Stimulation of Fire Ant Queen Fecundity by a Highly Specific Brood Stage

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ABSTRACT Fourth instars of the fire ant, Solenopsis invicta Buren, stimulate ovarian development and egg-laying in the queen. Fourth instars were separated into feeding, meconium-forming, postmeconial, and pupal stages, and each was tested for their effect on queen fecundity. Meconial-stage brood stimulated queens to lay eggs 60–170% faster than the other stages, which suggests that the stimulatory factor is released mainly at meconium formation. For brood to be effective, brood and queens must be present in the same nest. The effect of brood stage on ovarian development was significant only under some circumstances. Behavioral observations indicate that a certain group of workers repeatedly collects an unknown material from the anus of pharate pupae and proceeds more or less directly to the queen retinue, where they offer trophallactic exchange to the queen. Neither larva-tending nor most queen-tending workers shuttle in this manner. Results suggest that a specialized group of workers transfers a fecundity-stimulating anal factor from early postmeconial brood to the queen.

KEY WORDS Solenopsis invicta, larvae, oviposition, egg laying, metamorphosis, behavior

IN ANT COLONIES, egg-laying is carried out by the queen, but all other brood-rearing labor is carried out by different individuals, the workers, requiring that the rate of egg laying (fecundity) be coordinated with the availability of workers to rear the larvae that hatch from these eggs. Several factors regulating queen fecundity have been proposed. Most studies hypothesized that larvae had priority over the queen for food and worker attention and that larvae therefore depressed queen fertility (see Tschinkel 1988 for review). By contrast, the egg-laying rate of queens of the fire ant, Solenopsis invicta Buren, increased with the number of 4th instars, linking fecundity and larvae in a positive rather than negative feedback (Tschinkel 1988). Queen fertility also was positively related to the number of pupae in the black carpenter ant, Camponotus pennsyl vanicus (De Geer) (Gibson and Scott 1990), and the number of larvae in the Pharoah ant, Monomorium pharaonis (L.) (Borges en 1989, Borgesen and Jensen 1995).

Tschinkel (1988) showed that stimulation of oviposition in S. invicta was associated strongly with the end of the last instar and, using experiments with dried larvae, demonstrated that material moves from (especially) late 4th instars by way of workers to the queen and into the eggs. On the basis of these experiments, he suggested that stimulation of fecundity is associated with metamorphosis of larvae to pupae. The experiments in this report support this suggestion.

Materials and Methods

Methods were similar to those of Tschinkel (1988). Fire ant colonies were collected by methods generally similar to those described by Banks et al. (1981) and housed as stock colonies in 150-mm petri dishes with moist plaster floors. These were set in photo tray arenas with fluon-coated (Northeast, Woonsocket, RI) sides to prevent the escape of ants. Colonies were fed sugar water, liver, and tenebrionid beetle larvae and were maintained at 30°C. Most experimental queens were taken from stock colonies acclimated for several weeks in the laboratory.

Experiments were carried out in flat, plaster nests, each covered with a sheet of glass such that ants were =1 layer (3 mm) thick (Tschinkel 1988). These observation nests were set in trays coated with fluon and observed under a dissecting microscope capable of rolling freely in a horizontal plane.

Oviposition rate was determined by direct observation of queens for ½-h periods. Other variables also recorded included initial and final queen weight, egg volume (calculated from dimensions), and a census of nest contents at the end of the experiment. At that time, queens were dissected to determine their ovarian development.

Results

Staging and Timing of 4th Instars. Visual recognition and separation of the developmental stages through which larvae pass as they approach
Fig. 1. Early stages in the metamorphosis of 4th instars. Each stage is shown live and preserved in alcohol so that the nature of the pharate stage can be seen within the larval cuticle. (A) Feeding stage, live. (B) Feeding stage, alcohol. (C) Immediately after larval–pupal apolysis, live. (D) Same, in alcohol. (E) Meconium-forming stage, live. (F) Meconium-forming stage, alcohol. (G) Post-meconial stage, live, showing the shriveling resulting from passage of the meconium. (H) Post-meconial stage, alcohol.

