

1 Lack of aggression and apparent altruism towards
2 intruders in a primitive termite

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31 **Abstract**

32 In eusocial insects, the ability to discriminate nestmates from non-nestmates is
33 widespread and ensures that altruistic actions are directed towards kin and agonistic
34 actions are directed towards non-relatives. Most tests of nestmate recognition have
35 focused on hymenopterans, and suggest that cooperation typically evolves in tandem
36 with strong antagonism towards non-nestmates. Here we present evidence from a
37 phylogenetically and behaviourally basal termite species that workers discriminate
38 members of foreign colonies. However, contrary to our expectations, foreign intruders
39 were the recipients of more rather than less cooperative behaviour, and were not
40 subjected to elevated aggression. We suggest that relations between groups may be
41 much more peaceable in basal termites compared to eusocial hymenoptera, owing to
42 energetic and temporal constraints on colony growth, and the reduced incentive that
43 totipotent workers (who may inherit breeding status) have to contribute to self-
44 sacrificial intergroup conflict.

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46 Keywords: intergroup competition, cooperation, nestmate recognition, allogrooming,
47 aggression, *Pterotermes occidentis*

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51 **Introduction**

52 One of the key mechanisms proposed to explain the evolution of altruism is the ability to
53 direct care preferentially toward kin [1]. In primitively eusocial insects, colonies are
54 typically composed of close relatives, in which case the ability to distinguish nestmates
55 from non-nestmates may be a cost-effective rule-of thumb to ensure that altruism is
56 directed on average toward kin [2], and aggression toward non-kin [3]. In support of this
57 hypothesis, numerous eusocial hymenopterans [4,5,6], some termites [7,8,9,10,11], and
58 eusocial mole rats [12] exhibit extreme aggression toward non-nestmates. Intense and
59 violent intergroup competition is also common of mammalian cooperative breeders
60 [13].

61
62 Tests of the role of kin or nestmate discrimination as a promoter of cooperation in
63 insects have focused mainly on Hymenoptera [6,14,15], while termites, the second-
64 largest eusocial taxon, have been relatively neglected [16-18]. Studies of termites may
65 be particularly illuminating because they vary enormously in colony size and individual
66 specialization [16,19], possess fundamentally different life history traits from
67 Hymenoptera (such as hemimetabolous development), display radically different
68 feeding ecologies [16], and there are no known extant solitary species [20,21].
69 Moreover, termites are diploid and hence offer a chance to test the hypothesized role of
70 genetic architecture in the evolutionary origin and maintenance of eusociality
71 [19,20,22,23].

72
73 Among extant termites the wood-dwelling species (Termopsidae, Kalotermitidae) are
74 thought to display the most basal life-history traits [24,25] (but see [26]). In contrast to
75 the high morphological and behavioural diversity of derived termites, workers in wood-
76 dwellers tend to show relatively little division of labor and are monomorphic. Most
77 species lack a true worker caste, instead possessing 'false workers' or 'pseudergates'
78 which are developmentally plastic, and like social vertebrates possess the potential to
79 explore all caste options throughout their lives [17]. In the drywood termites
80 (Kalotermitidae) these 'workers' perform little or no brood care or nest maintenance
81 activities [19,27]. However, workers do altruistically feed soldiers (who cannot feed
82 themselves) [18], engage in proctodeal and anal trophallaxis [28], and maintain hygiene,
83 [29]. Because the wood these species inhabit serves simultaneously as a source of food,

84 shelter, and protection, colony members never voluntarily leave the nest, except as
85 dispersing alates [18,21,30]. However, it has been previously noted in other drywood
86 species that two or more colonies occupying the same piece of wood sometimes meet,
87 which can lead to intergroup aggression and colony fusion [27,30,31,32].

88

89 In this study we tested the hypothesis that drywood termites can recognize non-
90 nestmates and directing specific behaviours towards them. We performed a series of
91 behavioural assays using captive colonies of the drywood termite *Pterotermes occidentis*
92 (Kalotermitidae), indigenous to the Sonoran desert of the southwest USA [21].

93 Specifically, we introduced single individuals into foreign nests and observed and
94 measured subsequent interactions, such as allogrooming rates, which we interpreted as
95 a cooperative behaviour, and frequency of butting, which in other termites is taken as a
96 measure of aggression or dominance [33]. While other aggressive interactions (such as
97 wing pad biting have been observed in wood-dwelling termites [24], butting was the
98 only potentially aggressive behaviour observed in our study. We predicted that foreign
99 individuals would face increased aggression and receive less allogrooming than
100 individuals native to their own colony, providing evidence of discrimination between
101 nestmates and non-nestmates.

