminate between enantiomers of long hydrocarbons because of the difficulty in synthesizing these compounds in an enantiomerically pure form. In light of the robust ability of ants to detect and discriminate hydrocarbons, we decided to test enantiomeric discrimination and, to that end, first developed a reliable synthetic route to these 3-methyl hydrocarbons (Breit and Seiche, 2003). In this synthesis, the key step is a highly selective copper-mediated allylic substitution that introduces the methyl group at the stereogenic center. Hydroformylation of a long-chain alkene followed by standard coupling procedures completed the synthesis of both *R* and *S* 3-methylheptacosane (Supplemental Experimental Procedures; Figure S4).

Electrophysiology experiments showed that the *C. floridanus* worker basiconic sensilla (SBI and SBII classes) responded robustly to both enantiomers (*R* and *S*), although it was impossible to determine whether different neurons were responding in each case (Figure 6A). Although the electrophysiology informs us that the ant olfactory system can detect these compounds well, it does not address whether discrimination between these enantiomers is possible. To test this, we turned to the associative learning assay and found that worker ants were able to behaviorally discriminate well between the two enantiomers (Figure 6B). Taken together, these results indicate that the ant olfactory system can detect and discriminate between enantiomeric hydrocarbons.

The systematic analysis of the olfactory system indicates that social insects such as *Camponotus* have developed a broad-spectrum detection system for CHCs in their antennal basiconic sensilla. These CHCs are long-chain compounds of low volatility

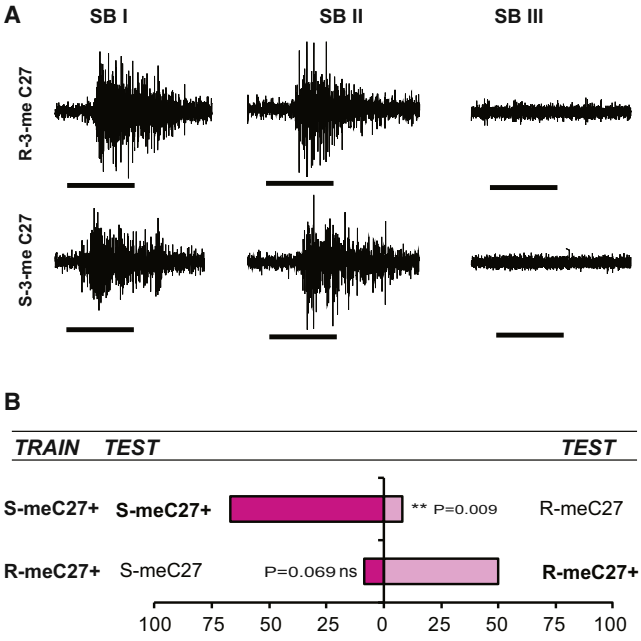

Figure 6. Ants Efficiently Detect and Discriminate Enantiomeric Cuticular Hydrocarbons

(A) Representative SB responses to the *R* and *S* enantiomers of 3-me C27.

(B) Discrimination experiments with the enantiomers with percentage of ants selecting hydrocarbon indicated. n = 11 and 12. Trained odor is indicated with a +. Statistics: chi-square test; ns, not significant.

and limited structural diversity that serve as important signature cues for social organization within a colony by indicating queen fertility and presence and marking other castes and, outside of the colony, for identifying non-nestmates. Although we could not predict a priori which CHCs were likely to be detected, our analysis identifies several CHCs from the queen and worker that elicit robust behavioral and electrophysiological responses. Because social insects often reside in large numbers in close proximity, they would require a chemical communication system that can recognize individual members only when in close proximity. Employing low-volatility CHCs as signature cues in pheromones could be very effective in close-range detection. Moreover, CHCs would also allow a large repertoire of chemicals to be used to mark the different castes. Solitary insects, on the other hand, may need to depend on more volatile pheromones that they can sense over large distances and, therefore, use other classes of chemicals.

