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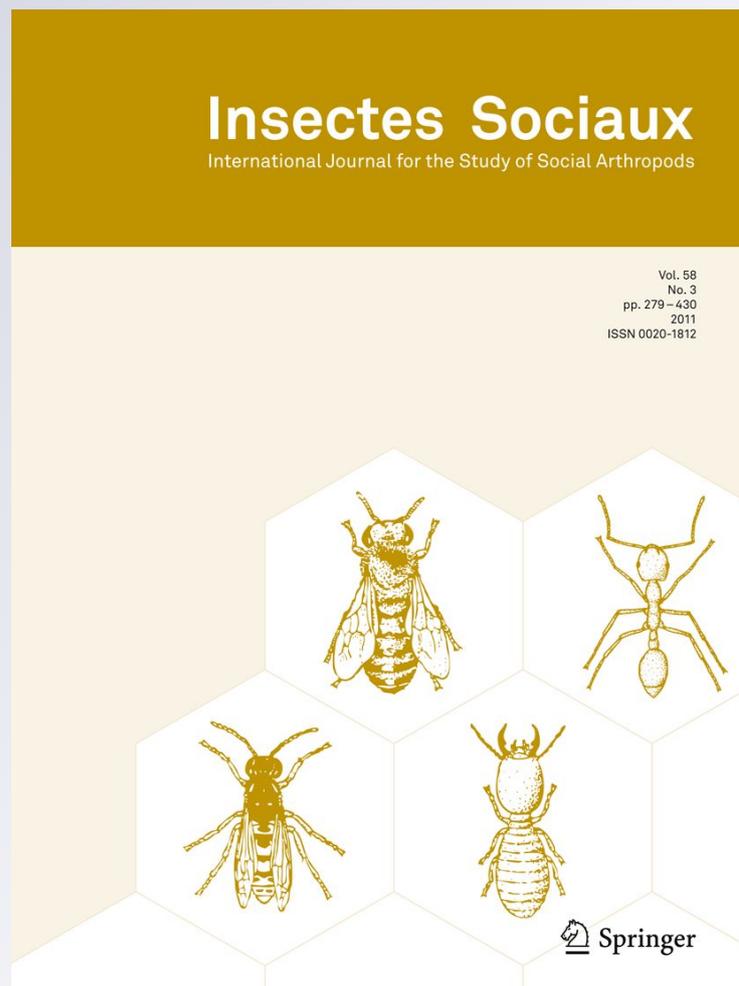
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Does lack of intraspecific aggression or absence of nymphs determine acceptance of foreign reproductives in *Macrotermes*?

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Abstract The rejection or acceptance of a foreign reproductive by an alien colony may not always be as straightforward as cue recognition between worker termites. This paper aims to determine whether adoption of foreign reproductives is caused simply by lack of intraspecific aggression or is contingent on the reproductive status of the host colony. In the fungus-culturing termites, *Macrotermes gilvus* (Hagen) and *Macrotermes carbonarius* (Hagen), major workers showed low intraspecific aggression towards non-nestmates irrespective of geographic distance between source colonies. Our results indicated that workers were hardly aggressive towards non-nestmates. In royal cell-swapping experiments, both species responded in a similar way: (1) in host colonies with nymphs present, the foreign reproductives were rejected; while (2) in host colonies without nymphs the foreign reproductives were either accepted and breeding resumed or the host colonies died eventually. Workers from the host colonies preferentially maintained offspring nymphs from which adultoid replacement reproductives develop rather than accepting foreign reproductives. There is no fitness gain for the queenless workers in accepting foreign reproductives; however, there is overall benefit to the newly born population.

Keywords *Macrotermes* · Kin discrimination · Fungus-culturing termites · Adultoid · Conspecific aggression · Colony circumstances

Introduction

Nestmate recognition is the ability to discriminate among nestmates and unrelated conspecifics. The phenomenon usually results in the acceptance or rejection by the host individuals, and could trigger territorial and colony defensive behaviours (Keller and Vargo, 1993). It is important to maintain colony integrity and to increase colony fitness indirectly by avoiding various costs associated with intrusions by conspecifics, for example, transmission of parasites and disease, and reproduction by the intruders (Lopez-Vaamonde et al., 2004). In the most social insects, such as termites, ants and bees, colony members often reject unrelated non-nestmates.