...and undergo metamorphosis were essential for this experiment. Careful scrutiny of color, texture, opacity, and posture suggested the following divisions (Figs. 1 and 2), the durations of which were determined from individuals or groups of synchronized individuals. (1) Feeding 4th instar (Fig. 1 A and B), lasting 6 d at 30°C (Porter 1988) and recognizable by head and mouthparts reflexed toward the venter or active feeding (or both) on morsels placed on the anteroventral region by workers (Petralia and Vinson 1978). The body wall is transparent, and the midgut is clearly visible as a large brownish, reddish, or greenish bag. (2) Apolysis stage (Fig. 1 C and D), recognizable in the living animal primarily by the retraction of the head away from the venter so the mouthparts are withdrawn from the venter. When this stage was preserved in alcohol to make the hypodermis opaque and to separate it from the cuticle, pupal hypodermis in the anterior region became clearly visible (Fig. 1D). This stage marked the beginning of the pharate pupal stage and lasted 1–3 h. (3) Meconial stage of the pharate pupa (Fig. 1 E and F), recognizable by the formation of the black meconial plug in the hindgut following connection of the pharate pupal hind and midguts. Preservation of such larvae in alcohol showed a well-advanced pharate pupa with clearly visible head and thoracic appendages (Fig. 1F). This stage could be recognized for 1–3 h (mean = 2.0 h, SD = 0.7), making it the best stage for precise synchronization of brood. The meconial stage lasted <5% of the total duration of the pharate pupal stage. (4) Early post-meconial stage of the pharate pupa (Fig. 1 G and
H): with the passing of the meconium via the anus, the larva loses so much volume that it shrivels dramatically, presenting a wrinkled and opaque white exterior. Alcohol preservation showed well-advanced pharate pupal development (Fig. 1H). (5) Late pharate pupal stage (Fig. 2A): as development of the pupa proceeds, the pharate pupa takes on a pupa-like shape, that is, becomes larger at the anterior and smaller at the posterior end. Alcohol preservation shows a complete pupa within the larval cuticle (Fig. 2A). (6) Pupal stage results from larval–pupal ecdisis (Fig. 2B and C).

The entire pharate stage, from apolysis to pupal ecdisis, lasted 2.7–3.0 d, agreeing well with the value of 2.7 d reported by Porter (1988). Because of its brevity, the meconial stage was the best stage for precise synchronization of brood stage.

**Effect of Precise Brood Stages on Queen Fertility.** Beginning 2 d before the experiment, postmeconial (white) stages were separated daily for 3 d from the brood of stock colonies and added to small groups of workers for tending. Two d after separation, those that were not yet in the pupal stage were designated as late pharate pupae (>24 h beyond the meconial stage). On the day of the experiment and daily for 3 d, feeding larvae, meconial stages, and pupae were selected from stock colonies. Together with the late pharate stages, these formed the 4 experimental treatments.

Worker source served as a blocking factor; that is, workers from each stock colony were divided into experimental nests such that each stock colony (worker source) was subjected to all treatments. Treatments were not replicated within blocks. Each experimental nest consisted of 1 g of workers in each of 2 separate observation nests, each in its own arena. At the beginning of the experiment (day 0), 100 individuals of the treatment brood and a queen chosen randomly from a stock colony were added to one of these nests. One day later, 100 freshly sorted treatment brood of the same type were added to the 2nd nest and the queen was transferred to it. Brood were removed from the 1st nest and preserved for census. These procedures were repeated daily so that the queen and brood alternated between the 2 nests. This procedure assured that brood and queen were always in nests >24 h following the last major disruption. On day 4, the egg-laying rate of the queen was determined by direct observation for ½ h, and the experiment was terminated. The queen was weighed and dissected. Each replicate set consisted of 4 treatments, 8 experimental nests, and 4 queens. Two sets were run simultaneously, and in total 8 replicates were completed. The last replicate sets lacked a pupal treatment. This experiment was repeated with two differences: queens were kept in broodless colonies for 1 wk before experimental use, and postmeconial larvae were not used. Six replicates were completed.

The dependent variables were number of eggs per ½ h, number of active ovarioles, total vitellogenic oocytes, and number of eggs contained in the calyx. In addition, the numbers and stages of
Fig. 3. Effect of specific brood stages on fecundity of fire ant queens. Brood added in the meconial stage stimulated 60–170% higher fecundity than did equal numbers of other stages. Treatments with the same letter were not significantly different. Results of brood-pretreated and nontreated experiments are combined. See text for details.

The brood removed during daily queen transfer were counted. Data were square root-transformed to stabilize the variance and analyzed by 2-way analysis of variance (ANOVA) with worker source colony as the blocking factor. Differences between treatments were assessed with the Tukey honestly significant difference (HSD) test. In addition, the 2 experiments were analyzed together as a 3-way ANOVA using brood–broodless pretreatment as the additional factor. In this combined analysis, the postmeconial treatment was eliminated.

