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RESEARCH ARTICLE

Worker-like behavioral and physiological phenotype in queens with removed wings in a ponerine ant

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ABSTRACT

Many highly eusocial insects are characterized by morphological differences between females, which are especially pronounced in ants. How these differences associate with particular behavioral and physiological phenotypes can illuminate early ant evolution. In ants, the morphological queen usually possesses a larger thorax with wings compared with a wingless worker. While queens specialize in reproduction, workers help with non-reproductive tasks and show various levels of reproductive degeneration. Here, we investigated the level of behavioral and physiological plasticity within queens in the ant species *Harpegnathos saltator*, which shows limited queen–worker dimorphism. We found that the experimental removal of wings led to the expression of worker behaviors and physiology, by examining young queens with wings, known as alate gynes, and those whose wings have been experimentally removed or naturally shed, known as dealate gynes. Compared with alate gynes, dealate gynes displayed higher frequencies of behaviors that are naturally shown by workers during reproductive competition. In addition, dealate gynes exhibited a worker-like range of ovarian activity. Like workers, they lacked the putative sex pheromones on their cuticle characteristic of dispersing gynes. Because gynes activate a worker-like phenotype after wing removal, the essential difference between the queen and worker in this species is a dispersal polyphenism. If the queen plasticity observed in *H. saltator* reflects the early stages of ant eusociality, a dispersal dimorphism rather than a distinct reproductive dimorphism might represent an early step in ant evolution.

KEY WORDS: Behavioral plasticity, Dealation, Queen–worker dimorphism, Dispersal polyphenism, Sex pheromones, Ovarian activity

INTRODUCTION

Phenotypic plasticity produces alternative behavioral and physiological phenotypes among individuals with the same genome, leading to the variation in morphological and behavioral phenotypes observed in many taxa (West-Eberhard, 1989). Alternative phenotypes, such as eyespots on wings, the presence or absence of wings, and variation in horn length, result from various mechanisms that may not be mutually exclusive, such as differences in gene expression, endocrine signaling and appendage development (Shapiro, 1976; Wheeler and Nijhout, 1983; Crespi, 1988; Emlen, 1994; Evans and Wheeler, 1999; Abouheif and Wray, 2002). In social insects, complex endocrine and molecular mechanisms regulate the development of the alternative

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morphological phenotypes of queens and workers or of the different worker sizes (Wheeler, 1986; Alvarado et al., 2015; Corona et al., 2016; Rajakumar et al., 2018). Within specific morphological phenotypes, however, behavioral phenotypes may vary (Calabi, 1988; Calabi and Rosengaus, 1988; Calabi and Traniello, 1989a,b; Gordon, 1989a,b; Seid and Traniello, 2006; Gordon, 2016). Therefore, similar behavioral phenotypes may be expressed in the multiple morphological phenotypes of social insects.

Within eusocial Hymenoptera, ants show the most dramatic morphological differences between the queen and worker. These morphological distinctions are often associated with specific behavioral and physiological traits (Hölldobler and Wilson, 1990), leading to phenotypes that may then be associated predominantly with the morph of the worker (worker-like) or with that of the queen (queen-like). In the most common case, the ant queen has a fully developed thorax with large muscles, wings to fly and somewhat developed ovaries before dispersing from the nest (Fletcher and Blum, 1981; Vieira et al., 2011; Monnin et al., 2018; Peeters et al., 2020). After flying away from the nest, the queen mates with males attracted to her sex pheromones (Ayasse et al., 2001). Once she finds a suitable nesting site, the queen sheds her wings and begins to lay eggs within several days, becoming the foundress of a new colony (Keller and Passera, 1990). The queen nurses her first generation of brood, and, in some species, also forages to provide them with food (Peeters and Ito, 2001; Cassill, 2002). Eventually, the adult worker offspring perform the bulk of non-reproductive labor in an established colony, such as brood care, foraging and defense, with thoracic muscles that favor ground-based locomotion (Hölldobler and Lumsden, 1980; Traniello, 1989; Keller et al., 2014; Walsh et al., 2018; Peeters et al., 2020). These queen- and worker-specific behavioral patterns are a classic representation of the reproductive division of labor in ants.

The association of some behaviors with the queen and others with the worker morph is not universal, however. In many natural contexts, the behavior and physiology of a social insect do not strictly associate with the queen or worker (Sumner et al., 2018). For instance, tasks are divided among wingless workers exclusively in ant species without queens (Peeters and Ito, 2001; Monnin and Peeters, 2008). In these and other species, reproductive workers signal their fecundity in similar ways to a queen (Bourke, 1988; Peeters and Liebig, 2009). When workers reproduce, they may show dominance behavior (Fletcher and Ross, 1985; Oliveira and Hölldobler, 1990, 1991; Ito and Higashi, 1991; Ito, 1993; Sommer et al., 1993; Monnin and Peeters, 1999), which can also be expressed by queens in some contexts (Hölldobler and Carlin, 1985; Oliveira and Hölldobler, 1991; Medeiros et al., 1992; Sommer and Hölldobler, 1992; Kolmer and Heinze, 2000; Yamauchi et al., 2007). Queens may also perform housekeeping tasks typically performed by workers, such as nest construction (Peeters and Andersen, 1989; Murakami, 2020) and defense

(Lachaud and Fresneau, 1985; Jerome et al., 1998). Given that some behavioral and physiological phenotypes can be expressed by both queens and workers, how is the expression of these phenotypes regulated in a morphological queen as opposed to a worker?

Although aerial dispersal aligns with a particular queen phenotype (Peeters and Ito, 2001), such queens nevertheless also express behavior in context-specific ways. Those queens founding a colony produce eggs and show frequencies of non-reproductive behaviors in line with their age and the developmental phase of their new colony (Julian and Gronenberg, 2002; Augustin et al., 2011). In contrast, gynes, those queens produced in the reproductive stage of a colony's development that have not yet dispersed from the nest, are generally young and unmated. For a period of time, gynes also show the wings and flight muscles to disperse aerially (Hölldobler and Wilson, 1990). Gynes demonstrate similar non-reproductive behaviors to foundresses, such as foraging (Plateaux, 1978; Della Lucia et al., 1993; Brown, 1999; Hora et al., 2005; Vieira et al., 2011; Araújo et al., 2016), nursing (Fresneau and Dupuy, 1988; Bourke, 1991; Ito et al., 1996; Rüppell et al., 2002; Vieira et al., 2011) and nest defense (Forder and Marsh, 1989). Therefore, if they fail to disperse, they can perform non-reproductive tasks in the colony. In these cases, are gynes activating behaviors from the ant's worker repertoire or from the behavioral program that queens show during colony founding?

