Cuticular hydrocarbon profile for queen recognition in the termite *Reticulitermes speratus*

Yuki Mitaka (ymitaka02@gmail.com)
Kyoto Institute of Technology: Kyoto Kogei Sen'i Daigaku https://orcid.org/0000-0002-3399-3470
Tadahide Fujita
Nippon Kayaku Co., Ltd.

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Abstract

Chemical communication underlies the sophisticated colony organization of social insects. In these insects, cuticular hydrocarbons (CHCs) play central roles in nestmate, task, and caste recognition, which contribute to maintenance of the social and reproductive division of labor. Queen-specific CHCs reflect queen fertility status and function as a queen recognition pheromone, triggering aggregation responses around the queens. However, there are only a few studies about the royal recognition mechanism in termites, and particularly, no study has reported about queen-specific CHCs in the species using asexual queen succession (AQS) system, in which the primary queen is replaced by neotenic queens produced parthenogenetically. In this study, we identified the CHC pheromone for neotenic queen recognition in the AQS termite species *Reticulitermes speratus*. Gas chromatography-mass spectrometry analyses revealed that the relative amount of *n*-pentacosane was disproportionately greater in the CHC profiles of queens than in the CHC profiles of kings, soldiers, and workers. Furthermore, we investigated the cuticular chemicals of the queen aggregate workers; bioassays demonstrated that *n*-pentacosane shows a worker arrestant activity in the presence of workers' cuticular extract. These results suggest that *R. speratus* workers identify whether each individual is a neotenic queen by recognizing the relatively higher ratio of *n*-pentacosane in the conspecific CHC background. Moreover, they suggest that termites have evolved queen recognition behavior, independently of social hymenopterans.

Introduction

The colony organization of social insects is maintained through a sophisticated chemical communication system, which is associated with various social activities (Wilson 1965). In bees, wasps, ants, and termites, cuticular hydrocarbons (CHCs) are used to prevent desiccation and microbial infections and also serve as nestmate, task, and caste recognition cues (Howard and Blomquist 2005; Blomquist and Bagnères 2010; Bagnères and Hanus 2015). One or more components of the mixture of CHCs are involved in these recognition cues; individual ants discriminate among species, families, and castes by recognizing differences in the composition or mixture ratio of the CHCs (Lahav et al. 1999; Akino et al. 2004; Ozaki et al. 2005; Blomquist and Bagnères 2010).

One hallmark of eusocial insects is the reproductive division of labor, which allows only one or a few queens to lay eggs (Wilson 1971). The organizational structure and stability of eusocial insects rely on pheromonal communication between queens and non-reproductive castes (i.e., workers and soldiers). In social hymenopterans, queen-specific compounds (e.g., saturated/unsaturated hydrocarbons in ants; unsaturated hydrocarbons, terpenes, esters, and fatty acids in honeybees) function as queen pheromones (Van Oystaeyen et al. 2014). The queen pheromones generally function both as a primer pheromone, which regulates worker reproduction, and as a releaser pheromone, which elicits queen retinue behavior in workers. The abundance of queen pheromone reflects each individual queen's fertility (Le Conte et al. 2008; Van Oystaeyen et al. 2014). Depending on the social hymenopteran species, the queen-specific components can function as a queen pheromone by themselves (Slessor et al. 2005; Holman et al. 2010,

2013; Van Oystaeyen et al. 2014) or only when presented with the nestmate CHC background (Smith et al. 2015).

Queen-specific compounds have been identified in some termite species. For example, volatile compounds were found in *Reticulitermes speratus* (Matsuura et al. 2010), *Nasutitermes takasagoensis* (Himuro et al. 2011), *Silvestritermes minutus* (Machara et al. 2018), *Embiratermes neotenicus* (Havlíčková et al. 2019), *Labiotermes labralis* (Havlíčková et al. 2019), and *Cyrilliotermes angulariceps* (Havlíčková et al. 2019); non-volatile queen-specific CHCs were found in *Cryptotermes secundus* (Weil et al. 2009). Also, royal-specific CHCs (that is, the CHCs that both kings and queens exclusively have) were found in *Zootermopsis nevadensis* (Liebig et al. 2009) and *Reticulitermes flavipes* (Funaro et al. 2018), but queen-specific compounds have not found in these two species. Pheromonal function was demonstrated only in *R. speratus*; a volatile queen pheromone (comprising butyl butyrate and 2-methyl-1-butanol) of this species has many functions, including inhibition of the differentiation of workers into neotenic queens (Matsuura et al. 2010; Yamamoto and Matsuura 2011; Matsuura and Matsunaga 2015; Suehiro and Matsuura 2015). However, it is unlikely that volatile compounds function as queen recognition pheromones when the colony members are in contact with each other. Volatile compounds easily saturate the colony space and can hamper nestmates' perception via sensory habituation; thus, the chemicals used for queen recognition should be non-volatile (Korb 2018; Hefetz 2019). Accordingly, a queen-specific, non-volatile compound (or a queen-specific cuticular profile) on the body surface of the queen is presumed to elicit queen retinue behavior by workers and/or soldiers in *R. speratus*.

R. speratus has a remarkable reproductive system, known as an asexual queen succession (AQS) system, in which queens use parthenogenesis to produce the next generation of queens but use normal sexual reproduction to produce alates (adult reproductives with wings) (Matsuura et al. 2009, 2018; Yashiro and Matsuura 2014). Each *R. speratus* colony typically produces numerous alates every spring. After swarming, a pair of male and female alates founds a new colony and then begins to produce offspring sexually as a primary king and a primary queen (PQ), respectively. As the PQ senesces, tens or hundreds of neotenic queens (secondary queens, SQs) are produced parthenogenetically by the PQ. SQs stay inside the nest to supplement egg production, eventually replacing the PQ; they are also able to parthenogenetically produce a subsequent generation of SQs. In contrast to the PQ, replacement of a primary king is rare in wild colonies (Matsuura et al. 2018), suggesting that primary kings have much greater longevity than PQs (Matsuura 2011). Consequently, in most wild *R. speratus* nests, the royal chamber usually includes one primary king and tens or hundreds of SQs (Matsuura et al. 2018). The SQs always gather densely at the royal chamber and are surrounded by workers and soldiers (Fig. 1). Even if individual SQs, soldiers, and workers are separately arranged in an artificial nest, SQs always congregate in one place, followed by soldiers and workers (Yanagihara et al. 2018). Therefore, it is likely that SQs, soldiers, and workers easily gather around SQs. It has been predicted that each individual identifies an SQ at this time using a non-volatile, queen-specific chemical (or cuticular profile) as a cue to initiate gathering around a queen.

In this study, we performed gas chromatography-mass spectrometry (GC-MS) analyses and discovered a neotenic queen-specific CHC profile for *R. speratus*. Furthermore, we conducted bioassays using glass dummies treated with the CHC profile of neotenic queens to test whether this CHC functioned as the neotenic queen recognition pheromone, eliciting aggregation around the queens.