The brood stage treatment had a significant effect \((F = 5.07; \text{df} = 2, 32; P < 0.02)\) on the egg-laying rate of the queen. Queens in the meconial treatment laid eggs at a significantly higher rate (Tukey HSD test, \(P < 0.05\)) than those with the midfeeding, postmeconial, or pupal treatment (Fig. 3). The brood type accounted for \(≈40\%\) of the variance in number of eggs per \(\frac{1}{2}\) h, whereas source colony (block) accounted for \(22\%\).

This effect of brood stage led to a significant regression \((P < 0.001)\) between the number of postmeconial brood at the end of each day and the fecundity of the queen. Most of these postmeconial brood had been added as meconial brood 24 h previously. The number of postmeconial brood accounted for 86% of the variation when queens were pretreated in broodless nests and 37% when they were not. Queen fecundity did not regress significantly on the number of feeding-stage larvae or meconial brood recovered at the end of each day. Together, these regressions confirmed the effects of the added brood stages.

When the queens were not pretreated in broodless nests, all experimental fecundity measures, including number of eggs per \(\frac{1}{2}\) h, were 40–60% higher. Overall, pretreatment reduced the significance of the treatments by reducing the magnitude of all fecundity measures without reducing the variance among blocks. Without pretreatment, the mean number of vitellogenic follicles per ovariole was significantly \((F = 3.88; \text{df} = 3, 27; P < 0.05)\) affected by brood stage treatment, and the total number of vitellogenic follicles approached significance \((P = 0.1)\). Both showed the same patterns as the number of eggs per \(\frac{1}{2}\) h. Treatments had no significant effect on ovarian measures in the pretreatment experiment.

Must the queen and larvae be present in the same nest for meconial brood to stimulate her fecundity? The answer would shed light on the mechanics of the system. A negative answer suggests that a bioassay could be developed. Three replicates of the following experiment tested this question. Each worker source colony yielded three 2-g groups of workers, 1 for each treatment. Each 2-g group of workers was housed in 2 experimental nests, 1 g in each. There were the following 3 treatments. (1) An adopted queen and 50 meconial brood were added to 1 nest of a pair. After 12 h, 50 fresh meconial brood were added to the other nest, the queen was immediately moved to this nest, and the 1st nest was debrooded. (2) Fifty meconial brood were added to 1 nest of a pair, the queen to the other. After 12 h, the brood was removed, and the queen was moved immediately to the freshly debrooded nest. At the same time, 50 fresh meconial brood were added to the nest from which the queen was taken. (3) Fifty pupae and a queen were added to 1 nest of a pair. After 12 h, the queen and fresh pupae were added to the other nest.

These procedures were repeated every 12 h for 4 d, so that queens alternated between the 2 nests and were either constantly in the presence of brood <12 h beyond the meconial stage, or they chased brood of this description with a 0–12 h lag, or they were constantly in the presence of fresh pupae. Once a day in all treatments, workers were lightly etherized to remove the brood. All treatment brood were collected to confirm stage. All treatment queens were selected randomly from stock colonies and were pretreated for 4 d in broodless nests. All worker source colonies had been broodless for at least 1 wk to assure that any stimulatory factor had disappeared.

The results were unequivocal. The egg-laying rate of queens in the presence of meconial brood averaged 20.7 eggs per \(\frac{1}{2}\) h, \(>4\) times that of queens that chased the meconial brood (3.7 eggs per \(\frac{1}{2}\) h). Queens in the pupal treatment laid 7.53 eggs per \(\frac{1}{2}\) h (ANOVA: \(F = 26.34; \text{df} = 2, 6; P < 0.005)\). For the stimulatory system to operate, the queen and brood must be physically present in the same nest. A lag of 0–12 h between encounters of workers with meconial brood and their contact with the queen eliminates the stimulatory effect.

Several interpretations are possible. Workers do not collect the factor from meconial brood in the
absence of the queen. The factor is highly labile and loses its effectiveness in 0–12 h. Workers do not share the factor with the queen in the absence of meconial brood. Workers do not store the factor for any appreciable period. Other possible explanations also exist.

