

**1 Origin, behaviour, and genetics of reproductive
2 workers in an invasive ant**

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12

13 **Abstract**

14 **Background:** Worker reproduction has an important influence on the social cohesion and
15 efficiency of social insect colonies, but its role in the success of invasive ants has been
16 neglected. We used observations of 233 captive colonies, laboratory experiments, and genetic
17 analyses to investigate the conditions for worker reproduction in the invasive *Anoplolepis*
18 *gracilipes* (yellow crazy ant) and its potential cost on interspecific defence. We determined
19 how common worker production of males is and whether it is triggered by queen absence;
20 whether physogastric workers with enlarged abdomens are more likely to be reproductive,
21 how normal workers and physogastric workers compare in their contributions to colony
22 labour and defence; and whether worker-produced males and males that could have been
23 queen- or worker-produced differ in their size and heterozygosity.

24 **Results:** Sixty-six of our 233 captive colonies produced males, and in 25 of these, some
25 males could only have been produced by workers. Colonies with more workers were more
26 likely to produce males, especially for queenless colonies. The average number of days
27 between the first appearance of eggs and adult males in our colonies was 54.1 ± 10.2 (mean \pm
28 SD, n=20). In our laboratory experiment, queen removal triggered an increase in the
29 proportion of physogastric workers. Physogastric workers were more likely to have yolked
30 oocytes (37-54.9%) than normal workers (2-25.6%), which is an indicator of fertile or trophic
31 egg production. Physogastric workers were less aggressive during interspecific aggression
32 tests and foraged less than normal workers. The head width and wing length of worker-
33 produced males were on average 4.0% and 4.3% greater respectively than those of males of
34 undetermined source. Our microsatellite DNA analyses indicate that 4.9% of worker-
35 produced males and 21.1% of males of undetermined source were heterozygous, which
36 suggests the presence of diploid males and/or genetic mosaics in *A. gracilipes*.

37 **Conclusions:** Our experimental work provides crucial information on worker reproduction in
38 *A. gracilipes* and its potential cost to colony defence. The ability of *A. gracilipes* workers to
39 produce males in the absence of queens may also contribute to its success as an invasive
40 species if intranidal mating can take place between virgin queens and worker-produced
41 males.

42

43 **Keywords:** *Anoplolepis gracilipes*, aggression tests, eusocial insects, haplodiploid,
44 Hymenoptera, *Oecophylla smaragdina*, oocytes, ovaries, worker conflict, worker
45 reproduction

46

47 **Background**

48 In social Hymenoptera, a caste of less reproductive individuals (i.e. workers) contributes to
49 colony labour while fecund individuals (i.e. queens) produce offspring. However, workers
50 sometimes challenge the reproductive primacy of the queen by producing male-destined eggs
51 [1,2]. In most species, including *Apis* honeybees, Meliponinae stingless bees, Vespinae
52 wasps, *Bombus* bumblebees, and most ants, workers possess ovaries but cannot mate [2].
53 Through the haplodiploid sex determination system of Hymenoptera, in which females (i.e.
54 workers and queens) are diploid and originate from fertilized eggs while males are haploid
55 and originate from unfertilised eggs (arrhenotoky), these workers can produce male-destined
56 eggs [3,4].

57 Despite worker reproduction not being beneficial to social Hymenoptera queens, workers
58 from queenright colonies produce males in 69 out of 90 taxonomically diverse studied
59 species (ants, sweat bees, bumblebees, honeybees, stingless bees, and wasps) for which

60 workers have functional ovaries [5]. Workers from most ant species have retained functional
61 ovaries and are able to lay male-destined eggs [2,6]. According to kin selection theory,
62 worker reproduction is beneficial at the worker level because workers tend to be more related
63 to their own sons (average degree of relatedness, $r=0.5$) than to their brothers (i.e. queen's
64 sons, $r=0.25$) [7]. However, in the case of queens mating multiple times, workers tend to be
65 more related to their brothers ($r=0.25$) than to other workers' sons ($r<0.25$) which favours
66 workers to police eggs laid by other workers [8,9]. The queen should always prefer to invest
67 in her own sons, which are more related to her ($r=0.5$) than her grandsons ($r=0.25$). Queens
68 from several ant species can inhibit worker fertility via pheromones and therefore, workers
69 are most reproductive in the absence of a queen ([2] e.g. *Neoponera apicalis* [10];
70 *Camponotus floridanus* [11]; *Lasius niger* [12]). If the queen dies, the production of males by
71 workers advantages both workers and queens because it is the last opportunity for the
72 deceased queen to contribute to the gene pool.

73 Ant workers with functional ovaries can also produce trophic eggs (unviable eggs fed to the
74 colony) [2,13]. Trophic eggs are used to transfer proteins and nutrients to members of the
75 colony (especially queens and larvae) and can be an important source of nutrition for colony
76 members [10,13–16]. In some species, workers switch from trophic egg to male-destined egg
77 production in the absence of queens [2]. For example, *Oecophylla longinoda* workers
78 produce trophic eggs in queenright colonies and begin laying male-destined eggs one to two
79 months after being separated from the queen [17].

80 The production of males by workers can be costly and disrupt the social organisation of the
81 colony [18]. Colony productivity may decrease due to workers laying male-destined eggs and
82 exhibiting high levels of aggression toward other workers instead of contributing to colony
83 labour [18–20]. For example, worker reproduction led to a 15% reduction in time spent on
84 brood care for queenless colonies of *Temnothorax allardycei*, while worker dominance

85 behaviour to regulate worker reproduction in queenless colonies of *Pachycondyla*
86 *obscuricornis* incurred an energetic cost and a reduction in colony labour [19,20]. In the case
87 of non-invasive ants, worker reproduction is often associated with queen death or colony
88 decline [2].

89 Given the presumed costs of worker reproduction, we would not expect invasive ants to have
90 reproductive workers. However, worker reproduction was recently reported for the first time
91 in the yellow crazy ant (*Anoplolepis gracilipes*) [21], one of the world's worst invaders and
92 for which the reproductive mode is not fully resolved [21–23]. Workers with an unusually
93 distended abdomen (i.e. physogastric), from queenless *A. gracilipes* colonies collected in
94 Taiwan, had ovaries that were more developed than those of other workers and laid male and
95 trophic eggs [21]. Worker-produced males may produce viable sperm, but we do not know
96 their relative fitness compared to queen-produced males. Ploidy and male size can be
97 indicators of fitness as diploid males tend to be sterile [3,4,24] and male size is correlated
98 with fitness in some ant species [25,26]. Results from several genetic studies suggest that
99 heterozygous males are common in this species across its range (Borneo [23], Christmas
100 Island [27], Arnhem Land in Australia [28,29], Taiwan [21]) which would suggest *A.*
101 *gracilipes* males are often diploid.

102 We do not know whether worker reproduction contributes to or hinders the invasive success
103 of *A. gracilipes*. Worker reproduction in this highly successful invader [22,30] may be too
104 rare to impose a cost on colony success, or the benefits of worker reproduction (e.g.
105 production of fertile males) may outweigh its costs (e.g. reduction in colony labour).
106 Understanding colony dynamics when the queen dies and/or the colony declines may provide
107 insights relevant to the management and control of this invasive species.

108 We used a combination of observations, experiments, microscopy, and genotyping to
109 investigate the attributes, potential triggers, and costs of worker reproduction in *A. gracilipes*.

110 Our specific aims were to determine 1) how common worker production of males is in *A.*
111 *gracilipes* colonies and whether it is triggered by queen absence; 2) whether physogastric
112 workers are more likely to be reproductive; 3) how physogastric and normal workers
113 compare in their contributions to colony labour and defence; and 4) whether worker-produced
114 and queen-produced males differ in their size and observed heterozygosity.

115

116 **Results**

117 **Dissections of worker's ovaries of entire colonies**

118 Workers with two to four exposed intersegmental membranes (hereafter physogastric
119 workers) had a conspicuously enlarged gaster that was more likely to contain yolked oocytes
120 (54.9%, N=56/102), which may indicate the presence of fertile or trophic eggs [13,31], than
121 workers that had zero to one exposed intersegmental membrane (hereafter normal workers,
122 25.6%, N=10/39, GLM: binomial, ANOVA: $\chi^2=4.7578$, df=1, p=0.0292). The proportion of
123 physogastric workers was higher in queenless (mean \pm SD: 78.6 \pm 10.4%) than in queenright
124 colonies (mean \pm SD: 53.9 \pm 11.6%, GLM: binomial, ANOVA: $\chi^2=14.093$, df=1, p=0.0002).
125 Yellow bodies, which are characteristic of fertile eggs but are sometimes observed in trophic
126 egg-layers [13,32–34], were only observed in physogastric workers (10.7% in queenright
127 colonies and 12.9% in queenless colonies).

128 **Colony observations**

129 We observed males in 66 out of our 233 captive colonies (28%). Of the 66 colonies in which
130 males were observed, 44 were queenless throughout their captivity, and 22 had at least one
131 queen at some time in their captivity. Of the 22, 14 had at least one queen present when males
132 were first observed. Of the 167 colonies that never produced males, 70 were always

133 queenless. Colonies with more workers were more likely to produce males, especially if these
134 colonies were queenless. Male production was not significantly predicted by time in captivity
135 regardless of whether a colony was ever queenright (Fig. 1, Table 1).

136 In 25 of the 66 colonies with males produced, we could attribute at least some of the adult
137 males to workers. All 25 of these colonies came into captivity without queens, so we could
138 not determine how long they were queenless before males were produced, but we could
139 discern that the brood that produced males later in captivity were from workers. Seven of
140 these 25 colonies also had males emerge earlier in captivity from brood with which they
141 entered captivity and for which we therefore could not rule out a queen origin. We also could
142 not rule out a queen origin of males for an additional 36 colonies that either came in
143 queenless (22 colonies) or became queenless during captivity (1 colony) or were never
144 queenless (14 colonies). In five of the 66 colonies, the males could have been produced by
145 workers or alate queens.

