

The Influence of Allogrooming Behavior on Individual Innate Immunity in the Subterranean Termite *Reticulitermes chinensis* (Isoptera: Rhinotermitidae)

Long Liu,² Wei Wang,² Yiliang Liu, Pengdong Sun, Chaoliang Lei, and Qiuying Huang¹

Hubei Insect Resources Utilization and Sustainable Pest Management Key Laboratory, Huazhong Agricultural University, Wuhan 430070, Hubei, China, and ¹Corresponding author, e-mail: qyhuang2006@mail.hzau.edu.cn

²These authors contributed equally to this work.

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Abstract

Insect societies have evolved a series of disease defenses against pathogens, including social sanitary behavior and individual innate immunity. However, whether sanitary behavior can affect individual innate immunity remains unknown. Here, we exposed the termite *Reticulitermes chinensis* Snyder to the entomopathogenic fungus *Metarhizium anisopliae* (Metchnikoff) Sorokin (Ascomycota: Hypocreales), and then measured their allogrooming behavior, conidia load, infection mortality, antifungal activity and immune gene expressions. Our results showed that most of the fungal conidia were fast removed from the cuticles of the grouped termites by intensive allogrooming behavior, resulting in low mortality. The antifungal activity and immune gene expressions (*termicin* and *transferrin*) in grouped exposed termites were significantly lower than those in single exposed termite but not significantly different from those in unexposed treatments. These results suggest that allogrooming behavior can fast remove fungal conidia from termite cuticles and then decrease their physiological investment in individual innate immunity.

Key words: termite, entomopathogenic fungus, survival, antifungal activity, immune gene

In a social context, pathogens can employ social interaction networks to transfer from infectious to susceptible individuals (Schmid-Hempel 1998, Cremer et al. 2018). For example, nesting in pathogen-rich environments, high densities, frequent social contact, and close relatives in some social insects can make disease easier to outbreak when compared with solitary insects (Cremer et al. 2007, Rosengaus et al. 2011, Cremer et al. 2018). To counteract these potential threats, social insects evolve many disease defenses at individual and colony levels (Fefferman et al. 2007, Rosengaus et al. 2011, Parker et al. 2012, Mitaka et al. 2016, Cremer et al. 2018).

Colony-level disease defenses were achieved by cooperation of group members, which can quickly react to the potential threats and benefit the whole colony fitness (Cremer et al. 2007, Rosengaus et al. 2011). For example, allogrooming behavior is one particularly important component of the colony-level sanitary behavior and can externally remove pathogens from cuticles and disinfect the cuticles to inhibit pathogen growth during early-stage infection (Yanagawa and Shimizu 2007, Hamilton and Bulmer 2012, Konrad et al. 2018). When pathogens survive from the external disinfection and invade the body cavity of the hosts, they will cause an internal infection that can induce individual innate immunity, along with an upregulation of immune genes and antifungal activity (Thompson et al.

2003, Bulmer and Crozier 2004, Konrad et al. 2012, Liu et al. 2015). However, the question of whether the decreased infection risk mediated by sanitary behavior affects the internal immune response of individuals remains unexplored.

The subterranean termite *Reticulitermes chinensis* Snyder is an economically important pest in China (Huang et al. 2013, Yin et al. 2013). The entomopathogenic fungus *Metarhizium anisopliae* (Metchnikoff) Sorokin (Ascomycota: Hypocreales) has been widely used in biological pest control and is an ideal experimental fungus in which to study social insect immunity (Rosengaus et al. 1998, Liu et al. 2015, Konrad et al. 2018). Here, we used the termite workers and the fungal conidia as a host–pathogen system and then analyzed allogrooming behavior, conidia load, infection mortality, antifungal activity, and immune gene expressions to determine whether sanitary behavior affects individual innate immunity.

Materials and Methods

Experimental Workers

Six colonies of the subterranean termite *R. chinensis* were collected from Wuhan City in Hubei Province, China. Termite workers were reared under laboratory conditions at a temperature of $25 \pm 1^\circ\text{C}$,

80% relative humidity, and 24-h darkness throughout all the tests. Only healthy workers, which were older than third instar and could normally perform behaviors, were selected for the following different experiments.

