Interplay between insulin signaling, juvenile hormone, and vitellogenin regulates maternal effects on polyphenism in ants

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Polyphenism is the phenomenon in which alternative phenotypes are produced by a single genotype in response to environmental cues. An extreme case is found in social insects, in which reproductive queens and sterile workers that greatly differ in morphology and behavior can arise from a single genotype. Experimental evidence for maternal effects on caste determination, the differential larval development toward the queen or worker caste, was recently documented in _Pogonomyrmex_ seed harvester ants, in which only colonies with a hibernated queen produce new queens. However, the proximate mechanisms behind these intergenerational effects have remained elusive. We used a combination of artificial hibernation, hormonal treatments, gene expression analyses, hormone measurements, and vitellogenin quantification to investigate how the combined effect of environmental cues and hormonal signaling affects the process of caste determination in _Pogonomyrmex rugosus_. The results show that the interplay between insulin signaling, juvenile hormone, and vitellogenin regulates maternal effects on the production of alternative phenotypes and set vitellogenin as a likely key player in the intergenerational transmission of information. This study reveals how hibernation triggers the production of new queens in _Pogonomyrmex_ ant colonies. More generally, it provides important information on maternal effects by showing how environmental cues experienced by one generation can translate into phenotypic variation in the next generation.

Many plants and animals can express specific adaptive responses to their environment through phenotypic plasticity, whereby a given genotype can develop into different phenotypes depending on environmental conditions (1, 2). Maternal effects, through which the environmental conditions experienced by the mother are translated into phenotypic variation in the offspring (3, 4), contribute to many phenotypic traits in a wide variety of taxa (5, 6) and have important ecological and evolutionary consequences (7, 8). Investigating the mechanisms of cross-generational transmission of information underlying maternal effects is needed to better understand the optimization of phenotypes in changing environments (6) and, more generally, the evolution of life history strategies (9).

In many insect species, maternal effects are known to affect polyphenism (3, 10), an extreme form of phenotypic plasticity characterized by the production of alternative and discrete phenotypes from a single genotype (1, 11–13). Such maternal effects allow adequate responses to environmental cues such as temperature, photoperiod, nutrition, and population density in many species (10). Examples of maternal effects on insect polyphenism include the production of sexual versus parthenogenetic morphs in aphids (14, 15), winged versus wingless morphs in firebugs (16), and dispersal versus solitary morphs in locusts (17, 18). The endocrine system was found to play a role in the regulation of some maternal effects on insect polyphenisms (19–21), but the nature of the physiological and genetic pathways interacting with the hormonal system to translate environmental cues into offspring polyphenism remains mostly unknown (22).

The most striking example of polyphenism is found in insect societies (23), where a reproductive division of labor leads to the coexistence of fertile queens and sterile workers that greatly differ in morphology and behavior (24, 25). Even though recent studies revealed genetic influences on caste determination in social insects (reviewed in ref. 26), female caste fate is primarily influenced by environmental factors in most species studied (27–39). In ants, several studies suggested that maternal factors such as temperature or queen age may affect caste determination (40–44). However, it is only recently that the first example of maternal effects on female caste polyphenism was documented experimentally (45). Cross-fostering of eggs between hibernated and nonhibernated _Pogonomyrmex_ colonies revealed strong maternal effects on caste production, as only eggs produced by a hibernated queen were able to develop into queens, irrespective of the hibernation status of the rest of the colony (45). Such maternal effects on the caste fate of the female offspring require that the hibernation triggers changes in the queen that affect polyphenism in the offspring. Hormones may be involved in this process in _Pogonomyrmex_ ants, as _Pogonomyrmex rugosus_ queen- and worker-destined eggs differed in their ecdysteroid content (45) and _Pogonomyrmex barbatus_ mature queens treated with juvenile hormone (JH) were recently found to produce larger workers (46).

