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► To cite this version:

Claudie Doums, Pierre Federici, Pascaline Chifflet-Belle, Thibaud Monnin. Worker thelytoky allows requeening of orphaned colonies but increases susceptibility to reproductive cheating in an ant. *Animal Behaviour*, 2018, 135, pp.109-119. 10.1016/j.anbehav.2017.11.013 . hal-01730713

HAL Id: hal-01730713

<https://hal.sorbonne-universite.fr/hal-01730713>

Submitted on 13 Mar 2018

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1 Worker thelytoky allows requeening of orphaned colonies but increases susceptibility to
2 reproductive cheating in an ant

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21 Word count: 7304 words (all included)

22

23 ABSTRACT:

24 In some social insects workers can produce females asexually through thelytokous
25 parthenogenesis. This allows them to produce replacement queens (i.e. requeening) if the
26 queen has died, but also to compete with the queen for producing females (i.e. reproductive
27 cheating). For the first time, we experimentally tested the role of worker thelytoky under
28 quasi-natural conditions in the ant *Cataglyphis cursor*, where the queen uses both sexual and
29 thelytokous reproduction. We reared pairs of orphaned and queenright colonies in enclosures
30 for almost three months, during which they competed for resources. Orphaned colonies lost
31 more workers than queenright colonies over the course of the experiment, presumably
32 because of the costs of reproductive conflicts between workers. Nevertheless, they produced
33 new queens through worker thelytoky and new colonies through colony fission. This is the
34 first unambiguous demonstration that worker thelytoky allows requeening under natural
35 conditions in this species. We further show that worker thelytoky results in reproductive
36 cheating in the form of a few workers reproducing in presence of the queen (in queenright
37 colonies) and of a few worker lineages producing more new queens than other lineages (in
38 orphaned colonies). In addition, it also results in rare instances of social parasitism i.e.
39 workers entering and reproducing in foreign colonies. These benefits to workers seem too
40 occasional and too low to drive the evolution of theytoky in this species. We argue that
41 thelytoky likely evolved in the queen caste, where it allows producing young queens and
42 confers frequent and massive benefits by increasing gene transmission, but is also expressed
43 in workers because of genetic correlations between the two castes.

44

45 Keywords: *Cataglyphis cursor*, cheating, cost of reproduction, thelytoky, social insects,
46 worker reproduction

47

48 INTRODUCTION

49

50 Cooperative systems are exposed to the apparition of selfish elements that benefit from the
51 common goods without paying the costs arising from their production (Chapuisat, 2009;
52 Dobata & Tsuji, 2013; Hughes & Boomsma, 2008). By doing so, cheaters increase their
53 fitness and decrease that of the other individuals. Cheating has evolved in a range of
54 cooperative organisms, from bacteria to mammals (Ghoul, Griffin, & West, 2014; Riehl &
55 Frederickson, 2016). In social hymenoptera, workers typically rear the offspring of their
56 mother queen instead of their own, yet a low level of worker reproduction in presence of the
57 queen (i.e. cheating) has been observed in various species (Barron, Oldroyd, & Ratnieks,
58 2001; Bourke, 2011; Ratnieks, Foster, & Wenseleers, 2006; Ratnieks & Wenseleers, 2008;
59 Sundström & Boomsma, 2001).

60

61 Because of the haplo-diploid sex determination system of hymenoptera, workers can
62 potentially produce haploid sons (arrhenotoky) in most species (e.g. Ratnieks et al. 2006;
63 Bourke 2011). However, worker policing (through oophagy and/or aggression) and self-
64 restraint usually lessen or even prevent intra-colonial cheating (Ratnieks, 1988; Ratnieks et
65 al., 2006). In addition, colony recognition similarly limits or prevents intraspecific social
66 parasitism, that is the reproduction of foreign workers (Beekman and Oldroyd 2008). Despite
67 this, low levels of intra-colonial cheating have been observed in many species (Barron et al.,
68 2001; Bourke, 2011; Ratnieks et al., 2006; Ratnieks & Wenseleers, 2008; Sundström &
69 Boomsma, 2001), and reproduction by drifting workers has also been detected in several
70 species (Chapman, Nanork, et al., 2009; Chapman, Beekman, & Oldroyd, 2010; Chapman,
71 Makinson, Beekman, & Oldroyd, 2009; Dobata et al., 2011; Nanork et al., 2007)

72

73 Another, more subtle, form of cheating can occur when there are many patriline
74 within a colony (i.e. the queen is mated with several males), given that patriline that are more
75 successful at producing queens are advantaged by individual selection. A differential success
76 at queen production could be achieved by various ways. Patriline may differ in their larval
77 propensity to develop into queens, so that caste is partially determined by genetic variation
78 among patriline (Anderson, Linksvayer, & Smith, 2008; Hughes & Boomsma, 2008;
79 Schwander, Lo, Beekman, Oldroyd, & Keller, 2010). Workers may also preferentially rear
80 larvae from their own patriline into queens. However, such nepotistic behaviours have rarely
81 been detected, and have been subject to controversy when detected (Hannonen & Sundström,
82 2003; Holzer, Kümmerli, Keller, & Chapuisat, 2006; Reeve & Keller, 1997; Tarpy, Gilley, &
83 Seeley, 2004).

84

85 Finally, patriline may engage in direct reproductive competition when workers
86 themselves are capable of producing new queens by thelytokous reproduction, as in some
87 honey bees (Holmes, Tan, Wang, Oldroyd, & Beekman, 2015; Verma & Ruttner, 1983) and
88 some thermophilic ants from the genus *Cataglyphis* (Aron, Mardulyn, & Leniaud, 2016).
89 Some patriline may gain a disproportionately higher reproductive success than others either
90 because a higher fraction of their workers are reproducing or because they are more fecund
91 (Goudie et al., 2012). The selective pressure for the evolution of cheating is more pronounced
92 when workers are able to produce females, because a worker that manages to produce the new
93 queen in some way reincarnates herself into that queen (Greeff, 1996). Quite surprisingly, and
94 although thelytokous reproduction has evolved independently in many social insect species
95 (Wenseleers & Van Oystaeyen, 2011), worker thelytoky in queenright colonies has been
96 observed in only two species, the Cape honey bee *A. m. capensis* (Goudie and Oldroyd 2014
97 for a review and references therein) and the ant *C. cursor* (Doums, Ruel, et al., 2013). In the

