MULTIFUNCTIONAL QUEEN PHEROMONE AND MAINTENANCE OF REPRODUCTIVE HARMONY IN TERMITE COLONIES

KENJI MATSUURA

Laboratory of Insect Ecology, Graduate School of Agriculture, Kyoto University
Kitashirakawa Oiwakecho, Kyoto 606-8502, JAPAN
E-mail: kenjijpn@kais.kyoto-u.ac.jp

Abstract

Pheromones are likely involved in all social activities of social insects including foraging, sexual behavior, defense, nestmate recognition, and caste regulation. Regulation of the number of fertile queens requires communication between reproductive and non-reproductive individuals. Queen-produced pheromones have long been believed to be the main factor inhibiting the differentiation of new reproductive individuals. However, since the discovery more than 50 years ago of the queen honeybee substance that inhibits the queen-rearing behavior of workers, very little progress has been made in the chemical identification of inhibitory queen pheromones in other social insects. The recent identification of a termite queen pheromone and subsequent studies have elucidated the multifaceted roles of volatile pheromones, including functions such as a fertility signal, worker attractant, queen–queen communication signal, and antimicrobial agent. The proximate origin and evolutionary parsimony of the termite queen pheromone are also discussed.

Keywords: termite queen pheromone, semiochemical, pheromone parsimony, caste differentiation, primer pheromone

INTRODUCTION

Termite (the order Isoptera) consists of over 2,600 species worldwide (Kambhanpati and Eggleton, 2000). They are grouped according to their feeding behavior in the categories: subterranean, soil-feeding, drywood, dampwood and grass eaters. Subterranean termites build an intricate network of tunnels in soil, connecting multiple nest sites and wood. Most pest species of subterranean termites in the Northern Hemisphere belong to the genera *Reticulitermes* and *Coptotermes*. Due to their cryptic habitat in soil, subterranean termites are difficult not only to control but also to study. Especially, their population ecology and reproductive biology largely remain to be understood.

The sophisticated colony organization of eusocial insects is maintained primarily through the utilization of pheromones. Queen-specific pheromones have several functions within the colony, serving as releaser pheromones (behavioral activators) and primer pheromones (physiological primers). Releaser pheromones, which change the probability of performing a certain behavior upon perception, can elicit queen-tending behavior in workers, ensuring that the queens are groomed and fed and that their eggs are cared for. They can also elicit aggressive policing behaviors in workers against individuals producing a competing reproductive signal within a colony. Primer pheromones have the potential to suppress nestmate fertility (Hoover et al. 2003; Le Conte and Hefetz 2008). Functional reproductives produce a chemical signature that identifies their level of fertility (Keller and Nonacs, 1993; Vargo, 1999; Peeters and

Liebig, 2009). When perceived by target individuals, these signals affect their reproductive development possibly by influencing the endocrine system (Danty et al. 1999; Grozinger and Robinson, 2007; Fussnecker et al. 2011). However, since the discovery more than 50 years ago of a queen honeybee substance (Butler et al., 1959), very little progress has been made in the chemical identification of queen pheromones in other social insects, especially termites.

Approximately 2,600 species of termites have been described (Kambhampati and Eggleton, 2000). Seven families defined within Isoptera are classically grouped into the lower termites (all families except the Termitidae) and the higher termites (Termitidae). The former group includes termites with hindgut protozoa, whereas the family Termitidae contains protozoa-independent species. Neoteny is a widespread phenomenon in lower termites. The term neotenic reproductive refers to any termite reproductive that is not derived from an alate (Thorne, 1996). Neotenic reproductives differentiate within their natal colony, never dispersing to outbreed. These neotenic individuals can differentiate from either nymphs to become ‘nymphoid’ reproductives with wing pads or from workers to become ‘ergatoid’ reproductives without wing pads. Neotenic reproductives have been reported in 61.7% of lower termite genera but in only 13.4% of higher termite genera (Termitidae) (Myles, 1999). Neotenics develop as replacement reproductives in response to orphaning or as supplementary reproductives even in the presence of functional reproductives. The mechanism controlling the decision between neotenic reproduction and other options within the network of developmental pathways remains unclear.

