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Reproductive development and ontogeny of queen pheromone production in the fire ant *Solenopsis invicta*

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Abstract. Queens of the fire ant, *Solenopsis invicta* Buren, produce several releaser and primer pheromones. Using bioassays, the ontogeny of three of these pheromones related to reproductive development was investigated. Virgin queens, in which the process of wing-shedding (dealation) serves as an indicator of the initiation of reproductive development, were studied. First, the production of two queen pheromones produced in the poison gland was examined. The pheromone responsible for initiation and maintenance of retinue formation, a releaser effect, was found to be produced in detectable quantities two days after dealation, at which time queens showed significant ovary development and many (30%) had started laying eggs. A primer pheromone that inhibits alate virgin queens from dealating was detected in queens three days following wing-shedding, when 80% of the queens were ovipositing. Second, I examined the onset of a pheromone of unknown glandular origin produced by reproductively active virgin queens which leads to their destruction in queenright colonies by stimulating workers to attack and kill them. This pheromone is secreted in quantities detectable by bioassay two days after dealation. Thus in *S. invicta*, the ontogeny of three distinct queen pheromones is tightly linked with ovary development and initiation of egg laying. These results demonstrate reproductive and communicative functions are closely associated during the transition from potential to functional queen.

Key words. Reproduction, virgin queen, poison gland, *Solenopsis invicta*

Introduction

Queens of eusocial insects generally are the principal, if not exclusive, reproductives in the colony. They also play an important communicative role, producing numerous pheromones that coordinate the activity of colony members. Although the reproductive and communicative functions of queens are generally linked, little is known about the physiological mechanisms connecting the two activities. A first step in understanding this connection is to determine the ontogeny of queen pheromone production as individuals assume the position of functional queen.

The best studied queen pheromone system in social insects is that of the honey bee *Apis mellifera* L. (reviewed in Winston & Slessor, 1992). The queen mandibular glands produce a mixture of five compounds - three aliphatic and two aromatic molecules - that when presented in their natural proportions elicit the full attraction and tending response of a crude glandular extract (Slessor *et al.*, 1988). In addition, the queen mandibular gland complex inhibits workers from constructing queen cells in which new queens are reared (Winston *et al.*, 1989, 1990) and can delay the hormonally-based behavioral development of workers (Pankiw *et al.*, 1998).

Slessor *et al.* (1990) studied the ontogeny of the different components of the honey bee queen pheromone. These authors found that the levels of two of the aliphatic compounds (9-keto-2-decenoic acid and (S,E)-(+)-9-hydroxy-2-decenoic acid) increase rapidly in virgin queens within the first week after eclosion, a finding consistent with their role as sex attractants during the mating flight (Gary, 1962; Butler & Fairey, 1964). The full blend of compounds, however, is produced only after a queen is mated and begins laying eggs. The physiological factors regulating reproductive development and pheromone production in honey bees are not known.

Reproductive activity has been associated with pheromone production by queens in several ant species (reviewed in Cariou-Etienne & Passera, 1993). In general, mated, egg-laying queens are attractive to workers, whereas young winged virgin queens which are not yet reproductively active elicit little or no response. However, there is little information on when pheromone production begins during the initiation of reproductive activity, and almost nothing is known about the physiological factors regulating either reproductive development or queen pheromone production in ants.

Among ants, the queen pheromone system of the fire ant *Solenopsis invicta* Buren is particularly well-studied. Both releaser (behavioral effect mediated by the nervous system) and primer (physiological effect on the reproductive and/or endocrine system) pheromones have been demonstrated in this species. A queen recognition (releaser) pheromone is stored in the poison sac (Vander Meer *et al.*, 1980), from which three compounds have been identified (Rocca *et al.*, 1983a, b). These compounds, when presented in combination, were reported to elicit a behavioral response from workers (Glancey *et al.*, 1984). Several primer effects of *S. invicta* queen pheromones have been demonstrated (reviewed in Vargo, 1998). Female sexuals (new, winged queens) are pheromonally inhibited from developing their ovaries and shedding their wings (dealating) in the nest (Fletcher & Blum, 1981a). Queens produce a pheromone that inhibits workers from rearing male and female sexuals (Vargo & Fletcher, 1986, 1987), as well as a pheromone that results in mutual suppression of egg production among cohabiting queens in multiple-queen colonies (Vargo, 1992). In addition to these effects, queen pheromones play a role in regulating the number of queens in a colony through execution of excess queens by

workers (Fletcher & Blum, 1983a).

