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1 **Pre-existing differences in putative fertility signals give workers the upper hand in ant**  
2 **reproductive hierarchies**

3

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18

19 In social groups, competition often gives rise to conflicts, which are regulated through a variety  
20 of mechanisms. In several social insect species, the conflict for male production that takes place  
21 between workers after queen loss, is regulated through the establishment of a reproductive  
22 hierarchy. A recent study of *Neoponera apicalis* showed that workers differ in their fertility  
23 levels in the presence of the queen and proposed that such idiosyncratic differences might  
24 influence access to the top of the hierarchy after queen loss. In this study, we therefore sought  
25 to characterize the influence of the initial heterogeneity in ovarian development and its chemical

26 and behavioural correlates on the establishment of reproductive hierarchies among orphaned  
27 workers, which can only produce males. We monitored the chemical profile before and after  
28 hierarchy establishment in four groups of orphaned workers of *N. apicalis* morph 6. The  
29 analysis of the cuticular profiles showed that tricosane (n-C<sub>23</sub>) was highly correlated with  
30 ovarian development and could consequently act as a fertility signal in this ant. The relative  
31 amount of tricosane on the cuticle, both before and after the establishment of the hierarchy, was  
32 also correlated with the rank achieved within the hierarchy and with the expression of agonistic  
33 behaviours. Thus, our study experimentally shows that idiosyncratic differences in a putative  
34 fertility signal (and therefore presumably in ovarian activity) between workers in the queen's  
35 presence reliably predict the outcome of reproductive conflict after queen loss. We propose that  
36 this signal (together with an increased agonistic motivation of the more fertile workers) could  
37 play a major role in the regulation of dominance/submission behaviours, enabling the most  
38 fertile individuals to rapidly access top ranks and monopolize reproduction, thereby  
39 maximizing the global reproductive success of all colony workers while minimizing the costs  
40 associated with the expression of agonistic behaviour.

41

42 **Key-words:** dominance behaviour, fertility signalling, idiosyncratic difference, ponerine ants,  
43 reproductive hierarchy.

44

45

46 Reproductive hierarchies often appear in social hymenopteran species when the queen of the  
47 colony disappears or her reproductive potential decreases. In most species, workers, although  
48 they cannot mate, maintain an ability to develop their ovaries and lay unfertilized male-destined  
49 eggs (Yagound, 2014). In these species, the establishment of reproductive hierarchies through  
50 ritualized agonistic interactions regulates the overt conflict for male production (Oliveira &

51 Hölldobler, 1990; Heinze et al., 1994). An individual's rank stems from several factors that are  
52 classically described as 'intrinsic' and 'extrinsic' and which are intertwined in a network of  
53 feedback loops. Intrinsic traits refer to the state of each individual (e.g. neuroendocrine titres,  
54 reproductive status and motivation to fight, as well as potential chemical cuticular correlates)  
55 that determine its absolute fighting ability (so-called resource-holding power, or RHP, after  
56 Parker, 1974). Extrinsic factors that play a role in establishing hierarchies include the effects of  
57 past experiences (Rutte et al., 2006) and social environment whose causal role is exerted  
58 through the modification of intrinsic factors, which in turn modify future experiences. These  
59 influences are notably reflected in winner–loser effects where the outcome of an encounter  
60 (victory or defeat) induces changes in the neuroendocrine titres (Hsu et al., 2006), thus  
61 influencing individual behaviour and the outcome of future encounters (Dugatkin & Earley,  
62 2004; Sasaki et al., 2016).

63 In ants, cuticular hydrocarbons (CHCs) are well known for indicating colonial  
64 affiliation, but they also convey more subtle social information about species, sex, caste,  
65 hierarchical status and reproductive status (Greene & Gordon, 2003; Liebig, 2010), thus  
66 constituting unique individual chemical profiles. This chemical signal results from quantitative  
67 or qualitative differences (or both) of endogenous and exogenous origins between one or more  
68 compounds across individuals, castes and colonies (d'Ettorre & Lenoir, 2010). The signal can  
69 therefore allow the recognition of a congener's idiosyncratic characteristics, and many studies  
70 have demonstrated the involvement of CHCs in fertility and/or dominance signalling (Smith et  
71 al., 2009; Holman et al., 2013; Holman et al., 2016; Smith et al., 2015; Abril et al., 2018). The  
72 perception of the signal modifies the behaviour of potential partners (reviewed in Leonhardt et  
73 al., 2016). When it reflects RHP and/or fertility, the signal is thought to be honest, and workers  
74 are accordingly expected to follow their own interests in response to the signal and promote  
75 their inclusive fitness (Keller & Nonacs, 1993; Heinze & d'Ettorre, 2009). Thus, in the case of

76 reproductive hierarchies in a queenless colony, the most fertile worker should be selected to  
77 access the top of the hierarchy and produce males. Selection of the reproductive individuals  
78 stems from a fine balance between direct fitness costs, indirect fitness benefits and relatedness  
79 (Keller & Nonacs, 1993). Yagound et al. (2014) have shown that workers of a Neotropical ant  
80 species, *Neoponera apicalis*, can use CHCs as an index of rank in workers' established  
81 reproductive hierarchies, the quantity of certain compounds functioning as a reliable signal of  
82 both individual ovarian development and social status.