Two possible mechanisms may regulate how termite reproductives inhibit nestmate fertility. One is direct physical manipulation through agonistic interactions. Another is inhibition through pheromones. Little evidence exists to support the former hypothesis (Roisin, 1994). Based on observations of *Reticulitermes hesperus*, Pickens (1932) first hypothesized that an inhibiting secretion, produced by a queen, prevented the development of other reproductives in the colony. Shortly afterward, studies of *Zootermopsis* offered strong evidence for the existence of inhibitory pheromones (Castle, 1934; Light, 1944). Lüscher (1961) proposed the famous model of social control, which posits that reigning reproductives (kings and queens) inhibit the development of neotenics by inhibitory substances distributed among nestmates through mutual contact. The absence of this inhibition results in the formation of neotenics. Pheromonal inhibition has been repeatedly reported in the reproductive division of labor within termite colonies (Castle, 1934; Light and Weesner, 1951). However, the chemistry of a putative queen pheromone was not identified in any termite species until very recently.

VOLATILE OR NON-VOLATILE?

In terrestrial environments, chemosignals can be either volatile or non-volatile. Colony members can recognize reproductives by reproductive-specific “surface pheromones” through direct physical contact. In addition to non-volatile surface pheromones, the utilization of volatile signals enables members to recognize the presence of reproductives at a distance. Colony size can affect the form of such chemical messages. In small colonies, direct interactions result in reproductive hierarchies (Fletcher and Ross, 1985). For example, in single-site wood nesters, including the families Termopsidae and Kalotermitidae, whose entire lives are spent at a single wood dwelling that serves as both shelter and food, maximum colony size is relatively small, ranging from 600 to 8,000 (reviewed by Lenz, 1994). In such single-site nesting species with small colony size, inhibition depends on direct physical contact and not on volatile substances (Lüscher, 1952; Springheti, 1972), and even a brief absence of functional reproductives triggers neotenic development (e.g., 48 h in *Zootermopsis angusticollis* [Stuart, 1979] and *Kalotermes flavicollis* [Kindl and Hrdý, 2005]).

When the society becomes large, it would be impossible for all colony members to contact the reigning queens directly. Only a small percentage of the members, i.e., retinue workers and soldiers, contact the reproductives. Thus, selection then favors the evolution of volatile queen pheromones, which transmit queen messages to many more individuals at one time within the colony. Early studies have suggested the possible existence of volatile queen pheromones for both lower termites (Lüscher, 1952; 1961) and higher termites (Camazine et al., 2003). However, the existence of volatile pheromones does not preclude the existence of surface pheromones. Non-volatile cues on the body surfaces of queens are essential for queen recognition, even after the evolution of volatile queen pheromones. Therefore, relatively primitive termites that live in small colonies would likely only use surface pheromones, whereas species that form large colonies should utilize both non-volatile and volatile queen pheromones.

About a half-century ago, Edward O. Wilson had already noted the discrepancy in the number of studies of volatile and non-volatile pheromones (Wilson, 1965). For various technical reasons, volatile pheromones have been relatively easy to extract and bioassay. On the other hand, non-volatile surface pheromones have proven far more refractory. When removed from other surface odors or when masked by alien substrates added during conventional extraction techniques, surface pheromones no longer produce pheromonal effects. In addition, bioassays for surface pheromones are also extremely difficult even if a queen-specific compound is found. To test the pheromone activity of a surface pheromone, a successful bioassay requires such a good dummy queen as to be recognized as a true queen by colony members. When we identified the termite egg recognition pheromone, i.e., egg surface pheromone, dummy eggs composed of glass beads induced egg-protection behavior by workers, as did true eggs (Matsuura et al., 2007). This bioassay was successful for egg recognition because physical cues are very simple, as the dummy items need only have the same size and smooth spherical surface as eggs. In the case of queen surface pheromones, however, physical cues would be far more complicated. Bioassays might be nearly impossible if they required behavioral cues.

