

1 **Early developmental processes limit socially mediated phenotypic plasticity in**
2 **an ant**

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20 ABSTRACT

21 Caste determination in social insects has long been considered to exemplify socially
22 mediated phenotypic plasticity: young larvae can develop into queens or workers
23 depending on the social environment. However, recent studies have challenged this
24 view by showing that, in some species, larval development can be biased early by
25 factors such as larval genotype. We analysed this issue in the ant species
26 *Aphaenogaster senilis*. First, we found that the probability that a larva develops into a
27 queen or a worker varies consistently among colonies. Next, we conducted a cross-
28 fostering experiment in which larvae from colonies with relatively low queen production
29 were transferred to colonies with relatively high queen production and vice versa. The
30 results show a strong significant interaction between early determination and worker
31 control of larval caste fate. Therefore, our study shows that socially mediated
32 phenotypic plasticity is limited by processes occurring at an early developmental stage
33 that possibly include direct or indirect genetic effects or non-genetic maternal effects.

34 Keywords: Social behavior – Caste development – Phenotypic plasticity – Colony level
35 selection

Significance statement

Colonies of ants are composed of two castes of females with different morphologies and tasks. The queens specialize in reproduction whereas the workers are sterile and contribute to domestic tasks such as food gathering and colony defence. For decades, the difference between workers and queens has been thought to stem from nutritional and other environmental differences during the larval stage. We indeed show marked differences between colonies in the capacity of workers to rear new queens. However, worker control on the destiny of diploid larvae is constrained by precocious larval pre-determination. These precocious effects possibly include direct or indirect genetic effects or non-genetic maternal effects. Overall, our study shows that colony-level

resource allocation to the production of new queens is the result of complex interactions between adults and larvae.

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38 INTRODUCTION

39 Phenotypic plasticity is an important phenomenon with broad implications for
40 organismal ecology and evolution (West-Eberhard 1989; Pigliucci 2001). In many
41 plants and animals, the biotic or abiotic environment in which an individual develops
42 conditions its phenotype. A particular case occurs in social animals, in which
43 phenotypic plasticity can be mediated by the social environment (Rodd et al. 1997;
44 Ramm & Stockley 2009; Dingemanse and Araya-Ajoy 2015). This is well exemplified
45 by caste determination in social insects. Hence, in many species, like the honeybee
46 *Apis mellifera*, bumblebees, and various ant species including *Aphaenogaster senilis*,
47 diploid larvae are totipotent up until a certain stage and will then develop into queens or
48 workers depending on social cues. Several lines of evidence suggest that larval
49 development is partly controlled by adult workers that provide a different nutrition
50 (particularly proteins) to the larvae that develop into queens (Pereboom et al. 2003;
51 Kamakura 2011; Caut et al. 2014 – see also Linksvayer 2011). Moreover, workers are
52 often inhibited from raising new queens whenever they perceive the pheromones
53 produced by their fertile queen (Goulson 2003; Boulay et al. 2007, 2009; Seeley 2009).

54 In contrast with this view, recent studies have shown that, in some species,
55 larval caste fate is determined very early on, thus limiting phenotypic plasticity and
56 worker control of larval development (Schwander et al. 2010). Queen-laid eggs may be
57 predisposed to follow certain developmental pathways due to hormonal effects (e.g.
58 *Passera* et al. 1980; Libbrecht et al. 2013). In *Pogonomyrmex*, the eggs laid by queens
59 after a period of hibernation have a greater chance of yielding queens (Schwander et
60 al. 2008). This phenomenon is mediated by an increase of queen juvenile hormone
61 that enhances the accumulation of vitellogenin in the eggs, thus increasing the
62 probability of yielding queens (Libbrecht et al., 2013). In addition, direct genetic
63 influences that range from plastic genotypes that are biased toward queen or worker
64 development (Hughes and Boomsma 2008) to strict genetic determination (Julian et al.

65 2002; Leniaud et al. 2012; Schwander and Keller 2012) may constrain caste fate. In
66 *Pogonomyrmex* ants and Argentine ants, some combinations of maternal and paternal
67 genotypes give rise to larvae that have a greater probability of becoming queens than
68 do other combinations (Schwander and Keller, 2008; Libbrecht et al 2011). Finally,
69 both direct and indirect genetic effects (i.e. effects of maternal and worker genotypes)
70 may influence larval development. Using a cross-fostering experiment Linksvayer
71 (2006) reached the conclusion that larval caste fate depends on larval genotypes (i.e.
72 direct genetic effect) as well as on maternal and worker genotypes (i.e. indirect genetic
73 effects) in *Temnothorax* species. Interactions between brood and worker genotypes
74 also induce differences in adult worker size in *Temnothorax* species (Linksvayer, 2007)
75 and in the clonal ant *Ceraphys biroi* (Teseo et al. 2014).

