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1 **Does cooperation mean kinship between spatially discrete**  
2 **ant nests?**

3

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15

16 **Running headline:** Cooperation and kinship between ant nests

17

## 18 **Summary**

- 19 1) Eusociality is one of the most complex forms of social organisation, characterised by  
20 cooperative and reproductive units termed colonies. Altruistic behaviour of workers within  
21 colonies is explained by inclusive fitness, with indirect fitness benefits accrued by helping  
22 kin. Members of a social insect colony are expected to be more closely related to one another  
23 than they are to other conspecifics.
- 24 2) In many social insects, the colony can extend to multiple socially-connected but spatially  
25 separate nests (polydomy). Social connections, such as trails between nests, promote  
26 cooperation and resource exchange, and we predict that workers from socially-connected  
27 nests will have higher inter-nest relatedness than those from socially unconnected, and non-  
28 cooperating, nests.
- 29 3) We measure social connections, resource exchange and inter-nest genetic relatedness in the  
30 polydomous wood ant *Formica lugubris* to test whether i) socially-connected but spatially  
31 separate nests cooperate, and ii) high inter-nest relatedness is the underlying driver of this  
32 cooperation.
- 33 4) Our results show that socially-connected nests exhibit movement of workers and resources,  
34 which suggests they do cooperate, whereas unconnected nests do not. However, we find no  
35 difference in inter-nest genetic relatedness between socially-connected and unconnected nest  
36 pairs, both show high kinship.
- 37 5) Our results suggest that neighbouring pairs of connected nests show a social and cooperative  
38 distinction, but no genetic distinction. We hypothesize that the loss of a social connection  
39 may initiate ecological divergence within colonies. Genetic divergence between neighbouring  
40 nests may build up only later, as a consequence rather than a cause of colony separation.

## 41 **Keywords:**

42 colony organisation, eusociality, *Formica lugubris*, kin selection, polydomy, social  
43 organisation

## 44 **Introduction**

45 Understanding how and why animal societies are organised in the way they are has long been a focus  
46 of biological research. Eusocial societies, characterised by cooperative brood care, overlapping  
47 generations and division of labour, are amongst the most complex forms of social organisation.  
48 Eusociality is found throughout the animal kingdom, for example: in mammals and crustaceans  
49 (Duffy et al., 2000; Jarvis and Bennett, 1993), but is particularly widespread in insects (Inward et al.,  
50 2007; Johnson et al., 2013; Smith et al., 2009; Stern, 1998). In eusocial organisms, the colony is a  
51 fundamental unit of social organisation; this reproductive and selective unit competes with other  
52 colonies within a population (Hölldobler and Wilson, 1990). Furthermore, the colony is also a  
53 cooperative unit; workers cooperate within colonies, collaboratively collecting resources and tending  
54 young, in order to produce the next generation.

55 Within a social insect colony, the workers do not themselves reproduce, and are therefore behaving  
56 altruistically by helping the queens reproduce. This altruism can be explained by inclusive fitness  
57 theory, with indirect fitness benefits to the workers accrued via the enhanced reproduction of kin  
58 (Bourke, 2011; Hamilton, 1964). Positive relatedness between interacting organisms is required for  
59 the evolution of altruism, and as such members of a social insect colony are expected to be more  
60 related to one another than they are to other individuals within the population. The positive effects of  
61 inclusive fitness can be further enhanced by ecological factors which result in higher benefits or lower  
62 costs of altruism (Bourke, 2011).

63 The traditional view of an ant colony is a single nest which contains a single queen and highly related  
64 workers. However this view is increasingly being shown to be overly simplistic (Heinze, 2008). Ant  
65 colonies can contain multiple reproducing queens at any one time, a trait known as “polygyny” (e.g.  
66 Pedersen & Boomsma 1999; Tsutsui & Case 2001; Holzer et al. 2006). In addition, the number of

67 nests that comprise an ant colony can differ. Spatially discrete nests can operate functionally as a  
68 single colony, a situation termed polydomy (Debout et al., 2007). Polydomy is found in widespread  
69 ecologically-important species (Ellis and Robinson, 2014), and is a feature of some of the world's  
70 most damaging invasive species (e.g. *Pheidole megacephala* Fournier et al. 2012; *Linepithema humile*  
71 Gordon and Heller 2014; *Anoplolepis gracilipes* Hoffmann 2014). The suggested benefits of  
72 polydomy to the colony include: risk spreading (van Wilgenburg and Elgar, 2007), efficient resource  
73 acquisition and exploitation (Cook et al., 2013; Schmolke, 2009), escape from the limitations of a  
74 single nest site (Cao, 2013), or release from the inefficiency of a very large nest (Kramer et al., 2014;  
75 Robinson, 2014). All of these potential benefits of polydomy follow logically from the assumption  
76 that the colony is a cooperative unit, and this is reinforced by empirical evidence of cooperation in the  
77 form of resource exchange between nests (Buczowski, 2012; Ellis et al., 2014; Ellis and Robinson,  
78 2016; Gordon and Heller, 2014). Different methods for delineating ant colony boundaries do not  
79 always draw the same colony boundaries (Ellis et al., In Review).

80 Polydomous colonies are defined as consisting of spatially separate nests linked by a social  
81 connection (Debout et al., 2007). Some ant species connect spatially separate nests with trails along  
82 which workers continually move back and forth, forming a clearly visible social connection (Ellis et  
83 al., 2014; Gordon and Heller, 2014; McIver, 1991). The strength of social connection between nests  
84 can be dramatic, with strong connections between nests involving hundreds of workers moving in  
85 either direction every minute (Skinner, 1980). Wood ants of the *Formica rufa* group, which includes  
86 *Formica lugubris*, use above ground trail networks extensively (Rosengren, 1971); no examples of  
87 subterranean trail networks between nests are known in this group. Polydomous trail networks are  
88 structured to allow efficient transport of resources within the colony (Cook et al., 2014). In the wood  
89 ant *F.lugubris*, nests which, due to their network position, experience a higher flow of resources are  
90 more likely to grow, reproduce and survive from year to year than those experiencing a lower  
91 resource flow (Ellis et al., in Review). Polydomous trail networks therefore represent connections  
92 between cooperating nests (which we use as a shorthand to mean cooperating ant communities, each

93 living within its own nest), sharing workers and resources, in line with the expectations of a social  
94 insect colony. In populations of *F. lugubris*, groups of nests connected by trails are often bordered by  
95 other nests to which they have no social connection, although the distance between unconnected nests  
96 can be similar to that between connected nests (D. Procter pers. obs.). Wood ant trails frequently  
97 persist in the same location for many years (Rosengren, 1971); furthermore, during mapping of trail  
98 networks of *F. lugubris* in the UK over three years, separate trail networks were never observed to  
99 become connected by trails (Ellis et al., in Review), indicating stability of distinct networks of  
100 connected nests. *Formica lugubris* exhibits variation in dispersal strategies across its range but in the  
101 UK new nests are formed by budding, whereby one or several queens split off from the parent nest  
102 with a subset of the workers and form a new nest nearby (Hughes, 2006). Budding nest formation  
103 could result in neighbouring nests with high genetic relatedness, allowing the formation of  
104 polydomous colonies.