98 Cape honey bee, reproductive workers can be considered cheaters in queenright colonies
99 where they produce up to 60% of new queens (Allsopp, Beekman, Gloag, & Oldroyd, 2010;
100 Jordan, Allsopp, Oldroyd, Wossler, & Beekman, 2008) whereas in *C. cursor*, the use of
101 thelytoky by workers is less clear (Chéron, Monnin, Fédérici, & Doums, 2011; Doums, Ruel,
102 et al., 2013). In addition, worker thelytoky may also confer benefits from intra-specific social
103 parasitism, and indeed social parasitism has been observed in several species with thelytokous
104 workers (Allsopp et al., 2010; Dobata et al., 2011; Moritz, Lattorff, Crous, & Hepburn, 2011).
105 In the Cape honey bee, workers enter foreign colonies where they produce 6 to 62 % of
106 queens (Härtel, Neumann, Raassen, Moritz, & Hepburn, 2006; Moritz et al., 2011). One
107 clonal worker lineage has even been found to parasitize a closely related subspecies, *A. m.*
108 *scutellata* where it behaves like pseudo-queens (reviewed in Goudie and Oldroyd 2014).

109

110 In social insects, the main force preventing the evolution of high levels of cheating is
111 thought to be the costs of worker reproduction and of conflicts over reproduction, even
112 though experimental evidences of such costs are scarce (Bourke, 2011; Ratnieks et al., 2006).
113 Two studies in queenless ants showed a direct energetic cost (Gobin et al., 2013), and a cost
114 on worker immune defence and on the time spent by workers to perform collective tasks
115 (Bocher, Doums, Millot, & Tirard, 2008). Species with worker thelytoky are similar to
116 queenless ants in that after the death (or experimental removal) of the reproductive
117 individuals, all workers have the potential to produce diploid eggs. This can generate intense
118 conflicts over reproduction, but it can also provide the major benefit of avoiding colony death
119 following the loss of the reproductive individual. Indeed, in most monogynous species, the
120 death of the queen induces the rearing of new queens from the young diploid brood already
121 present in the colony and the production of male by workers through arrhenotoky (Bourke,
122 2011). When no young diploid brood is available, the colony is hopelessly queenless and dies

123 gradually after a bunch of male production. By allowing requeening of orphaned colonies,
124 worker thelytoky can avoid colony death even when no brood is present (Holmes et al., 2010).

125

126 In this study, we investigated the level of intra- and inter-colonial worker reproduction
127 in the ant *C. cursor* and tested the capacity of queenless colonies to requeen in spite of a
128 potential cost of reproductive conflicts between workers. To do so, we orphaned colonies at
129 the end of the hibernation period and let them compete with queenright colonies in quasi-
130 natural conditions until the time of sexual production. In *C. cursor*, the queen combines or
131 alternates thelytokous and sexual reproduction to produce new queens (called “gynes” before
132 they mate and start their own colony), but she only uses sexual reproduction to produce
133 workers (Doums, Cronin, et al., 2013; Percy, Aron, Doums, & Keller, 2004). Workers from
134 orphaned colonies kept under laboratory conditions use thelytokous reproduction to produce
135 gynes at the end of the hibernation period, and to produce workers later in the season
136 (Cagniant, 1980; Chéron, Monnin, et al., 2011; Clémencet, Rome, Fédérici, & Doums, 2008).
137 Worker-produced gynes mate and monogyny is then restored by the killing of all but one
138 gyne. Theoretically, a colony could hence be immortal since workers allow both colony
139 growth and colony requeening. However, the extent of worker reproduction in the field is
140 unknown.

141

142 First, we measured queen production in orphaned colonies, as evidence of their
143 capacity to requeen, and compared the rate of worker survival of orphaned and queenright
144 colonies under competition. Queenless colonies may suffer dramatic reduction of colony
145 efficiency because of the costs associated with the massive worker reproduction, such as
146 agonistic interactions, oophagy and lowered investment in foraging and colony maintenance
147 tasks (Bocher et al., 2008; Clémencet et al., 2008; Malka, Shnieor, Katzav-Gozansky, &

148 Hefetz, 2008; Miller & Ratnieks, 2001). We therefore expected a lower worker survival rate
149 in orphaned than in queenright colonies.

150

151 Second, we tested for the occurrence of cheating lineages in both orphaned and
152 queenright colonies. In orphaned colonies, we compared the paternity frequencies in workers
153 and gynes. Differential reproductive successes of patrilineages would result in a discrepancy
154 between these two frequencies. Under laboratory conditions, a previous study show that some
155 patrilineages produced significantly higher fractions of gynes than as expected according to their
156 frequencies among workers, suggesting that they had a higher reproductive success than the
157 other patrilineages and that the frequency of cheating lineages was low (Chéron, Monnin, et al.,
158 2011). However, new queens were collected at the pupal stage and it thus remains possible
159 that cheating behaviours could take place at a later developmental stage, such as by selective
160 killing of new queens from other patrilineages, and thus possibly result in more pronounced
161 differential success of competing patrilineages and higher levels of cheating. On the other hand,
162 contrary to laboratory settings, selective killing of brood could be avoided in natural nests by
163 physically separating brood of different patrilineages. In our experiment, we determined in
164 queenright colonies whether some gynes had been produced by workers instead of by the
165 queen. In previous studies, no worker-produced gynes had been detected among more than
166 200 gynes produced by queenright colonies in the field (Doums, Cronin, et al., 2013).
167 However, some diploid males had been produced by worker thelytoky in queenright colonies,
168 showing that some workers attempted to reproduce in presence of the queen (Doums, Ruel, et
169 al., 2013).

170

171 Third, because orphanage may be a time of fragility with weakened colony integrity,
172 we checked whether drifting occurred and whether drifting workers reproduced, as suggested
173 in a previous study (Chéron, Monnin, et al., 2011).