76 In spite of the above-mentioned results, our understanding of caste
77 determination in ants and other social hymenopterans is still limited by the reduced
78 number of model systems on which these studies have been conducted. Here, we
79 analysed the relative importance of worker control and early determination on colony-
80 level queen production in the ant *A. senilis*. This strictly monogynous species is well
81 suited for such study because queen production is highly predictable in captive
82 condition. Most diploid brood develop into workers when a queen is present in the
83 colony. However, 1st and 2nd instar larvae can develop into queens if the current queen
84 is removed or dies (Ledoux 1976, 1981; Boulay et al., 2009). This phenomenon, which
85 relies on pheromonal communication among workers, larvae, and queens, allows the
86 adjustment of resource allocation to queen production to be fine-tuned (Ruel et al.,
87 2012, 2013). Because of monogyny, producing a few queens from the current brood
88 guarantees that one of them will succeed her mother; however, a massive allocation of
89 resources to queen production would just increase local resource competition. Indeed,
90 queenless colonies rarely produce more than 3-4 queens (Chéron et al. 2009),
91 irrespective of the number of totipotent larvae that are present (Villalta et al., 2015).

92 In order to analyse the early developmental processes that limit caste
93 differentiation, we first determined whether the proportion of larvae that develop into
94 queens is a phenotypic trait that is expressed at the colony level. If so, it should vary
95 more among colonies than among replicates within colonies. We also tested whether it
96 co-varied with demographic variables and the season (late winter vs. late spring).
97 These variables were shown to affect queen production in several species (e.g.
98 *Passera* 1980; Tschinkel 1993; Schwander et al. 2008). Second, we conducted a
99 cross-fostering experiment in order to determine whether the production of new queens
100 was a trait deriving from worker state, larval state or the interaction between both. To
101 that end, workers and larvae were swapped between colonies that demonstrated the
102 highest and lowest levels of queen production. The results provide clear evidence that
103 early caste determination limits worker control and larval developmental plasticity.

104 MATERIALS AND METHODS

105 *Aphaenogaster senilis* colonies are composed of 200-3,000 workers (Boulay et al.
106 2007). In 2012, we collected 16 large colonies from a single population near
107 Aznalcázar (south-western Spain) in late winter (March) and then again in late spring
108 (June). We immediately counted the number of workers per colony (hereafter colony
109 size) and weighed the queens (± 0.1 mg). Three experimental groups of 200 workers
110 and 20 recently hatched 1st instar larvae (Fig. 1A) were then taken from each colony
111 (total: 96 experimental groups). In a colony, 1st instar larvae are kept with the eggs and
112 receive similar treatment (pers. obs.). Workers have been hypothesised to feed the
113 larvae through proctodeal food (sensu Torossian 1958; Villalta et al. 2014). In the
114 experiments, the 1st instar larvae were preferred over eggs because the caste is not yet
115 determined at this stage (Boulay et al. 2009) and egg incubation lasts longer than the
116 1st larval stage (30 days vs. 10 days, respectively). Hence, starting the experiment with
117 the egg stage would have induced considerable asynchrony in the development of the

118 larvae. Moreover, eggs die at a much higher rate than do larvae—larvae frequently
119 cannibalize fertile eggs although this phenomenon does not affect their development
120 (Ruel et al. 2012). Over a period of seven weeks, we quantified the total number of
121 female larvae (i.e. workers + queens, FEM, hereafter), and then calculated the
122 proportion of queens among the female pupae ($P(Q)$, hereafter). The groups were fed
123 sliced mealworms twice a week, given water *ad libitum*, and kept at 25°C. At the end of
124 the experiment, we counted the number of remaining workers to estimate the survival
125 rate.