The behavior of a gyne may be controlled by the attachment of her wings as well as the titer of specific hormones. After experimental wing removal, known as artificial dealation, gynes show oviposition, nursing and defense (Jemielity et al., 2006; Nehring et al., 2012). In addition, juvenile hormone (JH) is linked to the dealation of ant queens in multiple species (Kearney et al., 1977; Vargo and Laurel, 1994; Burns et al., 2002; Brent and Vargo, 2003; Penick et al., 2011) and JH is associated with worker-like behavior in social insects (Robinson and Vargo, 1997; Giray et al., 2005; Lengyel et al., 2007; Dolezal et al., 2009). Therefore, an endocrine factor such as JH may operate in tandem with dealation to regulate gyne behavior.

Using gynes of the ponerine ant species *Harpegnathos saltator*, we evaluated whether the experimental removal of their wings induced worker-like behavior and physiology. Unlike gynes of other ant species that may be adopted as secondary reproductives (Boomsma et al., 2014), no evidence supports secondary polygyny in *H. saltator* (Peeters and Hölldobler, 1995). Gynes are produced at various colony sizes at the beginning of the monsoon season and then leave the nest to mate and found a new colony. No alate gynes have been found in colonies 3 months later and the presence of dealate unmated gynes in colonies with established reproductive individuals outside of the mating season is rare (Peeters et al., 2000). If a foundress queen dies in an established colony, some workers assume the roles of principal reproductives in the colony. These workers establish themselves through dominance behaviors. Specifically, they perform dueling, dominance biting and policing behaviors that lead to a social hierarchy composed of reproductive workers, known as gamergates (Peeters and Crewe, 1984), and non-reproductive workers that perform helper tasks (Peeters and Hölldobler, 1995; Sasaki et al., 2016). Among the behaviors involved in the formation of the reproductive hierarchy, a dominance bite involves one biting ant grasping a recipient ant underneath in its mandible and jerking quickly towards itself while dueling consists of at least two antennal boxing ants approaching and avoiding one another (Heinze et al., 1994). Because the dominance behaviors shown by workers during the establishment of a reproductive hierarchy have not been observed in *H. saltator*

queens (J.L., personal observation), these behaviors are characteristic of the worker phenotype in this species. In addition, the transformation of non-reproductive helpers into reproductively active workers suggests that a spectrum of reproductive phenotypes is a property of *H. saltator* workers (Liebig et al., 1998). Here, we measured the gyne's expression of worker dominance behavior during competition over reproduction to investigate whether these gynes are able to show behaviors that are regularly only displayed by workers.

If dealation of gynes is associated with the expression of worker-like behaviors and a shift to a worker-specific behavioral program, dealate gynes should display dueling and initiate dominance biting in contrast to queen-like alate gynes in a hierarchy-forming context. Because gynes eclose as alates and shed their wings thereafter (Hölldobler and Wilson, 1990), we compared the worker-like behaviors of similarly aged alate and naturally dealate gynes. To control for the latency of a gyne to shed her wings (Table 1), we artificially removed the wings from gynes and compared the frequency of their worker-like behavior with that of alate gynes.

In addition to assessing behavioral differences, we also compared the ovarian development, mating status and production of putative sex pheromones between alate and dealate gynes. As alate gynes have already begun to prepare for colony founding, their ovaries are partially activated (Fletcher and Blum, 1981; Vieira et al., 2011), which can be measured by the presence of yolky oocytes (Monnin et al., 2018). In contrast, non-reproductive workers do not usually have partially activated ovaries in the presence of other reproductives (Hölldobler and Wilson, 1990). To ensure that they breed with a male from another nest, alate gynes should avoid mating until they have flown away from the nest (Peeters et al., 2000). If dealate gynes show similar mating receptivity to workers that mate with their male relatives (Peeters and Hölldobler, 1995), then dealate gynes should also inbreed with their relatives in the nest. To assess the presence of a worker-like or a queen-like phenotype in gynes, we also evaluated the expression of putative sex pheromones on the cuticles of gynes that disappear after they have dispersed, mated, lost their wings and became foundresses (Liebig et al., 2000).

We predicted that alate gynes should show greater ovarian activity and putative sex pheromone production compared with dealate gynes, which express the phenotype of workers. Because most alate gynes in social isolation shed their wings shortly after becoming reproductively active (Table 1), we evaluated the reproductive physiology, mating status and pheromone production of gynes in nests with established reproductive individuals. We presumed that gynes in this context would not become reproductively active and would therefore be more likely to retain their wings. In these nests, we first compared older dealate gynes that shed their wings with younger alate gynes. Because physiological changes to *H. saltator* may require months to manifest (Ghaninia et al., 2017), we then compared similarly aged

Table 1. Characteristics of alate gynes in a foundress context

	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
Minimum	33	1	16
Maximum	39	33	87
n	17	17	14

*Three gynes that laid eggs never shed their wings.

alate gynes with artificially dealate gynes as well as dealate gynes that shed their wings in these same nests. In a weakly dimorphic ant species with workers that possess a high reproductive potential, the association of wing attachment with worker-like behaviors in *H. saltator* gynes informs how the behavioral phenotypes are regulated. The characterization of the behavior and physiological plasticity of gynes and their ability to express worker-like phenotypes in this regard helps to further characterize what defines a queen in a species that shows characteristics assumed to have existed shortly after ants transitioned from solitary to eusocial.

MATERIALS AND METHODS
Source colonies
Harpegnathos saltator (T. C. Jerdon 1851) source colonies have been reared in the laboratory since they were collected from southern India between 1995 and 1999 (Peeters et al., 2000). Where indicated, individuals and brood from multiple source colonies were combined in the laboratory. Unique source colonies are those whose combination of original colonies from the field is different.

Colonies were maintained in a laboratory setting with a USDA APHIS permit at Arizona State University at 25°C on a 12 h photoperiod. They were housed in plastic containers (Model 79C, Pioneer Plastics, Inc., Dixon, KY, USA) lined with Fluon (Fluoropolymer resin, Dupont, Torrance, CA, USA) with a plaster base (no. 50046289, Labstone Blue, Modern Materials, Rochester, IN, USA; or no. 985-1692, Dentalstone-Buff, Darby Group Companies, Jericho, NY, USA), where a rectangular volume was dug out beneath a smaller piece of glass. Two to three times a week, the plaster was moistened with deionized water, while frass and dead ants were removed. At these times, colonies were provided with live crickets and sawdust for larvae to pupate.

Established gamergates (mated and reproductive workers; Peeters and Crewe, 1984) or reproductive queens were identified in these source colonies from their reproductive behaviors and marked using Testor® paint (Rockford, IL, USA) and/or 34-gauge artistic wire marks according to the method of Haight (2012). After laying an egg, a reproductive individual would hold the egg in her mandibles and could be found near the colony's brood, where she moved slowly compared with non-reproductive individuals. In addition, a non-reproductive individual would sometimes recoil and assume a lower submissive posture after contacting a reproductive with antennae.

