

Longevity, trophallaxis, and allogrooming in *Macrotermes gilvus* soldiers infected by the parasitoid fly *Misotermes mindeni*

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Abstract

In this study, we documented the behavioral changes of major soldiers of the subterranean termite *Macrotermes gilvus* (Hagen) (Blattodea: Termitidae) infected with larvae of the phorid fly *Misotermes mindeni* Disney & Neoh (Diptera: Phoridae). During periods of starvation, the longevity of parasitized individuals was significantly greater than that of unparasitized individuals. Parasitized individuals received significantly more frequent trophallaxis from the workers than unparasitized individuals. The frequency of allogrooming of parasitized individuals by workers was also significantly greater than that of unparasitized individuals. The extent to which workers provided trophallaxis and allogrooming for parasitized individuals did not differ significantly between colonies with and without unparasitized individuals. Likewise, no significant difference in the frequency of trophallaxis between workers and unparasitized individuals was detected in the groups with and without parasitized individuals. However, distinct differences in allogrooming frequency between groups with and without parasitized individuals were detected. Workers/unparasitized individuals exhibited significantly more allogrooming behavior when parasitized individuals were present. Parasitized major soldiers and unparasitized, food-deprived major soldiers received significantly more often trophallaxis from the workers than unparasitized major soldiers. Presence of a parasitoid fly in major soldiers may promote the frequent feeding of parasitized individuals by workers. Based on these data, the behavioral adjustments appear to be beneficial to the parasitoids, as the changes clearly increase their chances of survival and their chances of completion of their larval development before the host dies.

Introduction

Macrotermes gilvus (Hagen) (Blattodea: Termitidae) is a common fungus-growing termite species predominantly distributed in Southeast Asia (Roonwal, 1970). It is a serious pest of wooden structures and agricultural crops such as rubber, oil palm, and sugar cane (Roonwal, 1970; Cowie et al., 1989; Rouland-Lefevre, 2011). It also becomes an important secondary pest in buildings and structures when

the dominant *Coptotermes* spp. are eliminated via a termite baiting system (Lee, 2002; Lee et al., 2007).

The larvae of a phorid fly, *Misotermes mindeni* Disney & Neoh (Diptera: Phoridae), have been found parasitizing *M. gilvus* (Disney et al., 2009; Neoh & Lee, 2010). The fly larva completes its first instar development in fourth instars and major presoldiers of *M. gilvus*, whereas the second and third instars of the fly develop entirely in major soldiers' head capsules and abdomens respectively (Foo et al., 2011a). The fly larva consumes the entire contents of the major soldier's head capsule and subsequently moves to the termite abdomen when it is about to pupate. To create a dry microenvironment for pupation, the fly larva uses its spiracles to apply sideways pressure to the termite abdomen, which causes the body fluid to ooze out of the abdomen. The host eventually dies of trauma or dehydration after pupation is completed (Neoh & Lee, 2010).

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Starvation resistance is a trait often associated with longevity (Harshman et al., 1999; Ballard et al., 2008). Greater starvation resistance in *Drosophila* spp. due to an increase in lipid levels is supported by results of several physiological studies (Chippindale et al., 1996; Hoffman et al., 2005; Sisodia & Singh, 2010).

The relationships between insect parasitoids and their hosts are intricate. Many of the host–parasite interactions are viewed as adaptive responses of the host to compensate for the effects of parasitism, or an adaptive modification of the host by the parasite to ensure reproductive success of the parasite (Poulin, 1995). Host and parasite manipulate each other and both can change in response to what the other does. Barber et al. (1988) reported that survival of the parasitoid relies on aspects of host nutrition, as the development of the parasitoid depends on resources accrued from a single host. For both hosts and parasites, numerous behavioral modifications have been reported. As proposed by Poulin (1995), changes in host behavior can only be considered adaptive if they satisfy the following conditions: (1) they must be complex; (2) they must show signs of purposive design; (3) they must have evolved independently in several lineages of hosts or parasites; and (4) they must have fitness relevance for either the host or the parasite. Examples of such behavioral modifications in the host include behavioral fever, foraging, feeding, reproduction, phototaxis, and various social interactions such as trophallaxis, grooming, and social avoidance (Loehle, 1995; Roy et al., 2006; Libersat et al., 2009; Neoh & Lee, 2010).