The electrophysiology analyses indicate that worker basiconic sensilla detect both nestmate and non-nestmate CHC extracts. This is consistent with whole-antenna recordings and antennal lobe imaging studies (Brandstaetter and Kleineidam, 2011; Brandstaetter et al., 2011) but contrary to previous reports using an unusual tip-recording technique adapted from gustatory sensilla electrophysiology that involved placing a wider glass electrode containing the solubilized CHC stimulus over the tip of the basiconic sensilla, which suggested that nestmate extracts are poorly detected (Ozaki et al., 2005). Our findings suggest that the ability to distinguish non-nestmates likely involves non-peripheral mechanisms such as the learned discrimination of subtle differences in cuticular hydrocarbons, as has also been suggested by behavioral experiments (Bos et al., 2010; Smith et al., 2013; van Wilgenburg et al., 2012).

Taken together these results suggest that ants have evolved specialized *Or* receptor repertoires to detect long-chain hydrocarbons. *orco* co-receptor antagonist blocks CHC detection, and the odorant receptor (*Or*) gene families of hymenopteran social insects are the largest found in insects and include major expansions of specific phylogenetic clades in the *Or* tree (Zhou et al., 2012). The ability to detect numerous CHCs is matched not only by an expanded *Or* family but also by the remarkable ability to discriminate among closely related hydrocarbons. With several genomes of eusocial insects now available, it will be interesting to uncover how chemoreceptors have evolved to detect these CHC cues and how the molecular and cellular pathways generate powerful behavioral programs underlying the mystery of communication within their societies.

EXPERIMENTAL PROCEDURES

Ant Rearing

Ant workers from two laboratory-established colonies of *C. floridanus* (D229 or D260, collected from Long Key, Florida, and D601, collected from Sugarloaf Key, Florida) were used for studies, for which the major workers were selected randomly from the artificial nest box from either colony. Ants for behavior experiments in Figures 4C and 4E were randomly picked from 12 laboratory *C. floridanus* colonies (Long Key: D229, D260, D215, D254, and D10; Grassy Key: D366, D341, and D355; Cudjoe Key: C124; Little Torch Key: D167; Middle Torch Key: D208; and Vaca Key: D471). The ants were provided with sugar water, an artificial diet and pieces of cricket (*Acheta domestica*) or beetle larvae (*Zophobas morio*) twice a week. The ant colonies were maintained at 25 ± 2°C and a 12:12 light:dark (LD) cycle. The *C. laevigatus* ant colonies (CL-1 and CL-2) were collected from their nests in the San Bernardino Mountains in California.

Extraction and Analysis of Cuticular Hydrocarbons

To determine whether the CHCs of *C. floridanus* from our study population can encode information regarding colony membership in workers, we compared the CHC profiles of 30 major workers from each of three different colonies. The colonies were selected at random, but the selection process was engineered so that two colonies originated from the same collection site (Long Key, Florida; collected in November 2006 and October 2007) and the third colony from 45 miles away (Sugarloaf Key, Florida; collected in July 2002) to better capture the range of inter-colony variation. Thirty ants from the same colony were immersed in 3 ml of *n*-pentane (300 μg/30 ants/3 ml). After 5 min, the pentane (containing the crude extract) was applied to a silica gel column (0.5 cm, 0.75 g of 70–230 mesh Fisher S 826-1 silica gel sorbent, flash chromatography-grade), and the CHC fractions were eluted using pentane. One microliter of the pentane extract was injected into an Agilent 6890N gas chromatograph (GC) (Agilent Technologies) coupled with an cAgilent 5975 mass-selective detector operated in electron impact ionization mode. The GC was operated in splitless injection mode with helium as the carrier gas at a 1 ml/min flow rate. It was fitted with a 30 m × 0.25 mm (ID) × 0.1 μm DB-1MS non-polar column (Agilent). The GC injection port was set to 280°C. The GC oven temperature was programmed from 60°C (isothermal from 2 min) and then was raised by 40°C/min up to 200°C. The temperature subsequently increased 5°C /min to 320°C and was then held constant for 5 min. A similar extraction was done with the *C. laevigatus* colonies collected locally from National Children's Forest, San Bernardino mountains, California (2012).

