

Cuticular hydrocarbon profiles indicate reproductive status in the termite *Zootermopsis nevadensis*

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Abstract Reproductive division of labor in social insects is accompanied by the reliable communication of individual fertility status. A central question is whether there exists a general mechanism underlying this communication system across species. The best way to produce reliable information is through physiological markers tightly associated with reproductive status. Cuticular hydrocarbons exhibit this link to individual fertility in several species of ants, bees, and wasps, and we present the first evidence for such a system in a non-Hymenopteran eusocial species. In the termite *Zootermopsis nevadensis*, we identified four polyunsaturated alkenes, which only occur in significant amounts on reproductives that are actively producing gametes. These compounds are either absent or only occur in small amounts in soldiers, worker-like larvae, and secondary reproductives with inactive gonads. In contrast to Hymenopteran social insects, both sexes express the reproductive peaks. The reproductive-specific hydrocarbons may promote tending behavior by worker-like larvae or act as a primer pheromone, inhibiting the reproductive development of immature conspecifics.

Keywords Fertility signal · Queen pheromone · Reproductive inhibition · Social insects · Isoptera · Dampwood termite

Introduction

Several insect groups have independently evolved eusociality (Wilson 1971; Danforth 2002), exhibiting a reproductive division of labor in which one or just a few individuals within a colony are reproductively active and the remaining members are functionally sterile (Wilson 1971; Gadagkar 1994; Sherman et al. 1995). The organizational structure and stability of eusocial colonies depend on communication between reproductive and non-reproductive individuals, often through pheromones (Keller and Nonacs 1993), to maintain the central division of labor. Elucidating the fundamental elements of such communication system will greatly enhance our understanding of the function and evolution of these insect societies.

Reproductive-specific pheromones can have several functions within the colony, serving as behavioral activators and physiological primers. As activators, they can elicit tending behavior in workers, ensuring that the reproductive individuals are groomed and fed and that their eggs are collected and cared for. They can also elicit aggressive policing behaviors in non-reproductives directed toward individuals producing a competing reproductive signal within a colony. As primers, pheromones have the potential to suppress nestmate fertility. The current model suggests that functional reproductives produce a chemical signature that identifies their level of fertility (Heinze 2004; Monnin 2006; Le Conte and Hefetz 2008; Peeters and Liebig 2009). When perceived by target individuals, these signals affect the activity of mechanisms controlling

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reproductive development and gametogenesis, possibly by influencing the endocrine system (Hartfelder and Emlen 2005; Grozinger and Robinson 2007). In the ultimate sense, these compounds may promote self-regulated inhibition simply by allowing conspecifics to recognize the proximity of functional reproductives (Keller and Nonacs 1993).

For many eusocial insects, there is mounting evidence that cuticular hydrocarbons may be the primary means of conveying reproductive-specific information. Only in the honey bee, *Apis mellifera*, and the fire ant, *Solenopsis invicta*, is there clear evidence of non-hydrocarbon pheromones being used in this capacity (Vargo 1997; Hoover et al. 2003; Slessor et al. 1988). Although the original function of the cuticular lipid layer of insects is to protect against pathogens and water loss, hydrocarbons also function as chemical messengers between and within species (Lahav et al. 1999; Thomas et al. 1999; Wagner et al. 2000; Akino et al. 2004; Howard and Blomquist 2005; Dani 2006; Martin et al. 2008). Queen-specific hydrocarbon signatures have been identified in ants (e.g., Monnin et al. 1998; Peeters et al. 1999; Liebig et al. 2000; Cuvillier-Hot et al. 2001; Hannonen et al. 2002; Heinze et al. 2002; Tentschert et al. 2002; Dietemann et al. 2003; de Biseau et al. 2004; Smith et al. 2008), a bee (Ayasse et al. 1995), and wasps (e.g., Bonavita-Cougourdan et al. 1991; Sledge et al. 2001) with up to 60% of the hydrocarbon profile specific to highly reproductive queens (de Biseau et al. 2004; Endler et al. 2004, 2006). In the ant *Aphaenogaster cockerelli*, it has been experimentally shown that a *n*-alkane reveals the reproductive status of workers (Smith et al. 2009). Similar caste encoding has also been found in the hydrocarbon profiles of eggs from several ants (Monnin and Peeters 1997; D'Ettorre et al. 2004; Endler et al. 2004, 2006; Dietemann et al. 2005), possibly regulating differential egg destruction and worker reproduction (Endler et al. 2004).

