

# Effects of termiticide exposure on mutual interactions between the treated and untreated workers of the Asian subterranean termite *Coptotermes gestroi*

Kok-Boon Neoh,<sup>†</sup> Ching-Chen Lee and Chow-Yang Lee\*

## Abstract

**BACKGROUND:** Mutual interactions, including reciprocal food sharing and grooming between chlorantraniliprole- and fipronil-treated, and untreated Asian subterranean termites, *Coptotermes gestroi* (Wasmann), were examined using rubidium as a tracer. Two questions were addressed in this study: (1) After insecticide treatment, does the mutual interaction between termiticide-treated termites and untreated nestmates increase? (2) Does the nutritional status of both termiticide-treated termites and untreated nestmates affect the mutual interaction?

**RESULTS:** The comparative data suggested that chlorantraniliprole-treated termites were more regularly attended by untreated termites than the fipronil-treated termites. Mutual interaction between the chlorantraniliprole-treated termites and untreated termites was not affected by their nutritional status. A high level of rubidium was present in the reciprocal exchange from fipronil-treated termites to starved untreated termites, indicating that intoxication induced alimentary or anal fluids served as a food source for starved termites.

**CONCLUSION:** The results of the present study indicated that termites exposed to chlorantraniliprole were more likely to cease feeding and then undergo starvation. Thus, the treated termites were subject to intensive reciprocal food exchange and frequent attention from untreated nestmates. In the fipronil treatment, starvation status facilitated the reciprocal food exchange rate from treated termites to starved untreated termites.

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**Keywords:** horizontal transfer; nutritional status; food tracer; slow-acting effect; repetitive transfer; feeding cessation

## 1 INTRODUCTION

Trophallaxis, in which either alimentary or anal materials are transferred between nestmates within a colony, is a unique behaviour in eusocial insect societies.<sup>1</sup> In termites, trophallaxis serves three main functions: transfer of the symbiotic bacteria between generations,<sup>2</sup> distribution of caste regulatory pheromones within a colony,<sup>3</sup> and as a nutrient source for dependent castes (larvae, nymphs and soldiers)<sup>4–6</sup> and nutrient-deficient workers.<sup>7</sup> Due to food sharing among termites,<sup>8</sup> trophallaxis could be involved in transferring toxicants among termites in soils treated with non-repellent termiticides.<sup>9–11</sup>

Previous studies of termiticide trials on termites have focused primarily on the mortality of donor and recipient workers.<sup>10,12–14</sup> In fact, the behaviours induced in workers after treatment are crucial, as this is to ensure maximal interaction between treated and unexposed healthy termites.<sup>15,16</sup> In this study, we examined the food sharing behaviour between termiticide-treated termites and untreated termites. This paper is also the continuation of a previous study, which showed that chlorantraniliprole-treated termites cease to feed after being exposed to the termiticide for as little as 1 h.<sup>17</sup> To some degree, feeding inhibition might cause the treated termites to experience starvation. This observation raised

the following questions: (1) After treatment, does the mutual interaction (i.e. trophallaxis and mutual grooming) between termiticide-exposed termites and untreated nestmates increase? and (2) Does the nutritional status of both termiticide-treated termites and untreated nestmates affect the interaction? In this study, the reciprocal exchange between treated and untreated termites was determined using non-radiolabelled rubidium (Rb) as a tracer, as it has proven to be an excellent marker for termites.<sup>4,5,18</sup>

## 2 MATERIALS AND METHODS

### 2.1 Termites

Two field colonies of *Coptotermes gestroi* were collected from in-ground termite monitoring stations at Air Itam (5° 23' N, 100° 16'

\* Correspondence to: Chow-Yang Lee, Urban Entomology Laboratory, Vector Control Research Unit, School of Biological Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia. E-mail: chowyang@usm.my

† Present address: Center for Southeast Asian Studies, Kyoto University, 46 Shimoadachi-cho, Yoshida, Sakyo-ku, Kyoto 606–8501 Japan

Urban Entomology Laboratory, Vector Control Research Unit, School of Biological Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia

E) and Bukit Gambir (5° 18' N, 100° 14' E), Penang Island, Malaysia. Only workers of the third instar stage and older were used in this study.