Identification of gynes in source colonies
Within 3 days of eclosing to adulthood, gynes in source colonies were marked and monitored. Pupae produced in the source colonies were checked 2–3 times per week for the eclosion of new adult gynes. Callow adult gynes were wire-marked using the method of Haight (2012) just anterior or posterior to the petiolar node and returned to their source colony with established reproductive individuals to limit their ovarian activity. The attachment of wings and the mortality of adult gynes was observed when the source colonies were provided with live crickets for food. Rarely, an adult gyne showed uninflated and shriveled wings. For consistency in our experiments, we only used gynes that showed four inflated wings. We defined the removal of any of the wings as the commencement of a dealate status. The cuticular hydrocarbons and number of yolky oocytes were measured from gynes in the source colonies using procedures described below.

Overview of experiments
We compared the ovarian activity and pheromone production between non-reproductive dealate and alate gynes in the source colonies, and we examined how the attachment of wings influences a gyne's behavior in two separate experiments. In behavioral experiment 1, we compared the behavior of dealate gynes that naturally shed their wings at various ages with the behavior of alate gynes in mixed groups of alate and dealate gynes. For behavioral experiment 2, in contrast, we compared the behavior of groups of alate gynes to the behavior of groups of dealate gynes whose wings had been experimentally removed on the same day. As the attachment of wings associates with a gyne's reproductive ontogeny, we performed two additional experiments with non-reproductive gynes in source colonies with well-established reproductive individuals to evaluate how alate status influenced a gyne's physiology independent of her reproductive development. In one experiment, we measured the ovarian activity and pheromone production of alate gynes and dealate gynes that had naturally shed their wings at various ages. In another experiment, we standardized the age at which the wings of gynes were experimentally removed. Here, we compared the pheromone production and ovarian activity of alate gynes with that of dealate gynes whose wings were experimentally removed when they were 1 month old. To compare the ovarian development of these non-reproductive gynes with that of reproductive ones, we also assessed the ovarian activity of dealate gynes used in behavioral experiment 2 that became reproductive in the months after that experiment ended.

Behavioral experiment 1
We recorded the dueling behavior of gynes of different alate status ($n=7$ total dealate gynes; $n=10$ total alate gynes) in two groups from different source colonies to determine how the attachment of wings to a gyne affected the display of worker-like aggressive behavior associated with hierarchy formation. Each of the two groups consisted of five wire-marked alate gynes (age at group formation: 7–40 days, median: 23 days) and three or four wire-marked dealate gynes who had shed at least one of their four wings before the first observation (age at group formation: 7–35 days, median: 24 days; age at dealation: 4–33 days, median: 12 days). Additionally, each group received two or three callow workers, three worker pupae and six male pupae in nests like those of their source colony. Because the source colonies for each of the experimental groups, F100 and SAFC15, contained established reproductive individuals, it is unlikely that the gynes or workers in the groups were reproductive at the beginning of the experiment. Three adult males were also added to the experimental group derived from the source colony SAFC15.

Ten days after the experimental groups were created, we compared the worker-like behavior between gynes of different alate status. To allow time for the gynes to express behavior after the experimental setup, we started to observe gynes in behavioral experiment 1, 10 days after experimental setup for the F100 group or 11 days after setup for the SAFC15 group because some gynes in behavioral experiment 2 did not begin dueling until 9 days after the creation of their nests (Fig. S1). The groups in behavioral experiment 1 were watered and provided with live crickets for food 2–3 times per week. At least 1 day after the groups were provided with food, we recorded the behavior of gynes during a 10 min scan observation period. A gyne was noted to be dueling if she did so with another gyne or worker at least once. The sample size for alate gynes declined because one alate gyne died 42 days after the beginning of the experiment and four gynes shed their wings 14–59 days after the experiment began. Once dealate, these four gynes remained in the nest but were not included in the analysis. Therefore, dealate gynes were observed more frequently

than alate gynes (n=10 alate gynes, range: 1–15 observations, 2–49 days, median: 10 observations, 28 days; n=7 dealate gynes, range: 20–47 observations, 59–185 days, median: 29 observations, 112 days).

Behavioral experiment 2

We also observed groups of alate gynes and groups of dealate gynes whose wings were removed at the same time because the dealate gynes in behavioral experiment 1 shed their wings at various ages in the mixed groups with alates. Therefore, we compared the frequency of worker-like behavior of groups of gynes whose wings we removed on the same day with that of groups of gynes whose wings remained attached in separate groups. For this, we used 11 pairs of nests containing either five alate or five artificially dealate gynes each N=2 nests per pair×11 nest pairs=22 nests). We assembled pairs of experimental nests where each nest was composed of a group of 10 workers and five gynes from one of five source colonies. The gynes in each nest of a pair represented one of two treatments: (i) artificial dealation: the day of group formation, all wings of a gyne were cut near the base with Vannas Spring Scissors (3 mm cutting edge, no. 15000-00, Fine Science Tools, Foster City, CA, USA) while the gyne was held with forceps; or (ii) maintenance of alation: the gyne was held similarly with forceps, but its wings were not removed. We used a similar age distribution for alate and artificially dealate groups. The pairs of experimental nests were filmed at 30 frames s^{-1} and 1920×1080 resolution with a video camera (Panasonic, model HC-VX981 or HC-V520, Newark, NJ, USA) under constant lighting with minimal glare for 2 weeks continuously. Two or three times per week, we saturated the nests' plaster with deionized water as needed and provided crickets that had been sting-paralyzed by workers in the source colony as food. It is unlikely that the gynes in the experimental nests were mated because all first adult offspring were male.

We restricted our analysis of the behavior of gynes in the experimental nests from 10 until 14 days after the start of filming because the gynes in several nests were dueling after 10 days, but after 2 weeks multiple gynes had shed their wings or died. To verify that the gynes do not start and then stop dominance behavior before the onset of filming 10 days after the beginning of the experiment, we reviewed a subset of three pairs of experimental nests from different source colonies since the onset of nest formation using QuickTime on a computer as fast as 30 times normal speed. We found that the gynes in four nests that showed worker-like behaviors within the first 10 days of filming continued to show dueling or dominance 10 days after the onset of filming (Fig. S1). Every day of filming and 2–3 times per week after filming, we recorded the mortality and wing attachment of alate gynes. Most alate gynes shed their wings after the end of filming; however, the first alate gyne shed her wings 12 days after the start of filming. Her subsequent dealate behavior was excluded from the analysis of that group. Two alate gynes and one artificially dealate gyne that died within the first 10 days of filming reduced the number of gynes in respective groups from five to four.

We detected the beginning of dueling involving a gyne as well as the first dominance bite initiated by a gyne by reviewing continuous video of the interactions in the nest during the 10–14 day interval. Regardless of whether the gyne's partner was another gyne or a worker, we counted the gyne-involved duels in the nest for 2 h after detecting the first one. We also counted the number of dominance bites initiated by a gyne for 6 h after the first observation of gyne-initiated dominance, regardless of whether the recipient was another gyne or a worker. Groups with gynes that did not show involvement

in dueling or dominance were continuously observed from 10 until 14 days as well.

