TITLE: Musty odor of entomopathogens enhances disease-prevention behaviors in the termite *Coptotermes formosanus*

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FOOTNOTES TO THE TITLE: Fungal odor enhances termite disease-prevention behavior.

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Key Words

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Abstract

Termites often eliminate pathogens directly through mutual grooming, and are thereby prevent infections from entomopathogenic fungi. Our previous study confirmed that the antennae of *Coptotermes formosanus* sensitively responded to the musty odor of entomopathogenic fungi. However, it is unclear if this odor has any effect on termite behavior. The purpose of this study was to clarify the effects of fungal odor on termite behavior, especially on conidia removal. The musty odor was prepared as an aqueous solution by immersing conidia in distilled water. When untreated termites were mixed with fungal-odor-treated termites at a ratio of 4:1, mutual grooming and attack of treated termites were frequently observed. This indicated that the fungal odor triggered these behavioral responses. While some components of the fungal odor were found in all of the entomopathogenic fungi tested, the odor profiles differed among the isolates.

1. Introduction

Hygiene behavior plays a key role in insect prevention against pathogens (Oi and Pereira, 1993; Swanson et al., 2009). Mutual grooming behavior, which has been well studied in termites, is a typical hygiene behavior (Kramm and West, 1982; Boucias et al., 1996; Shimizu and Yamaji, 2003). Through mutual grooming, termite workers ingest fungal conidia on the cuticle of a nestmate with the glossae and dispose of them through their alimentary tract (Yanagawa and Shimizu, 2007; Chouvenc et al., 2009). When together with nest mates, mutual grooming reduces the chances of termites getting infected by entomopathogenic fungi. This aspect of termite behavior is one of the key reasons that control of termite populations with entomopathogenic fungi had so far only limited if any effect. Since such biological approaches are an environment-friendly alternative to the current chemical control (Verma et al., 2009), it is important to identify the cues that induce termite hygiene behavior. Although microbes vary greatly with regard to competitive strength, attachment pattern, germination ability, environmental adaptability, and so on (Clarkson and Charnley, 1996), it is not yet clear what cues lead termites to notice the presence of entomopathogenic fungi on the cuticle of their nestmates.

Termite hygiene behaviors are most likely triggered by chemical information, since most termites are blind. Recent studies have revealed that termite antennae sensitively respond to the musty odors of entomopathogenic fungi (Yanagawa et al., 2009; Yanagawa et al., 2010). To understand the role played by chemical perception in *Coptotermes formosanus* behavior, we investigated whether odor from entomopathogenic fungi may be the cue that induces termite hygiene behavior. We report here the results from a laboratory study.

55 **2. Materials and methods**

56 *2.1 Insects*

57 Matured workers of *C. formosanus* were obtained from a laboratory colony maintained since 2002
58 (Okayama, Japan) in the dark at 28 ºC and more than 85% R.H. at Kyoto University, Japan. Termites were
59 separated into two groups, A and B, and each group was placed in a Petri dish (90 × 15 mm). At the center of the
60 dishes was a filter paper (about 90 mm in diam., Whatman No.1) that was impregnated with distilled water
61 (group A) or an aqueous solution of 0.05% (wt/wt) Nile blue A (group B). They were then kept at 25 ºC for 1 to 2
62 weeks before use in the bioassay. This treatment stained all of the termites in group B blue.

63

64 *2.2 Preparation for collecting fungal odor*

65 Three isolates of highly virulent entomopathogenic fungi, *M. anisopliae* 455, *I. fumosorosae* K3 and *B.*
66 *brongniartii* 782, and three low-virulence isolates, *M. anisopliae* UZ, *I. fumosorosae* 8555 and *B. bassiana*
67 F1214 were selected. Termites show 90–100% mortality on highly virulent fungi and 10-50% mortality on low
68 virulent fungi at 7 days after treatment, and there are 10- to 100-fold difference in LC_{50} between lower- and
69 higher- virulence fungi when 5 termites are kept in a dish (for further information see Yanagawa and Shimizu
70 2005; Yanagawa et al., submitted).

71 All of the *Metarhizium*, *Isaria* and *Beauveria* fungi were maintained on L-broth agar (1% polypeptone,
72 0.3% yeast extract, 2.0% sucrose, 0.5% NaCl, 2.0% agar) at 25 ºC. Entomopathogenic fungal conidia were

harvested with a brush from 10- to 15-day-old cultures and suspended in distilled water. About 3 ml of conidia suspensions, which contained $1.59 \times 10^7 - 3.94 \times 10^8$ conidia/ml, could be collected from one culture. The conidial suspensions were left overnight at 25 ºC and the conidia were then removed through a 0.2 μm filter unit (Dismic-13CP, Advantec, Japan). Volatiles trapped in these filtered solutions were used for fungal odor.