105 In this paper we ask whether social connections between nest pairs correlate with genetic distinctions.  
106 To answer this question we measure i) worker movement, ii) carbohydrate resource exchange and iii)  
107 genetic relatedness between neighbouring nest pairs, which are either connected or unconnected by  
108 worker trails. The social connections characterised by worker and resource movement appear  
109 cooperative, therefore we predict that they will be reflected by increased relatedness between socially  
110 connected nest pairs.

## 111 **Methods**

### 112 **Study species and population**

113 *Formica lugubris* Zetterstedt, 1838, is a member of the mound-building red wood ants of the *Formica*  
114 *rufa* group, common across the temperate and boreal forests of Europe and Asia (Goropashnaya et al.,  
115 2004; Stockan and Robinson, 2016). The species exhibits variation in social structure throughout its  
116 range but populations in Britain are polygynous and polydomous (Ellis and Robinson, 2014;  
117 Gyllenstrand and Seppä, 2003; Hughes, 2006). Red wood ants are ecologically dominant, a trait they

118 share with many other polydomous species (Fournier et al., 2012; Gordon and Heller, 2014;  
119 Hoffmann, 2014). *Formica lugubris* forms strong trails both between neighbouring nests and from  
120 their feeding grounds in aphid colonies in nearby trees to nests (Ellis et al., 2014; Sudd, 1983). The  
121 majority of the nutrient intake during the summer comes from honeydew from aphids (Rosengren and  
122 Sundström, 1991).

123 The study population is located in the southern half of the North York Moors National Park, in the  
124 North East of England, UK (Long/Lat 54.289, -1.059, Fig. 1). This landscape has undergone large  
125 increases in forest cover in the last 160 years, which has allowed concomitant expansions of the wood  
126 ant populations (Procter et al., 2015). The investigated population of *F. lugubris* contains  
127 approximately 3000 nests, across an area of 10.4km<sup>2</sup> (Procter et al., 2015). This population was  
128 chosen for this study because prior knowledge of its extent and the location of nests allowed the  
129 selection of randomly distributed sampling points throughout the population, with sufficient spacing  
130 that any given polydomous colony, defined by social connections between spatially separate nests, did  
131 not span multiple sample points.

132 The forest is dominated by non-native conifer plantations adjacent to sections of ancient broadleaf  
133 woodland. Commercial forests dominated by non-native conifers represent a much more dynamic  
134 habitat than that provided by ancient woodland, due to relatively short harvest cycles, early canopy  
135 closure and frequent management interventions. The more dynamic nature of commercial forests may  
136 cause faster nest turnover than in ancient woodland. Our sampling points cover both ancient woodland  
137 and commercial forestry plantations, allowing us to assess whether there was an effect of forest age on  
138 the inter-nest genetic relatedness patterns we see within nest pairs. The age of the forest had no effect  
139 on these patterns, therefore we present analyses only in the Supporting Information (Appendix S1).

140

#### 141 **Mapping test triplets**

142 The specific arrangement required for this study was a series of groups of three nests, where two nests  
143 in each triplet were connected by a trail of workers (arbitrarily termed the ‘base’ and the ‘connected’  
144 nests) and the third nest was not connected directly or indirectly to either of the other two nests  
145 (termed the ‘unconnected’ nest, Fig. 2). In order to locate appropriate triplets, we began by randomly  
146 choosing 40 nests from previous survey data. Taking each randomly selected nest in turn, we mapped  
147 all nests to which the selected nest was connected by trails, either directly or indirectly (via one or  
148 more other nests), which resulted in a mapped network of nests connected by trails. We then searched  
149 the area immediately surrounding the mapped network of connected nests to find a nest close by that  
150 had no trail connection to any of the mapped nests (Fig. 2 unconnected nest). If no appropriate  
151 unconnected nest was found, we moved on to the next randomly chosen nest and began again. We  
152 found the desired triplet arrangement on 24/40 occasions. The mapping took place in April and May  
153 2014. .

154 We attempted to find connected and unconnected nests for each triplet that were a similar distance  
155 from the base nest; however overall, unconnected nests were significantly further away from the base  
156 nest (connected mean  $8.9\text{m} \pm 8.3\text{ SD}$ , unconnected mean =  $15.8\text{m} \pm 9.3\text{ SD}$ , paired t-test,  $t=-4.59$ ,  
157  $df=23$ ,  $P<0.001$ ). To account for this difference in distance between the base nest and the connected  
158 or unconnected nest, the Euclidean distance, i.e. straight line distance, between nests was included as  
159 a covariate in generalised linear mixed models during analysis.

160 It could have been possible that nest size explained presence or absence of trails within triplets. For  
161 example, trails might only form between nests that are over or under a certain size. We therefore  
162 recorded nest volumes using the methods of Chen and Robinson (2013), which have been shown to  
163 correlate with worker populations i.e. the number of workers within the nest (Chen and Robinson,  
164 2013), and tested for size effects on the presence of trails. None of the size effects was statistically  
165 significant (Supporting Information, Appendix S2), so nest volumes were not included in further  
166 analyses.

167 **Worker movement**



168 We assessed worker movement between nests by mass marking ants on the nest surface with a single  
169 light application of spray paint (Painter's touch multi-purpose paint, Rust-oleum, Durham, blossom  
170 white and spa blue) on two nests in each of the 24 mapped triplets in June 2014. The paint brand was  
171 chosen because colours did not wear off, and the application of paint did not affect worker behaviour  
172 (D. S. Procter, pers. obs.). The paint colours were chosen because they were both distinguishable from  
173 one another and clearly visible on the ants themselves. The ants on the base nest (Fig. 2) were sprayed  
174 one colour and those on the unconnected nest were sprayed a second colour. The third nest within the  
175 triplet ('connected' in Fig. 2), was not mass marked, because we could only find two paint colours  
176 that were both easily visible on the ants and distinguishable from one another. Nest surfaces were  
177 agitated before spraying, so that many workers from the interior came out onto the nest surface and  
178 were also marked. Colours were alternated between base and the unconnected nests in different  
179 triplets. We then returned to the sprayed triplet 1, 2, 3, 14 and 30 days after marking, and counted the  
180 number of workers of each colour on each of the three nests within the triplet by systematically scan-  
181 sampling the surface of each nest. From this we ascertained the relative level of worker movement  
182 from the base nest to the connected nest, the base nest to the unconnected nest and the unconnected  
183 nest to the base nest (Fig. 2). We tested whether the number of workers moving between nest pairs  
184 was significantly greater than zero using Wilcoxon rank tests in R (R Core Team, 2015).