In many eusocial Hymenoptera, cuticular hydrocarbons provide information about the egg-laying ability of female reproductives (Peeters et al., 1999; Liebig et al., 2000; Cuvillier-Hot et al., 2001; Sledge et al. 2001; Hannonen et al., 2002; Heinze et al., 2002; Dietemann et al., 2003; Hartmann et al., 2005; Holman et al., 2010). 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. Recently, two independent studies have observed cuticular hydrocarbon profiles specific to functional neotenics in the kalotermitid termite *Cryptotermes* *secundus* (Weil et al., 2009) and the termopsid termite *Zootermopsis nevadensis* (Liebig et al., 2009). In addition, Hanus et al. (2010) reported that functional reproductives have sex-specific proteinaceous compounds in three termite species, *Prorhinotermes simplex*, *Reticulitermes santonensis*, and *Kalotermes flavicollis*. These studies have proposed cuticular hydrocarbons (Liebig et al., 2009; Weil et al., 2009) and proteinaceous compounds (Hanus et al., 2010) as candidate non-volatile queen pheromones. Unfortunately, there has been no evidence that these chemicals act as queen recognition and/or inhibition signals in termites, primarily due to the technical difficulties of the bioassays described above. Future technical developments might overcome these difficulties and reveal the exact functions of these non-volatile compounds.

FIRST IDENTIFICATION OF TERMITE QUEEN PHEROMONE

Lower termites of the genus *Reticulitermes* make good subjects for identifying queen pheromones, as sufficient numbers of reproductive queens can be collected because the primary queen (alate-derived) can be replaced by many neotenic secondary queens (Thorne et al. 1999; Vargo and Husseneder, 2009). *Reticulitermes speratus* is the most common termite in Japan. Colonies are usually founded by one king and one queen. Upon the death of the primary queen or king, neotenic reproductives are produced. Mature field colonies usually have a single primary king and an average of 55.4 female neotenic reproductives, which are almost exclusively produced parthenogenetically by the original primary queen (Matsuura et al., 2009). In addition, in natural colonies, neotenic reproductives differentiate exclusively from nymphs, never workers (n =

3,029), as nymphs have priority in becoming neotenics in this species (Matsuura et al., 2009, 2010; Matsuura, 2010).

To test for the existence of queen inhibitory pheromone, we first compared the number of replacement queens produced in groups containing nymphs, workers, and a reproductive queen and in similar groups without a queen. In these experiments, some secondary queens and kings were produced from nymphs (nymphoid queens and kings) but not from workers (ergatoid queens and kings). The differentiation of new nymphoid queens was greatly suppressed by the presence of a functional queen (Matsuura et al, 2010). In contrast, queen presence did not significantly affect the differentiation of nymphoid kings. In a second experiment, we similarly investigated the inhibitory power of reproductive queens, but we used experimental units containing only workers. Under such conditions, some workers differentiated into ergatoid secondary queens and males. As in the previous experiment, the presence of a fertile queen greatly suppressed the differentiation of new ergatoid queens but not ergatoid kings. Thus, queens exerted a strong sex-specific effect on the differentiation of neotenic reproductives regardless of developmental origin. Additional experiments in which queens were confined within double-mesh cages revealed that the inhibitory effect of queens was mediated by volatiles.

To identify the volatile inhibitory pheromone produced by queens, we collected a large number of *R. speratus* colonies and used the two largest for chemical analyses. Headspace-collected volatiles from 100 fully developed secondary queens were analyzed using gas chromatography followed by mass spectrometry (HS GC-MS). We found that the queen volatiles consist of an ester n-butyl-n-butyrate (nBnB; CAS No.

109-21-7) and an alcohol 2-methyl-1-butanol (2M1B; CAS No. 137-32-6) in a 2.14:1 ratio. In contrast to queens, workers and nymphs did not produce any nBnB or 2M1B.