146 We could attribute eggs, larvae, or pupae to workers in 35 colonies. On 20 occasions (in 19
147 colonies), we could trace the maturation of worker-produced brood from egg to adult male.
148 The number of days from the first sighting of worker-produced eggs to the first sighting of
149 adult males was 54.1 ± 10.2 days (mean \pm SD). The number of workers in the colony on the
150 day that worker-produced eggs were first observed was estimated as 10-541 (median = 180,
151 n=31).

152 We observed eight colonies in which one or more alate queens co-occurred with males. In six
153 of these the queens emerged in captivity so we knew there was no prior exposure to males. In
154 five of the six colonies in which the queens emerged in captivity, the queens subsequently
155 lost their wings (a possible indicator of mating) and in all of these, eggs appeared over
156 subsequent weeks. We could not determine whether these were laid by workers or queens.

157 **Male morphometry and genotyping**

158 Head width and wing length, but not Weber's length, or wing width, of worker-produced
159 males were significantly greater than for males of undetermined source (i.e. males that could
160 have been produced by dealate queens or alate queens or workers, Table 2). The Weber's
161 length of worker-produced males tended to be greater than for males of undetermined source
162 (Table 2). Six worker-produced males had conspicuous deformities: 2 out of 55 worker-
163 produced males for which we measured the head width had one eye that was oversized
164 compared to the other eye, and 4 out of 58 worker-produced males with intact wings had
165 abnormal wings (black and stubby wings or underdeveloped wing tips). All six of these
166 deformed males were hemizygous (Additional file 1: Table S1). Two out of 17 males of
167 undetermined origin for which we measured the head had one oversized eye and both were
168 heterozygous for at least one locus (Additional file 1: Table S1).

169 Most worker-produced males and males of undetermined source were hemizygous and more
170 than one worker produced males in at least one of the queenless colonies. We found that
171 4.9% of worker-produced males ($n=4/82$ males from 3/14 colonies), 21.1% of males that
172 could have been produced by dealate queens or workers ($n=4/19$ males from 3 colonies out of
173 4), and 0% of males that could have been produced by alate queens or workers ($n=0/9$ from 2
174 colonies) were heterozygous for at least one locus (Additional file 1: Table S1). In 11
175 colonies we genotyped more than one worker-produced male and found three different alleles
176 at Ano5 in one of these colonies (Additional file 1: Table S1), which indicates that more than
177 one worker produced males in this colony.

178 Most workers were heterozygous for at least one locus while most queens were homozygous.
179 We found that 98.1% ($n=53/54$ workers from 14/14 colonies) of genotyped workers from
180 colonies in which males were worker-produced were heterozygous. For colonies in which

181 males could have been produced by dealate queens or workers, 94.4% (n=17/18 workers
182 from 4/4 colonies) of workers genotyped were heterozygous while all workers (n=10/10
183 workers from 4/4 colonies) from colonies in which males could have been produced by alate
184 queens or workers were heterozygous (Additional file 1: Table S1). Eight queens out of nine
185 were homozygous at all loci and one queen was heterozygous at Ano4 (Additional file 1:
186 Table S1). Note that amplification failures (Additional file 1: Table S1) were due to some
187 individuals being already dead and potentially degraded at the time of collection.

188 Queen transfer experiment

189 The number of physogastric workers increased following queen removal (Table 1, Fig. 2,
190 Additional file 2: Fig. S1). We found similar proportions of physogastric workers in
191 queenright and queenless subcolonies from days 0 and 15 of the experiment (Fig. 2, Table 1,
192 post hoc tests p=0.1803 for day 0 and 0.1669 for day 15). At days 30, 45, and 60, there were
193 more physogastric workers in queenless than in queenright colonies (Fig. 2, Table 1, post hoc
194 tests p=0.0005 for day 30, p=0.0001 for day 45, and p<0.0001 for day 60).

195 Workers in the queenless subcolonies readily accepted the return of their corresponding
196 queen at day 60. The queens were either ignored or touched by workers for the first hour after
197 translocation (aggression score 0-1, [35]), and all six queens were tended by workers
198 (aggression score 1) inside a nest or under the egg carton after 24 hours. Three queens
199 survived until day 120.

200 The number of physogastric workers increased in the newly queenless subcolonies (hereafter
201 referred to as secondarily queenless) following queen transfer. Initially, secondarily queenless
202 subcolonies had significantly fewer physogastric workers compared to secondarily queenright
203 subcolonies (day 75 p=0.0071, day 90 p=0.0001, Additional file 2: Fig. S1). The trend
204 reversed from day 105 and physogastric workers became significantly more common in the

205 secondarily queenless subcolonies at day 120 (day 105 p=0.406, day 120 p=0.0481), although
206 there was large variation, probably resulting from the small number of surviving colonies
207 (n=3, Additional file 2: Fig. S1). At day 120, secondarily queenless subcolonies had 53 to
208 467 workers per colony and secondarily queenright subcolonies 42 to 113. Yolky oocytes
209 were not significantly more common in physogastric workers (mean ± SD: 41.7±30.5%,
210 n=119) than in normal workers (mean ± SD: 24.1±25.9%, n=52) dissected at 120 days
211 (GLMM: binomial, Type II Wald test: $\chi^2=3.5061$, df=1, p=0.0611). Only the ovaries of
212 physogastric workers had yellow bodies (7.8% had yellow bodies in queenright and 8.6% in
213 queenless colonies).

214 We observed a difference in behaviour between normal and physogastric workers. During
215 colony monitoring, we observed normal workers in the foraging area outside of the nesting
216 tubes more often than physogastric workers (in 81/100 observations of normal workers and
217 5/100 of physogastric workers) regardless of whether the colony was queenright or queenless
218 (GLMM: binomial, Type II Wald test, worker type: $\chi^2=55.5766$, df=1, p<0.0001, colony
219 status: $\chi^2=0.6344$, df=1, p=0.4257). We did not observe male production in any of the
220 colonies, including the six queenless colonies we monitored for the additional 60 days (i.e.
221 until day 180). These six colonies had 36 to 337 workers at the end of the observations (mean
222 ± SD=131.2 ± 97.9).

223 Aggression tests

224 Physogastric workers and workers from queenless colonies irrespective of whether they were
225 physogastric or normal were less aggressive than normal workers and workers from
226 queenright colonies. The highest aggression scores of trials with normal workers (Fig. 3a,
227 median: 5, range: 0-5, N=34) was higher than the aggression score of trials with physogastric
228 workers (median: 4, range: 0-5, N=36, Table 1). The highest aggression score of trials with

229 workers from queenright colonies was also higher (Fig. 3a, median: 5, range: 0-5, N=36) than
230 when the workers were from queenless colonies (median: 4, range: 0-5, N=34, Table 1). The
231 interaction between worker type and colony status was not significant (Table 1). *Anoplolepis*
232 *gracilipes* workers were more likely to initiate the fight in interspecific aggression tests
233 against *Oecophylla smaragdina* if they were from queenright colonies as opposed to
234 queenless or were normal workers as opposed to physogastric (Fig. 3b, Table 1). *Oecophylla*
235 *smaragdina* workers were less likely to survive if they were fighting against normal workers
236 vs. physogastric workers from queenright colonies, but physogastry did not affect their
237 survival if *A. gracilipes* workers originated from a queenless colony (post hoc tests p<0.05
238 between normal and physogastric workers from queenright colonies and p>0.05 from
239 queenless colonies) (Fig. 3c, Table 1). The survival of *A. gracilipes* was not influenced by
240 physogastry or whether they originated from a queenless or queenright colony (Fig. 3c, Table
241 1). Physogastric workers dissected after the aggression trials were more likely to have yolky
242 oocytes (N=40/108) than normal workers (N=2/102, Table 1). We only observed yellow
243 bodies in physogastric workers (9.3% had yellow bodies in queenright colonies and 12.3% in
244 queenless colonies).

245

246 **Discussion**

247 Our experimental work significantly improves our understanding of worker reproduction in
248 the invasive *A. gracilipes*. We could attribute male production to workers in 25 captive
249 colonies out of 66 male-producing colonies. In our queen transfer experiment, the absence of
250 a queen triggered an increase in physogastric workers suggesting the existence of queen
251 control over worker reproduction. Dissections of physogastric workers revealed that their

252 ovaries were more likely to contain yolked oocytes. We also found that physogastric workers
253 were less aggressive and less likely to forage than normal workers, which indicates that their
254 presence may be costly to colony foraging capacity and defence. The head width and wing
255 length of worker-produced males were slightly larger than males for which we could not rule
256 out a queen or worker origin. Finally, 4.9% of worker-produced males and 21.1% males that
257 could have been produced by dealate queens or workers were heterozygous. We found that
258 most queens were homozygous while most workers were heterozygous for at least one locus.
259 This indicates that the reproduction of *A. gracilipes* is unusual and may involve diploid males
260 and/or gynandromorphs, consistent with previous suggestions [23,29]. To the best of our
261 knowledge, this study is the first to test for the potential cost of worker reproduction in an
262 invasive ant species. Given the cost of worker reproduction, male-production by workers is
263 unexpected in a highly successful invasive species such as *A. gracilipes*, which may indicate
264 that worker reproduction has benefits.

265 **Frequency of worker reproduction and evidence of queen control**

266 Male production by workers occurred in 25 colonies but not in another 70 colonies that were
267 always queenless. Nor did we find male production by workers within any of the colonies
268 from the queen transfer experiment, including six queenless colonies that we monitored past
269 the end of the experiment and that had been queenless for 116 to 186 days. The absence of
270 male production in these colonies could be due to the low worker count, as we found from
271 our observations of 233 captive colonies that worker number was positively associated with
272 male production. In Taiwan, three queenless colony fragments out of nine produced male
273 brood after being kept for four months in the laboratory, and adult males were observed in
274 one of these fragments two months later [21]. This matches our observation that it took 54.1
275 \pm 10.2 days (mean \pm SD) for males to develop from eggs to adult. Our observations on male
276 production are all based on laboratory-kept colonies. Caution must be taken when

277 extrapolating to field colonies, which are not as likely to be queenless, although queenless
278 aggregations of *A. gracilipes* workers and brood are frequently observed in the field (personal
279 observation).