102

103 **Methods**

104 Whole colonies of *P. occidentis* termites were collected from standing *Cercidium*
105 *floridum* (Blue palo verde) trees in September 2011 and October 2013 in the Sonoran
106 desert, Arizona. Of the 6-colonies used in the experiment, 5 were collected in one region
107 (Mendoza Canyon: 31.972088, -111.470339) and one colony was collected within the
108 Tucson metropolitan area (32.273160, -110.905818). In the Sonoran desert, single
109 colonies of *Pterotermes occidentis* stage multiple (up to 40) dispersal flights on nights in
110 July and August [21]. Dispersal distance is unknown for this species, but in other
111 Kalotermitidae alates disperse up to a few km, apparently as an inbreeding avoidance
112 mechanism [34,35]. The five colonies collected from Mendoza Canyon were all located
113 at least 500m apart when found, and consequently are likely to be founded by unrelated
114 queens. Behavioural observations were conducted between December 2013 and July
115 2014. During the experiment all colonies were housed in plastic boxes containing tightly
116 arranged blocks of *C. floridum* wood from the colonies sites of origin and kept in an

117 incubator at a constant temperature of $26\pm 2^\circ\text{C}$, with 30% - 36% humidity and
118 permanent darkness.
119
120 15 size-matched treatment-control pairs of individuals were selected randomly from
121 each colony and marked with a unique tricolour code using enamel-based paint on the
122 head, thorax, and abdomen. Focal individuals were marked 3-days prior to
123 commencement of observations, allowing time for recovery from the procedure.
124 Marking was carried out under anaesthesia following exposure to 30-seconds of CO_2
125 [36]. During the 3-day period between marking and the first observations all termites
126 were returned to their native colonies in captivity.
127
128 Prior to each observation session, a random subset of 15 termites was selected from
129 either the home or foreign colony with which interactions could take place, depending
130 on the stage of the trial. 40-minute behavioural observations were conducted over four
131 stages for each focal termite during a 4-day period (figure 1). Observations were
132 conducted at $27\pm 2^\circ\text{C}$ in perspex observation arenas separate to the main colony
133 containers, and contained a single, colony-specific piece of *C. floridum* wood to avoid
134 cross-contamination of odor-carrying hydrocarbons. The limitation of this setup is that it
135 differed from the termites usual tightly confined tunnel networks. However, it did allow
136 for easy observation of individuals, and we observed typical behaviours such as eating
137 and trophallaxis similar to those observed in the main colony housing boxes, suggesting
138 low levels of disturbance in the observation arena. Treatment and control groups were
139 observed simultaneously using video cameras, and observation sessions were preceded
140 by a 30-minute calming period following transfer from the nest box. Focal termites were
141 placed into the observation arenas with colony subsets immediately prior to the onset of
142 this calming period and allowed to settle together.
143
144 Allogrooming was measured by timing the onset and end times of each occurrence in
145 seconds and summing the total for each 40-minute session. For butting behaviour,
146 which we defined as a vigorous shaking motion in response to disturbance or as a signal
147 of reproductive dominance [33], frequency data were recorded. Occasional periods
148 when the focal termite was hidden or obscured were deducted from each session time.
149 For both behaviours recorded, the focal termite could either be the actor or recipient,

150 and the distinction was noted.

151

152 We used general linear mixed-effects models GLMMs to predict how treatment affected
153 the proportion of time spent being groomed and frequency of butting interactions.

154 Models were fitted with stage of experiment and treatment as fixed effects, and in our
155 models of butting we also included observation time (which occasionally was less than
156 40-minutes due to the focal individual being obscured). In all models we used individual,
157 native colony of focal termite, and colony sample in which the observation was taking
158 place as random intercepts. In our models of butting behaviour we also included these
159 observation level random effects to control for overdispersion [37].

160

161 The response variables in grooming observations were the proportion of time spent
162 being groomed or performing grooming, and those models were fitted with a binomial
163 error structure. The response variables in butting models were the frequency of
164 received/performed butting observed, using a poisson log-normal error structure. We
165 then conducted post hoc Tukey comparisons between treatment and control groups in
166 each stage to control for multiple pairwise comparisons. To test whether genetic
167 relatedness between colonies (which could conceivably be > 0 for Mendoza Canyon
168 colonies) influenced our results we conducted a post-hoc Mann-Whitney U test to test
169 whether trials involving focal individuals from the single Tucson area colony differed
170 from those involving focal individuals from Mendoza Canyon colonies.