126 The eight late-winter and late-spring colonies with the highest average $P(Q)$
127 were classified as H_{Winter} and H_{Spring} , respectively. The remaining colonies were
128 classified as L_{Winter} and L_{Spring} (for “low” queen production). We then conducted a cross-
129 fostering experiment (Fig. 1B) in which batches of 20 newly hatched 1st instar larvae
130 from each H_{Winter} colony were transferred into groups of 200 workers from a different
131 H_{Winter} colony (hereafter H-H') or from an L_{Winter} colony (H-L). Conversely, larvae from
132 each L_{Winter} colony were transferred into groups of workers from a different L_{Winter} colony
133 (L-L') or from an H_{Winter} colony (L-H). In addition, groups of workers from each colony
134 received larvae from their own colonies (H-same and L-same). The same experiment
135 was carried out using the late-spring colonies. The cross-fostering experiments were
136 conducted in two sets that took place approximately 10 weeks after the colonies had
137 been collected. Overall there were 14 replicates for H-control, H-H and H-L conditions
138 and 15 replicates for L-control, L-H and LL conditions. Three colonies were eliminated
139 either because the queen had died or the number of workers was too small. As above,
140 we monitored larval development for seven weeks. To minimize observer bias, a
141 blinded method was used so that the person who monitored larval development had no
142 knowledge of the treatment.

143 All data were analysed using R (R Core Team). Within-colony consistency in
144 FEM and $P(Q)$ was tested by calculating the intraclass correlation coefficient (ICC)

145 using the irr package (Gamer et al. 2012). Moreover, variation in FEM and P(Q) was
146 analysed by fitting general linear mixed effects (glm) models using the nlme package.
147 For the two response variables, the variance components were extracted by including
148 the colony of origin as a random factor. The explanatory variables were collection
149 season (late winter vs. late spring), the size of the mother colony, queen mass, and
150 worker mortality rate. FEM was log-transformed (Bartlett 1947) while P(Q) was arc-sin
151 transformed (Anscombe 1948; Zar 1999). Models were simplified by removing non-
152 significant parameters (Crawley 2007). We then compared the number of workers in H
153 and L colonies by means of a glm.

154 The data from the cross-fostering experiment were analysed by fitting glms
155 using the results from the H-H', H-L, L-L', and L-H groups only. We tested the effects of
156 worker type (H or L), larva type (H or L), and their interaction on FEM (log-transformed)
157 and P(Q) (arc-sin transformed) using the lme4 package which allows including more
158 than one random factor in the models. The season, worker colony and larva colony
159 were each included as random factors, and worker survival rate was included as a
160 covariate. Finally, we ran glms to compare P(Q) (arc-sin transformed) among the H-H',
161 H-same, L-L', and L-same experimental groups to test the effect of larval adoption by
162 foreign workers on queen production.

163 RESULTS

164 In the first experiment, the groups composed of 200 workers and 20 1st instar larvae
165 produced, on average, 11.75 ± 1.04 (mean \pm SE) female pupae (FEM). A mean of just
166 0.875 ± 0.155 males were produced per group, which indicates that $37\% \pm 2\%$ of the
167 larvae that were initially provided died before the pupa stage (no larva remained at the
168 end of the seven weeks of monitoring). Queens represented $8.16\% \pm 1.02\%$ of the
169 females produced. The ICC analysis indicated that P(Q) was consistent within colonies
170 (Fig. 2: $F_{31, 64} = 4.63$, $P < 0.0001$) while FEM was not (Fig. 2: $F_{15, 32} = 1.37$, $P = 0.146$).

171 This result was confirmed by the variance components extracted from the linear mixed
172 effects models. Hence, while variation among colonies explained 51% of the total
173 variance for P(Q), it explained only 12% of the total variance for FEM (Fig. 2). The
174 remaining amount of variance was explained by differences among groups within
175 colonies. Of the fixed effects that we were able to test, only the size of the mother
176 colony was significantly correlated with P(Q) (Figure S1; Table 1; $F_{1,30} = 4.67$, $P =$
177 0.039 after removing non significant parameters). None of the factors were significantly
178 correlated with FEM (Table 1). Based on the previous results we identified H_{Winter} and
179 H_{Spring} as the colonies with the highest P(Q) in the winter and spring sessions,
180 respectively (range: 0.07-0.15 and 0.10-0.19, respectively). H colonies contained
181 significantly more workers than L colonies ($F_{1,30} = 4.79$, $P = 0.037$) but there was no
182 significant difference between seasons ($F_{1,29} = 0.61$, $P = 0.612$); the interaction
183 between colony type and season was not significant either ($F_{1,28} = 2.69$, $P = 0.112$).