## 2.2 Palatability and toxicity control

Palatability and toxicity control experiments were conducted prior to the main experiments to ensure that the Rb-treated paper had no effect on termites' palatability and survivorship. Termites were introduced into Petri dishes (diameter 15 cm) containing 0.1 g tissue paper treated with 1000, 2000, 4000 or 8000 mg kg<sup>-1</sup> (wt:wt) of RbCl<sub>2</sub> [(ReagentPlus® = 99.0% (metals basis)] (Sigma-Aldrich Co., St. Louis, MO, USA). Only distilled water was added to the control. Fifty termites were used for each of three replicates of the control and for each concentration tested. The test was terminated and mortality was recorded 3 days after termites were introduced to the dishes. Termites were then subjected to Rb analysis. Another new set of 50 workers of three replicates after feeding on 8000 mg kg<sup>-1</sup> Rb-treated tissue paper were washed by immersing them into deionised water for 5 min. The supernatant was analysed to determine the content of Rb on the body surface of the workers. This test was conducted to ascertain whether grooming activity might contribute to the Rb level in the tested termites.

## 2.3 Preparation of termite samples

To differentiate between two groups of termites, members of one group (the Rb recipients) were marked by feeding them with filter paper dyed with 0.5% Nile blue A (Aldrich, Milwaukee, WI, USA) for 7 days. The other group (the Rb donors) was fed Rb-treated tissue paper (at 8000 mg kg<sup>-1</sup>, concentration based on the results in the section 'Effects of rubidium on termite paper consumption, survivorship, and rubidium level') for 3 days. For fed termites, food was added *ad libitum*. To starve termites, they were placed in a Petri dish without food (tissue paper) for 3 days. Petri dishes were abraded with a stainless steel scouring pad to provide friction for the termites to walk normally on Petri dishes.

## 2.4 Experimental procedures

A simple donor–recipient model was used in this study. A commercial formulation of the anthranilic diamides termiticide chlorantraniliprole (Altriset® 20 SC; DuPont Crop Protection, Wilmington, DE, USA) was used in this study. Fipronil (Termidor® 2.5 EC; BASF Corp., Research Triangle Park, NC, USA), a broad-spectrum insecticide, was also used in this study as a reference standard, as its mode of action differs from that of

chlorantraniliprole. The termiticide-treated donor termites were exposed to sand with a moisture content of 20% that had been treated with either 50 mg kg<sup>-1</sup> chlorantraniliprole or 60 mg kg<sup>-1</sup> fipronil for 1 h. These concentrations were set based on the label rates for field application.

Next, 20 termiticide-treated donors were mixed with 20 healthy termites (termiticide-treated recipients) in a Petri dish (diameter 15 cm). The termites were mixed based on the following combinations, in which the arrow represents the food (Rb) being transferred from the donor to the recipient termites:

For the control, termites were allowed to walk on moist sand for 1 h. For all combinations, mortality was recorded daily. Dead termites were not removed from the Petri dish. The test was terminated at 3 days post-mixture. Each combination was replicated seven times. The recipients from three of the seven replications for each combination were randomly selected for Rb analysis.