One ml of distilled water was prepared as control solution W. Another distilled water solution was prepared by gently washing the surface of solid L-broth agar that had not been inoculated with entomopathogenic fungi as control solution L. These two control solutions were left overnight and filtered as described above.

2.3 Comparison of grooming behavior among the 6 isolates

In this assay, groups of four workers treated with control solution and one worker treated with fungus odor were kept in a Petri dish (35 × 15 mm) and their touching frequency was monitored. The odor-treated termites were taken from the blue-stained termite group B, all termites treated with control solution originated from the unstained group A. As a control, one termite in group B was treated with control solution W or L and added to four termites from group A treated with control solution W, and their touching frequency was estimated.

For treatment, termites were collected from the Petri dishes and put in 1 ml microcentrifuge tubes containing a fungus-odor solution. The termites were submerged in the solution with gentle swirling for 5 seconds and allowed to dry on Whatman filter paper. The treated termite groups were then partitioned into the

91 dishes. After treatment, five termites were placed in Petri dishes and covered with a cardboard box during the
92 experiment to reduce the effect of room light on termite movements. The termites were then left for 15 minutes
93 to reduce the impact of the artificial treatment. Since it was impractical to observe and estimate the level of
94 grooming behavior for the entire duration of termite activity, the frequency at which the termites touched each
95 other was counted on photographs taken every 30 sec for 15 min. Only termites for which the mouth parts
96 touched their nestmates were counted. A total of 30 photographs were taken per dish to clarify differences in
97 grooming behavior among the 6 isolates in addition to the two control solutions. Data were obtained from 20
98 replicates, thus 800 termites were used.
99
100 *2.4 Comparison of disease-preventive behaviors and other responses*
101 Daily observation of other hygiene behaviors was conducted using the same assay model as described
102 above; four termites treated with control solution from group A were allowed to contact a single fungus odor-
103 treated nestmate from group B, and their behaviors were observed. Dead individuals were not removed and the
104 responses of other termites to the dead individual were also observed. Attack, cannibalism, burial and death
105 caused by contact with the odor-treated termite in a dish were observed for a week, and the number of dead
106 individuals and the duration until the first dead individual was found were estimated. The death rate was
107 calculated both for all of the individuals in a dish and for only the odor-treated individual in a dish. Attacks were
108 determined by the loss of body parts, and therefore cannibalism only included the eating of a dead body. As a

control, one termite in group B treated with control solution W (distilled water solution) or L (distilled water solution treated with L-broth medium) and four termites in group A treated with distilled water solution were placed in a dish. Data were obtained from 20 replicates.

2.5 Estimaton of fungal volatiles

Volatiles from entomopathogenic fungal conidia collected in water were extracted with SPME fiber.

SPME fiber coated with 100 μm polydimethylsiloxane (Red: 100 m; Supelco, Bellefonte, PA, USA) was used to sample the volatiles in the solution. A SPME fiber was immersed in 3ml of each of the solutions containing odor substances for 30 min at room temperature.

The gas chromatography apparatus was a GC-14A equipped with a polar capillary column, DB-WAX (30m length, 0.25 mm diameter, 0.25 μm film thickness; J & W Scientific. Inc.) and a flame ionization detector. Helium was used as the carrier gas. The SPME fiber was inserted into the GC injection port kept at 200 ºC for 1 min in splitless mode with a detector temperature of 220 ºC. The column oven was programmed to hold at 40 ºC for 5 min, to increase at 10 ºC/min to 180 ºC and then 20 ºC/min to 220 ºC, and finally to hold for 10 min at 220 ºC. The substances collected from the solutions were also analyzed by a Shimadzu QP5000 GC-MS system (Shimadzu, Japan) equipped with a polar capillary column (DB-WAX polar column). Helium was used as a carrier gas at a flow rate of 50ml/min. The 70 eV EI spectra were recorded at a rate of 0.5s per scan. Volatile