The attachment of wings in relation to a gyne's foundress properties

We recorded the egg-laying, brood care and attachment of wings of isolated gynes with all wings attached to evaluate how the presence of a gyne's wings influences her foundress characteristics. We housed individual gynes from 10 source colonies (n=17 gynes) in small plastic containers (Model 29C, Pioneer Plastics, Inc.) with a plaster base lined with Fluon. The gynes were observed for the presence of brood, the removal of any wings and mortality 1 or 2 times per week before providing pre-stung crickets and saturating the plaster with deionized water. Gynes only shed their wings after they had begun to lay eggs (Table 1).

Ovarian activity and pheromone production of non-reproductive gynes in the source colonies

We compared non-reproductive alate gynes and dealate gynes that naturally shed their wings at various ages in source colonies with established reproductive individuals to determine how the attachment of wings to non-reproductive gynes associates with their abundance of cuticular hydrocarbons (CHC) and ovarian activity. Specifically, we assessed the number of yolky oocytes from alate gynes (n=8 gynes) and dealate gynes that shed their wings at various ages in five source colonies with established reproductive individuals (range: 53–165 days; median: 66 days, n=7 gynes). We also quantified the abundance of larger alkadienes on the cuticles between additional alate (n=8 gynes) and dealate gynes that shed their wings at various ages in a total of 11 source colonies with established reproductives (range: 16–383 days; median: 94 days, n=21 gynes).

Ovarian activity and pheromone production of non-reproductive gynes whose wings were removed on the same day in the source colonies

We also examined the reproductive physiology and pheromone production of non-reproductive gynes whose wings were experimentally removed at the same age because the naturally dealate gynes shed their wings at different ages. Specifically, we compared the abundance of CHCs of alate (n=13) and artificially dealate (n=13) gynes whose wings were removed when they were 1 month old in nine source colonies with established reproductive individuals. We also compared the ovarian activity and mating status from a subset of these gynes in nine colonies that included naturally dealated gynes (n=12 artificially dealate gynes; n=12 alate gynes, n=7 naturally dealate gynes). We first sampled the abundance of CHCs on alate gynes at 1 month of age to provide sufficient time for sclerotization and the associated changes in CHCs to emerge because callow gynes show a lower abundance of large alkadienes than those that are sclerotized (Liebig et al., 2000). Then, these 1 month old gynes were randomly assigned to one of two treatments to assess the effect of the attachment of wings on their ovarian development and CHC profiles: (i) artificial dealation: gynes were dealated as in behavioral experiment 2 and returned to the source colony; or (ii) maintenance of alation: gynes were handled similarly to the gynes in the artificial-dealation group, wings were not removed, and these gynes were returned to the source colony. In this way, the wings of gynes in the artificial-dealation group were removed at 1 month, whereas the gynes in the maintenance of alation group remained alate. One-month old gynes designated for the alate or artificially dealate treatments did not

differ in the abundance of pentatriacontadiene (C35:2) and heptatriacontadiene (C37:2) before the experimental manipulation (Fig. S2). We then sampled the abundance of CHCs from the gynes of both treatments 1 month later (i.e. 2 months old) to evaluate how rapidly a gyne's CHC profile was influenced by the removal of wings. Because 2 month old alate gynes showed a higher abundance of C37:2 but not C35:2 than artificially dealate gynes (Fig. S3), we sampled the gynes' CHCs yet again one additional month later (i.e. 3 months old) to allow more time to observe a difference in the abundance of both alkadienes. After sampling the CHCs at 3 months, a subset of the gynes were culled and their ovaries dissected to assess their mating status and the number of yolky oocytes (n=12 artificially dealate gynes; n=12 alate gynes).

Eight gynes in the alate group shed their wings before they were sampled 3 times, becoming a treatment of naturally dealate gynes. Five gynes became dealate after their CHCs were sampled the first time when they were 1 or 2 months old, while three gynes did not shed their wings until after their CHCs were sampled a second time when they were 2 months old. While all of the naturally dealate gynes were assessed for ovarian activity and pheromone production after their CHCs were sampled at least 3 times, technical obstacles during data collection limited our analysis to a subset (n=7 gynes).

Five naturally dealate gynes shed their wings 1–41 days after their CHCs were sampled the first time: four alate gynes were sampled when they were 1 month old; and one alate gyne was sampled when it was 2 months old. One month after the five gynes became dealate, their CHCs were sampled a second time when they were 68–106 days old. The CHCs of the same five gynes were also sampled a third time when the gynes were 103–167 days old: three gynes were sampled 2 months after they became dealate; and two gynes were sampled 3 months after they shed their wings. After their CHCs were sampled the third time, all five gynes were culled, and their yolky oocytes and mating status were assessed.

Three additional alate gynes became naturally dealate gynes when they shed their wings 16–30 days after they were sampled a second time when they were 2 months old. One month after becoming dealate, the CHCs of the three gynes were sampled a third time when they were 107–121 days old. Two months after becoming dealate, the CHCs of the three gynes were sampled a fourth time when they were 136–150 days old. The fourth CHC samples from only two of these three gynes were analyzed. After the fourth CHC sample, two of the three gynes were culled and their ovaries dissected. Then, the number of yolky oocytes and mating status were assessed from these gynes. While eight gynes from the alate group removed their wings without manipulation and became part of the naturally dealate group, only seven gynes were assessed for ovarian activity and pheromone production at the end of the experiment.

Two of the fibers used to sample the CHCs from naturally dealate gynes were stored in a refrigerator in a plastic box. Two weeks later, these fibers were allowed to warm to room temperature over a few minutes and alkadiene abundance was analyzed in the gas-chromatograph mass-spectrometer (GCMS). Visually comparing these chromatograms with others in the same treatment revealed no conspicuous differences.

Ovarian development of reproductive gynes
Up to a year after being filmed in behavioral experiment 2, the ovarian activity of reproductive dealate gynes from two source colonies (n=3 artificially dealate gynes+n=3 alate gynes that became dealate=n=6 total gynes) provided a reference to the ovarian activity of non-reproductive gynes. After behavioral

experiment 2 ended, we provided the experimental nests with live crickets 2 or 3 times per week until the alate gynes had shed their wings, noting the mortality of gynes at these times. To produce a hierarchy of reproductives and non-reproductives among the gynes, alate gynes that had become dealate in two experimental nests of the same source colony were combined with artificially dealate gynes from two experimental nests also from the same source colony. Reproductive hierarchies emerged in three of these nests 8–12 months later composed of 1–4 reproductive dealate gynes, up to four non-reproductive dealate gynes, and 20–55 workers. Reproductive dealate gynes were identified from observations of their reproductive behaviors in at least three 10 min observational scans on separate days. The number of yolky oocytes in the ovaries of the reproductive dealate gynes was assessed using the methods described below.

