

1 **Social coercion of larval development in an ant**

2 Irene Villalta<sup>ab\*</sup>, Sévrine Antille<sup>c</sup>, Fernando Amor<sup>a</sup>, Xim Cerdá<sup>a</sup> and Raphaël Boulay<sup>b</sup>

3

4 <sup>a</sup> Estación Biológica de Doñana, Consejo Superior de Investigaciones Cientificas, Seville,  
5 Spain.

6 <sup>b</sup> Institut de Recherche sur la Biologie de l’Insecte, Université François Rabelais, Tours,  
7 France.

8 <sup>c</sup> Department of Ecology and Evolution, University of Lausanne, Switzerland.

9

10 \*Author Correspondence: irenevillaltaalonso@gmail.com

11

12

13

14

15

16 *Keywords:* aggressions, ant, *Aphaenogaster senilis*, caste productions, cuticular

17 hydrocarbon, social policing

18

## 19 **Introduction**

20 Colonies of eusocial insects are characterized by elevated cooperation and division of labor  
21 whereby a large number of workers forego their own direct reproduction to participate in  
22 domestic tasks such as collecting food and rearing the brood (Wilson 1971; Heinze et al.  
23 1994). Yet, the apparent harmony of the colony is often punctuated of conflicts over who will  
24 reproduce and who will not (Chapuisat and Keller 1999). In some primitive ant species, for  
25 example, all young females are born with a spermatheca and could potentially mate. Why do  
26 then most individuals commit themselves to become helpers? It was shown that only  
27 behaviorally dominant females reproduce whereas challengers are coerced by the group to  
28 remain steriles (Peeters and Higashi 1989; Peeters et al. 1992; Ito 1993; Monnin and  
29 Peeters 1999; Tay and Crozier 2000; Monnin and Ratnieks 2001). In more evolved species,  
30 diploid brood sexualization gives rise to morphologically distinct worker and queen casts.  
31 This limits reproductive conflicts among adults since the worker has lost the capacity to mate.  
32 However, conflicts remain among larvae which are bipotent and can develop into either casts  
33 (Bourke and Ratnieks 1999). Hence, any diploid larva may expect a higher direct fitness by  
34 developing into a queen rather than into a worker. Adults, in contrast, should prefer a large  
35 amount of colony resources being allocated to the production of workers to guarantee colony  
36 survival and growth.

37 Conflicts over larval caste fate may be particularly exacerbated in species that  
38 disperse through colony fission (Pamilo 1991; Boulay et al. 2007). During fission, new  
39 colonies are formed when a young queen abandons the mother colony with a group of  
40 workers (Cronin et al. 2013). Thus, queens are protected by workers throughout their life  
41 and, from the adult perspective, the development of a very low proportion of diploid larvae  
42 into queens is sufficient to ensure colony reproduction. This, in turn, enhances competition  
43 between larvae to become a queen suggesting a complex social regulation of queen  
44 production may evolve (Bourke and Ratnieks 1999). For workers, one way of regulating the  
45 production of new queens is by controlling larval food intake, thus limiting larval growth

46 (Bonavita-Cougourdan and Passera 1978). This is supported by the observation that  
47 honeybee queen larvae are provisioned with specific proteins that are contained in the royal  
48 jelly (Kamakura 2011; Wolschin et al. 2011) and that queen-destined ant larvae receive a  
49 protein-enriched diet compared to worker-destined larvae (Smith et al. 2008; Smith and  
50 Suarez 2010; Caut et al. 2013). In addition of controlling food, the adults may control larvae  
51 sexualization by physically aggressing cheating larvae as in *Myrmica* species and  
52 *Harpegnathos saltator* (Brian 1973; Penick and Liebig 2012).

53         The role of the queen in the policing of larvae is controversial (Vargo and Passera  
54 1991). However, the implication of her pheromones in the regulation of sexual production is  
55 known in several species of ants and bees. These chemicals can directly inhibit larvae  
56 sexualization (Vargo and Passera 1991; Cnaani et al. 1997; Bourke and Ratnieks 1999) or  
57 indirectly modify worker behavior, provoking the policing of undesired queen larvae (Boulay  
58 et al. 2007). Finally, the queen may use chemical marks from her Dufour (or other) gland to  
59 mark challengers and encourage punishment by workers (Gilley 2001; Monnin et al. 2002;  
60 van Zweden and d’Ettorre 2010).

61         In the present study, we analyzed the social regulation of queen production in the  
62 colonies of the ant *Aphaenogaster senilis*, a common species that disperses by colony  
63 fission. While diploid larvae are bipotent until the second larval instar, in natural conditions,  
64 most larvae develop into workers (Boulay et al. 2009). Young queens are produced in very  
65 small numbers and only in rare occasions, when the colony is large enough to permit fission  
66 or if the fertile queen dies (Boulay et al. 2007; Boulay et al. 2009). In queenless condition, the  
67 production of new queens increases non-linearly with the number of bipotent larvae and  
68 never exceeds 6-7 individuals (Ruel et al. 2012; Villalta et al. 2015). To explain this, it has  
69 been hypothesized that the first queen-destined larvae could signal their presence in order to  
70 enforce younger larvae development into workers. We conducted bioassays to determine if  
71 workers are able to police undesired queen larvae and if this depended on the perception of  
72 queen pheromones. Then, we tested if queen-destined larvae had a negative effect on  
73 younger larvae sexualization. Finally, we performed chemical analyses to compare the

74 cuticular hydrocarbon (HCs) profiles of queen- and worker-destined larvae in order to  
75 determine if these compounds could serve to signal larval caste fate. Many authors point at  
76 cuticular HCs as the main fertility signal in adults (Liebig et al. 2000; Cuvillier-Hot et al. 2001;  
77 Endler et al. 2004; Le Conte and Hefetz 2008) but their role in the recognition of larval caste  
78 fate is still unknown.