## 2.5 Rubidium analysis

The Rb recipient termites (palatability control, *n* = 50; treatment, *n* = 20) were transferred to scintillation vials (30 mL) (Kimble Glass, Vineland, NJ, USA) and frozen at -18 °C. Frozen termites were then placed in air-tight containers containing silica beads and dried at 65 °C for 24 h. The dry weight of termites was measured to the nearest 0.1 mg using an analytical balance (Sartorius Extended ED2245; Sartorius AG, Göttingen, Germany). The dried termites were digested with 0.8 mL of 65% nitric acid (Sigma-Aldrich Co., St. Louis, MO, USA) for 24 h. The following day, 0.2 mL of 60% perchloric acid (Riedel-de Haën, Seelze, Germany) was added to each vial. The contents were heated in a 95 °C water bath for 30 min. After cooling, 20 mL of deionised water were added to each sample. The Rb content of each sample was determined by inductively coupled plasma optical emission spectrometry (ICP-MS) (Elan DRC-e; Perkin Elmer, Waltham, MA, USA) in a class 1000 clean room at a temperature of approximately 22 °C and relative humidity of 50%. Standard solutions of 5, 10, 20, 40 and 80 ppb Rb (a mixture of RbCl<sub>2</sub>, deionised water, nitric acid and perchloric acid) were prepared. Three scans of each standard solution and sample were conducted. The three scans of each sample were averaged and divided by the dry weight (mg) of the termites.

## 2.6 Statistical analysis

Because no significant variability in Rb level was detected between the two field colonies after feeding them Rb-treated tissue paper (see the section 'Effects of rubidium on termite paper consumption, survivorship, and rubidium level'), the data from the two colonies

For the treatment:

Rb-marked donor			Blue-dyed recipient
A	Untreated worker	→	Termiticide-treated worker
B	Untreated worker	→	Termiticide-treated worker (starved)
C	Termiticide-treated worker	→	Untreated worker
D	Termiticide-treated worker	→	Untreated worker (starved)

For the control:

A	Untreated worker	→	Untreated worker
B	Untreated worker	→	Untreated worker (starved)

**Table 1.** Paper consumption, survivorship, and rubidium level of worker termites feeding on various concentrations of Rb-treated tissue paper

Parameter	Control	Rb concentration of tissue paper (mg kg <sup>-1</sup> )			
		1000	2000	4000	8000
Mean of paper consumed (% ± SE)	11.3 ± 1.5 <sup>a</sup>	11.1 ± 1.7 <sup>a</sup>	10.2 ± 1.5 <sup>a</sup>	8.8 ± 1.2 <sup>a</sup>	11.8 ± 1.2 <sup>a</sup>
Mean survivorship (% ± SE)	97.0 ± 1.0 <sup>ab</sup>	99.0 ± 0.5 <sup>bc</sup>	99.2 ± 0.6 <sup>c</sup>	96.6 ± 0.8 <sup>ad</sup>	95.6 ± 0.8 <sup>ad</sup>
Mean ppb Rb/dry weight (mg) of termites (±SE)	1.7 ± 0.3 <sup>a</sup>	11.3 ± 1.8 <sup>b</sup>	16.9 ± 2.3 <sup>c</sup>	28.0 ± 4.2 <sup>d</sup>	59.6 ± 4.2 <sup>e</sup>

Means within the same row followed by different letters are significantly different (ANOVA, least significant difference,  $P < 0.05$ ).

were pooled. To normalise the data, the percentage of termite mortality was subjected to arcsine square root transformation, and Rb content/dry weight (mg) was logarithmically transformed. The transformed values were then analysed using analysis of variance (ANOVA,  $\alpha = 0.05$ ), and the means were separated using the least significant difference test (LSD). All analyses were performed using SPSS, v.11.0 (SPSS Inc., Chicago, IL, USA).

### 3 RESULTS

#### 3.1 Effects of rubidium on termite paper consumption, survivorship, and rubidium level

Feeding on Rb-treated tissue paper did not appear to affect the feeding activity of termites ( $F_{4,42} = 0.493$ ,  $P > 0.05$ ). The mean of survivorship of termites feeding on Rb-treated tissue paper differed significantly among the concentrations tested ( $F_{4,42} = 4.075$ ,  $P < 0.05$ ). However, the mean survivorship for the highest concentration tested (8000 mg kg<sup>-1</sup>) remained high throughout the 3 days of the experiment (mean ± SE, 95.6% ± 0.8) (Table 1) and did not differ significantly from that of the control set.