83         Here, we studied the establishment of reproductive hierarchies after queen loss in  
84 workers of *N. apicalis*. In this species, workers cannot mate and therefore are unable to produce  
85 female progeny (Fresneau, 1994). While queenright workers do not usually lay eggs, they start  
86 producing males soon after being orphaned (Dietemann & Peeters, 2000). Because there is no  
87 production of new workers, which would care for the brood or adopt a new queen, males must  
88 be produced rapidly after the queen's death or no nurses will be available. Behavioural  
89 mechanisms exploiting interindividual differences in queen presence for both reproductive  
90 physiology and chemical signalling (Yagound et al., 2015) would allow the rapid establishment  
91 of a reproductive hierarchy after queen loss and hence meet the evolutionary pressure to rapidly  
92 solve the conflict between workers over male production (Dietemann & Peeters, 2000).  
93 Namely, we propose that the most fertile workers could be more motivated to enter the  
94 reproductive race, and that fertility signalling could help resolve the ritualized agonistic  
95 encounters. Such behavioural processes would ensure the most fertile workers lead the  
96 hierarchy, thereby maximizing the inclusive fitness of the whole worker collective (Hamilton,  
97 1964; Keller & Nonacs, 1993). To test our hypothesis, we first correlated variation in cuticular  
98 profiles and ovarian development among workers to determine which compound might be the  
99 putative fertility signal in *N. apicalis* (Liebig et al., 2000; Monnin et al., 1998; Yagound et al.,  
100 2015). We then jointly monitored the development of this compound (as a noninvasive proxy

101 for ovarian development) and of ritualized agonistic behaviours by workers, from queen loss to  
102 the stabilization of the reproductive hierarchy. We predicted that the workers most fertile in the  
103 presence of the queen would be more active during the establishment of the hierarchy and  
104 therefore would access the top ranks and monopolize reproduction.

105

## 106 <H1>Methods

107

### 108 <H2>Ethical note

109 *Neoponera apicalis* is a common ant species in central-south American tropical forests. We  
110 obtained collection permits (No 47615) from the Chico Mendes Institute for Conservation and  
111 Biodiversity (ICMBio/SISBIO) from the Brazilian Ministry of the Environment (MMA). Our  
112 experimental design in the laboratory included the orphaning of four experimental groups of  
113 workers, the labelling and behavioural observation of individual ant workers, the monitoring of  
114 the cuticular compounds and the dissection of workers to record ovary development. Ants were  
115 kept in artificial nests which are commonly used in ant research and in which ants do not show  
116 abnormal or stereotypical behaviour. The whole range of expected behaviour was observed.  
117 Ants were manipulated with soft forceps, which prevent any damage, and marked with paint,  
118 which does not alter their behaviour in the long term. Ants were killed by freezing before  
119 dissection. All these procedures were conducted following the institutional guidelines of animal  
120 welfare of both Brazil and France.

121

### 122 *Ants*

123 We collected 18 colonies of *N. apicalis* in Brazil in November 2016: eight queenless and six  
124 with fewer than 20 workers (1–56 workers, mean 22.1 workers per queenright colony, SD 17.5).  
125 The fact that eight colonies were queenless thus suggests that queenlessness is common in this

126 species. Comparison of hierarchy establishment in the *N. apicalis* species complex showed that  
127 it occurs earlier, and agonistic behaviour is more pronounced, in monogynous species  
128 (Yagound 2014). This suggests that an increased chance of queenlessness selects for hierarchy  
129 establishment mechanisms allowing quick conflict resolution. In this study, we used four  
130 colonies: colony 1 was collected in Marituba, state of Para (1°21'18''S, 48°20'21''W), and  
131 colonies 8, 18 and 20 in Santa Barbara do Para (1°13'36''S, 48°17'43''W). Cytochrome C  
132 oxidase I sequence analysis revealed that our colonies belong to morph 6 of *N. apicalis*  
133 (Yagound et al., n.d.). *Neoponera apicalis* was divided into three morphospecies by Delabie et  
134 al. (2008) based on fine morphological differences in this complex of cryptic species. Ferreira  
135 et al. (2010) defined three additional morphs based on a set of morphological, acoustic,  
136 chemical and genetic data. Yagound (2014) added a seventh morph. Mackay and Mackay  
137 (2010) described morph 5 as *Neoponera cooki*, but the original numeration is kept in order to  
138 be consistent. Colonies were harvested in mid-October 2016 and installed in the laboratory a  
139 week later. The experiment started 2 months after their installation. During this acclimation  
140 period, workers remained with the queen. The ants were housed in plaster nests (18 x 14 cm)  
141 connected to an external environment of the same size. They were maintained at a temperature  
142 of  $25 \pm 2$  °C, a relative humidity of  $50 \pm 10\%$  and a day:night cycle of 12:12 h. Each colony  
143 was fed three times a week with an apple–honey mixture and thawed crickets (*Acheta*  
144 *domestica*), as well as water ad libitum.

145         Based on the study by Yagound et al. (2012), which showed that workers close to the  
146 queen were the first to reproduce at the onset of hierarchical competition, we assumed that the  
147 most fertile workers would stay next to the queen within the nest. We selected and individually  
148 marked 20 workers in the vicinity of the queen in each experimental colony. Each of these  
149 workers received a number label glued on the thorax and two coloured dots (Uni-ball marker).  
150 The chemical profile of ants before orphaning was extracted using SPME (see below). The

151 individuals were then released into the original nest. The following week, 15 of these 20  
152 selected workers were isolated and placed in another artificial nest of the same type, to mimic  
153 an orphaning process. We recorded agonistic interactions, that is, antennal boxing and bites, in  
154 the nest (see Dominance Hierarchy below). Antennal boxing consists of repeated and rapid  
155 strokes of one ant by another with the antennae. This behaviour is typical of many ant species  
156 and is often observed during the establishment of hierarchies. In established hierarchies,  
157 dominant individuals perform antennal boxing against subordinate individuals (Monnin &  
158 Peeters, 1999; Blacher et al., 2010, Yagound et al., 2014). Biting occurs when the individual  
159 uses its mandibles to grip a part of another individual's body. In most instances, biting was  
160 prolonged, thereby immobilizing the other individual with no apparent damage or cuts, and we  
161 consider this behaviour ritualized biting. On the 10th day of the experiment, workers were  
162 frozen for later extraction of their chemical profile after orphaning and measurements of their  
163 ovarian development (Fig. 1). Of the initial 60 ants, 59 survived to this stage.