Sampling CHC abundance
The abundance of alkadienes on the cuticle of gynes was assessed by sampling the CHCs without killing the animals. Gynes were immobilized without anesthesia in a paper cutout such that only their gaster remained exposed on one side of the paper. A solid-phase microextraction (SPME) fiber (Supelco, 30 μm, PDMS, fused silica, 23-gauge, Yellow, 57289-U, Bellefonte, PA, USA) was then rubbed across the gaster of the gyne's cuticle for 500 strokes to collect CHCs. For insects, this method has been shown to have similar effectiveness to hexane extraction for the detection of larger hydrocarbons (Moneti et al., 1997).

The cuticular hydrocarbons sampled with a SPME fiber were then analyzed in a GCMS. Except for the two cases where fibers were stored in a refrigerator (see above), the fiber was inserted immediately after sampling into a 280°C hot inlet of a gas-chromatograph (GC, Agilent 5860) connected to a mass-spectrometer (MS, Agilent 5975). The GC was equipped with a non-polar capillary column (Agilent J &W, GC column, DB-1MS, size 30.0 m×250 μm×0.25 μm nominal, Santa Clara, CA, USA). The oven of the GC was programmed to begin at 60°C for 2 min, increasing by 40°C min^{-1} to 200°C. At 200°C, the oven increased by only 5°C min^{-1} to 320°C over 15 min. Helium was used as the carrier gas with a flow rate of 1 ml min^{-1}. C35:2 and C37:2 eluted near the known elution times of the straight-chain alkane standards for pentatriacontane (C35) and heptatriacontane (C37) resuspended in pentane solvent that were subject to the same temperature program.

Analyzing CHC abundance
The alkadienes C35:2 and C37:2 were identified from elution peaks from the chromatogram of the CHCs of a gyne. Ion fragmentation patterns of the alkadienes show a base ion with a molecular weight of 67 (Kroiss et al., 2011) as well as the molecular ion of 488 (C35:2) or 516 (C37:2). Relative alkadiene amounts were calculated as a proportion of the sum of all peak areas of the remaining compounds larger than tricosane in the chromatograms that exceeded an initial peak width of 0.67 and an initial threshold of 14.8. Chromatographic analysis was performed with Agilent MSD ChemStation (F.01.03.2357).

Ovary dissection, yolky oocyte count and mating status
Ovaries were dissected to assess a gyne's ovarian development and mating status. As opposed to developing oocytes and trophocytes in ovarioles, yolky oocytes identified by their opaque yolk protein indicate the activity of ovaries (Peeters et al., 2000). The mating status of gynes was determined by the presence of a white opaque

disc in the center of the spermatheca from dissected ovaries (Peeters et al., 2000). Counts of yolky oocytes and the determination of mating status were assessed from photographs of dissected ovaries and spermathecae captured using SPOT Insight QE (Model 4.2, Diagnostic Instruments, Inc., Sterling Heights, MI, USA). An observer blind to the true identities of the gynes counted the number of yolky oocytes and determined the mating status of the gyne from these photographs. The blind estimates of mating showed a perfect correlation with the unblinded estimates taken soon after dissection by another observer. Blind counts of yolky oocytes by one observer were highly correlated with the unblinded counts estimated by another observer soon after dissection (Fig. S4).

Statistical analyses

Colony origin was a random-intercept effect in linear mixed models (LMM) fitted to the data, while treatment was the fixed effect because multiple gynes originated from the same source colony in the experiments. We used an F-test with the Kenward–Roger approximation for smaller sample sizes (Kenward and Roger, 1997) to compare the full model with a random-effects model to interpret the significance of treatment. When the model would not converge, the response variable was transformed using a Box–Cox transformation. If the model still did not converge after transformation, non-parametric Wilcoxon tests were used to compare across treatments. In these cases, non-parametric Kruskal–Wallis tests were used to exclude that the response variable was affected by the variance in source colonies. Significance was assessed at an alpha of 0.05. All mixed models were fitted with the function *lmer* (package: lme4; Bates et al., 2015), and all statistics were conducted in R* (v.4.1.3). The Box–Cox transformations were performed with the *boxcox* function (package: MASS).

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

RESULTS
Behavior

If wing removal induces a worker-like behavioral phenotype, dealate gynes should have been more involved in worker-like behaviors than alate gynes. Dealate gynes in behavioral experiment 1 that shed their wings at various ages (range: 4–33 days; median: 12 days; n=7 gynes) were found to duel more often than similarly aged alate gynes (n=10 gynes) in mixed groups from the same source colony (Fig. 1). In three cases, alate gynes expressed worker-like behavior. However, two of the three dueling alate gynes that shed their wings at the age of 46 or 66 days continued to duel as dealate gynes.

We then conducted behavioral experiment 2 to compare the worker-like behavior of artificially dealate gynes with that of alate gynes in separate groups because gynes in behavioral experiment 1 shed their wings at different times. Specifically, we compared the dueling and dominance behavior between groups with dealate gynes
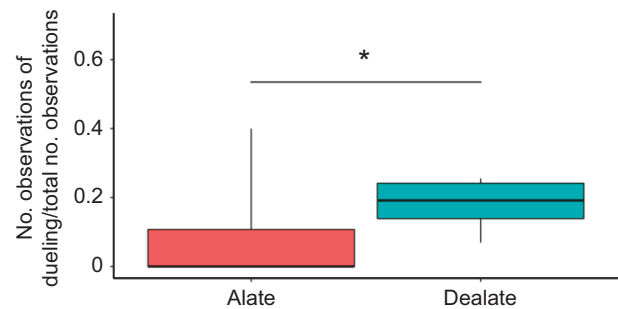

Fig. 1. Dueling by alate and dealate gynes of similar age in two mixed groups in behavioral experiment 1. Dealate gynes dueled in a larger proportion of 10 min scan observations than alate gynes. *P<0.05. Box shows median and interquartile range (IQR), while whiskers extend to the range of the data outside the IQR. Linear mixed models (LMM) on arc-sine square-root transformed data of Y (n=10 alate gynes; n=7 dealate gynes; $F_{1,14.04}$=7.67, P=0.015).

and workers and those groups with alate gynes and workers. After 10 days of filming, the groups with artificially dealate gynes (n=11 groups of 5 artificially dealate gynes each) were more frequently engaged in duels and initiated dominance bites more often than those with alate gynes (n=11 groups of 5 alate gynes each) (Fig. 2). In a subset of three artificially dealate gyne nests and three alate gyne nests, workers dueled and initiated dominance.

