

1 **Title: Loss of wings induces the expression of the worker-like phenotype in queens of a**
2 **ponerine ant**

3 **Running Title: Wing loss makes ant queens worker-like**

4 **Authors:** Benjamin Pyenson^{1*} Christopher Albin-Brooks¹ Corinne Burhyte¹ Jürgen Liebig¹

5 ***: Corresponding author:** bpyenson@asu.edu

6 **Affiliation:** ¹: School of Life Sciences, Arizona State University, Tempe, AZ, USA

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Loss of wings induces the expression of the worker-like phenotype in queens of a ponerine ant

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32 Summary Statement

33 Ant queens who have lost their wings express worker-like behaviors and physiology
34 including the display of dominance behavior during hierarchy establishment, which is normally a
35 worker-only behavior in this species.

36 Abstract

37 Many highly-eusocial insect species are characterized by morphological differences
38 between females. This is especially pronounced in ants where queens usually possess a fully
39 developed thorax with wings and are specialized for reproduction while workers have a reduced
40 thorax without wings and show various levels of reproductive degeneration that is associated
41 with their helper role in the colony. Despite their morphological differentiation, queens and
42 workers still show some plasticity leading to overlapping behavioral and physiological
43 phenotypes. We investigated the level of queen plasticity and the factor that induces a worker-
44 like phenotype in the ant species *Harpegnathos saltator* that has limited queen-worker
45 dimorphism and workers that can assume the reproductive role of a queen in the colony. By
46 comparing alate and dealate young queens, so-called gynes, we found that the loss of wings
47 initiated the expression of behavioral and physiological characteristics of ant workers. In contrast
48 to alate gynes, dealate gynes displayed higher frequencies of worker-like behaviors. In addition,
49 dealate gynes showed a worker-like range of reproductive states unlike alate gynes. Like
50 workers, dealate gynes lost the chemical signaling that is characteristic of alate gynes. Since
51 gynes can activate this worker-like phenotype after wing loss, the essential difference between
52 the ant queens and workers in this species with limited queen-worker dimorphism is a dispersal
53 polyphenism. If the phenotypic plasticity observed in *H. saltator* is representative of the early
54 stages of ant eusociality, an emerging dispersal dimorphism rather than a distinct reproductive
55 dimorphism might represent one of the first steps in ant evolution.

56 Introduction

57 Alternate behavioral and physiological phenotypes from individuals with the same
58 genome can be a response to environmental variation. This phenotypic plasticity is an important
59 mechanism of morphological and behavioral variation across many taxa (West-Eberhard, 1989).
60 Alternate phenotypes, such as eyespots on wings, the presence or absence of wings, and variation
61 in horn length, have been associated with various non-mutually exclusive mechanisms, like
62 differences in gene expression, endocrine signaling, and appendage development (Shapiro, 1976;

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63 Wheeler and Nijhout, 1983; Crespi, 1988; Emlen, 1994; Evans and Wheeler, 1999; Abouheif and
64 Wray, 2002). In social insects, complex endocrine and molecular mechanisms regulate the
65 development of the alternate morphological phenotypes of queens and workers or different sizes
66 of workers (Wheeler, 1986; Corona et al. 2016). In addition to the morphological variation,
67 plasticity in behavioral phenotypes within one morph may lead to an interaction between
68 morphological and behavioral variance.

69 Within Hymenoptera, the most distinct morphological differences between queen and
70 workers are present in ants (Hölldobler and Wilson, 1990). While queens and workers can be
71 distinguished by the degree to which anatomical modules are associated with each morphological
72 phenotype (Londe et al. 2015), we can also define behavioral and physiological phenotypes as
73 worker-like or queen-like depending on their association with the queen or with the worker
74 morph (Smith et al. 2018). In most cases, the ant queen has a fully developed thorax with large
75 muscles, wings to fly, and somewhat developed ovaries before dispersing from the nest (Fletcher
76 and Blum, 1981; Vieira et al. 2011; Monnin et al. 2018; Peeters et al. 2020). After flying from
77 the nest, the queen mates with males attracted by her sex pheromones (Ayasse et al. 2001). After
78 finding a new habitat, she sheds her wings and lays eggs within several days (Keller and Passera,
79 1990). The queen cares for her first generation of brood, and, in some species, also forages to
80 provision them with food (Cassill, 2002; Peeters and Ito, 2001). Eventually, the adult worker
81 offspring perform most of the non-reproductive labor of an established colony, such as brood
82 care, foraging, and defense, with thoracic muscles that favor ground-based locomotion
83 (Hölldobler and Lumsden, 1980; Traniello, 1989; Keller et al. 2014; Walsh et al. 2018; Peeters et
84 al. 2020). This clear-cut difference is nevertheless not universal.

85 In many natural contexts, the behavior and physiology of social insects does not strongly
86 associate with the specific queen or worker morphology (Sumner et al. 2018). In ant species
87 without queens, all tasks are divided among wingless workers (Peeters and Ito, 2001; Monnin
88 and Peeters, 2008). In these and other species, reproductive workers signal their fecundity in
89 similar ways to queens (Bourke, 1988; Peeters and Liebig, 2009). When workers reproduce,
90 they may show dominance interactions (Fletcher and Ross, 1985; Oliveira and Hölldobler, 1990;
91 Ito and Higashi, 1991; Ito, 1993; Monnin and Peeters, 1999) which are also expressed by queens
92 in some social systems (Oliveira and Hölldobler, 1991; Medeiros et al. 1992; Kolmer and
93 Heinze, 2000; Yamauchi et al. 2007). Furthermore, queens may be involved in nest construction

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94 (Peeters and Andersen, 1989; Murakami, 2020), and defense (Lachaud and Fresneau, 1985;
95 Jerome et al. 1997) which are housekeeping tasks typically performed by workers. Because ant
96 queens and workers show context-dependent degrees of association with specific behavioral and
97 physiological phenotypes, how is the expression of these worker-like instead of queen-like
98 phenotypes regulated in a morphological queen or worker?

99 Given that reproductive queens may show reduced behavioral and physiological
100 plasticity, we wanted to investigate this question in gynes, which are ant queens who have not
101 yet dispersed from the natal nest and mated. Queens are the only female ants capable of aerial
102 dispersal (Peeters and Ito, 2001). Therefore, the expression of queen-like phenotypes associated
103 with dispersal are impossible to induce in workers. After dispersal, the queen's behavior
104 changes as she and her new colony develop, making it challenging to assess the phenotype of a
105 foundress independent from her colony's size (Augustin et al. 2018). Gynes, however, are known
106 to show worker-like behaviors like foraging (Plateaux, 1978; Della Lucia et al. 1993; Brown,
107 1999; Hora et al. 2005; Vieira et al. 2011; Silva Araujo et al. 2016), care for the brood (Fresneau
108 and Dupuy, 1988; Bourke, 1991; Ito et al. 1996; Ruppel et al. 2002; Vieira et al. 2011), and nest
109 defense (Forder and Marsh, 1989). Since artificial dealation of gynes, the experimental removal
110 of their wings, in some species has activated the foundress behaviors of oviposition, nursing, and
111 defense, dealation may also initiate other behaviors more typically associated with workers
112 (Jemielity et al. 2006; Nehring et al. 2012).