126 compounds were identified by comparison to the mass spectra and retention times of authentic compounds,
127 which were purchased from Nacalai Tesque (Kyoto, Japan).
128
129 *2.6 Statistical analysis*
130 To compare the differences in the grooming behavior of termites, a Poisson regression (Proc
131 GENMOD, SAS institute, 1999) was applied. For the analysis of behavioral differences and the number of dead
132 individuals, a logistic regression was applied and the survival time of the first individual to die, i.e., the duration
133 until the first dead individual was found, was analyzed by a Cox regression model using JMP 6.0 software (SAS).
134 The differences between control groups, which were treated with distilled water and broth solution, and the odor-
135 treated groups were described in terms of fungal odor parameters, and the differences among the 6 isolates were
136 examined with respect to genera, isolates and virulence.
137
138 **3. Results**
139 *3.1 Comparison of grooming behavior among the 6 isolates*
140 Grooming behavior was estimated in terms of the touching frequency among 5 termites in a dish
141 consisting of one odor-treated and four untreated termites. The frequency of mutual touching in a group is
142 presented in Fig. 1 (A) and that toward one odor-treated termite by its four nestmates was presented in Fig 1(B).
143 There was no statistically significant difference in the touching frequency between the two control groups, which

144 included termites treated with control solutions W and L ($p = 0.077$ in Fig.1 A, $p = 0.517$ in Fig.1 B). On the
145 other hand, the touching frequencies in a dish containing a fungus odor-treated termite were significantly greater
146 than those of the two controls (Fig. 1 A. $p < 0.001$ in Table 1(a); fungal odor parameter). Significant differences
147 in touching frequency were observed among the genera (*Metarhizium*, *Isaria* and *Beauveria;* $p < 0.001$ in Table
148 1(a); genus parameter) and isolates (*M. anisopliae* UZ, *M. anisopliae* 455, *I. fumosorosae* 8555, *I. fumosorosae*
149 K3, *B. bassiana* F1214 and *B. brongniartii* 782; $p < 0.001$ in Table 1(a); isolate parameter) in odor-treated
150 groups. However, though the frequency at which other termites touched the treated termite in a mixed group was
151 clearly greater than that in the control (Fig. 1B, $p < 0.001$ in Table 1(b); fungal odor parameter), no difference
152 was seen among genera, isolates or the level of fungal virulence (Table 1(b); genus, isolate and virulence
153 parameters). The odor substances induced high levels of grooming activity.
154
155 *3.2 Comparison of disease-preventive behaviors and other responses*
156 The changes in termite behavior caused by the fungal odor over 7 days after treatment are illustrated
157 in Fig. 2, and the mortality rates and survival time are shown in Table 2. Attack, cannibalism and burial
158 behaviors were not observed in either of the two control groups. Attack behavior increased significantly upon
159 treatment (Table 3, $p < 0.015$; fungal odor parameter). While this increase in attack behavior was specific to the
160 isolates of entomopathogenic fungi, it was not influenced by the fungal genera or the level of virulence (Table 3,
161 $p = 0.026$; isolate parameter; $p = 0.826$ and $p = 0.613$; genus and virulence parameters, respectively). On the

other hand, treatment with fungal odor did not alter the burial and cannibalism behaviors (Table 3, $p = 0.055$ and $p = 0.169$; fungal odor parameter, respectively).

The number of dead individuals also depended on the specific fungal isolate (Fig. 3 and Table 3, $p < 0.001$; isolate parameter). As in the results regarding behavioral alterations, there was no difference in the number of deaths between the two controls ($p = 0.165$, t-test). Death here was caused by attack from their nestmates since conidia were removed from the solutions and no physical pathogen was present in this study. Therefore, the pattern for the number of dead individuals parallels that of attack behavior. No remarkable difference was seen in the survival time until the first dead individual was discovered (Fig. 4 and Table 3, $p = 0.166$; fungal odor parameter).

3.3 Estimation of fungal volatiles

Gas chromatograms obtained for volatiles from the 6 fungal isolates extracted by SPME fiber are shown in Fig. 1. 3-Octanone, 3-octanol and 1-octen-3-ol (1; RT = 9.2 min, 2; RT = 11.9 min, 3; RT = 12.8 min, respectively) were estimated by comparison to the mass spectra and retention times of authentic compounds. The most common chemical, which all fungi possess, was 1-octen-3-ol (No. 3 in Fig. 5, Retention time = 12.8 min). The peak at a retention time of 9.2 min (No. 1 in Fig. 5) was found with *M. anisopliae* 455, *I. fumosorosae* K3, *B. brongniartii* 782 and *B. bassiana* F1214, and was considered to be 3-octanone. There was also another peak at a retention time of 12.0 min (No. 2 and * in Fig.5) with *I. fumosorosae* K3, *B. brongniartii* 782 and *B.*

180 *bassiana* F1214. The results of mass spectrometry showed that the chemical was 3-octanol in the two isolates of

181 *Beauveria*, but the chemical peak in *I. fumosorosae* K3 could not been identified (* in Fig. 5).