## 79 **MATERIALS AND METHODS**

### 80 *Model system, colony collection and maintenance in the laboratory*

81 *Aphaenogaster senilis* is a monandrous and monogynous species that is distributed over  
82 most of the Iberian Peninsula. Source colonies were collected in the Doñana National Park,  
83 southwestern Spain, between June 2007 and September 2012. In the laboratory, they were  
84 housed in artificial nests composed of an open plastic box (28 x 18 x 11 cm), the internal wall  
85 of which was lined with Fluon to prevent escapes. The ants were allowed to shelter in five 20  
86 cm long test tubes filled in their first third with water retained by a cotton plug. They were  
87 maintained under controlled conditions (27°C, 50% humidity) and fed three times a week with  
88 sliced mealworms, *Tenebrio molitor*.

### 89 *Experiment 1: Worker and queen control on larvae sexualization.*

90 In order to obtain as many queen larvae as required to conduct the experiment, we first  
91 created two production groups from each of 25 collected colonies. Each production group  
92 was composed of 200 workers and 20 1<sup>st</sup> instar larvae. The production groups were checked  
93 daily and as soon as a queen-destined 3<sup>rd</sup> instar larva was detected she was immediately  
94 introduced together with a worker-destined 3<sup>rd</sup> instar larva in an observation group of 50  
95 workers from their respective source colony. The observation groups were placed in Petri  
96 dishes (9 cm diameter) in one of the following resident conditions: 1) the source colony  
97 queen was present and free to move in the dish (queenright, QR, n = 15); 2) the source  
98 colony queen was present in the dish but she was encaged in a 2 mL tube, the cap of which  
99 had been replaced by a wire mesh allowing chemical volatile exchange with the workers

100 (Qen, n = 12); 3) the workers were in contact with the queen in the source colony until the  
101 beginning of the experiment but not during the observation period (queenless, QL, n = 15); 4)  
102 the workers were already queenless for two weeks at the beginning of the observation period  
103 (QL2w, n = 9). Humidity was maintained in the Petri dishes by a moisten cotton plug.

104 Behavioral observations started 30 minutes after larval introduction and lasted 30  
105 minutes. Every minute, we recorded the number of ants that interacted with each larva and,  
106 among these interactions, the number of aggressive behaviors (bites and threats, i.e.  
107 opening the mandibles with the gaster bent below the thorax). We then monitored both  
108 larvae survival during 5 days. At the end of each experiment, all the adult ants, including the  
109 queen, were returned their respective source colony. Each of the 25 source colonies was  
110 used successively in one to four experiment corresponding to different resident conditions  
111 and with at least a month apart.

112 *Experiment 2: Effect of already determined larvae on younger larvae sexualization.*

113 As previously, we started by creating two production groups of 200 workers and 20 1<sup>st</sup> instar  
114 larvae from each of 20 recently collected source colonies. Larval development into worker or  
115 queen was monitored daily during three weeks. Once the first 3<sup>rd</sup> instar queen-destined larva  
116 was detected in the production group, we immediately created two experimental groups from  
117 the same source colonies, both composed of 200 workers and 20 1<sup>st</sup> instar larvae. One  
118 experimental group received the 3<sup>rd</sup> instar queen-destined larva while the other group  
119 received a 3<sup>rd</sup> instar worker-destined larva from either production groups from the same  
120 source colony. We then continued supplying both experimental groups with the same  
121 numbers of queen-destined or worker-destined 3<sup>rd</sup> instar larvae as they appeared in the  
122 production groups during the following 7 days. The total number of supplied larvae varied  
123 from 1 to 6 depending on the availability of queen-destined larvae in the production groups  
124 (the availability in worker larvae was not limiting). On the 8<sup>th</sup> day, all the 3<sup>rd</sup> instar supplied  
125 larvae were removed (some of them had already reached the pupal stage) and we then

126 monitored the development of the 1<sup>st</sup> instar larvae during the following 6 weeks at each  
127 experimental group.

### 128 *Chemical analyses*

129 Twelve worker-destined and 12 queen-destined larvae were selected from 12 different  
130 queenless colonies. The larvae were first photographed using a stereomicroscope equipped  
131 of digital camera. We used imageJ free software to measure the length (a) and width (c) of  
132 each larva. Both measurements were considered as the two semi axis of a prolate spheroid,  
133 the surface of which was calculated as:

$$134 S = 2 \pi a^2 (1 + a c/e \arcsin(e)) \text{ with } e = \sqrt{1 - a^2/c^2} \text{ (Beyer 1987)}$$

135 The larvae were killed and were immersed in 100  $\mu$ L of hexane during one hour to extract  
136 their cuticular HCs. We then removed the corpses and evaporated the samples under a  
137 nitrogen flow. The extracts were stored at 4°C until their injection into a gas chromatograph  
138 (GC 2010 Shimadzu) equipped with a Flame Ionization Detector. The temperature program  
139 ran from 130°C to 240°C at 15°C min<sup>-1</sup>, and then from 240°C to 300°C at 3°C min<sup>-1</sup>. Each  
140 sample received 20ng of C20 as an internal standard. Once all the samples were run in GC-  
141 FID, they were pooled by caste and injected into a Perkin Elmer TurboMass GC mass  
142 spectrometer with similar chromatographic conditions for peaks identification.