113 With gynes of the ponerine ant species *Harpegnathos saltator*, we can test whether the
114 attachment of wings mediates a worker-like phenotype and if it interacts with the morphological
115 phenotype of queens. Workers and queens are morphologically distinct (Peeters et al. 2000),
116 with gynes showing putative sex pheromones on their cuticles that disappear in foundresses and
117 are absent in workers (Liebig et al. 2000). After the death of a foundress, workers perform
118 dueling, dominance biting, and policing behaviors after which some workers become the new
119 reproductive individuals, while others occupy non-reproductive roles in the colony (Peeters and
120 Hlldobler, 1995; Sasaki et al. 2016). A dominance bite involves one biting ant grasping a
121 recipient ant underneath in its mandible and jerking quickly towards itself, while at least two
122 antennal boxing ants approaching and avoiding one another is a duel. Antennal boxing by one
123 ant towards another that is unreciprocated is not a duel. This reproductive hierarchy among
124 workers suggests that a high variance among reproductive phenotypes is a property of workers in

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125 this species (Liebig et al. 1998). In this study, we compared the expression of worker-like
126 behavior and physiology of *H. saltator* gynes from up to three groups: alate gynes; gynes who
127 have shed their wings; and gynes whose wings were artificially removed.

128 We predicted that the gynes lacking wings should duel and initiate dominance biting at
129 higher frequencies than alates if they express a worker-like phenotype. Because gynes eclose as
130 alates and shed their wings thereafter, we compared the worker-like behaviors of similarly-aged
131 alate and dealate gynes. To control for the varying latency of a gyne to shed her wings (Table 1),
132 we artificially removed the wings from gynes and compared the frequency of their worker-like
133 behavior to alate gynes.

134 In the presence of established reproductive individuals, we also predicted that dealate
135 gynes, like workers, show little ovarian activity and pheromone production compared to alate
136 gynes. We compared the reproductive physiology and pheromone production of alate and dealate
137 gynes in the presence of an established reproductive because alate gynes may lose their wings
138 after becoming reproductive (Table 1). Dealate gynes who shed their wings at various ages were
139 compared to younger alate gynes. Artificially-dealate gynes were also compared to gynes of
140 similar age who shed their wings as well as alates. Since reproductive status can require several
141 months to manifest physiological differences in *H. saltator* workers (Ghaninia et al. 2017), we
142 assessed these parameters two months after wing loss.

143 **Materials and methods:**

144 **Source Colonies**

145 *Harpegnathos saltator* source colonies were collected from southern India from 1992
146 through 1999 (Peeters et al. 2000) and have been reared in the laboratory continuously since
147 then. Individuals and brood from multiple source colonies have been bred in new combinations
148 in the laboratory, and notes document the combination of colony backgrounds comprising a new
149 laboratory source colony. Source colonies are distinct from one another if the combination of
150 their originating colonies sampled from the field are different.

151 Colonies were maintained in a laboratory setting with a USDA APHIS permit (Number:
152 P526P-20-01935) at Arizona State University at 25 degrees Celsius on a 12-hour photoperiod.
153 They were housed in plastic containers (Model 79C, Pioneer Plastics, Inc. Dixon, KY, USA)
154 lined with Fluon (Fluoropolymer resin, Dupont, Torrance, CA, USA) with a plaster base
155 moistened with deionized water (No. 50046289, Labstone Blue, Modern Materials, Rochester,

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156 IN, USA; or No. 985-1692, Dentalstone-Buff, Darby Group Companies, Jericho, NY, USA) with
157 an area of dugout plaster beneath a piece of glass. Two to three times a week, colonies were
158 provided live crickets and sawdust for larvae to pupate, when frass and dead individuals were
159 removed.

160 Established gamergates (mated, reproductive workers) or reproductive queens were
161 identified in these source colonies from their reproductive behaviors and marked using Testor's®
162 paint (Rockford, IL, USA) and/or 34-gauge artistic wire marks according to the method of
163 Haight (2012). After laying an egg, a reproductive individual holds the egg in her mandibles and
164 can be found near the colony's brood, where she moves slowly compared to non-reproductive
165 individuals. After antennal contact with a reproductive, a non-reproductive individual sometimes
166 recoils and assume a lower submissive posture. (Liebig, 1998).

167 Identification of Gynes

168 Pupae from source colonies were checked two to three times per week for eclosion of
169 new gynes. Upon eclosion, new gynes were wire-marked according to the method of Haight
170 (2012) just anterior or posterior of the petiolar node and returned to their source colony to remain
171 non-reproductive in the presence of established reproductive individuals. Attachment of wings
172 and mortality of these gynes was recorded when colonies were fed. In rare cases, *H. saltator*
173 gynes show uninflated and shriveled wings after eclosion. For consistency, all gynes used in this
174 study showed four inflated wings after eclosion. The loss of any number of wings was defined as
175 the beginning of a dealate status. The cuticular hydrocarbons and number of yolky oocytes of a
176 gyne were measured using procedures described below.

177 Behavioral Experiment #1:

178 To compare the worker-like dueling behavior of gynes of different alate status in the
179 absence of established reproductive individuals, groups of 10 wire-marked gynes (age 7-40 days
180 old, median=23 days old, $n=20$) comprised of alate gynes and dealate gynes who had already
181 shed their wings, two or three callow workers, three worker pupae, and six male pupae were
182 combined in a new experimental nest. One nest was created from gynes, workers, and pupae
183 from the source colony F100, whereas another nest was created from the gynes, workers, pupae,
184 and two males from the source colony SAFC15. Both F100 and SAFC15 showed established
185 reproductive individuals at the time that individuals and brood were removed. Both nests were
186 watered and fed two to three times per week and provided live crickets. Because dueling may

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187 not begin for groups of 15 individuals in a new nest until 9 days after group formation (Fig. S1),
188 both experimental nests were not first observed until after 10 (F100) or 11 days (SAFC15).
189 Those who had lost any of their 4 wings by the start of the analysis period were classified as
190 dealate (age at dealation: 4-33 days, median: 12 days, $n=7$ gynes).

191 Gynes who participated in at least one duel with either another gyne or a worker were
192 noted during scan observation periods of 10 minutes at least one day after feeding. Mortality and
193 the alate status of gynes was also recorded during these periods. Alate gynes were observed 1-16
194 times (median: 10 observations, $n=10$ gynes) before they died or shed their wings, while dealate
195 gynes were typically observed for more periods. Therefore, the number of periods where dealate
196 gynes showed dueling was analyzed over only the first 10 observations to conservatively
197 compare to the number of variable observations of alate gynes in the same nests. The gynes
198 compared from both groups were of similar age (median= 23 days old, $n=10$ alate gynes;
199 median=24 days old, $n=7$ dealate gynes).

200 Behavioral Experiment #2:

201 To control for the variance in the latency for gynes to shed their wings while determining
202 whether alate status influences the expression of worker-like behavior, five randomly-chosen
203 wire-marked gynes, one from each of five age-sorted pairs (0-26 days since eclosion, median: 10
204 days old, $n= 110$ gynes) from the same source colony, were placed into to a new plaster nest for
205 one of two treatments: i. artificial dealation: the day of group formation, the wings of gynes were
206 removed with Vannas Spring Scissors (3mm cutting edge, No. 15000-00, Fine Science Tools,
207 Foster City, CA, USA) while they were held with forceps without anesthesia; ii. maintenance of
208 alation: alate gynes were held similarly with forceps without anesthesia, but wings were not
209 removed. 10 foraging workers from the same source colony were added to each nest.