174

175

176 MATERIAL AND METHODS

177

178 *Colony collection and experiment*

179

180 At the end of the hibernation period (8th to 10th March 2011), 18 colonies were completely
181 excavated in the population of Argelès-sur-Mer near Perpignan (42.5722°N, 3.0437°E). They
182 contained an average of 961.8 workers (range: 427-2101) and no brood (Table A1). Two of
183 these colonies were collected with no queen. Whether the queen died during hibernation or
184 was lost during excavation is unclear. Colonies were then paired by colony size, with each
185 pair comprising one queenright and one queenless colony (either collected with no queen or
186 experimentally orphaned). Workers from colonies collected queenless should not behave
187 differently than workers from experimentally orphaned colonies because eggs laying had not
188 started at the time of collection. Removed queens were stored in 90% Ethanol (10% TE). The
189 queen of queenright colonies was marked with a dot of paint (UniPaint marker, Mitsubishi
190 pencil Co Ltd) and returned to its colony.

191

192 Colonies were transplanted into experimental enclosures located at the Mediterranean
193 Garden of Mas de la Serre (42.4740°N, 3.1161°E) in Banyuls-sur-mer, i.e. 12.5 Km from
194 their population of origin. Paired colonies were installed in the same enclosure (i.e. one pair
195 of colonies per enclosure) as described in Cronin et al. (2012). Enclosures were 10.8 m² in

196 area. They consisted of a circular, vertical, metal chamber 1 m high filled to a height of
197 approximately 0.5 m with a 1:1 sand:earth mix (see details in Cronin et al. 2012). Enclosures
198 have the same number than in Cronin et al. (2012), and enclosure #7 was not used.

199

200 Colonies were recovered from the enclosure at the time of sexual production (23rd to
201 29th of May 2011). In *C. cursor*, colony fission (i.e. the founding of new colonies by queens
202 with the help of workers) takes place soon after sexual production (Chéron, Cronin, et al.,
203 2011; Lenoir, Quérard, Pondicq, & Berton, 1988). We timed the collect of colonies to
204 maximize the probability that most sexuals would have emerged or be sufficiently advanced
205 in their development to be sexed by dissection of large cocoons. At the same time, we paid
206 attention to collect colonies before monogyny was restored (i.e. before all but one gynes were
207 killed) to avoid losing gynes necessary for genetic analyses. Although we were largely
208 successful and indeed collected a large number of sexuals, colony fission had started in five of
209 nine enclosures (Figure 1). All nests except one (the queenright nest in enclosure 2) were fully
210 excavated, and we censused the number of workers, gynes, males and large cocoons (i.e.
211 cocoons of sexual). Large cocoons were opened and all were at the pupal stage and could be
212 sexed by morphology. In enclosure 2, the nest with the marked queen not found in May was
213 discovered in September, when we checked the enclosure for another purpose. The nine
214 marked queens were therefore eventually all recovered. Colonies are identified by the number
215 of the enclosure followed by QR or QL for queenright and queenless (orphaned) colonies,
216 respectively, and a letter identifying colonies that were initially transplanted in the enclosure
217 (a) and new colonies that they produced by fission (b to d). For instance, 1-QR-a and 1-QL-b
218 design the queenright colony initially transplanted in enclosure 1 and a new colony produced
219 by fission of the orphaned colony of enclosure 1, respectively.

220

221 *Success of queenright and queenless colonies*

222

223 The queenright colony could be identified by the presence of the marked queen in all
224 enclosures, but when more than one queenless nest was recovered their origin was uncertain.
225 Queenless nests from the five enclosures where fission had started were assigned to either the
226 queenright or the orphaned colony using the genotypes of two workers per nest (see below
227 genetic analysis).

228

229 The ratio of workers lost during the course of the experiment was compared between
230 orphaned and queenright colonies using a non parametric Wilcoxon paired test. Colonies were
231 paired by enclosure to control for potential differential success among enclosures (Cronin et
232 al. 2012). Given that there is no hibernating brood and that worker emergence only starts at
233 the time or just after sexual production, colonies can only lose workers between March (time
234 of transplantation) and May (time of collection). We calculated the ratio of workers lost as the
235 difference in worker number between March and May divided by the number of worker in
236 March. When a colony had fissioned into several nests, we pooled the nests belonging to the
237 same colony (identified through the genetic analysis above) to obtain colony size in May and
238 reproductive success.

239

240 We compared sexuals production by orphaned and queenright colonies after parentage
241 identification of sexuals (see genetic analysis below) using a Wilcoxon paired test. As for the
242 ratio of workers lost, we pooled the sexuals from newly founded nests with those from the
243 corresponding mother colony. To determine the number of males produced, we removed the
244 foreign males identified by the genetic analysis (see below) from the number of males
245 collected.

246 Note that for enclosure 2, the queenright nest was not found in May but was collected
247 a few months later as explained above, hence enclosure 2 was removed to perform the
248 Wilcoxon paired tests.

249

250 *Genetic analysis*

251

252 To identify the mode of reproduction and the colony of origin of sexuals, we genotyped the
253 queens of experimentally orphaned (n=7) and queenright (n=9) colonies and all sexuals
254 collected (males: 128 adults and 29 pupae dissected out of large cocoons; gynes: 349 adults
255 and 56 pupae) at 8 microsatellites (Doums, Cronin, et al., 2013; Pearcy, Clémencet,
256 Chameron, Aron, & Doums, 2004) (multiplex 6: cc51, cc58, cc65, cc100 and multiplex 7:
257 cc61, cc11, cc46, cc99). In each enclosure, we genotyped around 20 workers from the
258 queenless nest that produced the more gynes, which was likely to be the transplanted
259 orphaned colony, to compare patriline distribution in workers and gynes. For enclosure 4, we
260 also genotyped 20 workers from the queenright nest because the results showed that three of
261 its ten gynes were produced by workers from the paired orphaned colony (see Results). We
262 also genotyped two workers from each smaller queenless nests, which were likely to be newly
263 founded nests, to assign them to either the initial orphaned or queenright colony as their
264 mother colony (see below). A total of 806 individuals were thus genotyped (16 queens, 227
265 workers, 157 males and 405 female sexuals, Table A1).