Methods And Materials

CHC analyses

To extract the CHCs, we collected four termite colonies (Colonies A, B, C, and D) from secondary forests. Colony A was collected from Shimotakakura-cho, Hitachiota city, Ibaraki prefecture, Japan, in July 2019. Colonies B and C were collected from Kabutoichiba, Kameyama city, Mie prefecture, Japan, in August 2019. Colony D was collected from Takaragaike park, Kyoto city, Kyoto prefecture, Japan, in July 2017. We identified the royal chambers in these colonies and took a primary king, SQs, and both sexes of soldiers and workers from each royal chamber. The chemical compounds from the whole-body extracts of the termites, one primary king, five SQs, five female soldiers, five male soldiers, and five female or male workers were extracted in a 3-mL glass tube containing 100 µL *n*-hexane (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) for 5 min. Next, each hexane extract was transferred into a 100-µL insert, evaporated without complete desiccation under a gentle stream of high-purity nitrogen, and redissolved in a 10-µL hexane solution containing 10 ng octadecane as an internal standard.

GC-MS analyses were performed on a JMS-Q1500GC mass spectrometer (JEOL Ltd., Tokyo, Japan) combined with a 7890B GC System (Agilent Technologies Japan Ltd., Tokyo, Japan) equipped with a DB-1MS column (30 m, 0.25 mm diameter, 0.25 µm film, Agilent Technologies). The column temperature was raised at 20°C/min, from 50°C (5 min) to 300°C (5 min). A volume of 1 µL hexane solution was injected for each sample in splitless mode, with helium as the carrier gas (1.2 mL/min). The injection port temperature was 250°C. Mass spectra data were obtained under the following conditions: ionization current, 50 µA; ionization energy, 70 eV; accelerating voltage, 2 kV; and scan range, 50–500 *m/z*, because the CHCs of *R. speratus* do not include hydrocarbons having 30 or more carbon atoms (Takematsu and Yamaoka 1999). The gas chromatographic system and mass spectrometer were controlled using msPrimo System Controller software ver. 1.06 (JEOL). Candidate compounds were predicted from the Mass Spectral Library (NIST11) using Escrime software ver. 2.04 (JEOL). Compounds were identified according to mass spectra and Kovats indices on the DB-1MS column. Methyl branch positions of mono- and multimethyl-branched alkanes were determined from characteristic fragmentation patterns of their mass spectra (Nelson et al. 1972) as well as by their calculated Kovats indices (Carlson et al. 1998). The positions of double bonds were not determined.

Principal component analysis

Using the chromatograms, we selected 98 relevant peaks, which were detected in any caste from all of the colonies, excluding internal standard (octadecane), and these were used to compare the chemical profiles among all of the castes. All peaks were normalized to the octadecane internal standard, and area

percentages were input into a principal component analysis (PCA) matrix. The significance of the component differences was determined via permutational multivariate analyses of variance (PERMANOVA) with Bray-Curtis similarities and 1000 permutations. In the PERMANOVA, six caste groups (primary kings, secondary queens, female soldiers, male soldiers, female workers, and male workers) were used as the main factor. PCA and PERMANOVA were performed using R software v.3.4.3 (R Core Team 2016).

Comparison of relative amounts of compounds among castes

Using the chromatograms, we first excluded colony-specific compounds in each of caste, and then remaining compounds were used to compare the relative amount to total CHC peaks among all castes. The relative amount of each compound was compared among castes using a generalized linear model (GLM) with binomial distribution, followed by Tukey's honestly significant difference (HSD) test. In this model, the relative amount was regarded as the responsible variable, and the castes were used as the explanatory variable. Each caste had 4 replications (4 colonies × 1 sample extract). Statistical analyses were performed using R software v. 4.0.0 (R Core Team 2016).

Bioassays

We collected three colonies (colonies E, F, and G) from secondary forests for the bioassays. Colony E was collected from Shimoyada-cho, Kameoka city, Kyoto prefecture, Japan, in April 2019. Colony F was collected from Saga-toriimoto-hukatani-cho, Ukyo-ku, Kyoto city, Kyoto prefecture, Japan, in August 2019. Colony G was collected from Saga-koshihata-kitano-cho, in April 2020. Workers, soldiers, and nymphs of each colony were kept in a plastic case with a mixed sawdust bait (Matsuura and Nishida 2001) for at least 1 month, to allow the nymphs to differentiate into SQs. We then confirmed that the newly differentiated SQs had laid hundreds of eggs, indicating that these queens had matured sufficiently to be fertile. Then, we collected the queens, the soldiers, and the workers at the periphery of the queens.

For the bioassays, we placed a group of five SQs, five soldiers, and five workers in a 40-mm plastic dish (assay dish). In each dish, two glass dummies (3 mm diameter, 0.9–1.3 mm long) were placed separately. Before the experiments, the bottoms of the assay dishes were roughened with a piece of sandpaper because termites are not good at walking on a smooth surface. The dishes were replaced to new ones every time. The 5-μL sample solution was applied to one dummy (sample dummy), and the same amount of solvent (*n*-hexane) was applied to the other dummy (solvent dummy). Both dummies were dried for approximately 5 min to remove the solvent before the lid was placed over the dish. For each treatment, we took vertical photographs of each assay dish after 3 min with an HD webcam (LOGITUBO L-920M3 Webcam 1080P, LOGITUBO, Shenzhen, China), then recorded the number of individuals aggregated within a 7.5 mm radius from the center of the dummy using Adobe Premiere Pro 2020 (Adobe Systems Incorporated, San Jose, CA, USA). Each termite group was tested with a rest period of more than 10 min between treatments.

First, we tested whether *n*-pentacosane (C25) applied to a glass dummy triggered aggregation of the queens, soldiers, and workers. Because we hypothesized that the workers' CHC profiles might enhance the behavioral responses by providing a conspecific chemical context to the termites (Funaro et al. 2018), C25 was tested alone and in combination with a worker extract (WE) (1 individual equivalent per dummy). Concurrently, we tested the WE alone and hexane as the control, and a queen extract (QE) (1 individual equivalent per dummy) as a positive control, respectively. QE, WE, and mixtures of WE and C25 (WE + 1, 10, or 100 ng C25 per dummy) were obtained as follows. Forty queens or 200 workers (derived from colony E) were extracted in a 5-mL glass vial containing 1 mL *n*-hexane for 5 min. Each extract was transferred into a new 1.5-mL glass vial. QE was concentrated to 200-μL aliquots using a gentle stream of nitrogen. When extracting the workers, we did not distinguish the sex of workers because there was no distinguished difference in the CHC profiles between female and male workers (see below). For solutions with C25 alone, a standard solution containing 20 μg authentic standard C25 (Fujifilm Wako Pure Chemical Corporation) dissolved in 1 mL hexane was prepared, along with 10- and 100-fold diluted solutions. To prepare the solutions containing WE and different doses of C25, a 4-μL hexane solution of C25 (10, 100, and 1,000 ng/μL) was introduced into a 1.5-mL glass vial, evaporated under a gentle stream of high-purity nitrogen, and dissolved in 200 μL of the above WE. In this experiment, we created 13 termite groups (5 groups from colony D, 3 groups from colony E, and 5 groups from colony G).