164

## 165 <H2>*Extraction and analysis of chemical profiles*

166 The individuals' chemical profiles were analysed before they were orphaned and after the  
167 establishment of the reproductive hierarchy. The initial chemical profile (before orphaning) was  
168 obtained by solid-phase microextraction (Monnin et al., 1998). This involved rubbing an SPME  
169 fibre (polydimethylsiloxane 100 µm) on the first segment of the abdomen for 2 min. The fibre  
170 was then desorbed in a Varian 3900 gas chromatograph with flame ionization detection (GC-  
171 FID). The carrier gas used was helium at 1 ml/min, with hydrogen streams at 30 ml/min and air  
172 at 300 ml/min. The programme was as follows: the initial temperature was 70 °C for 1 min,  
173 then it rose from 40 °C/min for 4 min to 250 °C, then increased by 1 °C/min for 8 min to 258  
174 °C and finally increased from 40 °C/min to 320 °C and stabilized at 320 °C for 3 min. The



175 temperature of the injector was maintained at 280 °C and that of the FID at 340 °C. Profiles  
176 were extracted with the Varian system control software Star Chromatography workstation  
177 version 6.2 (Varian, Palo Alto, CA, U.S.A.). The compounds were identified based on their  
178 retention time (Appendix Table A1), comparing them to standard hydrocarbons already  
179 identified in a gas chromatograph coupled to a mass spectrometer (GC-MS) as well as  
180 chromatograms of Yagound (2014) for *N. apicalis* morph 6. The advantage of this method was  
181 that it was not invasive; however, it was time consuming and did not allow quantification of  
182 compounds.

183         For temporal constraints, the chemical profile after orphaning was obtained by a liquid  
184 phase extraction. The head and thorax of each dissected ant were soaked in 200 µl of pentane  
185 containing 4 ng/µl of compound n-C<sub>17</sub> (representing our internal standard) in a vial tube for 5  
186 min. The tube was then left to evaporate. After the solution was completely evaporated, the  
187 sample was analysed by GC–MS (Agilent A7890), by injecting 2 µl of the extract resuspended  
188 in 80 µl of solvent (pentane), with electron impact ionization at 70 eV. The carrier gas was  
189 helium at 1 ml/min. The same analysis programme as above was applied. The chemical profiles  
190 were integrated using the MSD ChemStation software version E.02.01.1177 (Agilent  
191 Technologies Inc., Santa Clara, CA, U.S.A.). The compounds were identified by comparing  
192 their retention time and spectra with already known compounds. The internal standard allowed  
193 us to translate peak areas to absolute quantities for the related compounds.

194

## 195 <H2>*Dominance hierarchy*

196 Antennal boxing and bites were recorded, together with the identities of the interacting ants.  
197 The loser was the ant showing submissive behaviour, that is, hunching or dodging. Twelve  
198 observation sessions were carried out (15 h total) per colony: two of 1.5 h on the first, second

199 and third days after being orphaned, then two of 1 h on the fourth and fifth days and finally one  
200 of 1 h on the eighth and 10th days. During these sessions, all boxing and bites were recorded.  
201 Observations were made under red light to avoid biasing the ants' behaviour in the interior of  
202 the nest (Depickère et al., 2004).

203         The hierarchical rank of each worker in the orphaned colonies was obtained using the  
204 'Glicko-rating' method, which is a dynamic matched comparison model that calculates a score  
205 for each individual, based on the outcome of each individual's interactions (victory or defeat;  
206 Glickman, 1999). From this score, a ranking can be determined to deduce the hierarchy. The  
207 Glicko-rating algorithm includes a positive constant 'c', which governs the size of the standard  
208 deviation over time. This constant is defined by the user, an increased value of 'c' leading to a  
209 greater average deviation per individual over time. In our study, following the guidelines of  
210 Glickman (1999; and see So et al., 2015), we used a value of 1 for 'c'. We checked the impact  
211 of the 'c' value on our results by replicating the calculation over a range of 1–10. We obtained  
212 similar results for the hierarchical rankings over the whole range. Glicko-rating calculations  
213 were performed with the PlayerRatings package v1.0 (Stephenson & Sonas, 2014) in R 3.4.1  
214 (R Development Core Team, 2017). Data were compiled in chronological order of dyadic  
215 interactions. The same coefficient was attributed to antennal boxes and bites, so that in the  
216 calculation of the hierarchy the two types of agonistic behaviours had the same power.

217

## 218 <H2>*Fertility measurement*

219 With a graduated binocular microscope, we measured the ovarian development of the ants. The  
220 length of the three basal oocytes of the ovarioles of each ovary was measured. A fertility index  
221 was calculated by summing the lengths of the six basal oocytes (Yagound et al., 2014). We  
222 present this below as mean  $\pm$  SD.

223

224 <H2>Statistical analysis

225 <H3>Establishment of the hierarchy

226 The distribution of the average number of agonistic interactions per hour of observation was  
227 compared between colonies to compare the dynamics of hierarchy establishment. For this, a  
228 two-sample Kolmogorov–Smirnov test was performed between each pair of the four colonies.  
229 To compensate for multiple comparisons, *P* values were then adjusted to *P*' values following  
230 Holm (1979).