266

267 DNA was extracted from the head (half of the head for gynes) of each individual in
268 500 μ L of Chelex 10% with 20 μ L of protein K. The samples were incubated for 4h at 55°C
269 followed by 15 min at 100°C. After centrifugation, 20 μ L of supernatant was taken for
270 subsequent PCR. PCRs were carried out in a 10 μ L volume containing 1 μ L DNA solution

271 (10–40 ng of DNA), 150 μ M of each dNTP, 100 nM of each primer (except for Ccur65: 250
272 nM; Ccur99: 150 nM; Ccur100: 200 nM), 1X Taq buffer (with MgCl₂ 1.5 mM final) and 0.75
273 unit of Taq DNA polymerase (Q Biogen). Thermocycle conditions were as follows: 10 min at
274 94°C followed by 10 amplification cycles at 94°C for 15 s, 52°C for 15 s, 72°C for 30 s, 20
275 amplification cycles at 89°C for 15 s, 52°C for 15 s, 72°C for 30 s and a final elongation step
276 of 10 min at 72°C. Amplification products were loaded together on a 3500 Genetic Analyzer
277 (Applied Biosystems) and allele sizes were estimated using GenMapper 4.0 software. A
278 previous study detected no linkage disequilibrium or departure from Hardy-Weinberg
279 equilibrium in the population studied (Doums, Cronin, et al., 2013).

280

281 We used the software Colony (Jones & Wang, 2010) to assign workers to their mother
282 queen (and subsequently assigned the queenless nests in the enclosure in which fission had
283 started), determine worker patriline and, for the two colonies that had been collected
284 queenless, infer queen genotype. Colony was run assuming multiple mating by females, a
285 medium prior with a paternal sibship of four and a maternal sibship of 20. We provided the 16
286 available mother queens and assumed a probability of the mother being in these 16 queens of
287 0.9 (since two queens were missing). We were able to deduce the genotype of the mother
288 queen for only one of the two colonies that had been collected queenless (nest 3-QL-a).
289 Colony proposed up to eight mothers for the other queenless colony (nest 6-QL-a, see results)
290 hence we did not use it for comparing patriline distribution in workers and gynes.

291

292 *Origin and mode of production of gynes*

293

294 All gynes could unambiguously be identified as offspring of either the queen or workers of
295 the queenright or orphaned colony, because these queens had at least four diagnostic loci

296 (with no common alleles). The assignment was done manually because recombination during
297 thelytoky can lead to the transition from the heterozygous to the homozygous state (Pearcy et
298 al. 2011 for queens, Chéron, Monnin, et al. 2011 for workers). Hence at some loci, a worker-
299 produced gyne could be homozygous at a paternal allele and have no common allele with the
300 mother queen. We therefore only used heterozygote loci to assign the gynes to one of the two
301 possible colonies.

302

303 In queenright colonies, we assessed whether gynes were produced by sexual or
304 thelytokous reproduction of the mother queen, or by thelytokous reproduction of workers. We
305 compared the genotype of gynes with those of the mother queen. If all alleles of a gyne were
306 present in the queen, we concluded that the gyne had been produced parthenogenetically by
307 the queen. When at least one locus of a gyne was heterozygous with one allele present in the
308 mother queen and one allele not present in the mother queen, we concluded to sexual
309 reproduction of the queen. For a few gynes, we observed a maternal or non-maternal allele at
310 the homozygous state for one or two loci (see results) and concluded that the gyne had been
311 produced by thelytoky of a worker (see Doums, Cronin, et al. 2013 for additional
312 informations on the identification of worker produced gynes). In this last case, the PCR
313 amplification was performed a second time to confirm the genotype.

314

315 *Origin of males*

316

317 In *C. cursor*, workers do not produce males in presence of the queen (Doums, Ruel, et al.,
318 2013; Pearcy, Timmermans, Allard, & Aron, 2009). We therefore considered that males
319 carrying one or more alleles absent in the queen were foreigners. To investigate further the
320 origin of foreign males, we tested whether they could be the offspring of one of the other

321 queens from our enclosures using the software Colony. None of the 42 haploid foreign males
322 could be the offspring of a queen of our enclosures. Moreover, for six of the eight loci, the
323 allelic distribution was significantly different between the queens and the pool of foreign
324 males (fisher exact tests: $P = 0.02$ (L46); $P = 0.07$ (L51); $P < 0.001$ (L11); $P < 0.001$ (L58); P
325 $= 0.01$ (L61); $P < 0.001$ (L99); $P < 0.001$ (L100); $P = 0.27$ (L65)). Given that genetic
326 differentiation can be very high even at the scale of few kilometres in this species (Clémencet
327 et al., 2005), it is very likely that the foreign males originated from colonies naturally
328 occurring in the vicinity of the enclosures.

329

330 *Comparison of worker and gyne patriline distribution in queenless nests*

331

332 The genotypes of 201 workers were used for determining patriline distribution in orphaned
333 colonies and in the queenright colony of enclosure 4 using the software Colony.
334 Unfortunately, we could not consider nest 6-QL-a because it was collected queenless before
335 the onset of the experiment and it was not possible to deduce the genotype of the queen from
336 workers' genotypes (see above). We considered the diploid males as gynes since they are
337 produced by thelytokous reproduction of workers (Doums, Ruel, et al., 2013). We determined
338 the patriline of the diploid males visually (see above). The distribution of paternity frequency
339 was compared between workers and gynes using a Fisher exact test with the null hypothesis
340 that there was no difference in patriline distribution.

341

342 All statistical analyses were conducted using the R program (R Core Team, 2016) with
343 RStudio (RStudio Team, 2016), and graphics were drawn with ggplot2 package (Wickham,
344 2009).

345

346

347 RESULTS

348

349 *Success of queenright and queenless colonies*

350

351 At least two nests were found in each enclosure, and they matched the two colonies initially
352 transplanted in the enclosures. The process of colony fission had started at the time of colony
353 collection in five enclosures where more than two nests were recovered (Figure 1). Newly
354 founded nests contained fewer workers (median 56 workers, quartiles 40-103) and no or few
355 gynes and hence could not be confounded with the two colonies initially transplanted, which
356 were more populous (median 314 workers, quartiles 280-479) except for nest 8-QL-a (Figure
357 1). The genetic analysis of two workers from each of the 12 newly founded nests allowed to
358 unambiguously assign them to their mother nest. Nine (75%) originated from orphaned
359 colonies and three from queenright colonies (Figure 1). This demonstrates that orphaned
360 colonies can found new colonies by fission.

361

362 The ratio of workers lost during the 11 weeks spent in the enclosures varied from 0.32
363 to 0.68 (Figure 2a). Orphaned colonies lost a higher ratio of workers than paired queenright
364 colonies in all but one enclosure, and overall worker loss was significantly higher for
365 orphaned colonies (median of 0.58 vs. 0.41, respectively, Wilcoxon paired test : $V = 2$, $ddl =$
366 7 , $p = 0.023$; Figure 2a).