### 143 *Data analysis*

144 All statistical analyses were performed using the R software v. 2.14.1 (R Core Team 2012).  
145 The behavioral results of the first experiment (the average number of contacts and  
146 aggressions between the workers and the introduced worker-destined or queen-destined 3<sup>rd</sup>-  
147 instar larvae) were analyzed using linear mixed-effect models (Lmer; lme4 package) where  
148 the resident condition, the caste of the introduced larva and the interaction were fixed effects  
149 while the colony of origin was a random effect. Larval mortality rate after 5 days was  
150 compared between treatments by fitting a generalized linear model with the binomial family.  
151 Although this kind of model better fits to binary data such as death vs survival, they do not

152 provide accurate estimation of the main effects. Only the differences between levels are  
153 given in the result section.

154 For experiment 2, a linear mixed-effect model was fitted to test the effect of the  
155 number and caste of larvae added during the first week and the interaction between them on  
156 the total production of female pupae during the remaining six weeks of experiment. The  
157 colony of origin was included in the model as a random effect. A similar model was fitted  
158 using the proportion of queens among the produced female pupae as a response variable,  
159 while using the same fixed and random effects. In addition, we tested for the effect of the  
160 colony of origin by comparing both the total numbers of produced females and the proportion  
161 of queens in the groups receiving worker-destined and queen-destined 3<sup>rd</sup> larvae for the  
162 same source colony by means of the Pearson correlation test.

163 The amount of each HC was calculated based on the peak area compared with the  
164 internal standard and reported on the body surface. The Wilcoxon test was used to compare  
165 each compounds quantities in worker-destined and queen-destined larvae. The Holm-  
166 Bonferroni correction was used to control for family-wise multiple comparisons. Given that 10  
167 null hypotheses were tested, the lowest  $\alpha$  was set to 0.005 instead of 0.05.

## 168 **RESULTS**

### 169 *Experiment 1: Worker and queen control on larval sexualization.*

170 Worker policing towards undesired queen-destined larvae was contingent on the presence of  
171 the queen. The average number of contacts between the workers and the introduced larvae  
172 was a function of the resident condition (Fig. 1a; lmer:  $F_{3,91} = 31.40$ ,  $P < 0.0001$ ), the caste of  
173 the introduced larva ( $F_{1,78} = 38.10$ ,  $P < 0.0001$ ) and their interaction ( $F_{3,78} = 3.84$ ,  $P = 0.012$ ).  
174 Hence, there were overall more worker contacts directed towards queen-destined larvae  
175 than to worker-destined larvae in the QR, Qen and QL groups but not in the QL2w groups. A  
176 similar pattern occurred for aggressive interactions (Fig. 1b; lmer: resident condition:  $F_{3,87} =$   
177  $2.41$ ,  $P = 0.072$ ; intruder caste:  $F_{1,64} = 25.01$ ,  $P < 0.0001$ ; interaction:  $F_{3,64} = 2.99$ ,  $P = 0.037$ ).  
178 Although QR, Qen and QL workers behaved more aggressively towards queen-destined

179 larvae than to worker-destined larvae, there was no significant difference for QL2w ants (Fig.  
180 1b). Contacts and aggressions between QL2w workers and larvae were significantly lower  
181 than in other conditions irrespective of larval caste fate (Fig. 1a,b).

182 The relatively high aggression level observed in the QR group towards queen-  
183 destined larvae led to a high level of mortality compared to worker-destined larvae (Fig. 2;  
184 glmer:  $z = 3.513$ ,  $P = 0.0004$ ). However, in the other resident conditions including the Qen  
185 and QL conditions, there was no such significant difference between the mortality rate of  
186 worker and queen larvae. QL2w queen-destined larvae displayed similar survival rates to  
187 Qen and QL conditions (Fig. 2).

#### 188 *Experiment 2: Effect of already determined larvae on younger larvae sexualization.*

189 The results of the second experiment provided no evidence of a negative impact of the  
190 presence of queen-destined larvae on young larvae sexualization. Over the seven weeks of  
191 experiment, the number of 1<sup>st</sup> instar larvae that developed into worker or queen pupae was  
192 neither related to the caste nor to the number of the 3<sup>rd</sup> instar larvae that were provided  
193 during the first week (Fig. 3a; lmer: Caste:  $F_{1,18} = 0.04$ ,  $P = 0.85$ ; Number:  $F_{1,18} = 0.003$ ,  $P =$   
194  $0.96$ ). The interaction between the caste and the number of provided larvae was not  
195 significant either (lmer: Caste x Number:  $F_{1,18} = 0.05$ ,  $P = 0.83$ ). Only 7 male pupae were  
196 produced over the whole experiment. The proportion of queen pupae among the females  
197 decreased significantly with the number of 3<sup>rd</sup> instar larvae supplied (Fig. 3b; lmer: Number:  
198  $F_{1,18} = 25.92$ ,  $P > 0.0001$ ) but was not significantly associated with their caste (lmer: Caste:  
199  $F_{1,18} = 4.38$ ,  $P = 0.051$ ). The Caste x Number interaction did not affect significantly the  
200 proportion of queens among the female pupae (lmer: Caste x Number:  $F_{1,18} = 1.79$ ,  $P =$   
201  $0.20$ ).