210 A pair of experimental nests, one from each treatment, were filmed at 30 frames per
211 second at 1920 X 1080 resolution with a video camera (Panasonic, model HC-VX981 or HC-
212 V520, Newark, NJ, USA) under constant lighting but with minimal glare for 14 days
213 continuously. Two to three times per week, all nests were fed crickets that had been previously
214 paralyzed by workers and the nest's plaster nest was saturated with deionized water as needed.
215 Mortality and wing loss by alate gynes was recorded every day during filming as well as two to
216 three times per week in the weeks after filming. The first alate gyne shed her wings 12 days after
217 filming, while the second alate gyne lost her wings after 14 days. One alate gyne died after two

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218 days of filming, another died after 9 days, whereas one artificially-dealate gyne died four days
219 after filming. Two to four weeks after the onset of filming, two additional gynes died in each
220 treatment. To maximize the number of gynes showing behavior, the behavior from nests was
221 compared within two weeks from the start of filming.

222 The behavior of gynes in experiment nests was analyzed ten days after the start of filming
223 because both alate and dealate gynes will have begun to express worker-like behaviors.
224 Experimental nests were reviewed on a computer using QuickTime as fast as 30 times normal
225 speed from the beginning of filming to detect the first worker-like behaviors involving gynes.
226 Workers were involved in dueling and dominance in all nests ($n=6$), and artificially-dealate
227 gynes initiated dominance or were involved in dueling within six days since the start of filming
228 in their nests ($n=3$). Alate gynes in two of the three nests began dueling and/or biting behavior
229 within 9 days since the start of filming and continued to show these behaviors after 10 days (Fig.
230 S1).

231 Ten days after the start of filming, all groups were reviewed as fast as 30 times normal
232 speed for the first detection of a duel or dominance bite involving a gyne. After detecting the first
233 duel involving a gyne, regardless of whether the partner was another gyne or a worker, the
234 number of gyne-involved duels in the nest was counted for a subsequent two hours. The results
235 are presented for the count of gyne-involved duels over 30-minutes because non-parametric
236 comparisons showed similar effects of the counts of duels over 30-minutes to those over two
237 hours (Wilcoxon tests: 30-minutes: $W = 37.5$, p -value = 0.1272; 2-hour: $W = 36.5$, p -value =
238 0.1116) while a parametric model comparing counts over 2-hours was overfit. The number of
239 dominance bites initiated by a gyne in the same nest was counted for a subsequent six hours after
240 a gyne was observed initiating the first dominance bite after 10 days of filming. In the absence
241 of detecting at least one of these behaviors involving gynes, the interactions in a group were
242 observed for the entirety of a 10 to 14 day period.

243 Two weeks after filming, nests were provided live crickets two to three times per week,
244 at which times mortality and dealation was noted. The first offspring were male, suggesting that
245 the gynes in these nests were likely unmated. After several weeks, some of the gynes became
246 the established reproductive individuals in these nests. The ovary development ($n=6$) of these
247 reproductive gynes was assessed using methods described below.

248 **Cuticular hydrocarbon abundance and ovarian activity of non-reproductive gynes**

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249 To determine how the presence of wings affects a gyne's reproductive ontogeny as a
250 foundress, we isolated gynes in a small plastic container (Model 29C, Pioneer Plastics, Inc.
251 Dixon, KY, USA) with a blue plaster base lined with Fluon as above. The gynes were checked
252 for the presence of brood, wing loss, and mortality during observations one to two times per
253 week before providing them pre-stung crickets and saturating their plaster with deionized water.
254 Those gynes that shed their wings did so after laying eggs (Table 1). Because gynes become
255 dealate after becoming reproductive, it is difficult to interpret whether the phenotype of these
256 foundresses represents their alate status or their reproduction. To avoid this complication, we
257 compared the ovary development and abundance of cuticular hydrocarbons (CHC) from alate
258 gynes and dealate gynes in colonies with established reproductive individuals.

259 To control for the age at which a gyne loses her wings, we also non-destructively
260 sampled CHC abundance from gynes who remained in the nests of source colonies with
261 established reproductives at one, two, and three months of age before culling them, dissecting
262 their ovaries, and counting their yolky oocytes. Since callow gynes show a lower abundance of
263 large alkadienes compared to sclerotized alate gynes (Liebig et al. 2000), we sampled gynes at
264 one month to provide sufficient time for sclerotization and the associated changes in CHCs.

265 After non-destructive sampling of CHC abundance, one-month old gynes were randomly
266 assigned to one of two treatments: i. artificial-dealation: gynes were dealated as above and
267 returned to the source colony; ii. maintenance of alation: gynes were treated as above without
268 wing-removal and returned to the source colony. In this way, all gynes in the artificial-dealation
269 group lost wings at 1 month, while those in the control group remained alate. One-month old
270 gynes designated for alate or artificially-dealate treatments did not differ in the abundance of
271 pentatriacontadiene (C35:2) and heptatriacontadiene (C37:2) (Fig. S3). Gynes from these two
272 treatments were sampled again for CHC abundance one and two months later (i.e. at two- and
273 three-months old, respectively). After sampling the gynes for the third time, the ovaries of gynes
274 were dissected and the number of yolky oocytes counted.

275 In some cases (n=3), control alate gynes shed their wings after the initial sampling of
276 CHC abundance at one-month old. In other cases (n=3), control alate gynes shed their wings
277 after the second sampling of CHC abundance at two-months old. Two months after the loss of
278 their wings, gynes from these two groups, described as naturally-dealating gynes

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279 (NATLDEALATE), were pooled for analysis of CHC abundance as well as for the number of
280 yolky oocytes.

281 In rare cases (n=2), the fibers used to sample CHC abundance were stored in a
282 refrigerator in a plastic box after sampling. Later, the fibers were allowed to warm to room
283 temperature over a few minutes and alkadiene abundance was analyzed in the GCMS.
284 Specifically, the CHC abundance was sampled two months after dealation and analyzed in the
285 GC-MS 14 days later. Visually comparing these chromatograms to others in the same treatment
286 revealed no conspicuous difference due to storage in the refrigerator.

287 Sampling CHC abundance:

288 Gynes were immobilized without anesthesia in a paper cutout such that only its gaster
289 remained exposed on one side of the paper. To collect CHCs, a solid-phase microextraction
290 (SPME) fiber (Supelco, 30um, PDMS, fused silica, 23-gauge, Yellow, 57289-U, Bellefonte, PA,
291 USA) was rubbed across the gaster of the gyne's cuticle for 500 strokes. This method has been
292 shown to have similar effectiveness to hexane-extraction for detection of larger hydrocarbons
293 (Moneti et al. 1997).

294 Except for the rare cases (n=2) where fibers were stored in a refrigerator (see above), the
295 fiber was inserted immediately after sampling into a 280 degree Celsius inlet of a Gas-
296 Chromatograph/Mass-Spectrometer (GC-MS), and analyzed using helium as a carrier gas,
297 through a non-polar column (Agilent J & W, GC column, DB-1MS, size 30.0m X 250um X
298 0.25um nominal, Santa Clara, CA, USA), to ionization by a filament, and to the Mass-
299 Spectrometer detector. The oven of the GCMS was programmed to begin at 60 degrees for 2
300 minutes, increasing by 40 degrees per minute to 200 degrees Celsius. At 200 degrees Celsius,
301 the oven increased by only 5 degrees per minute Celsius to 320 degrees Celsius over 15 minutes.
302 C35:2 and C37:2 eluted near the known elution times of the straight-chain alkane standards for
303 pentatriacontane (C35) and heptatriacontane (C37) resuspended in pentane solvent that were
304 subject to the same temperature program.

