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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-C₂₃) 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 ± 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-C₁₇ (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-C₂₁, C_{23:1} and n-C₂₃, 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 ± 2.59 mm), followed by colony
290 8 (3.72 ± 3.10 mm), colony 1 (3.42 ± 2.25 mm) and finally colony 20 (2.97 ± 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-C₂₁, C_{23:1} and n-C₂₃) 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-C₂₃ 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-C₂₃) 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-C₂₃ 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-C₂₃ 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-C₂₃ 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-C₂₃ initial) and after queen removal (right, n-C₂₃ 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_s</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 ₂₁	0.48	0.001	42
C _{23:1}	0.34	0.03	42
n-C ₂₃	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-C₂₃) and before
 653 (initial n-C₂₃) queen removal and the mean deviation in amount of n-C₂₃ 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> _S	<i>P'</i>	<i>r</i> _S	<i>P'</i>	<i>N</i>
	(final n- C ₂₃)		(initial n- C ₂₃)		
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_s</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 ₁₇ (internal standard)
2	6.532	0.05	268	n-C ₁₉
3	6.915	0.08	282	n-C ₂₀
4	7.265	0.7	294	C _{21:1}
5	7.399	14.85	296	n-C ₂₁
6	7.563	0.06	140 196 295	9-MeC ₂₁
7	7.694	0.07	70 267 295	4-MeC ₂₁
8	7.785	1.41	308	C _{22:1}
9	7.906	0.97	310	n-C ₂₂
10	8.139	0.07	169 182 309	11-MeC ₂₂
11	8.523	62.42	322	C _{23:2} + C _{23:1}
12	8.633	11.13	324	n-C ₂₃
13	8.847	0.19	168 196 323	11-MeC ₂₃
14	8.962	0.16	85 252 281 323	5-MeC ₂₃
15	9.167	0.09	336	C _{24:1}
16	9.371	0.07	338	n-C ₂₄
17	10.135	0.99	350	C _{25:1}
18	10.392	1.4	352	n-C ₂₅
19	11.606	0.14	366	n-C ₂₆
20	12.756	0.04	378	C _{27:1}
21	13.168	2.45	380	n-C ₂₇
22	14.374	0.12	394	n-C ₂₈
23	14.824	0.33	365 393	2-MeC ₂₈
24	14.918	0.06	406	C _{29:1}
25	15.046	0.83	408	n-C ₂₉
26	15.225	0.24	168 196 252 281 407	11-16-diMeC ₂₈
27	15.961	0.2	393 421	2-MeC ₃₀
28	16.065	0.04	434	C _{31:1}
29	16.174	0.09	436	n-C ₃₁
30	16.352	0.32	168 196 224 252 281 309 435	11-13-15-MeC ₃₁
31	17.634	0.44	168 308 337 463	11-MeC ₃₃

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'
n-C₁₉	-0.51	0.009
n-C ₂₀	-0.15	1
C _{21:1}	-0.23	1
n-C ₂₁	0.18	1
9-MeC ₂₁	-0.28	1
4-MeC₂₁	0.44	0.05
C _{22:1}	0.07	1
n-C ₂₂	0.48	0.023
11-MeC ₂₂	0.41	0.14
C _{23:2} + C _{23:1}	0.27	1
n-C₂₃	0.63	1.5e-4
11-MeC ₂₃	0.07	1
5-MeC₂₃	0.61	2.9e-4
C _{24:1}	0.34	0.42
n-C ₂₄	0.3	1
C_{25:1}	0.45	0.05
n-C₂₅	0.48	0.03
n-C ₂₆	0.27	1
C _{27:1}	-0.01	1
n-C ₂₇	0.41	0.13
n-C ₂₈	0.16	1
2-MeC ₂₈	0.08	1
C _{29:1}	-0.22	1
n-C ₂₉	0.24	1
11-16-diMeC ₂₈	0.12	1
2-MeC ₃₀	0.05	1
C _{31:1}	-0.11	1

n-C ₃₁	-0.22	1
11-13-15-MeC ₃₁	0.05	1
11-MeC ₃₃	-0.06	1

672

673 For colony 18, only the compound n-C₂₇ 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.