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1 **The effects of disturbance threat on leaf-cutting ant colonies:**
2 **a laboratory study**

3

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10

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19

21 **Abstract**

22 The flexibility of organisms to respond plastically to their environment is fundamental
23 to their fitness and evolutionary success. Social insects provide some of the most
24 impressive examples of plasticity, with individuals exhibiting behavioural and
25 sometimes morphological adaptations for their specific roles in the colony, such as large
26 soldiers for nest defence. However, with the exception of the honey bee model
27 organism, there has been little investigation of the nature and effects of environmental
28 stimuli thought to instigate alternative phenotypes in social insects. Here we investigate
29 the effect of repeated threat disturbance over a prolonged (17 month) period on both
30 behavioural and morphological phenotypes, using phenotypically plastic leaf-cutting
31 ants (*Atta colombica*) as a model system. We found a rapid impact of threat disturbance
32 on the behavioural phenotype of individuals within threat-disturbed colonies becoming
33 more aggressive, threat-responsive and phototactic within as little as two weeks. We
34 found no effect of threat disturbance on morphological phenotypes, potentially because
35 constraints such as resource limitation outweighed the benefit for colonies of producing
36 larger individuals. The results suggest that plasticity in behavioural phenotypes can
37 enable insect societies to respond to threats even when constraints prevent alteration of
38 morphological phenotypes.

39

40 **Keywords**

41 Phenotypic plasticity, animal behaviour, castes, social insect, division of labour, animal
42 personality, behavioural syndromes

43

44 **Introduction**

45 The ability of organisms to respond flexibly to their environment is fundamental to their
46 evolutionary success. Adaptation towards a locally optimal phenotype can increase both
47 direct and indirect fitness (Via and Lande 1985; Lande 1985). One way in which this
48 can occur is via the production of size variation in response to environmental
49 conditions, such as climate or competition, which is seen in a wide variety of organisms
50 found over an environmental gradient (Rosenzweig 1968; McNab 1971; Lomolino
51 2005). However, many organisms show other morphological and behavioural
52 adaptations to environmental pressures that enable individuals to increase their fitness
53 over the course of their lifetime (Boag and Grant 1981; Engel and Tollrian 2009;
54 Torres-Dowdall et al. 2012). Understanding the biotic or abiotic environmental stimuli
55 involved in regulating the modification of the morphological or behavioural phenotype,
56 and any potential negative implications associated with this, are central to our
57 understanding of the evolution and dynamics of phenotypic plasticity.

58 Some of the most extreme examples of phenotypic plasticity are provided by the
59 social insects. In these societies it is often thought that the specialization of individuals
60 into behavioural and sometimes morphological phenotypes (castes) may make them
61 better adapted to their particular roles in the colony, thereby enhancing the division of
62 labour that is commonly thought to be key to their evolutionary success (Oster and
63 Wilson 1978; Bourke and Franks 1995). However, the degree to which specialists
64 actually outperform generalists is still highly contentious and our understanding of these
65 processes remains limited (Dornhaus 2008; Wright et al. 2014; Gordon 2015).
66 Individual workers are more-or-less sterile in species with advanced societies, such as
67 *Atta* leaf-cutting ants, and therefore gain their fitness indirectly, meaning that selection

68 may act simultaneously at both the individual and colony level, such that individual-
69 level and colony-level optimal caste ratios can be hypothesised to be the same
70 (Hamilton 1964; West-Eberhard 1989; Korb and Heinze 2004). The morphological
71 phenotype of adult social insects is determined during development in the eusocial
72 Hymenoptera (ants, some bees and some wasps). Both morphological and indeed
73 behavioural phenotypes are not simply determined by environmental conditions such as
74 nutrition and temperature (Wheeler 1986; Kamakura 2011), or by genotype (Robinson
75 and Page 1988; Hughes et al. 2003; Smith et al. 2008; Waddington et al. 2010), but
76 rather by the interaction of genotype and environment (Chapman et al. 2007; Hughes
77 and Boomsma 2007; Schwander et al. 2010). Surprisingly, however, we still have only a
78 limited understanding of how environmental conditions can drive the specialization of
79 different individuals. It is well established that social insect colonies vary in their
80 frequency distributions of morphological phenotypes or behavioural phenotype profiles
81 (Yang et al. 2004; Wray et al. 2011; Chapman et al. 2011; Scharf et al. 2012; Pinter-
82 Wollman et al. 2012; Gordon et al. 2013; Jandt et al. 2014; Wiernasz et al. 2014; Wills
83 et al. 2014). However, our understanding of the environmental conditions generating
84 such intercolony variation is still limited (Hui and Pinter-Wollman 2014; Keiser et al.
85 2014; Jandt et al. 2014).