305 Analyzing CHC abundance

306 By manually analyzing the ion fragmentation patterns of these candidate elution peaks,
307 the alkadiene can be identified with a base ion with a molecular weight of 67 (Kroiss et al. 2011)
308 as well as the molecular ion of 488 (C35:2) or 516 (C37:2). Once the alkadienes were identified
309 in the chromatogram, integration of the area under all elution peaks in the chromatogram was

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310 used to calculate the peak areas of C35:2 and C37:2 as a proportion of the total peak area of all
311 elution peaks in the chromatogram. Peak area for a given compound relative to all compounds
312 later than the retention time of C23 straight-chain alkane in the chromatogram was used as the
313 response variable because the total amount of hydrocarbons sampled from one gyne to the next
314 likely varies. Nonpolar compounds that elute earlier than C23 are not present on the *H. saltator*
315 cuticle in significant quantities (Liebig et al. 2000).

316 Ovary Dissection and Yolky Oocyte Count

317 Gasters were separated from the rest of the body of a gyne and tergites of cuticle were
318 separated from internal tissue in an anterior to posterior order in deionized water using forceps
319 on a Sylgard medium (Dow, Inc., Midland, MI, USA). The ovary was then isolated from
320 connected tissues to reveal exposed ovarioles and spermatheca. Mating status was confirmed by
321 identifying a white opaque in the center of the spermatheca (Peeters et al. 2000). Conspicuous
322 yellow bodies in the posterior portions of ovarioles indicated prior egg laying activity (Peeters
323 and Hölldober, 1995). Consistent with the literature (Peeters et al. 2000), “larger yolky oocytes”
324 was used as a proxy for ovarian activity, which could be distinguished from developing oocytes
325 and trophocytes by their: 1) opacity indicative of the presence of yolk protein and 2) ovoid shape
326 indicative of the chorion. Photographs of dissected ovaries and spermatheca were captured using
327 SPOT Insight QE (Model 4.2, Diagnostic Instruments, Inc. Sterling Heights, MI, USA) on a
328 Windows-based computer mounted on the trinocular port of a stereoscope (Leica MZ-125,
329 Buffalo Grove, IL, USA), while the stage was illuminated by dual-fiber gooseneck (NCL 150,
330 Volpi Mfg. Auburn, NY, USA). An observer blind to the true identities of the gynues counted the
331 number of yolky oocytes, the presence of yellow bodies, and the mating status of the gyne from
332 these photographs. The blind estimates of mating showed a perfect correlation with estimates
333 freshly after dissecting, while estimates of yolky oocytes were highly correlated with those
334 estimated by CAB (Fig. S2). All alate gynues (n=12) and most dealate gynues (n=12 of 13
335 artificially-dealate; n=4 of 5 naturally-dealate) were unmated based on the absence of a filled
336 spermatheca.

337 Statistical Analyses

338 To compare alate gynues and dealate gynues who shed their wings in source colonies,
339 Wilcoxon’s non-parametric test was used in the analysis of the number of yolky oocytes, the
340 abundance of cuticular hydrocarbons and age. Since gynues are produced from a limited number

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341 of source colonies, however, the response variables for experimental data were analyzed in a
342 linear mixed-model (LMM). In these cases, treatment was the fixed effect, while the source
343 colony identity was the random-intercept effect. To determine whether a mixed model will be a
344 better fit than a random-effects model, Akaike's Information Criterion was calculated and the
345 model structure with the lowest value was analyzed. An F-test with Kenward-Roger
346 approximation for smaller sample sizes (Kenward and Roger, 1997) was used to compare the full
347 model to a random-effects model to indicate the significance of the fixed effect. Significance
348 was assessed at an alpha of 0.05. All mixed models were fitted with the function *lmer* (Bates et
349 al. 2015), and all statistics were conducted in R*(v.3.2.3).

350 To check the assumptions of the mixed models, Pearson's residuals were checked for
351 normality with a Shapiro-Wilk test at an alpha of 0.01. For those with p-value between 0.05 and
352 0.01, linearity of residuals was visually evaluated with quantile-quantile plots. The homogeneity
353 of variance of Pearson's residuals were checked visually by plotting the residuals against fitted
354 values (Zuur et al 2009). In cases where models did not satisfy assumptions of normality, count
355 data was $\ln(Y+1)$ transformed, while proportion data was arc-sine-square root transformed. For
356 posthoc comparison of a fixed effect with more than two levels, estimated marginal means
357 (package: emmeans) evaluated which groups were significantly different using Tukey adjustment
358 of p-values for multiple comparisons.

359 **Results:**

360 **Behavior**

361 If dealation activates the worker-like phenotype, dealate gynes should be involved in
362 worker-like behaviors more frequently than alate gynes of similar age. From observations of
363 experimental nests with dealate gynes who shed their wings at various ages (range: 4-33 days;
364 median: 12 days; n=7) as well as similarly-aged alate gynes from the same source colony, the
365 dealate gynes were found to be dueling more often (**Fig. 1**). To control for the latency until
366 gynes become dealate, we removed the wings from groups of five gynes on the same day and
367 combined them with non-reproductive workers from the same source colony in a new nest in a
368 second experiment. These artificially-dealate gynes engaged in duels and initiated dominance
369 bites at a much higher level than alate gynes in similar groups (**Fig. 2**).

370 **Ovarian Activity**

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371 Alate queens showed yolky oocytes in the presence of established reproductives. If this is
372 associated with dispersal and dealation produces a worker-like reproductive state, then dealate
373 gynes in the presence of established reproductive individuals should show fewer yolky oocytes
374 than alate gynes. In source colonies with established reproductive individuals, gynes who
375 dealated at various ages (range: 53-165 days; median: 66 days, $n=7$) showed fewer yolky oocytes
376 (Alate, median: 5 yolky oocytes, $n=8$, Dealate, median: 0 yolky oocytes, $n=7$) and tended to be
377 older than alate gynes (**Fig. 3**). To control for the variance in age and latency of a gyne to lose
378 her wings present in the previous comparison, we removed the wings from one-month old gynes
379 (Artificially-Dealate) and compared their subsequent ovary development to that of alate gynes of
380 similar age in source colonies with established reproductive individuals. To account for the
381 effect of removing their wings, we also compared the ovaries of Artificially-Dealate gynes to
382 those of gynes who shed their wings at various ages (Naturally-Dealate). Two months after wing
383 loss, both Artificially-Dealate and Naturally-Dealate gynes showed fewer yolky oocytes in their
384 ovaries than three-month old alate gynes (**Fig. 4**). While the ovaries of these alate gynes were
385 active, they were still not as developed as those of older reproductive dealate gynes used in the
386 second behavioral experiment ($n=6$, age: 377-524 days, median: 497.5 days, 7-17 yolky oocytes,
387 median: 11.5 yolky oocytes).

388 **Cuticular Hydrocarbon Abundance**

389 If the hydrocarbon profile on a gyne's cuticle also depends on alate status, then the gynes
390 that have lost their wings should show a reduced abundance of the alkadienes that are
391 characteristic of non-callow gynes (Liebig et al. 2000). In colonies with established reproductive
392 individuals, gynes who shed their wings at various ages (range: 16-383 days; median: 94 days,
393 $n=21$) showed lower abundances of the cuticular hydrocarbons (CHC) pentatriacontadiene
394 (C35:2) and heptatriacontadiene (C37:2) than alate gynes (**Fig. 5**). Because of the large observed
395 latency of gynes to shed their wings, we also compared the abundance of CHCs of Artificially-
396 Dealate gynes, Naturally-Dealate gynes, and three-month old alate gynes. Two months after
397 wing loss, both Artificially-Dealate and Naturally-Dealate gynes showed reduced abundance of
398 C35:2 and C37:2 compared to three-month old alate gynes (**Fig. 6**).