The broad occurrence of this connection between hydrocarbons and the reproductive division of labor within the social Hymenoptera suggests that it may also occur in other non-hymenopteran social insects. To determine if that was the case, we examined the primitive dampwood termite *Zootermopsis nevadensis* (Order Isoptera) for hydrocarbon correlates of reproductive status. In addition to having a very different evolutionary origin, termites also provide an opportunity to examine whether reproductive-specific information is also sex specific. Both male and female reproductives occur in termite colonies, whereas in social Hymenoptera, the reproductive males do not return to the nest after leaving to mate. In *Z. nevadensis* and its sister species *Zootermopsis angusticollis*, there is evidence that functional reproductives are able to suppress the reproductive development and activity of their nestmates in a sex-specific manner (Heath 1903; Castle 1934; Light and Weesner 1951). This requires

that either they produce a sex-specific inhibitory pheromone or a sex-independent pheromone that is paired with a pheromone that indicates sex, e.g., from sternal glands (Pasteels 1972). Although pheromonal inhibition has been repeatedly implicated in the reproductive division of labor within termite colonies (Castle 1934; Light and Weesner 1951; Hewitt et al. 1972; Lüscher 1972; Greenberg and Stuart 1979; Lefeuvre and Bordereau 1984; Watson and Abbey 1985; Vieau 1990), research on the possible suppressive influence of volatile compounds has failed to demonstrate their efficacy or even their existence in termites. However, recent work on termite hydrocarbons has shown that there are caste- and species-specific differences (Clement and Bagnères 1998; Sevala et al. 2000), suggesting that these compounds might also encode other individual-specific information such as information about fertility affecting the behavior and development of nestmates. In the termite *Cryptotermes secundus*, hydrocarbon differences between neotenic queens and workers have been found (Weil et al. 2009). Whether these differences are due to caste-specific changes or are caused by differences in fertility is unknown, but the latter scenario is more likely, considering the frequency with which it has been found in hymenopteran species (Peeters and Liebig 2009).

In this study, we compared the hydrocarbon profiles of reproductively active males and females with those of non-reproductive individuals, the worker-like larvae and soldiers, in *Z. nevadensis*. We identified alkadienes and alkatrienes specific to the hydrocarbon profiles of all reproductive individuals, which may contribute to colony reproductive dynamics. This is the first demonstration of a linkage between fertility and a potential signaling mechanism in a termite.

Materials and methods

Experimental animals

Stock colonies of *Z. nevadensis* were collected in June of 2006, August 2007, and July 2008 from the Del Monte Forest in Pebble Beach, CA, USA. Colonies were removed from their natal nest and placed in a more accessible nest consisting of several pre-cavitated layers of moistened Douglas fir sheets bolted together. These nests were kept in closed plastic boxes at 25°C and were regularly sprayed with water to ensure high humidity. The termites were identified as the subspecies *nuttingi* according to location and their subspecies-specific hydrocarbon pattern (Haverty et al. 1988; Haverty and Thorne 1989).