184 In the cross-fostering experiment, FEM was significantly greater in the groups
185 composed of H larvae than in those composed of L larvae (Table 2; 12.52 ± 0.67 vs.
186 9.86 ± 0.75 , respectively; $F_{1,15} = 8.788$, $P = 0.009$). However, FEM depended neither
187 on worker type, on the interaction between worker and larvae type, nor on worker
188 survival (Table2). P(Q) was significantly affected by worker type, larvae type and their
189 interaction (Fig. 3; Table2). Hence, H workers produced a higher proportion of queens
190 than did L workers only when they were provided with H larvae (Fig. 3; $F_{1,15} = 5.122$, P
191 $= 0.038$). When they were provided with L and H workers produced a similar proportion
192 of queens. P(Q) did not vary significantly with worker survival ($F_{1,52} = 0.884$, $P = 0.351$)
193 and was not significantly different between the H-H' and H-same groups (Fig. 3; $t = -$
194 0.098 , $df = 2.42$, $P = 0.93$) or the L-L' and L-same groups ($t = -3.411$, $df = 2.36$, $P =$
195 0.060), which indicates that being reared by foreign workers did not significantly bias
196 larval fate.

197 DISCUSSION

198 Our results show that groups of workers and larvae taken from some colonies
199 consistently produced a higher proportion of queens than did others. The propensity to
200 produce new queens may therefore be considered a phenotypic trait that is expressed
201 at the colony level. What could explain colony-level variation in queen production? The
202 cross-fostering experiment reveals it likely results from a significant interaction between
203 the larvae and the adult workers that nurse them.

204 In the first experiment, in which we analysed the consistency in larval
205 development among colonies, $P(Q)$ was correlated with the size of the mother colony.
206 Large colonies are known to be more likely to produce new queens than small colonies
207 in many species of social insects (i.e. Brian 1957; Torossian 1958; Passera 1980;
208 Tschinkel 1993; Iwanishi et al. 2007; Boulay et al. 2007; Alaux et al. 2005; Smith et al.
209 2010). However, in our experiment, group size was identical in all the groups so that
210 queen production was not directly affected by the number of workers. Large colonies
211 differ from small colonies in many other ways that may affect queen production. For
212 example, the correlation between $P(Q)$ and colony size may be due to large colonies
213 being headed by relatively older queens as in bumblebees (Alaux et al. 2005). Even
214 though colony fission dissociates colony growth from queen aging compared to species
215 with independent colony-founding, newly formed colonies are likely composed of a
216 young queen and a smaller number of workers than mature colonies (Chéron et al.
217 2011; Amor et al. 2011). Another hypothesis may be that queen production is fuelled
218 by worker physiological condition (fat content), which increases with colony size as in
219 *Solenopsis invicta* and *Pogonomyrmex badius* (Tschinkel 1993, 1998). We might thus
220 expect the propensity to produce new queens to be a plastic trait that tends to increase
221 as a colony ages. The fact that there was no difference between colonies collected in
222 late winter and late spring contrasts with results obtained for *Pogonomyrmex*

223 (Schwander et al. 2008) but fits with field observations showing that, in the study
224 population, new queens can be produced from April to November (Boulay et al. 2007).

225 Previous studies have shown that requeening in *A. senilis* is possible only if 1st
226 or 2nd instar larvae are present in the colony when the mother queen is removed or dies
227 (Boulay et al. 2009). However, the cross-fostering experiment suggests that the
228 development of 1st instar larvae into queens is constrained by the social environment.
229 While the probability that H colonies produced new queens depended on the type of
230 workers that cared for them, the probability that L-colony produced queens was low,
231 irrespective of worker type. This important result is similar to what has been shown in
232 *Temnothorax* ants (Linksvayer 2006). Several mechanisms can explain it. First, given
233 the elevated mortality rate of the larvae, we cannot exclude the possibility that the low
234 production of queens in L colonies resulted from higher mortality of queen oriented
235 larvae than in H colonies. However, the result of previous studies showing that queen
236 production is independent of larval number and survival do not support this hypothesis
237 (Ruel et al. 2012; Villalta et al. 2015). Second, phenomena such as direct and indirect
238 genetic effects and non-genetic maternal effects can account for early developmental
239 determination. These effects are not mutually exclusive and may be additive or non-
240 additive. A genetic effect, whereby some larval genotypes have a higher probability of
241 becoming a queen, was suggested in *A. rudis* based on isozyme data (Crozier 1973).
242 Although more and more examples are being discovered (Schwander and Keller
243 2012), we still have no evidence that such genetic effect operates in *A. senilis*. Queens
244 may also control the development of their offspring through non-genetic maternal
245 effects. For example, in *Pheidole pallidula*, queen hormones are known to determine
246 brood sexualisation (Passera 1980; Suzzoni et al. 1980). In *Pogonomyrmex*, queen
247 hormones determine larval caste fate by affecting the accumulation of vitellogenin in
248 the egg (Libbrecht et al. 2013). The role of queen hormones on brood sexualisation
249 may prevail in *A. senilis*. Hence, the queen's endocrine titre, which is likely to vary with