280 Additional results from our colony observations suggest that queens may limit worker
281 reproduction, though we cannot rule out worker policing. In our queen transfer experiment,
282 removing queens triggered an increase in physogastric workers, and moving the queen back
283 after 60 days led to a decrease in physogastric workers. Observations from Lee et al. (2017)
284 [21] suggests that physogastric workers may switch from producing trophic eggs in
285 queenright conditions to producing viable male eggs in queenless conditions. Workers of
286 several ant species switch from trophic egg production to male egg production when the
287 queen dies or disappears (e.g. *Aphaenogaster senilis* [36]; *Aphaenogaster cockerelli* [37];
288 *Prolasius advena* [38]; *N. apicalis* [10]; *O. longinoda* and *O. smaragdina* [17], but to the best
289 of our knowledge, *A. gracilipes* is the only invasive ant species that has been found to do so.
290 Social insect queens can inhibit worker reproduction with queen pheromones, i.e. chemical
291 signals indicating the reproductive status of the queen [2,39]. Several experiments with ants,
292 wasps, and some bees have shown that applying synthetic queen pheromones to queenless
293 colonies inhibits worker reproduction by preventing workers from activating their ovaries and
294 by causing secondary oocyte resorption [12,39–41]. Worker reproduction could also be
295 controlled through the policing of reproductive workers [5,8]. For example, queens and
296 workers could behave aggressively towards egg layers or destroy worker-laid eggs [9].

297 Physogastric workers dissected as part of the dissections of entire colonies and following the
298 aggression tests were more likely to have yolked oocytes than normal workers. Yolked oocytes
299 indicate the presence of fertile or trophic eggs [13,31]. There was no difference in yolked
300 oocyte presence at the end of the queen transfer experiment, probably because colonies in the
301 queen transfer experiment had been queenless for a relatively short period (60-120 days vs

302 108 and 143 days for dissections of worker ovaries of entire colonies and 102-212 days for
303 aggression tests). We only observed yellow bodies in physogastric workers (7.8-12.9%).
304 Yellow bodies can indicate active oviposition of viable eggs, although they are sometimes
305 observed in trophic egg-layers [13,32–34]. Physogastric *A. gracilipes* workers originating
306 from Taiwan also had a higher reproductive potential than normal workers [21]. They had
307 more well-developed ovaries and more yolk oocytes than normal workers [21]. Yellow
308 bodies were also only observed in physogastric workers (13%) [21]. Histological sections of
309 the abdomen of physogastric workers indicated that fat bodies were more abundant in
310 physogastric than in normal workers [21]. The distended abdomen of physogastric workers
311 could therefore be due to the presence of fat bodies and well-developed ovaries. The
312 reproductive or trophic egg-layer status of individual workers can only be determined by
313 ovary dissections or by observations of egg-laying. Physogastric workers are more likely to
314 be reproductive than normal workers, but some non-reproductive workers may have a
315 temporary distended abdomen from feeding extensively on liquid diet. Additional research is
316 needed to determine an objective way to distinguish reproductive from non-reproductive
317 workers in live specimens.

318 **Costs of worker reproduction**

319 We found that behavioural differences between physogastric and normal workers could
320 decrease the competitive ability of the colony. Physogastric workers in queenright and
321 queenless colonies were infrequently observed in the foraging area and were mostly observed
322 inside the nesting tubes during the queen transfer experiment. These observations suggest that
323 physogastric workers do not contribute to colony labour as much as normal workers.
324 Physogastry may also affect the ability of workers to defend the colony during interspecific
325 conflicts. We found that physogastric workers were less aggressive towards *O. smaragdina*
326 workers and were less likely to engage in a fight than normal workers, which would reduce

327 the potential of *A. gracilipes* colonies with a large proportion of physogastric workers to
328 become behaviourally dominant. The production of males by workers also generates costs for
329 other ant species [18]. For example, in *Neoponera obscuricornis* colonies, two costs are
330 associated with worker reproduction following queen removal: an increase in energetic cost
331 associated with aggressive interactions between workers for egg-laying and a decrease in
332 colony labour due to reproductive workers spending less time working for the colony [19].
333 Costly worker conflicts about which workers become reproductive and which workers
334 continue to contribute to colony labour also take place in *Aphaenogaster senilis* [36]. We
335 have never observed aggressive interactions among workers so it is unlikely conflicts take
336 place among *A. gracilipes* workers as to which will become physogastric and which
337 physogastric workers will produce males. Adult males do not appear to originate from a
338 single dominant physogastric worker in queenless *A. gracilipes* colonies, as our genetic
339 results indicated that males originated from more than one worker in at least one of our
340 queenless colonies. This result is consistent with male genetic data for *A. gracilipes* in
341 Taiwan, which showed four different alleles at one locus (Ano10) in one queenless colony
342 fragment [21].

343 Without a queen, *A. gracilipes* colonies are doomed because reproductive workers are unable
344 to lay worker eggs due to their lack of spermatheca [21]. The only chance of survival for a
345 queenless colony would be to merge with a queenright colony and/or adopt a queen. Our
346 workers in queenless colonies readily accepted their original queen back in the nest after
347 being separated for 60 days. In the Northern Territory (Australia), laboratory-kept *A.*
348 *gracilipes* queenless colonies were successfully merged with queenright colonies from a
349 different source colony [42]. Orphaned colonies may therefore merge with other colonies
350 and/or adopt a queen from a different colony in the field. However, the increase in proportion
351 of physogastric workers, which have less competitive ability and do not contribute to colony

352 labour as much as normal workers, following queen death could precipitate the demise of
353 orphaned colonies before such opportunity arises.

354 **Potential benefits of worker reproduction**

355 Despite the costs associated with their lack of contribution to colony labour and defence, the
356 role of physogastric workers as trophic-egg layers in queenright colonies may be significant
357 [21]. Colony observations have shown that trophic eggs may represent a major part of the
358 larval diet in *A. gracilipes* [21]. We did not observe trophic eggs during the queen transfer
359 experiment, but any trophic eggs produced by physogastric workers would likely have been
360 fed to the queen and brood immediately after being laid, as observed in queenright *A.*
361 *gracilipes* colonies by Lee et al. (2017) [21].

362 Worker reproduction may also increase the fitness of deceased *A. gracilipes* queens and
363 orphaned workers because it is their last opportunity to contribute to the gene pool. In
364 Taiwan, the seminal vesicles of *A. gracilipes* worker-produced males contained viable sperm
365 suggesting that they are able to mate [21]. Although the reproductive mode of *A. gracilipes* is
366 unresolved, genetic data and laboratory observations suggest that intranidal mating is the
367 main mode of reproduction for this species [27,43]. In eight of our captive colonies in which
368 males were present, we observed alate queens with no prior exposure to males lose their
369 wings before observing eggs in the colony. If queen brood or virgin queens were present in
370 the colony at the time of the queen's death and did not inhibit the production of males by
371 workers, intranidal mating between worker-produced males and virgin queens could occur.
372 Such a strategy could prolong the life of a colony after the queen's death.

373 **Size and genotypes of males and implications for *A. gracilipes* reproduction**

374 The head width and wing length of worker-produced males were significantly larger (4.0-
375 4.3%) than for queen or worker-produced males and Weber's length tended to be larger,

376 which may provide worker-produced males with a competitive advantage [26]. We do not
377 know whether *A. gracilipes* queens select the males they mate with, whether this selection
378 involves male sizes, and whether larger males have a competitive advantage over smaller
379 ones. Larger males of some *Pogonomyrmex* harvester ants are more successful at mating than
380 smaller males because they can be more successful at gaining access to a mate and transfer a
381 greater proportion of their sperm [25,26]. It would be informative to test whether *A.*
382 *gracilipes* queens choose larger males, potentially selecting worker-produced over queen-
383 produced males.

384 We also found that 4.9% of worker-produced males and 21.1% of males that could have been
385 produced by dealate queens or workers were heterozygous. Our findings are different to those
386 of Lee et al. (2017) [21] who found all 14 *A. gracilipes* worker-produced males from a single
387 queenless colony fragment to be hemizygous, and most of the 20 males from one queenright
388 colony to be heterozygous. Elsewhere they have been genotyped, field-collected
389 heterozygous *A. gracilipes* males were found to be common (Borneo [23], Christmas Island
390 [27], Arnhem land Australia [29]. For example, about 50% of males collected in Borneo were
391 heterozygous [23]. A heterozygous genotype in males would typically indicate diploidy.

392 In ant populations, when a queen mates with a male sharing the same genotype at the sex
393 determination locus (or loci, i.e. match mating), half of the diploid offspring produced by the
394 queen will be homozygous at the sex determination locus (or loci) and develop into diploid
395 males instead of workers [4,24]. Diploid male production is especially common in ant
396 populations that have low genetic diversity (such as invasive populations), and hence low sex
397 determining allele diversity [4,24]. Intranidal mating may be common in *A. gracilipes* [27,43]
398 which would increase the chance of mating between related individuals and increase diploid
399 male production.

400 Heterozygous *A. gracilipes* males that were produced by queens can be diploid, but it is
401 unlikely that heterozygous males produced by workers are diploid. *Anoplolepis gracilipes*
402 workers do not possess a spermatheca and are unable to mate [21] so their male offspring
403 cannot be diploid through match mating. Instead, heterozygous males may be produced as a
404 result of genetic mosaicism in which an individual possess two sets of cells that are
405 genetically different [44]. In the case of *A. gracilipes* worker-produced males, heterozygous
406 individuals could be hemizygous but combine the two genomes of a single worker, which
407 would explain why they possess two different alleles at some loci.