86 Honey bees appear to show differing behavioural phenotypes corresponding to
87 length of disturbance, with short-term disturbance causing an upregulation in aggression
88 (Couvillon et al. 2008), but long-term disturbance decreasing aggressive responses
89 (Rittschof and Robinson 2013). Environmental factors, such as competition, or indeed
90 predation, are also thought to be important in morphological phenotype production in
91 other eusocial insects such as polyembryonic wasp, termite and social aphid species

92 which show a morphologically specialized soldier caste (Crespi 1992; Shiba0 1998;
93 Harvey et al. 2000; Shingleton and Foster 2000; Thorne et al. 2003; Smith et al. 2010).
94 In one of the only direct experimental investigations in ants, Passera et al. (1996)
95 demonstrated that exposure to volatiles from non-nestmate potential competitors caused
96 colonies of *Pheidole pallidula* ants to produce more soldiers within a remarkably short
97 seven week period. However, the environmental stimuli that interact with genotype to
98 produce different morphological or behavioural phenotypes in other social insects are
99 unknown.

100 Here we test experimentally the effect of a controlled environmental stimulus of
101 threat-disturbance on morphological and behavioural phenotypes in phenotypically
102 plastic *Atta* leaf-cutting ant societies. *Atta* are one of the most polymorphic of all ant
103 species, with larger workers including specialised soldiers playing the primary role in
104 defending their colonies against threats (Wilson 1980; Whitehouse and Jaffe 1996;
105 Hernández et al. 2002; Hölldobler and Wilson 2010). We test whether repeated threat
106 disturbance leads to colonies adjusting their behavioural phenotype, production of
107 morphological phenotypes, or both. We also explore if other traits are affected by such
108 threat-disturbance.

109

110 **Materials and methods**

111 Immature *Atta colombica* colonies, approximately 12 months old, were collected from
112 Gamboa, Panama in May 2013. At this age colonies are too small to produce soldiers
113 (Weber 1972). All colonies were kept in a controlled environment room at the
114 University of Sussex at $80 \pm 5\%$ relative humidity, $26 \pm 2^\circ\text{C}$ and a 12:12 h light/dark

115 cycle, and fed twice weekly on privet leaves (*Ligustrum* spp.) placed in a foraging pot
116 (100 mm x 80 mm x 60 mm), with water provided ad libitum. The mutualistic fungus
117 gardens were housed in plastic boxes (115 mm x 75 mm x 75 mm) covered with a
118 flower pot to maintain a dark and humid environment. The 13 colonies were randomly
119 assigned to either a threat disturbance (6 colonies) or control treatment (7 colonies)
120 group. The colonies were of similar size at the start of the experiment (mean \pm s.e.
121 volume of fungus garden: threat-disturbance colonies 107 ± 23.9 ml and control
122 colonies 156 ± 39 ml) and did not differ significantly in size throughout the experiment
123 (fig. S3). At the start of the experiment no soldiers or large workers (> 2 mm head
124 width) were observed in any of the colonies. Colonies undergoing the threat disturbance
125 treatment had their fungus gardens exposed by removing the flower pot and plastic box
126 lid (85 mm depth) for 2 min. Preliminary trials suggested that this exposure protocol
127 produced a maximal alarm response (increased activity and mandible gaping from
128 workers indicating a response to a threat) from the ants within the 2 min period. The
129 disturbance was carried out on 4-5 days per week for 17 months, while control colonies
130 were not disturbed in this way. Exposure of the vulnerable fungus garden and brood in
131 this way would only occur in nature during a vertebrate predation attempt (such as by
132 armadillos) and, regardless of cause, would represent a serious threat to colony survival,
133 stimulating a dramatic defensive response in leaf-cutting ant colonies in nature (Wilson
134 1980; Whitehouse and Jaffe 1996; Rao 2000). The long 17 month period gave colonies
135 ample time to alter the production of morphological phenotypes. Furthermore, given the
136 development time in *Atta* is about 2 months (Weber 1972) and that Passera et al (1996)
137 found changes in caste ratios after only 7 weeks, any changes in morphological
138 phenotype should be present after this time. At the end of the disturbance period, the