Studies on the mechanisms regulating insect polyphenisms (reviewed in ref. 10) suggest that the insulin/insulin-like growth factor signaling (IIS), JH, and vitellogenin (Vg) pathways, known to regulate reproduction in adult insects (47–51), play predominant roles in modulating larval development in response to environmental cues. A well-known example illustrating the role of these pathways is the caste fate of the female brood (queen or worker) in the honey bee _Apis mellifera_ (52–58). In this species, worker-triggered differences in larval diet induce changes in IIS that affect JH (57), possibly through the release of neuropeptides (e.g., allatostatin and allatotropin) that influence JH production by the corpus allatum, as found in _Drosophila_ (59). Changes in JH in turn affect the production of Vg (60–62), which may be involved in the process of caste determination (62, 63). Such effects of JH on Vg production, also reported in flies (64), locusts (65), and cockroaches (66), have been proposed to involve the action of ecdysteroids (62, 67–70). IIS, JH, and Vg may also play a role in the regulation of caste differentiation of larvae in ants, as caste-specific expressions

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of genes involved in the IIS pathway were documented in *Solenopsis invicta* (71) and *Diacamma* sp. (72). Interestingly, caste-specific differences in IIS, JH, and Vg were also documented in adult ants and bees (48, 73–78), suggesting further roles of these pathways in the regulation of social life (74, 79).

We propose that the interplay between IIS, JH, and Vg regulates maternal effects on caste polyphenism in ants by translating the environmental conditions experienced by the queen during hibernation into the production of alternative phenotypes in the offspring. Under this hypothesis, IIS would translate environmental cues into changes in JH, which would, in turn, affect the amount of Vg in queens and in eggs, thus possibly affecting the caste fate of the offspring (62, 63). This hypothesis makes four predictions. First, a pharmacological increase of JH in queens should mimic the effect of hibernation and stimulate the production of queens. Second, hibernation should affect IIS and the production of JH in queens. Third, both hibernation and a JH increase should stimulate the production of Vg in queens. Finally, Vg content should differ between queen- and worker-destined eggs. We tested these predictions by performing artificial hibernation, hormonal treatments, gene expression analyses, hormone measurements, and Vg quantification in *Pogonomyrmex rugosus*, an ant species in which temperature-triggered changes in the queen had previously been shown to affect the relative production of queens and workers. Each of the four predictions was confirmed by our experiments, thus revealing that the interplay between IIS, JH, and Vg regulates maternal effects on caste polyphenism in *P. rugosus*.

Results

To investigate the mechanisms of caste allocation, we compared the production of queens between control, hibernated, and methoprene-treated *P. rugosus* colonies. There was a great variation among colonies in the proportion of queens among the offspring produced, ranging from 0 to 0.47 (0.05 ± 0.11, mean $\pm$ SD). There was a significant effect of the treatments on the proportion of queens produced [$F_{(2,73)} = 40.51$, $P < 0.001$; Fig. 1]. Hibernation significantly increased the proportion of queens among the female offspring ($t = 2.06$, $P = 0.04$). The methoprene (JH analog) treatment had a similar—albeit stronger—effect, as the queen/worker ratio among the female offspring was significantly higher in colonies fed methoprene-treated food compared with control colonies ($t =$
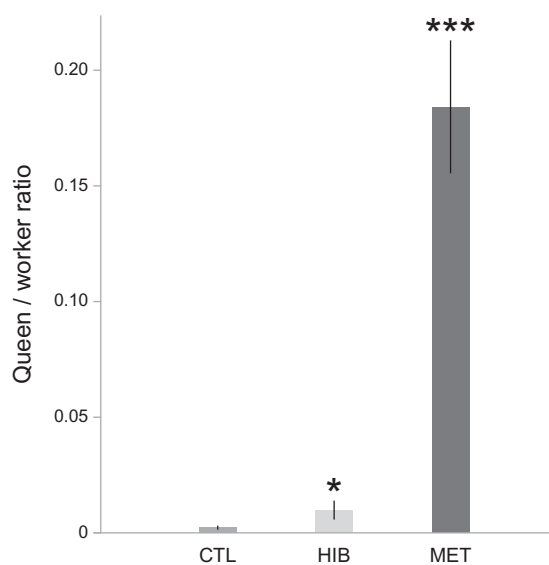

Fig. 1. The proportion of queens among the offspring produced (mean ± SE) was increased in hibernation (HIB) and methoprene (MET) treatments compared with control (CTL). *$P < 0.05$; ***$P < 0.001$.

5.39, $P < 0.001$). Interestingly, when only pupae that did not receive any treatment during larval development but were produced by treated queens (thus, those collected after week 11) were considered, there was also a significant difference between control and methoprene-treated colonies in the proportion of queens produced ($t = 5.56$, $P < 0.001$), showing that at least part of the observed effect of methoprene on caste determination was triggered by maternal effects.