As a rule, termite colonies are closed to conspecifics. Many studies have proven that termites act aggressively against unfamiliar conspecifics (Haverty and Thorne, 1989; Pearce et al., 1990; Su and Haverty, 1991; Huang et al., 2007). In other cases, a lack of intraspecific aggression in termites (Grace, 1996; Bulmer and Traniello, 2002; Harahap et al., 2005) has also been reported. Aggression levels can change with environmental conditions (Shelton and Grace, 1997), season and across the distribution range (Clément, 1986), and importantly with colony circumstances, such as worker to nymph ratios (Matsuura and Nishida, 2001). In polygynous termite societies workers are derived from several matrilineages, resulting in variable levels of within-colony genetic relatedness (Hacker et al., 2005); yet, these workers of mixed origin coexist peacefully (Kaib et al., 1996). This may also facilitate the acceptance rate of non-nestmates

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since genetic cues may no longer be as important for differentiating between nestmate and non-nestmate conspecifics. The fact is further supported by a study in facultatively polygynous *Nasutitermes corniger* (Motschulsky) (Adams et al., 2007).

On the other hand, the rejection or acceptance of a foreign reproductive may not always be as straightforward as cue recognition between sterile castes. In ants, factors such as colony condition (i.e., whether male or female producing) (Brown et al., 2003), the mating status of the queens (Fortelius et al., 1993; Sundström, 1997; Holzer et al., 2008) and primer pheromones (Vargo and Fletcher, 1986; Vargo and Passera, 1991; Keller and Nonacs, 1993; Vander Meer and Alonso, 2002) can determine the outcome of queen introductions into foreign colonies. Laboratory colonies of *Coptotermes gestroi* (Wasmann) accepted foreign queens readily (Guaraldo and Costa-Leonardo, 2009). The result was linked to the lack of conspecific aggression among steriles. The factors influencing the acceptance of reproductives in termites have rarely been studied in the field.

According to the reviews by Reeve (1989) and Keller (1997), conspecific acceptance is generally determined by the similarity of template-cues between the parties and the cost of any mistake in accepting non-nestmates. For example, a conspecific is accepted if the template-cue is within the acceptance threshold and the cost of making an error is less than the benefits derived from accepting a conspecific. A good example from termites is provided by Matsuura and Nishida (2001). They demonstrated that the host rejected intruders when the intruding colony had a higher nymph ratio than its own (raising nymphs to the alate stage is energetically costly). Having to provision the non-nestmate nymphs in addition to their own would increase the workload of the workers considerably; a cost the host is not prepared to bear.

The two fungus-culturing Macrotermitinae *Macrotermes gilvus* (Hagen) and *Macrotermes carbonarius* (Hagen) are a dominant element of the Southeast Asian termite fauna, ranging from Myanmar to peninsular Malaysia, Borneo, and the Philippines. Aspects of their breeding biology have been studied in recent years in peninsular Malaysia. Neoh et al. (2010) observed resumption of breeding in 30% of orphaned *M. carbonarius* colonies. Either the nests could be re-colonized by another conspecific termite colony or foreign conspecific alates or dealates could invade such queenless colonies and commence breeding with the help of host workers (Neoh et al., 2010). However, the rate of acceptance of foreign alates by existing termite colonies is unknown. To date, most of the relevant studies focused on intraspecific interactions of workers, and often were carried out over relatively short periods in the laboratory. Nevertheless, a mere finding of non-aggression among conspecifics may not be conclusive to predict the host response to conspecific reproductives or an actual colony

intrusion. Several main issues, such as whether the host colony can accept the foreign reproductives and how the colony structure is affected in the long term have to be considered.

As a first step to better understand the degree of nestmate recognition in *Macrotermes* spp. we investigated whether workers of the two sympatric species act aggressively towards non-nestmates from different locations. Further, foreign alates might be used as intruders into field colonies. However, since it would be impossible to trace or re-locate alates at later inspections of the nests, mature reproductives within their royal cells were used to study the colony response. Royal cells with the reproductives in them were switched between field colonies during times of year when nymphs were either present or absent to elucidate the factors determining the adoption of foreign reproductives in a colony.