139 morphological and behavioural phenotypes of colonies were determined. After the end
140 of this long-term experiment we also carried out a shorter disturbance experiment to test
141 at a finer-scale how quickly colonies could upregulate and down-regulate their
142 behavioural responses.

143

144 1. Alteration of morphological phenotype

145 To determine if threat-disturbance resulted in colonies producing larger individuals, the
146 50 largest workers from each colony after the 17 month disturbance period were
147 photographed dorsally using a Canon EOS 350d dSLR camera and Canon EF 100 mm
148 f/2.8 Macro lens under constant lighting conditions. Images were imported into Image J
149 and the size of each individual was quantified by measuring the width of the head
150 between the eyes, a commonly used measurement of size in ants including *Atta* (Wilson
151 1980; Hölldobler and Wilson 1990; Hughes and Goulson 2001; Holman et al. 2011).
152 The colonies were too young and small to produce soldiers in significant numbers, but
153 we also counted any soldiers present to compare the numbers of soldiers between
154 treatment and control colonies.

155

156 2. Alteration of behavioural phenotype

157 During the last month of the long-term disturbance, assays were carried out to compare
158 behavioural phenotypes of colonies that had either been disturbed, or not, in order to see
159 if threat disturbance affected the responsiveness of ants to threats, we carried out a
160 mandible opening response (MOR) assay (Guerrieri and D'Ettore 2008; Norman et al.
161 2014). Ants were chilled on ice until immobile and then harnessed in 0.2 ml pipette tips
162 (Starlab, Bucks, UK), cut at the apex through which the ant's head was passed and

163 secured with a thin strip of masking tape. Ants were left for 2 h in the harness to
164 acclimatize before being assayed. Three threat treatments were tested in a random order
165 on each ant: a freshly freeze-killed nestmate, a freshly-killed non-nestmate
166 (*Acromyrmex echinator*) or a burst of carbon dioxide. The latter treatment has been
167 used previously as specific stimulus for sampling defensive workers in *Atta* colonies by
168 simulating a vertebrate predation threat (Wilson 1980; Hölldobler and Wilson 2010).
169 The stimulus ants or carbon dioxide burst were gently placed in contact with an antenna
170 of the focal ant for 10 s, and the response of the focal ant recorded as either opening its
171 mandibles for > 1s (a positive MOR), or not responding (Norman et al. 2014). For focal
172 ants that showed a positive MOR, the duration of the response was also recorded. For
173 each colony, this assay was carried out for randomly selected soldiers (> 3mm head
174 width), large workers (2-3 mm head width), medium-sized workers (1.2-2.0 mm head
175 width), and small workers (< 1.2 mm head width) to test if specific castes responded
176 differently to defensive stimuli. Five individuals of each caste were tested in each
177 colony, or as many as the colony had for colonies which had very few large workers.

178 To determine the phototaxis of ants, workers were placed individually in a 90
179 mm Petri dish, half of which had been covered with black tape (Norman and Hughes
180 2016). The ants were allowed to acclimatize for 5 min and then filmed for the
181 subsequent 10 min. The proportion of time spent in the light half of the Petri dish was
182 recorded for each individual. This was repeated for 120 ants from threat-disturbed
183 colonies and 140 ants from control colonies (20 per colony in both cases), using
184 randomly selected medium-sized and medium-aged external workers. Worker age
185 correlates positively with a darkening of the cuticular colouration (Armitage and
186 Boomsma 2010), therefore medium-aged ants could be distinguished by their