Ovarian activity and mating status

Ovaries of alate queens contained yolky oocytes in the presence of established reproductive individuals (Peeters et al., 2000). If this ovarian activity is associated with dispersal and the removal of wings produces a worker-like reproductive state, then the dealate gynes in the presence of established reproductive individuals should have shown fewer yolky oocytes than alate gynes. In five source colonies with established reproductive individuals, the older gynes that shed their wings at various ages (range: 53–165 days; median: 66 days, n=7) showed fewer yolky oocytes (median: 0 yolky oocytes, n=7) than younger alate gynes (median: 5 yolky oocytes, n=8) (Fig. 3).

In source colonies with established reproductive individuals, we minimized the variance in the latency of a gyne to lose her wings by comparing the ovary development of gynes whose wings were experimentally removed when they were 1 month old (n=12 artificially dealate gynes) with that of alate gynes (n=12 gynes) of similar age. To account for the effect of artificially removing a gyne's wings, we also compared the ovaries of artificially dealate gynes with those of gynes that had already shed their wings at various ages (n=7 naturally dealate gynes) in the source colonies in the presence of established reproductives. Two months after the removal of wings, both artificially dealate and naturally dealate gynes showed fewer yolky oocytes in their ovaries than 3 month old alate gynes (Fig. 4). While the ovaries of 3 month old alate gynes were active, they were still not as developed as those of older reproductive dealate gynes that were previously used in behavioral experiment 2 (n=6 gynes, age when sampled: 377–524 days, median: 497.5 days; 7–17 yolky oocytes, median: 11.5 yolky oocytes).

Dealate gynes occasionally mated in the nest, unlike alate gynes. We found that two 3 month old artificially dealate gynes (n=12 artificially dealate gynes) were mated, whereas all alate gynes (n=12 alate gynes) and naturally dealate gynes (n=7 naturally dealate gynes) were unmated. This difference is not statistically significant, however (Fisher's exact test, P=0.33). Separately, the first adult
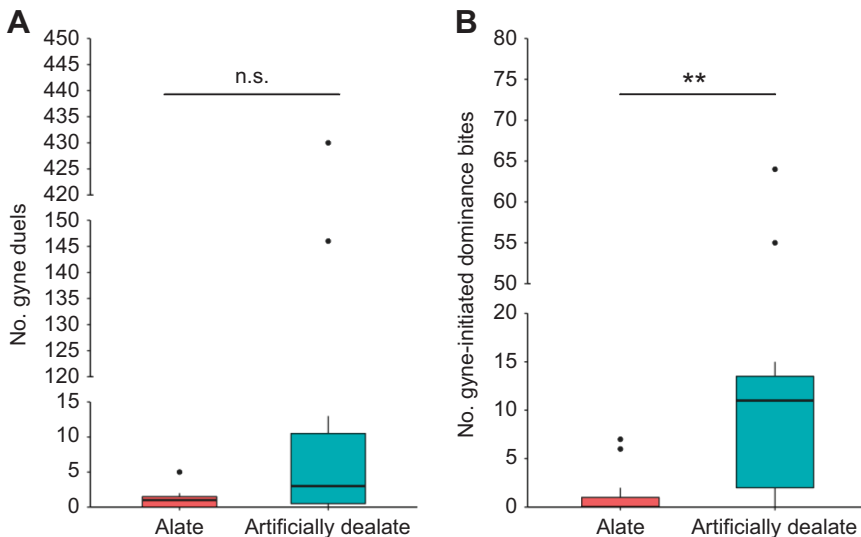
Fig. 2. Frequency of duels and dominance bites in separate experimental groups of alate gynes and artificially dealate gynes for behavioral experiment 2. (A) Groups of gynes in the artificially dealate treatment perform more duels than groups of gynes in the alate treatment. (B) Groups of gynes in the artificially dealate treatment initiate significantly more dominance bites than groups of gynes in the alate treatment. **$P<0.01$. Box shows median and IQR, while whiskers extend to 95% of data, and points are outliers [alate groups, $n=11$ nests each with 5 gynes; artificially dealate groups, $n=11$ nests each with 5 gynes for 22 nests total; A: LMM using $(1+Y)$ Box–Cox transformation on Y, $F_{1,16.06}=3.61$, $P=0.076$; B: LMM using $\ln(1+Y)$ transformation, $F_{1,16.04}=10.66$, $P=0.004$].

offspring produced from eight isolated alate gynes presented in Table 1 were male, suggesting that the gynes that produced these offspring were unmated.

CHC abundance

A gyne whose wings are removed should show a reduced abundance of the alkadienes like a non-callow alate gyne if the hydrocarbon profile on a gyne's cuticle is associated with the attachment of her wings (Liebig et al., 2000). In colonies with established reproductive individuals, dealate gynes that shed their wings at various ages (range: 16–383 days; median: 94 days, $n=21$ gynes) showed a lower abundance of the CHCs pentatriacontadiene (C35:2) and heptatriacontadiene (C37:2) than alate gynes ($n=8$ gynes) (Fig. 5). Although 1 month old gynes designated for different treatments (designated alate, $n=34$ gynes; designated artificially dealate, $n=17$ gynes) showed a similar abundance of C35:2 and C37:2 (Fig. S2), the 2 month old alate gynes ($n=21$ gynes) showed a higher abundance of C37:2 than the 2 month old artificially dealate gynes ($n=17$ gynes) whose wings were removed

1 month earlier (Fig. S3). Some of these alate gynes then shed their wings, becoming naturally dealate gynes at various ages (range: 36–90 days; median: 67.5 days, $n=7$ gynes). Two months after gyne wing removal, we compared the abundance of alkadienes from these naturally dealate gynes with that of 3 month old artificially dealate gynes whose wings were removed 2 months earlier ($n=13$ gynes) as well as 3 month old alate gynes ($n=13$ gynes). Both artificially dealate and naturally dealate gynes showed a reduced abundance of C35:2 and C37:2 compared with 3 month old alate gynes (Fig. 6).

DISCUSSION

Our results indicate that natural and artificial dealation of *H. saltator* gynes in a worker-specific environment is followed by a shift towards a behavioral and physiological phenotype that is usually only shown in workers. Specifically, dealate gynes displayed dominance behaviors that usually only occur in the context of hierarchy establishment of workers in this species.