### 185 **Resource movement**

186 We cannot assume that carbohydrate resource movement correlates with worker movement (Ellis et  
187 al., In Review); therefore, we assessed inter-nest resource movement independently of worker  
188 movement in a subset of 10 of the 24 mapped triplets in July 2014. We restricted the resource  
189 movement assessment to 10 of the triplets containing smaller nests, because in these smaller nests we  
190 could be confident of detecting the marked food using a sample size of 100 workers per nest. The  
191 sampling limit was imposed by logistical constraints. Ants transfer sugar solution between colony  
192 workers via trophallaxis, the exchange of food mouth to mouth or mouth to anus (Hölldobler and  
193 Wilson, 1990). There is a large amount of ant activity around nests that does not occur along the inter-

194 nest trails: therefore, trophallaxis between workers of different nests could hypothetically be  
195 independent of the trails of workers between nests. Using a food bait approach, we assessed resource  
196 movement within the triplets by mixing sugar solution with Rabbit Immunoglobulin IgG (Sigma-  
197 Aldrich) using the methods of Buczkowski and Bennet (2006). We focussed on the transfer of  
198 resources from the base nest to others within the triplet using a single label. Sucrose solution (70%) in  
199 1.5ml volumes with 0.5mg/ml IgG was placed in feeders made from inverted micro-centrifuge tubes  
200 placed on top of the base nest of each triplet. We used 10 feeders per baited nest. Feeders were topped  
201 up 24 hours after initial placement on the nest surface. Samples of 100 workers per nest from each  
202 nest within the triplet were collected 48 hours after sugar solution was initially provided and sampled  
203 ants were placed in a chilled cool box. Upon arrival at the laboratory, the chilled workers were killed  
204 by placing them in the freezer at -20°C, where they were retained prior to analysis. Each sampled  
205 worker was assayed for IgG presence using an ELISA assay, carried out as follows: a 96 well PCR  
206 plate was coated with 100µl of anti-rabbit IgG, diluted 1:500 in distilled water and incubated at 4°C  
207 for 2 hours. Once incubation was complete, the primary antibody was discarded and 280µl of 1% non-  
208 fat dry milk was added to each well as a blocker of any remaining non-specific binding sites. After 30  
209 minutes the milk was discarded. Individual ant samples were homogenised in 200µl phosphate  
210 buffered saline, vortexed, and 70µl of each sample was added to a well in the prepared plate and  
211 incubated for 1 hour at room temperature. Samples were then discarded and each well was washed  
212 three times with PBS Tween 20 (0.05%) and then twice with phosphate buffered saline. Anti-rabbit  
213 IgG conjugated to horseradish peroxidase diluted 1:1000 in 1% non-fat dry milk was added to each  
214 well, after which the plate was incubated at room temperature for 1 hour. All wells then received the  
215 five washes described above before adding 50µl of TMB (tetramethylbenzidine) HRP (horseradish  
216 peroxidase) substrate (New England Biolabs) and incubated for 30 minutes at room temperature.  
217 Samples were analysed on a BMG Labtech POLARstar OPTIMA microplate spectrophotometer set at  
218 an absorbance of 650nm. Six negative controls which contained ants without IgG and six blanks  
219 which contained no ant sample were run on each plate. Individual wells were scored as positive if  
220 their absorbance value was more than three standard deviations higher than the mean of the negative

221 controls (Buczowski and Bennett, 2007). We analysed differences in the number of workers testing  
222 positive for IgG between connected and unconnected nest pairs using a generalised linear mixed  
223 model (GLMM). The response variable was the number of workers testing positive for IgG and we  
224 used a Poisson error structure. The explanatory variables were whether or not the nest pair was  
225 connected by a trail and the Euclidean distance between nests. The triplet the nest pair came from was  
226 included as a random effect. We used the glmer function in the lme4 package of R (Bates et al., 2014).

### 227 **Aggression**

228 Aggression bioassays are a commonly used determinant of colony boundaries (e.g. Denis et al. 2006;  
229 Garnas et al. 2007; Hölldobler 1983; Kenne and Dejean 1999), based on the assumption that workers  
230 will behave aggressively towards workers from neighbouring colonies, but not their own colony  
231 mates. We conducted preliminary aggression studies in May 2014 (see Supporting Information,  
232 Appendix S3 for details) on *F. lugubris* in our study landscape, but found that aggression levels were  
233 so low that aggression tests could not even distinguish behaviourally between populations that were  
234 separated by tens of kilometres, let alone neighbouring colonies. We note that lack of aggression does  
235 not necessarily imply lack of colony-mate recognition (Björkman-Chiswell et al., 2008; Holzer et al.,  
236 2006), however we found no difference in antennation duration between tested workers from different  
237 locations (see Supporting Information, Appendix S3 for details). We therefore decided not to deploy  
238 aggression bioassays in the full study, because they were unlikely to be informative.

### 239 **Genetic distinctions between connected and unconnected nest pairs**

240 We collected 10 workers per nest from each nest within 20 of the 24 triplets throughout the landscape  
241 in July 2014. We excluded four of the triplets used to assess worker movement, due to damage during  
242 the study period. All 10 triplets used to assess resource movement were included within the 20  
243 sampled for genetic work. DNA was extracted using GeneJET Genomic DNA Purification kits  
244 following the manufacturer's instructions (Thermo Scientific). The sampled workers were each  
245 assessed for variation at the following 12 nuclear microsatellite loci: Fe7, Fe11, Fe13, Fe16, Fe17,

246 Fe19, Fe21, Fe37, Fe38 (developed for *Formica exsecta* Gyllenstrand et al., 2002), and Fl12, Fl20 and  
247 Fl21 (developed for *Formica paralugubris* Chapuisat 1996, known as *Formica lugubris* type B at the  
248 time), using the primers and PCR conditions specified in those papers. Each forward primer had a 5' –  
249 AGGTTTTCCCAGTCACGACGTT – 3' M13 sequence attached at the 5' end for subsequent  
250 detection purposes. DNA was amplified in a total volume of 20µl using the following reaction  
251 mixture: 1µl DNA, 1X PCR buffer (Bioron, Germany), 5µM of each primer (Integrated DNA  
252 Technologies), 0.2mM of each dNTP (VWR International), 0.25µM M13 oligo with either 700nm or  
253 800nm fluorescent dye attached (Li-Cor Biosciences), and 0.25U Taq DNA polymerase (Bioron).  
254 PCR products were diluted with formamide loading buffer and run on a Li-Cor 4300 (Li-Cor  
255 Biosciences, Lincoln, NE, USA). Allele sizes were scored by eye using a set of size standards for  
256 700nm and 800nm wavelengths. Analyses based on genetic differentiation assume that loci are at  
257 Hardy-Weinberg equilibrium and there is no linkage disequilibrium between loci. Therefore loci were  
258 tested for deviations from Hardy-Weinberg equilibrium and linkage disequilibrium within triplets in  
259 FSTAT 2.93 (Goudet, 1995).