187 colouration. To see if threat disturbance affected the aggressiveness of ants, individual
188 ants were carefully touched on the head with the tip of a toothpick, similar to
189 Pamminger et al. (2014). The reaction of the ant was ranked (0 = ignore, 1 = antennate,
190 2 = gape mandibles in a threat response, 3 = bite). This was repeated for 120 ants from
191 the disturbed colonies and 140 ants from the control colonies (20 ants per colony in both
192 cases), using randomly selected medium-sized and medium-aged external workers to
193 control for any differences between castes in aggression. Assays were carried out in the
194 order listed above during the final month of the 17 month disturbance. Ants were
195 returned to the colony after the assays with at least 5 days occurring between assays.
196 Given the number of workers per colony (ca. 5000, of which ca. 3,000 would be
197 medias, for colonies of the size used here (Weber 1972)), and that at least 5 days was
198 left between assays, the likelihood of resampling the same ant for multiple assays was
199 very low.

200

201 3. Potential effects on other traits

202 In order to explore whether changes in the behavioural or morphological phenotypes of
203 individuals in response to disturbance might affect other traits in ways that could be
204 potentially negative, we compared the foraging rate, individual immunity, and brood
205 care propensity of ants from threat-disturbed and control colonies. With the exception of
206 the immunity assays, ants were returned to their colonies after use; given the number of
207 workers per colony and that at least 5 days occurred between assays, it was unlikely that
208 ants were resampled for multiple assays.

209 Foraging rate was quantified four days following the last feed. The foraging pot
210 of each colony was filled with leaves and the initial mass of the pot recorded. Each pot

211 was then placed back with its respective colony for 1 h, after which the ants within the
212 pots were removed and counted, and the pots reweighed to determine the proportion of
213 leaf material that had been foraged during the 1 h foraging period. This was carried out
214 once for each colony.

215 To quantify brood care propensity, individual ants were placed in a 90 mm Petri
216 dish with a randomly selected nestmate larva, allowed to acclimatize for 5 min and then
217 filmed for 10 min. The proportion of time spent interacting with larvae during the 10
218 min period was recorded. This was repeated with 20 medium-sized and medium-aged
219 external workers from each colony. Using external workers avoided the disruption to
220 the fungus chamber that sampling within-nest workers would have involved, and
221 external workers have been shown previously in many ant species to pick up brood
222 found outside of the nest and transport it back into the colony (Robinson et al. 2012;
223 Tragust et al. 2013).

224 To determine the effect on individual-level immunity, we measured levels of the
225 phenoloxidase (PO) and prophenoloxidase (PPO) immune enzymes in haemolymph.
226 Haemolymph samples of 1 µl were collected from individual, freeze-killed ants using a
227 calibrated, pulled glass capillary inserted under the cuticle of the thorax. Haemolymph
228 was diluted 1:40 in ice-cold sodium cacodylate/CaCl₂ buffer (0.01 M Na-Cac, 0.005 M
229 CaCl₂), flash frozen in liquid nitrogen and stored at -80°C to disrupt haemocyte
230 membranes and release cellular PPO. All reactions were prepared in 96-well plates on
231 ice. 15 µL of diluted sample was placed in an individual well, together with 5 µL of
232 distilled water for PO reactions or 5 µL of the activation agent alpha-chymotrypsin
233 (5mg mL⁻¹, in distilled water; Sigma Aldrich™) for PPO reactions. Samples were then
234 incubated for 5 min at room temperature. To start the reaction 35 µL of L-DOPA (4 mg

235 mL⁻¹ in distilled water; prepared freshly and protected from light, Sigma Aldrich™) was
236 added to each well and the plate placed in a Molecular Devices VersaMax micro-plate
237 reader. Temperature was set to 30°C and the absorbance of each sample at 492 nm was
238 measured every 15 s over a period of 45 min using SoftMax Pro software. For each
239 sample the enzyme activity was calculated at the maximum slope (V_{max}) in the linear
240 phase of the reaction (usually 200-1000 s after the start of the reaction). Each plate had
241 a control well, which contained only buffer and no sample, and all controls displayed
242 essentially no enzyme activity during the reaction (<0.1 mOD min⁻¹). Two technical
243 replicates were carried out of each reaction and all samples where the reaction curved
244 showed irregularities were excluded, leaving measurements of PO and PPO for 58 and
245 67 ants from disturbed colonies, and 50 and 63 ants from control colonies, respectively.