Fungal Pathogen

Termites were exposed to the entomopathogenic fungus *M. anisopliae* (strain IBCCM321.93). The fungus was cultivated on potato dextrose agar (QDRS Biotec, China) for 2–4 weeks, and then it was collected with 0.1% Tween 80 to be made into a conidial suspension stored at 4°C for a maximum of 3–4 weeks. Before each experiment, we measured conidial germination and found that all conidial suspensions had a germination rate of >95%. Exposed termites were inoculated on their abdomen's surface with a 0.35- μ l droplet of the conidial suspension using a pipette (0.1–2.5 μ l, Eppendorf). Unexposed termites (controls) were treated with 0.35- μ l droplet of conidia-free 0.1% Tween 80 instead of conidia suspension. After being exposed to the conidia or the Tween 80, all treated termites were refrigerated at 4°C for an hour to slow their activity and to precipitate the conidia onto their cuticle before being used in the following experiments (Traniello et al. 2002).

Observation of Allogrooming Behavior

Allogrooming behavior is an effective behavioral adaptation to remove conidia from the cuticle of termites. Thus, it is important to determine the changes of this behavioral adaptation between fungus-exposed groups and control groups. For the fungus-exposed groups, two termites were exposed to 5×10^7 conidia/ml conidial suspension. For the control groups, two termites were treated with 0.1% Tween 80. According to the method of Hamilton et al. (2011), a pair of termites was placed in a cell culture dish (3.5 cm in diameter) with a disc of moist filter paper in darkness. Beginning 5 min after cultivation, we video-recorded the events of allogrooming behavior (licking body surface) for a pair of termites by the camera with a nightshot function (HDR-XR550E, SONY) for 30 min. There were nine replicates from three colonies for this experiment.

SEM Observation of Conidia on the Cuticles

Fungus-treated termites were exposed to 5×10^7 conidia/ml suspension. One fungus-treated termite or 20 fungus-treated termites were put in a Petri dish (9.0 cm in diameter) with a disc of moist filter paper. All the petri dishes were kept at $25 \pm 1^\circ\text{C}$ and 80% RH in darkness. After 1 or 12 h of *M. anisopliae* infection, three termites from each treatment were killed with ether fumes and then fixed in 2.5% glutaraldehyde solution at 4°C. Samples were dehydrated by ethanol method, followed by critical point drying method. The dried samples were fixed on a holder with conductive carbon adhesive tape, followed by coating with platinum. Conidia on the cuticles of the termite samples were observed by using scanning electron microscope (SEM) (JSM-6390, JEOL, Japan). Both unexposed single and unexposed grouped termites after 12-h cultivation were regarded as controls.

Survival

For the mortality assay of exposed single termites, termites were reared individually in Petri dishes (3.5 cm in diameter) with moist filter paper, then each was assigned to one of four treatments: exposed to 0.1% Tween 80 (control), 5×10^3 , 5×10^5 , and 5×10^7 conidia/ml. Each treatment contained 24 termites from three colonies (eight termites per treatment per colony). Mortality was recorded daily for 7 days and dead termites were removed when recording.

In addition, we compared the infection mortality between single termites and grouped termites under the 5×10^7 conidia/ml treatment. One and twenty termites were exposed and reared in the Petri dishes (9.0 cm in diameter) with moist filter paper for a week. Three colonies were used for this experiment (one exposed termite: 12 termites per colony, 36 termites in total; twenty exposed termites: 20 termites per colony, 60 termites in total). We recorded the number of dead termites every day and the dead termites were removed when recording.