Whole-body queen samples were used to measure the expression of genes involved in the IIS pathway (two insulin-like peptide genes: *ILP1* and *ILP2*), JH production (one gene coding for JH epoxidase: *JHepox*), and vitellogenesis (two Vg genes: *Vg1* and *Vg2*). The treatments significantly affected the expression of all of the genes tested [*ILP1*: $F_{(2,36)} = 5.30$, $P = 0.01$; *ILP2*: $F_{(2,36)} = 19.47$, $P < 0.001$; *JHepox*: $F_{(2,36)} = 4.12$, $P = 0.02$; *Vg1*: $F_{(2,36)} = 11.15$, $P < 0.001$; *Vg2*: $F_{(2,36)} = 7.93$, $P = 0.001$]. Compared with the control group, both hibernation and methoprene treatments up-regulated the expression of *ILP1* (hibernation: $t = 1.92$, $P = 0.06$; methoprene: $t = 3.24$, $P = 0.003$; Fig. 2), *ILP2* (hibernation: $t = 4.02$, $P < 0.001$; methoprene: $t = 6.14$, $P < 0.001$; Fig. 2), *JHepox* (hibernation: $t = 2.28$, $P = 0.03$; methoprene: $t = 2.65$, $P = 0.01$; Fig. 3), *Vg1* (hibernation: $t = 2.20$, $P = 0.03$; methoprene: $t = 4.72$, $P < 0.001$; Fig. 4), and *Vg2* (hibernation: $t = 2.15$, $P = 0.04$; methoprene: $t = 3.98$, $P < 0.001$; Fig. 4).

To determine whether ecdysteroids mediated the effect of JH on Vg genes expression, we compared the 20-hydroxyecdysone (20E) titer between queens from the control, hibernation, and methoprene groups. Although the 20E titer was lower in the methoprene group (3.38 ± 4.44 pg/mg) compared with the control (8.16 ± 8.47 pg/mg) and hibernation (8.18 ± 9.28 pg/mg) groups, the effect of the treatments was not significant (Kruskal–Wallis $\chi^2 = 2.76$, $P = 0.25$). However, there was a significant negative correlation between the 20E titer in queens and the proportion of queens in their brood (Spearman correlation test, $\rho = -0.40$, $P = 0.01$).

There was no significant difference between treatments in the number [$F_{(2,72)} = 1.35$, $P = 0.27$] and weight [$F_{(2,72)} = 1.09$, $P = 0.34$] of eggs produced. However, the treatments significantly affected the proportion of Vg among total proteins (Kruskal–Wallis $\chi^2 = 6.63$, $P = 0.04$; Fig. 5). The proportion of Vg in the protein content of eggs produced by both hibernated ($U = 42$, $P = 0.038$) and methoprene-treated ($U = 53.5$, $P = 0.026$) queens was significantly higher than in eggs produced by control queens. By contrast, this proportion did not differ significantly between eggs produced by hibernated and methoprene-treated queens ($U = 79$, $P = 0.93$).

Discussion

Each of the four predictions developed under the hypothesis that the interplay between IIS, JH, and Vg regulates maternal effects on caste polyphenism in *P. rugosus* was confirmed by this study. In line with the first prediction that an artificial increase of JH in queens should stimulate the production of queens, the feeding of *P. rugosus* colonies with a JH analog (methoprene) mimicked the effect of hibernation, with both hibernated and methoprene-treated colonies showing an increased production of queens. These results reveal a role of JH in the regulation of caste polyphenism in *P. rugosus*. In this species, maternal effects were previously found to stimulate the production of queens in response to hibernation, as only colonies headed by a hibernated queen produced queens, whether or not the workers had been exposed to cold (45). The exposure to cold therefore triggers changes in queens that make them more likely to lay queen-destined eggs. In this study, the methoprene treatment also targeted the queen, as evidenced by an increase in the proportion of queens among the offspring developing in a non–methoprene-treated environment from eggs laid by methoprene-treated queens. Similar results were found in *Pheidole pallidula*, in which direct topical application of JH on the queen stimulated the production of queens (80), and in
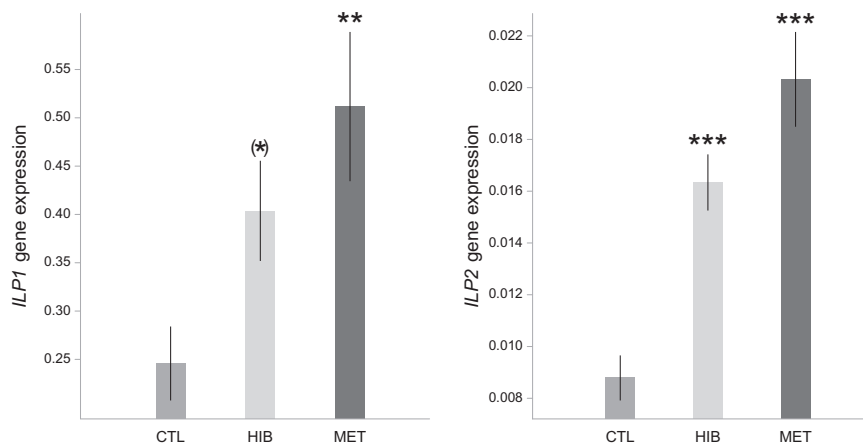
Fig. 2. *ILP1* and *ILP2* were up-regulated in hibernation and methoprene treatments. The *y* axis indicates the relative gene expression in queens, corresponding to the *ILP1* and *ILP2* mRNA levels relative to the *RP49* (control) mRNA level (mean ± SE). $^{(*)}P = 0.06$; $**P < 0.01$; $***P < 0.001$.