202 The identity of the mother colony accounted for an important partition of the total  
203 variance in the number of diploid pupae and in the proportion of queens among the females  
204 (70.2% and 60%, respectively). This was also evidenced by the significant correlation  
205 between both the number of females and the proportion of queens that were produced in

206 both experimental groups created from each mother colony (Fig. S1; Pearson correlation:  $t_{18}$   
207 = 4.25,  $P = 0.0004$  and  $t_{18} = 5.95$ ,  $P < 0.0001$ , respectively).

### 208 *Cuticular hydrocarbon analyses.*

209 Both worker and queen larvae were relatively depauperate in HCs. GC-MS analysis revealed  
210 only 10 peaks that corresponded to 11 HCs, ranging from 25 to 30 carbon chain-length  
211 (Table 1). Methyl-branched alkanes represented on average  $37 \pm 4\%$  and  $29 \pm 5\%$  of the  
212 total amount of HCs in queen and worker larvae, respectively. The remaining were linear  
213 alkanes. Dimethyl alkanes were completely absent. On average, queen larvae contained 1.7  
214 times more HCs than worker larvae ( $21.7 \pm 1.4 \text{ ng.larva}^{-1}$  vs  $12.6 \pm 0.8 \text{ ng.larva}^{-1}$ ,  
215 respectively; Wilcoxon test: ). However, when reported to larvae cuticular surface, the  
216 amount of HCs per  $\text{mm}^2$  was not significantly different between queen and worker larvae  
217 ( $5.58 \text{ ng.mm}^{-2}$  vs  $6.59 \text{ ng.mm}^{-2}$ ; Wilcoxon test:  $W = 57$ ,  $P = 0.41$ ). After the Holm-Bonferroni  
218 correction applied to control for family-wise multiple comparisons, no compound had different  
219 amounts per unit of surface between castes (Table1).

## 220 **DISCUSSION**

221 As it is probably the case in many species of social hymenoptera, young larvae of *A. senilis*  
222 are bipotent and can develop into workers or queens depending on environmental  
223 conditions. Theory predicts that an individual larva may expect a greater direct fitness by  
224 developing into queen (Bourke and Ratnieks 1999), so why do most larvae forgo  
225 reproduction to commit themselves to become helpers? Previous results have shown that  
226 workers may coerce larval development by controlling food quality and quantity (Ichinose et  
227 al. 2009). The present study shows that, in addition, workers may police cheating larvae and  
228 that this behavior depends on the perception of queen pheromones. However, in contrast to  
229 what was previously hypothesized, queen-destined larvae do not inhibit younger larvae  
230 sexualization. Finally, chemical analyses did not allow identifying a queen-specific HC profile  
231 at the larval stage.

232 Worker policing has been described as an efficient mechanism of controlling cheaters  
233 in insect societies (Wenseleers et al. 2004). Depending on the species, worker aggressions  
234 were shown to be directed toward individuals that could potentially challenge the  
235 reproductive monopoly of the queen (Kikuta and Tsuji 1999; Monnin and Ratnieks 2001;  
236 Ruel et al. 2013a). The results of experiment 1 show that worker policing also serves as a  
237 mechanism controlling larval development into worker. When a queen was present and free  
238 to move in the experimental Petri dish, the adult workers specifically aggressed the queen  
239 larva but not the worker larva. This led to the death of most of the queen larvae in less than 5  
240 days while the worker larvae survived. In contrast, when the adult workers had been  
241 queenless for two weeks before the beginning of the experiment (QL2w condition), they less  
242 interacted with and aggressed the larvae. As a result, both larvae survived well during 5  
243 days. Queen removal or isolation in a cage (QL and Qen conditions, respectively) had no  
244 short-term effect on workers' behavior: they were still highly aggressive towards queen  
245 larvae during the first hour. However, after 5 days, queen larvae mortality was low in both  
246 experimental conditions and not significantly different from worker larvae mortality. Although  
247 3rd instar larvae did not experience aggression from workers in the QL2w condition, their  
248 mortality rates were similar to those of Qen and QL 3<sup>rd</sup> instar larvae irrespective of their  
249 caste. A first possible explanation for this result is that policing is contingent of the perception  
250 of queen pheromones, the effect of which lasts even after the queen has disappeared (). A  
251 second possible non exclusive explanation is that the queen actively participates to the  
252 policing behavior. We were not able to observe direct aggressive interactions between the  
253 adult queen and the queen larvae, adult queens are known to attack young unmated queens  
254 (Ruel et al. 2013a). They also spray the content of their Dufour gland on adult challengers,  
255 which stimulates aggression by workers (Ruel et al. 2013a). A similar behavior was shown in  
256 other species (Heinze et al. 1994; Monnin and Ratnieks 2001; van Zweden and d'Ettorre  
257 2010; Smith et al. 2012).