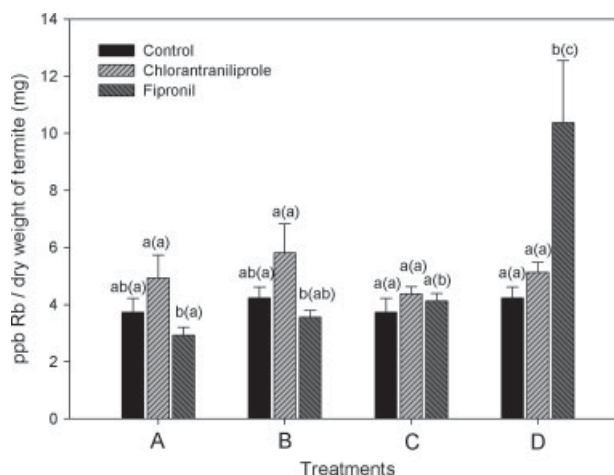
No significant difference in Rb level was found between the two field colonies tested in the 1000 ( $T = 0.905$ ,  $df = 4$ ,  $P > 0.05$ ), 2000 ( $T = 1.717$ ,  $df = 4$ ,  $P > 0.05$ ), 4000 ( $T = 1.281$ ,  $df = 4$ ,  $P > 0.05$ ), and 8000 mg kg<sup>-1</sup> Rb-treated tissue paper categories ( $T = 0.598$ ,  $df = 4$ ,  $P > 0.05$ ). The mean background level of Rb ± SE for workers was 1.36 ± 0.09 ppb Rb/termite, which is equivalent to a mean Rb/dry weight (mg) of termite of 1.75 ± 0.27. There were constant increases in the Rb level in workers across the increasing concentrations tested ( $F_{4,25} = 97.201$ ,  $P < 0.05$ ) (Table 1). Seeing that the high survival rate of termites was achieved in the 8000 mg kg<sup>-1</sup> Rb-treated tissue paper category, thus, we used the concentration for the remainder of the experiment.

After feeding on 8000 mg kg<sup>-1</sup> Rb-treated tissue paper, the mean Rb concentration on the termite body surface was 0.55 ± 0.09 ppb mg<sup>-1</sup>, which was three-fold lower than the mean background content of worker termites (1.75 ± 0.27 ppb mg<sup>-1</sup>). Even so, we cannot rule out the likelihood that Rb might also be transferred via grooming activity.

#### 3.2 Mutual interaction between untreated workers and termiticide-treated workers

##### 3.2.1 Treatment with chlorantraniliprole and fipronil

Overall, chlorantraniliprole-treated ( $F_{2,15} = 3.109$ ,  $P > 0.05$ ) and fipronil-treated workers ( $F_{2,15} = 2.363$ ,  $P > 0.05$ ) showed no significant increase of Rb transfer from untreated workers when compared to that of the control (Fig. 1). However, the post-hoc tests revealed that the chlorantraniliprole-treated workers in both the fed and starving conditions had a significantly higher Rb level compared to the fipronil-treated workers. This result indicates