Materials and methods

Study site

The study was conducted on Penang Island, located on the northwestern coast of peninsular Malaysia. Penang experiences an equatorial climate that is uniformly warm (temperature: 26–35°C) and humid (relative humidity: 70–90%) throughout the year. The rainfall is evenly distributed throughout the year, although January–February and June–July are considered to be the driest periods of the year (see <http://www.met.gov.my>).

Termites

We collected workers of *M. gilvus* and *M. carbonarius* from the following sites in Penang: Universiti Sains Malaysia Minden campus (USM) (5° 21'N, 100° 18'E), Bayan Lepas (BL) (5° 18'N, 100° 16'E), Penang Youth Park (PYP) (5° 25'N, 100° 17'E) and Teluk Bahang (TB) (5° 27'N, 100° 12'E). The distances between collection sites ranged from 6 to 18 km.

Only active mature colonies (mounds with a height: 0.35–0.70 m, diameter: 0.80–1.60 m; and with recently built extensions) were selected for the study. The mounds were carefully opened up to minimize injuries to termites. The termites (e.g., workers, soldiers, larvae) with some fungus comb were collected and kept in a plastic container (30 cm × 20 cm × 15 cm). The termites were maintained in the laboratory at 24.3 ± 0.8°C and 50.1 ± 2.0% RH and in complete darkness.

Agonistic test

The termites were obtained by tapping them from pieces of nest material and fungus comb onto trays. The test arena

consisted of a plastic dish (10 cm in diameter) lined with a thin layer of sand with a water content of 20% (150 g of sterilized sand sieved through 40-mesh). The test arena was equally divided into two areas by a plastic card.

We assessed the level of worker–worker aggression among all pairs of colonies (i.e., USM–BL, USM–PYP, TB–PYP, BL–PYP, TB–USM, TB–BL) with five replicates for each combination for both species of *Macrotermes*. Nestmates from each collection site were also paired as the control. For each combination, major workers were used in this study as they far outnumber the soldiers in a colony and they form the potential defensive unit in termite–termite encounters (Thorne, 1982b). Five workers from each colony were paired. Workers from one colony in a pair were marked with a dot of silver paint (Zig Painty[®], Nara, Japan) on the head capsule to distinguish them from the workers of the other colony. Laboratory tests prior to the aggression experiments showed that the paint caused no significant behavioural changes or mortality in termites. Termites were acclimatized in the test arena for 5 min before the separating plastic card was removed. The behaviours during encounters were video recorded (Nikon Coolpix S600, Bangkok, Thailand) for 5 min. The videos were reviewed and analysed for agonistic reactions. The aggression behaviours were scored from 1 to 3 based on the definition described by Jmhasly and Leuthold (1999) with slight modifications: (1) examination or antennation: contact between antenna, head or body (absence of intraspecific aggressiveness); (2) alarm reaction or avoidance: jerking, chasing and/or being chased (moderate intraspecific aggressiveness); (3) aggression: mandibles open, seizing or biting (high intraspecific aggressiveness). The highest intraspecific aggressiveness (or the highest score) shown in the interaction for every 10 s intervals was recorded (thus, 30 observations for each pair). The scores of aggression behaviour in every 10 s intervals were pooled and averaged. The results obtained were compared between colony pairs, using Kruskal–Wallis one-way analysis of variance

(Statistix[®] 7, 2000 Analytical Software). The correlations between mean aggression score and distance were analysed by Spearman's rank order correlation test.

Royal cell swap

This study was conducted at the Minden campus of USM, from May 2009 until September 2010. The chosen dates (Table 2) followed the production season of nymphs in a given species (though there are exceptional cases in which mature colonies do not produce nymphs) (Neoh and Lee, 2009b). The distances between all pairs of treated nests ranged from 20 to 1,000 m for both species. The nests were selected using the criteria described above.