Next, we tested whether *n*-tricosane (C23) applied to a glass dummy triggered aggregation of queens, soldiers, and workers. C23 was chosen as a control hydrocarbon because it was also contained in the extracts of all castes and colonies (Table S1), and the chain length of C23 is similar to that of C25. C23 was tested alone and in combination with WE (1 individual equivalent per dummy). Concurrently, we tested the WE alone and hexane as the control. Mixtures of WE and C23 (WE + 1, 10, or 100 ng C23 per dummy) were obtained using a method similar to that described above. For solutions with C23 alone, a standard solution containing 20 μg of authentic standard C23 (Fujifilm Wako Pure Chemical Corporation) dissolved in 1 mL hexane was prepared, along with 10- and 100-fold-diluted solutions. To prepare solutions containing WE and different doses of C23, a 4-μL hexane solution of C23 (10, 100, and 1,000 ng/μL) was introduced into a 1.5-mL glass vial, evaporated under a gentle stream of high-purity nitrogen, and dissolved in 200 μL of the above WE. In this experiment, we created 10 termite groups (5 groups from colony D, and 5 groups from colony G).

We compared the total numbers of aggregated individuals between the sample dummies and the solvent dummies for each treatment, using sign test with Bonferroni correction. When comparing the proportion of individuals aggregated around the sample dummies among treatments, we used a GLM approach. In the model, the proportions of workers around the sample dummies were regarded as the responsible variable, assuming a binomial distribution, and the treatments were used as the explanatory variable. The colonies, termite group IDs, and the interaction between treatments and colonies were initially used as the explanatory variable, but as they had no effects on the proportions of workers around the sample dummies ($P > 0.05$), they were excluded from further analysis. Tukey's HSD test was used to perform pairwise comparisons between the treatments. Comparison of the proportion of individuals aggregated around the sample dummies between queens and workers in the same treatment was also conducted by

using a GLM approach. In this model, the proportion was regarded as the responsible variable, assuming a binomial distribution. The castes, the colonies, and the interaction between the castes and the colonies were used as the explanatory variables. All statistical analyses were performed using R software v. 4.0.0 (R Core Team 2016).

Ethics

No specific permits were required for the described field studies, and no specific permissions were required for the locations and termite sampling activities because these fields are public areas, and *R. speratus* is a common species in Japan.

Results

Comparison of cuticular profiles among castes

Using GC-MS analyses, a total of 98 compounds (excluding octadecane internal standard) was detected from the hexane extracts of the primary kings, SQs, soldiers, and workers derived from four colonies (Fig. 2 and see Table S1 in the Electronic Supplementary Material for the list of all of the compounds). A PCA of the 98 compounds detected in any caste in all of the colonies revealed that the soldier group was completely segregated from the other castes (PERMANOVA, $P < 0.001$; Fig. 3). The first two principal components accounted for 82% of the total compound variation. The eigenvector that contributed most to the separation between soldiers and other castes suggested that *β*-elemene and geranyl linalool were primary indicators of soldiers, while C25 was a primary indicator of other castes. But, PK, SQ, and worker groups were not clearly separated from each other, probably because these castes did not have any caste-specific components. This suggests that the chemical component of the cuticular profile is shared among PKs, SQs and workers, but that different composition ratios are involved in the discrimination between kings, queens, and workers.

Among these 98 compounds, 90 compounds were colony-specific, and only eight compounds including *β*-elemene, C23, *n*-tetracosane (C24), 2-methyltetracosane (2meC24), C25, 11-methylpentacosane (11meC25), 2-methylpentacosane (2meC25), and 3-methylpentacosane (3meC25) were commonly detected in all of the colonies in any castes (Fig. 2 and Table S1 in the Electronic Supplementary Material). C23, C25, and 11meC25 were shared in all of the castes from all of the colonies, C24 and 2meC24 were commonly shared only in male soldiers and SQs, and 2meC25 and 3meC25 were shared only in SQs. *β*-elemene was detected only in soldiers, and it is known as a soldier pheromone component in *R. speratus* (Mitaka et al. 2017). Therefore, we focused on the seven CHCs (C23, C24, 2meC24, C25, 11meC25, 2meC25, and 3meC25). Comparisons of the relative amounts of the seven CHCs in total CHCs among castes, the relative amount of C25 was significantly higher in the SQs than in other castes (GLM followed by Tukey's HSD test, $P < 0.05$; Fig. 4, and Table S1 in the Electronic Supplementary Material), but the relative amounts of the other six CHCs did not significantly differ among castes. In addition, butyl butyrate and 2-methyl-1-butanol, which are the components of the volatile queen pheromone in *R.*

speratus (Matsuura et al. 2010), were not detected in QEs. We predicted that the relative amounts of C25 in the cuticular profiles contribute to discrimination of SQs from other castes.

CHC pheromone for queen recognition

To test whether C25 functioned as the queen recognition pheromone, we performed two-choice tests. We observed whether groups of SQs, soldiers, and workers aggregated at the sample dummies coated with 1–100 ng C25 or at the solvent dummies coated with *n*-hexane in bioassay devices. We also tested the sample dummies coated with a mixture of WE and different doses of C25 (hereafter, WE + C25), because we predicted that C25 functioned as the queen recognition pheromone under the condition wherein all *R. speratus* CHC components were complete. We found that SQs aggregated at the WE, WE + C25, and QE, while workers aggregated only at the WE + C25 and the QE (Fig. 5). SQs aggregated significantly more at the sample dummies than at the solvent dummies in the WE, WE + C25 (10–100 ng), and QE treatments (sign test with Bonferroni correction, $P < 0.001/9$); these treatments showed significantly greater aggregation levels (the proportions of individuals aggregated at the sample dummies) than the negative control and C25 (1–100 ng) treatments (GLM followed by Tukey's HSD test, $P < 0.05$). Conversely, workers aggregated at the sample dummies more than at the solvent dummies in the QE and WE + C25 (10–100 ng) treatments (sign test with Bonferroni correction, $P < 0.05/9$); the WE + C25 (10 ng) treatments showed greater aggregation levels than the negative control and WE treatments (GLM followed by Tukey's HSD test, $P < 0.05$). This was consistent with the finding that SQs have 45 ± 13 ng (mean ± standard error of the mean) of C25 per capita on their body surface (Table S1 in the Electronic Supplementary Material). Also, in the WE and WE + C25 (10 ng) treatments, the aggregation level of the workers was lower than that of the queens, and this trend was seen to be common to all of the colonies (GLM, caste: $P < 0.05$, colony: $P > 0.05$, caste × colony: $P > 0.05$). Soldiers did not aggregate at the sample dummies in any treatments (sign test with Bonferroni correction, $P < 0.05/9$), and the aggregation level did not differ among all treatments (GLM followed by Tukey's HSD test, $P > 0.05$).

Furthermore, we investigated whether termites aggregated at the dummies coated with WE and/or C23, which contains a different carbon-chain length from C25 (Fig. 6). Although queens aggregated at the sample dummies more than at the solvent dummies in the WE and WE + C23 (10 ng) treatments (sign test with Bonferroni correction, $P < 0.01/9$), the aggregation levels of these treatments did not differ from that of the negative control (GLM followed by Tukey's HSD test, $P > 0.05$). Soldiers and workers did not significantly aggregate at the sample dummies in any treatment.

Additionally, *R. speratus* SQs, soldiers, and workers showed scarcely any shaking behavior against the glass dummies during the experimental periods.