Antifungal Activity

For antifungal activity assay (the ability of tissue fluid to inhibit fungal growth) of exposed single termites, termites were exposed to 0.1% Tween 80, 5×10^3 , 5×10^5 , and 5×10^7 conidia/ml, respectively. They were isolated from their nestmates and reared in Petri dishes (3.5 cm in diameter) with moist filter paper. There were five replicates (each replicate containing a pool of five termites) from five colonies for this experiment. The antifungal activity was tested after 3 d of fungal infection. The result showed that 5×10^5 conidia/ml was sufficient to upregulate immunity (Fig. 4a) with lower mortality in single termites when compared with the single termites exposed to 5×10^7 conidia/ml (Fig. 3a). Thus, we chose 5×10^5 conidia/ml for the following test.

To measure the antifungal activity of single termites and grouped termites, one and twenty exposed termites were exposed to 5×10^5 conidia/mL, and then reared in Petri dishes (9.0 cm in diameter) with moist filter paper. For the control groups, 1 and 20 unexposed termites were treated with 0.1% Tween 80. There were five replicates (each replicate containing a pool of seven termites) from five colonies for this experiment. The antifungal activity was tested after 3 d of fungal infection. Samples were crushed in 1.5-ml centrifuge tube with liquid nitrogen and then macerated with phosphate buffered saline (PBS) in a proportion of 1 mg of body weight to 5 μ l of PBS. About 20 μ l supernatants were extracted after centrifugation of the homogenates at 6,000 \times g for 5 min at 4°C (Thermo Scientific Sorvall Legend Micro 21R). Repeated the protocol and then 10- μ l supernatants were extracted and stored at -80°C until the antifungal activity assay; 96-well microplates were used for the antifungal activity assay and each well contained 50- μ l potato dextrose (PD), 2- μ l blastospores (about 10^6 spores/ml), and 2- μ l supernatants. Additionally, we used 50- μ l PD, 2- μ l blastospores, 2- μ l PBS per well for spores-growth control, 50- μ l PD, and 4- μ l PBS per well for standards. After about 24-h incubation in constant temperature shaker (200 rpm; $25 \pm 1^\circ\text{C}$), the absorbance of each well was measured by the microplate spectrophotometer (Bio-Rad xMark; 600 nm) (Konrad et al. 2012).

Expression of Immune Genes

To determine immune-gene expressions of single termites and grouped termites, 1 termite and 20 exposed termites were exposed to 5×10^5 conidia/ml and then reared in Petri dishes (9.0 cm in diameter) with moist filter paper. For the control groups, 1 termite and 20 unexposed termites were treated with 0.1% Tween 80. There were three replicates (each replicate containing a pool of seven termites) from three colonies for this experiment. The immune gene expressions were tested after 5 d of fungal infection. Samples were crushed in 1.5-ml centrifuge tube with liquid nitrogen, using sterilized disposable tissue grinding pestles. Total RNA was extracted from the samples using RNAiso Plus according to manufacturer protocol (TaKaRa, Dalian, China). The purity and concentration of the extracted RNA were determined by Thermo NANO DROP

2000 Spectrophotometer. Approximately, 1- μ g RNA was treated by PrimeScriptTM RT Reagent Kit with gDNA Eraser (Perfect Real Time; TaKaRa). The cDNA products were diluted in a proportion of 1 μ l of cDNA to 20 μ l of deionized water as templates for the real-time polymerase chain reaction (PCR) of *termicin* (Bulmer and Crozier 2004), *transferrin* (Thompson et al. 2003), and β -*actin* (reference gene) (Hamilton and Bulmer 2012). The quantitative reaction was performed on My IQ 2 Two color Real-Time PCR Detection System (Bio-Rad). Reaction mixtures, containing 10 μ l of SYBR Premix Ex Taq II (TaKaRa), 7.2 μ l of deionized water, 0.4 μ l of forward primer (10 μ M), 0.4 μ l of reserve primer (10 μ M), and 2 μ l of template cDNA, were performed as the following reaction condition: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 58°C for 30 s.

The primers used for real-time PCR are listed in Table 1. The expressions of groups exposed to fungus were calibrated by those of groups exposed to 0.1% Tween 80. The relative gene expressions were normalized by the method of $2^{-\Delta\Delta C_t}$ (Livak and Schmittgen 2001). The real-time PCR was performed on three biological replicates, each containing three technical replicates (Liu et al. 2015).