399 **Discussion:**

400 The loss of wings of *H. saltator* gynes within an established colony induced the
401 expression of a behavioral and physiological phenotype that is usually only shown by workers.

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402 Specifically, dealate gynes displayed behaviors that are commonly expressed by workers during
403 the establishment of a reproductive hierarchy but not by dealate queens that follow the trajectory
404 of founding a colony and becoming an established queen. Within a colony, alate gynes
405 occasionally showed such behaviors as well but to a much lower extent than dealate gynes. The
406 latter showed additional worker-like features such as low ovarian activity in the presence of
407 reproductive individuals, but activated their ovaries in the absence of established reproductives.
408 In contrast, alate gynes in the presence of reproductive individuals demonstrated ovarian activity
409 associated with their preparation for a mating flight that was intermediate to non-reproductive
410 and highly-reproductive dealate gynes. Alate gynes also expressed a higher abundance of
411 putative sex pheromones on their cuticle that was reduced in dealate gynes as well as in workers
412 (see also Liebig et al. 2000). These differences help identify queen-specific traits in species with
413 little queen-worker dimorphism expected early after the transition to ant eusociality.

414 The presence of wings determines the expression of true worker-like or queen-like
415 behavior in *H. saltator* gynes. In *H. saltator* and other ants, foundresses perform brood care,
416 defense, and foraging in a laboratory setting and the field (Table 1; Peeters et al. 2000) which is
417 also part of the task repertoire of workers (Peeters and Hölldobler, 1995; Haight, 2012). Brood
418 care behavior can also be induced by dealation in other ants (Jemielity et al. 2006; Nehring et al.
419 2012). Thus, it is not clear if dealate queens express a worker-like phenotype or part of the
420 foundress phenotype when displaying brood care behavior. In contrast, dealate gynes of *H.*
421 *saltator* engaged in behaviors that are only shown by workers during the establishment of their
422 reproductive hierarchy (Sasaki et al. 2016). Thus, the expression of these hierarchy-related
423 worker behaviors indicate that queens switch to a true behavioral worker phenotype upon
424 dealation within a colony. Workers on the other hand switch to a foundress phenotype that
425 includes foraging and brood care in parallel with reproduction when isolated on their own which
426 is a phenotype that is normally not expressed (Liebig et al. 1998). Thus, the only exclusive
427 queen-specific phenotype is related to flying during dispersal (Hakala et al. 2019).

428 Wing attachment also determined the ovarian activity of a gyne. Dealate *H. saltator*
429 gynes, like workers, showed ovarian activity that depended on social context: they were non-
430 reproductive in the presence of reproductive inhibition; or they were highly reproductive in the
431 absence of such inhibition (Liebig et al. 1998). However, ovaries of alate *H. saltator* gynes
432 contained developing eggs most likely in preparation for founding a nest after the mating flight.

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433 In fact, dispersing unmated gynes of *H. saltator* have been found to have ovaries containing
434 mature oocytes (Peeters et al. 2000). While *H. saltator* workers show different behavioral and
435 physiological phenotypes depending on their reproductive status (Liebig et al. 1998; Penick et al.
436 2021), these results collectively suggest that the attachment of wings to a queen determines
437 whether her ovarian activity oscillates as a function of social context, like workers in this
438 species.

439 Unlike dealate gynes and workers, alate gynes produced a high abundance of C35:2 and
440 C37:2 on their cuticles, which may function to attract mates (Liebig et al. 2000). Insects use sex
441 pheromones to signal mating receptivity (Ayasse et al. 2001), but female-specific sex
442 pheromones have only been identified from a limited number of ant species (Walter et al. 1993;
443 Greenberg et al. 2007; Castracani et al. 2008; Greenberg et al. 2018; Iwamoto et al. 2020). While
444 social insect queens produce various compounds that function as sex pheromones (Gary, 1962,
445 Ayasse et al., 1999, Niehuis et al. 2013; Wen et al. 2017), sex-specific alkadienes mediate
446 mating in some Hymenopterans (Syvertsen et al. 1995; Krokos et al. 2001) and male *H. saltator*
447 perceive larger hydrocarbons (Ghaninia et al. 2018). Therefore, the higher abundance of
448 alkadienes on alate *H. saltator* gynes may be a contact sex pheromone for use during the mating
449 flight.

450 How might the loss of wings alter the gyne's phenotype? While mating status and the
451 attachment of wings influence the behavior and physiology of *Monomorium pharaonis* queens
452 (Nagel et al. 2020), it is unlikely that mating mediates the phenotypic changes following
453 dealation in the *H. saltator* gyne since nearly all gynes were unmated. Instead, if the wings of *H.*
454 *saltator* gynes are innervated similarly to the gemmae of *Diacamma* (Gronenberg and Peeters,
455 1993), then these neurons may also mediate the effect of dealation on gyne behavior and
456 physiology.

457 Our results indicate that queens can express the behavioral phenotype of a worker even in
458 conditions that do normally not occur. Conversely, workers can express a queen-like phenotype
459 as reproductive workers in a colony and in isolation similar to a founding queen (Liebig et al.
460 1998, Liebig et al, 2000). This means that both queen and worker morphs can express each
461 other's behavioral and physiological phenotype given the respective context. This also means
462 that the main difference between queens and workers in this species is the ability of queens to
463 disperse due to their possession of a winged thorax and flight capability. In the evolution of ants

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464 from a solitary wasp ancestor, the reproductive specialization of queens compared to workers
465 and the loss of wings and associated change in the thoracic musculature of workers could have
466 evolved simultaneously or one feature could have evolved first (Hanna and Abouheif, 2021). If
467 our results apply to other species with weak queen-worker dimorphism, the difference between
468 the queen and workers early in ant evolution might be defined by dispersal ability rather than a
469 distinct reproductive dimorphism.

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475 **Competing Interests**

476 No competing interests declared.

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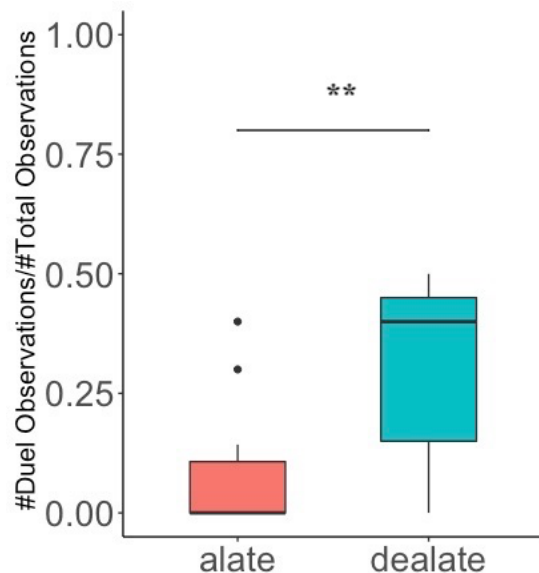
Loss of wings induces the expression of the worker-like phenotype in queens of a ponerine ant