367 All orphaned colonies produced gynes, and these gynes were all thelytokous offspring
368 of natal workers (i.e. workers that were themselves daughters of the mother queen that we
369 experimentally removed or inferred). In *C. cursor*, worker thelytoky allows requeening in the
370 laboratory (Cagniant, 1980; Chéron, Monnin, et al., 2011). This is the first unambiguous

371 demonstration that it also allows requeening under semi-natural conditions and despite the
372 presence of a queenright competitor colony.

373

374 We compared sexuals production by orphaned and queenright colonies after parentage
375 identification of sexuals (see below). As for the ratio of workers lost, we pooled the sexuals
376 from newly founded nests with those from the corresponding mother colony. A total of 405
377 gynes were produced, with 79% produced by orphaned colonies. All orphaned colonies
378 produced gynes whereas less than half queenright colonies did (Figure 1). Six out of eight
379 orphaned colonies produced more gynes than the paired queenright colony, even though this
380 was marginally not significant (Wilcoxon paired test: $V = 4$, $ddl = 7$, $p = 0.055$; Figure 2b).
381 On the opposite, orphaned colonies produced only eight males and they were all diploid (see
382 below), while two queenright colonies produced a total of 98 haploid males (57 and 41)
383 (Figure 1).

384

385 Orphaned colonies that produced many sexuals experienced more worker reproduction
386 and associated conflicts than colonies that produced few sexuals. If reproduction and conflicts
387 are costly the former suffered a higher cost which may have resulted in a higher loss of
388 workers. Supporting this idea, we found a positive significant correlation between the rate of
389 worker loss and the number of sexuals produced in orphaned (Wilcoxon paired test, $P =$
390 0.004) but not in queenright colonies (Wilcoxon paired test, $P = 0.11$).

391

392 *Origin and mode of production of gynes*

393

394 Orphaned colonies produced numerous gynes (320) and all were thelytokously-produced by
395 workers. In contrast, in the four queenright colonies that produced gynes most gynes were

396 thelytokously-produced by the mother queen (72 out of the 85 gynes i.e. 85%, including all
397 gynes from nests 1-QR-a, 3-QR-a and 5-QR-a, Figure 1). The remaining 12 gynes were non-
398 clonal and found in two nests. Two from the newly founded nest 3-QR-b were produced by
399 worker thelytoky. However, it is unclear if these gynes were produced in presence of the
400 queen. Indeed, nest 3-QR-b is a small new nest and it is possible that it had been founded by
401 workers (by accidental colony fission) following the implantation of colonies in the enclosure.
402 Ten non-clonal gynes were found in nest 4-QR-a. Five were likely worker-produced, two
403 were either worker-produced or sexually-produced by the queen, and the remaining three
404 were offspring of foreign workers (they had at least four heterozygote loci with no alleles in
405 common with the mother queen, Table A2). It is telling that these three foreign gynes
406 belonged to two patriline (P1 and P3) found in the orphaned colony from the same enclosure
407 (4-QL-a). This strongly suggests that worker drifting and intra-specific social parasitism had
408 occurred, and this is corroborated by the finding that one of the 19 workers screened in
409 queenright colony 4-QR-a also belonged to the same patriline (P1) from the orphaned colony.
410 There is therefore little doubt that drifting occurred from the orphaned to the queenright
411 colony and was followed by reproduction, i.e. that social parasitism occurred.

412

413

414 *Origin of males*

415

416 Out of the 121 males found in queenright colonies, 15 could be considered as foreigners as
417 they had six to seven alleles different from those of the queen. Out of the remaining 106
418 males, one was diploid (nest 1-QR-a) and had a genotype similar to gynes, suggesting that it
419 resulted from inbreeding linked to thelytokous reproduction by the queen. The remaining 105

420 males were found in nests 1-QR-a, 2-QR-b and 8-QR-a and resulted from queen arrhenotoky
421 (Figure 1).

422

423 Far fewer males (36) were found in orphaned colonies. All haploid males (27) were
424 likely foreigners as they harboured one to eight alleles absent from workers. All these foreign
425 males were adults and collected in nests producing gynes. Nine males were diploid, of which
426 eight belonged to the same patriline as gynes and could have been produced by worker
427 thelytoky while the remaining one was probably a foreigner.

428

429 *Comparison of worker and gyne patriline distribution in queenless nests*

430

431 A total of 201 workers were used to determine patriline distribution (nest 6-QL-a was
432 excluded as the genotype of the mother queen could not be determined, see M&M). All but
433 two were offspring of the mother queen. One of these two foreign workers (in nest 8-QL-a)
434 could not be the offspring of any of our sampled queens. The second foreign worker (in nest
435 4-QR-a) came from the orphaned colony of the same enclosure (nest 4-QL-a). The
436 distribution of patrilines significantly differed between gynes and workers in three out of the
437 eight colonies tested (Figure 3). In one of these three nests, most gynes belonged to patrilines
438 not sampled in workers (Figure 3, nest 2-QL-a).

439

440 DISCUSSION

441

442 In many species of social insects, orphanage results in colony death. In others, the lost queen
443 is replaced and the colony carries on, yet orphanage is a crisis situation during which colony
444 functioning is disturbed and poor colony growth ensues. In addition, orphaned colonies are

445 predicted to be particularly prone to reproductive conflicts in polyandrous species (Châline,
446 Arnold, Papin, & Ratnieks, 2003; Chéron, Monnin, et al., 2011; Hughes & Boomsma, 2008),
447 especially in species where workers can reproduce by thelytokous parthenogenesis (Greeff,
448 1996). Our experiment under semi-natural conditions shows that in the thelytokous ant *C.*
449 *cursor*, orphaned colonies lost a larger ratio of workers than queenright colonies, yet
450 produced many gynes as well as new incipient nests. This is to our knowledge the first
451 unambiguous demonstration that orphaned *C. cursor* colonies can requeen and fission under
452 quasi-natural conditions even though worker reproduction was costly. The results also suggest
453 the occurrence of cheating patriline at a low frequency, in both orphaned and queenright
454 colonies, as well as the occurrence of intra-specific social parasitism, with some workers from
455 one orphaned colony drifting into the paired queenright colony and producing gynes.