Royal cells [containing queen(s) and king(s) as well as larvae, workers, soldiers] from a mound pair were extracted as described by Neoh et al. (2010) and exchanged within 30 min ($n = 15$ for *M. gilvus*; $n = 21$ for *M. carbonarius*). The royal cell was usually located from a depth of 0.25 m up to ground level. The fungus combs and crevices were also surveyed for the presence of nymphs. The royal cells were placed back in the same position within the mound or 10 cm higher than where the original cells were located. After the introduction of conspecific reproductives, nest material and outer casing were used to seal all breaches of the mound to prevent predation and the impact of unfavourable weather. All mounds were dissected and re-examined after 3 months. The inspection interval was set shorter than the duration required for female alates to become physogastric (6 months) to exclude any misleading result that might result from re-colonization by conspecific species or reproductive replacement (see Neoh et al., 2010). Samples of larvae were subjected to morphometric analysis to determine the larval stages (Neoh and Lee, 2009a). Colony status was determined as (1) healthy, viz. foreign reproductives were accepted; (2) survived, but no signs of reproduction taking place, and (3) dead, viz. foreign reproductives had been rejected (Table 1).

Table 1 Criteria for assessing the status of colonies of *Macrotermes gilvus* and *M. carbonarius* at re-examination time following an exchange of reproductives (royal cell swap) 3 months prior

Colony status	Contents of royal cell	Reproduction	Nursery zone	Appearance of workers	Fungus comb
Healthy	Reproductives present	Yes (eggs, young larvae, workers, soldiers)	Present	Healthy, with none to a few with white, speckled body	Often intact to modestly consumed
Survived	Empty or reproductives highly mutilated/dead	No (only late stage larvae, workers, soldiers)	Absent	Many workers with white, speckled body	Moderately-to-highly consumed or overgrown with fast-growing fungus
Dead	Empty, reproductives dead	No termites present	Absent	N.A.	Absent

N.A. not available

Results

Agonistic test

In *M. gilvus*, the mean aggression scores differed significantly, $\chi^2(6, n = 50) = 18.865, P < 0.05$ between control (1.00) and colony pairs of USM–PYP (1.23 ± 0.03), USM–TB (1.23 ± 0.02) and BL–TB (1.25 ± 0.03) (Fig. 1a). However, in most instances, *M. gilvus* major workers showed very low intraspecific aggression, with scores ranging from 1.13 to 1.36 with a possible maximum score of high intraspecific aggression of 3. Workers performed acts of allogrooming/ignoring and jerking towards the non-nestmate workers. However, in colony pair USM–BL,

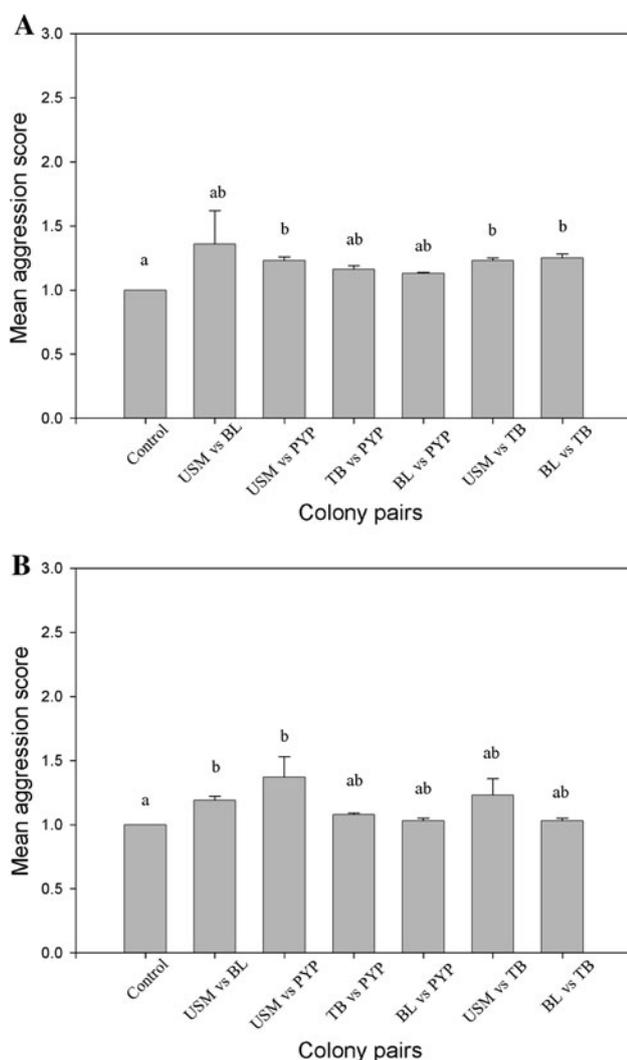


Fig. 1 Mean worker–worker aggression scores (\pm SE) for four colony pairs each of *Macrotermes gilvus* (a) and *M. carbonarius* (b). Mean values followed by different letters are significantly different ($P < 0.05$) between colony pairs. Both control data sets were the combination of control trials from four sampling sites

workers acted occasionally aggressively towards non-nestmates.

