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RESEARCH ARTICLE

Queen pheromone promotes production of salivary lysozyme by workers in a termite

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Abstract Social insects have highly elaborated communication systems. In particular, communication via pheromones is important for maintaining complex social roles and behaviors. Brood care is a typical activity that involves pheromonal communication among colony members. In termites, eggs cannot survive without grooming by workers. The workers coat the eggs with their saliva, which contains an antibacterial protein lysozyme that protects the eggs against bacterial pathogens. The more eggs a colony has, the more salivary lysozyme the workers need to produce for egg grooming. However, it is unknown how termite workers regulate their lysozyme production. Here we show that the queen pheromone, which is emitted by both queens and eggs, promotes lysozyme production by workers in the termite *Reticulitermes speratus*. Exposure to artificial queen pheromone significantly increased the production of salivary lysozyme by workers as well as the artificial addition of eggs. Furthermore, our survey of field colonies revealed clear seasonality in the production of salivary lysozyme. The seasonal pattern of lysozyme production matched well with the seasonal change of the number of eggs per colony. In addition to the known function of the queen pheromone as an inhibitor of neotenic queen differentiation, this study reveals that the same pheromone also acts as a promoter of lysozyme production in workers. We describe a novel function of the multifunctional queen pheromone in termites and provide new insights into the evolutionary parsimony of social insect pheromones.

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Introduction

Communication via pheromones is important in all social activities including foraging, sexual behavior, defense, nestmate recognition, and caste regulation in social insects (Vander Meer et al. 1998). Pheromone compounds exhibit great chemical parsimony. Multifunctional pheromones have proven to be especially characteristic of eusocial insects, where secondary uses of chemical compounds that have evolved for other primary functions occur in various species (reviewed by Blum 1996; Steiger et al. 2011). For example, cuticular hydrocarbons, which originated as antidesiccation compounds, have been secondarily utilized as cues for nestmate recognition (Howard 1993; Vander Meer et al. 1998; Akino et al. 2004), brood recognition (Endler et al. 2004; D'Ettorre et al. 2004), and fertility signals (Monnin and Peeters 1998; Peeters et al. 1999; Heinze et al. 2002; de Biseau et al. 2004) in the social Hymenoptera. Pheromone parsimony is also well developed in termites (reviewed by Bordereau and Pasteels 2011). In many species, the same compound is acting as a trail-following pheromone and a sex pheromone (Pasteels and Bordereau 1998; Bordereau et al. 2002; Robert et al. 2004). Cuticular hydrocarbons have multiple functions as interspecific recognition (Bagnères et al. 1991), caste-specific signals (Clément and Bagnères 1998), cues for reproductive status (Liebig et al. 2009) and might be involved in nestmate recognition (Clément and Bagnères 1998) in termites.

Recently, a volatile blend consisting of *n*-butyl-*n*-butyrate (nBnB) and 2-methyl-1-butanol (2M1B) was

identified as the queen pheromone in the subterranean termite *Reticulitermes speratus* (Matsuura et al. 2010). This queen pheromone plays a variety of roles including inhibition of reproductive differentiation (Matsuura et al. 2010) and regulation of colony-level egg production (Yamamoto and Matsuura 2011). Importantly, the two volatiles in the queen pheromone, 2M1B and nBnB, are also emitted by eggs (Matsuura et al. 2010). These compounds function as an orientation pheromone causing workers to care for eggs. This dual production of an inhibitory pheromone by both reproductive females and eggs may provide a mechanism for ensuring honest signaling of reproductive status, with a tight coupling between fertility and inhibitory power.