456

457 Thelytokous worker reproduction allows requeening of colonies that become
458 queenless during overwintering. However, orphaned colonies lost a larger ratio of workers
459 than queenright colonies, suggesting that their functioning was impaired. It is likely that many
460 workers attempted to produce gynes following orphanage and that this was costly. In the
461 laboratory, orphanage results in many workers laying eggs and the production of a huge
462 amount of eggs (Cagniant, 1980; Clémencet et al., 2008). How the classic
463 reproductive/survival trade-off (Harshman & Zera, 2007; Stearns, 1992) applies at the
464 individual level in social insect is not clear (Kramer, Schrempf, Scheuerlein, & Heinze, 2015),
465 yet within the worker caste the high investment in reproduction may reduce survival.
466 Moreover, conflicts over reproduction may be costly at the colony level (Bocher et al., 2008;
467 Gobin, Heinze, Strätz, & Roces, 2003). For instance, the time devoted to reproduction cannot
468 be used for colony maintenance and laying workers may avoid the risk of foraging outside
469 (Roth et al., 2014), which could lead to a lack of resources. Such ergonomic costs of worker

470 reproduction are intuitive but hard to demonstrate (e.g. Bourke, 2011). Some indirect
471 evidence comes from the fact that colonies control worker reproduction in clonal species
472 reproducing asexually, where worker reproduction has no genetic cost (Hartmann, Wantia,
473 Torres, & Heinze, 2003; Teseo, Kronauer, Jaisson, & Châline, 2013). Also, in another species
474 workers are not policed but refrain from reproducing in incipient colonies, presumably
475 because the ergonomic costs of worker reproduction would be prohibitively costly in such
476 small colonies (Moore & Liebig, 2013). Direct costs of conflicts and of reproduction on
477 worker survival or on colony resources may therefore explain why orphaned colonies suffered
478 a higher worker loss than queenright colonies.

479

480 The number of new colonies that can be produced by colony fission is more limited by
481 the number of workers available than by the number of gynes (Cronin, Molet, Doums,
482 Monnin, & Peeters, 2013). In *C. cursor*, colonies fission into 4.0 ± 1.3 (mean \pm SD) colonies
483 in the field (Chéron, Cronin, et al., 2011), or into 2.35 ± 0.88 colonies in our experimental
484 enclosures where competition for resources may be more severe owing to the limited space
485 (Cronin et al., 2012). In spite of this, orphaned colonies produced twice as many gynes in the
486 current experiment (36 on average) than colonies fissioning in the field (14 on average,
487 Doums, Cronin, et al. 2013). This suggests that they produced more gynes than as required for
488 requeening and colony fission. We argue that this is because worker thelytoky allows
489 requeening but also results in reproductive competition between workers, with the effect of an
490 excess production of gynes. Consequently, most gynes die when monogyny is restored in new
491 colonies, or else many new colonies are produced but they are small hence have reduced
492 competitive abilities. Either way, the mass production of gynes appears non-optimal at the
493 colony level for queenless colonies. This may be because of the conflict over reproduction

494 between workers, or because selection for an efficient requeening strategy is too low which
495 could occur for instance if orphanage is rare.

496

497 Reproductive cheating occurred in three out of nine orphaned colonies, where some
498 patriline produced more gynes than their fair share. A similar proportion (3/13) had
499 previously been observed under laboratory conditions and at an earlier pupal stage (Chéron,
500 Monnin, et al., 2011). Comparison of patriline distribution in workers and gynes is not
501 sufficient to clearly demonstrate cheating. Other factors such as differential survival of
502 patrilines in workers (Clémencet et al., 2008) or queen turnover could also generate
503 differences in patriline distribution. The latter probably occurs in nest 2-QL-a, where 14
504 patrilines were detected with clear difference between worker and gyne patrilines distribution.
505 Cheating is also suggested by the occurrence of worker reproduction in one queenright
506 colony. These results are in apparent contrast with another study where workers produced no
507 gyne in presence of the queen (all gynes were queen-produced, by sex or thelytoky, Doums,
508 Cronin, et al. 2013), even though attempts of worker reproduction nevertheless occurred as
509 revealed by the presence of worker-produced diploid males (Doums, Ruel, et al., 2013).
510 Interestingly, the queenright colony where workers reproduced was the only one with a higher
511 rate of workers loss than in the paired queenless colony (Figure 2, enclosure 4). This suggests
512 that worker reproductive conflicts may have occurred in this colony, as it did in queenless
513 colonies. Overall, our results thus suggest the occurrence of cheating at low frequencies, and
514 in both queenright and queenless contexts. This is expected on relatedness grounds because
515 the pattern of selection for gyne production through worker thelytoky parallels that for male
516 production in polyandrous species (Ratnieks, 1988; Wenseleers & Ratnieks, 2006). Workers
517 are collectively not expected to reproduce because they are equally or more related to queen-
518 produced gynes (by sex, $r=0.4$; by thelytoky, $r=0.5$) than to worker-produced gynes ($r=0.4$)

519 (Doums, Cronin, et al., 2013). However, cheaters can occur at a low frequency because they
520 are more related to clonal female offspring of their patriline (0.75) than to any other female
521 offspring (own patriline > mother queen > other patrilines). Because cheaters exploit the
522 benefits of cooperation they should increase in frequency, until they reach an equilibrium with
523 cooperators if the fitness of cheaters is negatively frequency dependent, or until they invade
524 the population and cooperation stops, at which points cheaters cease to exist as such as there
525 is no more sociality to exploit (Ghoul et al., 2014). Here, the benefits of cheaters are clearly
526 frequency dependent in regards of relatedness, and they would decrease if too many workers
527 were to reproduce. Consistently with this, the cheating lineages detected so far have always
528 been found to be rare in colonies and/or in populations (Härtel et al. 2006; Hughes and
529 Boomsma 2008; Chéron, Monnin, et al. 2011, this study). Another factor limiting the
530 frequency of cheaters is that they likely trade-off their investment in colony maintenance and
531 brood rearing towards reproduction (Roth et al., 2014). This cost may be negligible when
532 cheaters are rare but may become important if they increase in frequency. One factor that may
533 favour cheaters is that they may be more likely to engage in social parasitism than other
534 lineages, in which case they would provide some inclusive fitness to the other worker
535 patrilines.