Trophallaxis is the transfer of food or fluids among colony members through stomodeal (mouth to mouth) and proctodeal (anus to mouth) contact. Only stomodeal feeding occurs in *M. gilvus* (McMahan, 1969). Termite soldiers cannot feed themselves and are completely dependent on workers for nutrition because the soldier mandibles are modified for defense (Sieber & Leuthold, 1981; Su & La Fage, 1987; Huang et al., 2008). According to Su & La Fage (1987), trophallactic exchange is an unsolicited food-offering event by worker donors to satisfy the nutritional need of soldiers. These authors hypothesized that workers are responsible for mediating the transfer of food to soldiers and that the process is largely independent of the nutritional status of the soldiers due to their small proportion within a colony. However, McMahan (1963) and Whitman & Forschler (2007) suggested that trophallaxis is initiated by starved individuals via antennation to solicit food from prospective donors. In this scenario, the soliciting termite touches the head of the prospective donor with its antennae or taps the donor's buccal region with its antennae (McMahan, 1969).

Allogrooming is an activity in which an individual in a colony cleans the body surface and appendices of another individual (Maistrello & Sbrenna, 1996). This behavior is believed to reduce disease risk in insects because it effectively removes foreign particles (Loehle, 1995). For example, the cricket *Gryllus texensis* Cade & Otte prevents planidia of the parasitoid *Ormia ochracea* (Ortnia) from burrowing into its body by increasing grooming activity (Vincent & Bertram, 2010). The repertoire of grooming behaviors reported in termites such as *Zootermopsis angusticollis* (Hagen) (Rosengaus & Traniello, 2001), *Reticulitermes speratus* Kolbe (Shimizu & Yamaji, 2003), and *Coptotermes formosanus* Shiraki (Yanagawa & Shimizu, 2005) reduces their susceptibility to fungal infections.

The objectives of this study were to: (1) assess the longevity of parasitized and unparasitized major soldiers during periods of starvation; (2) examine and compare the frequencies of trophallaxis and allogrooming between workers/parasitized major soldiers and workers/unparasitized major soldiers; (3) determine whether the frequencies of trophallaxis and allogrooming between workers and unparasitized and parasitized major soldiers were affected by the presence of parasitized or unparasitized major soldiers in the colony; and (4) test whether the frequency of food exchange between workers and parasitized major soldiers is similar to that between workers and unparasitized food-deprived major soldiers.

Materials and methods

Termite collection

Parasitized and unparasitized major soldiers were randomly collected from previously surveyed parasitized *M. gilvus* colonies located on Minden Campus of Universiti Sains Malaysia (5°21'N, 100°18'E), Bayan Lepas (5°17'N, 100°15'E), Gelugor (5°22'N, 100°18'E), and Ayer Itam (5°23'N, 100°17'E), Penang, in northern Peninsular Malaysia (Foo et al., 2011c). Parasitized individuals were identified based on the presence of a rounded head capsule with notably short mandibles (Neoh & Lee, 2010). Parasitized *M. gilvus* mounds were broken up by digging a trench around the base of the nest. The mound casing was carefully removed to avoid injury to parasitized termites, which are often found congregated at the peripheral zone of the mound (Neoh & Lee, 2010; Foo et al., 2011c). The collected termites were then brought to the laboratory.

Longevity assay

To assess the longevity of parasitized and unparasitized individuals during a starvation period, 31, 32, and 33 parasitized individuals and 30, 30, and 25 unparasitized individuals from three colonies, respectively, were placed in six

cylindrical polyethylene containers (9.5 cm diameter, 6.5 cm high) to which 20 g of moistened sand had been added to serve as substrate and water source. The containers were maintained in an incubator (Incucell; MMM Medcenter Einrichtungen, Munich, Germany) in total darkness at 30 ± 1 °C and 90% r.h. Mortality was recorded daily until no major soldiers remained alive.