While queens and workers can reproduce in *H. saltator* colonies, they achieve this in different ways. Queens become reproductive foundresses by independently starting a colony alone (Hakala et al., 2019). Workers, in contrast, establish themselves as new
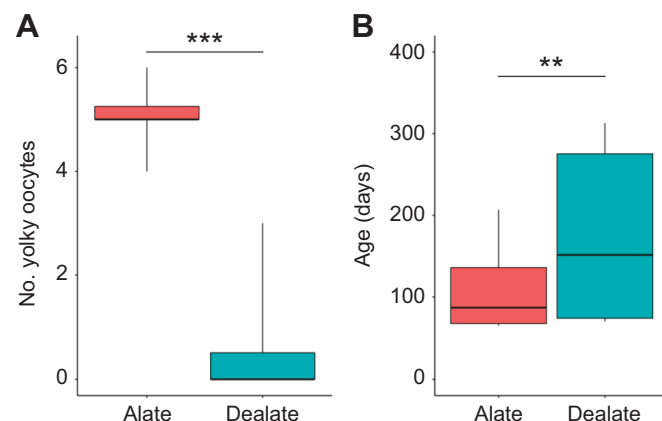

Fig. 3. Ovarian activity of non-reproductive alate and dealate gynes that shed their wings at various ages in five colonies with established reproductives. (A) Alate gynes show more yolky oocytes (as counted from photographs of their dissected ovaries) than dealate gynes. (B) Alate gynes tend to be younger than dealate gynes. ***$P<0.001$; **$P<0.01$. Box shows median and IQR, while whiskers extend to all data outside the IQR (alate gynes, $n=8$; dealate gynes, $n=7$; A: Wilcoxon test by status, $W=56$, $P=0.0009$; B: LMM, $F_{1,22.786}=11.072$, $P=0.003$).
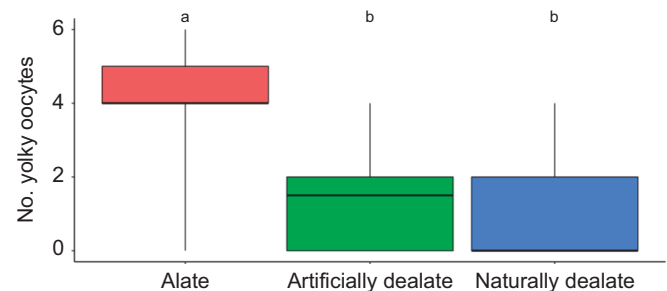

Fig. 4. Ovary development of non-reproductive gynes in nine colonies with established reproductives whose abundance of CHCs was sampled at least 3 times. Two months after wing loss, the ovaries of dealate gynes have fewer yolky oocytes than those of alate gynes of similar age. Box shows median and IQR, while whiskers extend to all data outside the IQR (alate: $n=12$ gynes, median age: 91.5 days; artificially dealate: $n=12$ gynes, median age: 90.5 days; naturally dealate: $n=7$ gynes, median age: 136 days; LMM: $F_{2,23.915}=10.871$, $P=0.0004$). Estimated marginal mean differences for multiple comparisons are shown with different letters above the boxes.
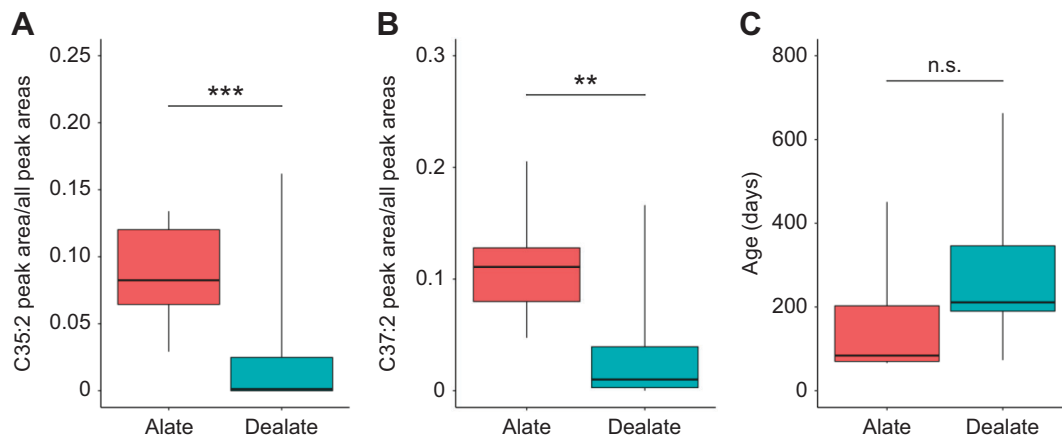
Fig. 5. Abundance of long-chain alkadienes from non-reproductive alate and dealate gynes that shed their wings at various ages in 11 colonies with established reproductive individuals. (A) C35:2 abundance; (B) C37:2 abundance; and (C) age of gynes in colonies with established reproductive individuals. **$P<0.01$; ***$P<0.001$. Box shows median and IQR, while whiskers extend to all data outside the IQR (alate gynes, $n=8$; dealate gynes, $n=21$; A: Wilcoxon test by status, $W=153$, $P=0.0006$; B: LMM on arc-sine square-root transformation of Y: $F_{1,26.428}=12.319$, $P=0.0012$; C: LMM, $F_{1,26.9956}=0.93$, $P=0.34$).

reproductive individuals in a colony where the principal reproductives, either the queen or workers, died or were experimentally removed (Peeters and Hölldobler, 1995). The associated process of hierarchy formation with its specific dominance behaviors is only shown by workers and results in multiple reproductives of equal rank (Sasaki et al., 2016). By isolating artificially or naturally delated queens together with a group of non-reproductive workers, we were able to induce the expression of dominance behaviors in gynes.

Such dominance behaviors are normally not shown by gynes in a colony of *H. saltator*. In addition, multiple reproductive queens do not regularly co-exist in colonies. In contrast, the establishment of workers as reproductives always occurs in groups (Peeters and Hölldobler, 1995). If gynes use these behaviors to establish themselves as reproductives in existing colonies, we would expect to see multiple reproductive queens in natural colonies. Out of 48 queen-right colonies collected in the field, however, only one of them contained two instead of one reproductive queen (Peeters et al., 2000). Furthermore, alate gynes displayed dominance behavior at a much lower level in our experiments than dealate queens. Therefore, it is unlikely that alate queens in natural colonies

of *H. saltator* engage in dominance behaviors rather than preparing for their mating flight. Thus, we conclude that the expression of dominance behavior by gynes is most likely very rare in natural conditions.