246

247 4. Speed of behavioural plasticity

248 In order to see how quickly *Atta colombica* colonies altered their behavioural phenotype
249 to disturbance we carried out a finer-scale threat disturbance experiment. Four months
250 after the end of the long-term experiment (the length of at least one generation of adult
251 workers (Weber 1972), the remaining seven control colonies were split into equally
252 sized subcolonies (ca. 500ml fungus per subcolony) that were randomly assigned to
253 either short-term disturbance or control group. Colonies were monogynous so the
254 queen-right sub-colony was randomly assigned between treatments. Disturbed
255 subcolonies were disturbed in the same way as in the long-term experiment but for a
256 period of only 2 weeks, with the behavioural phenotype of colonies being determined
257 using the phototactic (N = 126 per treatment group; 18 ants per subcolony), aggression
258 (N = 140 per treatment group; 20 ants per subcolony) and MOR assays using two

259 stimuli treatment of a nestmate and a non-nestmate (N = 126 and N = 136 for the threat-
260 disturbed and control treatment groups respectively; 18-20 ants per subcolony).

261 Colonies were then left undisturbed for a period of 2 weeks after which the assays were
262 repeated with the same numbers of individuals in order to determine if colonies would
263 then down-regulate behaviours to match their prevailing environmental conditions.

264

265 Statistical analyses

266 The size and behaviours of ants were compared between threat-disturbed and control
267 colonies using generalized linear mixed models (GLMM), which included colony-of-
268 origin as a random factor. The head widths of ants, length of MOR, phototaxis, brood
269 care and proportion of leaves harvested in the foraging assays were compared using
270 models with gamma distributions and log link function, while the propensities of ants to
271 exhibit a MOR or aggressive response were compared with a binomial distribution and
272 logit link function, and aggression scores using a multinomial distribution and probit
273 link function. The number of ants in each foraging pot in the foraging assay was
274 analysed using a Poisson distribution with a log link function. Levels of PO and PPO
275 were both log transformed to ensure normality and analysed in a GLMM with a
276 Gaussian distribution and identity link function. Colony size, measured as volume of
277 fungus garden, was included as a covariate in all models to control for variation in
278 colony sizes. Best fitting models were selected by comparison of AIC values.

279 Overdispersion was checked for in all cases by calculating a dispersion parameter and
280 none of the models were overdispersed. Nonsignificant interaction terms were removed
281 stepwise in all cases to obtain the minimum adequate models. All statistics were
282 performed in SPSS (v.20 SPSS Inc., Chicago, IL, USA).

283

284

285 **Results**

286 Long-term disturbance

287 There was no significant difference in the size of the largest 50 workers in the threat-
288 disturbed and control colonies ($F_{1, 648} = 0.665$; $P = 0.415$; Fig. 1a). The number of
289 soldiers produced was minimal over the 17 month experimental period (4 and 8 from all
290 threat-disturbed and control colonies respectively). There were no differences between
291 treatment and control colonies in the numbers of large workers or soldiers in them over
292 the last six months (Fig. S4), and no indication anecdotally of differences before that
293 either. However, there was an effect of the disturbance treatment on the behaviour of
294 ants in the colonies. Ants from the threat-disturbed colonies spent significantly more
295 time in the lightened half of a Petri-dish compared to ants from control colonies ($F_{1, 258}$
296 $= 17.09$; $P < 0.001$; Fig. 1b). In the MOR assay, ants from the threat-disturbed colonies
297 were also significantly more threat responsive compared to those from the control
298 colonies ($F_{1, 719} = 4.15$; $P = 0.042$; Fig. 1c), and individuals that gaped their mandibles
299 did so for significantly longer ($F_{1, 182} = 6.42$; $P = 0.012$; Fig. 1d). Overall, all ants from
300 both treatment groups, showed significantly different responses between the three
301 stimuli in both MOR propensity and duration ($F_{2, 719} = 17.1$ $P < 0.001$; Fig. S1a; $F_{2, 182} =$
302 16.7 ; $P < 0.001$; Fig. S1b, respectively). Size of the focal ant showed no significant
303 relationship with either propensity or duration of MOR response ($F_{3, 719} = 1.08$, $P =$
304 0.36 , and $F_{3, 182} = 0.99$, $P = 0.4$, respectively). In the aggression assay, ants from the
305 threat-disturbed colonies did not show a difference in propensity to be aggressive
306 compared to ants from the control colonies ($F_{1, 128} = 0.634$; $P = 0.427$; Fig. 1f), but