In queenless colonies of *S. invicta*, female alates will quickly shed their wings, develop their ovaries and lay eggs (Fletcher & Blum, 1981a). These virgin replacement queens assume the role of the queen, including the production of queen pheromones. Two separate studies have investigated the initiation of pheromone secretion by virgin replacement queens. Glancey *et al.* (1981) found that virgin queens removed from the mother colony and kept in isolation became attractive to workers 9-12 d after dealation. These authors examined the virgin queens only every third day and they did not quantify ovary development, leaving unclear the relationship between the onset of reproductive activity and pheromone production. Willer & Fletcher (1986) showed that virgin replacement queens kept in a queenless colony fragment for 1 wk produced detectable amounts of the inhibitory primer pheromone that suppresses dealation and ovary development in female alates, although they did not look earlier than 1 wk to see if the pheromone was produced before this time, nor did they know the age of the virgin replacement queens tested.

Experiments presently reported were designed to reveal more precisely the ontogeny of *S. invicta* queen pheromones during the initiation of egg production. Using the act of dealation as an accurate marker for the onset of ovary development, virgin replacement queens were examined for their ability to produce both releaser and primer effects.

Methods

Maintenance of ants

Ants of the monogyne form (single queen per colony) were excavated from established mounds on the dates given below. The colonies were removed from the soil by flooding (Jouvenaz *et al.*, 1977) and housed in the laboratory according to standard procedures (Vargo & Laurel, 1994). Food was provided in the form of frozen crickets (*Acheta domesticus*); water and sugar water were available ad libitum.

Experimental procedure

To determine the progression of ovary development following dealation, colonies were collected from Blanco County, Texas, near Johnson City on 25 October 1993 and from Calcasieu Parish, Louisiana, near Sulfur on 11 November 1993. These colonies also served as the source of most of the colonies for the experiments on the ontogeny of the queen recognition pheromone and the onset of queen execution by workers (see below). Colony fragments consisting of 10 g workers and brood (approximately 5500 adult worker, 4000 worker pupae and 8500 larvae of all stages) and 20 winged virgin queens, but no mother queen, were separated from the source colony. The colony fragments were housed in rearing trays ($24 \times 17.5 \times 8$ cm) containing a single nest (14-cm Petri dish half-filled with damp plaster). Colony fragments were checked daily for the presence of dealates during the 7-day experimental period. Degree of ovary development was assessed by dissecting ants under a microscope and counting the number of fully developed (chorionated) oocytes present in both ovaries and the common oviduct. Sample size was 10 individuals for each day, with 2 individuals taken from each of 5 colonies.

To determine when dealates begin to lay eggs, queenless fragments of 5 colonies collected from Calcasieu Parish, Louisiana on 4 May 1997, were set up as above. Dealates were removed daily and subjected to an oviposition test (Fletcher & Blum, 1983b), in which individuals were placed in separate plaster-bottomed cups, and the number of eggs laid after 5 h was recorded.

To study the onset of production of queen retinue pheromone, queenless colony fragments of monogyne colonies were set up as described above. Dealates were collected from the colony fragments, placed in separate glass vials and then frozen at -20 °C until tested in the recognition bioassay. A slightly modified version of a bioassay from Vander Meer *et al.* (1980) was used to measure attraction of workers to queens. Twenty workers were placed into a test arena, consisting of a small Petri dish (9 cm diam) with a plaster base and flouon coated sides. An observation square (2.5×2.5 cm) was marked in the center. Twenty workers and 3 pieces of brood (last instars or pupae) were taken from a queenright colony fraction and introduced to the test arena. Corpses of the test dealates or alate controls were thawed for 20 min and then introduced to the center of the observation square. Virgin queen corpses were tested for attraction using nestmate workers from the queenright fraction of their common natal colony. The number of workers inside the square was counted at 1-min intervals for 5 min. The number of brood inside the square was also counted and recorded separately. At the end of the 5-min test period, the number of workers and the number of brood inside the test square were summed for the 5 counts, giving a total possible score of 100 for worker attraction and 15 for brood deposition. The same colonies used for determination of ovary development were used for the retinue bioassay. There were 10 replicates for each age tested, 2 from each of 5 colonies.