Evaluation of trophallaxis and allogrooming behaviors

The following termite groups were established to examine whether the presence of parasitized individuals or unparasitized individuals in the colony affects the frequencies of trophallaxis and allogrooming of workers/unparasitized individuals and workers/parasitized individuals respectively. For the first experiment, six parasitized individuals and six unparasitized individuals were maintained together with 100 workers (42 major and 58 minor workers) in the containers described above. In the second experiment, 100 workers were combined with either 12 parasitized individuals or 12 unparasitized individuals. The caste composition in both experimental groups was set according to Lee et al. (2012). Each container received 20 g of moist sand and 3 g of fungus comb for food. The containers were subsequently placed in an opaque plastic box ($35 \times 23.5 \times 19.5$ cm) with a glass cover for ease of observation and maintained in the dark under constant conditions of 30 ± 1 °C and 90% r.h. Three days after the experimental units were established, trophallaxis and allogrooming behaviors of termites were video-recorded using a digital camera (Digital Ixus 80 IS; Canon, Tokyo, Japan) at 07:00, 12:00, and 17:00 hours daily for 3 days. Each video recording was performed for 30 min under red light, starting 10 min after the light was turned on to allow termites to acclimate to the observation conditions. Trophallaxis was considered to have taken place if the mouthparts of the workers and major soldiers were held in close contact for at least 30 s (Sieber & Leuthold, 1981). Workers were considered to be allogrooming if they moved their mouthparts to palpate and clean the body surface and appendices of the major soldiers (Maistrello & Sbrenna, 1996). The frequency of occurrence of each behavior in every sample period (30 min) was recorded. A total of eight groups originating from eight colonies were observed for each group composition.

Worker response to starved soldiers

The occurrence of food exchange from workers to parasitized individuals was significantly greater than that to unparasitized individuals, and we hypothesized that the greater nutritional requirements of parasitized individuals

may be a cause of elevated feeding activity. To test this premise, we compared the rate at which unparasitized food-deprived major soldiers, parasitized major soldiers, and unparasitized major soldiers received food from the workers. Eight replicates with six individuals for each major soldier type of *M. gilvus* were established. Soldiers were taken at random from eight colonies. Unparasitized food-deprived individuals, parasitized individuals, and unparasitized individuals were established separately in the aforementioned polyethylene containers, but no fungus comb and no workers were provided for unparasitized food-deprived individuals for 3 days. According to Huang et al. (2008), a 3-day period is sufficient to starve the termites. The containers were placed in an opaque plastic box with a glass cover and maintained under constant conditions (30 ± 1 °C and 90% r.h.) in complete darkness. After 3 days, unparasitized food-deprived individuals were introduced into containers with fully fed workers. Observations of trophallactic exchange events between workers and unparasitized food-deprived individuals, parasitized individuals, and unparasitized individuals were made using videotape recordings under red light taken at 07:00 hours for 30 min.

Statistical analysis

For the longevity assay, cumulative mortality was calculated and subjected to probit analysis to determine the values of LT_{50} and LT_{95} for parasitized and unparasitized individuals. Non-parametric tests were used in this study due to violations of normality and equal variance assumptions in the data. The frequencies of occurrence of trophallaxis and allogrooming behaviors between workers/parasitized individuals and workers/unparasitized individuals were compared. Because there were no differences in trophallaxis and allogrooming frequencies based on time (07:00, 12:00, and 17:00 hours) and over 3 days of observations, the data were pooled and analyzed using Mann–Whitney U-test. This test was also applied to examine whether the presence of parasitized or unparasitized individuals affected the frequencies of trophallaxis and allogrooming behaviors of workers/unparasitized individuals and workers/parasitized individuals respectively. Kruskal–Wallis test was used to analyze the differences in frequency of occurrence of trophallactic behavior among unparasitized food-deprived individuals, parasitized individuals, and unparasitized individuals. Comparison of mean ranks was used to assess which groups of major soldiers differed significantly from each other. All analyses were performed at $\alpha = 0.05$ using SPSS version 16.0 (SPSS, Chicago, IL, USA), with the exception of the Kruskal–Wallis test, for which STATISTIX version 7.0 was used (Analytical Software, Tallahassee, FL, USA).