536

537 Our experiment showed that workers from one orphaned colony (nest 4-QL-a) drifted
538 into the paired queenright colony (4-QR-a) where they produced gynes (Figure 1). This is the
539 first unambiguous demonstration of intra-specific social parasitism in *C. cursor* under natural
540 conditions, and it corroborates the finding of a previous study under laboratory conditions in
541 which foreign workers present in one colony produced a disproportionably high number of
542 gynes (Chéron, Monnin, et al., 2011). The drifting of workers into unrelated colonies is well
543 known in social insects, including in *C. cursor* (Chéron, Monnin, et al., 2011; Doums, Cronin,

544 et al., 2013). However, it remains largely unclear whether it is an accidental phenomenon, as
545 in the thelytokous Cape honey bees (reviewed in Goudie and Oldroyd 2014), or an active
546 process, as in *Bombus terrestris* (Blacher et al., 2013). Orphaned colonies may be more
547 susceptible to social parasitism by foreign workers. They are weakened by the loss of many
548 workers and disorganised by the onset of worker reproduction, so that foreign workers may
549 succeed entering colonies more often. In addition, orphaned colonies stop policing worker
550 reproduction hence foreign workers that have succeeded entering the colony are more likely
551 to reproduce unchecked. In our experiment, orphaned colonies did not suffer more from social
552 parasitism as we found only one foreign worker in one of the nine orphaned colonies, which
553 is less than in the field where foreign workers occurred in 6 out of 13 colonies (Chéron,
554 Monnin, et al., 2011). This may be because colonies react to orphanage by tightening the
555 scrutiny of individuals entering the nest, as occurs in orphaned honeybee colonies that face a
556 similar threat from social parasitism and respond by being more discriminatory against
557 foreign workers (Chapman, Makinson, et al., 2009). Alternatively, orphaned colonies may be
558 a source of social parasitic workers if the intense reproductive conflicts between workers
559 result in some workers absconding or being expelled from the colony. That is, social
560 parasitism may be a side effect of the massive worker reproduction in orphaned colonies
561 rather than an evolved parasitic strategy (Blacher et al., 2013). Our data support this
562 hypothesis to some extent as we found gynes produced by workers from the orphaned colony
563 in one of the four queenright colonies that produced gynes (colony 4-QR-a).

564

565 Finally, our results could also shed some light on the potential factors affecting the
566 evolution of thelytokous parthenogenesis in this species. In general, thelytoky provides some
567 benefits compared to sexual reproduction, such as the avoidance of the twofold cost of sex, a
568 reproductive insurance under low population density and the maintenance of co-adapted gene

569 pool (Bell, 1982; Hurst & Peck, 1996; Meirmans, Meirmans, & Kirkendall, 2012). These
570 benefits also occur in social organisms if the queen reproduces through thelytoky, but other
571 selective forces come into play when workers use thelytoky as in *C. cursor*. At the colony
572 level, worker thelytoky is beneficial by allowing requeening after the death of the queen
573 (Lenoir et al., 1988). Our results show that this factor could indeed play a role in *C. cursor*,
574 even though the frequency of colony orphanage is unknown. Queens are often replaced by
575 young queens during colony fission (Chéron, Cronin, et al., 2011) so we surmise that old
576 queens may be relatively rare and hence orphanage may be relatively uncommon. Assuming
577 that colonies collected queenless result from natural orphanage and not from the loss of the
578 queen during collection, we can estimate an upper limit to the frequency of orphanage at the
579 end of hibernation. Based on this paper and on two other field collections carried out in
580 March, we found 5.5% of queenless colonies at collection (2/18 queenless colonies in 2011
581 (this paper); 0/13 in 2013; 1/19 in 2015 (Doums & Monnin, n.d.)). Whether worker thelytoky
582 could allow requeening or survival of colonies that become orphaned at other times than early
583 spring, when sexuals are produced, is unclear because gynes would remain virgin. Orphaned
584 colonies would thus have to produce the workers necessary for their survival by thelytoky (of
585 gynes and/or workers) until the next mating season, but the high worker loss experienced by
586 orphaned colonies in our experiment suggests this is unlikely. Therefore, the benefits obtained
587 by requeening may be insufficient to explain the evolution of worker thelytoky. At the
588 individual level, worker thelytoky may be favoured by the benefits obtained by cheating,
589 allowing workers to rewind the tape of social evolution in some ways (Gadagkar, 1997).
590 However, worker reproduction in presence of the queen and cheating patriline in queenless
591 colonies are less frequent than in the Cape honey bees (Allsopp et al., 2010; Goudie &
592 Oldroyd, 2014; Moritz et al., 2011). Ultimately, worker thelytoky may be better explained by
593 the benefits thelytoky confers to the queen caste (Doums, Cronin, et al., 2013; Pearcy, Aron,

594 et al., 2004). Indeed, intercaste genetic correlations could constrain the evolution of queen-
595 worker dimorphism such that worker thelytoky may be a nonadaptive “caste load” from
596 positively selected thelytoky in queens (Holman et al 2013). More studies are required to
597 confirm the cost and benefits of thelytoky in both castes.
598

599 DATA ACCESSIBILITY STATEMENT

600 Genetic analyses reported in this article can be reproduced using the data published in
601 Mendeley (doi:10.17632/83j7xtw6fm.2).

602

603

604 ACKNOWLEDGEMENTS

605 We thank the Laboratoire Arago (Observatoire Océanologique de Banyuls-sur-mer,
606 Université Pierre et Marie Curie) for permitting this study to take place on the experimental
607 grounds of the Mediterranean Garden of Mas de la Serre. We thank David Sillam-Dussès,
608 Fabien Aubrun, Romain Péronnet and Claire Tirard for helping in collecting colonies.