P. barbatus, in which it affected the size of the workers produced (46). Overall, the observed effects of hibernation and methoprene treatments show that hibernation-triggered JH changes in queens are involved in the production of queens in *P. rugosus*.

The second prediction was that hibernation should affect IIS and JH in queens. In line with this prediction, our results revealed that genes involved in IIS (*ILP1* and *ILP2*) were up-regulated in *P. rugosus* hibernated queens. This suggests that hibernation can translate into changes in the IIS pathway. Low temperature or the associated photoperiod changes could directly affect IIS, as reported in the regulation of insect diapause (81). Alternatively, the effect of exposure to cold could have been mediated by a change in the queen nutritional status due to decreased activity and metabolism (82) or lower food intake during hibernation. Such effects of nutrition on IIS have been reported in *Drosophila* (83–85). Changes in IIS usually result in the release of neuropeptides (e.g., allatostatin, allatotropin) that influence
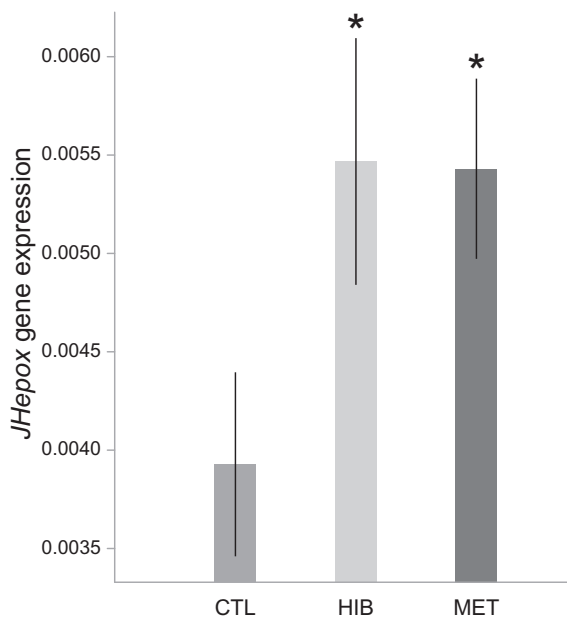
the production of JH by the corpus allatum (59, 64). Accordingly, the exposure to cold also up-regulated the expression of the *JHepox* gene, which encodes JH epoxidase, the enzyme that catalyzes the oxidation of methyl farnesoate into JH III (86, 87), the last step in the JH biosynthesis in most insects (88–91).

The finding that the expression of IIS genes was also affected by the methoprene treatment could be explained by JH translating environmental cues into IIS changes rather than the opposite. This is consistent with the report that RNAi-mediated manipulation of JH production affects IIS in *Tribolium* beetles (49). However, the effect of methoprene on IIS is not incompatible with IIS regulating JH production, as it may have been mediated by the associated changes in Vg (48, 92), of which levels are known to affect IIS through the target-of-rapamycin pathway in bees (55, 57, 78). Furthermore, IIS is known to regulate the production of JH in flies (59, 64). Although our data and the available literature do not provide a definite answer on the directionality of the relationship between IIS and JH in ants, our results clearly show interactions between these pathways in response to environmental changes such as those experienced during hibernation.

The third prediction was that both hibernation and an artificial increase in JH should stimulate the production of Vg. In our experiments, both hibernation and methoprene treatments stimulated the production of queens and up-regulated the expression of Vg genes (*Vg1* and *Vg2*) in queens. The effect of hibernation on vitellogenesis is likely to have been triggered by the increase in JH production. This is supported by the finding that the methoprene treatment also up-regulated Vg expression. These results show that JH-regulated vitellogenesis in adult *P. rugosus* queens is involved in the regulation of caste polyphenism.