171

172 All analyses were performed using R statistical software [38] using packages lme4 and
173 lsmeans [39,40], with the exception of the Mann-Whitney U tests which performed in
174 Microsoft Excel 2011.

175

176 **Results**

177 Treatment affected the amount of grooming the focal individuals received (Table 1).
178 Contrary to our predictions however, grooming towards the foreign individuals
179 increased upon introduction to a foreign colony (Stage 2). This effect disappeared after 4
180 days within the host colony (Stage 3, see figure1). Post-hoc testing revealed no
181 significant difference between trials involving focal individuals from the Tucson area
182 colony and those involving focal individuals from the 5 Mendoza Canyon colonies

183 (Supplementary Material). In contrast to our results for grooming behaviour, there was
184 no difference between control and treatment individuals in the amount of butting
185 received or given. Introduction to a foreign colony also had no significant effect on the
186 amount of grooming performed by focal individuals. Finally, there was no significant
187 difference in behaviour (rates of grooming or butting) of Treatment or Control
188 individuals after reintroduction to their original colony, immediately after Stage 3 (Table
189 1).

190

191 **Discussion**

192 The intense levels of grooming directed toward individuals from foreign colonies
193 suggests that *P. occidentis* workers can distinguish nestmates from non-nestmates.
194 However, the results were opposite to our original predictions: foreign intruders were
195 not subject to greater levels of aggression (butting), and were the beneficiaries of
196 significantly higher levels of allogrooming, which in social organisms is usually
197 interpreted as a form of cooperation. After 4 days the rate at which intruders were
198 groomed had dropped back to the same rate as control individuals in their native colony
199 (figure 3, stage 3).

200

201 A plausible explanation for these results is that *P. occidentis* workers use allogrooming
202 to maintain a recognizable colony odor, most likely mediated by cuticular hydrocarbons
203 (CHCs). In fact allogrooming behaviour may be the primary, and perhaps sole
204 mechanism of transferring in-group chemical profiles between colony members [5,18].
205 Over the course of the 4-days spent integrating with the foreign host colony, elevated
206 levels of allogrooming received by an intruder may reduce any dissimilarity in CHC
207 profile between itself and the members of its host colony, so that by day 4 of our
208 experiment the foreign termite is no longer recognized as an intruder. This hypothesis
209 would predict that reintroduction to the focal individual's own colony should again lead
210 to elevated levels of grooming compared to controls. In our limited sample, levels of
211 grooming upon reintroduction were elevated for treatment individuals, but not
212 significantly so ($p = 0.07$; Table 1). To test this hypothesis further will require further
213 experiments using non-destructive CHC sampling techniques (such as the use of SPME
214 fibres [41]) to determine whether the profile of excluded individuals drifts from that of
215 their native colony over time; and whether allogrooming functions to homogenise the

216 CHC profile of intruders.
217
218 Why should *P. occidentis* group members actively try to integrate foreign individuals into
219 a colony (through allogrooming), rather than repelling or attacking them as commonly
220 occurs in many insect and vertebrate societies? Encounters between colonies may be
221 frequent in wood-dwelling termites such as *P. occidentis*, because suitable nesting trees
222 usually contain multiple colonies [30]. With colony growth and expansion of nest
223 galleries, colony contact can occur when adjacent cavities meet [22,23,27,32]. Genetic
224 studies of within-colony relatedness in wild populations have found evidence for
225 mergers in several species of both wood-dwelling [27,32,42,43,44] and external foraging
226 termites [45,46,47,48,49], with one study finding evidence of multiple mergers in
227 several colonies of the drywood termite *Kaloterms flavicollis* [32]. In laboratory studies
228 of the drywood termite *Cryptotermes secundus*, peaceful colony mergers are associated
229 with increased colony survival and an increased production of new reproductives,
230 suggesting that individual workers may stand to benefit (in terms of direct fitness) when
231 colonies fuse [27]. Similarly, in the dampwood termite *Zootermopsis nevadensis*, colony
232 fusion creates opportunities for workers to inherit reproductive status [30]. Theoretical
233 models predict that an increase in the probability of inheritance should favour lower
234 investment in self-sacrificial, colony-beneficial behaviour, such as intergroup conflict
235 [50,51] (note this prediction does not necessarily hold if invaders represent a threat to
236 an individual's inheritance rank [52]). In addition, since the indirect fitness benefits of
237 helping appear to be relatively low in wood-dwelling termites [18], workers have less
238 incentive to invest in colony defence to maintain kinship within groups at the expense of
239 their own potential future fecundity. Together, these ecological and social factors may
240 explain why in wood-dwelling termites individual workers may be selected to detect and
241 integrate foreign individuals into the group rather than attack them.

