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±2° 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±2° 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 *Kalotermes 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.

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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.

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269 **Data accessibility**

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

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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.

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429

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.

450

		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

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Stage 1, Day 1

Treatment and control termites observed in separate containers, each with 15 individuals from their native colony
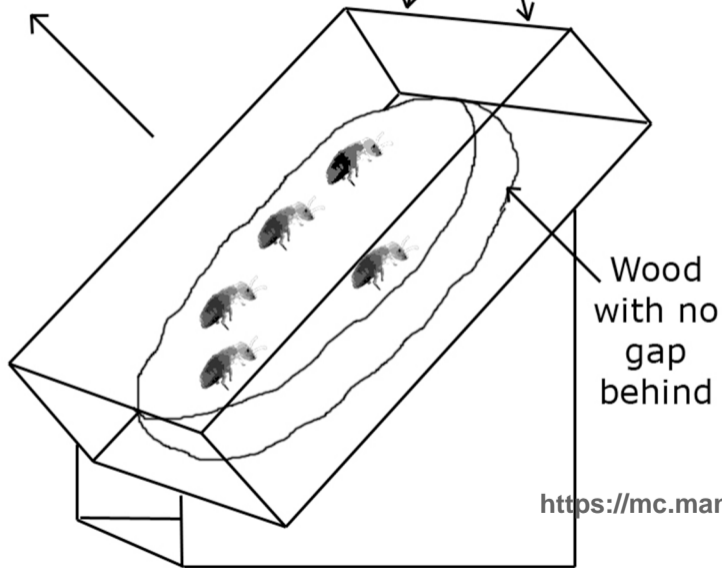
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Stage 2, Day 1

- Treatment termite introduced to foreign colony sample containing 15 individuals.

- Control termite transferred to new sample of 15 individuals from native colony

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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

Observer

Perspex box

Wood with no gap behind

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