Statistical Analysis

All of the data were analyzed with IBM SPSS (Statistical Package for the Social Sciences) Statistics 19.0 software. Behavioral observations were analyzed with paired *t*-test. Survival was analyzed with a Cox proportional regression for assessing variables affecting survival and then was analyzed with Kaplan–Meier methods for life span under different conidial concentrations and termite quantities. For antifungal activity and immune-gene expression, data analysis was performed with one-way ANOVAs using Tukey's HSD test for significant differences.

Table 1. Primers used for real-time RT-PCR in this study

| Gene name | Orientation | (5'→3') Primer sequence |
|---------------------------------|-------------|-------------------------|
| <i>β-actin</i> | Forward | ctcaggtagtggtgtctc |
| | Reverse | caggtagtcggcaagtc |
| <i>termicin</i> | Forward | tcgtcttctgctgctgtagt |
| | Reverse | cagtgggatagagatgata |
| <i>transferrin</i> | Forward | atgcacggcctgctgttat |
| | Reverse | tgtgtgcctctgtgagca |

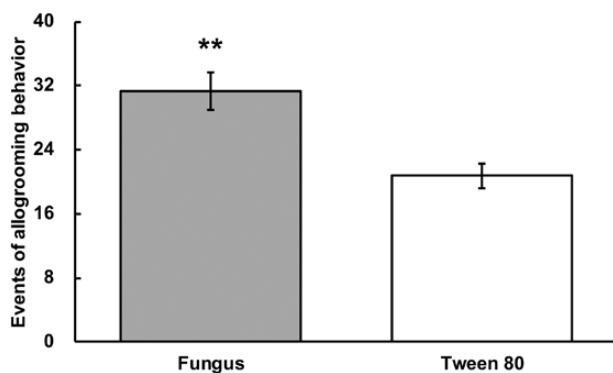


Fig. 1. Events of allogrooming behavior. A pair of termites was exposed to fungal conidia (gray) or 0.1% Tween 80 (White). Error bars represent mean \pm SEM. Asterisks denote significant differences (** $P < 0.01$, paired *t*-test).

Results

Observation of Allogrooming Behavior

Allogrooming behavior occurred significantly more frequently in the fungus-treated groups than in the controls (Fig. 1; $T = 3.777$; $df = 8$; $P = 0.005$).

SEM Observation of Conidia on the Cuticles

Compared with the conidia on the cuticle of single exposed termites (Fig. 2a and c), most of the conidia on the cuticles of grouped exposed termites were removed by intensive allogrooming behavior after 1- and 12-h infection (Fig. 2b and d). After 12-h cultivation, there were no conidia in unexposed single and grouped termites (Fig. 2e and f).

Survival

The exposed termites show concentration-dependent susceptibility to *M. anisopliae*. For the survival of individuals under different conidial concentrations, the colony had the negligible effect on termite survival (Wald = 0.001; $df = 1$; $P = 0.970$), whereas the conidial concentrations had the significant effect (Wald = 20.605; $df = 1$; $P < 0.001$). The survival under the 5×10^7 conidia/ml treatment was significantly lower than under the other treatments (Fig. 3a; 10^7 vs 10^5 : $\chi^2 = 6.559$, $P = 0.01$; 10^7 vs 10^3 : $\chi^2 = 24.269$, $P < 0.001$; 10^7 vs Tween 80: $\chi^2 = 24.269$, $P < 0.001$). The survival under the 10^5 conidia/ml treatment was significantly lower than under the 5×10^3 conidia/ml and 0.1% Tween 80 treatments (Fig. 3a; 10^5 vs 10^3 : $\chi^2 = 8.054$, $P = 0.005$; 10^5 vs Tween 80: $\chi^2 = 8.054$, $P = 0.005$).

For the survival between single termite and grouped termites, the termite quantity (Wald = 21.913; $df = 1$; $P < 0.001$) rather than colony (Wald = 0.090; $df = 1$; $P = 0.764$) had significantly impact on termite survival. The survival of grouped exposed termites was significantly higher than that of single exposed termites (Fig. 3b; $\chi^2 = 38.498$, $P < 0.001$).