231 The linearity '*h*' within our four colonies was calculated between the 15 orphaned  
232 workers by the de Vries method (1995) using software R (package *compete*, Curley 2016).

233 To verify whether worker isolation led to the establishment of a reproductive hierarchy,  
234 we investigated the link between the hierarchical rank and fertility of individuals using  
235 Spearman correlations for the 59 orphaned ants dissected at the end of the experiment.

236

237 <H3>Chemical data analysis

238 Although some intercolonial heterogeneity is expected in the proportion of each compound in  
239 the cuticular profiles, a principal coordinate analysis (PCo) and an analysis of similarity  
240 (ANOSIM) were performed to verify whether our experimental colonies (59 workers) shared a  
241 similar chemotype, due to the potential presence of cryptic morphs, differing in chemical  
242 profiles. For this, we used the PERMANOVA+ for PRIMER software (Anderson et al., 2008)  
243 using a Euclidean distance matrix calculated on square-root-transformed percentages.

244 Using two different methods to extract the chemical profiles was a potential source of  
245 methodological variability. To verify whether our methods were reliable, we used Spearman  
246 correlations to compare the profiles before and after orphaning using the proportions of the  
247 major compounds, namely n-C<sub>21</sub>, C<sub>23:1</sub> and n-C<sub>23</sub>, with a Bonferroni–Holm adjustment for  
248 multiple tests on the same data set (Holm, 1979). Significant correlations would indicate  
249 reliability of the two methods (even if distinct methods can generate a slight chemical distance  
250 between the profiles before and after orphaning). This calculation could be done only for three  
251 colonies (see Results for details). In addition, two individuals from colony 20 could not be  
252 included in these chemical analyses because of a technical problem when acquiring the profile  
253 before orphaning (missing data). In colony 20 there were only 14 individuals because a worker  
254 died during the experiment. We thus analysed a total of 42 workers. For these three colonies  
255 we also performed a Mantel test (package *ecodist* in R) between the Euclidean distance matrix  
256 of the square-root-transformed percentages of the chemical profiles before and after orphaning  
257 to evaluate global concordance between the two methods.

258 To identify the cuticular compound(s) potentially acting as a fertility signal in our study,  
259 we used Spearman rank correlation (on 44 workers) to assess the relationship between the  
260 fertility index measured at the end of the experiment and the final quantities of cuticular  
261 compounds. Once the putative fertility signal was identified, we also checked for correlations  
262 with the proportions before orphaning to verify pre-existing heterogeneity between the workers  
263 in the presence of the queen (42 workers). *P* values were adjusted to account for multiple testing  
264 of the same data (Holm, 1979).

265 Last, we investigated the presence of a correlation between the putative fertility signal  
266 and the observed behaviour using Spearman correlation. The deviation from the mean quantity  
267 of fertility-related compound(s) (within each colony) was correlated with the different  
268 behaviours expressed by each individual (42 workers). Using a mean deviation index allowed

269 us to buffer the effect of intercolonial variation in the quantity of compound.

270

## 271 <H1>Results

### 272 <H2>Setting up reproductive hierarchies

### 273 <H3>Dynamics of agonistic behaviours

274 Despite some variability among colonies in the intensity of agonistic displays (Fig. 2), the  
275 dynamics of agonistic behaviour did not differ significantly between them. No two by two  
276 comparison between colonies was significant (Kolmogorov–Smirnov test with Bonferroni–  
277 Holm correction: colony 1–8:  $D = 0.571$ ,  $P' = 0.203$ ; colony 1–18:  $D = 0.571$ ,  $P' = 0.203$ ;  
278 colony 1–20:  $D = 0.571$ ,  $P' = 0.212$ ; colony 8–18:  $D = 0.571$ ,  $P' = 0.203$ ; colony 8–20:  $D =$   
279  $0.571$ ,  $P' = 0.203$ ; colony 18–20:  $D = 0.429$ ,  $P' = 0.575$ ). Agonistic interactions rose rapidly  
280 within the first 2 days of being orphaned and then returned to basal level.

281

### 282 <H3>Establishment of hierarchies

283 Hierarchy established in colonies 8, 18 and 20 had linearities of  $h' = 0.52$  ( $P = 0.001$ ),  $h' = 0.66$   
284 ( $P < 0.001$ ) and  $h' = 0.65$  ( $P < 0.001$ ), respectively. Hierarchy in colony 1 did not show a  
285 significant linearity ( $h' = 0.19$ ,  $P = 0.48$ ).

286

### 287 <H3>Rank and ovarian development

288 All but 10 of our 59 workers had activated ovaries. The average ovarian development, measured  
289 at the end of the experiment, was highest for colony 18 ( $4.16 \pm 2.59$  mm), followed by colony  
290 8 ( $3.72 \pm 3.10$  mm), colony 1 ( $3.42 \pm 2.25$  mm) and finally colony 20 ( $2.97 \pm 2.06$  mm). Within

291 each colony the fertility index of workers was significantly correlated with their hierarchical  
292 rank from the first day of being orphaned (Table 1).

293

294 <H2>Chemical profiles, fertility and behaviours

295 <H3>Chemical analyses of profiles after orphaning

296

297 The chemical profiles were grouped by colony in the PCo and with the ANOSIM, suggesting  
298 the existence of a characteristic colonial signature (Appendix Fig. A1). Considering the average  
299 chemical distance calculated between colonies, colony 18 was very different from the others  
300 (0.75 on average with colony 18, against 0.20 between the other three colonies; Appendix Table  
301 A2).