258 Both in natural and laboratory conditions, the lack of a queen in a colony of *A. senilis*  
259 stimulates the production of a few new queens (Ruel et al. 2013a; Villalta et al. 2015). The

260 production of only a few young queens after the queen death is adaptive at the colony level  
261 because only one of them is likely to succeed to her mother while the others will be rapidly  
262 eliminated by the workers. Therefore, allocating a lot of resources to the production of many  
263 queens would counterproductive for a colony. However, this tends to increase competition  
264 among bipotent larvae for who will become the next queen. The underlying mechanism is  
265 complex and our results do not support the hypothesis according to which queen-destined  
266 larvae inhibit the development of younger bipotent larvae into queens. However, they  
267 highlight the cost of producing queens with respect to workers: the more 3<sup>rd</sup> instar larvae  
268 were added in the first week of experiment 2, the lower was the proportion of queens among  
269 the produced pupae during the following weeks. In addition, there was a strong effect of the  
270 source colony on the capacity to rear larvae and to produce new queens. This may result  
271 from colony-level behavioral syndromes whereby workers of some colonies are more  
272 efficient in nursing larvae than others (Blight et al. 2015).

273         Several studies have highlighted the role of cuticular HCs in ant communication both  
274 within and between colonies. Within colonies, the queen and her eggs arbor specific HCs  
275 profiles that differ from workers and worker-laid eggs (Endler et al. 2004; Ruel et al. 2013a;  
276 Ruel et al. 2013b). Quiet surprisingly, the larvae possessed very few HCs and no significant  
277 qualitative or quantitative difference could be detected. In particular, specific dimethylalkanes  
278 that abound on adult queens and on their eggs were completely absent on the larvae. In  
279 addition, the total amount of HCs detected on the larvae ( $21.7 \pm 1.4$  ng vs  $12.6 \pm 0.8$  ng, for  
280 queens and workers, respectively) was smaller than on the eggs ( $87 \pm 9$  ng vs  $73 \pm 13$  ng)  
281 which volume is approximately 10 times lower (Ruel et al. 2012). HCs are also involved in  
282 nestmate recognition and their very low concentration on larval cuticle is in line with larval  
283 “chemical insignificance”, that is, the fact that they can easily be exchanged between  
284 colonies without being aggressed (Signorotti et al. 2014). This also suggests that workers are  
285 able to discriminate between worker and queen larvae using other cues, which may include  
286 volatile pheromones, or non chemical signals like the behavior or the morphology.

287           In conclusion, the results of this study clearly show that workers of *A. senilis* are able  
288 to detect and eliminate larvae that would selfishly develop into queens in small queenright  
289 colonies. This phenomenon, which depends on the presence of and contact with the free  
290 queen, is similar in many points to the worker policing observed in primitive queenless  
291 species. Further studies should intend to identify the signals allowing adult workers to detect  
292 and eliminate queen-destined larvae.

293

#### 294 **ACKNOWLEDGEMENTS**

295 We wish to thank Ana Carvajal for helping carry out some of the experiments and Jessica  
296 Pearce for her English editing services. Irene Villalta's contract and this project were funded  
297 by grant number CGL2012-36181 and CGL2009-12472/BOS (from the Spanish Ministry of  
298 Economy and Competitiveness and the FEDER), awarded to Xim Cerdá and Raphaël  
299 Boulay.

300

301

302 REFERENCES

- 303 Beyer WH (1987) CRC Standard Mathematical Tables, 28th ed. CRC Press, Boca Raton,  
304 FL.
- 305 Blight O, Díaz-Mariblanca GA, Cerdá X, Boulay R (2015). A proactive–reactive syndrome  
306 affects group success in an ant species. *Behav Ecol*: (in press)
- 307 Bonavita-Cougourdan A, Passera L (1978). Étude comparative au moyen d'or radio-actif de  
308 l'alimentation des larves d'ouvrières et des larves de reine chez la fourmi *Plagiolepis*  
309 *pygmaea* Latr. *Insectes Soc* 25: 275-287
- 310 Boulay R, Cerda X, Fertin A, Ichinose K, Lenoir A (2009). Brood development into sexual  
311 females depends on the presence of a queen but not on temperature in an ant  
312 dispersing by colony fission, *Aphaenogaster senilis*. *Ecol Entomol* 34: 595-602
- 313 Boulay R, Hefetz A, Cerdá X, Devers S, Francke W, Twele R, Lenoir A (2007). Production of  
314 sexuals in a fission-performing ant: dual effects of queen pheromones and colony  
315 size. *Behav Ecol Sociobiol* 61: 1531-1541
- 316 Bourke AF, Ratnieks FL (1999). Kin conflict over caste determination in social Hymenoptera.  
317 *Behav Ecol Sociobiol* 46: 287-297
- 318 Brian M (1973). Caste control through worker attack in the ant *Myrmica*. *Insectes Soc* 20: 87-  
319 102
- 320 Caut S, Jowers MJ, Cerda X, Boulay RR (2013). Questioning the mutual benefits of  
321 myrmecochory: a stable isotope-based experimental approach. *Ecol Entomol* 38:  
322 390-399
- 323 Chapuisat M, Keller L (1999). Extended family structure in the ant *Formica paralugubris*: the  
324 role of the breeding system. *Behav Ecol Sociobiol* 46: 405-412
- 325 Cnaani J, Borst DW, Huang Z-Y, Robinson GE, Hefetz A (1997). Caste determination in  
326 *Bombus terrestris*: differences in development and rates of JH biosynthesis between  
327 queen and worker larvae. *J Insect Physiol* 43: 373-381
- 328 Cronin AL, Molet M, Doums C, Monnin T, Peeters C (2013). Recurrent evolution of  
329 dependent colony foundation across eusocial insects. *Annu Rev Entomol* 58: 37-55