Antifungal Activity

After 3 d of cultivation, significant differences were detected in antifungal activity of single termite under different conidial concentrations (Fig. 4a; $F = 8.598$, $df = 3, 16$; $P = 0.001$). Antifungal activity of single termite exposed to 5×10^7 and 5×10^5 conidia/ml was significantly higher than that of single termite exposed to 5×10^3 conidia/ml (Fig. 4a; $P < 0.05$) and 0.1% Tween 80 (Fig. 4a; $P < 0.05$). Antifungal activity of single termite exposed to 5×10^3 conidia/ml was not significantly higher than that of single termites exposed to 0.1% Tween 80 (Fig. 4a; $P > 0.05$).

After 3 d of cultivation, termite quantity had a significant effect on antifungal activity of individuals under 5×10^5 conidia/ml (Fig. 4b; $F = 8.598$, $df = 3, 16$; $P = 0.001$). Antifungal activity of single exposed termite was significantly higher than that of single unexposed termite, and grouped exposed and unexposed termites (Fig. 4b; $P < 0.05$). There were no significant differences in antifungal activity among single unexposed termite, and grouped exposed and unexposed termites (Fig. 4b; $P > 0.05$).

Expression of Immune Genes

After 5 d of cultivation, the expression of the immune gene *termicin* in single exposed termite significantly higher than that in single unexposed termite, and grouped exposed and unexposed termites (Fig. 5a; $F = 115.992$; $df = 3, 8$; $P < 0.001$). Also, the expression of the immune gene *transferrin* in single exposed termite was significantly upregulated when compared with the other three treatments (Fig. 5b; $F = 67.517$; $df = 3, 8$; $P < 0.001$). However, the two immune