302 The cuticular profile of each ant was composed of 28–30 peaks and included several  
303 series of n-alkanes, branched mono and dimethyl-alkanes and alkenes, with carbon atom  
304 numbers ranging from 19 to 33. The majority of compounds were linear alkanes and alkenes.  
305 Consistent with the chemical distance results, colonies 1, 8 and 20 displayed a qualitatively  
306 distinct chemotype from colony 18 (Appendix Fig. A2). Colony 18 was thus excluded from  
307 correlation analysis with the chemical profiles. The chemical profiles of the workers from  
308 colony 18 were heterogeneous, some appearing separated and others represented among the  
309 other colonies' profiles (Appendix Fig. A1). As several morphs of *N. apicalis* occur in the  
310 collection area, this unusual result could thus be a consequence of a chance hybridization  
311 between two morphs (i.e. a male from another morph), which cannot be detected using nuclear  
312 DNA. Although interesting, we have no additional means to explain this discrepant chemotype.

313

314 <H3>Reliability between the two chemical extraction methods

315

316 The proportions of the three major compounds (n-C<sub>21</sub>, C<sub>23:1</sub> and n-C<sub>23</sub>) in the SPME samples  
317 analysed by GC–FID before orphaning were significantly correlated with those analysed by  
318 GC–MS after orphaning (Table 2). The Mantel test between the two chemical profile matrices  
319 before and after orphaning were significantly correlated ( $P < 0.001$ ) with a Mantel value of  
320 0.70, which, considering the potential variation due to fertility and environmental changes  
321 between the two analyses, is sufficient to validate the use of the two methods. Initial and final  
322 n-C<sub>23</sub> proportions were also correlated (Spearman correlation:  $r_s = 0.75$ ,  $P < 0.001$ ).

323

### 324 <H3>Correlation between chemical profiles and fertility

325

326 The amount of the alkane tricosane (n-C<sub>23</sub>) after orphaning was significantly correlated with the  
327 fertility index for each individual (Spearman correlation:  $r_s = 0.63$ ,  $N = 44$ ,  $P < 0.001$ ; Fig. 3a),  
328 as was the proportion of n-C<sub>23</sub> in the chemical profile before orphaning (Spearman correlation:  
329  $r_s = 0.69$ ,  $N = 42$ ,  $P < 0.001$ ; Fig. 3b). This compound was the component of the chemical  
330 profile that best correlated with ovarian development. Correlation values of the other  
331 compounds are presented in Appendix Table A3.

332

### 333 <H3>Fertility and behaviour

334

335 We focused on n-C<sub>23</sub> which was the best correlated compound with fertility. Both the number  
336 of fights won by an ant and the number of interactions it was involved in were significantly  
337 correlated with the quantity of n-C<sub>23</sub> after orphaning (Table 3). The same results were obtained  
338 when considering only the first 2 days of interaction. Last, behaviours at the beginning of the  
339 experiment (first 2 days) were highly correlated with all behaviours observed during the whole

340 10 days of the experiment (Table 4), showing that the hierarchy was established during the first  
341 48 h after queen loss.

342

### 343 <H1>Discussion

344 Our results confirmed the rapidity of hierarchy establishment over a period of 48 h after queen  
345 loss. The number of agonistic behaviours decreased drastically after this period, which is typical  
346 of a stabilized hierarchy. The cuticular profiles analysis showed tricosane to be highly  
347 correlated with ovarian development, therefore putatively acting as a fertility signal.  
348 Interestingly, the relative amounts of tricosane on the cuticle both before and after the  
349 establishment of the hierarchy were also correlated with (1) the rank achieved within the  
350 hierarchy and (2) the frequency of the agonistic behaviours displayed.

351 These results constitute the first experimental evidence that differences in ovarian activity  
352 (estimated by an indirect method) between workers in the presence of the queen accurately  
353 predict the outcome of the reproductive hierarchy, which is a consequence of a tournament  
354 between workers. They also fully support our hypothesis that physiological differences between  
355 workers are mirrored in modulated motivations to fight (Stevenson et al., 2000). The outcome  
356 of the dominance/submission could then be facilitated with chemical signalling (Yagound et  
357 al., 2015).

358 Fertility signals have been identified in other morphs of *N. apicalis* (Yagound, 2014),  
359 but not in morph 6. In three colonies (the fourth having a different chemotype) tricosane was  
360 the cuticular compound best correlated with fertility. Thus, tricosane is the most probable  
361 fertility signal in these colonies. We used tricosane as a proxy for the fertility signal, but we are  
362 aware that it may also be part of a mixture of compounds used for fertility recognition. This  
363 does not, however, change the conclusions of our study. In his comparative study of fertility  
364 signalling in the *N. apicalis* complex, Yagound (2014) found that an alkene was best correlated



365 with fertility in morph 6 ( $r_s = 0.75, P < 10^{-4}$ ). However, Yagound could analyse only one colony  
366 and he also found that tricosane was correlated with ovarian development ( $r_s = 0.51, P < 0.01$ ).  
367 This finding and the larger sample that we analysed legitimize the interpretation of tricosane as  
368 a putative signal of fertility for *N. apicalis* morph 6. Other compounds were also correlated with  
369 fertility in our sample but tricosane appeared to be the best correlated compound with both  
370 fertility and behavioural parameters (the expression of agonistic behaviours and the social ranks  
371 achieved) during the establishment of the hierarchy. This consistency between physiological  
372 and behavioural data fulfils the condition for tricosane to be an index of fertility. The correlation  
373 between the putative fertility signal and the hierarchical ranks also strengthens the idea of it  
374 being an honest signal (Heinze & d’Ettorre, 2009). A reliable index of fertility allows adequate  
375 reproductive decision making, depending on individual interests in terms of inclusive fitness  
376 (Yagound, 2014). This signal would allow workers to identify the best potential reproducer  
377 within the colony.