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707 **Figure Legends**

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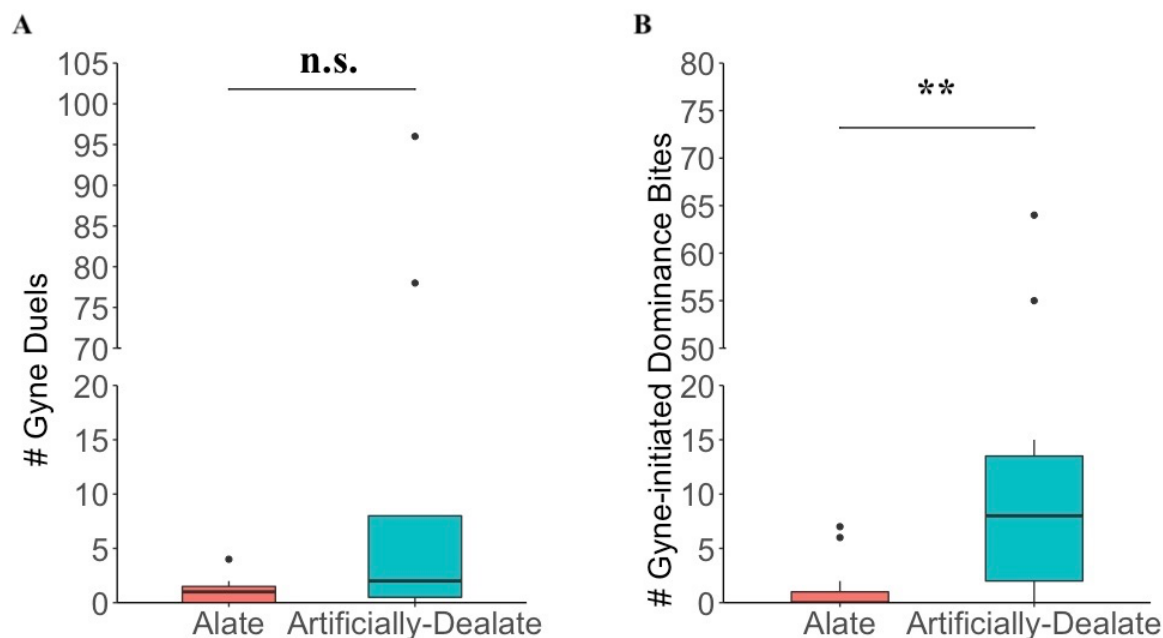


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Loss of wings induces the expression of the worker-like phenotype in queens of a ponerine ant

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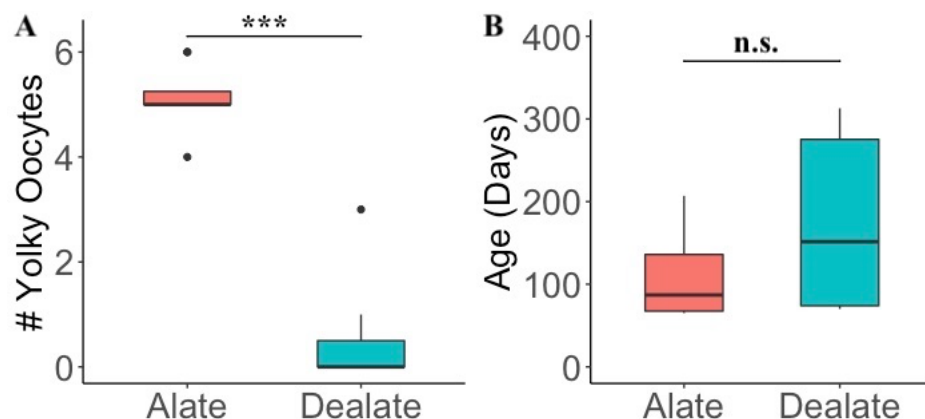
710 **Fig. 1. Dueling by alate and dealate gynes.** Dealate gynes were observed to be dueling over a
711 larger proportion of 10-minute scan observations than alate gynes of similar age. Box shows
712 median and interquartile range, while whiskers extend to 95% of data, and points are outliers. **:
713 p-value < 0.01. LMM of arc-sine-square-root-transformed data with Kenward-Roger
714 approximation for small samples (alate, $n=10$; dealate, $n=7$; $F_{1, 14.03} = 9.36$, $p=0.008$).
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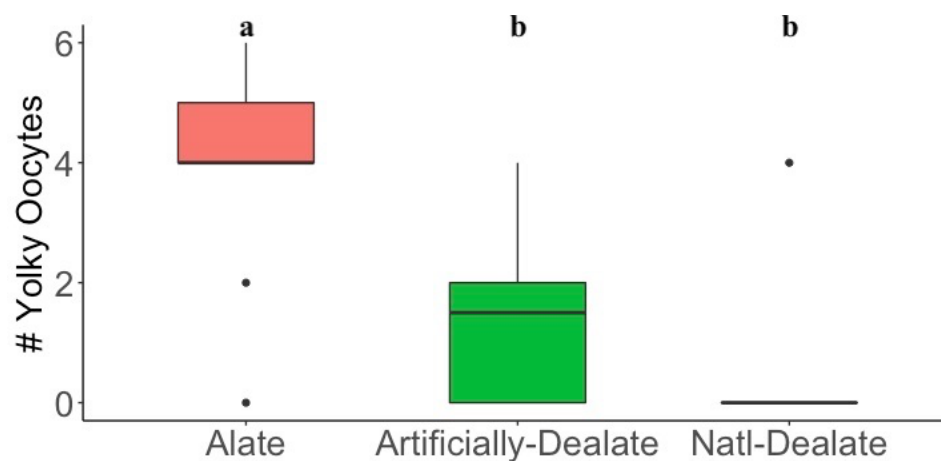
716 **Fig. 2. Frequency of duels and dominance bites involving gynes in the alate and artificially-**
717 **dealate experimental nests. A)** Gynes in the artificially-dealate groups are involved in more
718 duels than the alate gynes in the alate groups. **B)** Gynes in the artificially-dealate groups initiate
719 significantly more dominance bites. Box shows median and interquartile range, while whiskers
720 extend to 95% of data, and points are outliers. **: p-value < 0.01. LMMs on $\ln(Y+1)$ -
721 transformed data using F-test with Kenward-Roger approximation.
722 (Alate groups, $n=11$; Artificially-Dealate groups, $n=11$; **A)** $F_{1, 16.05} = 4.05$, $p=0.061$; **B)** $F_{1, 16.04} = 10.66$, $p=0.005$.)
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Loss of wings induces the expression of the worker-like phenotype in queens of a ponerine ant