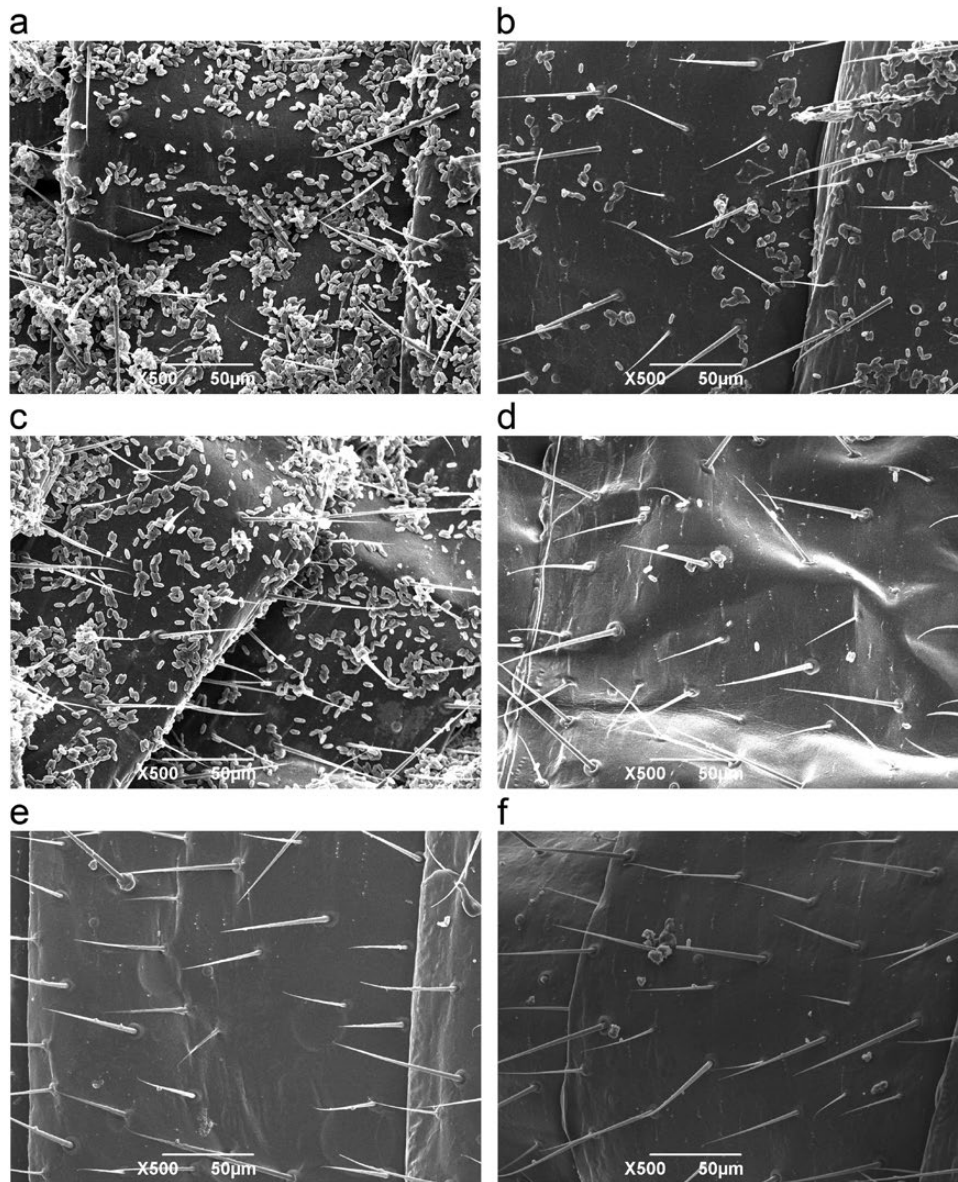


Fig. 2. The fungal conidia on termite cuticles. Conidia on the cuticle of single exposed termites after 1 h (a) and 12 h (c) infection. Conidia on the cuticles of grouped exposed termites after 1 h (b) and 12 h (d) infection. After 12 h cultivation, unexposed single (e) and grouped (f) termites were regarded as controls.

genes exhibited no significant differences among single unexposed termite, and grouped exposed and unexposed termites ($P > 0.05$).

Discussion

Sanitary behaviors can effectively reduce the risk of disease for pathogen-exposed individuals (Yanagawa and Shimizu 2007, Konrad et al. 2018). When insects somehow contact the pathogenic fungus, the fungal conidia may transfer to their cuticles, and hence, the insects become pathogen-exposed individuals. During early-stage infection, the conidia those attach to the insect cuticles loosely can be cleared by sanitary behaviors among insects (Rosengaus et al. 1998, Calleri et al 2006, Yanagawa and Shimizu 2007, Cremer et al. 2007, Konrad et al. 2018). However, when the conidia germinate and fully attach to the cuticles, it will be very difficult to remove them. If the pathogens cause disease in exposed individuals, innate immunity will be activated to kill pathogens or inhibit pathogen growth in vivo

(Hamilton et al. 2011, Konrad et al. 2012, Zeng et al. 2016, Liu et al. 2015). In this study, we found that grouped termites immediately increased allogrooming behavior when they were exposed to the pathogen, resulting in apparently decreased pathogen loads. Since termites exhibited concentration-dependent susceptibility to the pathogens, the decreased pathogen loads had less impact on infection mortality of termites under group conditions. Therefore, allogrooming behavior among termite nestmates during early infection was an effective mechanism to reduce the risk of disease and improve the survival of group members.

However, when pathogens successfully invade the body cavities of termites, individual innate immunity will work (Thompson et al. 2003, Bulmer and Crozier 2004). Termites exposed to pathogens exhibit the upregulation of antifungal activity and expression of immune proteins to kill pathogens or inhibit pathogen growth (Bulmer and Crozier, 2004, Rosengaus et al. 2007; Zeng et al. 2016, Liu et al. 2015). Among these immune proteins, termicin is a