182

183 **4. Discussion**

184 The volatile odor of pathogenic fungi significantly increased termite grooming and attack behavior, which

185 resulted in an increased number of dead individuals. On the other hand, there were no changes in cannibalism or

186 burial behavior. This suggests that cannibalism and burial behavior are induced by signals after infection, rather

187 than by the pathogen itself. These results indicate that odor information affects behavior in the termite *C.*

188 *formosanus.* Presence of spores on a termite body may also trigger grooming and attack behaviors. There are

189 certainly differences in response to *Metarhizium* spore by nestmates depending on whether the isolate is virulent

190 or less virulent. This could also be odor related. In other words, some of the behaviors could also be induced

191 prior to infection. However odor triggers the remarkable responses as detailed in this study.

192 Grooming behavior was remarkably enhanced by the odor stimuli (Fig. 1 and Table 1 (a)(b); fungal

193 odor parameters). The termites showed specific changes in behavior related to fungal genera and isolates (Fig. 1

194 A and Table 1 (a); $p < 0.001$; genus and isolate parameters), but all termites carrying odor were groomed at

195 similar high rates irrespective of pathogen source (Fig. 1 B and Table 1 b; $p > 0.05$; genus, isolate and virulence

196 parameters). This may have been because the termites could not find an actual target to groom since there were

197 no conidia for the termites to remove from the surface of their nestmate. However, they still sensed something

198 unusual in the population, and thus there was an increase in overall grooming behavior. Rosengaus et al. (1999)
199 reported an increase in pathogen alarm behavior by the termite *Zootermopsis angusticollis* using *M. anisopliae.*
200 In their study, termites were not allowed to directly contact treated individuals, and they showed an enhanced
201 vibration display. This increase in the vibration display may have been caused by chemical perception of the
202 pathogen. In our study, attack behavior and the number of dead individuals also increased by having the fungal
203 odor-treated nestmate in the population (Table 3; fungal odor parameter for attacks and number of deaths).
204 According to Myles (2002), alarm, grooming, attack and burial behaviors occur as an interactional sequence. In
205 contrast, our results suggest that these behaviors may be regulated by different neural mechanisms, since burial
206 and cannibalism behaviors were not enhanced by treatment with the fungal odor solution (Table 3, $p = 0.055$ and
207 $p = 0.169$; fungal odor parameter for burial and cannibalism, respectively). For grooming to be effective as a
208 preventive strategy, it has to be triggered as early as possible. Most effective would be to respond simply due to
209 the presence of fungal conidia or their odor. Grooming and attacking behaviors are probably enhanced by signals
210 before infection, and cannibalism and burial behavior are enhanced by signals after infection. These chemical
211 compounds, which induce aggressive behavior in termites, may lead to new strategies for managing termites.
212 A statistical analysis showed that the number of dead individuals was correlated with virulence (Table
213 3, $p = 0.038$; virulence parameter for the number of deaths). This correlation, however, appeared to be very
214 complicated. For example, in *Metarhizium*, dead individuals were only found with the weak virulent UZ isolate,
215 while in *Isaria* and *Beauveria*, more termites died due to treatment with the odor of the high-virulence isolates

K3 and 782 (Fig. 3). While several studies have reported that termites are repelled by the odor of strongly virulent entomopathogenic fungi (Myles 2002; Mburu et al., 2009), the more isolates one examines the more complicated the picture can become. Contrasting outcomes between studies are common; it often comes down to which isolates were used. Our previous study showed that fungal virulence did not significantly affect termite behavior towards these conidia (Yanagawa et al., submitted). The solutions, however, also contain exotoxins of the pathogens and high molecular compounds, which will be recognized by gustatory receptors and thus the interaction between all these factors is still ambiguous. Our results here suggest just that termites cannot identify differences in virulence solely from their odor. Besides, the observation indicates that odor of dead individuals affects the behavior of the termites. Although the biological control using fungal agent has not been successful yet (Chouvenc and Su, 2010), if we learn more of the termite habitat, it may bring new possibilities to use entomopathogenic fungi for the biological control of termites.

Although the SPME technique has its limitations (Pedrini et al., 2007), it provided the best chance to estimate soluble chemical volatiles in the applied solution. While the substances on the surface of entomopathogenic fungi have been well examined by HPLC (Hallsworth and Magan 1993, 1997; Crespo and Cafferata 2000), little information is available regarding the volatile compounds of entomopathogenic fungi. This study used a novel approach to examine the effects of entomopathogenic fungi on termites by using an odor solution. Among the volatile compounds themselves, 3-octanol was only seen with *Beauveria*. We could only identify a few chemicals, but were not able to determine at what quantities they were present. It seems that the

odor emission of fungi changes readily depending e.g. on growing conditions, age of the culture etc. Although the chemical composition was quite stable, it was difficult to quantify the collected odors. The methods for quantification and standardization of the fungal odor are necessary for future studies.