In insects, effects of JH on Vg production have been proposed to be mediated by the ecdysteroid pathway (62, 67–70). Our results do not provide evidence for such a role of ecdysteroids, as the 20E titer in queens did not differ significantly among treatments. Interestingly, the results revealed a trend toward a reduction of 20E titer in methoprene-treated queens and a significant negative relationship between the 20E titer in queens and the proportion of queens in their offspring. This suggests that ecdysteroids may be involved in the process of caste determination (45, 93).

Finally, the fourth prediction was that the Vg content in eggs should correlate positively with their likelihood of developing into queens. This prediction was also supported by our data. Although neither the number nor the weight of eggs produced differed between control, hibernated, and methoprene-treated queens, the proportion of Vg in the protein content was significantly higher in eggs produced by both hibernated and methoprene-treated queens than by control queens. It is likely that the increased production of

Fig. 3. *JHepox* was up-regulated in hibernation and methoprene treatments. The *y* axis indicates the relative gene expression in queens, corresponding to the *JHepox* mRNA level relative to the *RP49* (control) mRNA level (mean ± SE). $*P < 0.05$.

Fig. 4. *Vg1* and *Vg2* were up-regulated in hibernation and methoprene treatments. The *y* axis indicates the relative gene expression in queens, corresponding to the *Vg1* and *Vg2* mRNA levels relative to the *RP49* (control) mRNA level (mean ± SE). *$P < 0.05$; ***$P < 0.001$.

Vg in hibernated and methoprene-treated queens translated into a higher Vg content in the eggs, increasing their likelihood of developing into queens. How the Vg content in eggs alters the caste fate remains to be investigated, but, as Vg is thought to act as a nutritive source for the embryo (94), more Vg in the egg could result in more energy during early development, facilitating the path toward queen development. The finding of a higher proportion of Vg in eggs produced by queen-producing hibernated and methoprene-treated queens is consistent with our fourth prediction, and shows that the quantity of Vg injected in the eggs is involved in the early regulation of caste allocation and plays a role in the intergenerational transmission of information required for maternal effects on polyphenism to happen.

Overall, this study describes the mechanisms that allow the environmental cues experienced by one generation to be translated into phenotypic variation in the next generation. The interaction between IIS and JH in queens translates environmental cues into

changes in Vg production. This affects the quantity of Vg injected into the eggs produced, influencing their development toward the queen or worker caste. In addition to the insights provided on the regulation of caste determination in social insects, this study raises the possibility that the interplay between IIS, JH, and Vg is also involved in the maternal regulation of other insect polyphenisms. More generally, this study provides routes to study the proximate mechanisms regulating maternal effects on any phenotypic traits.

Methods

Pogonomyrmex rugosus founding queens were collected during nuptial flights on July 15, 2008, in Bowie, AZ (N32°18′54″/W109°29′03″). Although it is affected by genetic compatibility effects, caste determination in this species remains mostly environmental with very strong maternal effects (26, 32, 45). After worker eclosion, the colonies were kept in laboratory conditions (30 °C, 60% humidity, and 12-h/12-h light:dark cycle) in plastic boxes containing a nest, a foraging area, and water tubes, and were fed once a week with grass seeds and a mixture of eggs, honey, and crushed mealworms. The experiments were performed on 92 2.5-y-old colonies that had never been exposed to cold and never produced queens. The colonies were divided in three groups: control ($n = 26$), hibernation ($n = 25$), and methoprene ($n = 26$).

The experimental manipulations were divided in two phases (Fig. S1). The first phase was set up to test the effect of an exposure to cold. Colonies from the hibernation group were kept for 2.5 mo in a dark climate chamber at 13 °C ± 1 °C and 60% humidity. The transition to and out of hibernation was done over a period of 2 wk by progressively decreasing or increasing temperature in a 8-h/16-h light:dark cycle. All of the other colonies (control and methoprene groups) were kept in the usual laboratory conditions (30 °C, 60% humidity, and 12-h/12-h light:dark cycle). The first phase terminated at week 0, when the second phase started. The second phase was set up to test the effect of JH treatment. To do so, we used methoprene (Sigma-Aldrich), a synthetic analog of JH. The colonies from the methoprene group were fed four mealworms crushed with 0.1 mg of methoprene in 0.1 mL of acetone each week for 8 wk (from week 0 to week 7), whereas colonies from the hibernation and control groups received four mealworms crushed in 0.1 mL of acetone. There is a drawback to this whole-colony approach because it affects all of the individuals in the colony. To circumvent this problem and determine whether the methoprene treatment acted on the queens to affect caste determination, we performed an additional analysis restricted to the offspring that pupated after week 11. These individuals were produced by methoprene-treated queens, but were not exposed to methoprene during larval development. The experimental design also required to control for a potential effect of acetone: feeding colonies mealworms crushed in acetone had no significant effect on the proportion of queens produced (food with acetone: $n = 26$; food without acetone: $n = 15$; Mann–Whitney *U* test: $U = 204$, $P = 0.15$).