408 Some heterozygous males produced by queens may also not be diploid but genetic mosaics.
409 Diploid males tend to be sterile [3,4,24], but in some ant species, a low proportion of diploid
410 males produce sperm and can father triploid progeny [45,46]. In *A. gracilipes*, dissections of
411 the seminal vesicles of 16 putative diploid males revealed that all of them possessed motile
412 sperm, which suggests that they are not sterile [21]. Given that heterozygous males
413 (putatively diploid) are apparently common for this species [this study, 23,27,29], we would
414 expect a high prevalence of triploid workers resulting from successful mating between a
415 queen and a diploid male. However, evidence of triploid *A. gracilipes* individuals has never
416 been reported [21,23,27,29,47]. Heterozygous males may therefore be genetic mosaics with
417 both maternal and paternal cells (i.e. gynandromorphs) [29]. Gynandromorphs, can occur in
418 Hymenoptera and may combine the morphological features of males and females [48–50].
419 Some gynandromorphs can have bilateral symmetry with one side female and the other male,
420 while other gynandromorphs are mosaics with male and female tissues spread across the
421 body [51,52]. Our four males which had one eye that was oversized compared to the other
422 eye may be sex mosaics with a conspicuous phenotype. In ants, sex mosaics sometimes
423 present an enlarged eye (female) on one side of the head and a smaller eye (male) on the
424 other side [49,51].

425 In addition to gynandromorphs, *A. gracilipes* reproduction may also involve a caste
426 determination system. We found that most genotyped workers were heterozygous for at least
427 one locus, and that most queens were homozygous. This genetic pattern is typical of *A.*
428 *gracilipes* populations and suggests that female castes are determined by a genetic component
429 for this species [21,23,27,29,47]. A potential caste determination system could be linked to
430 gynandromorphy in males. Queen-produced gynandromorph males could produce sperm
431 from their inherited paternal or maternal cells, and female castes could be determined by a
432 combination of male and female alleles [29]. The reproductive mode of *A. gracilipes* may
433 contribute to the ecological dominance of this ant by maintaining a high number of
434 heterozygous workers that may be better adapted to human-modified environments, as has
435 been suggested for another invasive ant species, *Wasmannia auropunctata* (the little fire ant
436 or electric ant) [53,54].

437

438 **Conclusions**

439 We found that workers produced males in at least 25 of our 233 captive *A. gracilipes*
440 colonies. Our work suggests that queen removal triggers workers to become physogastric and
441 potentially reproductive. Physogastric workers can be costly to colony foraging capacity and
442 defence because they forage less and are less aggressive in interspecific conflicts than normal
443 workers. However, reproductive workers may also benefit the colony when the queen dies
444 because worker-produced males are the last opportunity for workers and the deceased queen
445 to contribute to the gene pool. Worker-produced males were slightly larger and less likely to
446 be heterozygous than males that could have been produced by queens or workers. Our results
447 are consistent with those of other studies that suggest that the reproductive mode of *A.*
448 *gracilipes* is unusual and may involve gynandromorphy and/or a caste determination system
449 [23,29,47]. Additional investigations into the reproductive mode of *A. gracilipes* are

450 necessary to resolve current uncertainties on worker and queen caste determination as well as
451 the occurrence of gynandromorphs and to determine whether this potentially unusual
452 reproductive mode contributes to the invasive success of *A. gracilipes*.

453

454 **Methods**

455 **Colony collection and worker dissections**

456 Colonies of *A. gracilipes* used in all our experiments were collected in Queensland, Australia,
457 which is part of the invasive range of this species (Additional file 1: Table S2). Nests were
458 visually located and partially excavated to collect queens, workers and brood. For each
459 worker's dissections in our experiments, we counted the number of ovarioles and yolk (i.e.
460 opaque) oocytes and determined the presence of yellow bodies. We avoided bias for all ovary
461 dissections by keeping the dissector (PL in all cases) blind to colony status (queenless or
462 queenright) and aggression test outcome (see aggression tests section) for the dissections
463 following aggression tests.

464 **Dissections of worker's ovaries of entire colonies**

465 We dissected the ovaries of all the workers from two queenright colonies (26 and 29 workers
466 per colony containing 6 and 8 queens, Additional file 1: Table S3). We also dissected all the
467 workers (22 and 64 workers) from two colonies that had been queenless for 108 and 143 days
468 following queen's death (Additional file 1: Table S3). Our aim was to objectively define
469 physogastry and determine whether there is a link between workers' physogastry and their
470 reproductive capability.

471 Colonies were reared in a 150x220x320mm (height x width x depth) box with two 50ml
472 nesting tubes (length x diameter: 93x60mm) containing moist cotton and a 35x100x150mm
473 (height x width x depth) piece of cardboard egg carton. The colonies were kept in a constant
474 temperature room at $26\pm0.2^{\circ}\text{C}$ (mean \pm SD) and $59.5\pm4.4\%$ humidity.

475 The colonies were alternately fed either a mealworm or a cricket twice a week and 25% sugar
476 water ad-libitum. Prior to dissecting each worker, we visually evaluated its physogastry by
477 counting the number of exposed intersegmental membranes on its gaster (Fig. 4). In
478 accordance with our results (see Results section), we define physogastric workers as having a
479 conspicuously enlarged gaster with two to four exposed intersegmental membranes. We used
480 this definition of physogastric workers for the queen transfer experiment and the aggression
481 tests.

482 Colony observations

483 We conducted observations from May 2016 to December 2019 on 419 colonies collected
484 from May 2016 to June 2019 at 23 sites in Queensland (20 near Cairns, 2 near Townsville, 1
485 near Hervey Bay, Additional file 1: Table S2 and S4). Most colonies were collected by
486 placing transects of bamboo segments (approximately 300 mm long, 40-60mm width of
487 opening) at multiple sites and collecting pieces that *A. gracilipes* had colonized. Colonies
488 were kept in the same conditions as described in the Dissections of worker's ovaries of entire
489 colonies section. We observed colonies every 1-3 weeks during which we counted the
490 number of queens (alate and dealate) and males, categorized the number eggs, larvae, and
491 pupae (0, 11-20, 21-50 and 51+). We did not distinguish between trophic eggs and
492 reproductive eggs. To estimate the total number of workers during each observation, we
493 categorized the number of workers (0-10, 11-20, 21-50, 51-100, 101-200 and 200+) engaged
494 in each of five different activities (in the nest tube, in the sugar tube, walking around,

495 standing still, other) and then summed the mid-points of the recorded categories across the
496 five activities. To check for the presence of males, we scrutinized the nest and checked dead
497 ant piles. We excluded from analyses colonies that had more than 21 days between any two
498 observations, had fewer than 4 observations, had been in captivity for fewer than 26 days, or
499 that were used for other experiments. The remaining 233 colonies had been observed 5-73
500 times (median=32) for at least 29 and up to 747 days (median=237) at intervals of 8-21 days
501 (median=10). The number of observations varies among colonies because it is dependent on
502 their collection date and longevity.

503 For colonies that produced males, we categorized the likely male source based on the
504 preceding observations of brood and queens. We concluded that any brood produced during a
505 queenless state after at least three weeks in which no brood had been observed had been
506 produced by workers. We could then usually trace the development from egg to larvae to
507 pupae over successive weeks of observations and conclude that any males that emerged were
508 worker-produced. Two colonies collected without queens had more disjunct patterns of
509 observable eggs, but we concluded that their males were worker-produced considering they
510 were in captivity queenless for 140 and 146 days and male brood does not enter diapause in
511 constant laboratory conditions [55]. We approximated the time to development as the number
512 of days from when we first observed a worker-produced egg to when we first observed an
513 adult male on 20 occasions in 19 colonies.

514 Males that emerged in colonies in which queens had emerged, and in which brood appeared
515 after at least three weeks in which no brood had been observed, were considered to have been
516 produced by either workers or alate queens. Males that emerged in colonies (either queenless
517 or queenright) in which brood was present continuously following collection, were
518 considered to be either queen- or worker-produced. We collected some live males for
519 genotyping, but otherwise left males in colonies so as to disrupt functioning as little as

520 possible. Workers and queens were collected dead opportunistically to minimize effects on
521 colony dynamics.

522 **Morphometry and genotyping**

523 **Measurements**

524 Live and dead adult males that were found during the colony observations (protocol
525 described above) were collected, placed in ethanol, and measured under a Leica M165C
526 stereomicroscope at 20-80x magnification. We measured the head width (i.e. maximum width
527 of the head in full face view including the eyes), Weber's length, wing width and wing length
528 of 34 males that could have been produced by queens (alate or dealate) or workers from 8
529 colonies and 86 worker-produced males from 13 queenless colonies. See Colony observations
530 section for details on how male source was determined. Not all the measurements could be
531 obtained from some males that were collected dead because of missing or damaged parts (e.g.
532 missing head or indented alitrunk). Wing width and length were always measured using the
533 same veins as reference (see Additional file 2: Fig. S2) to ensure consistency between
534 individuals. Deformed wings were not measured as they did not show wing venation. All the
535 colonies from which the samples originated were fed the same diet and were kept in the same
536 rearing conditions (see Dissections of worker's ovaries of entire colonies section for more
537 details on the diet and rearing conditions).

538 **Microsatellite analysis**

539 We conducted microsatellite analysis to determine whether the observed heterozygosity of
540 queen- and worker-produced males differed. We genotyped 28 males that could have been
541 produced by queens (dealate or alate) or workers from 6 colonies and 82 worker-produced
542 males from 14 colonies. We also genotyped 3-5 workers from 6 of these colonies with queen-
543 or worker-produced males (N=28 workers), 1-5 workers from 14 of the colonies with worker-

544 produced males (N=54), 1-3 queens from 4 of the colonies with queen- or worker-produced
545 males to determine their observed heterozygosity (n=9 queens in total, details of the
546 genotyping protocol in Additional file 2: Appendix S1).