Discussion

Our GC-MS analysis revealed that the relative abundance of C25 in the total CHCs was greater in SQs than other castes in *R. speratus* (Fig. 4). Although SQs of *R. speratus* emit the volatile pheromone (a mixuture of butyl butyrate and 2-methyl-1-butanol), which is a true signal of the queen's fertility status

and presence (Matsuura et al. 2010; Matsuura and Yamamoto 2011; Yamamoto and Matsuura 2011), the cuticular extract of SQs did not contain these pheromone components (Tables S1 and S2 in the Electronic Supplementary Material); this suggests that these volatiles do not remain on the queen's body surface and that termites use C25-enriched CHC profile to recognize SQs when they make contact with each other. Our behavioral experiments demonstrated that workers aggregated at dummies treated with QE and WE + C25 (10 ng), rather than at dummies treated with WE alone (Fig. 5). On the other hand, workers did not more aggregate at the dummies treated with WE + C23 than at the dummies treated with WE alone (Fig. 6). These results suggest that workers highly respond to the C25-enriched CHC profile, rather than the C23-enriched CHC profile or the worker's CHC profile. Furthermore, in WE and WE + C25 (10 ng) treatments respectively, the mean number of workers aggregated around the sample dummies was less than that of queens (Fig. 5). This means that workers are less arrested by the WE and the WE + C25 than SQs, and that the workers did not accompany the living SQs. We estimated that the queen's C25-enriched CHC mixtures acts like an arrestant pheromone, not a queen retinue pheromone, for workers. Worker might use the queen's CHC profile as a contact chemical cue for starting to feed or groom the queens or to care for eggs laid from them, depending on the queen' condition. Moreover, because the aggregation level slightly decreased in the WE + C25 (1 ng) and WE + C25 (100 ng) treatments (Fig. 5), the proportion of C25 to total cuticular compounds of each individual might be important information for workers to discriminate queens and workers. Although C25 is also used as one of the components of a worker aggregation pheromone in *R. speratus* (Mitaka et al. 2020), this aggregation pheromone is released out of worker's body, and then workers recognize the released pheromone in different behavioral context such as exploiting new nest areas far from a royal chamber (a room for queens and a king) (Mitaka et al. 2020). Thus, the use and the action mechanism of the aggregation pheromone is different from queen recognition pheromone. Also, if increasing the ratio of C25 in the aggregation pheromone than the usual mixture ratio, workers evade from the C25-enriched aggregation pheromone blend (Mitaka et al. 2020). Therefore, recognition mechanisms of both the proportion of C25 and the chemical context play an important role in queen-worker chemical communication in *R. speratus*.

SQs aggregated at dummies treated with QE, WE + C25, or WE (Fig. 5), indicating that the queens aggregate where the workers are located, as well as where other queens are located. The aggregation around workers is reasonable for SQs because each SQ can more easily be fed and groomed by the workers. Moreover, the assembly of SQs may increase their survival rate. If SQs densely aggregate at the royal chamber, they may increase their tolerance to water loss (Klok and Chown 1999). In addition, aggregation of SQs should increase the concentration of antifungal volatile pheromone (unless its emission is controlled by a negative feedback system) because the *R. speratus* volatile queen pheromone inhibits the germination and growth of entomopathogenic and parasitic fungi (Matsuura and Matsunaga 2015). Reinforcement of anti-desiccation and antimicrobial resistance is expected to improve queen survival rates, thereby enhancing egg productivity. Further studies are needed to investigate how and why the SQ aggregation behavior has developed in this species, and whether this behavior is common in AQS termite species and/or the species that neotenic kings and queens always occur in the colony after one or both of the primary reproductives senesces or dies.

Unlike other termite species, *R. speratus* soldiers, workers, and queens scarcely showed any shaking behavior under all treatments. Previous studies have shown that workers (and soldiers) often perform shaking behavior near the queens and kings in some termite species, including *Z. nevadensis* (Penick et al. 2013), *C. secundus* (Korb et al. 2009; Hoffmann et al. 2014), and *R. flavipes* (Funaro et al. 2018, 2019). Although it is presumed that *Z. nevadensis* uses C29–33 alkadienes and alkatriens as a royal (i.e., both king and queen) recognition pheromone (Liebig et al. 2009, 2012) and *C. secundus* uses C25–35 alkanes, methyl-branched alkanes, alkenes, and dienes as a queen recognition pheromone (Hoffmann et al. 2014; Korb 2018), the activities of these CHCs have not been demonstrated. In *R. flavipes*, a royal-specific CHC, *n*-heneicosane, elicits the shaking behavior of workers and soldiers under the presence of a full set of worker CHC components (Funaro et al. 2018). Although shaking behavior is used in various contexts, including alarm, feeding, and hygiene behavior, in many termite species (Howse 1965; Reinhard et al. 2002; Evans et al. 2005; Whitman and Forschler 2007; Ohmura et al. 2009; Sun et al. 2017), *R. speratus* workers show this behavior when they inform nestmates of the presence of predators (Matsuura 2002). This implies that a manner of royal recognition differs among termite species.

In *R. speratus*, C25 is present on the cuticles of all castes across all colonies, but the relative amount of this hydrocarbon is remarkably higher in queens than other castes (Fig. 4, and Tables S1 and S2 in the Electronic Supplementary Material). And C25 functions as a neotenic queen recognition signal only when a blend of conspecific cuticular compounds is included (Fig. 5). These results show that the development process of queen recognition signals in *R. speratus* is partly different from the related species, *R. flavipes*. Both of these two species require conspecific chemical background for neotenic queen recognition However, neotenic queens (and also kings) of *R. flavipes* develop royal-specific hydrocarbon, which is not present on the cuticles of primary reproductives and non-reproductive castes (Funaro et al. 2018; Eyer et al. 2021), while queens of *R. speratus* increase the relative amount of one of the CHCs shared among all castes. These facts suggest that it depends on termite species whether queens synthesize a queen-specific hydrocarbon or increase the relative amount of one of the pre-existing hydrocarbons as a queen signal. Previous studies reported that chemical context was considered as a major factor in queen recognition for the trap-jaw ant *Odontomachus brunneus*. In this ant, (*Z*)-9-nonacosene is shared between queens and workers but is relatively more abundant in queens than workers. And (*Z*)-9-nonacosene functions as a queen pheromone only under the presence of nestmate or near-nestmate chemical background (Smith et al. 2012, 2015). But in some eusocial hymenopterans, queen-specific hydrocarbons function as queen pheromones without conspecific chemical backgrounds; for example, 3-methylhentriacontane in *Lasius niger* (Holman et al. 2010) and *Lasius flavus* (Holman et al. 2013), n-heptacosane, *n*-nonacosane, and 3-methylnonacosane in *Cataglyphis iberica* (Van Oystaeyen et al. 2014), and n-heptacosane, *n*-octacosane, *n*-nonacosane, 3-methylheptacosane, and 3-methylnonacosane in *Vespula vulgaris* (Van Oystaeyen et al. 2014). Therefore, future studies will probably discover a queen-specific hydrocarbon functioning as a queen recognition pheromone without chemical context in a certain termite species.