307 when they did show an aggressive response, they showed a significantly higher
308 aggression score ($F_{2, 256} = 3.39$; $P = 0.035$; Fig. 1e). Colony size (volume of fungus)
309 showed no relationships with the size of workers, the propensity or duration of MORs,
310 phototaxis or aggression ($F_{1, 257} = 0.912$, $P = 0.340$; $F_{1, 962} = 0.001$, $P = 0.983$; $F_{1, 321} =$
311 0.758 , $P = 0.385$; $F_{1, 258} = 0.546$, $P = 0.461$; $F_{2, 253} = 0.250$, $P = 0.779$, respectively).

312 In the assays exploring potential effects on other traits, there was no difference
313 between the threat-disturbed and control colonies in the number of ants in the foraging
314 pots after 1 h ($F_{1, 11} = 0.172$; $P = 0.686$; Fig. 2a), but the threat-disturbed colonies
315 nevertheless harvested significantly less leaf material than the control colonies during
316 the 1 h period ($F_{1, 11} = 31.8$; $P < 0.001$; Fig. 2b). Colony size showed no significant
317 relationship with either the number of ants in the foraging pot, nor (marginally) on the
318 amount of leaf material they harvested ($F_{1, 10} = 2.16$; $P = 0.172$; $F_{1, 10} = 4.67$, $P = 0.056$,
319 respectively). There was no significant effect of disturbance on the immunocompetence
320 of ants in terms of either PO or PPO activity ($F_{2, 105} = 0.165$; $P = 0.848$, and $F_{2, 126} =$
321 0.144 ; $P = 0.866$; Fig. 2c), nor on the propensity of ants to pick up brood ($F_{1, 248} =$
322 0.076 ; $P = 0.784$; Fig. 2d). There was no significant difference in size of colonies at the
323 end of the experiment ($F_{1, 11} = 0.20$; $P = 0.663$; mean \pm s.e. size of threat-disturbance
324 and control colonies were 1087 ± 182 ml and 1176 ± 120 ml of fungus garden,
325 respectively), or over the course of the experiment (fig. S3).

326

327 Speed of behavioural plasticity

328 After two weeks of disturbance, ants from disturbed subcolonies spent significantly
329 more time in the light half of a Petri dish and were significantly more threat responsive
330 than ants from control colonies ($F_{1, 250} = 6.36$, $P = 0.012$, Fig. 3a and $F_{1, 527} = 20.6$, P

331 <0.001, Fig. 3c, respectively). They were also significantly more aggressive than ants
332 from control colonies, both in the propensity to show an aggressive response ($F_{1, 278} =$
333 14.15; $P < 0.001$; Fig. 3e) and in the aggressiveness of responses ($F_{2, 184} = 3.19$; $P =$
334 0.044; Fig. 3g). Two weeks after this short-term disturbance had ended, there was no
335 difference between ants from disturbed and control colonies in their threat response
336 behaviour ($F_{1, 530} = 1.91$; $P = 0.179$; Fig. 3d), propensity to be aggressive ($F_{1, 278} =$
337 0.758; $P = 0.385$; Fig. 3f) or aggression score ($F_{1, 176} = 0.671$; $P = 0.414$; Fig. 3h), and
338 ants from disturbed colonies spent less, not more, time in the light half of a Petri-dish
339 ($F_{1, 250} = 7.98$; $P = 0.005$; Fig. 3b).