In the course of our trial to analyze queen volatiles, we realized that these queen-specific volatiles are truly honest fertility signals. Soon after isolation from the nest, the physogastric queens maintained in Petri dishes with workers reduced oviposition, likely due to poor provisioning. After they ceased egg-laying, the queen-specific volatiles were no longer detectable (Matsuura et al, 2010). This result appears to support the hypothesis that queen pheromones act as honest signals of queen fertility (Keller and Nonacs, 1993).

To investigate the effect of nBnB and 2M1B on queen differentiation, we developed a new protocol for gradually and continuously exposing termites to the volatile compounds. The compounds, once absorbed by an unglazed ceramic ball, would slowly volatilize in an outer Petri dish and then enter an inner Petri dish through a small opening on the lid. A 2:1 blend of commercial nBnB and 2M1B, matching the naturally occurring ratio, strongly suppressed the differentiation of workers into ergatoid queens (Matsuura et al, 2010; Matsuura and Yamamoto, 2011), whereas the substances had no significant effect on the differentiation of ergatoid kings. In addition, each single compound alone had no effect on queen differentiation compared to a control treatment (Matsuura et al, 2010; Yamamoto et al. 2011).

In chemistry, a molecule that is non-superposable on its mirror image is considered to be chiral, and the two mirror images of a chiral molecule are called enantiomers. The relationships between absolute configuration and pheromone activity are diverse and complicated (reviewed by Mori, 1998, 2007). 2M1B is the first chiral molecule identified as a primer pheromone in social insects, which presents the intriguing question of whether enantiomeric composition plays a role in caste regulation. To determine whether the (*R*)/(*S*) configuration of 2M1B influences the inhibitory activity in *R. speratus*, the (*R*)- and (*S*)-enantiomers of 2M1B were synthesized, and the number of female neotenic reproductives differentiated from workers under exposure to (*R*)-, (*S*)-, and the racemic mixture of 2M1B in combination with nBnB was compared. The (*R*)- and (*S*)-enantiomers and the racemic mixture of 2M1B showed significant inhibitory effects on the differentiation of new female neotenics in combination with nBnB, and no significant differences in inhibitory activity were observed among these treatments (Yamamoto et al., 2011). This suggests that termites recognize 2M1B as a queen signal, but they do not distinguish among the stereostructures of the enantiomers, although the absolute configuration of the naturally occurring queen pheromone remains to be identified.

EGG VOLATILES AS ATTRACTANT AND FERTILITY SIGNAL

In addition to queens themselves, the existence of brood (eggs and larvae) may act as a direct signal of queen fertility. In the honeybee *Apis mellifera*, old larvae provide the primary signals that prevent ovary activation in workers (Trouiller et al., 1991; Arnold et al., 1994; Mohammedi et al., 1998; Oldroyd et al., 2001; Pankiw and Garza, 2007; Maisonnasse et al., 2010), and in the ant *Pachycondyla apicalis*, larvae affect worker reproduction in queenless groups (Heinze et al., 1996). In the ant *Myrmica rubra*, queen-laid eggs inhibit worker ovary development (Brian and Rigby, 1978). A recent study of the ant *Camponotus floridanus* demonstrated that queen-derived hydrocarbon-labeled eggs inform workers of the presence of a fertile queen, suppressing worker reproduction (Endler et al., 2004).

To test whether eggs may also have an inhibitory effect in *R. speratus*, we compared the differentiation of neotenic queens in colonies without queens, in which eggs were either absent or continuously added at a rate of 100 or 20 eggs/day. Differentiation rates significantly differed between colonies receiving 100 eggs/day and colonies without eggs, whereas colonies receiving 20 eggs/day and colonies without eggs did not significantly differ (Matsuura et al., 2010). This indicates that not only queens themselves, but also eggs in sufficiently large numbers, have a strong inhibitory effect on the differentiation of neotenic queens in this termite.