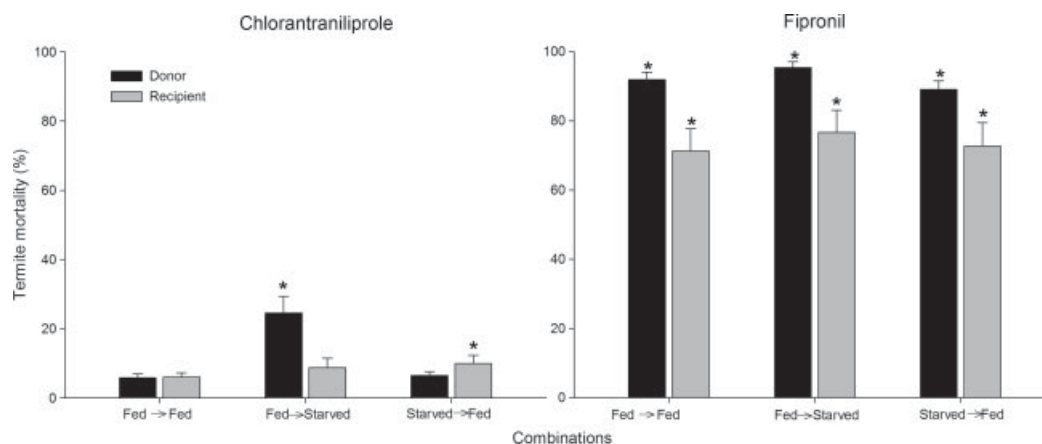
**Figure 1.** Comparison of rubidium transfer between untreated workers and workers exposed to chlorantraniliprole and fipronil. Treatment A: untreated workers → termiticide-treated workers; treatment B: untreated workers → starved termiticide-treated workers; treatment C: termiticide-treated workers → untreated workers; treatment D: termiticide-treated workers → starved untreated workers. Error bars represent the standard error. Means followed by different letters within the same treatment were significantly different. Letters in parentheses were compared between treatments of the same termiticides (ANOVA, least significant difference,  $P < 0.05$ ).

that the chlorantraniliprole-treated workers were attended more frequently by untreated workers compared to fipronil-treated workers (Fig. 1, treatments A and B).

When termiticide-treated workers were the donors and untreated workers were the recipients, the same amount of Rb was transferred to untreated unstarved termites from the donors with either termiticides ( $F_{2,15} = 1.143$ ,  $P > 0.05$ ) (Fig. 1, treatment C). In contrast, starved untreated workers that encountered fipronil-treated workers has a significantly higher Rb content compared to those that encountered chlorantraniliprole-treated workers ( $F_{2,15} = 7.288$ ,  $P < 0.05$ ) (Fig. 1, treatment D).

##### 3.2.2 Effects of nutritional status

When comparing the Rb transfer between untreated workers and termiticide-treated workers under fed and starved conditions, no significant difference in the Rb content was found in the chlorantraniliprole treatment ( $F_{3,20} = 0.402$ ,  $P > 0.05$ ) (Fig. 1). This result indicated that the nutritional status of chlorantraniliprole-treated workers or untreated workers had a minimal effect on the intensity of mutual interaction among workers. In the fipronil treatment, however, a significant increase in the Rb content was found ( $F_{3,20} = 14.861$ ,  $P < 0.05$ ), particularly regarded to the Rb transfer from starving treated worker to starving untreated worker, and from treated worker to starving untreated worker (Fig. 1).



**Figure 2.** Mortality of donor and recipient workers at day 3 after being exposed to chlorantraniliprole- and fipronil-treated sand under various combinations of fed and/or starved conditions. Error bars represent the standard error. \*, indicates the means of mortality were significantly different when compared between the control and treated termites under similar nutritional status (ANOVA, least significant difference,  $P < 0.05$ ).

### 3.3 Mortality of termiticide donors and recipients

Significant difference in mortality was detected between control termites and treated termites in fed condition ( $F_{4,95} = 16.435$ ,  $P < 0.05$ ) at day 3 post-mixture. Nevertheless, overall, the mortalities of chlorantraniliprole donor and recipient workers were generally low, in which less than 25% mortality was recorded, regardless of nutritional status (Fig. 2). In contrast, mortalities greater than 89% and 65% were recorded for donor and recipient workers, respectively, in the fipronil treatment.

## 4 DISCUSSION AND CONCLUSION

Chlorantraniliprole is a relatively new anthranilic diamide insecticide. It is highly selective for insect ryanodine receptors, and it works by diminishing the contraction of insect muscles and causing feeding cessation before death. Prior to moribundity, treated termites generally cease to feed after being exposed to chlorantraniliprole-treated sand for a minimum of 1 h.<sup>17</sup> Neoh *et al.*<sup>17</sup> hypothesised that such symptoms would put termites into a starving state, and thus they likely would be frequently attended by untreated workers for care-taking activities include reciprocal food exchange and grooming. The comparative data accumulated in this study support this hypothesis. Though the  $F$ -values were low to assume the existence of a statistical difference compared to those of controls, generally the mutual interactions of chlorantraniliprole-treated termites and untreated termites regardless the recipients' nutritional status, remained high. In further, the Rb contents in the chlorantraniliprole-treated termites in both the fed and starving conditions were significantly higher than those in fipronil-treated termites, which suggest that chlorantraniliprole-treated termites were more regularly attended by untreated termites compared to fipronil-treated termites.