340

341

342 **Discussion**

343 Repeated threat disturbance of colonies over a prolonged period did not affect the
344 investment by small leaf-cutting ant colonies into morphological phenotypes, but it did
345 alter the behavioural phenotypes of colonies. Ants from disturbed colonies were
346 significantly more threat responsive, aggressive and phototactic than ants from control
347 colonies, and this change in behavioural phenotype occurred after as little as two weeks
348 of threat disturbance, showing how rapidly social insect colonies can behaviourally
349 buffer themselves in the face of environmental perturbation.

350 The lack of any effect of frequent, and quite substantial, nest disturbances over
351 such a prolonged period of time (17 months) on the production of soldiers or the size of
352 large workers is at first sight surprising. Colonies of many ant species, including *Atta*
353 leaf-cutting ants, show considerable variation within and between populations in the
354 frequency distributions of morphological phenotypes (Davidson 1978; Oster and Wilson

1978; Wetterer 1995; Yang et al. 2004; Hölldobler and Wilson 2010; Wills et al. 2014), and it seems plausible that such intercolony variation may at least in part be a response to environmental conditions. Pheidole ant colonies have been shown experimentally to produce more soldiers within only seven weeks when under greater perceived threat from competitors (Passera et al. 1996). It is unlikely that the 17 month duration of disturbance in the experiment here was insufficient for a shift in caste investment given that the development time in *Atta* is about two months (Weber 1972), and that the disruption of morphological phenotypes can produce a change in allocation after eight weeks in *Acromyrmex* leaf-cutting ants (Hughes and Boomsma 2007). It is also unlikely that the leaf-cutting ant colonies studied here were not genetically capable of producing larger workers or soldiers, given the relatively high intracolony genetic diversity and genotypic variation in size propensity shown by this species (Helmkamp et al. 2008; Evison and Hughes 2011; Holman et al. 2011). The lack of an alteration to threat disturbance in the morphological phenotype distributions of colonies indicates that some other factor or constraint outweighed the stimulus from the disturbance. The exact cause is unknown, but while all colonies were healthy and old enough to produce larger workers, they were relatively small (Weber 1972). The production of larger phenotypes, particularly soldiers, will require the investment of substantially more resources than smaller workers (Oster and Wilson 1978; Segers et al. 2015). That resource limitation prevented the colonies from increasing their production of larger defensive individuals is therefore one possible explanation for the results.

Although constraints such as resource limitation may therefore limit the ability of social insect colonies to alter their morphological phenotypes, insect societies have other routes to phenotypic plasticity available to them and in this case showed an

379 alteration of their behavioural phenotype. The results from the MOR assay indicate that
380 all castes upregulate their individual-level threat responsiveness in response to colony
381 threat disturbance rather than this response being limited to certain castes. Furthermore,
382 medium sized workers also showed an upregulation of aggression, phototaxis and threat
383 response behaviour (Chapman et al. 2011; Bengston and Dornhaus 2014). This
384 behavioural flexibility, particularly where aggressive or defensive behaviours are
385 involved, could therefore offer a more adaptable and plastic alternative to a costly
386 investment in a morphological defensive phenotype (West-Eberhard 1989; Tufto 2000;
387 Sih et al. 2004). The short-term disturbance experiment showed that the behavioural
388 upregulation was dynamic, being upregulated after only two weeks of disturbance, and
389 down-regulated again with two weeks of the disturbance ending. For most variables,
390 two weeks after the disturbance had ended the behaviour of disturbed colonies was
391 downregulated to levels similar to those of control colonies, although phototaxis
392 appeared to be downregulated further. This highlights the highly plastic nature of
393 behavioural phenotypes in social insect colonies as well as the exceptional capabilities
394 of colonies to behaviourally buffer themselves in response to environmental disturbance
395 (Robinson 1992; Pamminger et al. 2011; Gordon et al. 2013; Yan et al. 2014).