Distinguishing a true worker behavior from a queen behavior is not straightforward, however. In *H. saltator* and other ants, foundresses perform brood care, defense and foraging behavior in the laboratory and in the field (Peeters et al., 2000), which is part of the task repertoire of workers (Peeters and Hölldobler, 1995; Haight, 2012). Thus, the expression of such behaviors in dealate queens (e.g. in *Acromyrmex* queens; Nehring et al., 2012) is not necessarily a worker behavior, but part of the queen behavioral program shown during foundation. In other ant species, queens may naturally exhibit dominance behavior during the establishment of linear hierarchies (e.g. Hölldobler and Carlin, 1985; Oliveira and Hölldobler, 1991; Medeiros et al., 1992; Sommer and Hölldobler, 1992; Ortius and Heinze, 1999; Kolmer and Heinze, 2000; Yamauchi et al., 2007). In *H. saltator*, however, queens express dominance behavior such as dueling only in artificial conditions with other gynes. Thus, dominance behavior such as dueling is not part of the regular behavioral repertoire of queens but is a distinct
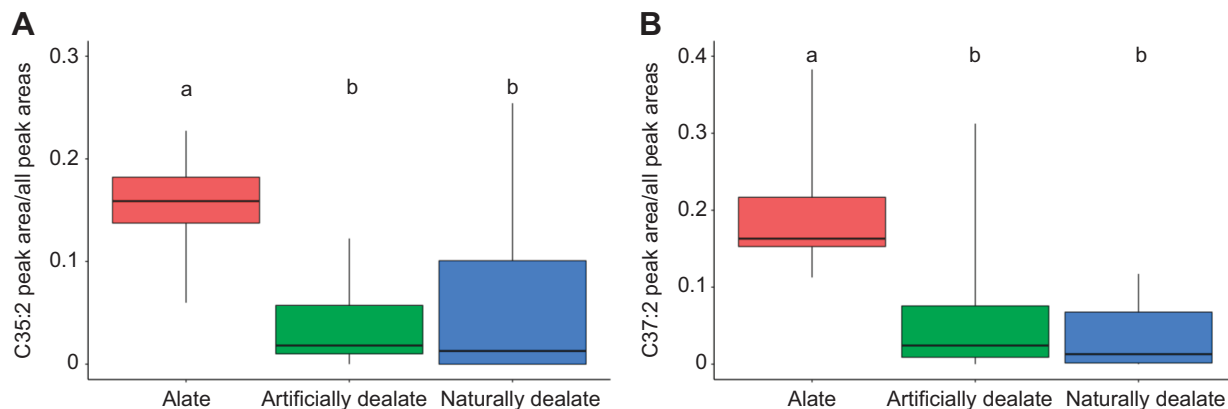

Fig. 6. Abundance of long-chain alkadienes on the cuticles of non-reproductive gynes in nine colonies with established reproductives sampled at least 3 times. Alate gynes show a higher abundance of C35:2 and C37:2 than gynes of similar age that have been dealate for 2 months. Box shows median and IQR, while whiskers show all data outside the IQR (alate: $n=13$ gynes, median age: 91 days; artificially dealate: $n=13$ gynes, median age: 90 days; naturally dealate: $n=7$ gynes, median age: 140 days; LMM of arc-sine square-root transformed data; A: $F_{2,28.59}=13.30$, $P=8.3\mathrm{e}{-}05$; B: $F_{2,28.40}=14.15$, $P=5.5\mathrm{e}{-}05$). Estimated marginal mean differences are shown with different letters above the boxes.

worker behavior shown during hierarchy establishment as part of the regular colony ontogeny.

Although dominance behavior was primarily displayed by dealate gynes, the expression of such behavior from alate gynes to a lower extent suggests that an endocrine mechanism operates in parallel to the attachment of wings. Furthermore, some alate queens that showed dueling behavior later shed their wings and dueled as dealate queens. This suggests that endocrine factors act in concert with dealation to induce the expression of dominance behavior. JH may be one of these endocrine factors, as it is associated with dealation in other ants (Kearney et al., 1977; Burns et al., 2002; Brent and Vargo, 2003; Penick et al., 2011). In addition, JH regulates the sensitivity of insects to stimuli (Koudele et al., 1987; Robinson and Ratnieks, 1987; Stout et al., 1991; 1998; Anton and Gadenne, 1999; Ignell et al., 2001; Narbonne and Pollack, 2008). If the attachment of wings and endocrine changes are closely associated, wing shedding may follow endocrine changes whereas artificial dealation may precipitate an endocrine change. Both scenarios would involve the same physiological network that is associated with a shift to worker-like behavior.

Besides the behavioral shift, we also observed changes in the gyne's physiology and cuticular chemical signatures towards a more worker-like state after wing removal. In our laboratory colonies with reproductive queens or gamergates as well as in colonies in the field (Peeters et al., 2000), the ovaries of alate *H. saltator* gynes contained developing eggs most likely to prepare for founding a nest after the mating flight. Workers of many ant species, in contrast, do not show active ovaries with developing oocytes in the presence of a reproductive queen (Bourke, 1988; Choe, 1988). Like non-reproductive workers, the gynes which shed their wings or were artificially dealate in their parental colonies showed a significant reduction in their ovarian activity. Thus, we conclude that the ovarian activity of gynes that dealated in a regular colony follows a worker-like pattern (Bourke, 1988; Choe, 1988; Van Oystaeyen et al., 2014; Holman, 2018). Interestingly, the difference in ovarian activity between alate and dealate gynes suggests that the presence of established reproductives has a lower inhibitory effect on ovaries of alate gynes than on those of dealate gynes and workers. Such a difference in sensitivity to a queen signal and the physiological response might also be important for the evolution of reproductive division of labor at the beginning of ant social evolution.

The cuticular chemical signature also followed a worker-like pattern. Alate gynes from regular colonies produced a high abundance of the long-chain alkadienes C35:2 and C37:2 on their cuticles, which may function to attract mates (Liebig et al., 2000; Ghaninia et al., 2018). These compounds have not been found on workers, regardless of their reproductive status (Liebig et al., 2000). While all alate queens investigated showed a prominent presence of these two alkadienes on their cuticle, naturally or artificially dealate queens had either not-detectable or significantly reduced levels of these compounds on their cuticle at the same age, suggesting that they resemble a worker-like state. The higher abundance of alkadienes on alate *H. saltator* gynes may be a contact sex pheromone for use during the mating flight. In fact, sex pheromones are commonly used by insects for signaling mating activity (Ayasse et al., 2001). However general, female-specific sex pheromones have only been identified from a limited number of ant species (Walter et al., 1993; Greenberg et al., 2007; Castracani et al., 2008; Greenberg et al., 2018; Iwamoto et al., 2020). While social insect queens produce various compounds that function as sex pheromones (Gary, 1962; Ayasse et al., 1999; Niehuis et al., 2013; Wen et al., 2017), sex-specific alkadienes mediate mating in

some hymenopterans (Syvertsen et al., 1995; Krokos et al., 2001) and male *H. saltator* perceive larger hydrocarbons (Ghaninia et al., 2018).

Our results suggest that gynes can switch from a queen-specific to a worker-specific behavioral and physiological state. Furthermore, workers can be induced to act like single foundresses when isolated on their own (Liebig et al., 1998). Thus, behavioral and physiological properties of queens and workers can be expressed to a large extent by the alternative morph. This indicates that in this weakly dimorphic ant species, the major difference that defines the dimorphism is the presence of wings and a thorax that support flight capability in queens. If the high reproductive potential of workers in *H. saltator* and the weak queen–worker dimorphism represents similar conditions to the early evolution of ant dimorphism, the dispersal dimorphism may have preceded a reproductive dimorphism in ant evolution.

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