This study demonstrated that *R. speratus* uses a queen-specific CHC profile as a queen recognition pheromone to trigger aggregation of the workers, and suggests that *R. speratus* queens use both volatile

compounds (as shown in Matsuura et al. 2010) and non-volatile compounds as a signal to indicate the presence of the queen. Our study findings strongly suggest that termites and social hymenopterans independently evolved the use of multicomponent queen signals by parsimonious use of CHCs for other purposes, enabling them to perform multifunctional roles (Le Conte et al. 2008; Matsuura 2012; Leonhardt et al. 2016; Hefetz 2019; Mitaka and Akino 2021). On the other hand, it is still unclear how AQS termites recognize the presence of a primary king in a colony because our GC-MS analysis did not detect any king-specific or king-biased compounds (Tables S1 and S2 in the Electronic Supplementary Material). Therefore, further studies will be required for elucidating the mechanism of king recognition in AQS termites. A comparison of the chemical components and functions of royal's pheromones between termites and social hymenopterans will deepen our understanding of the evolutionary process of the chemical communication that underlies the reproductive division of labor in social insects.

Declarations

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Conflicts of interest

We declare that we have no competing interests.

Availability of data and material

Data supporting the findings of this study are available within the manuscript and online resource.

Code availability

Not applicable.

Authors' contributions

YM designed the experiments. TF collected the wild termites for the GC-MS analyses. YM reared the termites for the bioassays, performed the GC-MS analyses and bioassays, and wrote the manuscript. YM and TF edited the manuscript.

Ethics Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Figures

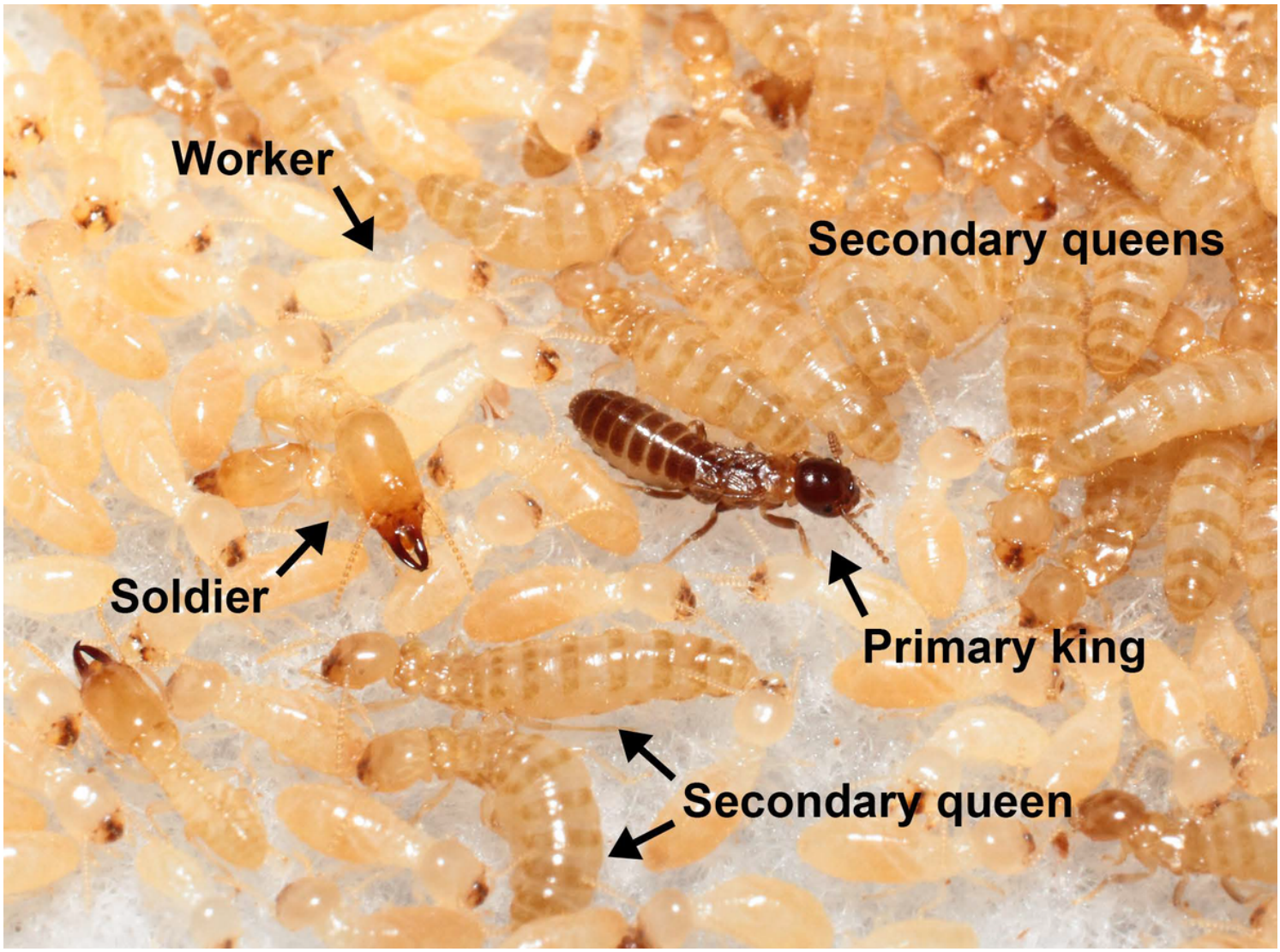

Figure 1

R. speratus colony members. The black individual is the primary king. Brown individuals with enlarged abdomens are secondary queens. Individuals with orange heads and sharp mandibles are soldiers. White

individuals are workers. In this species, while there are tens or hundreds of queens per colony, there is only one king per colony.