242

243 An alternative hypothesis to explain our results is that foreign individuals are perceived
244 as potentially harboring pathogens, with allogrooming being an adaptive, selfish
245 response to maintain social immunity and colony health [53]. Rosengaus et al. [29]
246 observed that drywood termites tend to have low pathogen loads, but whether this is
247 linked to allogrooming behaviour is unknown. In dampwood termites, elevated levels of
248 allogrooming behaviour have been observed following exposure to fungal pathogens

249 [29,54], and substantially lowered external pathogen loads have been observed
250 following experimental exposure to fungal spores and subsequent grooming [46].
251 Experimental manipulation of pathogen load in *P.occidentis* could be used to test this
252 hypothesis.

253

254 In summary, the basal life history traits of *P.occidentis* [31] make the species an
255 excellent system to investigate the behavioural factors and evolutionary processes
256 associated with the apparently unique origin of termite sociality. Future fitness benefits,
257 low levels of helping behaviour, and strict ecological and temporal constraints on colony
258 growth may explain why relations between groups in basal termites are less fractious
259 and violent than is typically the case in eusocial Hymenoptera and cooperatively
260 breeding vertebrates. We suggest that further studies of intergroup interactions in basal
261 termites may contribute to an improved understanding of the role of between-group
262 competition in social evolution.

263

264

265 **Ethics**

266 All procedures received prior written approval from the Ethics Committee of the College
267 of Life and Environmental Sciences, University of Exeter, UK.

268

269 **Data accessibility**

270 Our data are deposited at Dryad: <http://datadryad.org>

271

272 **Competing interests**

273 The authors declare no competing interests.

274

275 **Author's contributions**

276 M.C., N.G., F.C., and R.S. conceived the study. N.G., M.C. and R.S. collected colonies.

277 N.G. and F.C. maintained colonies. F.C. collected data. H.M., N.G. and E.V. analysed data.

278 All authors contributed to drafting the manuscript.

279

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288

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430 **Table Legend**

431 Table 1. Tukey's honest significant difference tests comparing the allogrooming and
432 butting that control and treatment individuals received and gave in each stage. In each
433 stage the parameters estimates for treatment individuals are compared to control
434 individuals. * $p < 0.05$.

435

436 **Figure Legends**

437 Figure 1. Design and sequence of the experiment.

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440 Figure 2a. Diagram of the experimental setup as it was during observations.

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443 Figure 2b. Still image of the arena taken from one of the observation videos.

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446 Figure 3. Proportion of observation session for which focal individuals were groomed

447 when introduced to a foreign colony (treatment: black line) or to their own colony

448 (control: gray line). N=15 trials between 6 colonies in both treatment and control

449 categories. Points show means, bars show standard error.

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			Behaviour received				Behaviour given			
			Beta	s.e.	z	P-Value	beta	s.e.	z	P-Value
Grooming										
Stage 1	C		-2.53	0.44			-3.52	0.67		
	T		0.03	0.22	0.14	0.44	-0.013	0.82	0.017	0.49
Stage 2	C		-2.24	0.44			-4.07	0.67		
	T		0.75	0.22	3.34*	<0.01*	0.91	0.82	-1.11	0.134
Stage 3	C		-2.31	0.44			-3.59	0.67		
	T		-0.11	0.23	0.51	0.31	-0.5	0.82	0.62	0.27
Reintroduction	C		-2.07	0.44			-3.39	0.67		
	T		0.34	0.22	1.49	0.07	-0.64	0.82	0.78	0.22
Butting										
Stage 1	C		1.7	0.55			1.27	0.62		
	T		-0.041	0.49	0.084	0.46	-0.7	0.83	0.84	0.2
Stage 2	C		1.57	0.55			0.45	0.66		
	T		0.4	0.49	0.82	0.21	-0.24	0.86	0.28	0.39
Stage 3	C		1.65	0.55			1.29	0.63		
	T		0.28	0.49	0.56	0.29	-0.54	0.83	0.65	0.26
Reintroduction	C		1.59	0.54			1.24	0.63		
	T		-0.03	0.48	0.061	0.48	-0.55	0.82	0.66	0.25