250 age (and thus co-vary with colony size) may have a major effect on larval caste fate.
251 The treatment received at the egg and early larval developmental stages may further
252 constrain caste fate. Workers care for eggs and 1st instar larvae by maintaining them
253 together in a specific part of the nest and in close contact with the queen. The quality
254 and quantity of care at this very early stage may therefore affect further development.

255 Even though the probability of becoming a queen is partly determined at an
256 early stage, workers exercise a great deal of control over larval caste fate at later
257 developmental stages. Larvae that become queens are bigger than those that become
258 workers and must therefore receive more protein-enriched food (Smith and Suarez
259 2010; Caut et al. 2014). The rate at which workers nourish larvae may also influence
260 caste differentiation, as is the case in some bumblebee species (Pereboom et al.
261 2003). More generally, the amount and quality of care a larva receives shapes its
262 development. Interestingly, behavioural variation in care among colonies has been
263 reported in several species of ants (Chapman et al. 2011; Jandt et al. 2014; Blight et al.
264 2015). So far, the fitness consequences thereof are poorly understood (Moldmeier et
265 al. 2012), but they may be related to worker capacity to rear new queens.

266 To conclude, although the social environment influences many aspects of
267 animals' phenotypes, including behaviour, morphology, and physiology, our results
268 clearly show that phenomena occurring at very early stages can constrain socially
269 mediated phenotypic plasticity. These phenomena may include direct genetic effects
270 (i.e. directly caused by the larval genotype) and indirect genetic effects (i.e. caused
271 from the maternal or sib-social genotypes) as well as non-genetic effects (Linksvayer
272 2006). Ants constitute important model systems for the study of socially mediated
273 phenotypic plasticity but further studies are needed to disentangle the physiological
274 and behavioural factors affecting caste determination.

275

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281

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420

421 FIGURE CAPTIONS

422

423 Figure 1: A) Larval development in *A. senilis*—Larvae are bipotent until larva stage II;

424 B) experimental design used in the cross-fostering experiment. H and L refer to

425 colonies with high and low levels of queen production in experiment 1, respectively.

426

427 Figure 2: Within-colony consistency in queen production. Intraclass Correlation

428 Coefficient (triangles \pm 95 confidence intervals) and percentage of variance explained

429 by differences among colonies (blue) and among groups within colonies (red) for the

430 number of female pupae (FEM) and the proportion of queens among the females

431 produced (P(Q)).

432

433 Figure 3: Proportion of queens produced (P(Q); mean \pm SE) by groups of L and H

434 workers provided with L (red symbols) or H (blue symbols) larvae from different

435 colonies. The green symbols stand for groups of L and H workers provided with larvae

436 from their own colonies. H and L refer to individuals from colonies with high and low

437 levels of queen production, respectively.

438

439 **Table 1:** Result of the linear mixed effect models analyzing the main effects of colony
 440 size, queen mass, worker survival and the season of collection on the number of
 441 female produced (FEM) and the proportion of queens among females (P(Q)).

	FEM			P(Q)		
	Df (num, den)	F	P	Df (num, den)	F	P
Colony size	1, 28	0.005	0.943	1, 28	4.237	0.049
Queen mass	1, 28	0.533	0.471	1, 28	0.185	0.671
Worker survival	1, 63	0.585	0.448	1, 63	0.105	0.747
Season	1, 28	0.005	0.449	1, 28	0.108	0.745

442

443

444 **Table 2:** Result of the linear mixed effect models analyzing the main effects of worker
 445 type, larvae type, their interaction and worker survival on the number of female
 446 produced (FEM) and the proportion of queens among females (P(Q)).

	FEM			P(Q)		
	Df (num, den)	F	P	Df (num, den)	F	P
Worker type	1, 25	0.585	0.461	1, 22	7.724	0.011
Larvae type	1, 16	8.788	0.009	1, 23	22.303	0.001
Worker survival	1, 53	2.430	0.125	1, 52	0.884	0.351
Worker x larvae type	1, 14	3.138	0.098	1, 15	5.122	0.038

447

448