To determine the onset of the pheromone produced by virgin queen dealates that stimulates attack by workers, live, virgin dealate queens of known age post-dealation were introduced in groups of 2-10 into a fragment of their natal colony containing the mother queen. There was a total of 2-7 colonies used per treatment (mean \pm SD = 4.8 ± 1.8), with 20-25 dealates introduced per treatment. The colony fragments were inspected hourly for 8 h for worker attacks of virgin queens and for virgin queen mortality. Attacks were easy to recognize; workers cluster around the victim stretching her legs and aggressively biting her. The attacked

virgin queens respond by curling up and lying still. Virgin queens under attack were gently picked up with forceps. Those showing any movement of limbs or appendages were returned, whereas those showing no movement were recorded as dead and discarded. Controls consisted of alate virgin queens that had been placed in a queenless colony fragment for 24 h but did not dealate.

To determine the ontogeny of the primer pheromone inhibiting alates from shedding their wings, corpses of virgin queens of known post-dealation age were tested in the bioassay of Fletcher & Blum (1981b). Four colonies were used; three colonies were collected from Calcasieu Parish, Louisiana on 14 May 1997 and one colony from Blanco County, Texas on 29 April 1997. Virgin queen alates were induced to dealate as described above, and dealates of known age were collected, frozen individually and held at -20°C until bioassay. The bioassay consists of placing two alates in small colony fragments (2.5 ml workers and brood). There were five replicates per treatment for each of the four colonies. Treatments consisted of a single corpse of a virgin queen dealate, from 1- to 7-days post-dealation, originating from the same nest as the test alates. There also was a control which received the corpse of a freshly killed nestmate alate. The colony fragments were inspected every 8 h for dealation. The time lapsed before at least one of the virgin queens dealated was recorded. The data were normalized by the square-root transformation and analyzed by a two-way ANOVA, using both treatment and colony as the dependent variables. The Dunnett test ($P < 0.05$) was used to indicate which treatments differed significantly from the control.

Results

Assessment of reproductive development

Ovary development steadily progressed following dealation (Fig. 1), rising from a mean of 1 chorionated oocyte for alates (0 day post-dealation) to over 50 fully developed eggs for dealates 7 days after wing shedding.

As shown in Fig. 2, egg laying began 2 days after wing shedding, when 30% of the dealates oviposited during the test. At 3 days post-dealation, 80% of the individuals were laying eggs, averaging 2-3 eggs during the test. Egg production steadily climbed after this point, with 100 % of the virgin queens laying an average of about 30 eggs by 7 days post-dealation.

Onset of production of queen retinue pheromone

Although there was little change in attractiveness between alate controls and virgin queens 1 day after dealation (Fig. 3), there were detectable amounts of the recognition pheromone present beginning on day 2 post-dealation. The number of brood deposited was highly correlated with the number of workers attracted ($r = 0.71$, $P < 0.001$). Because no additional, independent information concerning the ability of virgin queens to attract a retinue was evident from the brood deposition data, only the number of workers was used in the analysis.

Onset of pheromonal basis of virgin queen execution

There was no observable hostility toward the alate controls, although two of the 25 (8%), one from each of two colonies, were missing after 24 h and presumed dead. There was little hostile treatment of virgin queens who had just shed their wings. Attacks of 1-d dealates were observed in only two of the six colony fragments in which they were tested, occurring at 2 and 4 h after introduction of the virgin queens. No deaths were observed in the first 8 h in any fragment, and only four of the 25 (16%) 1-d dealates were found dead after 24 h, one from each of two colonies and two from a third colony.