Hydrocarbons and Other Chemicals Used for Odor Stimulation

For hydrocarbons, *n*-pentane was used as a solvent, and all chemicals were tested at 1 μg/μl dilution. The following hydrocarbons were purchased from Sigma-Aldrich: *n*-tricosane (C_{23}, 99%, Sigma-Aldrich), *n*-pentacosane

(C_{25}, 99%, Sigma-Aldrich), *n*-hexacosane (C_{26}, 99%, Sigma-Aldrich), *n*-heptacosane (C_{27}, ≥99.5%, Fluka), *n*-octacosane (C_{28}- 99%- Aldrich), *n*-nonacosane (C_{29}- ≥ 99%- Fluka), *n*-triacontane (C_{30}, 98%, Sigma-Aldrich), *n*-hentriacontane (C_{31}, ≥99%, Fluka), *n*-dotriacontane (C_{32}), and *n*-tritriacontane (C_{33}, 98%, Sigma-Aldrich). The following racemic mixtures of methyl-branched CHCs were synthesized in addition to the enantiomer-pure *R*-3-methyl heptacosane (*R*-3-me C_{27}) and *S*-3-methyl heptacosane (*S*-3-me C_{27}): 9-methyl heptacosane (9-me-C_{27}), 13-methyl heptacosane (13-me C_{27}), 3,7-dimethyl heptacosane (3,7-dime C_{27}), and 3,9-dimethyl heptacosane (3,9-dime C_{27}). For these low-volatility hydrocarbon stimuli and extracts, 1 μl of the 1 μg/μl solution was applied in a Pasteur pipette using a precision syringe. Immediately before delivery, the outside of the pipette was briefly heated with a butane torch for ~1 s, and a controlled 0.5-s stimulus of air (5 ml/s) was puffed through the odor cartridge containing vapors into a humidified airstream (10 ml/s) that was passed over the ant antenna.

The following hydrocarbons with a higher predicted volatility were also prepared in *n*-pentane at 10 μg/μl dilution (20 μl): *n*-decane (C_{10}, ≥99%, Sigma-Aldrich), *n*-undecane (C_{11}, ≥99.8%, Fluka), *n*-tridecane (C_{13}, ≥99%, Sigma-Aldrich), *n*-pentadecane (C_{15}, ≥99%, Sigma-Aldrich), and alkane mix (C_7–C_{40}, Supelco). Other volatile odorants were dissolved in paraffin oil at 10 μg/μl concentration (20 μl): 4-methyl-3-heptenol (4-ME-3-HEP-OL), 6-methyl-5-hepten-2-one (6-ME-5-HEP-2-ON), 2,3-butanedione (D4ON, 97%, Sigma-Aldrich), 2-heptanone (7-ON, 98%, Sigma-Aldrich), *E*-2-hexenal (E2al, 98%, Sigma-Aldrich), geranyl acetate (GerAC, 98%, Sigma-Aldrich), methyl salicylate (MetS, 99%,Sigma-Aldrich), 1-octen-3-ol (1-oct-3-ol, 98%, Sigma-Aldrich), isopentyl acetate (Iso 5Ac, 98%, Sigma-Aldrich), propanal (3al, ≥97%, Sigma-Aldrich), and Z-9-tricosene (Z-9-C_{23}, 97%, Sigma-Aldrich). Formic acid and acetic acid were prepared at 10 μg/μl dilution in distilled water (20 μl). Blank, heat blank, pentane (20 μl), and paraffin oil were used as controls (20 μl). To deliver the test volatile odorants, a 20-μl aliquot was applied to a strip (1 cm × 1 cm) of Whatman filter paper no. 1.