Except for one pair of physogastric primary reproductives, all of these colonies were headed by multiple neotenic replacement reproductives. These individuals

were identified by cuticles that were much more darkly pigmented than those of their nestmates' (Heath 1903; Castle 1934). Putative reproductive and non-reproductive individuals were periodically sampled from 26 colonies for determination of hydrocarbon profiles (females: secondary reproductives—14 from 10 colonies, worker-like larvae—nine from eight colonies, soldiers—19 from 12 colonies; males: secondary reproductives—12 from nine colonies, worker-like larvae—10 from nine colonies, soldiers—12 from eight colonies). Individual reproductive status was verified by dissection under a stereomicroscope (Leica MZ125). Females were examined for the presence or absence of distinct yellow bodies (follicular remnants) as an indicator of recent ovipositional activity. Male testis diameter, an indicator of sperm production capacity (Brent and Traniello 2001a), was measured using an ocular micrometer.

Chemical extraction and gas chromatography for profile determination

The hydrocarbon profiles of male and female workers and reproductives were obtained by hexane extraction. The whole insect was shaken in a Teflon capped borosilicate glass vial containing 200 μ l hexane (Sigma-Aldrich, St. Louis, MO, USA) for 2 min. These hexane extracts were dried with high-purity nitrogen, then resuspended with 30 μ l hexane. One microliter aliquots of the hexane extracts were injected into an Agilent 6890N GC (Agilent, Santa Clara, CA, USA) coupled with an Agilent 5975 mass selective detector, operated in the electron impact ionization mode. The GC was operated in splitless injection mode with helium as carrier gas at 1 ml/min flow rate. It was fitted with a 30 m \times 0.25 mm (ID) \times 0.1 μ m DB-1MS non-polar column (Agilent). The oven temperature was programmed to rise from 60°C to 200°C at 40°C min⁻¹ after an initial delay of 2 min, including a splitless time of 0.5 min. Subsequently, the temperature rose from 200°C to 320°C at 5°C min⁻¹. Injector temperature was 260°C, MS quad 150°C, MS source 230°C, and transfer line 300°C.

Statistical analysis

For the comparison of peak areas and to exclude compounds that are at the threshold of detection by gas chromatography, we included only peaks that are present in at least 50% of members of the following six groups: reproductive females, reproductive males, non-reproductive females, non-reproductive males, and female and male soldiers. These peaks were standardized to 100% and relative peak areas refer to this set of compounds. We compared the relative amount of the four compounds that were typical for reproductives between the different female castes using a Kruskal–Wallis test with subsequent pairwise

comparison using Mann–Whitney *U* test. Although darker coloration of reproductives is already a criterion for their identification, we used the presence of yellow bodies as an indication of long-term reproduction for the classification of secondary reproductives versus workers. Not all secondary reproductives are active gamete producers, so verification of fertility status is crucial. Soldier identification is clear due to their distinct head morphology. For male reproductives, we used a product–moment correlation between compound amounts and testis development (STATISTICA 7.1, Statsoft). Sex-specific differences and quantitative differences between reproductives, workers, and soldiers were analyzed using non-parametric multidimensional scaling (PRIMER 6.0). For the analysis of quantitative differences, we excluded the four peaks typical for reproductives and reset the remaining 10 peaks again to 100%. We used Euclidean distances in the analysis.

Chemical identification of reproductive compounds

Reproductive individuals possessing the reproductive-specific hydrocarbons were freeze-killed, and their cuticular lipids were extracted in 200 μ l hexane (Sigma-Aldrich) for 1–2 min. The extract was fractionated by flash column chromatography over 200 mg of 10% silver-nitrate silica gel (70–230 mesh; Fisher Scientific, Fair Lawn, NJ, USA) and eluted with 2 ml each of hexane, ether, ethyl acetate, and methanol (Sigma-Aldrich). Each fraction was tested for the presence of the reproductive-specific compounds by GC-MS analysis (see below). The fraction containing the compounds was then subjected to dimethyl disulfide (DMDS; Alfa Aesar, Ward Hill, MA, USA) derivatization to locate double-bond positions in alkadienes and alkatrienes. The fraction was dried under N₂ in a 0.3-ml conical reaction vial to ~10 μ l. Ten microliters of 5% iodine (Alfa Aesar) in diethyl ether and 1 μ l of DMDS were added and left at room temperature for 24 h. A 5% aqueous solution of sodium thiosulfate (Alfa Aesar) was added to the reaction vial until the solution became clear, and then the organic phase was transferred to a new vial. The aqueous phase was washed with hexane twice, and organic phases were pooled and analyzed by GC-MS. For alkatrienes, derivatization by DMDS was not sufficient for determining the position of the double bonds, and ethanethiol (Fisher Scientific) derivatization was used for further indication. A fraction containing the reproductive-specific compounds was similarly dried under N₂ in a 0.3-ml conical reaction vial to ~25 μ l. Twenty-five microliters of 5% iodine in diethyl ether and 40 μ l of ethanethiol were added and incubated in a 50°C bead bath for 48 h. A minimum amount of 5% aqueous solution of sodium thiosulfate was then added to the reaction vial, and the organic phase was transferred to