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291

292 **FIGURE LEGENDS**

293 Fig. 1. Comparison of grooming activity in termite groups of 5 individuals which included a termite treated with
294 the odor substances of 6 different fungal isolates. A: Frequency of touching between all 5 termites in a dish. B:
295 Frequency at which 4 untreated termites touched one odor-treated termite. Vertical bar represents standard
296 deviations (n = 20).

297 Fig. 2. Behavioral changes observed in termites treated with the odor substances of 6 fungal isolates.

298 ■: Number of attacked termites in a dish (attack), □: Number of eaten dead bodies in a dish (cannibalism), ■:
299 Number of buried dead bodies in a dish (burial). Vertical bar represents standard deviations (n = 20).

300 Fig. 3. Mortality of termites in a dish at a 4:1 mixing ratio of untreated and odor-treated termites. Vertical bar
301 represents standard deviations (n = 20).

302 Fig. 4 Number of days until the first dead individual was found in a dish at a 4:1 mixing ratio of untreated and
303 odor-treated termites (survival time).

304 Fig. 5. Gas chromatogram of volatiles from A: an empty glass container. B: a glass container containing control

305 solution L. C: a glass container containing *M. anisopliae* 455 odor-solution. D: a glass container containing *M.*

306 *anisopliae* UZ odor-solution. E: a glass container containing *I. fumosorosae* K3 odor-solution. F: a glass

307 container containing *I. fumosorosae* 8555 odor-solution. G: a glass container containing *B. bassiana* 782 odor-

308 solution. H: a glass container containing *B. brongniartii* F1214 odor-solution.

309

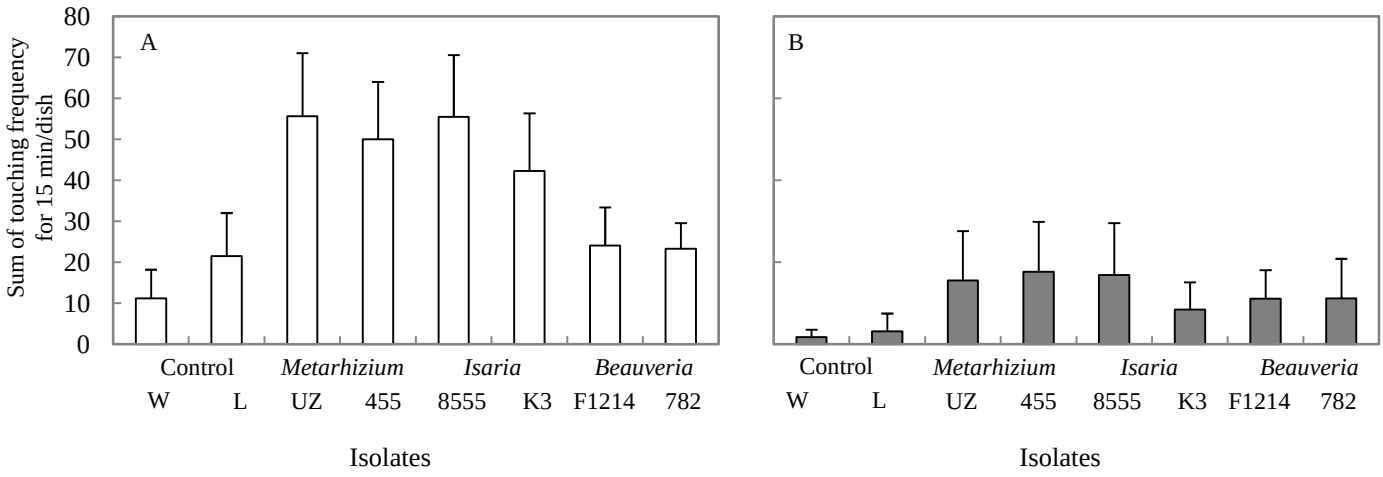
A
B
Sum of touching frequency for 15 min/dish
80
70
60
50
40
30
20
10
0
Control
Metarhizium
Isaria
Beauveria
W
L
UZ
455
8555
K3
F1214
782
Isolates