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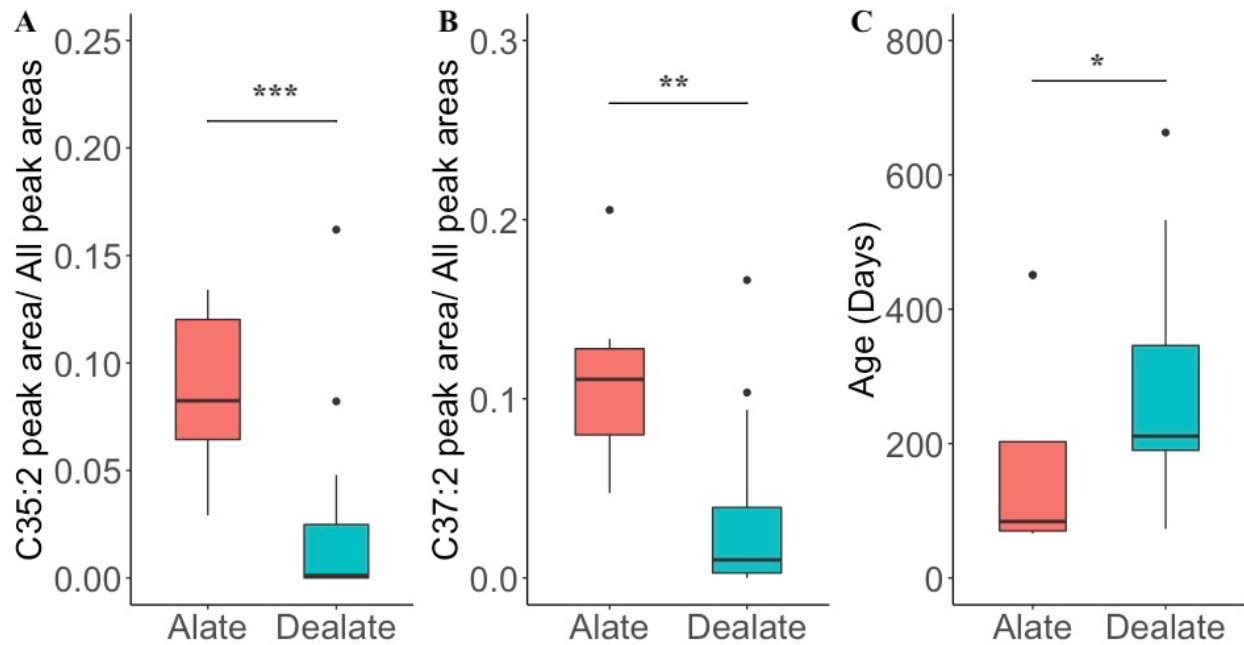
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727 **Fig. 3. Ovarian activity of gynes of different alate status and age in colonies with**
728 **established reproductive females.** A) alate gynes show more yolky oocytes as counted from
729 photographs of their dissected ovaries than dealate gynes. B) The alate gynes tend to be younger
730 than the dealate gynes. ***: p -value < 0.001. Box shows median and interquartile range, while
731 whiskers extend to 95% of data, and points are outliers (Alate gynes, $n=8$; Dealate gynes, $n=7$;
732 Wilcoxon tests: A) $W = 56$, $p = 0.0009$; B) $W = 59$, $p = 0.076$).
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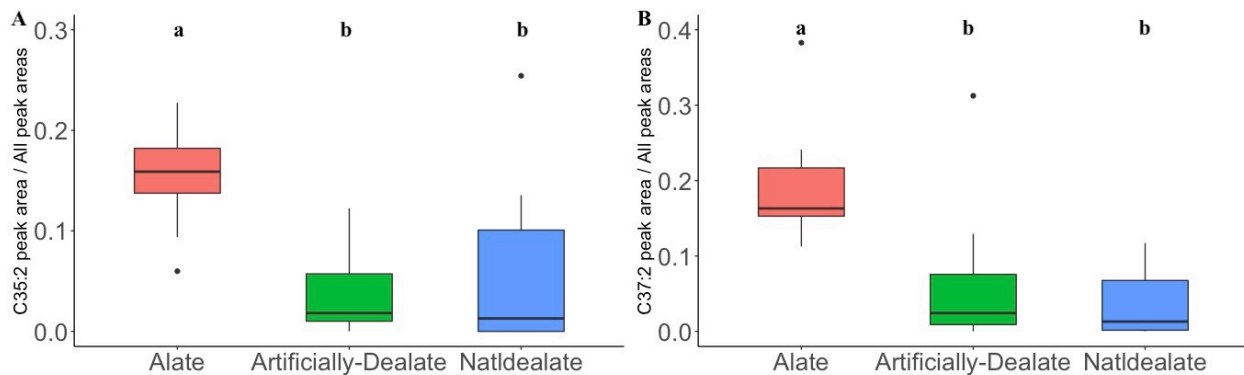
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735 **Fig. 4. Ovary development of non-reproductive gynes depends on alate status.** 2 months
736 after wing loss, the ovaries of dealate gynes have fewer yolky oocytes than 3-month old alate
737 gynes. Box shows median and interquartile range, while whiskers extend to 95% of data, and
738 points are outliers (LMM, F-test with Kenward-Roger approximation, $F_{2, 22.88} = 11.875$, $p =$
739 0.0002901 . Estimated marginal mean differences with Tukey adjustment of p -values for multiple
740 comparisons, shown with different letters above. Alate: $n = 12$, median age: 91.5 days;
741 Artificially-Dealate: $n = 12$, median age: 90.5 days; Natl-Dealate: $n = 5$, median age: 128.5
742 days).
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Loss of wings induces the expression of the worker-like phenotype in queens of a ponerine ant

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746 **Fig. 5. Abundance of larger alkadienes on the cuticles of gynes of different alate status and**
747 **age in colonies with established reproductive individuals.** The abundance of A) C35:2; B)
748 C37:2; and C) the age of the gynes in colonies with established reproductive individuals. Box
749 shows median and interquartile range, while whiskers extend to 95% of data, and points are
750 outliers*:p-value<0.05; **p-value<0.01; ***: p-value< 0.001. (Alate $n=8$; Dealate
751 $n=21$; Wilcoxon tests: A) $W=153$, ; B) $W=151$, $p=0.001$; C) $W=41.5$, $p=0.040$).
752



753
754 **Fig. 6. Abundance of larger alkadienes on the cuticles of gynes 2 months after wing loss or**
755 **of alate gynes that are 3 months old.** Alate gynes show a higher abundance of C35:2 and C37:2
756 than dealate gynes 2 months after wing loss. Box shows median and interquartile range, while
757 whiskers extend to 95% of data, and points are outliers (Alate: $n=13$, median age: 91 days;
758 Artificially-Dealate: $n=13$, median age: 90 days; NatlDealate: $n=7$, median age: 129 days).
759 LMM of arc-sine-square root-transformed data, F-test with Kenward-Roger approximation. A)
760 $F_{2, 28.59}=13.30$, $p=8.3e-05$; B) $F_{2, 28.40}=14.15$, $p=5.5e-05$. Estimated marginal mean differences
761 with Tukey adjustment of p-values for multiple comparisons, shown with different letters above.)
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Table 1. Characteristics of alate gynes in a foundress context
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Loss of wings induces the expression of the worker-like phenotype in queens of a ponerine ant