Results

Longevity assay

Parasitized individuals lived almost $1.5\times$ ($LT_{50} = 4.8$ days, range = 4.25–5.34 days; $LT_{95} = 10.8$ days; range = 9.21–13.76 days; $n = 3$) longer than unparasitized individuals ($LT_{50} = 3.3$ days; range = 2.99–3.65 days; $LT_{95} = 7.69$ days; range = 6.75–9.17 days; $n = 3$) under starvation conditions.

Differences in trophallaxis and allogrooming behaviors

The rate of food transfer between workers and parasitized individuals differed significantly from that between workers and unparasitized individuals. Parasitized individuals received more frequent trophallaxis than unparasitized individuals (Mann–Whitney U-test: $Z = -3.361$ and -3.363 , respectively; both $P < 0.01$; Table 1). Similarly, the frequency of allogrooming between workers and parasitized individuals also was greater than that between workers and unparasitized individuals ($Z = -3.361$ and -3.363 , respectively; both $P < 0.01$; Table 1).

Worker behavior in groups with or without parasitized or unparasitized individuals

The frequencies with which workers performed trophallaxis and allogrooming behaviors for parasitized individuals were similar irrespective of whether unparasitized individuals were present or not (Mann–Whitney U-test: $Z = -0.735$ and -1.785 , respectively; both $P > 0.05$; Table 2). Likewise, no difference in trophallaxis frequency between workers and unparasitized individuals was detected in the groups of termites with or without parasitized individuals ($Z = -0.170$, $P > 0.05$; Table 3). However, there were differences in the allogrooming frequency between groups of unparasitized individuals with and without parasitized individuals. Workers/unparasitized individuals exhibited significantly higher allogrooming

frequency when parasitized individuals were present ($Z = -2.312$, $P < 0.05$; Table 3).

Worker response to starved soldiers

A significant difference in the frequency of trophallaxis was found between unparasitized major soldiers, unparasitized food-deprived major soldiers, and parasitized soldiers (Comparison of mean ranks: $Z = 15.488$, $P < 0.01$). Trophallaxis occurred more often for unparasitized food-deprived individuals and parasitized individuals than for unparasitized individuals [mean \pm SE = 0.08 ± 0.02 (range = 0.07–0.12), 0.09 ± 0.03 (0.06–0.13), and 0.01 ± 0.01 (0.00–0.03), respectively; all $n = 8$].

Discussion

Resistance to starvation differed significantly between parasitized major soldiers and unparasitized major soldiers. Parasitized individuals lived almost $1.5\times$ longer than unparasitized individuals when starved. There is a strong likelihood that greater lipid levels (Foo et al., 2011b) account for the longer survival of parasitized individuals. The importance of lipid as an energy reserve during starvation has been well-documented in other insects, particularly in *Drosophila* species. For example, Harshman et al. (1999) found a positive correlation between starvation resistance and the proportion of body lipid in the fruit fly *Drosophila melanogaster* Meigen, and Ballard et al. (2008) reported that starvation-resistant *Drosophila simulans* Sturtevant contained higher levels of lipids. In another study, Forsman (1991) stated that larger individuals tend to survive longer than smaller ones because they can store greater quantities of energy reserves and function at a lower metabolic rate. Thus, body size may have played a role in the starvation resistance of parasitized individuals, as they were substantially larger (41.1%) than unparasitized individuals (Foo et al., 2011b). The increase in host size

Table 1 Frequencies of trophallaxis and allogrooming of *Macrotermes gilvus* workers/major soldiers, of which the major soldiers are parasitized or unparasitized by *Misotermes mindeni* flies

Experimental group	Behavior	Major soldier	n	Mean \pm SE	Range	Z	P
Workers together with parasitized and unparasitized major soldiers	Trophallaxis	Parasitized	8	0.10 ± 0.05	0.04–0.18	-3.361	<0.01
		Unparasitized	8	0.01 ± 0.01	0.00–0.02		
	Allogrooming	Parasitized	8	0.52 ± 0.17	0.32–0.81	-3.361	<0.01
		Unparasitized	8	0.14 ± 0.06	0.06–0.23		
Workers with only parasitized major soldiers or only unparasitized major soldiers	Trophallaxis	Parasitized	8	0.07 ± 0.01	0.05–0.09	-3.363	<0.01
		Unparasitized	8	0.01 ± 0.01	0.00–0.02		
	Allogrooming	Parasitized	8	0.39 ± 0.04	0.31–0.46	-3.363	<0.01
		Unparasitized	8	0.08 ± 0.02	0.05–0.11		

Mann–Whitney U-test.