Conversely, a low level of Rb exchange from untreated donors to fipronil-treated recipient termites was observed. This result suggests that the fipronil-treated termites were rarely attended by untreated workers. Another explanation for the low Rb transfer is that the food exchange to treated workers could have ceased at day 1 post-exposure due to the death of fipronil-treated workers, since maximal uptake of fipronil by termites can occur within 24 h.<sup>19</sup> In contrast, the Rb transfer from fipronil-treated termites to starving untreated termites was threefold greater compared

the exchange with fed untreated termites. It is worth noting that termites treated with fipronil excrete stomodeal and proctodeal fluids,<sup>20</sup> which might serve as food for starving untreated termites.

Previous experiments used test insects in the fed condition.<sup>21,22</sup> This test condition might not represent in a natural context, as it is commonly known that termites residing in a nest may, to a certain degree, be in a hunger state and readily receive food and attention from foragers. An increased Rb exchange when fipronil-treated termites encounter starving untreated termites shown in the present study hinted the mutual interaction could be higher than previously thought. Although termites treated with chlorantraniliprole reportedly excrete proctodeal fluid,<sup>20</sup> limited or no proctodeal fluid was observed on treated termites at day 3 in the current study. This can be explained by the slow-acting effect of chlorantraniliprole against termites. The onset of moribundity after being exposed to chlorantraniliprole-treated sand is slower than that of fipronil.<sup>20</sup> This was in agreement with Neoh *et al.*,<sup>17</sup> who reported that the donor and recipient termites began to die 14 days after treatment with chlorantraniliprole, but in the fipronil treatment moribundity usually occurred after only 24 h. Nevertheless, we do not rule out that proctodeal transfer activity could occur as the number of days after chlorantraniliprole treatment increases.

Quarcoo *et al.*<sup>15</sup> noted the importance of toxicant-induced behaviour in termites exposed to a soil treatment. It is important to ensure that termiticide-exposed termites are capable of walking back to their own nest to interact with other nestmates before death or the onset of abnormal behaviour that may hinder activity. For fipronil treatment in the present study, though food exchange from untreated workers to treated workers was rarely observed, stomodeal and proctodeal fluids excreted by treated worker were always treated as a food source by starved untreated workers. This indirectly increased the mutual interaction between the untreated and treated workers. However, one of the main drawbacks of fipronil treatment is the fast onset of the toxicological effect. Fipronil is highly potent to termites.<sup>17,21,23–25</sup> Uptake of a mere 0.9 ng was sufficient to kill 50% of test insects of *C. gestroi* in 24 h.<sup>17</sup> A recent study demonstrated that fipronil-treated termites exhibited greatly reduced walking and tunnelling capacity with increased exposure time and termiticide concentration.<sup>26</sup> This explains why the successful control of termite with fipronil was restricted to the colonies at the vicinity of the treated

zones.<sup>27,28</sup> In addition, foraging treated termites may die along the pathway to treated areas and thus create a hindrance that disallows other termites further access to the treated areas.<sup>29,30</sup> The present study strongly supports the premise that termites exposed to chlorantraniliprole experienced starvation and were frequently attended by untreated termites. Together with the slow-acting effect of this termiticide,<sup>17,31</sup> this situation could enhance mutual interaction between chlorantraniliprole-treated termites and untreated termites and ultimately lead to a cascade effect due to repetitive transfer from recipient termites (as secondary donors) to other termites.<sup>8</sup>

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