547 **Queen transfer experiment**

548 **Experimental design**

549 We conducted a laboratory experiment to determine whether the absence of a queen triggers
550 workers to become physogastric and lay eggs. We evenly split workers and brood from 14
551 colonies into two subcolonies and randomly assigned one of each pair to house a queen while
552 the other was queenless. Each of the resulting 28 subcolonies had 121 to 200 workers
553 depending on the size of the original colony (Additional file 1: Table S3). Each subcolony
554 had several pieces of brood at the various stages present in the colony at that time. The
555 colonies were collected from September to November 2017 at five sites in Queensland
556 (Additional file 1: Table S3 and S4). Each subcolony was housed in a 150x220x320mm
557 (height x width x depth) box with two 50ml nesting tubes (length x diameter: 93x60mm)
558 containing moist cotton and a 35x100x150mm piece of cardboard egg carton and maintained
559 at $23.7 \pm 0.78^{\circ}\text{C}$ (mean \pm SD) and at ambient photoperiod. They were fed one mealworm
560 biweekly and were provided with 25% sugar water ad libitum.

561 Eight queens (out of 14) died before or on day 60 while six queens survived past day 60 and
562 were moved to their corresponding queenless subcolony pair to determine whether the queen
563 would be accepted, and whether moving the original queen back to the queenless treatment
564 reduced the percentage of physogastric workers and stopped workers from laying eggs. When
565 moving the queen between subcolonies, we recorded worker behaviour towards the queen
566 every 10 minutes for 1 hour and recorded the queen's position within the colony box after
567 24h. We scored behaviour toward the queen according to Lai et al. (2015) [35] as: 0 ignoring,

568 1 touching, 2 avoiding, 3 holding, 4 aggression, and 5 fighting. At day 120 for all but the six
569 queenless subcolonies with the most workers, we dissected the ovaries of 15 workers selected
570 haphazardly among all the remaining workers, or all the workers if the subcolony was smaller
571 than 15 workers. We continued monitoring six queenless colonies with the most remaining
572 workers for another 60 days to determine whether they would produce males.

573 **Colony observations**

574 We counted the number of dead workers, brood (eggs, larvae, pupae) and trophic eggs (sub-
575 spherically shaped eggs, [21]) weekly, and recorded the position (outside, under the egg
576 carton or inside the nesting tubes) of physogastric and normal workers every two weeks to
577 determine whether the behaviour of physogastric and normal workers differ.

578 **Aggression tests**

579 We conducted aggression trials between *A. gracilipes* and native green tree ant (*Oecophylla*
580 *smaragdina*) workers to determine whether *A. gracilipes* worker aggression toward a
581 competitor differed depending on physogastric state (physogastric or normal) and colony
582 state (queenless or queenright) in a fully factorial design. *Oecophylla smaragdina* is a native
583 dominant species that had similar competitive ability of *A. gracilipes* in aggression tests with
584 various *A. gracilipes*:*O. smaragdina* worker ratios in Borneo [56], although *A. gracilipes*
585 displaces *O. smaragdina* in northern Australia [57]. Workers from queenless *A. gracilipes*
586 colonies originated from the six colonies we had kept monitoring for 60 days after the end of
587 the queen transfer experiment (Additional file 1: Table S3). These colonies were collected
588 around Cairns and had been queenless for three to seven months. We did not have remaining
589 queenright colonies from the queen transfer experiment, so we used six laboratory queenright
590 colonies also collected around Cairns at the same dates as the queenless colonies (Additional
591 file 1: Table S3) to avoid variation caused by time spent in the laboratory and colony origin.

592 Workers from these colonies were approximately 4mm in length. We collected *O.*
593 *smaragdina* workers before each trial from a single tree on the James Cook University
594 campus in Cairns to eliminate variation in *O. smaragdina* worker aggression due to workers
595 originating from a different colony. We only selected minor workers (approximately 8mm in
596 length) that exhibited defensive behaviour (i.e. lifting their gaster to spray acid) when
597 approached by our forceps. We let the *O. smaragdina* workers acclimatize to the laboratory
598 for 10 minutes after collection. We used each *A. gracilipes* and *O. smaragdina* worker only
599 once.

600 We measured aggressive interactions between three *A. gracilipes* workers that were either all
601 physogastric or normal and a single *O. smaragdina* worker. We conducted a pilot experiment
602 in which *O. smaragdina* workers always overcame *A. gracilipes* in 1:1 or 1:2 interactions.
603 We therefore decided on a 1:3 ratio to enable us to detect differences between the aggression
604 level and survival of physogastric and normal *A. gracilipes* workers. We replicated the
605 aggression tests three times for both physogastric states (normal or physogastric) for each of
606 the six queenless colonies and six queenright colonies. Only three normal workers were
607 present in one queenless colony. Therefore, we ran only one aggression test between normal
608 workers and one *O. smaragdina* worker for this colony. Thus, we ran 18 tests each of normal
609 workers and physogastric workers from queenright colonies and physogastric workers from
610 queenless colonies, and 16 tests for normal workers from queenless colonies (n=70 tests in
611 total).

612 We conducted the 60-minute aggression tests in fluon-coated 60x93mm (diameter x height)
613 PVC cylinders separated into two halves with a laminated paper card. The arenas were placed
614 on a plastic tray that was washed with non-scented soap after each trial. We placed one *O.*
615 *smaragdina* worker on one side and three *A. gracilipes* workers on the other side and let the
616 ants acclimatize for five minutes before removing the dividing wall. For the first five

617 minutes, we noted the highest aggression score between the two species at 30s intervals
618 according to the method used in Lai et al. (2015) [35] and described above. We then checked
619 the arena every 5 minutes for the remaining 55 minutes and recorded whether *O. smaragdina*
620 or *A. gracilipes* workers had died. At the end of the trial, we collected the three *A. gracilipes*
621 workers (dead or alive), placed them in ethanol, and dissected their ovaries.

622 Data analysis

623 We analysed our data in R version 3.5.0 [58] and used functions from the stats package (R
624 Development Core Team 2009) unless specified otherwise. All the models used are
625 summarised in Table 3. We used generalized linear model (GLM, glm function) followed by
626 likelihood ratio tests (Anova function in the car package [59] and generalized linear mixed
627 model (GLMM, glmer function in the lme4 package [60], followed by Type III Wald χ^2 tests
628 (Anova function). We also used linear mixed-effects model (LMM, lmer function in the lme4
629 package) followed by Type II Wald χ^2 tests and cumulative link mixed models (CLMM,
630 clmm2 function in the package ordinal, [61] followed by likelihood ratio tests [62]. We used
631 post-hoc Tukey tests to make pairwise comparisons (emmeans function, in the package
632 emmeans, [63]) and tested the data for overdispersion where appropriate. We used an
633 observation-level random intercept to re-evaluate models with over-dispersion in the queen
634 transfer experiment and aggression tests and also changed the distribution to negative
635 binomial for one of the aggression tests models (Table 3) because the first method was not
636 sufficient to resolve overdispersion issues. For the queen transfer experiment, we analysed
637 data from replicates in which the queens survived until or past day 60 (10 queenright and 10
638 queenless colonies) separately from data obtained from replicates in which the queens
639 survived until day 120 (3 queenright and 3 queenless colonies) because of the low queen
640 survival rate at day 120.

641

642 **Declarations**

643 **Ethics approval and consent to participate**

644 Not applicable

645 **Consent for publication**

646 Not applicable

647 **Availability of data and material**

648 The datasets supporting the conclusions of this article will be available in the Research Data
649 (Tropical Data Hub) repository at James Cook University (DOI to be added upon
650 publication). They are available from PL on request during the reviewing process.

651 **Competing interests**

652 The authors declare that they have no competing interests.

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659 observation of some of the colonies.

660 **Authors' contribution**

661 PL, LL and HF conceived the study. PY, BH, and WT assisted with the study design. PL, AS,
662 MG and AH carried out the field and laboratory work. LL analysed the colony observation

663 data; PL analysed the other data sets and drafted the manuscript. LL, HF, PY, BH, and WT
664 assisted with the data analysis. All authors edited the manuscript and LL coordinated the
665 study.

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671 **Additional files**

672 Additional file 1:

673 Table S1. Genotyping results of males, workers, and queens.

674 Table S2. Site coordinates of where colonies were collected

675 Table S3. Additional information on the colonies used in dissections of worker's ovaries of
676 entire colonies, the queen transfer experiment, and the aggression tests.