Fig1

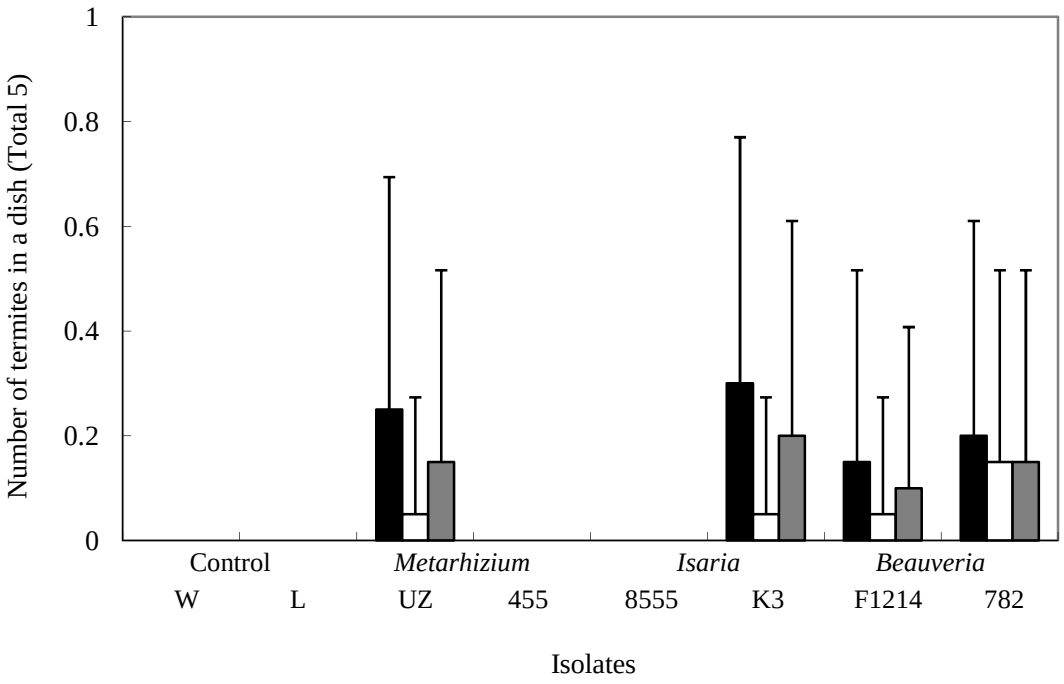
Number of termites in a dish (Total 5)
1
0.8
0.6
0.4
0.2
0
Control
Metarhizium
Isaria
Beauveria
W
L
UZ
455
8555
K3
F1214
782
Isolates

Fig2

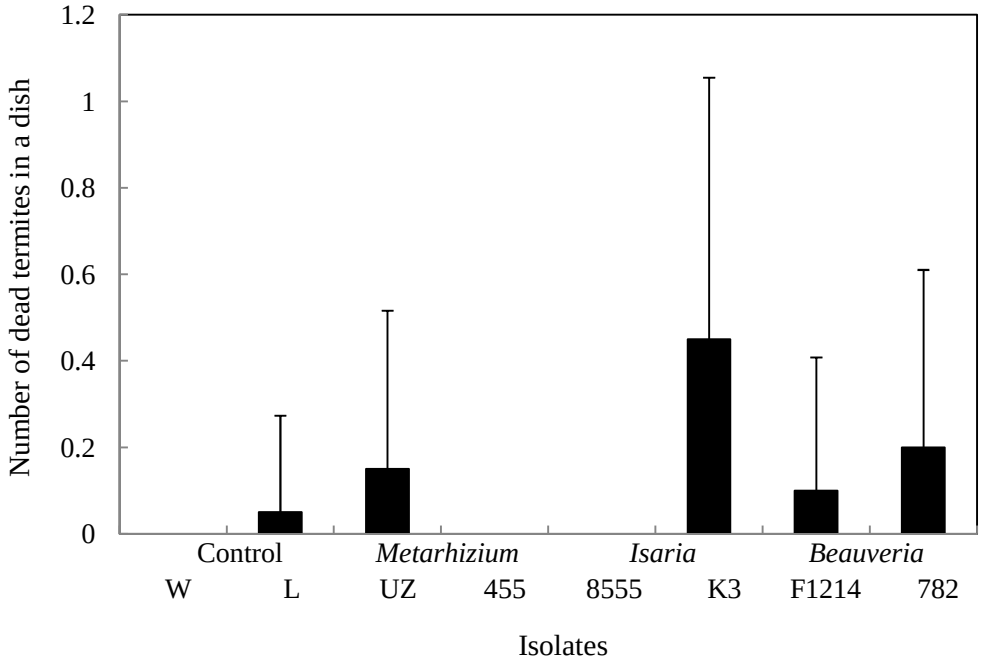
1.2
1
0.8
0.6
0.4
0.2
0
Number of dead termites in a dish
Control
Metarhizium
Isaria
Beauveria
W
L
UZ
455
8555
K3
F1214
782
Isolates