260 We calculated pairwise genetic relatedness between all sampled workers in each triplet using  
261 the Triadic likelihood estimator of relatedness of Wang (2007) in the Coancestry 1.0.1.5 program  
262 (Wang, 2011), allowing for inbreeding in the population. Differences in inter-nest genetic relatedness  
263 between workers from connected and unconnected nest pairs were analysed as a generalised linear  
264 mixed model (GLMM) with binomial errors, because response values are constrained between 0 and  
265 1. The response variable was the pairwise inter-nest genetic relatedness between workers with  
266 explanatory variables being the nest pair on which the inter-nest relatedness value was based  
267 (connected or unconnected) and the Euclidean distance between the pair of nests. Triplet identity was  
268 included as a random effect. The GLMM used the glmer function in the lme4 package of R (Bates et  
269 al., 2014).

270 We could not expect to see any differentiation between adjacent nests if there is no  
271 differentiation in the population as a whole. In order to confirm that there was differentiation within

272 the population we assessed isolation by distance for the 60 sampled nests within the population as a  
273 whole by measuring all pairwise  $F_{ST}$  scores between nests using the `fst.pp` function of the `hierfstat`  
274 package of R (Goudet, 2005). We then assessed whether there was a significant relationship between  
275 genetic distance ( $F_{ST}/1-F_{ST}$ ) and Euclidean distance between nests using a Mantel test with 9999  
276 permutations, using the `mantel.rtest` function in the `ade4` package of R (Chessel et al., 2004). We also  
277 analysed genetic differentiation between connected and unconnected nest pairs using hierarchical F-  
278 statistics in the `hierfstat` package of R (Goudet, 2005). We separated the data into three hierarchical  
279 levels. Firstly the differentiation among workers within nests, which we term  $F_{Nest}$ , secondly the  
280 differentiation between nests connected and unconnected by trails within triplets, termed  $F_{Trail}$ , and  
281 lastly the differentiation between triplets within the population, termed  $F_{Trip}$ .  $F_{Trail}$  is the differentiation  
282 between those nests that share a social connection or do not, which is the value we are interested in in  
283 this study. Statistical significance of the different hierarchical levels was determined by permutation  
284 tests with 1000 permutations (Goudet, 2005).

285 Non-significant results indicate that there is no effect greater than that which is possible to  
286 detect given the experimental design employed. We conducted a power analysis in order to test the  
287 minimum level of difference in genetic relatedness we would be able to detect between connected and  
288 unconnected nest pairs. We simulated inter-nest relatedness for the two treatments (pairs of connected  
289 and pairs of unconnected nests) based on characteristics of preliminary genetic data (mean relatedness  
290 0.131, standard deviation = 0.055). We varied the difference in mean inter-nest relatedness between  
291 connected and unconnected nest pairs between 0.001 and 0.1, at steps of 0.001. We simulated 1000  
292 variables per level of difference in treatments. Using 20 repeats, we achieved a power of 80%  
293 whenever the difference in relatedness between treatments was greater than 0.05, in other words, a  
294 significant difference ( $P < 0.05$ ) between treatments was found in 80% of simulations. We were  
295 therefore confident that we could detect a significant difference in inter-nest genetic relatedness  
296 between connected and unconnected nest pairs whenever the magnitude of the difference in  
297 relatedness was 0.05 or greater.

298

## 299 **Results**

### 300 **Worker movement**

301 The number of ants detected to have moved between the base and connected nests in each triplet (Fig.  
302 2), was significantly greater than zero on all counting visits: 1, 2, 3, 14 and 30 days after paint  
303 marking (Wilcoxon rank test,  $W=171-253$ , all  $P<0.001$ , Fig. 3a). In contrast, the number of ants that  
304 moved from the base nest to the unconnected nest did not significantly differ from zero on any  
305 counting visit (Wilcoxon rank test,  $W=0-1$ , all  $P=1$ , Fig. 3b). Similarly, the number of ants moving  
306 from the unconnected nest to the base nest did not differ significantly from zero on any counting visit  
307 (Wilcoxon rank test,  $W=0-3$ ,  $P=0.346-1$ , Fig. 3c). Therefore, the presence of trails between nests does  
308 indicate a greater movement of workers and the absence of trails does appear to mean a lack of social  
309 connection. The number of workers detected to have moved between connected nests on different  
310 days did not significantly differ (Kruskal-Wallis,  $df=4$ ,  $\chi=1.46$ ,  $P=0.83$ , Fig. 3).

### 311 **Resource movement**

312 After 48 hours of IgG marked sucrose being made available for ant feeding on the base nest, we  
313 detected a total of 279 out of 3000 collected workers positive for IgG. Of these, 252 were found on  
314 the baited base nest themselves, 22 on the connected nest and only 5 on the unconnected nest. There  
315 were significantly more workers that tested positive for IgG on the connected nest than on the  
316 unconnected nest (GLMM,  $df=1,4$ ,  $\chi=9.34$ ,  $P<0.001$ , Fig. 4a). There was no significant effect of  
317 Euclidean distance between nests on the number of workers testing positive for IgG (GLMM,  $df=1,4$ ,  
318  $\chi=0.24$ ,  $P=0.62$ )

319

### 320 **Genetic distinctions between connected and unconnected nest pairs**

321 Diversity across the 12 microsatellite loci used ranged from low to high. Three of the loci displayed  
322 low variability (2-3 alleles, expected heterozygosity 0.16-0.51), with the remaining nine loci being  
323 more variable (4-19 alleles, expected heterozygosity 0.67-0.89). None of the loci showed significant  
324 deviations from Hardy-Weinberg equilibrium or significant linkage disequilibrium within samples, so  
325 all loci were retained for the analysis. The 60 nests making up the 20 triplets of nests in which  
326 workers were genotyped, displayed significant isolation by distance, with genetic distance, measured  
327 by  $F_{ST}/1-F_{ST}$ , increasing significantly as distance between nests increased (Mantel test,  $r=0.36$ ,  
328  $P<0.001$ , Fig. 5).