In contrast, workers quickly killed all dealates that had shed their wings more than 24 h before testing. In half of the 6 fragments in which 2-d dealates were tested, workers began attacking virgin queens within 1 h of introduction; in the other half, attacks began by 2 h after introduction. Tests with all dealates older than this resulted in vigorous worker attacks within 1 h of introduction. All 2-d dealates and older were killed within 24 h, many much faster than this. Fig. 4 shows the time between introduction of dealates and the appearance of the first dead virgin queen as well as the time required for at least 50% of the dealates to be executed. Because 1-d dealates were killed in only a minority of the colony fragments, they were excluded from the statistical analysis. There was a significant difference among treatments for both the time between introduction and first killed ($F_{5,22} = 3.25$, $P < 0.025$) and the time to at least 50% killed ($F_{5,22} = 4.11$, $P < 0.01$). In both cases, the workers were slower to kill 2-day dealates; this treatment was significantly slower than some but not all older dealates for both measures. Thus workers appear to be able to detect the pheromonal cues issued by reproducing virgin queens as early as 24 h after wing shedding in some cases, but strong universal response appeared with virgin queen dealates that were two or more days post-dealation.

Onset of the primer pheromone that inhibits dealation

The inhibitory capability of virgin dealates increased steadily with age post-dealation (Fig. 5), with a significant effect evident at 3-d post-dealation and older. There was a significant effect of treatment ($F_{7, 128} = 4.61$, $P < 0.0001$) but not of colony ($F_{3, 128} = 1.89$, $P > 0.1$) or of the interaction term ($F_{21, 128} = 0.66$, $P > 0.8$).

Discussion

These results demonstrate that, in *S. invicta*, the production of both primer and releaser pheromones by virgin replacement queens is tightly linked with the initiation of ovary development and oviposition. Indeed, they give a clearer picture of when different queen pheromones are produced in relation to reproductive development and in relation to each other, highlighting the close association between the queen's reproductive and communicative roles. Previous work has shown the close coupling of oviposition and pheromone secretion in *S. invicta* queens. Both the queen recognition pheromone and the inhibitory primer pheromone originate in the poison sac (Vander Meer *et al.*, 1980; Vargo, 1997). Vander Meer & Morel (1995) demonstrated that fire ant queens dispense a small droplet of poison sac contents during the act of oviposition, thereby linking egg-laying and pheromone secretion. However, recent results of experiments in which queen poison sacs were surgically removed (unpublished data) indicate that

there are additional glandular sources of the queen recognition and inhibitory primer pheromone in *S. invicta*. Thus queen pheromone production appears to be linked with egg development, whereas pheromone secretion, to the extent that pheromones emanate from the poison sac, is linked with oviposition.

The association between reproductive development and pheromone production raises the question of whether both processes are regulated by common hormonal triggers. In a few insects, e.g., *Blattella germanica* (Chase *et al.*, 1992) and *Pseudaletia unipuncta* (Cusson & McNeil, 1989), juvenile hormone (JH) controls egg development and pheromone production. JH appears to regulate egg development in *S. invicta* queens (Barker, 1978; Vargo, 1992; Vargo & Laurel, 1994), but what role, if any, JH might play in queen pheromone production is unknown.

The queen recognition pheromone and the pheromone underlying queen execution by workers was detectable by bioassay a day earlier than the primer pheromone. The primer pheromone may thus be secreted slightly later in the reproductive development of the queen. Alternatively, the production of all three pheromones could occur simultaneously, but the bioassay used to detect the inhibitory pheromone, a primer, could be less sensitive. Distinguishing between these two possibilities will require identification of the active compounds responsible for the different effects and quantification of the compounds during the ontogeny of queen pheromone production.

Queens of *S. invicta* have a number of reproductive options open to them, with or without the help of workers (Tschinkel, 1998). The present study investigated the ontogeny of queen pheromone production in virgin queens that initiated ovary development and egg-laying in the nest following removal of the queen. In the field, queen loss can lead to some virgin queens dealating and undergoing reproductive development in the natal colony (Tschinkel & Howard, 1978). Such virgin replacement queens do not mate and therefore produce only unfertilized eggs that give rise to males (Tschinkel, 1998). The present results may reflect the onset of queen pheromone production under these conditions.