In *M. carbonarius*, the mean aggression scores showed a significant difference particularly between control pair and the combinations of USM–BL and USM–PYP, $\chi^2(6, n = 50) = 23.601, P < 0.05$. However, *M. carbonarius* demonstrated little aggression towards non-nestmates with the mean aggression scores ranging from 1.03 to 1.34 (Fig. 1b). Similarly, antennation, allogrooming and jerking behaviours were commonly observed in most colony pairs.

No evidence of geographical differences was found in *M. gilvus*, $r(7) = 0.3063, P > 0.05$ and *M. carbonarius*, $r(7) = 0.0721, P > 0.05$.

Acceptance rate of foreign reproductives

Of 15 colonies of *M. gilvus* with the royal cell exchanged, three (20%) with no nymphs present at the time of the royal cell swap contained first-to-third larval instars, eggs, pre-soldiers, soldiers and workers, indicating the occurrence of reproduction. These colonies contained a well-defined nursery area and intact fungus comb. Overall, the colonies without nymphs did not have a marked tendency towards the acceptance of foreign reproductives, $\chi^2(1, n = 15) = 3.28, P > 0.05$.

Eleven colonies (73.3%) survived but showed no signs of the occurrence of reproduction. At inspection time only workers and soldiers were present. The colonies lacked a nursery. The fungus combs were consumed partially-to-highly and often overgrown with fast-growing fungi. The reproductives were either found dead and the bodies decayed in the royal cells or the royal cells were empty with large holes in the walls. In all likelihood, the royal pairs had been killed. In seven of those colonies that were alive, nymphs (i.e., 2nd–4th nymphal instars) that were present at the time of the royal cell swap, had developed to alates within the 3 months. One colony (6.7%) had died out completely and the mound was occupied by *M. carbonarius* (Table 2).

In *M. carbonarius*, 10 out of 21 colonies (47.6%) had resumed egg production and castes comprised first-to-third larval instars, soldiers and workers at the time of re-examination (Table 3). However, in Colony 1, 7, 14, the reproductives could not be located anywhere in the mound. Importantly, the colonies which had accepted the foreign reproductives did not contain nymphs at the time the royal cells were exchanged, $\chi^2(1, n = 21) = 6.46, P < 0.05$.

Similar observations to those for *M. gilvus* were made on 11 colonies of *M. carbonarius* classed as ‘survived’: only workers and soldiers populated the mounds, fungus combs had been consumed, and a nursery zone was lacking. However, in seven of those colonies that survived (Colony 15–21), numerous alates were retained in the parental colonies. Although a few late larval instars were found, most

Table 2 Caste composition of *Macrotermes gilvus* colonies after 3 months following introduction of non-nestmate reproductives

Colony no.	Date of royal cell exchange	Date of re-examination of colony	Presence of nymphs/alates at the time of cell exchange	Colony composition at time of re-examination					Status
				Larvae	Workers	Soldiers	Alates	Reproductives	
1	05.03.09	05.06.09	–	+	+	+	–	+	R
3	08.03.09	08.06.09	–	+	+	+	–	+	R
4	08.03.09	08.06.09	–	+	+	+	–	+	R
2	05.03.09	05.06.09	–	–	+	+	–	–	S
11	29.01.10	29.04.10	–	–	+	+	–	–	S
7	21.01.10	21.04.10	–	–	+	+	–	–	S
9	21.01.10	21.04.10	–	–	+	+	–	–	S
8	21.01.10	21.04.10	+	–	+	+	+	–	S
10	29.01.10	29.04.10	+	–	+	+	+	–	S
5	21.01.10	21.04.10	+	–	+	+	+	–	S
6	21.01.10	21.04.10	+	–	+	+	+	–	S
13	29.01.10	29.04.10	+	–	+	+	+	–	S
14	23.02.10	23.05.10	+	–	+	+	+	–	S
15	23.02.10	23.05.10	+	–	+	+	+	–	S
12	29.01.10	29.04.10	–	–	–	–	–	–	D