329 Inter-nest genetic relatedness between workers from connected nest pairs did not differ significantly  
330 from inter-nest genetic relatedness between workers from unconnected nest pairs (connected pair  
331 mean= 0.17, unconnected mean= 0.16, GLMM,  $df=1,3$ ,  $\chi=0.122$ ,  $P=0.73$ , Fig. 4b). There was no  
332 relationship between inter-nest genetic relatedness and Euclidean distance within triplets (GLMM,  
333  $df=1,3$ ,  $\chi=0.81$ ,  $P=0.36$ ). The majority of differentiation was explained by the highest hierarchical  
334 level of organisation of the data: the differentiation between different triplet groups i.e. the  
335 differentiation due to landscape patterns, which was significantly greater than zero ( $F_{Trip}=0.055$ ,  
336  $P=0.001$ ). There was negligible differentiation between connected and unconnected pairs within  
337 triplets ( $F_{Trail}=0.001$ ), or within nests ( $F_{Nest}=0.004$ ), neither of which were significantly greater than 0  
338 ( $F_{Trail} P=0.683$ ,  $F_{Nest} P=0.087$ ). The negligible value of  $F_{Trail}$  supports the lack of difference in  
339 relatedness between connected and unconnected nest pairs, and high within triplet relatedness, that  
340 our relatedness analyses report.

## 341 **Discussion**

342 A social insect colony is expected to be a cooperative, reproductive and selective unit, where  
343 members are more related to one another than to other members of the population. However, we have  
344 shown that workers from nests of *F. lugubris* that appear to cooperate are no more genetically related  
345 to one another than workers from nests that do not cooperate. Cooperation between ant nests involves

346 the exchange of workers and resources. We have shown that both workers and resources move  
347 between connected nest pairs, whereas workers do not detectably move between unconnected nest  
348 pairs, and significantly fewer resources are exchanged. Nest pairs with an apparently cooperative  
349 connection neither differ in their inter-nest genetic relatedness from unconnected nest pairs, nor do  
350 they display significant genetic differentiation from unconnected nests. The difference we observe in  
351 apparent cooperation therefore cannot be explained by a genetic difference.

352 Our results suggest that spatially separate nests in *F. lugubris* are cooperative units when connected  
353 by trails. Firstly, we have confirmed that trails between nests do constitute a social connection,  
354 because workers move between connected nest pairs but, more importantly, workers are not  
355 exchanged between unconnected nest pairs. Substantially rarer movement between unconnected nests  
356 than connected nests is consistent with previous findings in a related species (O'Neill, 1988), and is  
357 expected if nests are solely cooperating within one colony. Secondly, we have shown that connected  
358 nests exchange significantly more resources than unconnected nests. Movement of resources between  
359 nests could be interpreted as either cooperation or stealing, but with stealing, we would expect  
360 competitive interactions. The strong social connections we observe, without aggression, suggest  
361 cooperation rather than competition. Existing evidence from other ant species suggests that new nests  
362 within polydomous colonies are placed near food sources (Holway and Case, 2000; Lanan et al.,  
363 2011). In *F. lugubris* this does not appear to be the case; however, nests with workers that forage are  
364 more likely to survive than non-foraging nests (Ellis and Robinson, 2015). In *F. lugubris* colonies,  
365 workers appear to use nests they are connected to by trails as a foraging resource, which could be  
366 interpreted as a form of intraspecific kleptoparasitism (Ellis and Robinson, 2016). However,  
367 polydomous nest networks in wood ants and other species are structured to allow efficient transport of  
368 resources (Cook et al., 2014), suggesting there may be a colony-level benefit to allowing, and even  
369 promoting, resource transfer between nests. In *F. lugubris* a nest's position within the colony resource  
370 flow predicts nest survival (Ellis et al., in Review), and nests within a network that do not forage are  
371 more likely to be abandoned (Ellis and Robinson, 2015). There is, therefore, an advantage to be



372 connected to multiple nests, which should elicit competition between nests if connections are not  
373 cooperative. However we find no aggression between neighbours in our population (see Supporting  
374 Information, Appendix S3). Resource movement between spatially separate nests therefore suggests  
375 active cooperation between socially connected nests, as we predicted.

376 Our results clearly demonstrate that there is significantly higher resource transfer between nests  
377 connected by trails of workers than between unconnected nests. However, in three out of ten trials we  
378 did see carbohydrate resource transfer between unconnected nests, albeit at a low level. The few  
379 workers that were found to be positive for IgG on the unconnected nest may have acquired resources  
380 from the baited nest via non-cooperative means. The non-cooperative acquisition of food could  
381 involve stealing from the baited nest or possibly inducing trophallaxis from workers from the baited  
382 nest. Trophallaxis is a standard method by which resources are transferred between workers of the  
383 same colony and is normally thought of as a sign of cooperation, but trophallaxis can also occur  
384 between species which do not cooperate (Bhatkar and Kloft, 1977). Under these circumstances,  
385 trophallaxis acts as a means of reducing inter-species aggression. Therefore, the exchange of  
386 resources seen in this study, could be an activity that reduces aggression between colonies, analogous  
387 to reducing aggression between species. Resource movement can either correlate well with social  
388 connections (Heller et al., 2008; VanWeelden et al., 2015) or can operate at a different spatial scale  
389 (Buczowski, 2012); therefore, the slight disparity between worker movement and resource  
390 movement in our results agrees with the literature: future studies should be cautious in assuming that  
391 social connections and resource movement are always closely correlated.

392 Workers themselves must also be considered resources for ant colonies, because they contribute to the  
393 production of the next generation. Our data support movement of workers between connected nests,  
394 which could be genuine worker exchange if the ants perform beneficial acts such as brood care or  
395 foraging for the recipient nests. While our current study does not investigate the behaviour of the  
396 workers that move, worker movement may also be a form of resource exchange, and arguably be  
397 more important than the exchange of carbohydrate, because carbohydrate maintains only the current

398 generation of ants. Total resource exchange between nests is therefore a combination of worker  
399 exchange and exchange of food. Viewed in this way, the resource exchange between socially  
400 connected nests far exceeds the resource exchange between socially unconnected nests, and represents  
401 a real cooperative distinction if the workers are behaving beneficially in the recipient nest.