378         The initial heterogeneity between ants in queenright colonies could be amplified during  
379 subsequent agonistic interactions. Idiosyncratic variations could initially reflect the differences  
380 in workers’ ages affecting their physiological and hormonal states, and hence their ovarian  
381 activation (Yagound et al., 2015). Workers with an already partially active ovarian system  
382 would have a clear advantage during the establishment of hierarchies. Lamba et al. (2007)  
383 hypothesized that in other eusocial insects (wasps) fighting could be used not to exclude the  
384 other females from dominance, but rather to speed up the development of the ovarian system  
385 of the future reproductive (via an action on biogenic amines) and so facilitate the  
386 monopolization of colony reproduction. Aggressive behaviours also lead to a decrease in  
387 juvenile hormone titre (usually positively correlated with fertility) in subordinate individuals  
388 (Tibbetts et al., 2018). Physical contact between workers in the ant *Diacamma* has also been  
389 shown to affect dopamine secretion in the worker’s brain and to regulate reproduction inside

390 the nest (Shimoji et al., 2017). Agonistic interactions in *Neoponera* may thus impact ovarian  
391 development through similar neuroendocrine changes.

392 Hierarchical status discrimination based on the putative fertility signal can generate a  
393 linear hierarchy. Fertility signalling would be involved in both the establishment (Yagound et  
394 al., 2015) and the maintenance (Heinze et al., 2002) of the reproductive hierarchy. Agonistic  
395 interactions acting on the physiological and hormonal secretions would reinforce the pre-  
396 existing differences in fertility between individuals, and this would accelerate cooperation  
397 within the nest. Subordinates would maximize their fitness by quickly resolving conflicts by  
398 reducing their ability to reproduce (Tibbetts et al., 2018).

399 Interestingly, our results showed a strong correlation between fertility and the number of  
400 fights an individual is involved in (whatever the outcome). This result suggests two mutually  
401 nonexclusive hypotheses. First, tricosane could be correlated with both fertility and motivation  
402 to fight and/or involvement in the colony's hierarchy. Biogenic amines such as octopamine or  
403 dopamine could possibly be involved in this process. Indeed, biogenic amines mediate changes  
404 in dominance behaviour linked with fertility in the ant *Harpegnathos saltator* (Penick et al.,  
405 2014). Moreover, it has been demonstrated in the cricket *Gryllus bimaculatus* that these  
406 bioamines are necessary to trigger aggressive behaviour (Stevenson et al., 2000). Second,  
407 tricosane could act as a fertility signal and thus attract aggression from competitors attempting  
408 to gain dominance. Such behaviour where workers attack congeners that display fertility signals  
409 has been shown, for example, in the context of worker policing in social insects (ants: Hartmann  
410 et al., 2005; Monnin & Peeters, 1999; Smith et al., 2009; bees: Visscher and Dukas, 1995;  
411 wasps: Wenseleers et al., 2005). This mechanism could thus ensure the fertility signal has  
412 similar functions in the contexts of worker policing and establishment of the reproductive  
413 hierarchy, namely regulating reproduction at the level of the worker collective.

414 The fact that tricosane was also correlated with the percentage of fights won favours the  
415 first explanation. Attacked individuals in the case of worker policing are indeed more likely to  
416 be defeated (and their reproductive activity suppressed; Monnin & Peeters, 1999), while highly  
417 motivated animals could have an advantage in a tournament system. Tricosane could thus both  
418 reflect ovarian development and be correlated with a network of neuroendocrine activity that  
419 ensures fighting motivation and, maybe more generally, the ability to mobilize resources (RHP;  
420 Parker, 1974). One mechanism ensuring the honesty of the fertility signal (and its role in the  
421 reproductive hierarchy) could be the strong links between the neuroendocrine networks  
422 involved in the regulation of reproduction, agonistic behaviour and fighting abilities. A second  
423 mechanism could be that individuals ‘motivated’ to fight, but lacking the skills required to  
424 occupy the top of the hierarchy, would be defeated by others workers. This mechanism would  
425 be in line with theories proposing that the costs (both physiological and social) of maintaining  
426 a signal ensure its honesty (Zahavi, 1975; Heinze & d’Ettorre, 2009).

427 The loss of the queen probably lifts an inhibition for already fertile individuals which very  
428 quickly start competing to reproduce. The highest motivation for fighting of these individuals  
429 probably drives the expression of ritualized agonistic encounters within the colony. The impact  
430 of social experience and especially winner–loser effects would then help amplify the pre-  
431 existing differences at the physiological (Oliveira et al. 2009), cognitive and behavioural (Hsu  
432 & Wolf, 2000; Rutte et al., 2006) levels. The social system would then develop from the  
433 queenright state, where all workers refrain from reproducing, to the establishment of the  
434 reproductive hierarchy based on self-organized processes. After a short period of social  
435 perturbation with intense fighting behaviour, the social system stabilizes again with the  
436 selection of a new reproductive individual and the disappearance of agonistic interactions.