a new vial. The aqueous phase was washed with hexane twice, and the organic phases were pooled and analyzed by GC-MS, as described above. The oven temperature was programmed to rise from 60°C to 250°C at 20°C min⁻¹ after an initial delay of 2 min and from 250°C to 320°C at 5°C min⁻¹ and held at 320°C for 15 min. Injector temperature was 260°C, MS quad 150°C, MS source 230°C, and transfer line 250°C.

Results

Chemical identification

In *Z. nevadensis*, both female (Fig. 1a) and male (Fig. 1c) reproductives have cuticular hydrocarbon profiles with four polyunsaturated alkenes that are not found in significant amounts in worker-like larvae or soldiers (Figs. 1b, d and 2). Compound 1 had fragmentation characteristics and a retention index identical to an authentic 6,9-nonacosadiene (courtesy of Thomas Schmidt), both in its natural and its DMDS-derivatized state. The identification of compounds 2–4 is based solely on fragmentation patterns of natural and derivatized forms, and final confirmation awaits comparison with authentic compounds. They are tentatively identified as 6,9-hentriacontane (2), 6,9,17-dotriacontane (3), and 6,9,17-tritriacontane (4).

Compounds 5 to 14 were identified by their typical fragmentation patterns and in comparison with alkane standards and with previously published data (Haverty et al. 1988). We found one difference in the identification. Compound 9 was consistently assigned to x,y-tricosadiene with the typical fragmentation pattern and a molecular ion

mass of 320. Haverty et al. (1988) reported 2- or 4-methyltricosane at this position.

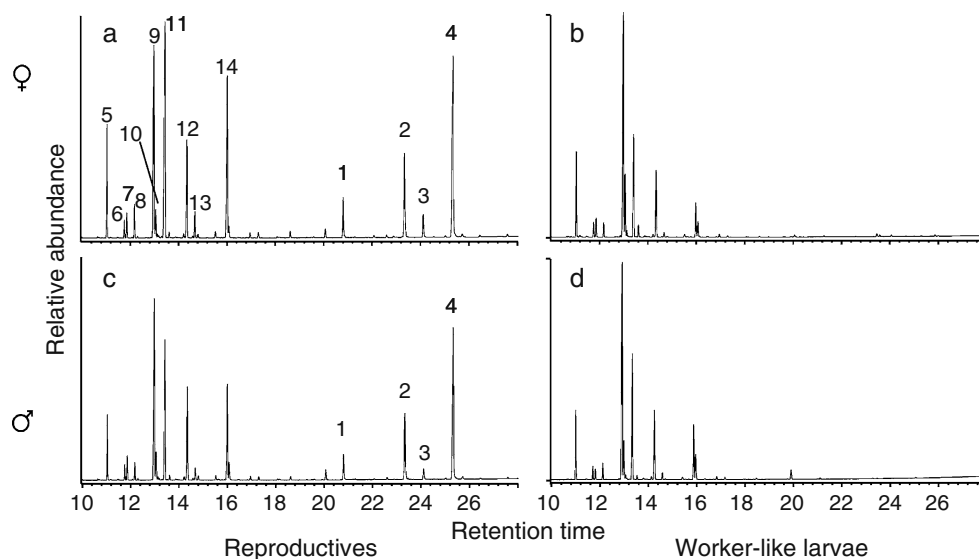
Cuticular hydrocarbon profiles and reproductive status