Fig3

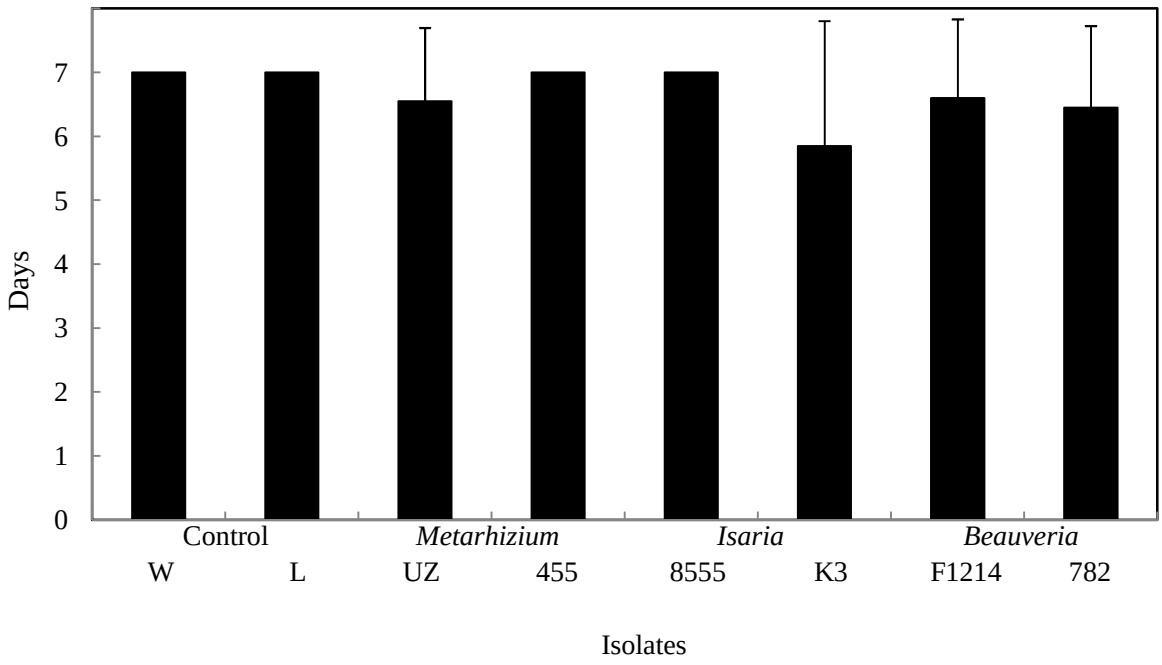
Days
0
1
2
3
4
5
6
7
Control
Metarhizium
Isaria
Beauveria
W
L
UZ
455
8555
K3
F1214
782
Isolates