Samples were collected in each colony to assess the proportion of queens among the female offspring produced, the number, weight, and Vg content of eggs produced, the expression of candidate genes, and the ecdysteroid titers in queens. All of the pupae produced were collected from week 3 until no brood remained (Fig. S1) and observations of size and morphology allowed the assignation of each pupa to the queen or worker caste. The proportion of

Fig. 5. The proportion of Vg among total proteins (mean ± SE) was increased in eggs produced in hibernation and methoprene treatments. *$P < 0.05$.

queens among the offspring produced was then calculated for each colony (except one that did not produce enough offspring; control: $n = 26$; hibernation: $n = 25$; methoprene: $n = 25$). At week 4, the queen of each colony was isolated for 24 h in a 2-mL plastic tube closed with wire mesh and placed in the colony. Thus, the queen could still communicate with workers, reducing the stress of isolation. This method allowed us to collect and count the number of eggs produced by each queen in 24 h (control: $n = 26$; hibernation: $n = 25$; methoprene: $n = 25$). At week 5, a batch of eggs was collected in each colony (between 5 and 52 eggs per colony; 26.1 ± 8.9, mean $\pm$ SD) and weighed using a microbalance (Mettler Toledo MT5) to a precision of 1 μg (control: $n = 26$; hibernation: $n = 25$; methoprene: $n = 25$). The eggs were then stored at –80 °C for further measurement of Vg content, successfully performed on eggs produced by 40 colonies (control: $n = 15$; hibernation: $n = 11$; methoprene: $n = 14$). At week 7, the queen was collected in each colony. One-half of the queens were flash-frozen in liquid nitrogen and stored at –80 °C for later RNA extraction (control: $n = 13$; hibernation: $n = 13$; methoprene: $n = 13$), whereas the other half was used for ecdysteroid measurement (control: $n = 12$; hibernation: $n = 12$; methoprene: $n = 13$).

RNA extractions from whole-body queen samples were performed using a modified protocol including the use of TRIzol (Invitrogen) for the initial homogenization and the RNeasy Plus Micro extraction kit (Qiagen). For each individual queen, cDNAs were synthesized using 500 ng of total RNA, random hexamers, and Applied Biosystems reagents. Levels of mRNA were quantified by quantitative real-time PCR (qRT-PCR) using ABI Prism 7900 sequence detector and SYBR Green. All qRT-PCR assays were performed in triplicate and subjected to the heat-dissociation protocol following the final cycle of the qRT-PCR to check for amplification specificity. qRT-PCR values of each gene were normalized by using an internal control ribosomal protein 49 (*RP49*)

gene. Paralog-specific primers (Table S1) were designed using sequence alignment (95) and primer analysis (96) programs. Primer sequences overlapped coding regions split by introns, allowing the specific amplification of cDNA levels over potential genomic DNA contaminations. Transcript quantification calculations were performed by using the ΔΔCT method (97).

The ecdysteroid titer in queens was determined using the liquid chromatography–mass spectrometry method developed by Westerlund and Hoffmann (98), with some minor modifications (see *SI Text* for details). The amount of Vg in eggs was measured by dot-blotting using *Ectatomma tuberculatum* (Formicidae: Ectatomminae) anti-Vg antibodies (99) (see *SI Text* for details).

To test for the effect of the treatments on the proportion of queens among the offspring, gene expression, and egg number and weight, we conducted ANOVAs on models optimized to fit our data. The proportion of queens was fit using a generalized linear model with quasi-binomial errors. The gene expression data were fit using a general linear model with normal errors. The ecdysteroid and Vg data could not be normalized and were analyzed using Kruskal–Wallis and Mann–Whitney nonparametric tests. The correlation between the ecdysteroid titer and the proportion of queens produced was tested using a Spearman rank correlation test. All statistical analyses were performed with R (www.R-project.org).

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