402 We have shown that the apparent cooperative distinction we found is not reflected by a genetic  
403 distinction, however, we are not claiming that genetic factors are not important within ant colonies.  
404 The altruistic acts of workers within an ant colony are explained by inclusive fitness (Bourke, 2011;  
405 Hamilton, 1964), which includes both a benefit and cost term, as well as genetic relatedness. Genetic  
406 relatedness between the unconnected, non-cooperative, nest pairs is remarkably high (mean = 0.16),  
407 indeed higher than is often observed within single nests of other ant species (e.g. in another *Formica*  
408 species as low as 0.01: Pamilo et al. 2005; and in other ant species 0.04: Goodisman and Ross 1997;  
409 and 0.05 Pedersen and Boomsma 1999). There is therefore, no genetic reason why cooperative  
410 interactions should not occur. In *F. lugubris*, interactions between nests appear to be based on the  
411 movement of resources through the colony; ant nests that differ most in the amount of foraging that  
412 they perform are linked by stronger trails than those nests that had a more equal foraging effort (Ellis  
413 et al., 2014). In this study we did not assess foraging in sufficient detail to determine the costs and  
414 benefits to each nest. If both nests within an unconnected pair forage sufficiently to support their  
415 worker force, then there may be no benefit to be gained from the presence of a trail between nests, and  
416 therefore no reason to maintain a trail. Alternatively, because aphids are abundant in the vicinity of  
417 wood ant colonies, the exchange of carbohydrate between neighbouring nests may incur only a tiny  
418 cost. With a small enough cost of resource exchange, there will be minimal evolutionary pressure to  
419 eliminate trails that are remnants of the nest formation event. Some trails may be lost by chance, while  
420 others are maintained, without a penalty to those that remain connected. We assume that the cost of  
421 the trail between nests is proportional to the length of that trail and account for trail length in our  
422 analyses. However, there may be other factors, such as desiccation or predation risk, that mean that  
423 trails between unconnected nest pairs are more costly than between connected nest pairs and preclude

424 trail formation. We therefore suggest the distinction between connected and unconnected nest pairs is  
425 not caused by a genetic distinction, but by some unmeasured ecological or stochastic process.

426 Ants use cuticular hydrocarbons (CHCs) for nestmate recognition (Hölldobler and Wilson, 1990). The  
427 extent to which genetic and environmental patterns affect hydrocarbon profiles varies between ant  
428 species (Buczkowski and Silverman, 2006; van Zweden et al., 2009) but in wood ants, experimental  
429 separation has been shown to alter CHC profiles (Sorvari et al., 2008). It may therefore be that once a  
430 social connection has been lost for long enough for hydrocarbon profiles to diverge, genetically  
431 similar ants will no longer recognise one another as colony-mates and the division becomes more  
432 permanent. Further studies may wish to assay CHC profiles alongside social connection methods to  
433 ascertain whether this is the driving factor.

434 The study landscape is dominated by commercial forests, which are both recently planted and highly  
435 dynamic in comparison to natural woodland. The addition of these commercial forests has benefitted  
436 the wood ants, allowing large population expansions (Procter et al., 2015). Due to these recent  
437 population expansions, we cannot expect the ant populations to be at equilibrium. It is possible that  
438 the recent range expansions of *F. lugubris* on the North York Moors have resulted in neighbouring  
439 colonies exhibiting the high inter-nest relatedness that we see. However, our sampled triplets were  
440 located in both ancient woodland and recently planted conifer plantations, and all showed the same  
441 lack of genetic distinction between connected and unconnected nest pairs (Supporting Information,  
442 Appendix S1). We therefore think it is unlikely that the dynamic landscape will have masked any  
443 possible distinctions, but it would still be interesting to compare our results with a similar study in a  
444 less disturbed forest system.

445 The genetic patterns we report are based solely on nuclear DNA variation. Many ant species are  
446 known to exhibit sex-biased dispersal, whereby males disperse larger distances than females. This  
447 results in differentiation in biparentally inherited nuclear genetic differentiation at a larger spatial  
448 scale than is seen for maternally inherited markers such as those located on mitochondrial DNA (e.g.  
449 Clémencet et al., 2005; Doums et al., 2002; Soare et al., 2014). If there is a similar pattern of sex-

450 biased dispersal in this population, the division between connected and unconnected nest pairs may  
451 become exposed if mitochondrial DNA markers are used, because different matrilineal lines within the  
452 connected and unconnected nest may be resolved. However, preliminary surveys of fragments of  
453 mitochondrial COI DNA showed only two haplotypes within this population, with variation never  
454 present within a sampled triplet (D. S. Procter unpubl. data).

455 In the studied population, *F. lugubris* colonies reproduce by budding; this method of dispersal often  
456 results in strong spatial genetic structuring of populations, meaning that nests close to one another are  
457 more genetically similar irrespective of colony divisions (Sundström et al., 2005). Budding dispersal  
458 could therefore mean that all three of the nests in each of our triplets share common descent. Wood  
459 ant trails can be stable over long time periods (Rosengren, 1971). The trail structures within this  
460 population have not been mapped over multiple years, so we do not know how long the unconnected  
461 nests have been unconnected. However, in another *F. lugubris* population in the UK, trails have been  
462 mapped over multiple years: trail turnover does occur but new connections were not formed between  
463 separate trail networks, nor did trail networks separate and then reconnect (Ellis et al., in Review).  
464 Therefore there does appear to be a genuine separation between neighbouring nest networks in *F.*  
465 *lugubris*. If unconnected nest pairs were connected until recently then our results indicate there has  
466 been insufficient time for genetic distinctions to build up between unconnected nests.

467 A social insect colony is expected to be a cooperative, reproductive and selective unit, which should  
468 apply whether the colony occupies a single nest or multiple spatially separate nests. In a polydomous  
469 species, we suggest that there are cooperative divisions within genetically homogenous groupings. In  
470 some eusocial insects, social organisation is to a degree controlled by environmental factors (Eickwort  
471 et al., 1996; Richards, 2000). Similarly, we suggest that it is ecology rather than genetics that is  
472 driving the polydomous nest organisation that we observe here. Our findings support the polydomous  
473 colony as a cooperative entity, but not one that is genetically distinct from its neighbour. Our study  
474 also suggests that ecology plays a large role in determining social organisation in this, and likely  
475 other, ant species.