Fig4

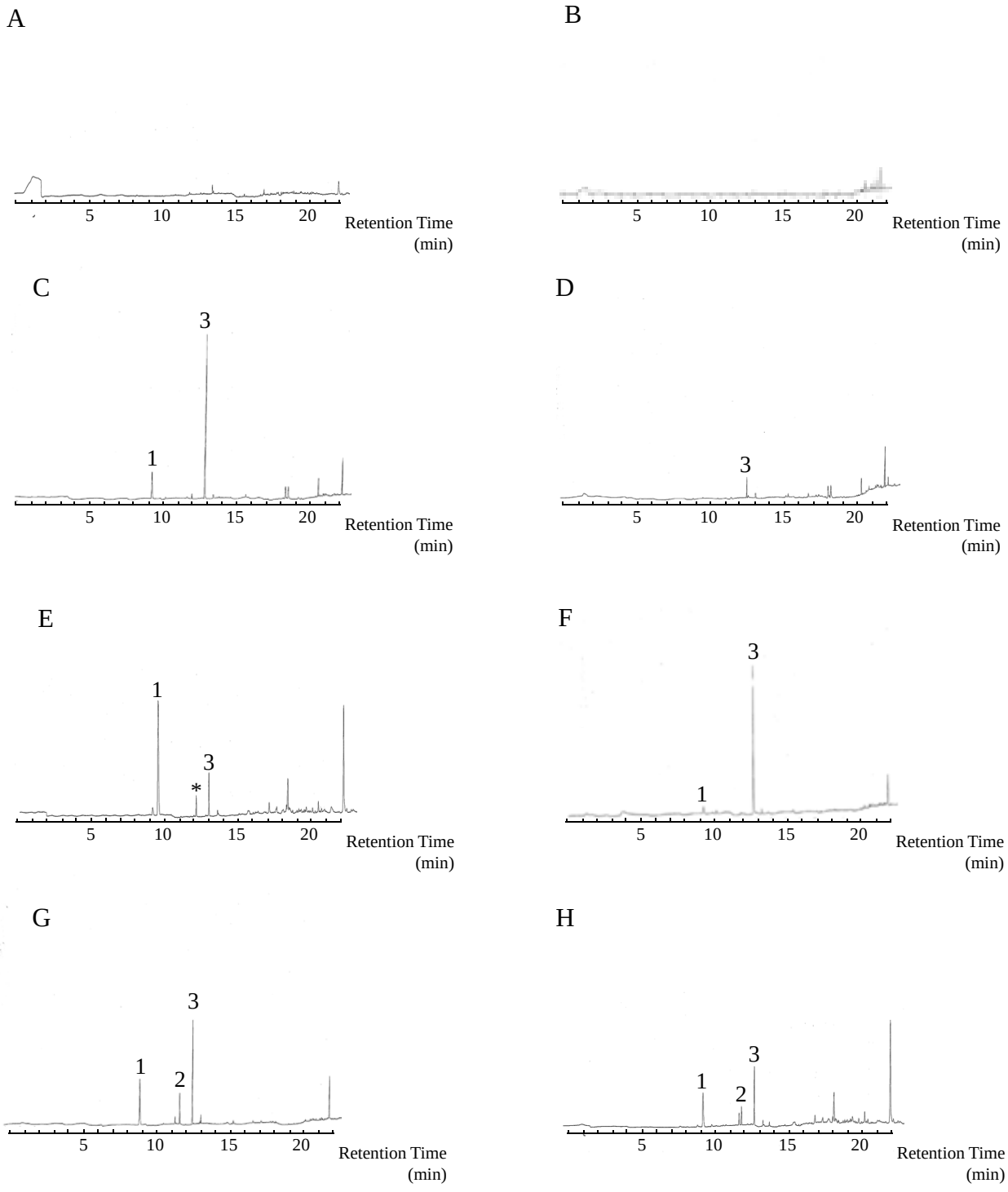

Fig5

Table 1 Comparison of the frequency of touching (a) between all 5 termites in a dish and (b) of one termite treated with odor from 6 fungal isolates by 4 untreated termites.
Results of Poisson regression

Parameter	DF	Estimate	Standard error	Wald 95% confident limit		X2	Pr > <chi>2
(a) Touching frequency of all nestmates in a dish							
Fungal odor	1	0.938	0.186	0.574	1.303	25.45	<0.001
Genus	1	-0356	0.080	-0.513	-0.120	19.92	<0.001
Isolate	1	-0.176	0.038	-0.251	-0.102	21.45	<0.001
Virulence	1	-0.157	0.127	-0.406	0.091	1.54	0.215
(b) Touching frequency to one treated termites in a dish							
Fungal odor	1	1.714	0.468	0.798	2.631	13.45	<0.001
Genus	1	-0.204	0.138	-0.474	0.066	2.19	0.139
Isolate	1	-0.107	0.066	-0.236	0.022	2.62	0.105
Virulence	1	-0.156	0223	-0.594	0.281	0.49	0.484

Table 2 Occurrence of dead termites in 20 replicates

			Isolates					
			Metarhizium		*Peacilomyces*		*Beauveria*	
	control	LB	UZ	455	K3	8555	F1214	782
Total of dead individuals (n= 100)	0	1	3	0	9	0	2	4
First dead individuals								
Treated termite	0	0	1/3	-	3/8	-	0	2/4
Untreated termite	0	1/1	2/3	-	5/8	-	2/2	2/4
Dead individuals; n / total dead individuals (rate in termite group%)								
Treated termite (n=20)	0(0)	0(0)	1/3(5)	0(0)	3/9(15)	0(0)	0(0)	2/4(10)
Untreated termite (n=80)	0(0)	1/1(1.25)	2/3(2.5)	0(0)	6/9(7.5)	0(0)	2/2(2.5)	2/4(2.5)

Table 3 Comparison of differences in termite responses to odors from 6 fungal isolates.
Results of logistic regression.

	Fungal odor	Parameters		
	(control - treated termite group)	Genus	Isolate	Virulence
Behavioral response				
Attack	$p = 0.015$	$p = 0.826$	$p = 0.026$	$p = 0.613$
Burial	$p = 0.055$	$p = 0.762$	$p = 0.171$	$p = 0.547$
Cannibalism	$p = 0.169$	$p = 0.231$	$p = 0.227$	$p = 0.398$
Other factors				
Number of death	$p = 0.015$	$p = 0.160$	$p < 0.001$	$p = 0.038$
Time until first death	$p = 0.166$	$p = 0.421$	$p = 0.519$	$p = 0.524$