The close link between ovary development and pheromone production in virgin replacement queens creates a positive feedback loop, setting in motion an escalation in both egg production and pheromone secretion. Thus those virgin replacement queens that initiate reproductive activity early upon the loss of the queen prevent other, competing virgin queens from laying eggs in the nest. By monopolizing the colony's resources, the virgin replacement queens may be able to secure some measure of direct reproductive success through the production of males before the colony dies out.

Fire ant queens can initiate reproductive development with the help of workers under two other circumstances. In monogyne colonies, virgin queens that have overwintered can leave the nest in early spring, mate and gain entrance into orphaned colonies, becoming a mated replacement queen (Tschinkel, 1996; DeHeer & Tschinkel, 1998). *S. invicta* queens issuing from polygyne colonies will initiate reproduction either in their natal nest or a foreign one (Glancey & Lofgren, 1988; Porter, 1991; Ross *et al.*, 1996); although most of these will be mated, a substantial proportion, as many as 30-50%, can be unmated (Vargo & Fletcher, 1989; Ross *et al.*, 1996). Interestingly, queens of a particular genotype are executed by workers as they initiate

reproduction in polygyne colonies (Keller & Ross, 1993), suggesting an effect of genotype on the quality and/or quantity of queen pheromone production.

The reproductive option exercised by most fire ant queens is to found new colonies without the aid of workers, relying on body reserves instead. Such independent founding queens undergo much slower reproductive development than do dependent founding queens (Keller & Passera, 1990; Tschinkel, 1998). If, as the present results suggest, pheromone production were strictly linked with ovary development in fire ant queens, then independent founding queens would produce pheromones at a correspondingly slower pace than queens initiating reproduction in the presence of workers.

Studies of other ant species also suggest a link between reproductive development and queen pheromone production and/or release. In *Myrmica rubra* (Brian, 1973), *Linepithema humile* (Cariou-Etienne & Passera, 1993), and *Camponotus pennsylvanicus* (Fowler & Roberts, 1982), winged, virgin queens are not attractive to workers, or much less so, compared to mated, laying queens. In polygyne colonies of *M. rubra*, Cammaerts & Scanu (1985) found a positive correlation between ovary development and attractiveness of functional nestmate queens. In *Dinoponera quadriceps*, an ant that lacks a queen caste, Monnin *et al.* (1998) showed that the reproductively active and aggressively dominant worker, the alpha ant, contained about 7 times more of a C₃₁ hydrocarbon - 9-hentriacontene - than did the reproductively inactive subordinates, suggesting this compound may be a component of a recognition pheromone.

Evidence of queen primer pheromone production associated with reproductive activity comes from studies in which winged, virgin queens fail to produce the effects of functional queens. This has been shown in *M. rubra* with respect to the queen's ability to inhibit larval growth (Carr, 1962; Brian, 1973), and in *L. humile* (Vargo & Passera, 1991) and *S. invicta* (Vargo & Fletcher, 1986) for the inhibitory effect of queens on the production of sexuals. The present study demonstrates a close link between reproduction and pheromone production in *S. invicta* queens. Other studies would tell if this situation is general among ants.

