

Scent of a queen—cuticular hydrocarbons specific for female reproductives in lower termites

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Abstract In social insects, it is assumed that signals of the queen inform nestmates about her reproductive status. Thus, workers forego their own reproduction if the queen signals high fertility. In hemimetabolous termites, little is known about reproductive inhibition, but evidence exists for a royal-pair control. Workers of lower termites exhibit a high developmental flexibility and are potentially able to become reproductives, but the presence of a fertile reproductive restrains them from reaching sexual maturity. The nature of this control, however, remains unknown. Here, we report on qualitative differences in cuticular hydrocarbon profiles between queens and workers of the basal drywood termite *Cryptotermes secundus*. Queens were characterized by a shift to long-chained and branched hydrocarbons. Most remarkably, similar chemical patterns are regarded as fertility cues of reproductives in social Hymenoptera. This might suggest that both groups of

social insects convergently evolved similar chemical signatures. The present study provides deeper insights into how termites might have socially exploited these signatures from sexual communication in their cockroach-like ancestor.

Keywords Termites · Cuticular hydrocarbons · Fertility signals · Chemical communication · Queen signal

Introduction

Reproductive division of labor is a key characteristic of social insects. In colonies of basal termites, all ontogenetically totipotent immatures (false workers, formerly also called pseudergates) have the possibility to develop via a single molt into neotenic replacement reproductives (Korb and Hartfelder 2008). Nevertheless, the queen is the sole egg-layer within the colony, and the development of a false worker into a neotenic reproductive only occurs when a colony's reproductive of the same sex dies or becomes unhealthy. The mechanism that prevents colony members from becoming a reproductive is poorly understood. In eusocial insects, the maintenance of the reproductive hierarchy is generally assured by the recognition of a fecund queen due to her characteristic chemical signature that indicates her ovarian activity (e.g., Peeters et al. 1999; Cuvillier-Hot et al. 2001; Heinze et al. 2002; Dietemann et al. 2003; Sledge et al. 2004; Cuvillier-Hot et al. 2005; Hartmann et al. 2005; Lommelen et al. 2006; Sramkova et al. 2008). Such chemical compounds of the queen's cuticular are often regarded as honest signals that inform nestmates of the presence of a fertile and healthy queen and therefore ensure an evolutionary stable regulation of worker sterility (Keller and Nonacs 1993).

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Wood-dwelling termites spend their entire life in the darkness of a single piece of wood, and most colony members are blind; thus, communication via scent is conceivable. It was shown that chemical communication occurs in termite colonies (e.g., Howard et al. 1982; Haverty et al. 1996; Haverty et al. 1997; Bagnères et al. 1998; Sevala et al. 2000; Dronnet et al. 2006). However, the relationship between cuticular hydrocarbon (CHC) profiles and fertility was not investigated in termites so far.

To study whether chemical cues exist that signal the presence of an established and fecund queen, we compared the CHC profiles of female neotenic and false workers of the drywood termite *Cryptotermes secundus*.

Materials and methods

Termites

Colonies of *C. secundus* were collected in mangroves near Darwin (NT, Australia). Colony rearing and the generation of neotenic queens were performed as previously described (Weil et al. 2007).

Chemical analyses

CHCs were obtained by solid phase microextraction (SPME) of 80 *C. secundus* termites, 40 false workers and neotenic queens, respectively. We used queens of established colonies with young larvae and eggs showing that the analyzed queens were reproducing. The reproductive system of false workers can be well developed in lower termites, but false workers never lay eggs (Grassé 1982). They are immature incapable of reproducing. The cuticular surface of an individual was gently rubbed for 5 min with a Supleco (Deisenhofen, Germany) polydimethylsiloxane fiber for SPME (df 7 μm for gas chromatography (GC) and df 100 μm for GC-mass spectrometry (GC-MS)) and then immediately placed in the injection port of a GC.