Interestingly, we determined that the two volatiles, 2M1B and nBnB, in the queen pheromone are also emitted by eggs (Matsuura et al., 2010). Egg volatiles might be transferred from queens through egg marking or could be emitted by the eggs themselves. An antibacterial protein lysozyme, which also functions as an egg-recognition signal, is synthesized in eggs (Matsuura et al., 2007), implying that termite eggs are biosynthetically active. If the volatiles are transferred from queens to the egg surface, the volatiles should be detected even after the eggs are killed by freezing. However, frozen-killed eggs show no volatile emission, indicating that the volatiles are actively produced by the eggs themselves.

Why do termite eggs emit volatiles identical to the queen pheromone? We investigated whether the volatile components emitted by eggs had additional functions. Termite eggs cannot survive without protection by workers (Matsuura et al., 2000, Matsuura, 2006). Soon after being laid, eggs are carried into nursery chambers where they are groomed by workers and coated with saliva and antibiotic substances. Workers recognize eggs by morphological cues and a termite egg recognition pheromone (TERP) consisting of an antibacterial protein lysozyme (Matsuura et al., 2007) and a digestive β-glucosidase enzyme (Matsuura et al., 2009). These two compounds can only be detected when workers directly touch the egg surface. Our preliminary experiments demonstrated that workers aggregated around egg piles confined in a stainless steel mesh cage, suggesting that workers are attracted to eggs by volatile cues. To determine whether the egg volatiles nBnB and 2M1B are used by workers to locate eggs, we conducted bioassays using dummy eggs made of glass beads. Dummy eggs coated with TERP together with nBnB and 2M1B were carried into egg piles at a significantly higher rate than dummy eggs without nBnB and 2M1B. Without TERP, dummy eggs were not recognized as eggs even when coated with nBnB and 2M1B, indicating that egg volatiles act as an attractant but not as a recognition pheromone.

QUEEN–QUEEN INTERACTION VIA VOLATILE PHEROMONE

The question of resource allocation has long been a core issue in the study of social insects. When and how resources are allocated to growth, maintenance, and reproduction determines colony survival and growth (Oster and Wilson, 1978). In eusocial insects, young broods need tending and nourishment by workers. Therefore, egg production must be adjusted in proportion to the capacity of the colony to rear the brood to maximize productivity (Tschinkel, 1988; Matsuura and Kobayashi, 2010). To accomplish optimal resource allocation to queens and the brood, colony members must adjust investment to queens in accordance with the current reproductive power of the queens. In the presence of multiple queens, regulation of colony-level egg production involves interactions among the queens. For example, Vargo (1992) demonstrated that queen-produced pheromones were involved in inhibition of the reproductive output of coexisting queens in the ant *Solenopsis invicta*.

To determine whether the queen pheromone influences the reproductive rate of queens in *R. speratus,* we compared the number of eggs produced by queens with and without exposure to an artificial queen pheromone. We kept either one or three mature female neotenic reproductives with 200 workers on a block of mixed sawdust food in a Petri dish and then compared the number of eggs produced between monogynous (single-queen) and polygynous (multiple-queen) groups. Exposure to the artificial queen pheromone significantly reduced the total number of eggs produced by the queens in each group, whereas no significant difference was observed in the total number of eggs produced between single-queen and three-queen groups (Yamamoto and Matsuura, 2011). The average number of eggs produced per queen in groups with multiple queens was significantly lower than in groups with a single queen. The number of eggs laid per queen was also reduced by exposure to the artificial queen pheromone. Hence, the total number of eggs produced in single-queen and multiple-queen groups did not significantly differ when these colonies had an equal number of workers, because the egg production of one queen negatively affected that of any other queens in a colony (Yamamoto and Matsuura, 2011). Reduced egg production under exposure to the artificial queen pheromone suggests that this mutual inhibition could be caused by the volatile chemical emitted by the queens and eggs.