437 To our knowledge, this is the first study to monitor the development of the chemical profile  
438 from the queenright state to the stabilization of a reproductive hierarchy by orphaned ant

439 workers. Our study supports the hypothesis that the pre-existing fertility differences between  
440 individuals in the queenright condition predict the destiny of workers in the reproductive  
441 hierarchy. The most fertile workers reach the high ranks and produce males. The selective  
442 pressures are strong after queen loss, with a short time window for producing the last batch of  
443 reproductive ants (Dietemann & Peeters, 2000). In response to these strong ecological  
444 constraints, ants have developed a recognition system based on cuticular hydrocarbons related  
445 to ovarian development and acting as a fertility signal (Yagound et al., 2015). This fertility  
446 signal, already perceptible in the presence of the queen, makes it possible for workers to  
447 evaluate the interindividual differences and, subsequently, agonistic interactions help to  
448 establish and stabilize the reproductive hierarchy (especially with winner–loser effects; Chase  
449 et al., 2002). All these mechanisms allow a quick resolution of the overt conflict and ensure  
450 division of reproductive and ergonomic tasks inside orphaned colonies to allow the production  
451 of males.

452

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605

606 **Figure legends**

607 **Figure 1.** Timeline of the experiment. The queen was removed from each colony 7 days after  
608 individuals were marked and the experiment ran for 10 days. ‘Initial profile’ corresponds to the  
609 workers’ chemical profile in the presence of the queen (determined by flame ionization  
610 detection, GC–FID); ‘final profile’ corresponds to the chemical profile at the end of the  
611 experiment (determined by gas chromatography–mass spectrometry, GC–MS). Agonistic  
612 interactions (antennal boxing and bite) were used to calculate the hierarchical rank of the 15  
613 orphaned individuals per colony.

614

615 **Figure 2.** Number of agonistic interactions per hour of observation as a function of observation  
616 day during the 10 days after the queen was removed.

617

618 **Figure 3.** Variation in the fertility index (summed lengths of the six basal oocytes in the ovary,  
619 mm) as a function of (a) the amount of tricosane in the ants’ chemical profile after queen  
620 removal and (b) the proportion of tricosane in the profile before queen removal.

621

622 **Figure A1.** PCO (principal coordinate analysis) of the chemical profiles of the four colonies  
623 (based on the Bray–Curtis similarity matrix calculated with the square-root-transformed  
624 proportions).  $N = 15$  individuals in each colony.

625

626 **Figure A2.** Representative examples of the chemical profiles from (a) colony 1 (chemotype A)  
627 and (b) colony 18 (chemotype B).

628

629 **Figure A3.** Plot of the data used in the Spearman correlations presented in Table 3. Correlations  
630 are shown between the recorded behaviours and the mean deviation of tricosane per colony

631 before (left, n-C<sub>23</sub> initial) and after queen removal (right, n-C<sub>23</sub> final). (a) Hierarchical rank on  
632 the 10th day (the end of the experiment), (b) the number of fights won for days 1 and 2, (c) the  
633 total number of fights from day 1 to day 10, (d) the percentage of fights won for days 1 and 2,  
634 (e) the total percentage of fights won from day 1 to day 10, (f) the number of fights individuals  
635 were involved in for days 1 and 2 and (g) the total number of fights individuals were involved  
636 in from day 1 to day 10.

637

638 **Table 1.** Spearman correlations between an individual ant's fertility index and hierarchical rank  
639 on the first, second and 10th (final) day of the experiment

640

641

	<i>r<sub>s</sub></i>	<i>P'</i>	<i>N</i>
642 Day 1 rank	-0.3	0.03	59
643 Day 2 rank	-0.39	0.006	59
644 Day 10 rank	-0.44	0.002	59

645 To compensate for multiple comparisons, *P* values were adjusted to *P'* values following Holm  
646 (1979).

647 **Table 2.** Spearman correlations between the proportions of the three main compounds within  
648 individual ants' chemical profiles before and after queen removal  
649

Cuticular hydrocarbons	$r_s$	$P'$	$N$
n-C <sub>21</sub>	0.48	0.001	42
C <sub>23:1</sub>	0.34	0.03	42
n-C <sub>23</sub>	0.74	2.1e-8	42

650 To compensate for multiple comparisons,  $P$  values were adjusted to  $P'$  values following Holm  
651 (1979).

652 **Table 3.** Spearman correlations between the behaviours expressed after (final n-C<sub>23</sub>) and before  
 653 (initial n-C<sub>23</sub>) queen removal and the mean deviation in amount of n-C<sub>23</sub> between individuals of  
 654 the same colony

655 To compensate for multiple comparisons, *P* values were then adjusted to *P'* values following

	<i>r</i> <sub>s</sub>	<i>P'</i>	<i>r</i> <sub>s</sub>	<i>P'</i>	<i>N</i>
	(final n- C <sub>23</sub> )		(initial n- C <sub>23</sub> )		
Final hierarchical rank	-0.52	0.002	-0.48	0.003	42
No. of fights won during days 1 and 2	0.46	0.008	0.53	0.002	42
Total no. of fights won	0.53	0.001	0.59	2.5e-4	42
Percentage of fights won during days 1 and 2	0.37	0.01	0.43	0.008	42
Total percentage of fights won	0.44	0.008	0.44	0.008	42
Fight number during days 1 and 2	0.45	0.008	0.51	0.002	42
Fight number total	0.5	0.002	0.59	2.2e-4	42

656 Holm (1979). ‘Final’ and ‘total’ correspond to the behaviours expressed from day 1 to day 10  
 657 of being orphaned. ‘Fight number’ corresponds to the number of fights an individual was  
 658 involved in. Data dispersions are presented in Fig. A3.