Brood protection is a fundamental behavior in social insects (Hölldobler and Wilson 1990; Ayasse and Paxton 2002; Tallamy 2005). Egg recognition and protection are especially important in termites because their egg cannot survive without being tended by workers. Without worker care, termite eggs are easily killed by naturally occurring pathogens or the egg-mimicking parasite termite-ball fungus in *R. speratus* (Matsuura et al. 2000, 2009; Matsuura 2006). In this species, it has been demonstrated that workers frequently lick eggs and smear their salivary gland lysozyme (SGL) on the egg surfaces (Matsuura et al. 2007a). Lysozyme is known to be a major antibacterial protein in the saliva of various termite species (Mednikova and Tiunova 1984; Fujita et al. 2001; Yuki et al. 2008). It is known that lysozyme protects the eggs against bacterial pathogens and also functions as a compound of the egg recognition pheromone in *R. speratus* (Matsuura et al. 2007a). The more eggs a colony has, the more SGL the workers need to produce for egg care. Therefore, workers should promote SGL production when they are exposed to a large amount of queen and egg volatiles containing 2M1B and nBnB, which indicate the egg production by a colony.

In this paper, we investigated a novel function of the termite queen pheromone as a signal of egg production for workers. We tested whether the queen pheromone and eggs influence SGL production by workers. First, we compared the amount of SGL produced by workers with and without exposure to an artificial queen pheromone or artificial addition of eggs. Second, we investigated seasonal variation in SGL production in field-collected colonies to assess the factors influencing the SGL.

Materials and methods

Queen pheromone bioassay

This experiment was designed to test whether the queen pheromone signals the number of eggs and, in turn, affects SGL production by workers. Three nest woods of *R. speratus* (colonies A, B, and C) were collected in a forest in June 2010 in the City of Okayama, Okayama Prefecture, western Japan. In the laboratory, we opened the nest woods and extracted the workers. We placed 100 workers in a 30-mm Petri dish lined with moist filter paper. Each dish was assigned to any one of the three treatments: the queen pheromone treatment, the egg treatment, or the negative control treatment. We made five replications for each of the three treatments and repeated the experiment using three colonies. Five dishes were arranged concentrically in a plastic container (82 × 178 × 31 mm) and an unglazed ceramic ball (6-mm diameter) was placed at the center of the plastic container as a pheromone source so as to be located with an equal distance from each Petri dish. We made a small opening (1.0-mm diameter) in the lid of each Petri dish to allow the pheromone to enter. In the queen pheromone treatment, we added 1 μL of artificial queen pheromone, consisting of 2 μl *n*-butyl-*n*-butyrate (Sigma–Aldrich Japan, Tokyo, Japan) and 1 μL 2-methyl-1-butanol (Wako, Osaka, Japan), to the unglazed ceramic ball every 24 h. In the egg treatment, we initially placed 1000 eggs in the Petri dishes and added 100 eggs every 24 h. In the control treatment, the ceramic ball was treated with 1 μL of distilled water every 24 h. After 4 days, we measured SGL activity using the methods below.

Lysozyme activity

We measured lysozyme activity according to the method of Matsuura et al. (2007b) with slight modifications. We collected salivary glands from 10 workers, which were randomly chosen from each Petri dish, and placed them in a single Eppendorf tube on ice. Samples were homogenized in 0.1 M phosphate buffer (pH 6.0) on ice and then centrifuged at 10,000×*g* for 10 min at 4 °C. We then collected the supernatant as enzyme extract. The lytic activity of the enzyme extract was measured against 0.2 mg/ml *Micrococcus lysodeikticus* (Sigma–Aldrich Japan, Tokyo, Japan) suspended in phosphate buffer. We measured lysis of *M. lysodeikticus* at 450 nm with a spectrophotometer (SmartSpec Plus, BioRad, Hercules, CA) at 30 °C. We added only extraction buffer to the negative control.

Seasonal patterns of SGL activity in field colonies

The goal of this experiment was to investigate the relationship between seasonal egg production and SGL production in the field. We collected five nest woods of *R. speratus* every half month from November 2009 to November 2010 in the forests in Okayama City. In the laboratory, we opened the nest woods and sampled workers. We measured SGL activity of workers within 24 h of collection. The average temperature of each month was

obtained from the annual report of the Okayama Local Meteorological Observatory (http://www.osaka-jma.go.jp/okayama/okayam1.htm).