GC analysis was performed with an Agilent Technologies 6890N GC equipped with a *split-splitless* injector (280°C; purge valve opened after 5 min), a flame-ionization detector (FID), and a HP-5 fused silica capillary column (30 m \times 0.32 mm ID; df 0.25 μm , J&W Scientific) using Helium as carrier gas (constant flow, 1 ml min^{-1}). The temperature programming was as follows: 120–150°C at 30°C min^{-1} , 150–280°C at 4°C min^{-1} , and 280–300°C at 10°C min^{-1} .

GC-MS was performed with an Agilent Technologies 6890N GC coupled to an Agilent 5973 inert mass selective detector (70 eV ionization voltage). The GC was equipped with a RH-5 ms+ fused silica capillary column (30 m \times 0.25 mm ID; df 0.25 μm , J&W Scientific). GC conditions were set as mentioned above, but the *split-splitless* injector

was set 250°C with the purge valve opened after 60 s. For GC-MS analyses, we used both SPME and hexane extractions. For the latter, ten false workers or three queens were pooled and extracted in 400 μl of distilled hexane for 5 min. The extracts were reduced to a final volume of 50 μl by a gentle stream of nitrogen at room temperature.

MSD ChemStation software (Agilent Technologies) was used for data acquisition. Linear compounds were identified by retention times, NIST MS library (Gaithersburg, MD, USA), and fragmentation patterns. Methyl alkanes were identified by diagnostic ions, standard MS databases (see above), and by determining Kovats indices using the method of Carlson et al. (1998).

Statistical analyses

In total, we found 55 peaks. However, there were large qualitative differences in the chemical profiles between both castes with several peaks being only present in one caste. To analyze these qualitative differences between false workers and neotenic queens, a multiple correspondence analysis (MCA, XLSTAT 2008, Addinsoft, Andernach, Germany) was performed. MCA is an extension of correspondence analysis, which allows one to analyze the pattern of relationships of several categorical dependent variables. Therefore, all peaks were classified as being present (1) or absent (0) for each tested individual. Peaks that contributed highly to the difference between the studied castes were selected. Differences between castes for the detected compounds were compared with Mann–Whitney *U* tests using SPSS 15.0 (SPSS, Chicago, IL, USA). All tests were two-tailed. The significance level α was corrected for the number of tests using Bonferroni adjustments.

Results

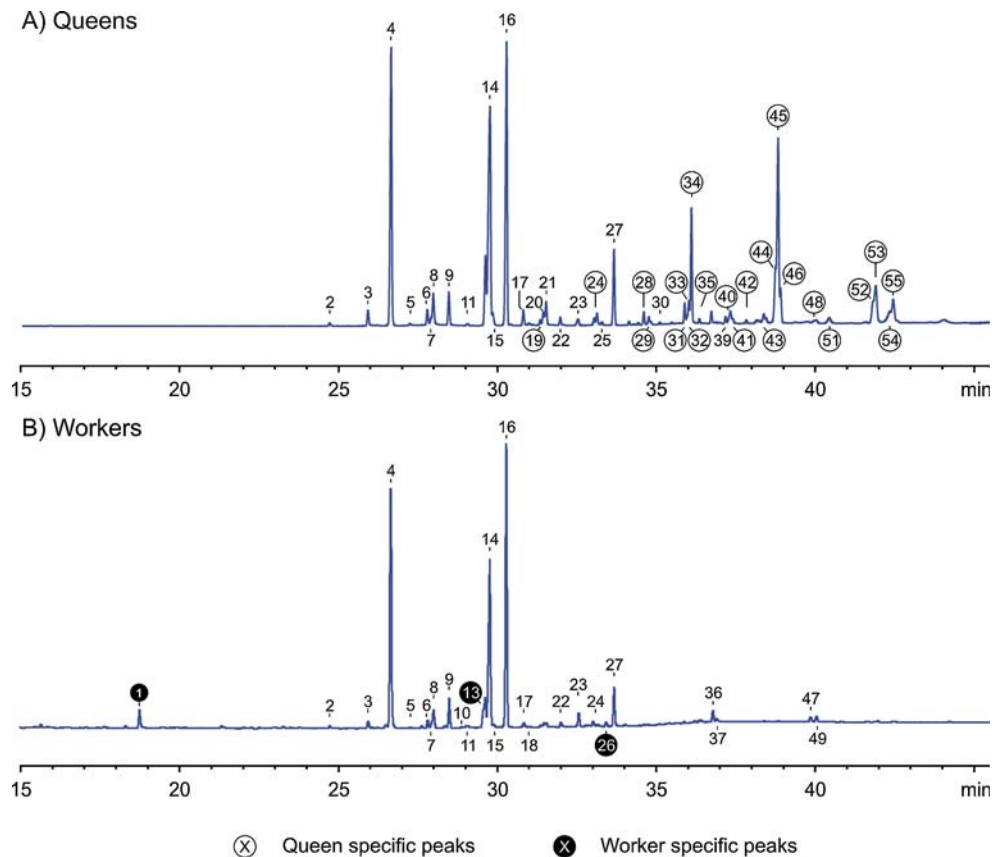
C. secundus neotenic queens and false workers exhibit different CHC profiles as shown in Fig. 1. For the 80 analyzed individuals, 55 peaks were obtained by GC analysis (FID). Using SPME and hexane extractions, 38 substances could be identified with GC-MS according to their characteristic mass spectral fragmentation patterns. The general CHC profile of *C. secundus* consisted of linear alkanes, monomethyl-branched alkanes, and linear alkenes with chain lengths from C21 to C35 (Fig. 1). It was dominated by *n*-C25, *n*-C27, C27:1, and *n*-C29 (Fig. 1).