677 Table S4 Additional information on the colonies used in the colony observations

678 Additional file 2:

679 Figure S1. Boxplot showing the proportion of normal workers in the queen transfer
680 experiment by colony status and number of days since the start of the experiment until day
681 120

682 Figure S2. Forewing of a male *A. gracilipes* showing which veins were used to measure wing
683 width and wing length

684 Appendix S1. Additional information on the genotyping protocol

686 **References**

- 687 1. Wilson EO. *The insect societies*. Cambridge: Belknap Press of Harvard University Press; 1971.
- 688 2. Bourke AFG. Worker reproduction in the higher eusocial Hymenoptera. *Q Rev Biol*. 1988;63:291–311.
- 689
- 690 3. Heimpel GE, de Boer JG. Sex determination in the Hymenoptera. *Annu Rev Entomol*. 2008;53:209–30.
- 691
- 692 4. Crozier RH. Evolutionary genetics of the Hymenoptera. *Annu Rev Entomol*. 1977;22:263–88.
- 693 5. Wenseleers T, Ratnieks FLW. Comparative analysis of worker reproduction and policing in eusocial Hymenoptera supports relatedness theory. *Am Nat*. 2013;168:E163–E179.
- 694
- 695 6. Hammond RL, Keller L. Conflict over male parentage in social insects. *PLoS Biol*. 2004;2.
- 696 7. Hamilton WD. The genetical evolution of social behavior I, II. *J Theor Biol*. 1964;7:1–52.
- 697 8. Wenseleers T, Hart AG, Ratnieks FLW. When resistance is useless: policing and the evolution of reproductive acquiescence in insect societies. *Am Nat*. 2004;146.
- 698
- 699 9. Ratnieks FLW, Foster KR, Wenseleers T. Conflict resolution in insect societies. *Annu Rev Entomol*. 2006;51:581–608.
- 700
- 701 10. Dietemann V, Peeters C. Queen influence on the shift from trophic to reproductive eggs laid by workers of the ponerine ant *Pachycondyla apicalis*. *Insectes Soc*. 2000;47:223–8.
- 702
- 703 11. Endler A, Liebig J, Schmitt T, Parker JE, Jones GR, Schreier P, et al. Surface hydrocarbons of queen eggs regulate worker reproduction in a social insect. *Proc Natl Acad Sci*. 2004;101:2945–50.
- 704
- 705 12. Holman L, Jørgensen CG, Nielsen J, D'Ettorre P. Identification of an ant queen pheromone regulating worker sterility. *Proc R Soc B Biol Sci*. 2010;277:3793–800.
- 706
- 707 13. Tay WT, Crozier RH. Nestmate interactions and egg-laying behaviour in the queenless ponerine ant *Rhytidoponera sp. 12*. *Insectes Soc*. 2000;47:133–40.
- 708
- 709 14. Wheeler DE. Nourishment in ants: patterns in individuals and societies. *Nourishment Evol Insect Soc*. 1994. p. 245–78.
- 710
- 711 15. Khila A, Abouheif E. Reproductive constraint is a developmental mechanism that maintains social harmony in advanced ant societies. *Proc Natl Acad Sci*. 2008;105:17884–17889.
- 712
- 713 16. Hölldobler B, Carlin NF. Colony founding, queen control and worker reproduction in the ant *Aphaenogaster (= Novomessor) cockerelli* (Hymenoptera: Formicidae). *Psyche A J Entomol*. 1989;3–4:131–51.
- 714
- 715
- 716 17. Hölldobler B, Wilson EO. Queen control in colonies of weaver ants (Hymenoptera: Formicidae). *Ann Entomol Soc Am*. 1983;76:235–8.
- 717
- 718 18. Heinze J. The demise of the standard ant (Hymenoptera : Formicidae). *Myrmecological News*. 2008;11:9–20.
- 719
- 720 19. Gobin B, Heinze J, Strätz M, Roces F. The energetic cost of reproductive conflicts in the ant *Pachycondyla obscuricornis*. *J Insect Physiol*. 2003;49:747–52.
- 721

- 722 20. Cole BJ. The social behavior of *Leptothorax allardycei* (Hymenoptera, Formicidae): time budgets
723 and the evolution of worker reproduction. *Behav Ecol Sociobiol.* 1986;18:165–73.
- 724 21. Lee C-C, Nakao H, Tseng S-P, Hsu H-W, Lin G-L, Tay J-W, et al. Worker reproduction of the
725 invasive yellow crazy ant *Anoplolepis gracilipes*. *Front Zool.* 2017;14:1–12.
- 726 22. Holway DA, Lach L, Suarez A V., Tsutsui ND, Case TJ. The causes and consequences of ant
727 invasions. *Annu Rev Ecol Syst.* 2002;33:181–233.
- 728 23. Drescher J, Blüthgen N, Feldhaar H. Population structure and intraspecific aggression in the
729 invasive ant species *Anoplolepis gracilipes* in Malaysian Borneo. *Mol Ecol.* 2007;16:1453–65.
- 730 24. Crozier RH. Heterozygosity and sex determination in haplo-diploidy. *Am Nat.* 1971;105:399–412.
- 731 25. Wiernasz DC, Sater AK, Abell AJ, Cole BJ. Male size, sperm transfer, and colony fitness in the
732 western harvester ant, *Pogonomyrmex occidentalis*. *Evolution (N Y).* 2001;55:324–9.
- 733 26. Davidson DW. Sexual selection in harvester ants (Hymenoptera: Formicidae: *Pogonomyrmex*).
734 *Behav Ecol Sociobiol.* 1982;10:245–50.
- 735 27. Thomas ML, Becker K, Abbott KL, Feldhaar H. Supercolony mosaics: Two different invasions by
736 the yellow crazy ant, *Anoplolepis gracilipes*, on Christmas Island, Indian Ocean. *Biol Invasions.*
737 2010;12:677–87.
- 738 28. Gruber MAM. Genetic factors associated with variation in abundance of the invasive yellow crazy
739 ant (*Anoplolepis gracilipes*). PhD dissertation, Victoria University of Wellington, Wellington, New
740 Zealand; 2012.
- 741 29. Gruber MAM, Hoffmann BD, Ritchie PA, Lester PJ. The conundrum of the yellow crazy ant
742 (*Anoplolepis gracilipes*) reproductive mode: No evidence for dependent lineage genetic caste
743 determination. *Insectes Soc.* 2013;60:135–45.
- 744 30. Wetterer JK. Worldwide distribution and potential spread of the long-legged ant, *Anoplolepis*
745 *gracilipes* (Hymenoptera: Formicidae). *Sociobiology.* 2005;45:77–97.
- 746 31. Peeters CP. The reproductive division of labour in the queenless ponerine ant *Rhytidoponera* sp.
747 12. *Insectes Soc.* 1987;34:75–86.
- 748 32. Billen J. Ultrastructure of the workers ovarioles in *Formica* ants (Hymenoptera: Formicidae). *Int J*
749 *Insect Morphol Embryol.* 1985;14:21–32.
- 750 33. Dietemann V, Hölldobler B, Peeters C. Caste specialization and differentiation in reproductive
751 potential in the phylogenetically primitive ant *Myrmecia gulosa*. *Insectes Soc.* 2002;49:289–298.
- 752 34. Gobin B, Billen J, Peeters C. Policing behaviour towards virgin egg layers in a polygynous ponerine
753 ant. *Anim Behav.* 1999;58:1117–22.
- 754 35. Lai L, Hua KH, Wu WJ. Intraspecific and interspecific aggressive interactions between two species
755 of fire ants, *Solenopsis geminata* and *S. invicta* (Hymenoptera: Formicidae), in Taiwan. *J Asia Pac*
756 *Entomol.* 2015;18:93–8.
- 757 36. Ichinose K, Lenoir A. Reproductive conflict between laying workers in the ant *Aphaenogaster*
758 *senilis*. *J Ethol.* 2009;27:475–81.
- 759 37. Smith AA, Hölldobler B, Liebig J. Hydrocarbon signals explain the pattern of worker and egg
760 policing in the ant *Aphaenogaster cockerelli*. *J Chem Ecol.* 2008;34:1275–82.
- 761 38. Grangier J, Avril A, Lester PJ. Male production by workers in the polygynous ant *Prolasius*
762 *advenus*. *Insectes Soc.* 2013;60:303–8.

- 763 39. Holman L. Queen pheromones and reproductive division of labor: a meta-analysis. *Behav Ecol.*
764 2018;29:1199–209.
- 765 40. Van Oystaeyen A, Oliveira RC, Holman L, Van Zweden JS, Romero C, Oi CA, et al. Conserved class
766 of queen pheromones stops social insect workers from reproducing. *Science* (80-). 2014;343:287–
767 90.
- 768 41. Holman L, Lanfear R, D'Ettorre P. The evolution of queen pheromones in the ant genus *Lasius*. *J*
769 *Evol Biol.* 2013;26:1549–58.
- 770 42. Hoffmann BD. Quantification of supercolonial traits in the yellow crazy ant, *Anoplolepis*
771 *gracilipes*. *J Insect Sci.* 2014;14:1–21.
- 772 43. Drescher J, Blüthgen N, Schmitt T, Bühler J, Feldhaar H. Societies drifting apart? Behavioural,
773 genetic and chemical differentiation between supercolonies in the yellow crazy ant *Anoplolepis*
774 *gracilipes*. *PLoS One.* 2010;5.
- 775 44. Wheeler WM. Mosaics and other anomalies among ants. Harvard University Press; 2013.
- 776 45. Krieger MJB, Ross KG, Chang CWY, Keller L. Frequency and origin of triploidy in the fire ant
777 *Solenopsis invicta*. *Heredity* (Edinb). 1999;82:142–50.
- 778 46. Cournault L, Aron S. Diploid males, diploid sperm production, and triploid females in the ant
779 *Tapinoma erraticum*. *Naturwissenschaften*. 2009;96:1393–400.
- 780 47. Gruber MAM, Hoffmann BD, Ritchie PA, Lester PJ. Genetic diversity is positively associated with
781 fine-scale momentary abundance of an invasive ant. *Ecol Evol.* 2012;2:2213–22.
- 782 48. Jones SR, Phillips Jr. SA. Gynandromorphism in the ant *Pheidole dentata* MAYR (Hymenoptera:
783 Formicidae). *Proc Entomol Soc Washingt.* 1985;87:583–6.
- 784 49. Skvarla MJ, Dowling APG. First report of gynandromorphism in *Temnothorax curvispinosus*
785 (Mayr, 1866) (Hymenoptera : Formicidae). *Entomol Soc Washingt.* 2014;116:349–53.
- 786 50. Wcislo WT, Gonzalez VH, Arneson L. A review of deviant phenotypes in bees in relation to brood
787 parasitism, and a gynandromorph of *Megalopta genalis* (Hymenoptera: Halictidae). *J Nat Hist.*
788 2004;38:1443–57.
- 789 51. Yoshizawa J, Mimori K, Yamauchi K, Tsuchida K. Sex mosaics in a male dimorphic ant
790 *Cardiocondyla kagutsuchi*. *Naturwissenschaften*. 2009;96:49–55.
- 791 52. de Campos AE, Kato LM, de Maria Zarzuela FM. Occurrence of different gynandromorphs and
792 ergatandromorphs in laboratory colonies of the urban ant *Monomorium floricola*. *J Insect Sci.*
793 2011;11.
- 794 53. Foucaud J, Orivel J, Fournier D, Delabie JHC, Loiseau A, Le Breton J, et al. Reproductive system,
795 social organization, human disturbance and ecological dominance in native populations of the little
796 fire ant, *Wasmannia auropunctata*. *Mol Ecol.* 2009;18:5059–73.
- 797 54. Chifflet L, Guzmán NV, Rey O, Confalonieri VA, Calcaterra LA. Southern expansion of the invasive
798 ant *Wasmannia auropunctata* within its native range and its relation with clonality and human
799 activity. *PLoS One.* 2018;13.
- 800 55. Kipyatkov VE. Seasonal life cycles and the forms of dormancy in ants (Hymenoptera:
801 Formicoidea). *Acta Soc Zool Bohemicae.* 2001;65:211–38.
- 802 56. Drescher J, Feldhaar H, Blüthgen N. Interspecific aggression and resource monopolization of the
803 invasive ant *Anoplolepis gracilipes* in Malaysian Borneo. *Biotropica.* 2011;43:93–9.