Stage 1, Day 1

Treatment and control termites observed in separate containers, each with 15 individuals from their native colony

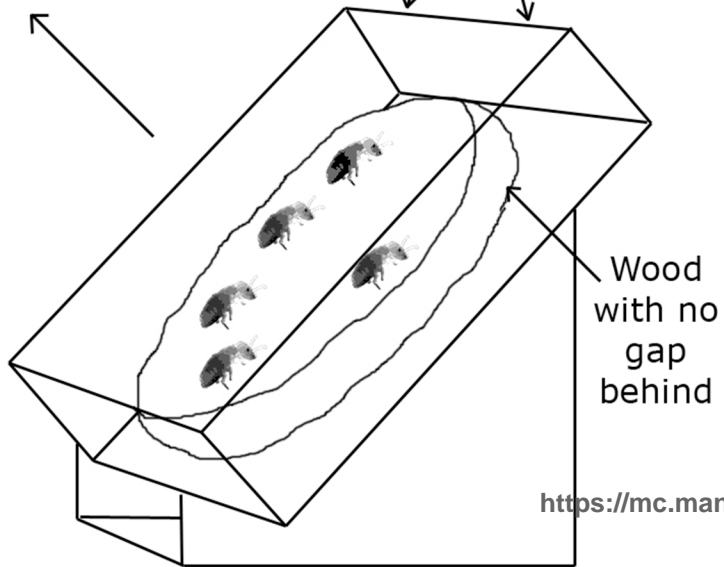
Stage 2, Day 1

- Treatment termite introduced to foreign colony sample containing 15 individuals.
 - Control termite transferred to new sample of 15 individuals from native colony
- <https://mc.manuscriptcentral.com/rsos>

Stage 3, Day 4

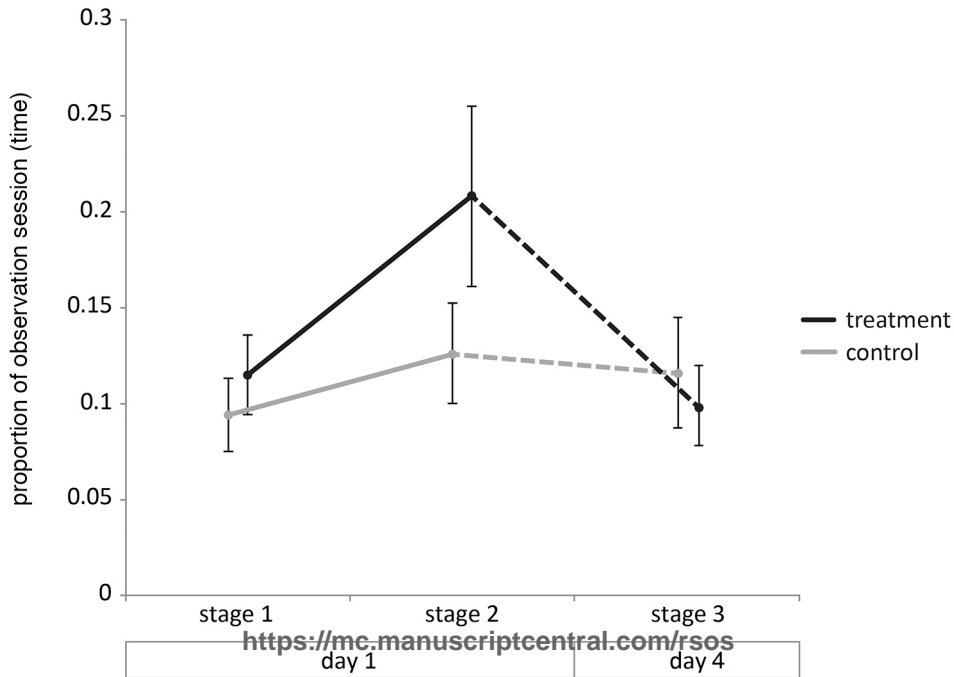
- Treatment termite observed with foreign colony sample of 15 individuals after 4-days within foreign colony
- Control termite observed with 15 termites from native colony

Perspex box



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