We propose three possible mechanisms for this process: 1) Queens make a decision, whereby nourishment by workers could be adjusted based on queen behavior such as begging frequency; 2) workers make a decision, whereby they control the amount of food supplied to each queen based on pheromonal information; or 3) the queen-produced pheromone acts directly on the queen's neuroendocrine system to affect egg production. Careful observation of queen feeding by workers with and without exposure to the queen pheromone might distinguish between hypotheses 1) and 2). Inactivation of the pheromone receptor of queens or workers may also be key to a better understanding of the regulatory mechanism.

QUEEN-SPECIFIC VOLATILE IN A HIGHER TERMITE

To understand the evolution of queen pheromones and social regulation in termites, it is essential to compare pheromone compounds among diverse taxa. As the first step for a comparative study, we performed chemical analysis of the queen volatiles of the higher termite *Nasutitermes takasagoensis* (Termitidae), which is phylogenetically distant from *R. speratus*. The foraging behavior of *N. takasagoensis* is a separate type, whereas that of *R. speratus* is an intermediate type (Abe, 1987). Both species are parasitized by egg-mimicking fungi, although the parasitism evolved independently in each genus (Matsuura and Yashiro, 2010).

To determine the queen-specific volatile compounds in *N. takasagoensis*, headspace-collected volatiles from individual fully physogastric primary queens were analyzed using HS GC-MS. We identified a single queen-specific compound, phenylethanol (Himuro et al., 2011), which differed from the volatile queen-pheromone identified in *R. speratus*, suggesting that the chemical compositions of queen pheromones may vary greatly among termite taxa.

Phenylethanol is a very common semiochemical that can be found in a broad range of insect species. It serves a variety of behavioral functions, for example, as a component of the aggregation pheromone in the cerambycid beetle *Megacyllene caryae* (Lacey et al., 2008), of the sex pheromone in male cabbage moths (Bestmann et al., 1977; Jacquin et al., 1991), and of the alarm pheromone in the ant *Crematogaster nigriceps* (Wood et al., 2002). Interestingly, phenylethanol is one of the queen-specific compounds in the European honeybee *Apis mellifera*, although its function is unknown (Gilley et al., 2006).

The exact functions of the queen-specific volatile of *N. takasagoensis* remain to be determined. Ergatoid reproductives reportedly develop upon the death of primary queens or after a reduction in the reproductive power of old primary queens in *N. takasagoensis* (Miura and Matsumoto, 1996), suggesting that pheromones produced by reigning queens inhibit the differentiation of new neotenic reproductives. The queen-specific volatile phenylethanol may function as a reproductive inhibitory pheromone in this termite. However, direct proof of reproductive inhibition is particularly difficult to obtain in higher termites for technical reasons. We were unable to apply the inhibition bioassay used for *R. speratus* to *N. takasagoensis*, as it was difficult to keep workers and nymphs alive after separation from the nest structure. Maintaining the nests in the laboratory is also very difficult. Therefore, another type of bioassay is needed to determine the function of queen-specific volatiles in higher termites. In the higher termite *Macrotermes subhyalinus*, queen volatiles serve as releaser pheromones for construction of the royal chamber by workers (Bruinsma, 1979). Such building behavior might be useful for developing a queen pheromone bioassay for higher termites.

PROXIMATE ORIGIN AND EVOLUTIONARY PARSIMONY

As discussed in the review by Murray S. Blum (1996), a wide variety of insects have adapted their own semiochemicals to subserve multiple functions in diverse contexts. Because the number of candidate compounds available to regulate multiple systems may be biosynthetically finite, strong selection favors the use of single natural products for many purposes. Secondary use of chemical compounds that have evolved for other primary functions occurs in various social insects (Blum and Brand, 1972; Turillazzi et al., 2006; Cremer et al., 2007). Identification of the termite egg-recognition pheromone elucidated such evolutionary parsimony (Matsuura et al., 2007, 2009). The primary functions of the pheromone components lysozyme and β-glucosidase are as an antibacterial defense agent and a digestion enzyme, respectively. Termites have evolved to use the preexisting chemicals on the egg surface as egg-recognition signals, without the evolution of any additional specific chemical for this purpose.