In females, expression of the reproductive-specific hydrocarbons at high concentrations is linked with the appearance of yellow bodies (Fig. 2). These residual tissues are reliable indicators of recent reproductive activity, and their presence in the ovaries is strongly associated with the production of vitellogenic oocytes. Six out of seven females with yellow bodies also had vitellogenic oocytes (median 21, range 0 to 42), while the size of the yellow bodies in the seventh female indicated that she had recently stopped laying eggs. Neotenic females without visible yellow bodies also lacked vitellogenic oocytes and expressed significantly less of the reproductive-specific hydrocarbons.

The reproductive-specific hydrocarbons accounted for 16% to 38% (median 25%) of the total amount of hydrocarbons in the profiles of all reproductives. The profile of workers and soldiers averaged close to 0% for these compounds. The difference in peak area between the four groups was statistically significant (Fig. 2, $N=42$, Kruskal–Wallis test, $H=24.9$, $p=0.0001$). In male reproductives, the relative quantities of these compounds are correlated with testis volume (Fig. 3; $N=12$, product–moment correlation, $r=0.86$, $p=0.0003$, $r^2=0.74$), which has been found to be a reliable indicator of sperm production capacity (Brent and Traniello 2001a). Soldier testis development was at the lowest end of the range.

The hydrocarbon profiles did not provide sex-specific information about individuals from each of these three castes. Multidimensional scaling, using profiles that did

Fig. 1 Abundance of hydrocarbons in female reproductives (a) and worker-like larvae (b) and male reproductives (c) and worker-like larvae (d) of the termite *Z. nevadensis*. There are significant differences between castes, particularly at the four polyunsaturated alkane peaks (numbered), which are only found on reproductives (see text for details). Chromatograms are representative of these categories. Identification of compounds: 5 C21, 6 2- or 4-MeC21, 7 3-MeC21, 8 C22, 9 x,y-C23:2, 10 9-C23:1, 11 C23, 12 3-MeC23, 13 C24, 14 C25



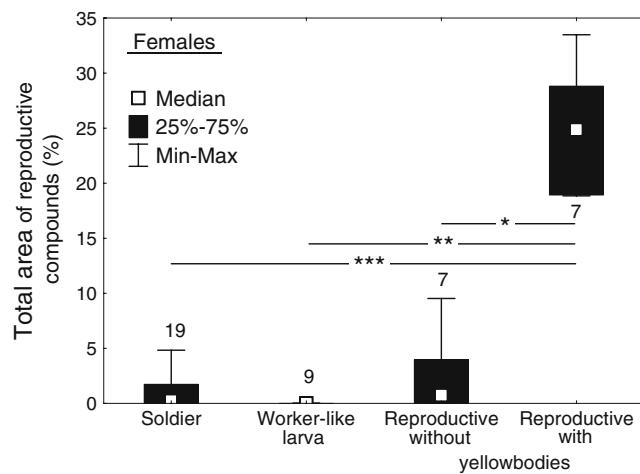


Fig. 2 Percent expression of reproductive-specific hydrocarbons in female secondary reproductives with and without yellow bodies, worker-like larvae, and soldiers. Comparison between secondary reproductives with yellow bodies and the other groups with Bonferroni adjusted alpha-level of 0.017, * $p < 0.002$, ** $p < 0.0003$, *** $p < 0.0001$. Numbers next to the box indicate sample size

(Fig. 4a) or did not (Fig. 4b) include reproductive-specific peaks, failed to separate females and males from each other.