According to the MCA analysis of the 55 compounds, false workers were clearly separated from neotenic queens by their CHCs (Fig. 2). There was no misclassification; all individuals of one caste clustered together (Fig. 2). The neotenic queens were separated from false workers on the

Fig. 1 Representative chromatograms of cuticular hydrocarbons extracted by SPME from a neotenic queen (a) and a false worker (b) of *C. secundus*.

Peaks 12, 38, and 50 are not labeled as they are not visible in these specific chromatograms. Only identified peaks are listed:

1 *n*-C21; 2 *n*-C24; 3 4meC24; 4 *n*-C25; 5 13meC25; 6 4meC25; 7 C26:1; 8 3meC25; 9 *n*-C26; 13 4meC26; 14 C27:1; 15 C27:1 16 *n*-C27; 17 13meC27; 19 4meC27; 20 C28:1; 21 3meC27; 22 *n*-C28; 24 4meC28 + C29:1; 25 C29:1; 27 *n*-C29; 28 4meC29; 29 3meC29; 30 *n*-C30; 31 4meC30; 33 C31:1; 34 C31:1; 35 *n*-C31; 36 13meC31; 39 4meC31; 41 3meC31; 44 C33:1; 45 C33:1; 46 C33:1; 52 C35:2; 53 C35:2; 54 C35:1; 55 C35:1



basis of function 1 (F1), which explained 88.9% of the total variance (Fig. 2). According to their contributions and cosine² values of the variables to function 1, 25 peaks were selected. Twenty two of these peaks were queen specific, while three peaks were specific for false workers. Besides long-chained alkenes and *n*-C31, mainly monomethyl-branched alkanes were characteristic for neotenic queens (Fig. 1a). Six queen specific compounds could not be

identified due to small amounts (peaks: 32, 40, 42, 43, 48, 51; Fig. 1a). *n*-C21, 4meC26, and an unknown compound (peak 26) were specific for false workers (Fig. 1b). All 25 compounds differed significantly between the tested castes (Mann–Whitney *U* test $P \leq 0.0001$; < corrected $\alpha = 0.002$; Supplementary Table 1).