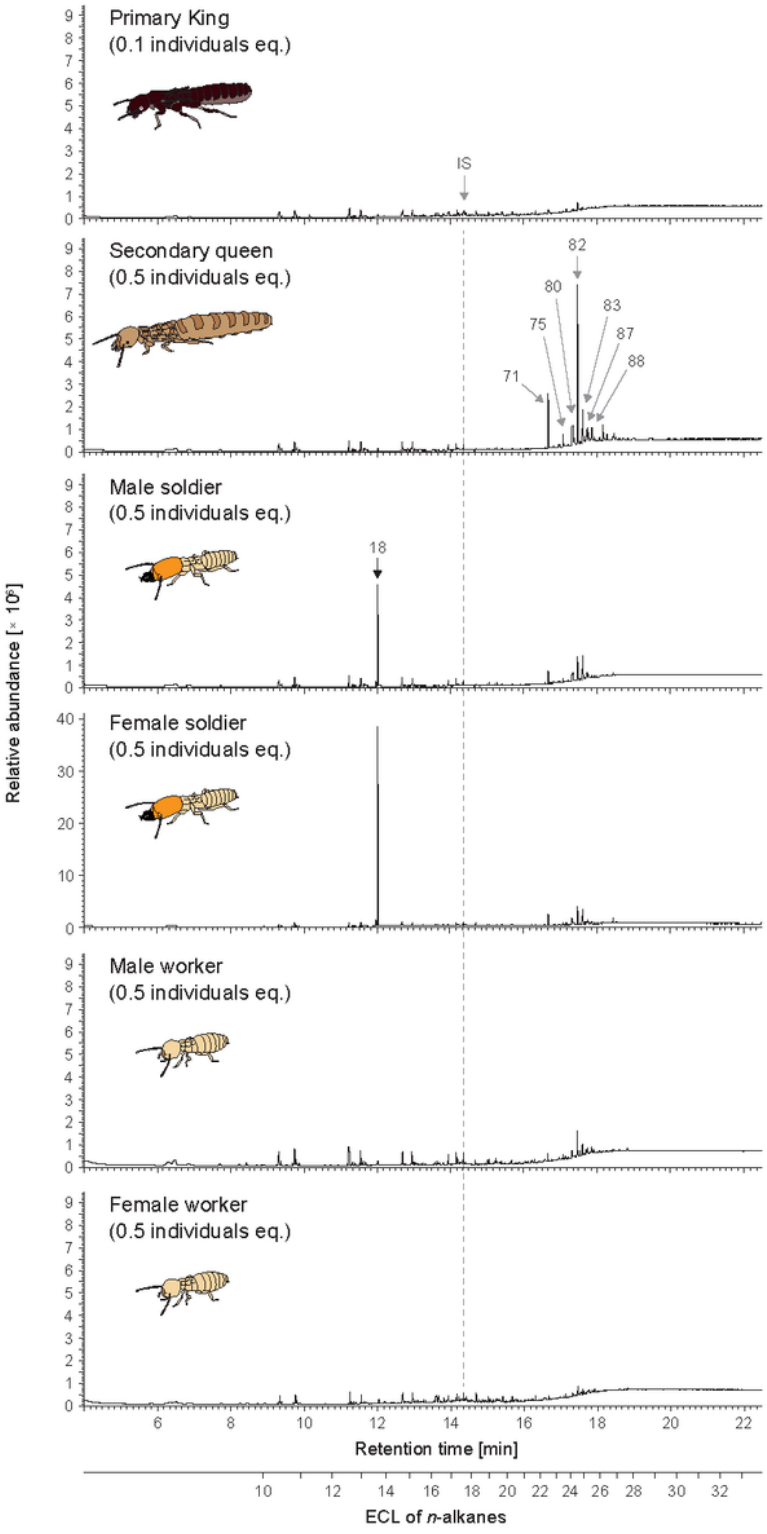

Figure 2

Examples of gas chromatography profiles of the whole-body extracts of the king, queen, and both sexes of soldiers and workers. These gas chromatographs were derived from the same colony (colony C). eq.: equivalent, IS: internal standard (octadecane). Peak numbers correspond to the compound list in Table

S1. Only secondary queens had a disproportionate amount of compound 82 (n-pentacosane). Compound 18 (β-elemene) was specifically detected in both female and male soldiers in all colonies. Compounds 71 (n-tricosane), 75 (n-tetracosane), 80 (2-methyltetracosane), 83 (11-methylpentacosane), 87 (2-methylpentacosane), and 88 (3-methylpentacosane) were detected in all of the castes.

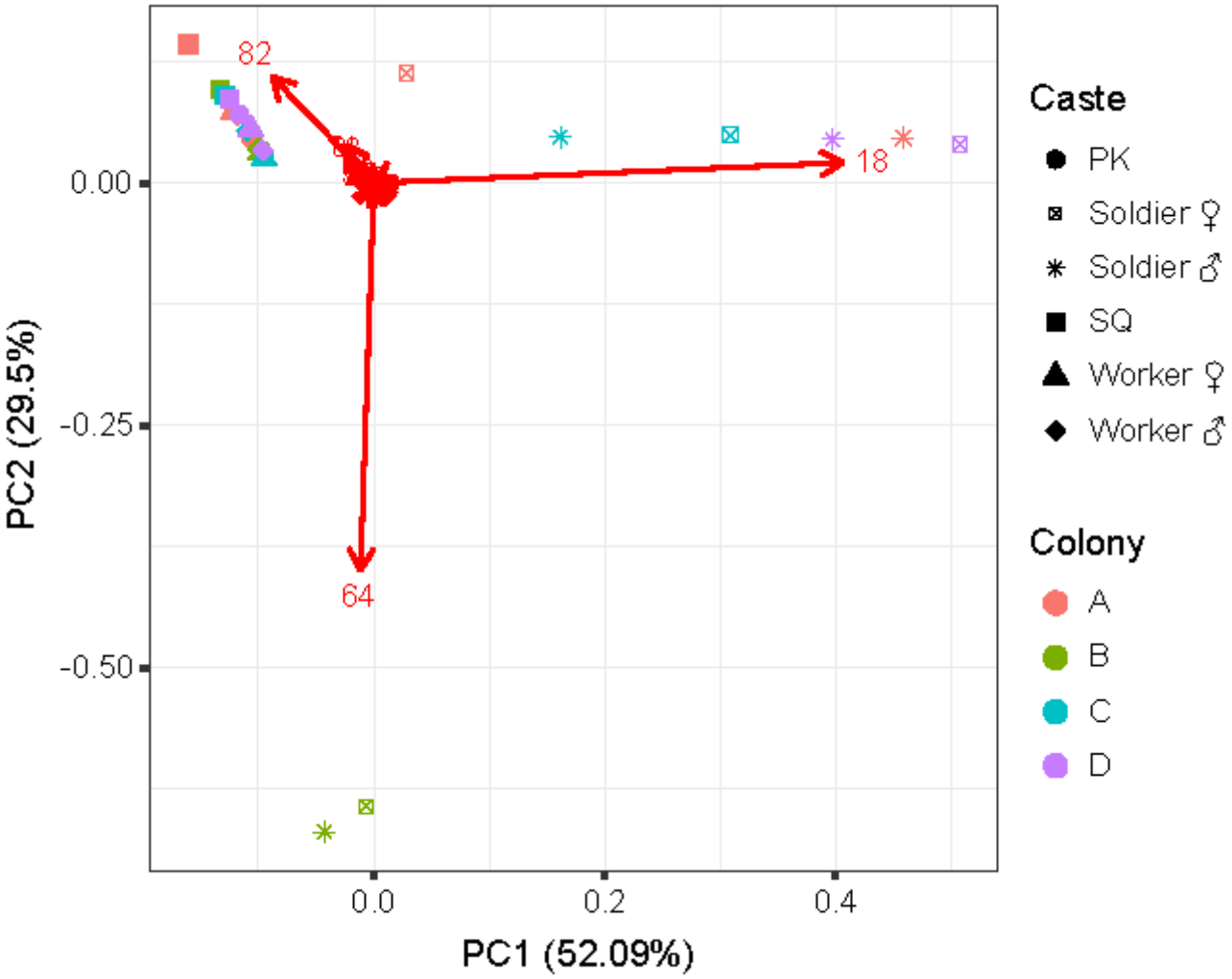

Figure 3

A PCA plot of the gas chromatograms from all castes. Different shapes represent different castes, and each color corresponds to each colony. The percent of variance explained by each principal component is indicated on the horizontal and vertical axes. Numbers near the eigenvectors correspond to the compound numbers. Principal component ordination of 99 GC peaks from primary kings, secondary queens, female and male soldiers, and workers. Compound 18 is β-elemene, compound 64 is geranyl linalool, and compound 82 is n-pentacosane (C25).