476 **Data Accessibility**

477 Microsatellite data, sampling locations, worker movement data and resource movement data will be  
478 archived in Dryad

479

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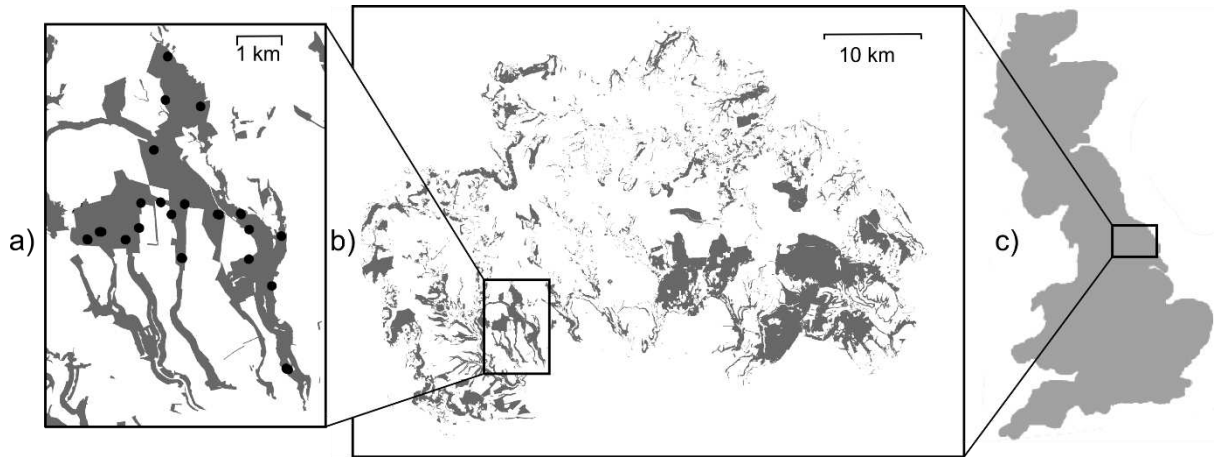
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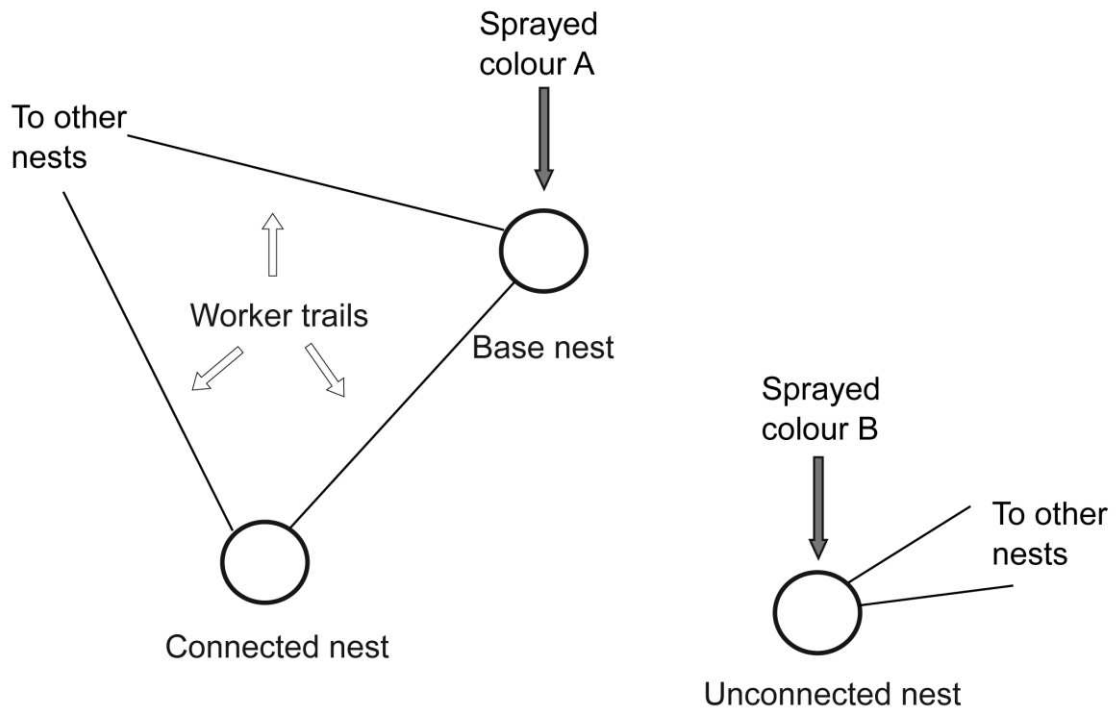


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671 **Fig. 1. a)** The study *F. lugubris* population: grey polygons are forest cover and black circles are  
 672 sampled triplet locations. Boxes denote **b)** the population's location within the North York Moors  
 673 National Park (again grey polygons are current forest cover) and **c)** the location of the North York  
 674 Moors within Britain.

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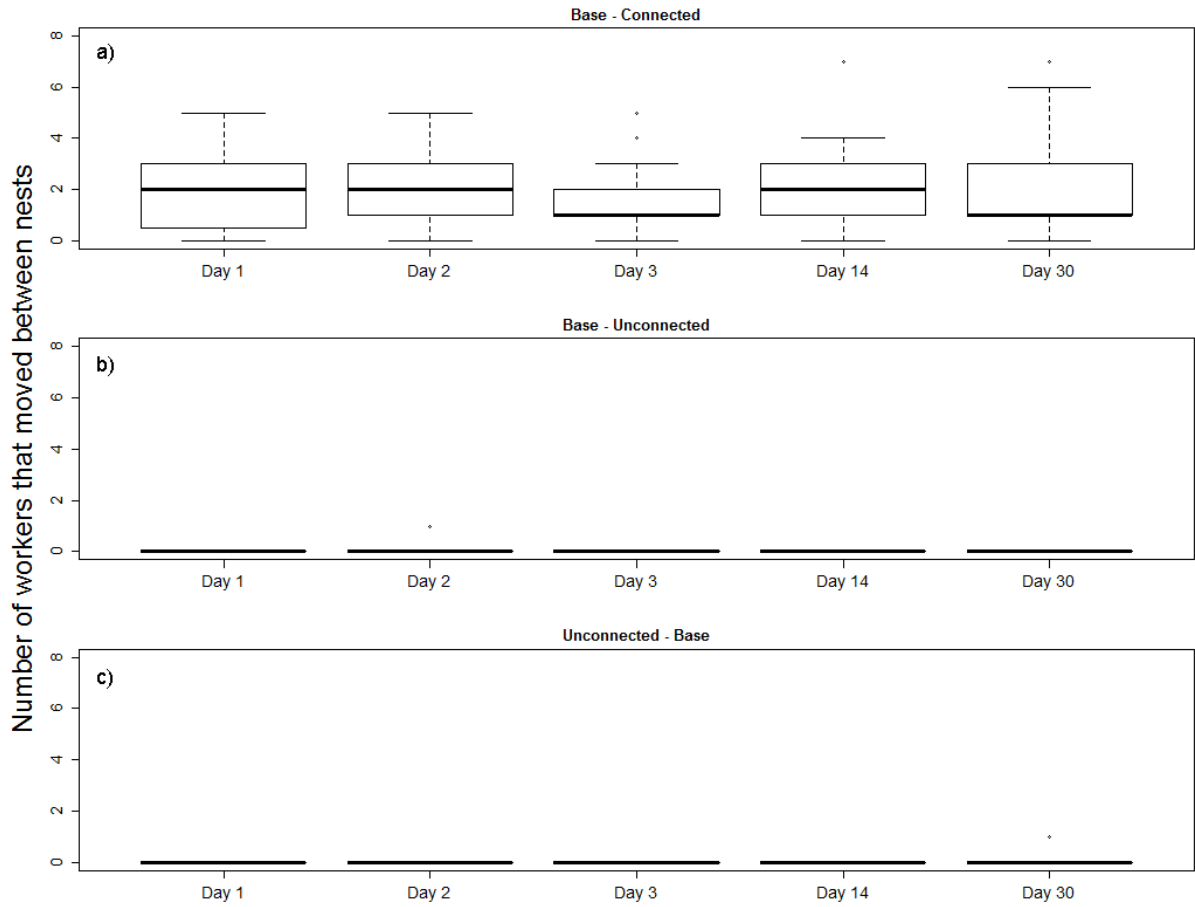
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678 **Fig. 2.** A schematic of the design for triplets used in this study: two nests connected by trails  
679 (arbitrarily termed 'base' and 'connected' nest) and a third nest (termed 'unconnected'), a similar  
680 distance away but not connected by a trail. Spraying the base nest colour A and the unconnected nest  
681 colour B allows us to track worker movement from the base to connected nest, from the base to the  
682 unconnected nest and from the unconnected nest to the base or connected nest. The unconnected nest  
683 was in some, but not all, cases connected to a separate nest network.