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825

826 FIGURE CAPTIONS

827 Figure 1: The nine enclosures are depicted as black rectangles with their number between
828 parentheses (enclosure #7 was not used). Circles represent colonies. In each enclosure, the
829 two circles at the top of the rectangle are the two transplanted colonies. The red and crowned
830 circle is the queenright colony and the green circle is the orphaned colony (# identify the two
831 colonies that were collected queenless). Queenright and orphaned colonies were identified by
832 the presence of the queen and by their large production of gynes, respectively. Smaller circles
833 show propagules with the same colour than their mother colony (as determined by genetic
834 analysis, see Results). The areas of circles are proportional to colony size at the time of
835 recollection, and are standardised relative to the largest nest within each enclosure. The
836 number of sexuals collected in each colony is given in light orange for gynes, light and dark
837 blue for haploid and diploid males, respectively (all diploid males were produced by
838 thelytoky), and grey for foreign males. All sexuals from queenright colonies were produced
839 asexually by the queen except in enclosure 4 where they were non clonal (*) and where seven
840 were worker-produced, including three originating from the queenless nest. ** shows worker-
841 produced gynes.

842

843

844 Figure 2: Worker loss ratio (a) and gynes production (b) in queenright (QR) and queenless
845 (QL) colonies. Worker loss ratio is the ratio of the number of workers lost during the 11
846 weeks spent in the enclosures divided by the initial colony size. Each dot represents one
847 enclosure labelled with its number (enclosure #7 was not used). Dotted lines correspond to an
848 equal ratio of worker loss and of gyne production, i.e. dots below the lines correspond to
849 enclosures where QR colonies had higher worker loss (a) or higher gyne production (b) than
850 QL colonies (and conversely above the lines).

851 Figure 3: Distribution of patriline in workers (dark grey) and gynes (light grey) for the eight
852 orphaned colonies and for the queenright colony of enclosure 4. The data are depicted as the
853 number of workers or gynes for each nest. The probability value of the Fisher test testing the
854 null hypothesis of no difference in patriline distribution is given between parentheses in the
855 title for each nest. In nest 4-QR-a, patriline 4AP1 (marked with ****) corresponds to patriline
856 1 of neighbouring nest 4-QL-a.

857

858

859 **Table A1:** Size and composition of colonies.

Enclosure	Nests	Workers implanted	Workers recollected	Worker genotyped	Sexuals recollected
1	QRa	1,570	824	0	86
	QLa	1,868	465	20	66
	QLb		167	2	1
	QLc		61	2	1
	QLd		82	2	0
2	QRa*	884	632	0	0
	QRb		448	2	7
	QLa	1,298	665	20	75
3	QRa	886	484	0	52
	QRb		38	2	2
	QLa [#]	960	277	20	36
	QLb		45	2	2
	QLc		60	2	1
4	QRa	652	267	20 (1 from A-QL)	12
	QLa	682	320	20	6
5	QRa	505	221	0	5
	QRb		53	2	1
	QLa	1,048	462	21	67
	QLb		9	2	0
6	QRa	606	410	0	0
	QLa [#]	427	135	20	13
8	QRa	1,760	1073	0	41

	QLa	2,101	298	23 (1 foreign)	41
	QLb		433	2	9
	QLc		41	2	0
	QLd		13	2	0
9	QRa	511	298	0	0
	QLa	564	309	20	27
10	QRa	429	269	0	0
	QLa	562	290	20	11

860 The table gives the number of workers implanted in enclosures in March (enclosure #7 was
861 not used), recollected from enclosures in May and genotyped, as well as the number of
862 sexuals collected (all sexuals were genotyped). Nests recollected in enclosures were assigned
863 to the corresponding implanted queenright (QR) or queenless (QL) colony based on the
864 presence of the marked queen or on the genetic analysis of workers (see M&M). Queenright
865 colonies and the new nests they produced are highlighted in bold. * this nest was not found in
866 may but collected in September. # these nests were collected orphaned.
867

868 **Table A2:** genotypes of the queens of nests 4QRa and 4QLa, of their mates inferred from worker genotypes, and of the 10 gynes produced by
 869 nest 4QRa.

Individuals	L46	L51	L11	L58	L61	L99	L100	L65	origin
Nest 4QRa									
Queen	161/161	196/198	225/245	150/150	245/255	126/126	189/193	248/248	
Mate1	153	216	235	150	265	128	195	248	
Mate2	153	206	235	171	253	128	177	248	
Mate3	153	198	235	150	217	130	193	256	
Mate4	157	204	235	150	241	118	193	248	
Mate5	153	196	235	150	225 or 245	130	193	256	
Mate6	161	188	239	171	251	126	177	250	
Mate7	155	198	225 or 245	150	225	126	191	250	
Gyne G1	153/161	198/198	235/235	165/178	231/253	118/130	191/195	248/250	4QL mate1
Gyne G9	157/157	198/200	235/235	165/178	?/?	118/118	191/191	248/250	4QL mate3
Gyne G11	153/153	196/198	235/259	165/178	231/243	118/130	191/195	246/248	4QL mate1
Gyne G10	<u>161/161</u>	198/216	235/245	150/150	?/?	126/128	189/195	248/248	<u>4QR mate1</u>

Gyne G2	155/161	196/198	<u>245/245</u>	150/150	225/255	126/126	189/191	248/250	<u>4QR mate7</u>
Gyne G4	153/161	198/216	235/245	150/150	255/265	<u>128/128</u>	193/195	248/248	<u>4QR mate1</u>
Gyne G5	153/161	196/216	<u>225/225</u>	150/150	255/265	126/128	193/195	248/248	<u>4QR mate1</u>
Gyne G6	157/161	196/204	225/235	150/150	241/255	118/126	189/193	248/248	4QR mate4
Gyne G7	<u>161/161</u>	198/216	235/245	150/150	??	126/128	193/195	248/248	<u>4QR mate1</u>
Gyne G8	153/161	198/216	235/245	150/150	245/265	126/128	193/195	248/248	4QR mate1

Nest 4QLa

Queen	153/157	196/198	235/259	165/171	243/253	118/118	185/191	250/246
Mate1	161	198	235	178	231	130	195	248
Mate2	157	200	235	178	233	118	191	248
Mate3	157 or 153	196	235	150	253	118	193	248
Mate4	153	200	235	150	233	138	193	248
Mate5	157 or 153	196 or 198	235 or 239	150	255	116	193	250 or 246

870 All gynes were sexually produced. Three were daughters of workers from the queenless nest 4QLa (highlighted in bold), five were probably
871 produced by workers of the queenright nest 4QRa, and two could have been produced either sexually by the queen or by workers of the
872 queenright nest 4QRa. The homozygous genotypes showing that gynes were probably parthenogenetically produced by workers are underlined.