330 Cuvillier-Hot V, Cobb M, Malosse C, Peeters C (2001). Sex, age and ovarian activity affect  
331 cuticular hydrocarbons in *Diacamma ceylonense*, a queenless ant. *J Insect Physiol*  
332 47: 485-493

333 Ender A, Liebig J, Schmitt T, Parker JE, Jones GR, Schreier P, Hölldobler B (2004). Surface  
334 hydrocarbons of queen eggs regulate worker reproduction in a social insect. *Proc*  
335 *Natl Acad Sci USA* 101: 2945-2950

336 Gilley DC (2001). The behavior of honey bees (*Apis mellifera ligustica*) during queen duels.  
337 *Ethology* 107: 601-622

338 Heinze J, Hölldobler B, Peeters C (1994). Conflict and cooperation in ant societies  
339 *Naturwissenschaften* 81: 489-497. doi 10.1007/s001140050114

340 Ichinose K, Boulay R, Cerdá X, Lenoir A (2009). Influence of queen and diet on nestmate  
341 recognition and cuticular hydrocarbon differentiation in a fission-dispersing ant,  
342 *Aphaenogaster senilis*. *Zool Sci* 26: 681-685

343 Ito F (1993). Social organization in a primitive ponerine ant: queenless reproduction,  
344 dominance hierarchy and functional polygyny in *Amblyopone* sp.(reclinata  
345 group)(Hymenoptera: Formicidae: Ponerinae). *J Nat Hist* 27: 1315-1324

346 Kamakura M (2011). Royalactin induces queen differentiation in honeybees. *Nature* 473:  
347 478-483

348 Kikuta N, Tsuji K (1999). Queen and worker policing in the monogynous and monandrous  
349 ant, *Diacamma* sp. *Behav Ecol Sociobiol* 46: 180-189

350 Le Conte Y, Hefetz A (2008). Primer pheromones in social hymenoptera. *Annu Rev Entomol*  
351 53: 523-542

352 Liebig J, Peeters C, Oldham NJ, Markstädter C, Hölldobler B (2000). Are variations in  
353 cuticular hydrocarbons of queens and workers a reliable signal of fertility in the ant  
354 *Harpegnathos saltator*? *Proc Natl Acad Sci USA* 97: 4124-4131

355 Monnin T, Peeters C (1999). Dominance hierarchy and reproductive conflicts among  
356 subordinates in a monogynous queenless ant. *Behav Ecol* 10: 323-332

357 Monnin T, Ratnieks FL (2001). Policing in queenless ponerine ants. *Behav Ecol Sociobiol* 50:  
358 97-108

359 Monnin T, Ratnieks FL, Jones GR, Beard R (2002). Pretender punishment induced by  
360 chemical signalling in a queenless ant. *Nature* 419: 61-65

361 Pamilo P (1991). Evolution of colony characteristics in social insects. I. Sex allocation. *Am*  
362 *Nat* 137: 83-107

363 Peeters C, Billen J, Hölldobler B (1992). Alternative dominance mechanisms regulating  
364 monogyny in the queenless ant genus *Diacamma*. *Naturwissenschaften* 79: 572-573

365 Peeters C, Higashi S (1989). Reproductive dominance controlled by mutilation in the  
366 queenless ant *Diacamma australe*. *Naturwissenschaften* 76: 177-180

367 Penick CA, Liebig J (2012). Regulation of queen development through worker aggression in  
368 a predatory ant. *Behav Ecol* 23: 992-998

369 R Core Team (2012). R: A language and environment for statistical computing.

370 Ruel C, Cerdá X, Boulay R (2012). Behaviour-mediated group size effect constrains  
371 reproductive decisions in a social insect. *Anim Behav* 84: 853-860

372 Ruel C, Hefetz A, Cerdá X, Boulay R (2013a). Recognition of caste and mating status  
373 maintains monogyny in the ant *Aphaenogaster senilis*. *Behav Ecol Sociobiol* 67:  
374 1295-1305

375 Ruel C, Lenoir A, Cerdá X, Boulay R (2013b). Surface lipids of queen-laid eggs do not  
376 regulate queen production in a fission-performing ant. *Naturwissenschaften* 100: 91-  
377 100

378 Signorotti L, Jaisson P, d'Ettorre P (2014). Larval memory affects adult nest-mate recognition  
379 in the ant *Aphaenogaster senilis*. *Proc R Soc B* 281: 20132579

380 Smith CR, Mutti NS, Jasper WC, Naidu A, Smith CD, Gadau J (2012). Patterns of DNA  
381 methylation in development, division of labor and hybridization in an ant with genetic  
382 caste determination. *PLoS One* 7: e42433-e42433

383 Smith CR, Suarez AV (2010). The trophic ecology of castes in harvester ant colonies. *Funct*  
384 *Ecol* 24: 122-130

385 Smith CR, Toth AL, Suarez AV, Robinson GE (2008). Genetic and genomic analyses of the  
386 division of labour in insect societies. *Nature Reviews Genetics* 9: 735-748

387 Tay WT, Crozier RH (2000). Nestmate interactions and egg-laying behaviour in the  
388 queenless ponerine ant *Rhytidoponera* sp. 12. *Insectes Soc* 47: 133-140