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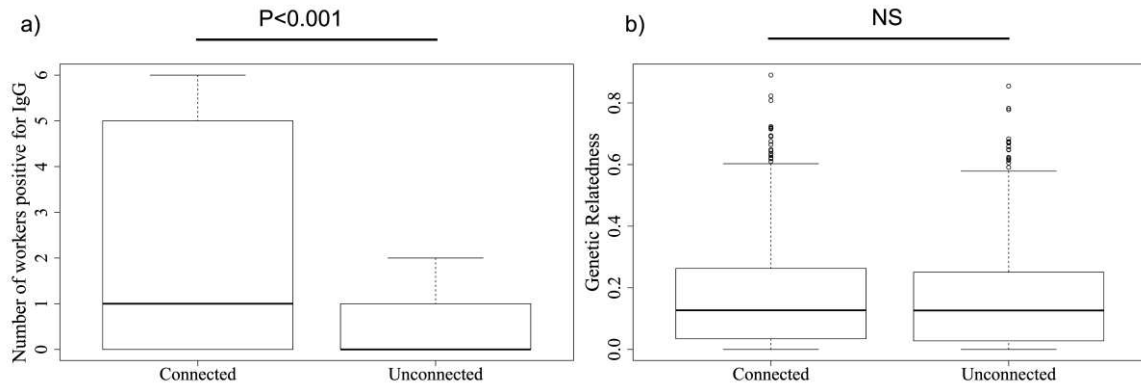


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686 **Fig. 3.** Number of workers that had moved from **a)** the base nest to the connected nest, **b)** the base  
 687 nest to the unconnected nest, **c)** the unconnected nest to the base nest, for each day of re-counting for  
 688 24 triplets of nests. Boxes display 1<sup>st</sup> quartile, median and 3<sup>rd</sup> quartile, whiskers extend to 1.5 IQ, and  
 689 outliers are displayed as points.

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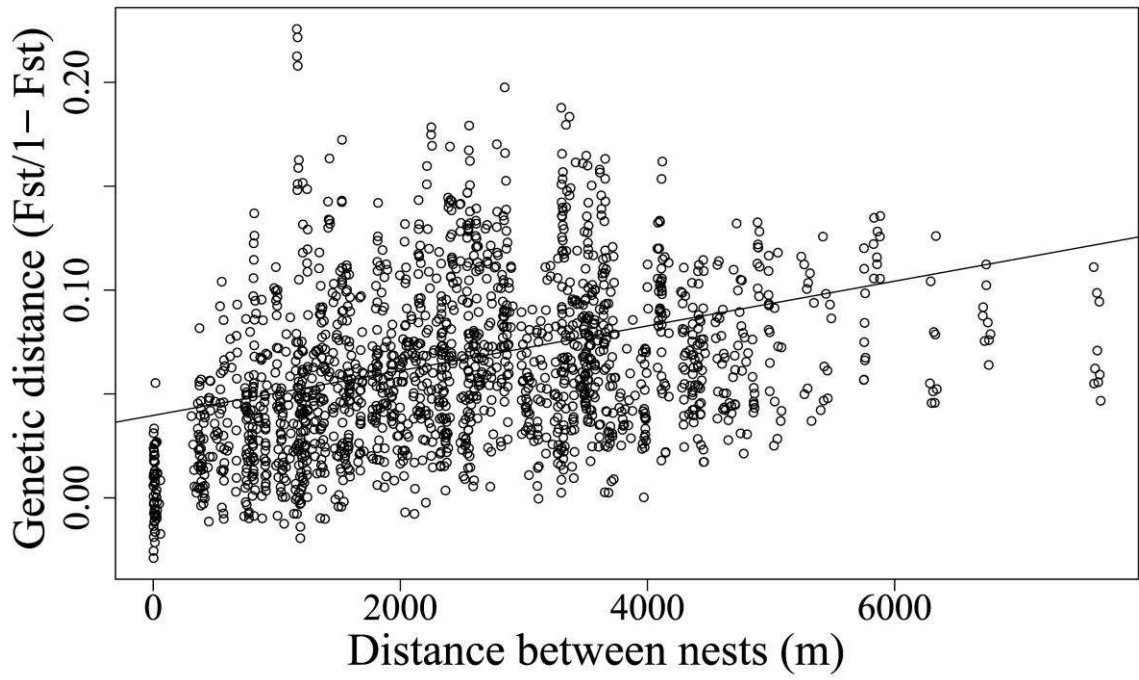
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693 **Fig. 4.** Comparisons between the connected and unconnected nest pair for **a)** The number of workers  
694 testing positive for IgG (10 triplets) **b)** Inter-nest genetic relatedness (20 triplets). Boxes display 1<sup>st</sup>  
695 quartile, median and 3<sup>rd</sup> quartile, whiskers extend to all points within 1.5 IQ, outliers are displayed as  
696 points.





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699

700 **Fig. 5.** Genetic distance, measured by  $F_{ST}/1-F_{ST}$ , against distance between all the sampled nest pairs in  
701 the population as a whole. The line displays a linear relationship between genetic distance and  
702 Euclidean distance between nests; significance was tested using a Mantel test.

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