- 804 57. Lach L, Hoffmann BD. Are invasive ants better plant-defense mutualists? A comparison of foliage
805 patrolling and herbivory in sites with invasive yellow crazy ants and native weaver ants. Oikos.
806 2011;120:9–16.
- 807 58. R Core Team. R: A language and environment for statistical computing [Internet]. R Found. Stat.
808 Comput. Vienna, Austria; 2018. Available from: <https://www.r-project.org/>
- 809 59. Fox J, Weisberg S. An {R} companion to applied regression. Sage Publ. 2002;2–3.
- 810 60. Bates D, Maechler M, Bolker B, Walker S. Fitting linear mixed-effects models using lme4. J
811 statisitical Softw. 2015;67:1–48.
- 812 61. Christensen RHB. Package “Ordinal.” R Packag. version 2018.8-25. 2018.
- 813 62. Christensen RHB. A tutorial on fitting Cumulative Link Mixed Models with clmm2 from the
814 ordinal Package. Vienna, Austria; 2015.
- 815 63. Lenth R, Singmann H, Love J, Buerkner P, Hervé M. Emmeans: estimated marginal means, aka
816 least-squares means. R Packag version 124. 2018;
- 817
- 818

820 **Table 1.** Summary of generalized linear mixed model (GLMM) or cumulative link mixed
 821 models (CLMM) results for each response variable for analyses of 1) colony observations, 2)
 822 the queen transfer experiment, and 3) aggression tests. ‘x’ represents the interaction term.

Response and explanatory variables	df	χ^2 or LRstat	p
1. Male production, GLMM, n=233 colonies			
Whether the colony ever had a queen	1	2.28	0.013
Maximum number of workers	1	35.35	<0.0001***
Days in captivity	1	3.17	0.08
Whether the colony ever had a queen x max. number of workers	1	7.58	<0.0059**
Whether the colony ever had a queen x days in captivity	1	3.04	0.08
2. Proportion of normal workers until day 60, GLMM, n=100 observations and 10 colonies			
Colony status (queenright or queenless)	1	1.80	0.18
Time (since the beginning of the experiment)	4	15.57	0.0037**
Colony status x Time	4	14.49	0.0059**
Proportion of normal workers until day 120, GLMM, n=54 observations and 3 colonies			
Colony status (queenright or queenless)	1	0.0001	0.99
Time (since the beginning of the experiment)	8	47.26	<0.0001***
Colony status x Time	8	37.92	<0.0001***
3. Maximal aggression score, CLMM, n=70			
Colony status	1	5.66	0.0173*
Worker type	1	6.65	0.0099**
Colony status x Worker type	1	1.30	0.25
Survival of all three <i>A. gracilipes</i> workers, GLMM negative binomial, n=70			
Colony status	1	2.45	0.12
Worker type	1	0.33	0.57

Colony status x Worker type 1 1.83 0.18

Survival of the *O. smaragdina* worker, GLMM binomial, n=70

Colony status 1 14.45 <0.0001***

Worker type 1 1.53 0.22

Colony status x Worker type 1 6.19 <0.0129*

Species initiating the fight, GLM binomial, n=51

Colony status 1 4.12 0.0423*

Worker type 1 5.25 0.0220*

Colony status x Worker type 1 0 0.99

Presence absence of mature oocytes, GLMM binomial, n=210

Colony status 1 5.52 0.01876*

Worker type 1 20.40 <0.0001***

823

824

825

826 **Table 2.** Trait measurements in mm of worker-produced and males of undetermined source¹
 827 (mean \pm SD) and results from type II Wald tests on LMM (df=1) * p<0.05, ** p<0.01

Trait	Worker-produced		Undetermined source ¹		χ^2	P
Head width	0.77 \pm 0.04	n=55	0.74 \pm 0.03	n=24	10.423	0.0012**
Weber's length	1.79 \pm 0.12	n=83	1.72 \pm 0.12	n=30	3.7443	0.053
Wing width	1.37 \pm 0.10	n=50	1.30 \pm 0.10	n=30	0.2135	0.644
Wing length	3.11 \pm 0.20	n=54	2.98 \pm 0.22	n=21	4.3492	0.037*

828 ¹ Males that could have been produced by queen (dealate or alate) or workers

829

830 **Table 3.** Summary table of final models and parameters. 'x' represents the interaction terms.
 831 Explanatory variables are fixed unless specified otherwise. Colony status: queenless or
 832 queenright, worker type: physogastric or normal *A. gracilipes*.

833

Section	Model and distribution	Response variable	Explanatory variables
Dissections of worker's ovaries of entire colonies	GLM binomial	Presence or absence of mature oocytes	Colony status Worker type
	GLM binomial	Worker type	Colony status
Colony observations	GLMM binomial	Whether the colony ever produced males	Whether the colony ever had a queen Maximum number of workers
			Whether the colony ever had a queen x Max. number of workers Number of days in captivity (random)
Morphometry and genotyping	LMM	Head width or Weber's length or wing width or wing length	Male source Colony of origin (random)
	GLMM binomial	Proportion of normal workers until day 60	Time ¹ Colony status Time ¹ x Colony status Colony of origin (random)
	GLMM binomial	Proportion of normal workers until day 120	Time ¹ Colony status Time ¹ x Colony status Colony of origin (random)
Queen transfer experiment	GLMM binomial	Presence or absence of workers in the foraging area	Worker type Colony status Colony of origin (random)
	GLMM binomial	Presence absence mature oocytes	Colony status Worker type Colony of origin (random)
	CLMM	Maximal aggression score	Colony status Worker type Colony status x Worker type Colony of origin (random)
Aggression tests	GLMM negative binomial	Survival of all three <i>A. gracilipes</i> workers	Colony status Worker type Colony status x Worker type Colony of origin (random)
	GLMM binomial	Survival of the <i>O. smaragdina</i> worker	Colony status Worker type

		Colony status x Worker type
		Colony of origin (random)
	GLM binomial	Species initiating the fight
		Colony status
		Worker type
		Colony status x Worker type
	GLMM binomial	Presence absence of mature oocytes
		Colony status
		Worker type
		Colony of origin (random)