389 van Zweden JS, d’Ettorre P (2010). Nestmate recognition in social insects and the role of  
390 hydrocarbons. *Insect hydrocarbons: biology, biochemistry and chemical ecology* 11:  
391 222-243

392 Vargo EL, Passera L (1991). Pheromonal and behavioral queen control over the production  
393 of gynes in the Argentine ant *Iridomyrmex humilis* (Mayr). *Behav Ecol Sociobiol* 28:  
394 161-169

395 Villalta I, Angulo E, Devers S, Cerdá X, Boulay R (2015). Regulation of worker egg laying by  
396 larvae in a fission-performing ant. *Anim Behav* 106: 149-156

397 Wenseleers T, Helanterä H, Hart A, Ratnieks FL (2004). Worker reproduction and policing in  
398 insect societies: an ESS analysis. *J Evol Biol* 17: 1035-1047

399 Wilson EO (1971) *The Insect Societies*. Harvard, MS.

400 Wolschin F, Mutti NS, Amdam GV (2011). Insulin receptor substrate influences female caste  
401 development in honeybees. *Biol Lett* 7: 112-115

402  
403  
404  
405  
406  
407  
408  
409  
410

---

Compounds	Queens	Workers	P values
-----------	--------	---------	----------

---

C25	0.30 ± 0.06	0.57 ± 0.14	0.14
C26	0.25 ± 0.07	0.26 ± 0.07	0.98
C27	1.09 ± 0.31	1.45 ± 0.42	0.04
3MeC27	0.72 ± 0.21	0.88 ± 0.26	0.71
C28	0.12 ± 0.03	0.02 ± 0.01	0.02
10 + 12MeC28	0.17 ± 0.05	0.20 ± 0.06	0.86
C29	2.09 ± 0.60	2.54 ± 0.73	0.18
11MeC29	0.25 ± 0.07	0.14 ± 0.04	0.09
5MeC29	0.36 ± 0.10	0.31 ± 0.09	0.44
C30	0.24 ± 0.07	0.22 ± 0.06	1.00
Sum	5.58 ± 1.61	6.59 ± 1.90	0.41

411

412 Table 1: HCs composition of queen- and worker-destined larvae. Compounds are ordered by  
 413 retention time. Quantities expressed in ng per larva are means ± SE. The  $\alpha$  threshold of  
 414 significance is 0.005 after the Holm Bonferroni correction.

415

416

417 Figure legends:

418

419 Fig. 1: Contacts (a) and aggressions (b) (mean  $\pm$  SE) between workers and larvae during the  
420 30 min observation period in the different resident conditions. Latin and greek letters denote  
421 significant differences between worker-destined larvae queen-destined larvae, respectively,  
422 across experimental conditions. Stars denote differences between larval castes within  
423 resident conditions. Dashed and white histograms represent groups that received queen and  
424 worker 3rd instar larvae, respectively.

425

426

427

428 Fig 2: Survival probability of worker-destined (white bars) and queen-destined (dashed bars)  
429 larvae (mean  $\pm$  SE) after 5 days in four resident conditions. Greek letters denote significant  
430 differences across experimental conditions for the queen-destined larvae. Stars denote  
431 significant differences between worker and queen destined larvae among resident  
432 conditions.

433

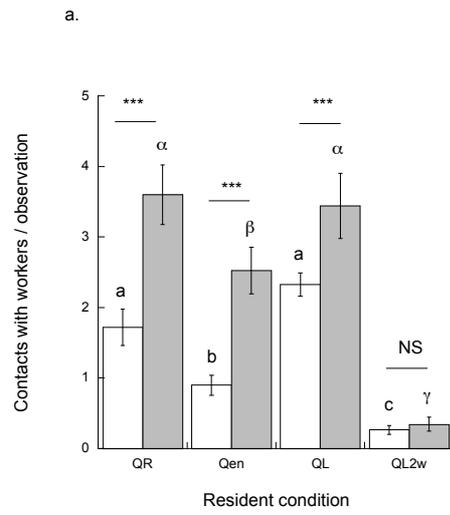
434

435 Fig. 3: Total production of pupae (a) and proportion of queens among the diploid pupae (b)  
436 that developed from 1<sup>st</sup> instar larvae as a function of the number of 3rd instar larvae added  
437 during the first week of experiment. Black symbols / solid line and white symbols / dotted line  
438 represent groups that received queen-destined and worker-destined 3rd instar larvae,  
439 respectively.