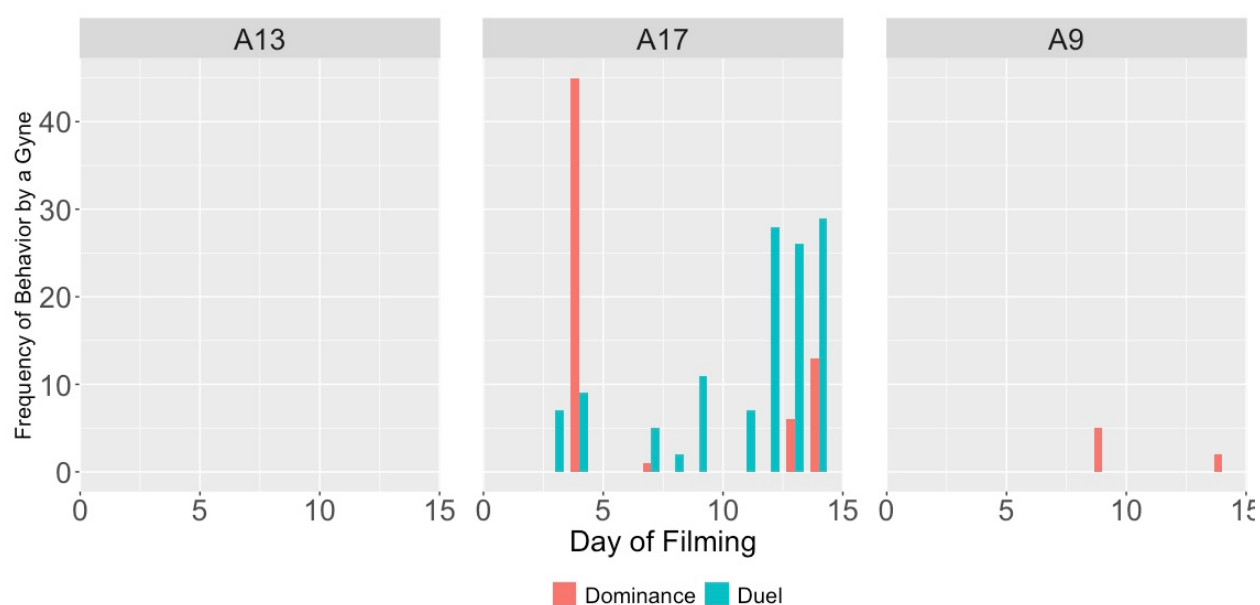
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	Age at isolation (days)	Latency from isolation until 1st egg laid (days)	Latency from 1st egg laid until loss of wings* (days)
Median	35	7.5	36
Min	33	1	16
Max	39	33	87
<i>n</i>	17	17	14

763 * 3 gynes who laid eggs never shed their wings.

764 Supplemental Figures

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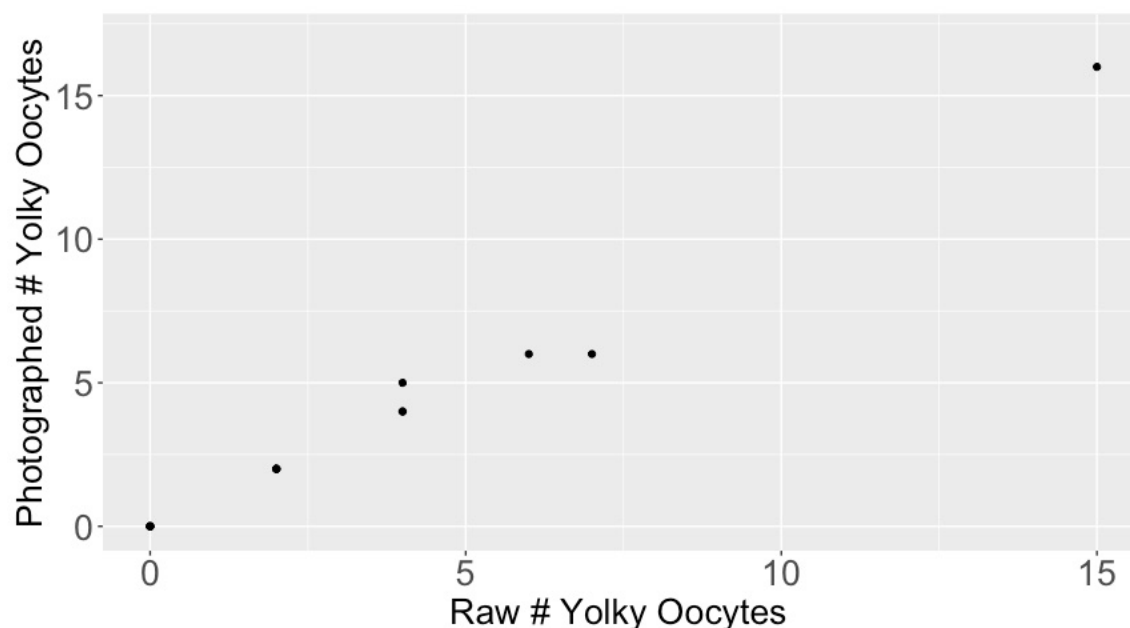
767 **Fig. S1.** Sum of alate gyne-initiated behaviors within each day of filming over 2 weeks for 3
768 groups of alate gynes (A9, A13, A17) used in the second behavioral experiment.

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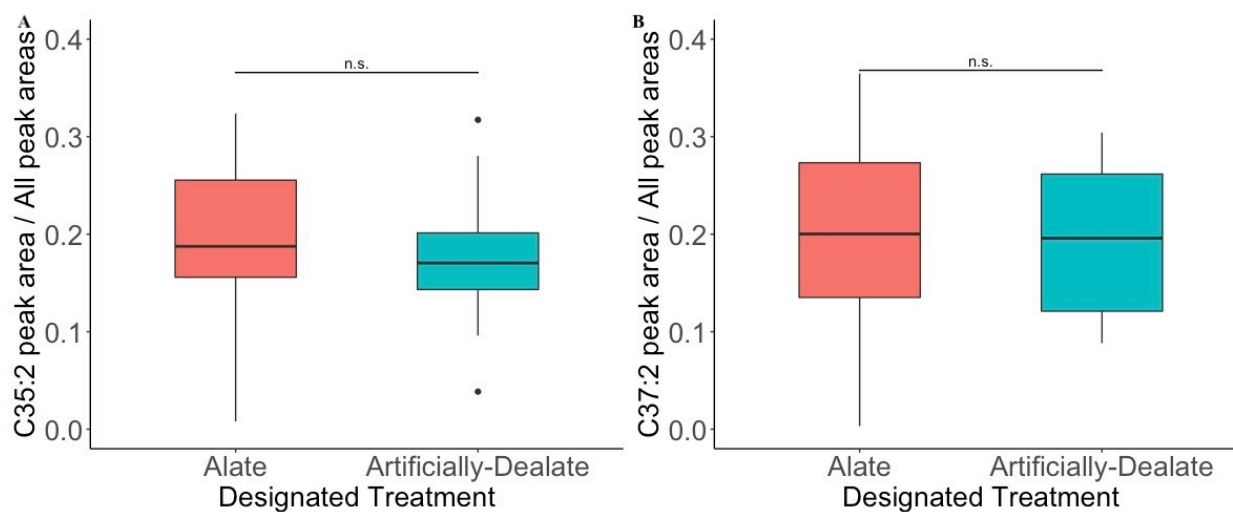
Loss of wings induces the expression of the worker-like phenotype in queens of a ponerine ant

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Fig. S2. The correlation between the number of yolky oocytes of blind estimates from photographs of ovary dissections and the raw counts made by another observer of the same ovaries when they were fresh (n= 14). There is a high correlation between the number of yolky oocytes of the blind estimates from photographs of ovary dissections and the raw counts of the same ovaries when they were fresh (Pearson's correlation: 0.99, n= 14).



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Fig. S3. One-month old alate gynes designated for alate or artificially-dealate treatments do not differ in abundance of C35:2 or C37:2. (Alate $n=34$; Artificially-Dealate $n= 17$; Wilcoxon tests, A) C35:2, $W = 306$, $p\text{-value} = 0.27$; B) Wilcoxon test, C37:2, $W = 264$, $p\text{-value} = 0.85$).