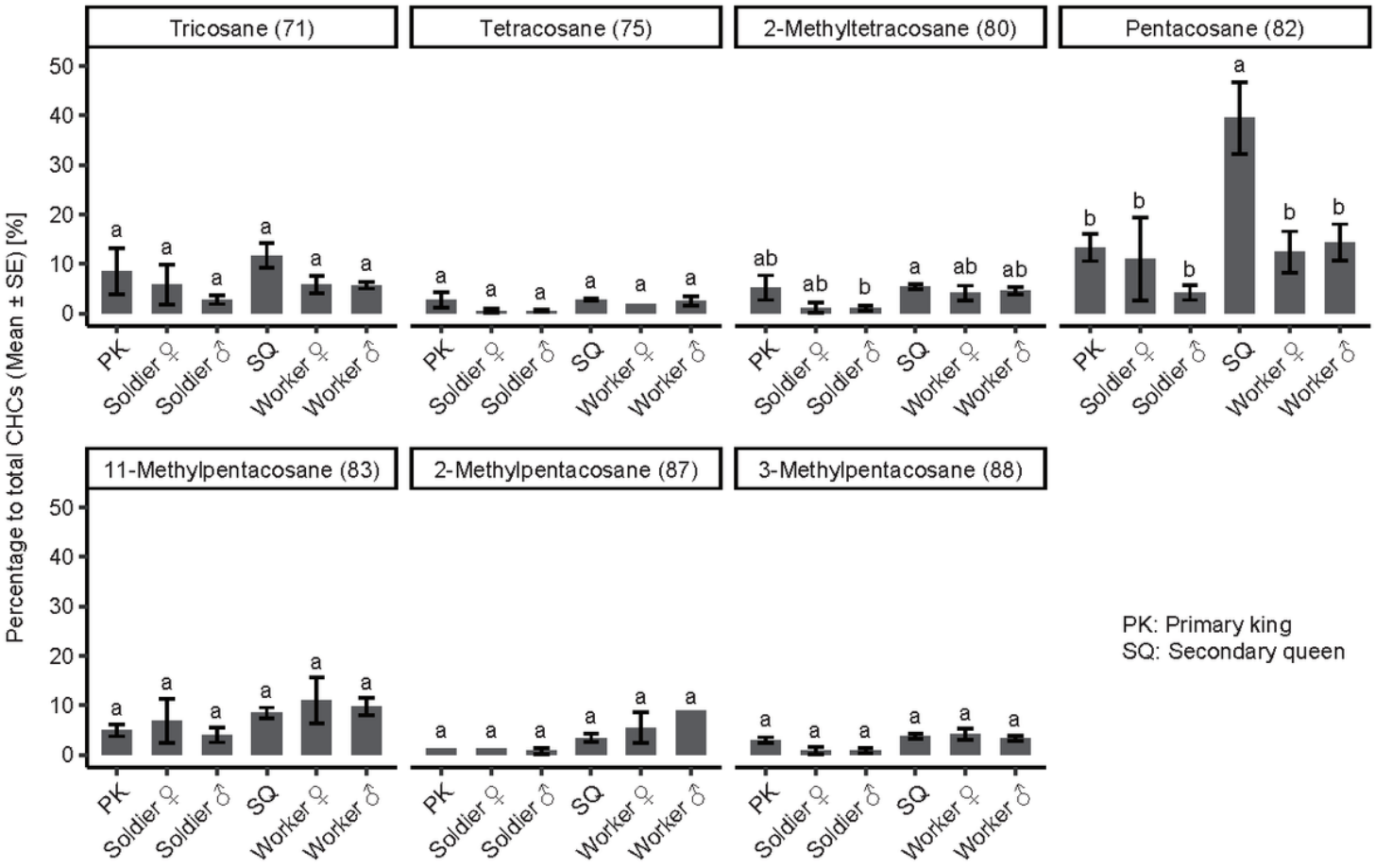

Figure 4

Percentages of seven common CHCs in total cuticular mass of numbered peaks corresponding to peaks listed in Table S1 (numbers after compound names indicate GC peak numbers). Error bars indicate mean ± standard error of the mean. Sample sizes are n = 4 for each caste. Bars without error bars indicate that standard errors could not be calculated because the compound was detected only in one colony in the caste. Different letters indicate significant differences (GLM followed by Tukey's HSD test, $P < 0.05$). Only the percentage of C25 was significantly higher in secondary queens than in the other castes. PK: primary king, SQ: secondary queen.