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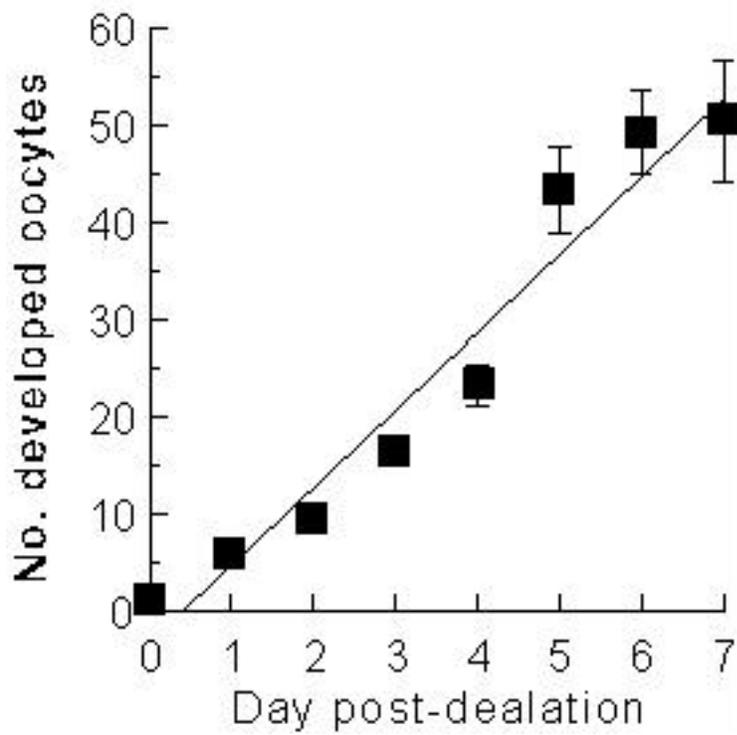


Fig. 1. Ovary development in relation to age post-dealation in virgin replacement queens. The line is described by the equation $y = 7.99x - 3.15$ ($R^2 = 0.947$; $F_{1,6} = 107.5$; $P < 0.001$).

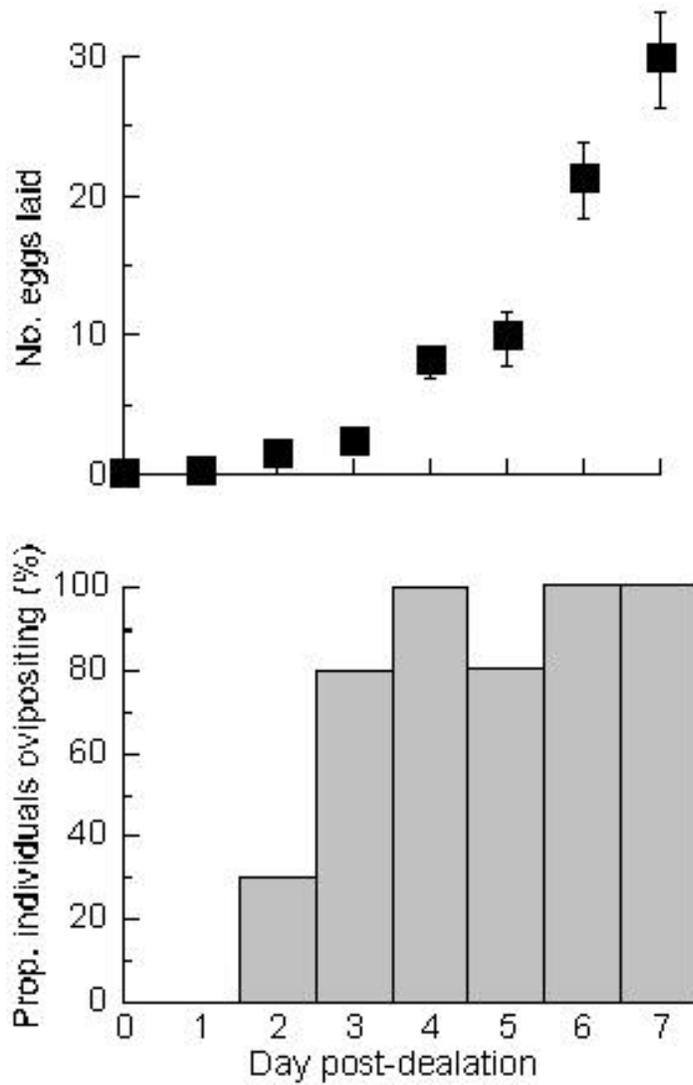


Fig. 2. Ovipositional activity in relation to age post-dealation in virgin replacement queens as determined by a 5-h oviposition test. Above, mean (\pm SE) no. eggs laid by queens at each age; below proportion of ovipositing individuals in each age class.