659



660 **Table 4.** Spearman correlations between behaviours during combined days 1 and 2 and from  
661 day 1 to day 10 of the experiment

	<i>r<sub>s</sub></i>	<i>P</i>	<i>N</i>
Hierarchical rank	0.86	<2.2e-16	59
No. of fights won	0.96	<2.2e-16	59
Percentage of fights won	0.90	<2.2e-16	59
No. of fights	0.95	<2.2e-16	59

662

663 **Table A1.** Identification of cuticular hydrocarbons (CHCs) on *N. apicalis* morph 6 for a  
 664 moderately fertile individual (corresponding to chemotype A in Fig. A2)

Peak	Retention time	Relative abundance	Characteristic fragments	CHC ID
1	5.876	-	-	n-C <sub>17</sub> (internal standard)
2	6.532	0.05	268	n-C <sub>19</sub>
3	6.915	0.08	282	n-C <sub>20</sub>
4	7.265	0.7	294	C <sub>21:1</sub>
5	7.399	14.85	296	n-C <sub>21</sub>
6	7.563	0.06	140 196 295	9-MeC <sub>21</sub>
7	7.694	0.07	70 267 295	4-MeC <sub>21</sub>
8	7.785	1.41	308	C <sub>22:1</sub>
9	7.906	0.97	310	n-C <sub>22</sub>
10	8.139	0.07	169 182 309	11-MeC <sub>22</sub>
11	8.523	62.42	322	C <sub>23:2</sub> + C <sub>23:1</sub>
12	8.633	11.13	324	n-C <sub>23</sub>
13	8.847	0.19	168 196 323	11-MeC <sub>23</sub>
14	8.962	0.16	85 252 281 323	5-MeC <sub>23</sub>
15	9.167	0.09	336	C <sub>24:1</sub>
16	9.371	0.07	338	n-C <sub>24</sub>
17	10.135	0.99	350	C <sub>25:1</sub>
18	10.392	1.4	352	n-C <sub>25</sub>
19	11.606	0.14	366	n-C <sub>26</sub>
20	12.756	0.04	378	C <sub>27:1</sub>
21	13.168	2.45	380	n-C <sub>27</sub>
22	14.374	0.12	394	n-C <sub>28</sub>
23	14.824	0.33	365 393	2-MeC <sub>28</sub>
24	14.918	0.06	406	C <sub>29:1</sub>
25	15.046	0.83	408	n-C <sub>29</sub>
26	15.225	0.24	168 196 252 281 407	11-16-diMeC <sub>28</sub>
27	15.961	0.2	393 421	2-MeC <sub>30</sub>
28	16.065	0.04	434	C <sub>31:1</sub>
29	16.174	0.09	436	n-C <sub>31</sub>
30	16.352	0.32	168 196 224 252 281 309 435	11-13-15-MeC <sub>31</sub>
31	17.634	0.44	168 308 337 463	11-MeC <sub>33</sub>

665

666 **Table A2.** Analysis of similarity between the chemical profiles of the four colonies

Groups	<i>R</i>	<i>P</i>	Permutations
1, 8	0.148	0.01	9999
1, 18	0.758	0.0001	9999
1, 20	0.208	0.0002	9999
8, 18	0.771	0.0001	9999
8, 20	0.281	0.0001	9999
18, 20	0.747	0.0001	9999

667 The global test of the analysis of similarity gives a global *R* of 0.549 (*P* = 0.0001, number of  
668 permutations = 9999). Pairwise test results are given in the table.

669 **Table A3.** Spearman correlations (with Bonferroni–Holm adjustment for multiple  
 670 comparisons) between the fertility index and the compounds present in the chemical profile  
 671 after orphaning of colonies 1, 8 and 20 ( $N = 44$ )

Cuticular hydrocarbons	$r_s$	$P'$
<b>n-C<sub>19</sub></b>	<b>-0.51</b>	<b>0.009</b>
n-C <sub>20</sub>	-0.15	1
C <sub>21:1</sub>	-0.23	1
n-C <sub>21</sub>	0.18	1
9-MeC <sub>21</sub>	-0.28	1
<b>4-MeC<sub>21</sub></b>	<b>0.44</b>	<b>0.05</b>
C <sub>22:1</sub>	0.07	1
n-C <sub>22</sub>	0.48	0.023
11-MeC <sub>22</sub>	0.41	0.14
C <sub>23:2</sub> + C <sub>23:1</sub>	0.27	1
<b>n-C<sub>23</sub></b>	<b>0.63</b>	<b>1.5e-4</b>
11-MeC <sub>23</sub>	0.07	1
<b>5-MeC<sub>23</sub></b>	<b>0.61</b>	<b>2.9e-4</b>
C <sub>24:1</sub>	0.34	0.42
n-C <sub>24</sub>	0.3	1
<b>C<sub>25:1</sub></b>	<b>0.45</b>	<b>0.05</b>
<b>n-C<sub>25</sub></b>	<b>0.48</b>	<b>0.03</b>
n-C <sub>26</sub>	0.27	1
C <sub>27:1</sub>	-0.01	1
n-C <sub>27</sub>	0.41	0.13
n-C <sub>28</sub>	0.16	1
2-MeC <sub>28</sub>	0.08	1
C <sub>29:1</sub>	-0.22	1
n-C <sub>29</sub>	0.24	1
11-16-diMeC <sub>28</sub>	0.12	1
2-MeC <sub>30</sub>	0.05	1
C <sub>31:1</sub>	-0.11	1

n-C <sub>31</sub>	-0.22	1
11-13-15-MeC <sub>31</sub>	0.05	1
11-MeC <sub>33</sub>	-0.06	1

672

673 For colony 18, only the compound n-C<sub>27</sub> was correlated with fertility ( $r_s = -0.5$ ,  $P' = 0.057$ ,  $N$   
674 = 15). To compensate for multiple comparisons,  $P$  values were adjusted to  $P'$  values following  
675 Holm (1979). Significant values are highlighted in bold.