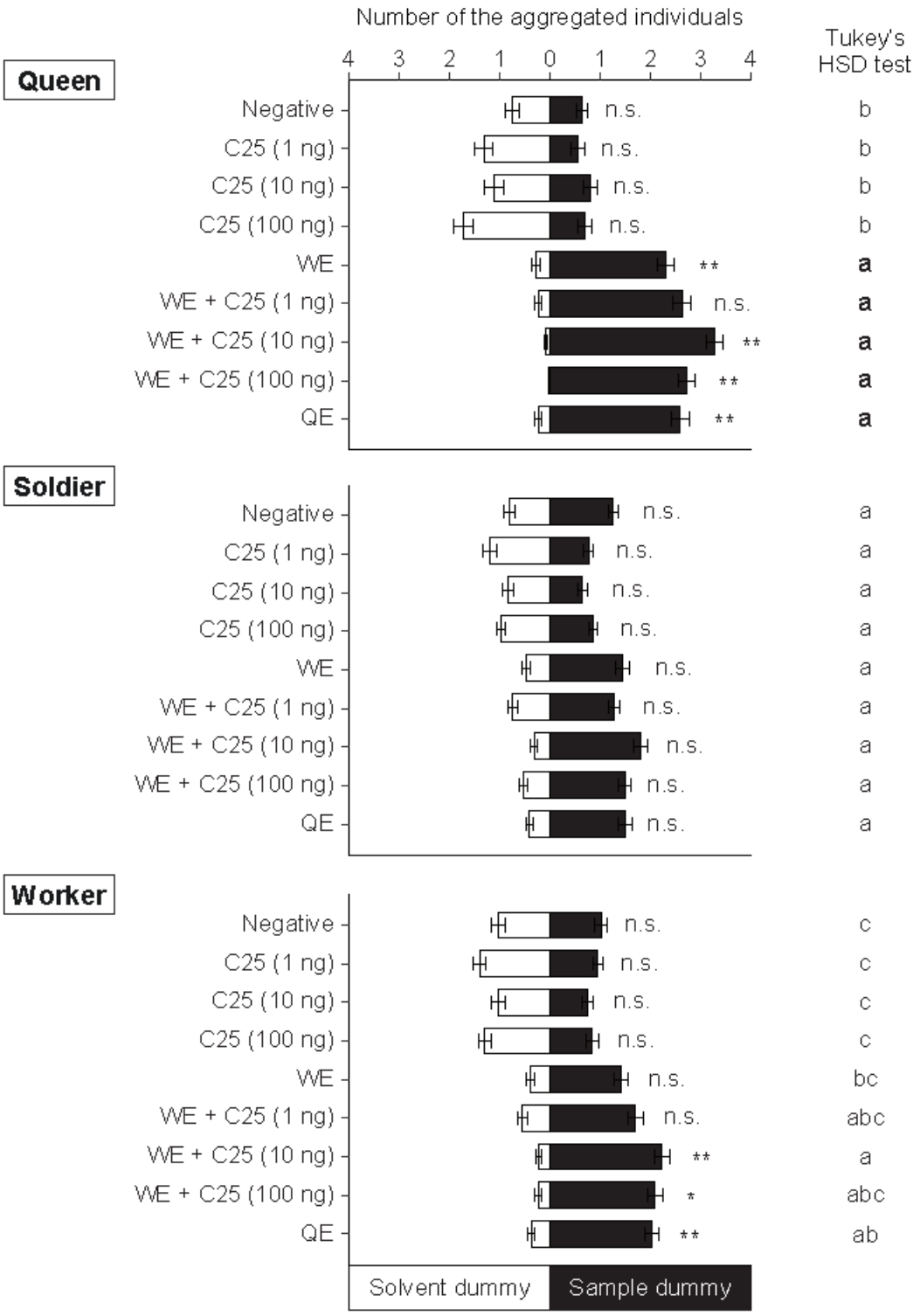

Figure 5

Aggregation responses of secondary queens, soldiers, and workers to glass dummies treated with n-pentacosane (C25) alone and together with worker extract (WE) after 3 min. Each treatment has 13 replications (5 replication from colony D, 3 replications from colony E, and 5 replications from colony G). Right (black) bars indicate mean numbers of individuals aggregated at sample dummies, while left (white) bars indicate mean numbers of individuals aggregated at solvent dummies. Error bars indicate

mean ± standard error of the mean. Asterisks adjacent to bars indicate significant differences between total number of individuals aggregated at sample dummies and total number of individuals aggregated at solvent dummies for each treatment (sign test with Bonferroni correction, *: $P < 0.05$, **: $P < 0.01$, n.s.: not significant). Different alphabets on right side of plots indicate significant differences in the mean proportion of individuals aggregated at sample dummies among treatments (GLM followed by Tukey's HSD test, $P < 0.05$). Queens significantly aggregated at sample dummies treated with worker extract (WE), a mixture of WE and 10–100 ng of C25 (WE + C25), or queen extract (QE) (GLM followed by Tukey's HSD test, $P < 0.05$). Workers significantly aggregated at sample dummies treated with QE or a mixture of WE and 10 ng of C25 (GLM followed by Tukey's HSD test, $P < 0.05$).

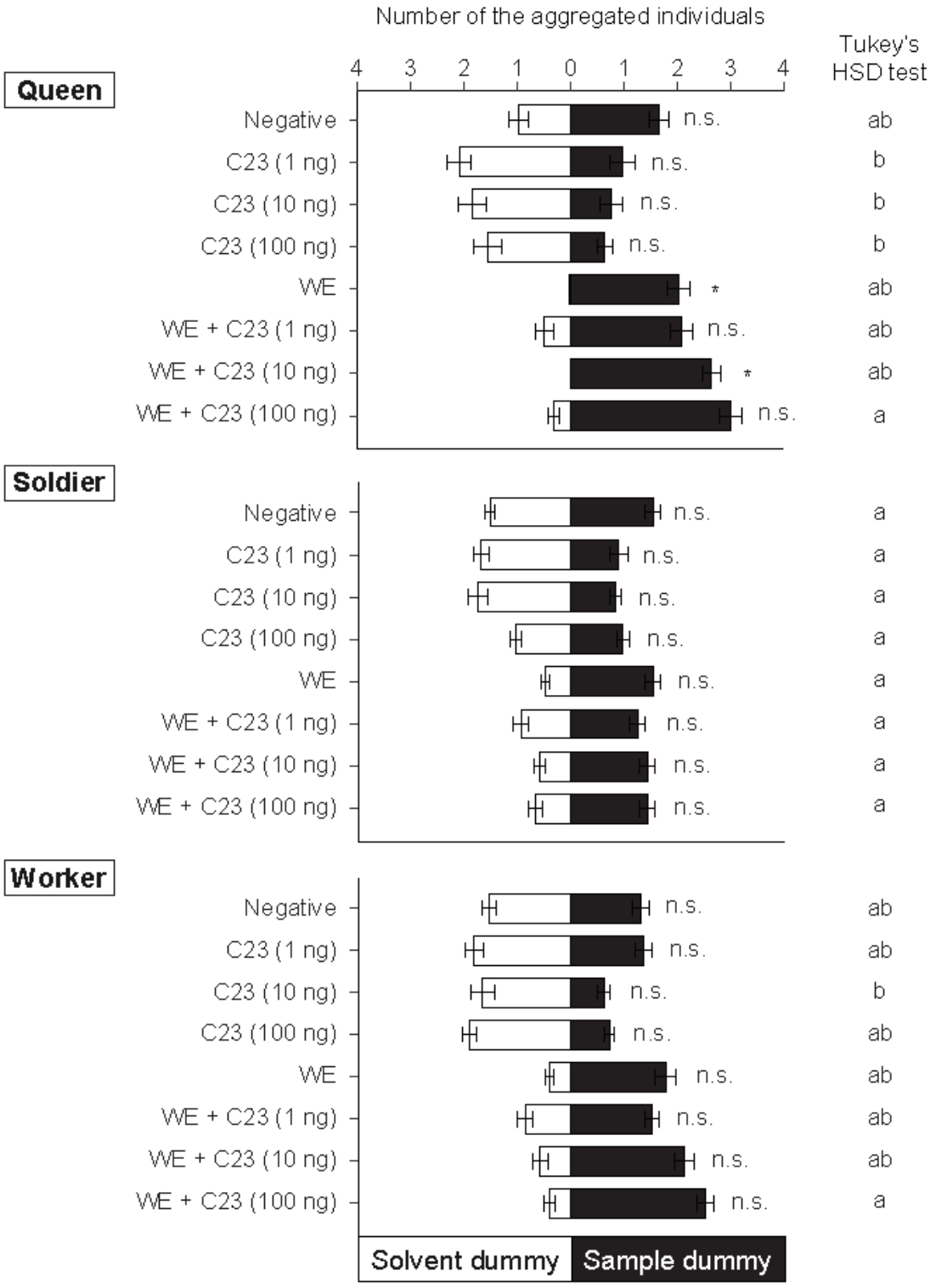

Figure 6

Aggregation responses of secondary queens, soldiers, and workers to glass dummies treated with n-tricosane (C23) alone and together with worker extract (WE) after 3 min. Each treatment has 10 replications (5 replications from colony D, and 5 replications from colony G). Right (black) bars indicate mean numbers of individuals aggregated at sample dummies, while left (white) bars indicate mean numbers of individuals aggregated at solvent dummies. Error bars indicate mean ± standard error of the

mean. Asterisks adjacent to bars indicate significant differences between total number of individuals aggregated at sample dummies and total number of individuals aggregated at solvent dummies for each treatment (sign test with Bonferroni correction, *: $P < 0.05$, n.s.: not significant). Different alphabets on right side of the plots indicate significant differences in the mean proportion of individuals aggregated at sample dummies among treatments (GLM followed by Tukey's HSD test, $P < 0.05$). None of the castes aggregated significantly more at sample dummies treated with WE or a mixture of WE and C23 (WE + C23), compared to sample dummies treated with the negative control.

Supplementary Files

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