Discussion

The comparison of the cuticular hydrocarbon profiles of reproductive individuals, non-reproductive worker-like larvae, and soldiers in the dampwood termite *Z. nevadensis* demonstrated the presence of reproductive-specific compounds in a non-Hymenopteran social insect. Four polyunsaturated alkenes occur in the profiles of reproductive males and females in high concentrations, while

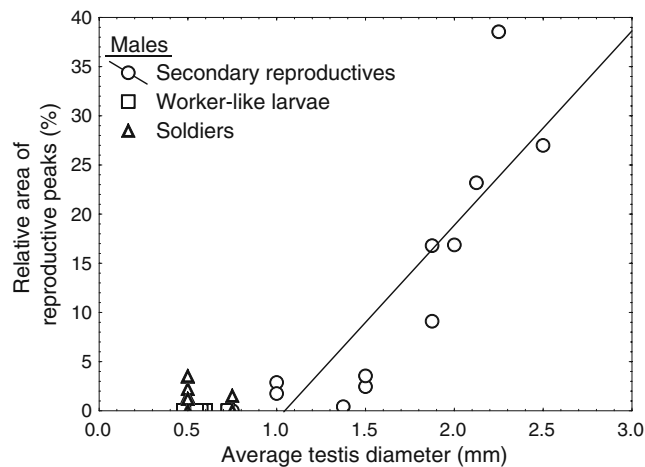


Fig. 3 Correlation between testis diameter and relative area of the four reproductive-specific peaks. Only secondary reproductives were used for calculating the correlation. Soldiers ($N = 12$) and worker-like larvae ($N = 10$) were added for comparison

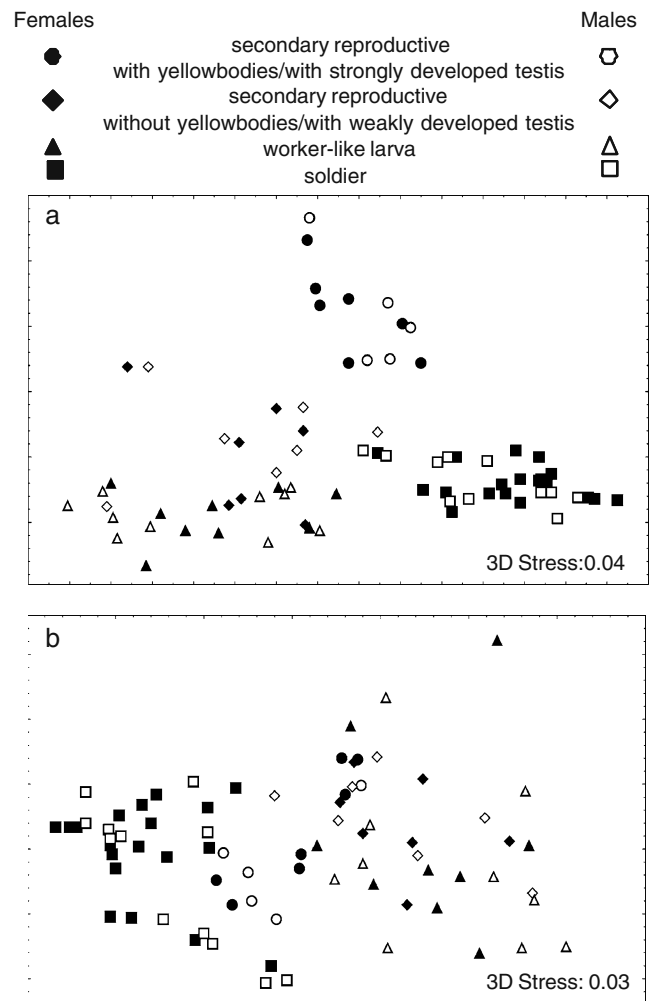


Fig. 4 Non-parametric multidimensional scaling of the hydrocarbon profiles of different termites grouped by caste and sex. Neither the inclusion of the reproductive-specific peaks (a) nor their exclusion (b) led to a separation of the sexes. Sample size is the same as in Figs. 2 and 3. Male reproductives were divided into strongly reproductive (testis development above 1.7 mm) and weakly reproductive. Stress values below 0.05 are considered to provide an excellent reflection of the data structure (Clarke and Warwick 2001). Adding the third dimension did not change separation

worker-like larvae and soldiers of both sexes either lack these compounds or express them in minor amounts. In fact, the relative proportions of these polyunsaturated alkenes correlate significantly with the extent of gonad development, indicating that the expression may be scaled with the relative fertility of these individuals.