VUANT1 (VU0183254-2-[[4-ethyl-5-(2-furanyl)-4H-1,2,4-triazol-3-yl]thio]-1-(10H-phenothiazin-10-yl)-ethanone, Chemical Abstract Series [CAS] no. 663412-40-6) was purchased from Molport, and a 10^{-2} M solution was made in DMSO. 25 μl of DMSO and VUANT1 were applied to a filter paper strip and placed inside a glass Pasteur pipette. However, for *n*-pentane, C_{26}, C_7-C_{40} mix, and D601-CHC extract, 1 μl of the sample was placed into the Pasteur pipette with a precision syringe. These odors (*n*-pentane, DMSO, C_7-C_{40} mix, D601 colony-CHC extract, and VUANT1) were followed by heat blank and delivered by heating the pipette with a butane torch right at the place of treatment for ~1 s before mixing odorants with the continuous airstream. All recordings were performed on SBI after confirming its identity by stimulating it with C_{26}.

All odors were delivered by puffing the odor cartridges with a controlled 0.5-s stimulus of air (5 ml/s) into a humidified airstream (10 ml/s), which was passed over the ant antenna. When *n*-pentane solvent was used, the filter paper was allowed to evaporate for ~1 min in a fume hood before inserting it into a glass Pasteur pipette odor cartridge.

Electrophysiology

Major workers of *C. floridanus* (from either the D229 or D260 colony) were taken and anesthetized using brief exposure to CO_2. A holding chamber for the ant was fashioned from a cut 200-μl pipette tip with an opening in the middle to insert the reference electrode into the abdomen. The ant was inserted into the pipette tip with the head facing the pointed end. Modeling clay was used to immobilize the head and body of the ant, and a coverslip was used to create a platform to support the antenna for recordings. An antenna was maneuvered and affixed on to double-sided sticky tape on the slide. The antennal segments were visualized under a compound light microscope (Olympus BX40) mounted on a vibration isolation table. Extracellular single-unit recordings were performed using glass capillary electrodes from the shaft near the base of the basiconic sensillum. Additionally, an audio amplifier was used to listen to the spike train while making the recordings to differentiate the muscle potential activity or the activity in the nervous system or body parts.

Electrophysiological data were analyzed with Clampfit 10.3 software (Axon Instruments). Each spike train was analyzed by expanding the trace and setting a count threshold above baseline/background noise when counting the spikes using Clampfit. The responses for each stimulus were quantified by counting all spikes recorded (all amplitudes) from an individual sensillum in response to odorant puffed onto the antenna during a 0.5-s window and doubled, from which we subtracted the number of spontaneous spikes in a 1-s window before stimulation. To obtain a solvent-corrected response, we subtracted the spike frequency evoked by the corresponding solvent for each odorant. Classification of datasets into classes of sensilla was made with a hierarchical cluster analysis using Euclidean distances and JMP software (SAS Institute) (de Bruyne et al., 1999).

To generate tuning curves, odorants are arranged on the x axis according to the strength of the response they elicit from each sensillum type. The odorants that elicit the strongest responses are placed near the center of the distribution, and those that elicit the weakest responses are placed near the edges. Therefore, the order of odorants is different for each sensillum type, and the K value, a statistical measure of "peakedness," is located in the upper right corner of each plot.

Dose-Response of the Experiments

It is difficult to test the CHCs in the same concentration as they are found in nature. The CHC profile changes from one caste to other (based on task allocation), from one colony to another (qualitative and quantitative differences), and under different weather conditions (based on higher or lower temperatures) (Hefetz, 2007). We tested four doses (0.01, 0.1, 1, and 10 μg/μl) of alkane mix (C_7-C_{40}) and obtained electrophysiology recordings from SBI to collect a response profile from low to high doses. SBI sensilla were confirmed first by testing the response to C_{26} (1 μl of a 1 μg/μl solution). Sensilla that strongly responded to C_{26} were identified and subsequently tested with the complete odor panel.

Orco Antagonist Experiment

VUANT1 has been reported as an insect Orco antagonist (Jones et al., 2012; Pask et al., 2011). Two sets of experiments were performed on SBI sensilla to find out whether the responses to CHCs are mediated olfactorily. The experiments were performed to test the effect of the solvent control (DMSO) in which the Orco antagonist (VUANT1) was dissolved in and the effect of antagonist on the response profile of SBI to CHCs. The details of these two experiments were as follows.