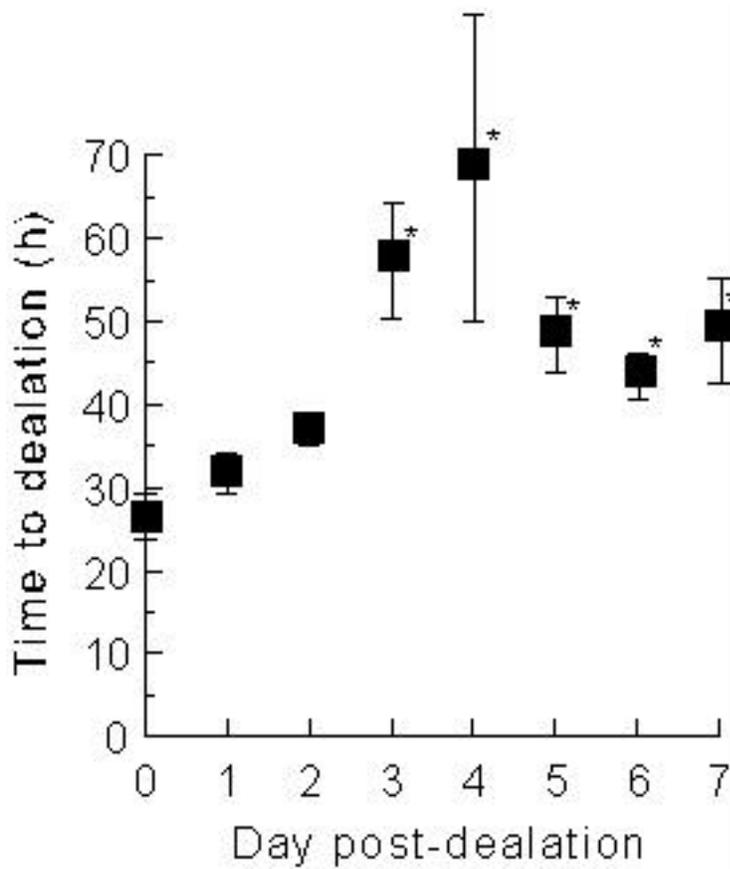


Fig. 3. Relative strength of the queen recognition pheromone produced by virgin replacement queens. Mean (\pm SE) number of workers out of a possible total of 100 attracted to the corpses of virgin replacement queens of different ages. Difference among treatments was significant ($F_{7,72} = 6.6$, $P < 0.0001$). Treatments marked with an asterisk differed significantly from the 0-day control ($P < 0.05$, Dunnett's test).