Statistical analysis

All statistical analyses were performed using R software version 2.15.1. We used two-way ANOVA to examine the effects of the artificial queen pheromone and the existence of eggs on the quantity of SGL. We used Tukey's HSD to test for differences among groups only when the main effect of the ANOVA was significant.

We used multiple regression to analyze the effects of the average temperature, the proportion of egg-present colonies (no. of colonies harboring eggs/total no. of colonies investigated), and the average number of eggs per colony at each half month on SGL activity. The data of the proportion of egg-present colonies and the average number of eggs obtained in 2005 (Matsuura et al. 2007a) were used in this analysis under the assumption that the seasonal pattern of egg production would not change very much among years in the same population.

Results

There were significant differences in SGL activity among the chemical treatments (colony $F_{2,\ 36} = 103.73$, $P < 0.001$; chemical treatment $F_{2,\ 36} = 8.45$, $P < 0.001$; colony × chemical treatment $F_{2,\ 36} = 103.73$, $P = 0.048$; two-way ANOVA; Fig. 1), showing that both artificial queen pheromone ($P < 0.001$) and eggs ($P = 0.012$, Tukey's HSD) significantly increased SGL activity in comparison to the control (Fig. 1). The difference between the queen pheromone and the egg treatment was not significant ($P = 0.107$, Tukey's HSD; Fig. 1). There were significant differences in the activity of control treatment among colonies ($F_{2,\ 12} = 77.44$, $P < 0.0001$; one-way ANOVA), where colony B showed higher control level than other colonies ($P < 0.0001$, Tukey's HSD). In colony B, there was a significant difference between the queen pheromone treatment and control ($P < 0.05$, Tukey's HSD), while no significant difference between the egg treatment and control ($P = 0.99$, Tukey's HSD).

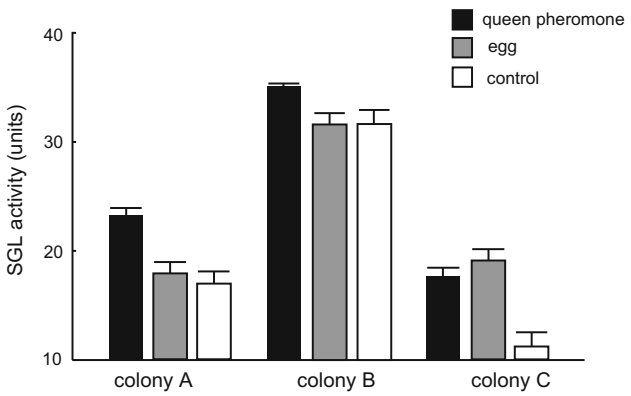

Fig. 1 Effect of queen pheromone and eggs on SGL production by workers. Both artificial queen pheromone ($P < 0.001$) and eggs ($P = 0.012$, Tukey's HSD) significantly promoted SGL production in comparison with the control treatment. There was no significant difference between the queen pheromone and egg treatment on SGL production ($P = 0.107$, Tukey's HSD)

In field colonies, there was a significant effect of the average number of eggs per colony on SGL activity of workers at each half month (Wald's $t = 6.802$, $P < 0.001$, multiple regression; Fig. 2). The average temperature (Wald's $t = 1.315$, $P = 0.190$) and the proportion of egg-present colonies (Wald's $t = 1.934$, $P = 0.054$) had no significant effect on SGL production.