Discussion

Our analyses revealed distinct, qualitative differences in the CHC profiles of non-reproducing false workers and neotenic queens of *C. secundus*. Especially branched hydrocarbons were characteristic for the queen's CHCs. We observed a shift to hydrocarbons with longer chain lengths in neotenic queens. These two characteristic chemical patterns have been discussed as fertility cues of reproductives in social Hymenoptera (Cuvillier-Hot et al. 2001; Heinze et al. 2002; Diemann et al. 2003; Hartmann et al. 2005; Liebig et al. 2000; Lommelen et al. 2006). The similarity may indicate that this CHC pattern also functions as a fertility signal in *C. secundus*. Possibly, a similar fertility signature independently evolved in hemimetabolous termites and holometabolous social Hymenoptera despite their different ancestry. Methyl-branched hydrocarbons are known from a wide range of insects, including solitary species

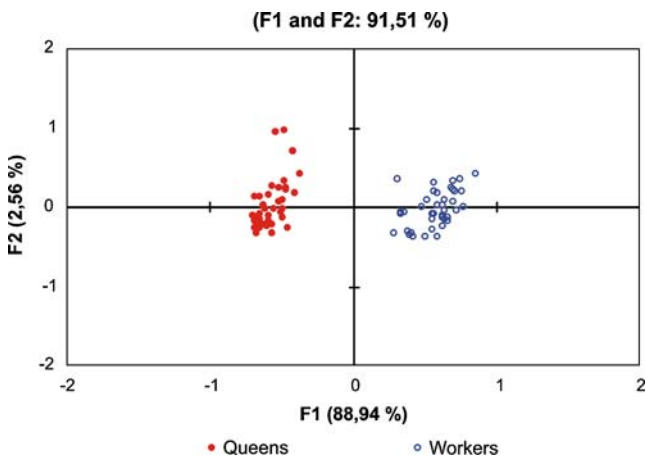


Fig. 2 Correspondence map depicting MCA results. Neotenic queens are completely separated from false workers on the basis of function 1 (F1), which explains 88.94% of the variance

(Howard and Blomquist 1982). Thus, they might have been good potential candidates for contact pheromone signals in social hymenoptera and in termites that were exploited during social evolution.

In termites, caste-specific variations in CHCs were described mainly for non-reproductives (workers, soldiers and nymphs) and “hopeful” reproductives (alates before colony foundation) (Howard et al. 1982; Haverty et al. 1996; Haverty et al. 1997; Bagneres et al. 1998; Sevala et al. 2000; Dronnet et al. 2006). Former studies also included unsexed neotenic reproductives in their analyses, but did not achieve good discrimination between reproductive and non-reproductive castes, likely due to the small sample sizes used (Howard et al. 1982; Bagneres et al. 1998). Furthermore, the main emphasis of these studies was on analyzing developmental stages rather than on fertility signals.

It is unknown whether the queen’s scent is sufficient to maintain her reproductive primacy in termites. Similar to Lüscher (1974), Brent et al. (2005) suggested that the termite queen releases inhibitory stimuli, which result in a reversible endocrine inhibition of the sexual development in nestmates and thus maintains the queen’s reproductive dominance (Lüscher 1974; Brent et al. 2005). So far, clear evidence is lacking whether the inhibitory signal is spread throughout the termite colony via proctodeal trophallaxis or whether it has an olfactory basis (Bordereau 1985; Noirot 1990; Korb 2005). In cockroaches (Blattodea), to which the termites belong (Inward et al. 2007), the epicuticle of sexual mature females contain contact pheromones, which elicit courtship response upon contact with the male’s antennae. Interestingly, methylbranched C27 and C29 carbons are active components of the female contact sex pheromone (Eliyahu et al. 2008). Stimulatory tergal compounds are also discussed for termites as sex-specific cues (Park et al. 2004). This might suggest that similar sex-specific contact pheromones were exploited by termites during social evolution.

In summary, our results demonstrate that CHCs differed significantly among neotenic queens and false workers of *C. secundus* and therefore might provide information about the presence of a reproductive. Interestingly, among holo- and hemimetabolous social insects, female reproductives reveal similarities in the signature of CHCs, which might reflect common mechanisms in maintaining and/or signaling reproductive status.

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