396 In contrast to some other studies (Rittschof and Robinson 2013), the behavioural
397 upregulation showed no evidence of habituation, with the level of upregulation after 17
398 months being very similar to that after two weeks. Anecdotally there was no evidence of
399 morphological habituation either, with the number of large workers remaining small in
400 both treatment groups over the course of the experiment (figure S4). The lack of
401 habituation may perhaps be due to the severity of the disturbance in the experiment, and
402 of the predator threat that exposure of the fungus garden would indicate in nature (Rao

403 2000). Interestingly, there was some evidence from our limited assays that the change in
404 behavioural phenotype may affect other traits, with workers from threat-disturbed
405 colonies harvesting less material than those from control colonies during the brief 1 h
406 foraging assay, in spite of the same number of ants having been recruited to the food.
407 There was no difference in the size of fungus gardens of threat-disturbed and control
408 colonies over the course of the experiment, showing that any effect on foraging rate did
409 not have an effect on colony growth in the competitor-free, food-rich environment of
410 the experiment. It would therefore be interesting to see whether threat-disturbed
411 colonies have lower foraging rate over a longer time period and whether this would
412 impact colony growth either when competitors are present or when food is more
413 transiently available.

414 This study highlights that changes in behavioural phenotype may offer a more
415 rapid and flexible alternative to changes in morphological phenotypes. Social organisms
416 are particularly interesting in this regard because phenotypes can be expressed at both
417 individual and group levels (Korb and Heinze 2004; Chapman et al. 2011; Dornhaus et
418 al. 2012), and further investigation of the dynamic relationships between phenotypes
419 and environmental cues is likely to be very useful in elucidating the evolution and
420 dynamics of phenotypic plasticity.

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611

612 **Figure legends**

613

614 **Fig. 1** The mean \pm s.e. morphological and behavioural phenotypes of *Atta* leaf-cutting
615 ant workers from threat-disturbed colonies (grey) and control colonies (white): A) head
616 width of the 50 largest workers in each colony, B) proportion of time spent in the light
617 half of a half-blackened Petri-dish, C) proportion of positive mandible opening
618 responses (MORs) to threat stimuli, D) length of MOR to three threat stimuli (different
619 letters indicate significantly different responses between treatment stimuli), E)
620 aggression score (ranging from 0 = no aggression to 3 = bite), and F) proportion of
621 aggressive interactions. Colonies either received a substantial threat-disturbance every
622 week for 17 months or were not disturbed in this way (control colonies). Asterisks
623 indicate a significant difference between threat-disturbed and control colonies (* = $P <$
624 0.05, ** = $P <$ 0.01, *** = $P <$ 0.001)

625

626 **Fig. 2** The mean \pm s.e. effects of threat disturbance on: A) number of ants present in a
627 foraging pot after 1 h, B) proportion of leaf material harvested after 1 h, C) activity of
628 the phenyloxidase (PO) and prophenyloxidase (PPO) immune enzymes, and D) the
629 proportion of time ants spent showing brood care behaviours over a 10 min period.
630 Colonies either received a substantial threat-disturbance every week for 17 months or
631 were not disturbed in this way (control colonies). Asterisks indicate a significant
632 difference between threat-disturbed and control colonies ($P <$ 0.001)

633

634 **Fig. 3** The mean \pm s.e. behavioural phenotypes of *Atta* leaf-cutting ant workers from
635 threat disturbed colonies (grey bars) and control colonies (white bars) after short term
636 disturbance. Left hand graphs (A, C, E & G) indicate behavioural assays after two

637 weeks of threat disturbance. Right hand graphs indicate (B, D, F & H) indicate
638 behavioural phenotypes two weeks after disturbance had ended. Figures A) and B) show
639 proportion of time in the light half of a half-blackened Petri-dish, C) and D) the
640 proportion of positive mandible opening responses (MORs) to threat stimuli (pooled
641 responses to nestmates and non-nestmates), and E) and F) the average proportion of
642 aggressive interactions and G) and H) the average aggression score. Colonies either
643 received a substantial threat-disturbance every day for two weeks or were not disturbed
644 in this way. Asterisks indicate a significant difference between threat-disturbed and
645 control colonies (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$) while different letters
646 above columns in C and D indicate behavioural stimuli which differed significantly
647 from one another in pairwise comparisons

648