Discussion

We investigated a novel function of the termite queen pheromone as a promoter of lysozyme production by workers from the point of view of evolutionary parsimony. The SGL production by workers was significantly promoted by exposure to the artificial queen pheromone. In our survey of field colonies, the production of SGL by workers showed clear seasonality. The seasonal pattern of SGL production matched well with the seasonal change of the number of eggs per colony. One possibility for increased SGL production is simply that termites need more lysozyme for digestion under higher temperatures because lysozyme is also used to digest bacteria for nutrition (Fujita et al. 2001) and feeding is dependent on temperature (Nakayama et al. 2004). However, our data analysis showed that temperature alone does not explain seasonal fluctuation of SGL production. Although an earlier study on the seasonality of egg production showed a significant effect of temperature on egg production (Matsuura et al. 2007a), the effect of temperature on SGL production was not significant after removal of the effect of egg production. Therefore, we conclude that higher SGL production during the egg producing season is due to the increased need for SGL to encourage egg grooming. These results provide evidence of parsimonious use of the queen pheromone, which acts as both an inhibitor of reproductive differentiation and a promoter of SGL production by workers in response to the presence of eggs.

All living organisms require nitrogen for the synthesis of proteins and nucleic acids. Woody tissue, which is the primary food of termites, contains only 0.03–0.7 % nitrogen, and its C/N ratio is 70/500, whereas termite bodies contain about 10 % nitrogen, and their C/N ratio is 4/12 (Tayasu