+, present; –, absent; R, reproduction occurrence; S, survived; D, dead (see Table 1)

likely the introduced reproductives had been rejected. The fact was further proven by the observation made on Colony 21 which still contained the queen, but its extremities and body were severely mutilated: she was hardly alive.

Royal cell

It was not uncommon to find remnants of abandoned royal cells (7 observations in *M. gilvus*; 10 observations in *M. carbonarius*) with large holes in the walls (diameter: 1.1–2.5 cm compared with 0.4–0.7 cm in a normal royal cell) in both healthy and surviving colonies. In healthy colonies, new royal cells [containing the physogastric queen(s) and king(s)] ($n = 8$, total from both species) were built at a new location within the mound either slightly below (≈ 10 cm) or adjacent (distance: 5–30 cm) to the original position of the royal cell.

Discussion

Interactions between conspecific workers

This study demonstrated that workers of both *M. gilvus* and *M. carbonarius* exhibited limited intraspecific aggression. We also excluded the possibility of geographical differences (within a distance range of 18 km) that may affect intraspecific agonism in these *Macrotermes*. These results with Southeast Asian species are in line with those for African

species of *Macrotermes*, for example, *M. michaelsoni* (Sjöstedt) (Schuurman and Dangerfield, 1995), *M. subhyalinus* and *M. bellicosus* (Jmhasly and Leuthold, 1999). Kaib et al. (2004) tested several factors, such as cuticular hydrocarbons, morphological features, genetic polymorphism and geographical distribution. Among these, the latter contributed the least to the nestmate recognition system of *Macrotermes*.

Species of *Macrotermes* form very populous colonies. Encounters between termites from neighbouring colonies would be frequent in areas of higher mound density as is the case for *M. carbonarius* and *M. gilvus* at our study sites (Wong, 2006). If conditions are favourable for foraging, several colonies of *M. carbonarius* can often be found to forage simultaneously on the surface on widespread items of plant litter at the experimental site. However, foragers from one party can be placed among those of another party without causing an adverse reaction. Territorial fights among such large foraging groups would be very costly for both parties, due to heavy losses of individuals and reduced foraging efficiency. Hence, reduced aggression among conspecifics in *Macrotermes* might be an adaptive tactic. Colonies may employ other measures to stay within their specific foraging territories without interfering with each other, such as reliance on trail pheromones (Arab et al., 2006) and foraging along paved surface tracks (Inoue et al., 2001). Further, their underground passages are separated horizontally and vertically from those of neighbouring colonies (Inoue et al., 2001).

Table 3 Caste composition of *Macrotermes carbonarius* colonies after 3 months following introduction of non-nestmate reproductives

Colony no.	Date of royal cell exchange	Date of re-examination of colony	Presence of nymphs/alates at the time of cell exchange	Colony composition at time of re-examination					Status
				Larvae	Workers	Soldiers	Alates	Reproductives	
1	04.03.09	04.06.09	–	+	+	+	–	Not found	R
3	04.03.09	08.06.09	–	+	+	+	–	+	R
4	04.03.09	08.06.09	–	+	+	+	–	+	R
7	20.01.10	20.04.10	–	+	+	+	–	Not found	R
11	28.01.10	28.04.10	–	+	+	+	–	+	R
12	28.01.10	28.04.10	–	+	+	+	–	+	R
13	28.01.10	28.04.10	–	+	+	+	–	+	R
14	28.01.10	28.04.10	–	+	+	+	–	Not found	R
5	20.01.10	28.04.10	–	–	+	+	–	–	S
6	20.01.10	28.04.10	–	–	+	+	–	–	S
2	04.03.09	04.06.09	–	–	+	+	–	–	S
8	20.01.10	20.04.10	–	–	+	+	–	–	S
9	20.01.10	20.04.10	–	–	+	+	–	–	S
10	20.01.10	20.04.10	–	–	+	+	–	–	S
15	02.09.10	02.12.10	+	Late instar	+	+	+	–	S
16	02.09.10	02.12.10	+	Late instar	+	+	+	–	S
17	02.09.10	02.12.10	+	Late instar	+	+	+	–	S
18	02.09.10	02.12.10	+	Late instar	+	+	+	–	S
19	02.09.10	02.12.10	+	Late instar	+	+	+	–	S
20	21.09.10	21.12.10	+	Late instar	+	+	+	–	S
21	21.09.10	21.12.10	+	Late instar	+	+	+	Severely mutilated	S