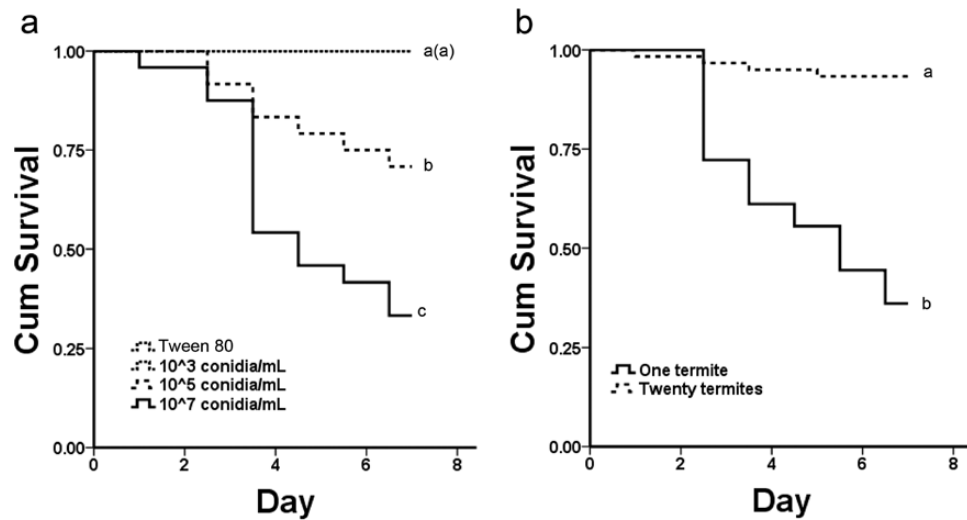


Fig. 3. The life span of the termites among different treatments. (a) The life span of the termites exposed to 5×10^7 , 5×10^5 , 5×10^3 conidia/ml, and 0.1% Tween 80, respectively; (b) the life span of single termites and grouped termites exposed to 5×10^7 conidia/ml. The curves of controls and 5×10^3 conidia/ml treatments largely overlapped and displayed as a single dotted line. Different letters indicated significant differences ($P < 0.05$, Kaplan-Meier methods).

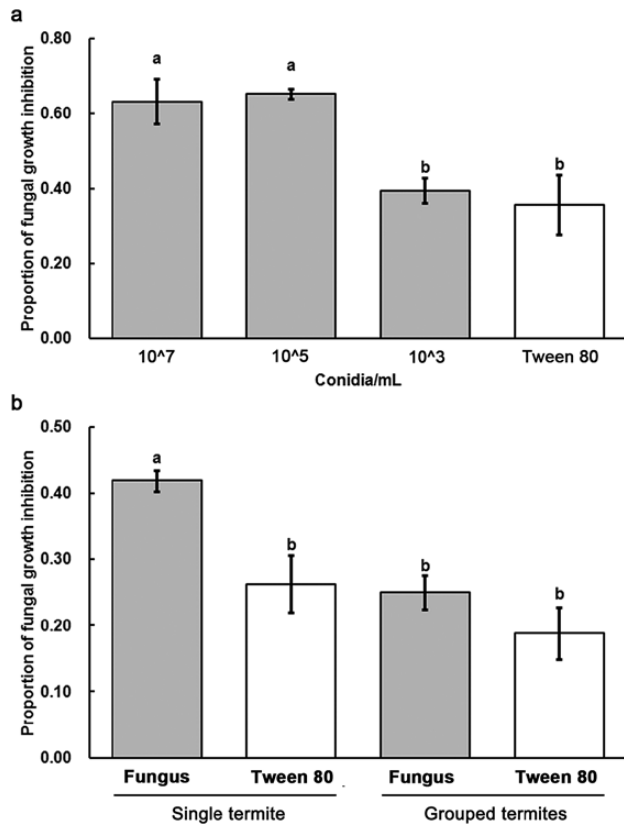


Fig. 4. Antifungal activity of the termites among different treatments. (a) The antifungal activity of single termites exposed to 5×10^7 , 5×10^5 , 5×10^3 conidia/ml, and 0.1% Tween 80, respectively; (b) The antifungal activity of single and grouped termites exposed to 5×10^5 conidia/ml, and single and grouped termites treated by 0.1% Tween 80. Error bars represent mean \pm SEM. Different letters over the bars denote significant differences ($P < 0.05$, Tukey's HSD).

cysteine-rich antimicrobial peptide, exhibiting strong killing activity against fungal pathogens (Bulmer and Crozier 2004). Transferrin may protect free iron ions from being utilized by fungus so as to inhibit fungal growth in termite hemocoel (Thompson et al. 2003).