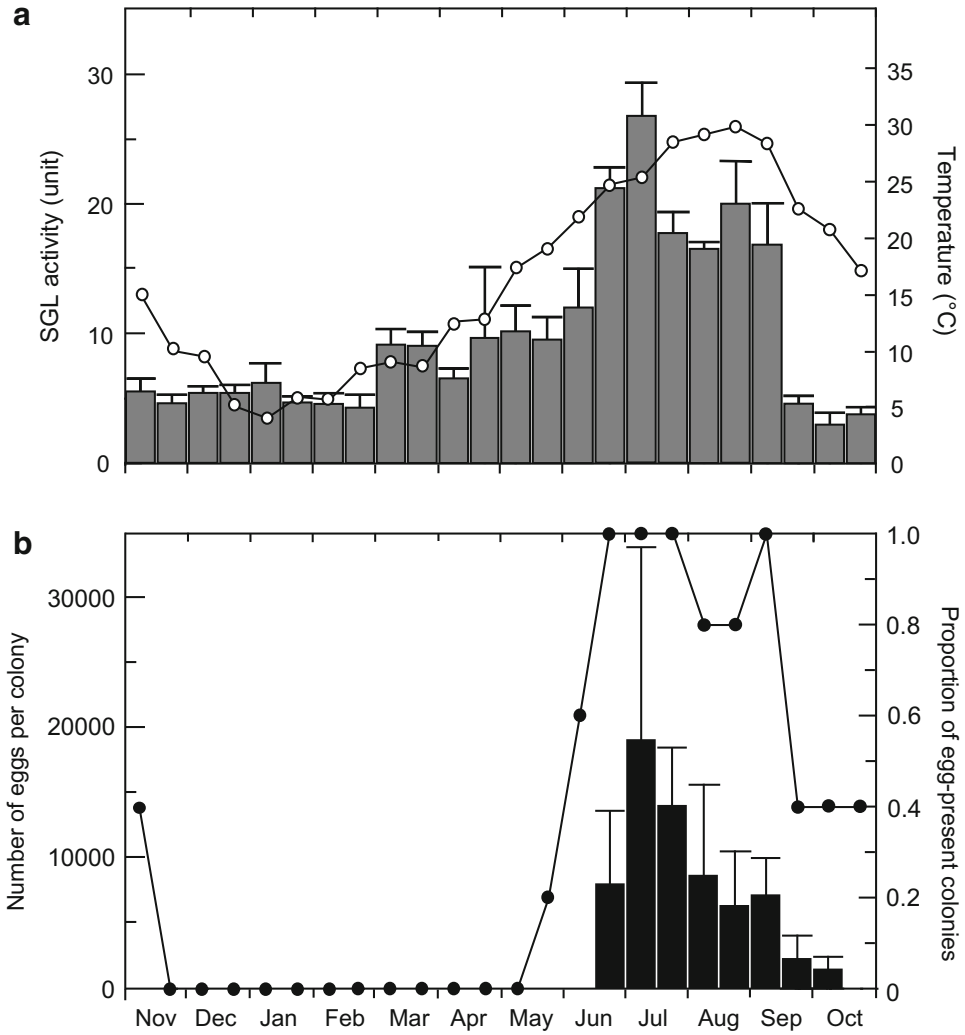

Fig. 2 Seasonal pattern of SGL production by workers with the change of the amount of eggs per colony. **a** Annual pattern of SGL activity (mean ± SE) of workers. Five colonies were collected in the first half and another five colonies in the second half of each month. SGL activity is indicated by *gray bars*. Mean monthly temperature is indicated by *open circles*. **b** Annual change of the number of eggs per colony (mean ± SE) and the proportion of egg-present colonies (*closed circles*). The number of eggs is indicated by *closed bars*. The number of eggs had a significant effect on SGL activity at each half month ($P < 0.001$), while temperature ($P = 0.190$) and the proportion of egg-present colonies ($P = 0.054$) showed no significant effect

et al. 1994). Therefore, xylophagous termites must acquire a much higher concentration of nitrogen than that is present in their food source. Conservation of nitrogen should contribute to colony productivity, even though termites have evolved the ability to fix atmospheric nitrogen (Benemann 1973; Breznak et al. 1973) and recycle nitrogen from uric acid with the help of symbiotic bacteria in their hindguts (Potrikus and Breznak 1981; Chappell and Slaytor 1993). Our result showing that termites strictly regulate the production of SGL, which includes high levels of nitrogen, indicates that termites have evolved to minimize their use of nitrogen. Although we demonstrated that the queen pheromone promotes SGL production by workers, the quantitative relationship between the amount of queen pheromone and SGL production remains unknown. The facilitation of SGL production by eggs varied among colonies. In colony B, we found no significant difference between the egg treatment and control. The baseline activity of SGL in colony B was much higher than other colonies. The large difference of control activity among colonies seems likely due to environmental differences among their nests. It is known that exposure to bacteria increases expression of lysozyme in workers of the termite *Coptotermes formosanus* (Hussain et al. 2013). Therefore, colonies living in environments rich with bacterial pathogens might have originally had high lysozyme activity, and thus, artificial addition of eggs might have had relatively low impact on such colonies. In addition, it is most likely that the workers in the egg treatment transferred lysozyme on eggs by egg grooming and thus the lysozyme production in the egg treatment may have been underestimated compared to the queen pheromone treatment and control.

The capacity to synthesize pheromonal compounds is biosynthetically finite, potentially leading to strong evolutionary pressure to use single products parsimoniously for multiple purposes (Blum 1996; Steiger et al. 2011). The queen pheromone is a multifunctional pheromone that plays various roles associated with reproduction and worker

behavior in *R. speratus* (reviewed in Matsuura 2012). As demonstrated in this paper, the queen pheromone also acts as a primer pheromone inducing a physiological change in workers and thus increasing their SGL production. These functions of the queen pheromone appear to have evolved with the development of social behavior. A more recent study demonstrated that volatile compounds of the queen pheromone have antimicrobial activity independent of their signaling functions (Matsuura and Matsunaga 2015). Therefore, the most parsimonious explanation is that the volatile compounds, which originated as defensive chemicals against pathogens, were secondarily adopted as pheromones that inform of the presence of the queen and of eggs.

As insects evolved into social systems, they required sophisticated communication systems and signals. Among the social insects, the number of exocrine glands described in termites is relatively limited in comparison with the considerably higher variety of the exocrine system in the social Hymenoptera (Gonçalves et al. 2010). Only 17 exocrine glands have been described in termites, whereas 28 abdominal exocrine glands have been identified even in a single ant *Pachycondyla tridentata* (Jessen and Maschwitz 1983). Exaptation of available chemicals, that is, the evolution of multifaceted roles of secretions, might explain why termites have highly sophisticated social systems similar to social hymenoptera but many fewer exocrine glands. This study provides significant insight into our understanding of the evolutionary trajectories of signal evolution in social insects.

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