+, present; –, absent; R, reproduction occurrence; S, survived; D, dead (see Table 1)

Response to foreign reproductives

The lack of intraspecific aggression among *Macrotermes* workers may create the precondition for foreign reproductives to enter alien colonies without impediment. However, in some situations the foreign reproductives are rejected, in others it is not, indicating that they are clearly identified as foreigners by the host colony. The approach by the host colony to the foreign reproductives would appear to be influenced mainly by colony-level factors, especially the presence or absence of nymphs.

In this study, all the foreign reproductives were rejected in colonies containing nymphs at the time of the cell swap. In these colonies numerous mature alates had developed by the time of the re-examination. The alates that largely should have dispersed in November were retained in the parental colony (Neoh and Lee, 2009b). As pointed out by Neoh et al. (2010), nymphs that gave rise to adultoids replace primary reproductives in both experimentally and naturally orphaned colonies of *Macrotermes*. In the current experiment, we could not re-sample colonies a second time (e.g., Colony 1–3 for *M. gilvus* and Colony 15–21 for *M. carbonarius*) because the nests had to be completely taken apart when they were checked for the fate of the exchanged

reproductives. However, we can assume that the nymphs that were retained in the parental colonies would have become adultoids, i.e., replacement reproductives. In other words, if after losing their own reproductives, the colonies have any chance to produce replacement reproductives of their own, i.e., contain the precursor nymphs among their castes, they will reject foreign reproductives. This decision to be “closed” to foreign conspecifics (Clément, 1986; Matsuura and Nishida, 2001) comes at a cost. While the nymphs mature to adultoids, no further recruitment to the colony population occurs and foraging is more restricted as the depleted fungus combs indicate (Tables 1 and 2). The benefit is of course that the colony can retain its genetic identity and keep propagating its own genes once it is able to resume reproduction with adultoids produced from among their own. However, the success of adultoid production in a colony either with nymphs or without nymphs after the reproductive removal is not necessarily guaranteed, in fact the success rate can be low (Neoh et al., 2010).

In contrast, colonies without nymphs at the time of the cell swap accepted the foreign reproductives at the rates of 37.5% for *M. gilvus* and 57.1% for *M. carbonarius*. However, no marked tendency towards the acceptance of foreign reproductives in colonies without nymphs was found in the

present study. Queenless colonies without nymphs were unable to rear relatives, and thus the colonies would gain no inclusive fitness. The original offspring of the removed queen will die out or would be displaced by the offspring of the new reproductives in due course. However, the foreign conspecifics may gain an immediate fitness benefit from the large labour force of existing workers, ensuring that foraging activity is maintained at adequate levels, as indicated from the good condition of the fungus combs (Table 1). The colony investment into the mound structure and associated resources is not placed at risk. Conditions for the newly born population stay favourable. Given these considerations, unlike the case of 'closed' colonies, selection pressure does not seem to mediate the decision to be 'open' to foreign conspecifics in the colonies without nymphs.

Nevertheless, the results should be viewed with some caution as they are based on the swapping of mature reproductives instead of introducing foreign alates. As outlined in the "Introduction" it is not possible to trace such alates during re-sampling of the colonies. It is known that the reproductive status of mature reproductives is profoundly different from that of unmated non-physogastric alates. This may also be a determinant in accepting a foreign reproductive. For example, in an earlier laboratory study with a polygynous colony of *N. corniger* fully matured foreign reproductives were given care but alates were attacked by the host workers (Thorne, 1982a). Currently, we do not know how the recognition cues change as alates and de-alates mature to fully functional reproductives.

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