The correlation of hydrocarbon profiles with testis development in males and their association with the yellow bodies in females strongly suggest that these profiles are used to communicate reproductive status to other colony members, similar to many Hymenopteran social insects (Monnin 2006; Le Conte and Hefetz 2008; Peeters and Liebig 2009). If the expression pattern does signal to

nestmates an individual's relative fertility, it has the potential to attract tending behavior and to be used as a regulatory mechanism for colony reproductive dynamics.

Hydrocarbons are known to be used by many insects for intra- and interspecific signaling (Howard and Blomquist 2005), and caste- and sex-specific differences have already been found in other termite species (Pasteels and Bordereau 1998; Uva et al. 2004). Most recently, qualitative differences in the cuticular hydrocarbon profiles have been found between neotenic females and workers in the termite species *C. secundus* (Weil et al. 2009). Although those results suggest a linkage between fertility and profile, they do not provide sufficient physiological evidence to resolve whether that is the case or whether there is simply a caste-specific difference in hydrocarbon expression. Nevertheless, these findings coupled with our own data suggest that the correlation between fertility and cuticular hydrocarbon expression may be a general pattern found in social insects.

The exact functions of the reproductive-specific hydrocarbons of *Z. nevadensis* remain to be determined. Two possible roles are as promoters of tending behaviors or as inhibitors of gonad development or activity in non-reproductive nestmates, which are not necessarily mutually exclusive. Among the eusocial Hymenoptera, there are several examples of a tending function. In the ant *Myrmecia gulosa*, extracts of the hydrocarbon profile of reproductive queens are more attractive to workers than similar extracts from non-reproductive workers (Dietemann et al. 2003), suggesting that they may trigger grooming or feeding. More fertile queens are tended more frequently in the ants *Leptothorax* sp. A and *Formica fusca* (Ortius and Heinze 1999; Hannonen et al. 2002). In the *Formica* species, higher attraction was also associated with differences in their cuticular hydrocarbon profiles. So far, a similar association between cuticular hydrocarbon profiles and queen tending behavior has not been established in termites.

The second proposed function, as an inhibitor of nestmate reproductive activity, is equally likely. Currently, there are two proposed mechanisms for how termite reproductives might inhibit nestmate fertility: direct manipulation through agonistic interactions (Zimmerman 1983) or indirect inhibition through pheromones (Pickens 1932). There is little evidence to support the former argument (Roisin 1994; Korb 2005), and previous experiments with *Zootermopsis* give no indication of aggression or direct physical manipulation (Rosengaus and Traniello 1993; Brent and Traniello 2001a, b). This suggests pheromonal regulation in *Zootermopsis* (Pickens 1932; Castle 1934; Lüscher 1972), although previous tests for volatile primer pheromones have been inconclusive. However, it may not be necessary for pheromones to operate over long distance to be effective throughout a colony; nestmate inhibition by contact may also succeed. This is particularly plausible for

species like *Zootermopsis* and other basal termites, in which colonies are composed of at most a few thousand members and are contained in small, discrete nests with high contact rates. The strongest candidate compounds for such a short-range recognition function in termites are hydrocarbons such as we identified on the reproductive males and females. Potential distribution mechanisms are currently being explored by the authors.