Table 2 Frequencies of trophallaxis and allogrooming of *Macrotermes gilvus* workers/parasitized major soldiers in colonies with or without major soldiers unparasitized by *Misotermes mindeni* flies

Behavior	Unparasitized major soldiers	n	Mean \pm SE	Range	Z	P
Trophallaxis	Present	8	0.10 \pm 0.05	0.04–0.18	–0.735	>0.05
	Absent	8	0.07 \pm 0.01	0.05–0.09		
Allogrooming	Present	8	0.52 \pm 0.17	0.32–0.81	–1.785	>0.05
	Absent	8	0.39 \pm 0.04	0.31–0.46		

Mann–Whitney U-test.

Table 3 Frequencies of trophallaxis and allogrooming of *Macrotermes gilvus* workers/unparasitized major soldiers in colonies with or without major soldiers parasitized by *Misotermes mindeni* flies

Behavior	Parasitized major soldiers	n	Mean \pm SE	Range ¹	Z	P
Trophallaxis	Present	8	0.01 \pm 0.01	0.00–0.02	–0.170	>0.05
	Absent	8	0.01 \pm 0.01	0.00–0.02		
Allogrooming	Present	8	0.14 \pm 0.06	0.06–0.23	–2.312	<0.05
	Absent	8	0.08 \pm 0.02	0.05–0.11		

Mann–Whitney U-test.

¹0.00 indicates that no behavior was observed.

may be the biggest manipulation of the host by the parasitoid.

Greater lipid reserves may be attributed to the higher feeding activity of parasitized individuals. Host feeding behaviors can be manipulated by parasites (Lenz & Kimbrough, 1982; Wise de Valdez, 2006; Heintschel et al., 2007). In the present study, we found that trophallaxis between workers and parasitized individuals was greater than that between workers and unparasitized individuals. An increase in feeding activity was also demonstrated in larvae of the mosquito *Aedes aegypti* (L.) infected with mermithid nematodes (Wise de Valdez, 2006) and in the mosquito *Anopheles stephensi* Liston infected with *Plasmodium yoelii nigeriensis* sporozoites (Anderson et al., 1999). We postulate that the increased food demands of parasitized individuals could be an adaptive strategy for both the parasitoid and the host because the parasitoid has to ensure that it has enough food for development and the host has to ensure that it has enough food for itself in addition to what the parasitoid takes. When the feeding activity of parasitized individuals was compared with that of unparasitized food-deprived individuals and unparasitized individuals, both parasitized individuals and unparasitized food-deprived individuals received trophallaxis more often than unparasitized individuals. Our results are consistent with the hypothesis that high energy/food demands caused by parasitization may be the underlying mechanism that triggers the frequent trophallaxis between workers and parasitized individuals.

At the same time, the frequent trophallactic exchanges observed between workers and parasitized individuals were a parasitized individuals-mediated behavior. This is supported by evidence from the termite feeding studies conducted by McMahan (1963) and Whitman & Forschler (2007), who suggested that trophallactic exchange was initiated by food-deprived individuals. Because parasitized individuals were able to survive longer than unparasitized individuals under starvation conditions, it is possible that the parasitoids cause the hosts to obtain more food than needed for basic needs to ensure that the parasitoid has a good chance of survival. Another possibility for greater food exchange between workers and parasitized individuals compared to unparasitized individuals is that parasitoids may require more food and use up stored host lipid reserves, especially toward the end of the larval development. These scenarios suggest that the parasitoid modified the food-acquisition behavior of parasitized individuals. Although behavioral modifications of parasitized individuals by larval parasitoids (i.e., increased frequency of trophallaxis) might appear to be adaptive, it is also possible that these behavioral changes are due to the pathological side effects of infection. The three modes of pathologic responses that could lead to host behavioral modifications are organ malfunctions due to direct tissue damage, modulation of neuroendocrine control systems, and impaired nutrition (Holmes & Zohar, 1990).