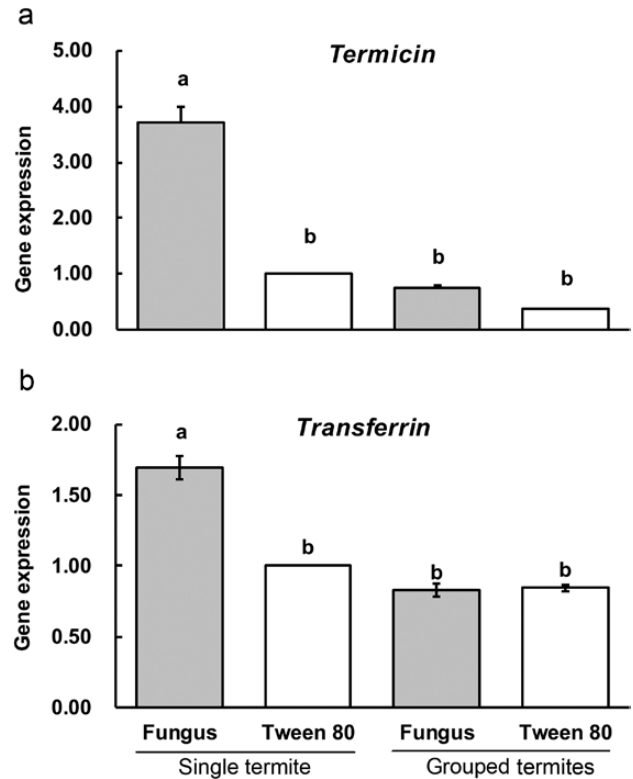


Fig. 5. Expressions of the two immune genes among different treatments. Single and grouped termites were treated by 5×10^5 conidia/ml or 0.1% Tween 80. Error bars represent mean \pm SEM. Different letters over the bars denote significant differences ($P < 0.05$, Tukey's HSD).

However, grouped fungus-exposed termites in this study did not upregulate their antifungal activities and expression of the immune genes *termicin* and *transferrin*, which might be caused by social sanitary behaviors. Since allogrooming behavior is an important component of sanitary cares to disinfect insect cuticles and clear pathogens (Yanagawa and Shimizu 2007, Hamilton and Bulmer 2012, Konrad et al. 2018), it may be difficult for the pathogens to succeed in invading into the termite hemocoel. Hence, the host immune system in

vivo is unnecessary to be activated for recognizing the pathogens, upregulating immune proteins, and increasing antifungal activity. Therefore, we inferred that allogrooming behavior decreased physiological investments in individual innate immunity by preventing pathogens from invading hosts.

In social insects, allogrooming can not only remove pathogens from the cuticle to reduce the risk of disease (Cremer et al. 2007, Yanagawa and Shimizu 2007) but also may result in the transfer sublethal doses of pathogens to naïve nestmates to immunize them against pathogens (Traniello et al. 2002, Konrad et al. 2012, Liu et al. 2015). This kind of social immunization can decrease susceptibility of social insects to the same pathogens (Traniello et al. 2002, Konrad et al. 2012, Liu et al. 2015). However, recent work has shown that low-level infected individuals by social immunization are more susceptible to a different pathogen, so the immunized social insects reduced the allogrooming frequency toward the individuals exposed to a different pathogen to avoid superinfections (Konrad et al. 2018). Thus, social insects always choose the optimum defensive strategies to resist infections of different pathogens (Cremer et al. 2007). In the future, more studies on the immune system in social insects need be carried out to deeply understand the evolution of social immunity (Cremer et al. 2018).

Acknowledgments

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