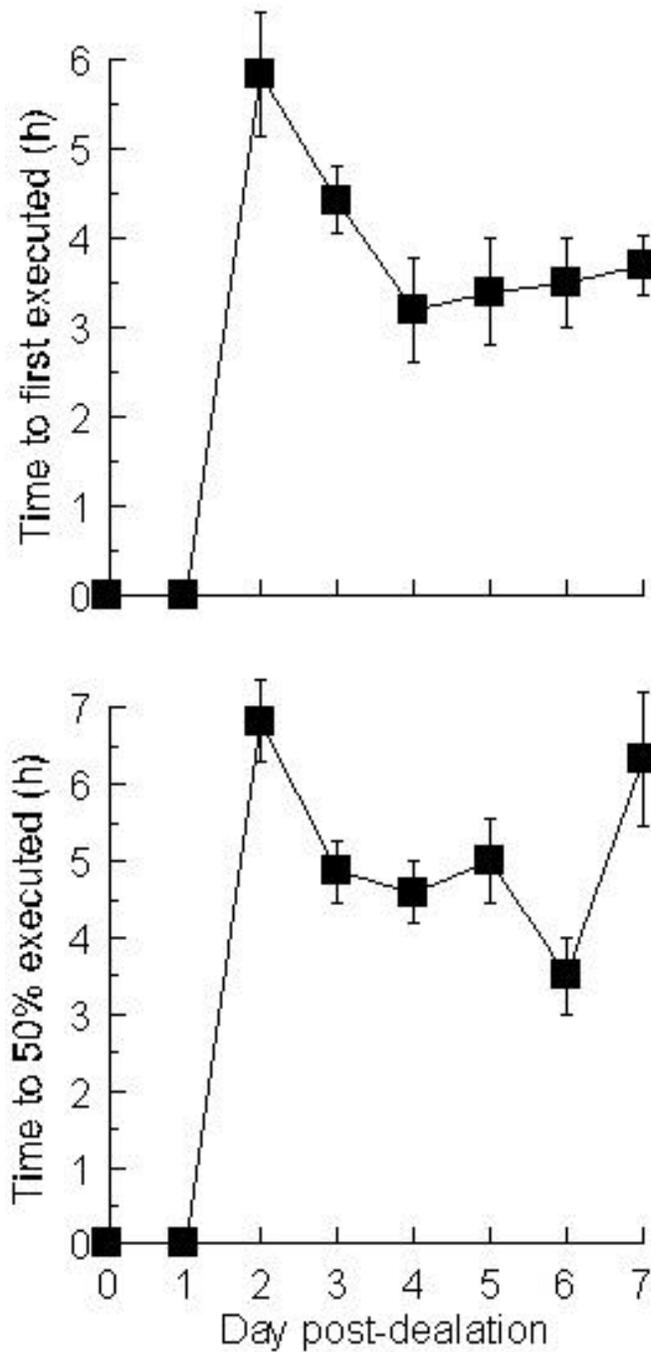


Fig. 4. Response of workers to virgin replacement queens of different ages in the presence of the mother queen. Virgin queens were introduced to the queenright colonies in groups of 2-10. Above, mean (\pm SE) time lapsed between introduction and execution of at least one virgin queen in each group; below, mean (\pm SE) time lapsed between introduction and execution of at least 50% of the virgin queens in each group.

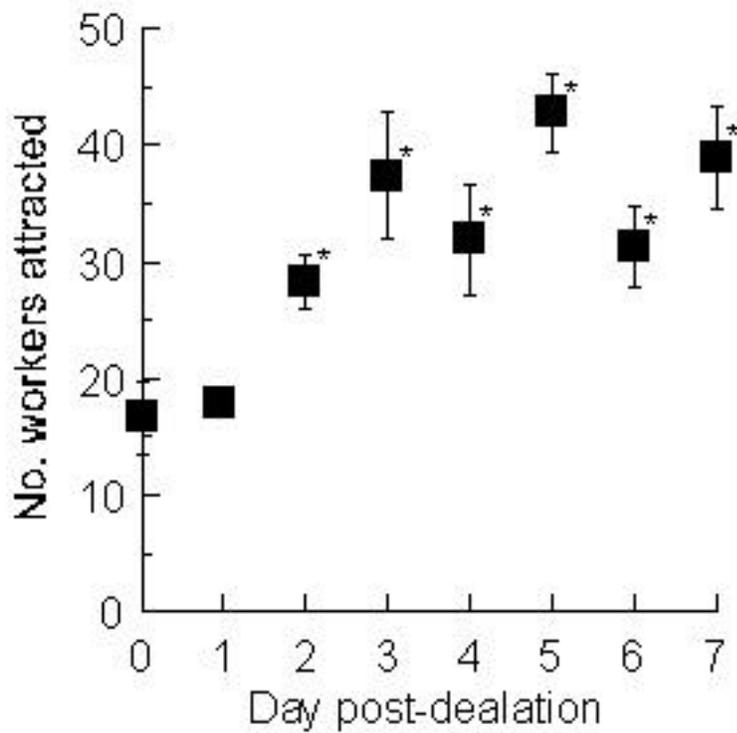


Fig. 5. Production of inhibitory primer pheromone by virgin replacement queens. Mean (\pm SE) time taken by winged virgin queens to shed their wings during exposure to corpses of virgin replacement queens of different ages. Treatments marked with an asterisk differed significantly from the 0 day control ($P < 0.05$, Dunnett's test).