Observations of Marked Workers. Fecundity control emanates from larvae and acts on the queen through the mediation of workers (Tschinkel 1988). To establish the nature of this mediation, workers were lightly anesthetized with ether, then lightly sprayed with diluted paint (Dyckem, St. Louis, MO). Observation nests of such workers contained a large majority with paint spots. The large variety of placement, size, and shape of paint spots facilitated visual tracking of individual workers for long periods. Observation nests included a queen and a mixture of all brood stages.

Observations of workers began as they engaged in one of the following five initial behaviors: (1) in the queen retinue, tending the queen (n = 12 workers; total observation time, 280 min); (2) feeding 3rd or 4th instars by trophallaxis (n = 10; total time, 237 min); (3) collecting anal exudate from feeding-stage larvae (n = 10; total time, 262 min); (4) collecting anal exudate from pharate pupae (n = 16; total time, 487 min); (5) processing solid food at nest perimeter (n = 6; total time, 114 min).

Workers were observed for as long as possible, and the following five behaviors were recorded on an event recorder: (1) trophallaxis with queen, (2) joining retinue, (3) oral trophallaxis with feeding-stage larvae, (4) collecting anal exudate from feeding-stage larvae, and (5) collecting anal exudate from pharate pupae. Also recorded but not included in the analysis were walking, stationary, and all combinations of grooming. Because workers were chosen on the basis of initial act, this act was subtracted from the frequency of their subsequent acts before analysis. Chi-square analysis was applied to the frequencies of subsequent acts, summed over workers for each initial act. The initial act processing food was eliminated from the analysis because it showed no relationship to other behaviors and contributed too many low expected values to the chi-square table.

When the 5 possible acts that followed the 4 remaining initial acts were subjected to a chi-square analysis, frequencies of subsequent acts were highly nonrandomly distributed ($x^2 = 123$, df = 12, $P < 0.001$). Workers fell into three groups (Table 1): (1) Workers first seen in oral or anal exchange with feeding larvae (categories 2 and 3) showed lower than expected frequencies of joining the retinue, feeding the queen, or collecting anal exudate from pharate pupae. These 2 categories were therefore combined as tend larvae for the analysis in Table 1. (2) Workers first seen tending the queen had much higher than expected frequencies of further queen tending and of feeding the queen, but fed larvae less frequently. (3) Workers first seen collecting anal exudate from pharate pupae (pharate-tending) entered the retinue or collected additional anal exudate more frequently than expected but collected anal exudate from feeding-stage larvae less frequently. They fed the queen as expected.

Together, these patterns suggested that there are 3 types of workers: queen-tenders, not involved in care of feeding-stage larvae; larva-tenders, not involved in queen care; and pharate tenders, more similar to those engaged in queen care, although there are some differences. A comparison of pharate and larval tenders showed frequencies are highly non-random ($x^2 = 82.2$, df = 3, $P < 0.001$), the larval tenders showing much lower than expected frequencies of queen care and collection from pharate pupae but much higher larval care. Pharate pupal tenders showed much higher frequencies of queen care and further pharate pupal tending but lower frequencies of care of feeding-stage larvae.

When pharate pupal tenders were compared with queen tenders, the differences were much less marked, although they were still significant ($x^2 = 9.23$, df = 3, $P < 0.05$); most of this significance was the result of higher frequency of queen feeding and lower frequency of larval care by queen tenders (Table 1).

When the comparisons were reduced to these 3 types (larva-tenders, pharate-pupa tenders, queen-tenders as the sum of the first 2 columns in Table 1), the workers first seen tending the queen showed much higher subsequent frequencies of the same behaviors and lower larval tending. Those first seen collecting pharate anal exudate showed higher frequencies of doing more of the same and

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<th>Table 1. Observed and expected frequencies of worker behaviors associated with worker–brood–queen trophallactic flow</th>
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<td>Initial behavior</td>
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$^a x^2 > 15.$

$^b x^2 = 5.15.$

$^c$ Not different than expected.
of tending the queen. Those first seen tending feeding-stage larvae did more of the same and much less tending of the queen. Together, these frequencies were highly nonrandom ($\chi^2 = 113$, df = 4, $P < 0.001$) (Table 1).

These data show there was a group of workers that collected material from the anal end of pharaate pupae and then joined the retinue, and that these workers were distinct from other workers already in the retinue in that they were more likely to repeat this initial behavior. This group of workers also was very distinct from those tending feeding-stage larvae, with almost complementary duties. Workers were observed for 30–75 min, suggesting that the scale of task fidelity may be $\geq 1$ h.