Although the identified reproductive-specific hydrocarbons may play a role in regulating the reproductive development of nestmates, they alone are not sufficient to induce the sex-specific inhibitory effect observed in *Zootermopsis* (Light and Weesner 1951). We did not find differences in the expression of cuticular hydrocarbons beyond those related to reproduction in any of the types of individuals we investigated. The lack of divergence between the profiles of reproductive males and females is also remarkable in that it indicates a shared physiological basis that lies upstream of their sex-specific physiology. However, if the fertility-related hydrocarbons regulate reproduction in a single sex, an additional sex-specific pheromone, e.g., from sternal glands (Pasteels, 1972), is required.

It is not surprising that the phylogenetically distant Hymenoptera and Isoptera show a similar link between reproductive physiology and cuticular hydrocarbon profiles. Hydrocarbon expression is a primitive system that is likely to have been conserved throughout the insects and other arthropods. Although it may have originally evolved as a water conservation mechanism (Gibbs 1998), it has acquired additional communicative functionality. The sensory machinery for hydrocarbon detection is present in solitary insects and is extensively used for species and mate recognition (Howard and Blomquist 2005). For some solitary species, the mate recognition functionality of hydrocarbons appears to have a physiological basis similar to that of fertility signaling in social insects. The onset of ovarian activity is correlated with changes in the cuticular hydrocarbon profiles in houseflies (Dillwith et al. 1983), burying beetles (Steiger et al. 2007), and spiders (Prouvost et al. 1999), enabling males to determine which females are ready to mate.

Furthermore, the hydrocarbon profile is quite likely to convey reliable information about reproductive state, so it would be evolutionarily favored. Current evidence suggests that the mechanisms governing hydrocarbon synthesis and expression share the same endocrine controls as those regulating reproductive capability. Strong links between hydrocarbon biosynthesis, reproductive status, and endocrine activity have already been described for the ant *Streblognathus peetersi* (Cuvillier-Hot et al. 2004; Brent et al. 2006), the flies *Calliphora vomitoria* (Trabalon et al. 1994) and *Musca domestica* (Dillwith et al. 1983), and the cockroach *Blattella germanica* (Schal et al. 1993, 1994, 1997; Sevala et al. 1999; Fan et al. 2002; Holbrook et al. 2000). Termites are

closely related to roaches (Kambhampati 1995) and are likely to share many of the same regulatory mechanisms. In *Zootermopsis*, juvenile hormone titers shift with changing reproductive status and appear to drive gametogenesis (Greenberg and Tobe 1985; Brent et al. 2005). Also in these termites, hydrocarbons are delivered to the cuticle and other tissues via a lipophorin carrier system (Schal et al. 1998; Sevala et al. 2000). Termite lipophorins also have a high affinity for juvenile hormone III (Okot-Kotber and Prestwich 1991a, b). Binding to JH may change the conformation of the lipophorin, which in turn can change the hydrocarbons it can transport to the cuticle (Sevala et al. 2000). Because of this interlinking system of controls, any change in termite reproductive status would be accompanied by a change in the hydrocarbon profile, keeping nestmates accurately informed and able to produce appropriate physiological and behavioral responses.

Finally, the structural diversity of cuticular hydrocarbons has most likely served as a preadaptation for communicative functions. There is tremendous variation across species in the hydrocarbons expressed and the functions that they have acquired. Just within the social Hymenoptera, the hydrocarbons correlated with reproductive status include *n*-alkanes, alkenes, and methyl-branched alkanes (Monnin 2006; Liebig unpublished). Variations in the composition of this complex profile, either via qualitative or quantitative differences, allow a social insect to simultaneously encode a wide array of information, including reproductive status and colony membership (Denis et al. 2006). Cuticular hydrocarbon profiles can also contain information about morphological and behavioral caste, sex, and age (Howard and Blomquist 2005).

The adaptability and potential for complex communication of the hydrocarbon signaling system may have led to the convergence on this mechanism in the evolution of social arthropods. If that is the case, we predict that similar usage of cuticular hydrocarbons will be found throughout the termites.

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