Allogrooming behavior of workers/parasitized individuals exhibited all of the components of normal allogrooming of workers/unparasitized individuals, but it differed in

the frequency with which it occurred. In the current study, workers groomed parasitized individuals more frequently than they groomed unparasitized individuals. Extensive allogrooming was also documented in the American cockroach, *Periplaneta americana* (L.), after it was parasitized by the parasitoid wasp *Ampulex compressa* (Fabricius) (Weisel-Eichler et al., 1999; Libersat et al., 2009). Intensive allogrooming may be required to ensure the removal of ectoparasites (e.g., fungi, bacteria, mites) from the host's outer body surface, which may be detrimental to the developing parasitoid larvae. Ectoparasites can be extremely costly, as they can suppress development and maturation rates and reduce survival of the host insect (Vincent & Bertram, 2010). Thus, various social behaviors, such as allogrooming, are effective at minimizing pathogen infection (Loehle, 1995; Pettis & Pankiw, 1998; Reber et al., 2011) in the host and promoting survival of parasitoid larvae.

The colony, in general, should have limited or no interest in looking after parasitized individuals because the host will die at the end of the period of larval parasitoid development. However, the current results show that workers fed and groomed parasitized individuals more frequently than they did unparasitized individuals, indicating that parasitoids affect worker behavior via the demands of parasitized individuals. However, how parasitized individuals induce extensive allogrooming behavior by workers remains unclear. We postulate that the increase in allogrooming behavior of workers for parasitized individuals is elicited by frequent antennal contact. Yanagawa et al. (2009) suggested that the antennal sensory organs play an essential role in controlling the occurrence and persistence of grooming in termites. If greater antennal contact does indeed induce higher allogrooming behavior, the party responsible for initiating this social interaction needs to be identified. Typically, there are two types of allogrooming behaviors: active and passive. During active allogrooming, the true initiator of the interaction is the one that constantly grooms the partner, whereas during passive allogrooming the party to be groomed is the instigator (Maistrello & Sbrenna, 1996). In our study, passive allogrooming behavior seemed to be occurring between workers and parasitized individuals. However, it is also possible that sensory organs on other appendages such as the labium may be utilized to initiate allogrooming behavior (Yanagawa et al., 2009). Alternatively, certain chemicals emitted by parasitized individuals or originating from the parasitoid may have stimulated the extensive allogrooming behavior by workers, as the key mode of communication among colony members in termites is through chemical signals (Maistrello & Sbrenna, 1996). All of these possibilities merit further investigation.

We found notably more allogrooming behavior of workers/unparasitized individuals in the presence of parasitized individuals than without parasitized individuals present. This could be interpreted as a notable host adaptation to the presence of the parasitoid and a possible attempt to avoid parasitism. If the fly eggs are deposited or the larvae latch directly onto the termites, allogrooming would be needed to remove them before the parasitoid could enter the termite. The presence of parasitized individuals may raise the alarm levels in the workers. Vincent & Bertram (2010) reported that crickets (*G. texensis*) increased their grooming activity when placed in an arena filled with *O. ochracea* planidia-laden grass in order to remove the parasitoids before the latter had a chance to burrow into their body. Allogrooming is generally a constitutive defense, in that it is also applied to individuals regardless of the actual presence of parasites (Reber et al., 2011).

In conclusion, the results of this study show that in addition to inducing a significant increase in host body size (Foo et al., 2011b), the larval parasitoid also modifies trophallaxis and allogrooming behaviors of workers in relation to parasitized major soldiers. Increased solicitation of feeding activity of parasitized individuals may result in a build-up of lipid reserves in the hosts, which in turn ensure the development of the parasitoid and sustain survival of the host until the parasitoid completes its development. However, further studies are necessary to confirm this hypothesis. Increased allogrooming behaviors between workers and parasitized individuals may have enhanced the fitness of the larval parasitoids by minimizing pathogen infections of their hosts. The behavioral adjustments that occur in parasitized *M. gilvus* appear to be beneficial to the parasitoids as the changes clearly increase their chances of survival and their chances of completing their larval development before the host dies.

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