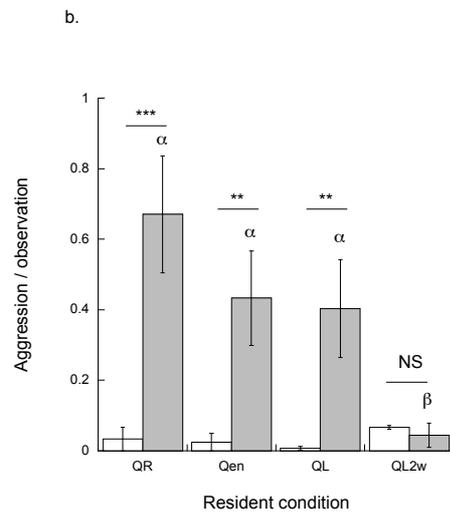
440

441

Figure 1



442



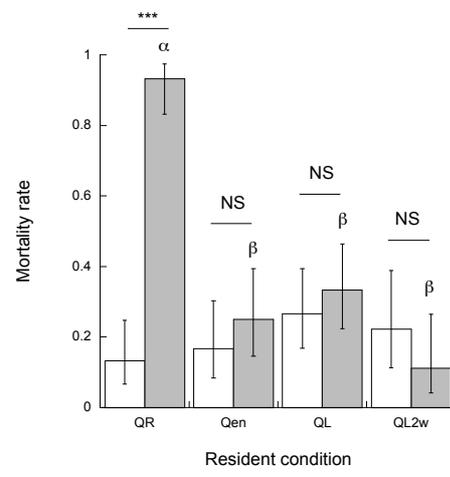
443

444

445

446

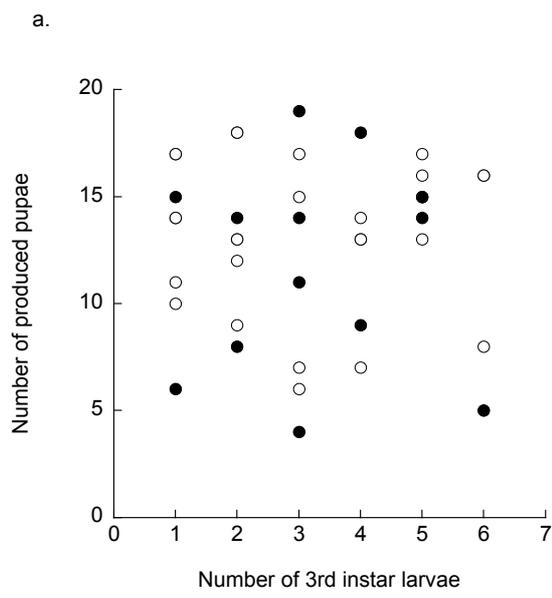
Figure 2



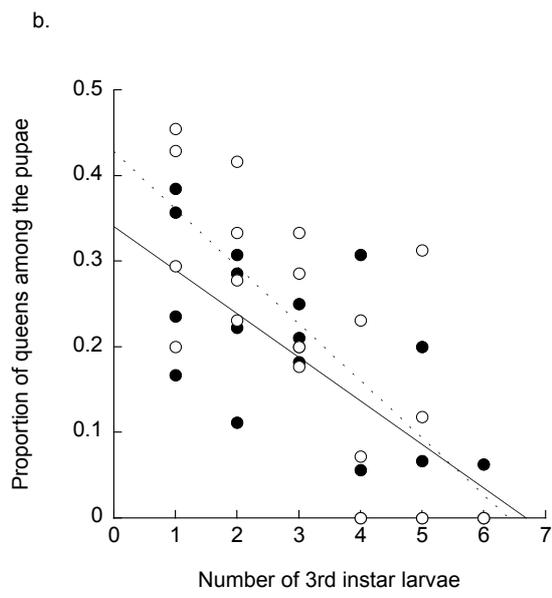
447

448

Figure 3



450

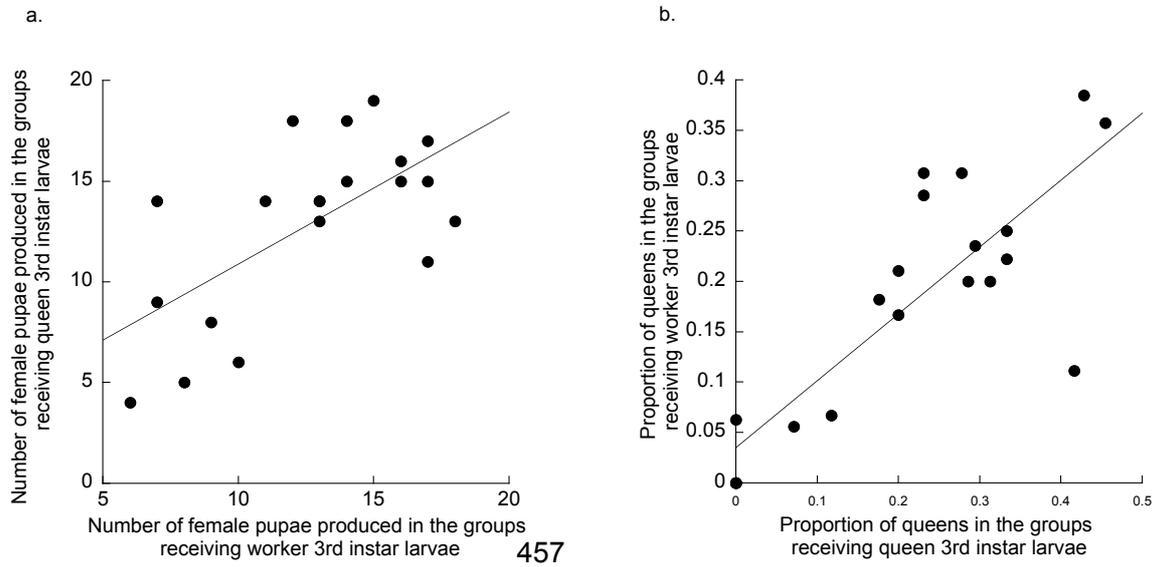


451

452

453 Supplementary material

454 Fig. S1. Relation between the number of females and the proportion of queens among the  
455 females produced in the groups that received worker (x axes) and queen (y axes) larvae  
456 during the first week of experiment for the same source colony.



458

459