Therefore, it would be reasonable to hypothesize that the volatile compounds of termite queen pheromones, 2M1B and nBnB, would also have practical functions other than their pheromonal roles. The nesting and feeding ecology of termites expose colony members to a great variety of microbes including bacteria, fungi, protozoa, viruses, spirochetes, and nematodes (Roseggaus and Traniello, 2001; Traniello et al., 2002). Therefore, one of the most important selection pressures on termites is how they cope with various microorganisms, resulting in the evolution of behavioral and physiological adaptations. Because an infection of a queen is fatal to colony survivorship, the queen is especially protected against incoming parasites (Cremer et al., 2007). The exudates of the physogastric queen of the termite *Termes redemanni* have known antimicrobial properties (Sannasi and Sundara Rajulu, 1967).

Recently, we determined that 2M1B and nBnB have inhibitory effects on the germination and mycelial growth of the “termite ball” fungus (K. Matsuura and T. Matsunaga, unpublished data). Termite ball is a sclerotium of an athelioid fungus (Basidiomycota, Agaricomycotina) of the genus *Fibularhizoctonia* (Matsuura et al., 2000; Yashiro and Matsuura; 2007; Matsuura and Yashiro, 2009). Termite balls mimic termite eggs so as to be tended by termites in the egg piles, whereby the fungus gains a competitor-free habitat in termite nests. Most termite balls are inhibited from germination in egg piles. However, if termite balls germinate in egg piles, the fungus consumes surrounding eggs (Matsuura et al., 2000; Matsuura, 2006). Therefore, an antifungal function of the egg volatiles seems reasonable. Interestingly, the inhibition activities of 2M1B and nBnB vary among strains of the fungus: 2M1B shows a stronger inhibitory effect than nBnB on one fungal strain, but the opposite is true of another strain. This result is indicative of an arms race between the parasitic fungus and termites and might explain why the queens and eggs emit multiple volatiles.

FUTURE PERSPECTIVE

To understand the evolution of multifunctional queen pheromone in termites, I would guess two further directions of this study including 1) comparison of queen substances among a variety of species and 2) identification of the molecular basis of pheromone activities. For comparative approach, queen pheromone needs to be identified in much more species of many taxa. The difficulties both in collecting adequate queen substances and in testing the inhibitory activity have concealed queen pheromone. A sure way of obtaining a sufficient amount of queen volatiles is to collect physogastric queens from mature field colonies. It is important to note that the volatiles need to be collected from the queens soon after extraction from the nest (hopefully within 24 hours) because isolation from the colony reduces egg production and volatile emission very quickly. In the bioassay, it is essential to expose termites to the volatile compounds gradually and continuously. To this end we developed a new protocol where the compounds once absorbed by an unglazed ceramic ball slowly volatilize in the outer Petri dish and then enter the inner Petri dish through a small opening on the lid (Matsuura et al., 2010). This device would be useful to test the inhibitory activity of volatiles in a species in which neotenics easily develop after orphaning.

Identification of a queen pheromone suppressing differentiation of new neotenic queens opens up exciting new possibilities to investigate how exocrine signals interact with the endocrine system to regulate caste differentiation. Recent studies have begun to shed light on the molecular basis for division of labor and caste determination in termites. The relationship between juvenile hormone (JH) synthesis and egg production was demonstrated in *Zootermopsis angusticollis* (Greenberg and Tobe, 1985; Brent et al., 2005), *Z. nevadensis* (Miura et al. 2003), *R. flavipes* (Scharf et al., 2005) and *R. speratus* (Maekawa et al., 2010). A gene *Neofem*2, which is overexpressed in queens but not in kings and workers, is necessary for the queen to suppress worker reproduction in *Cryptotermes secundus* (Korb et al. 2009). Characterization of the pheromone receptors and their downstream targets, as well as the elucidation of pheromone biosynthesis, should provide important new insights into how reproductive and non-reproductive developmental pathways are regulated in termite colonies.

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