834 ¹ days since the start of the queen transfer experiment

835

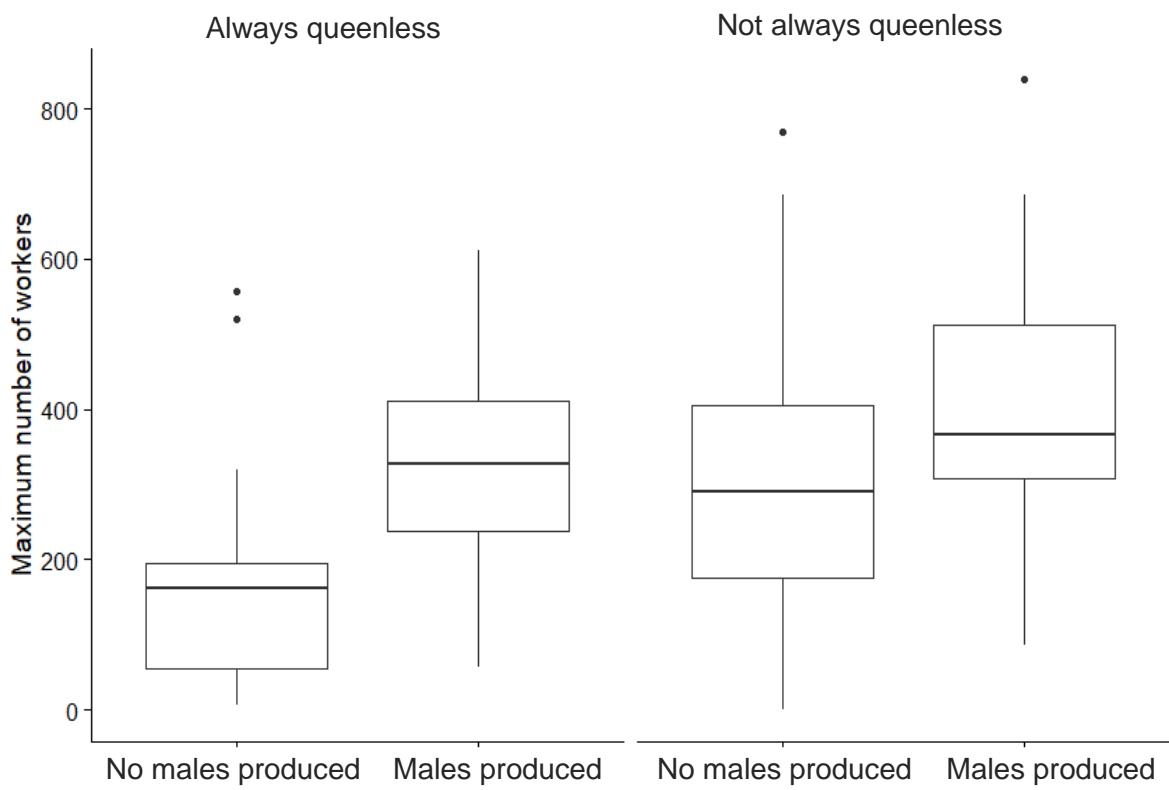
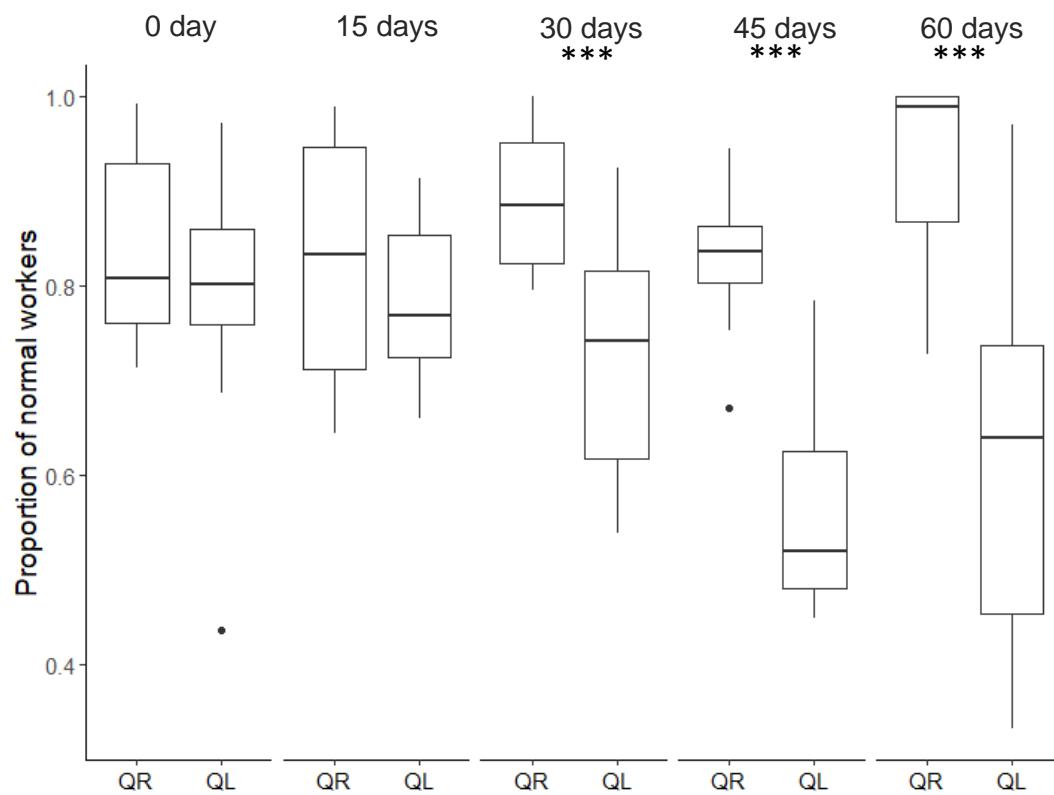


Figure 1. Male production in the 233 observed laboratory colonies by whether colonies were queenless and the maximum number of workers in the colony during captivity. See Table 1 for statistical results.

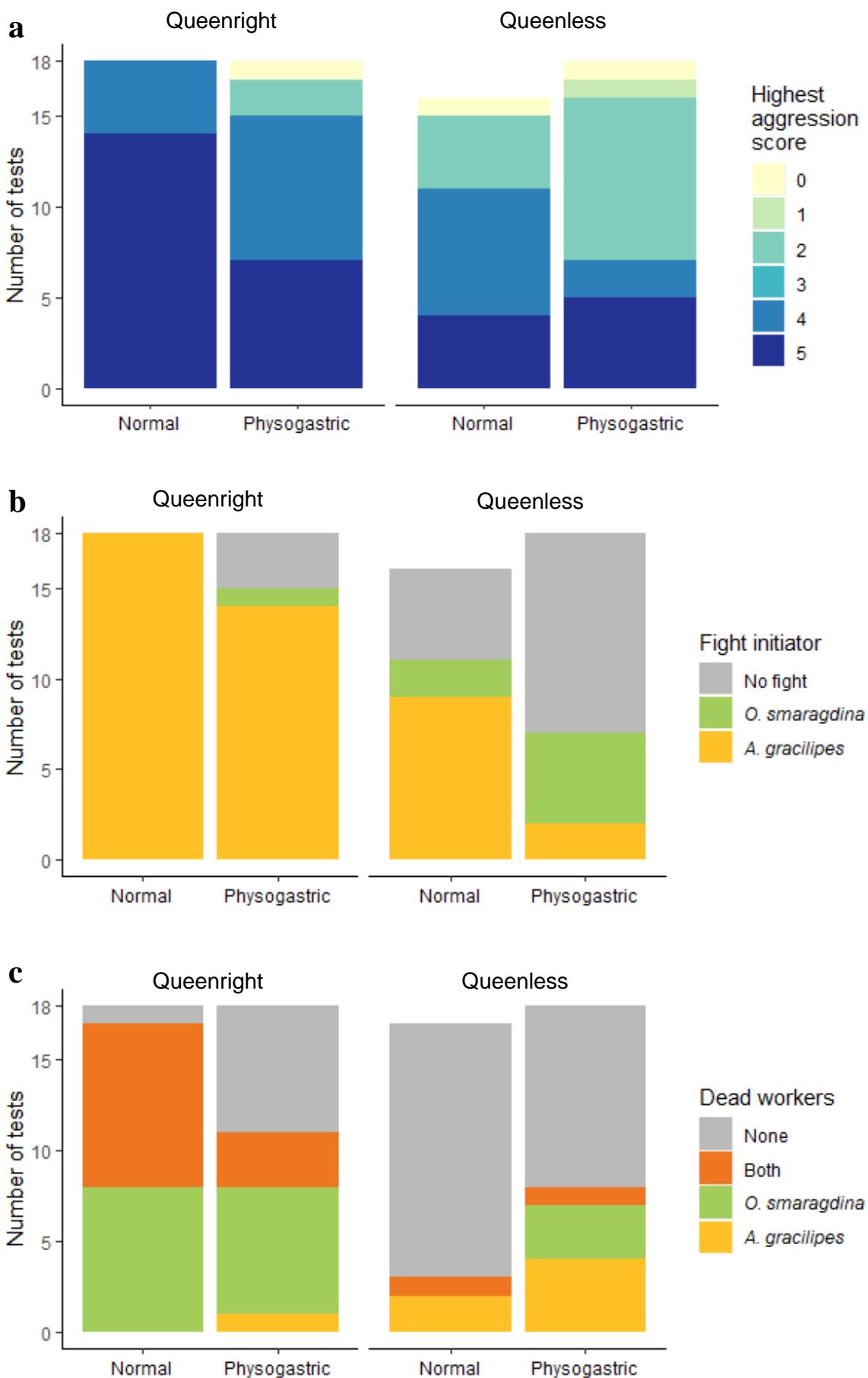
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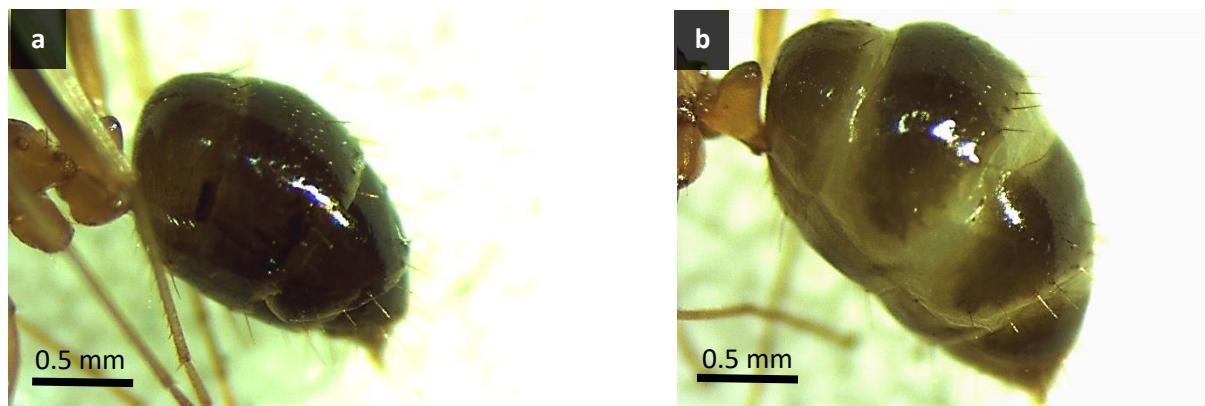
844 **Figure 2.** Proportion of normal workers in the queen transfer experiment by colony status
845 (QR=queenright, QL=queenless, N=10 for each) and number of days since the start of the
846 experiment until day 60. *** indicates a significant difference between queenright and
847 queenless colonies for the corresponding time (GLMM: binomial, Table 1, post-hoc tests
848 *** $p<0.001$).

849



851 **Figure 3.** Results from the aggression tests between *O. smaragdina* workers and *A. gracilipes*
852 normal or physogastric workers from queenright or queenless colonies. a: Highest aggression
853 score for each trial, b: Number of fights initiated by *A. gracilipes* and *O. smaragdina*
854 workers, c: Number of fights that resulted in the death of the *O. smaragdina* worker and/or
855 the death of one of the three *A. gracilipes* workers. See Table 1 for statistical results.
856

857



858

859 **Figure 4.** External morphology of the abdomen of (a) a normal worker and (b) a physogastric
860 worker. Note the exposed intersegmental membranes of the physogastric worker.

861