The Effect of the DMSO Solvent Control

This experiment was performed to confirm the effect of the solvent control on the response profile of odorants. The following stimuli were presented to sensilla in the following order (pre-treatment): heat blank, *n*-pentane (1 μl), C_7-C_{40} mix (1 μl), CHC extract from a colony of *C. floridanus* (D601) (1 μl), carbon dioxide (0.5%), and DMSO (25 μl), followed by additional stimuli (post-solvent) applied after a delay of 1 min each (C_7-C_{40} mix, D601-CHC extract, and carbon dioxide at the same concentration).

The Effect of the Antagonist VUANT1 at 10^{-2} M in 25 μl DMSO

The purpose of this experiment was to investigate whether the responses to CHCs from SBs are mediated by Orco-dependent Or family receptors. VUANT1 is an Orco antagonist that inhibits the activity of olfactory sensilla (Jones et al., 2012). The sensillum was stimulated with the same odor as in the first experiment, and the only difference between these two experiments was that VUANT1 was used in place of the DMSO stimulus, and rest of the odors were delivered in the same sequence as in the DMSO experiment.

Behavioral Discrimination Assay

We prepared 1 μg/μl stock solutions in *n*-pentane or *n*-hexane and then diluted them to prepare 1 μg/25 μl solutions. All experiments were performed using 10-cm diameter arenas: circular plastic petri dishes in Figure 5B, and circular enclosures with a glass slide inside for Figures 5C and 5E. On the underside of each petri dish or slide, two circles were drawn ~2.75 cm apart using two different color pens, which were randomized across trials. Differential olfactory conditioning assays were conducted where one hydrocarbon was rewarded (CS^+) and one hydrocarbon was unrewarded (CS^-). We starved groups of ants (majors and minors) for 3 days on water. However, only minors were used for the discrimination assays. During the training phase, we applied 12.5 μl of the CS^+ hydrocarbon in the middle of one of the drawn circles and 12.5 μl of the CS^- hydrocarbon in the middle of the other circle. We waited

to let the solvent evaporate completely. 1 μl of 50% sucrose solution was added to the middle of the CS^+-treated circle and 1 μl of distilled H_2O (dH_2O) to the middle of the CS^- circle. Then we carefully placed the ant in the arena and allowed it to drink sugar water and contact the CS^- at least twice. The ant was then removed to a separate box containing nestmate workers for 2 min so that it could clear its crop via trophallaxis. We repeated the same training procedure four more times, changing the position of CS^+ between the right and left.

For the test, we applied 12.5 μl of CS^+ on one side and CS^- on the other side, randomizing the orientation across trials. No sugar or water was applied in the test. A single trained ant was released in the arena and videotaped for 5 min. The videos were analyzed offline, blind to the treatments, and analysis time started after the initial 20-s period when then ant was introduced. A positive choice for a hydrocarbon was characterized by a characteristic antennation pattern that involved pointing both antennae together at acute angles (Movie S1). We counted the total number of choices for each hydrocarbon. The number of ants preferring CS^+ over CS^- was subjected to chi-square analysis in a 2 × 2 contingency table with Yates correction assuming a 50:50 distribution (Sokal and Rohlf, 1981) using SigmaStat V 3.5 (http://www.sigmaplot.com). Details about ant rearing, hydrocarbon analyses, odor experimentation, synthesis of methyl-branched CHCs, and extraction and analyses of ant CHCs are described in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, two tables, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.07.031.

AUTHOR CONTRIBUTIONS

K.R.S. performed the electrophysiology and behavior experiments, analyzed the data, and helped write the manuscript. A.R. supervised the project and wrote the manuscript. B.L.E. performed a portion of the behavior experiments with help from D.M. and J.L. G.R.J, Y.S, J.P., and B.B. synthesized hydrocarbons. D.R., S.L.B, L.J.Z., J.L., and A.R. managed the project and obtained funding.

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