A more detailed comparison shows that, whereas only 2 of the 20 larval-tending workers entered the retinue (5 entries), 13 of the 16 pharaate-tenders joined the retinue a total of 53 times. Seven of the 13 joined the retinue immediately after leaving the pharaate pupa, and the other 6 joined within 8 acts or less. Of these 13 joiners, 6 offered food or fed the queen a total of 10 times, about the same rate as the 9 queen-feedings by the 12 queen-tending workers. Thus, pharaate-tending workers appear to be shuttles between the pharaate pupae and the queen, possibly transferring fecundity-stimulating material. The reverse is less true; queen tenders do not show a tendency to seek out pharaate pupae, suggesting that the retinue membership of pharaate tenders may be less constant than that of queen tenders, as might be expected of workers with a shuttle-like function.

Overall, the data suggest the existence of a group of workers that shuttles back and forth between pharaate pupae and the queen, collecting an anal material from the former and feeding the latter. These workers continue in this role for $\geq 1$ h and form a group behaviorally distinct from other workers. They are the prime candidates for the mode of conveyance by which fecundity-stimulating material moves from the pharaate pupae to the queen.

**Discussion**

The current experiments and observations suggest that $< 24$ h after forming the meconium, brood peak in the production or release of a fecundity-stimulating factor. This factor is collected from the anus by a specialized group of workers and is carried and offered to the queen. This maximally stimulating period lasts only $\approx 30\%$ of the pharaate pupal period or 10% of the period from the last larval–larval ecodysis to the larval–pupal ecodysis. All other phases of this period were significantly less stimulating.

Tschinkel (1988) showed the relationship between number of larvae and queen egg-laying rate to be logarithmic (log eggs per $\frac{1}{2}$ h = 0.15 + 0.37 log larvae). If we assume each larva in his experiments produced the same amount of stimulating material, irrespective of the number of larvae present, and solve his equation for larval-equivalents using the data from his figure 2, we find that meconial-stage brood produce 3 times as much fecundity-stimulating material as do feeding larvae and 13 times as much as do pupae.

The existence of a specialized group of workers shuttling the factor to the queen suggests that a large part of the specificity of fecundity stimulation is the result of worker behavior. If this is so, the same material fed to colonies in the absence of brood might lack fecundity-enhancing activity because the behavioral cue leading to direct queen-feeding is missing. This may be the reason why the chase treatment failed to stimulate queen fecundity.

Behavior may also account for the decline of 2½ orders of magnitude in efficiency (eggs per $\frac{1}{2}$ h per larva) reported by Tschinkel (1988) as larval numbers increased from 1 to 10,000. Of the 16 workers observed collecting anal material from pharaate pupae, 3 did not join the retinue and 6 engaged in other behavior, including feeding other workers and larvae, before they joined the retinue and offered to the queen. Such prior feeding of others could reduce the amount of stimulating factor later offered to the queen. It is conceivable that as colonies grow, the incidence of failing to join the retinue or of prior feeding of other nestmates would increase, leading to a declining effectiveness of fecundity stimulation on a per-larva basis.

The possible role of specific worker behavior and the requirement that pharaate brood must be present in the same nest with queens make development of a bioassay for the stimulating factor difficult. Preliminary bioassays on broodless nests fed on the crop contents of workers maintained with early pharaate pupae, feeding larvae, or pupae showed no significant effect of brood stage on queen fertility (which remained low). Although this was not conclusive, development of a bioassay was not pursued.

Gibson and Scott (1990) found that cocoons of 2 species of carpenter ants stimulate egg-laying in founding queens. The cocoon is spun by the last instar, which passes the pharaate pupal and pupal stage within it. It is thus possible that in carpenter ants, as in fire ants, fecundity stimulation is associated with the early-pharaate pupal stage. On the other hand, Gibson and Scott (1990) indicate that the stimulation of cocoons is long-lived, unlike that of fire ant brood.

Brood also stimulate fecundity in *M. pharaonis*, but queens of this polygynous species appear to feed directly from larvae as well as through the intercession of workers. Special replete workers acted as temporary reservoirs for larval secretions (Borjesen and Jensen 1995), and may represent an analog to the shuttle workers of *S. invicta*. Although large larvae were shown to stimulate fecundity, it was not clear whether the stimulation emanated
from the feeding or early pharate stages. Queens of *M. pharaonis* were observed to collect anal material from meconium-passing brood with considerable avidity (Borgesen 1989).

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