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Juvenile hormone induces queen development in late-stage larvae of the ant *Harpegnathos saltator*

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ABSTRACT

A link between hormones and developmental plasticity has long been established, but understanding how evolution has shaped the physiological systems underlying plasticity remains a major question. Within the eusocial insects, developmental plasticity helps define a reproductive division of labor through the production of distinct queen and worker castes. Caste determination may be triggered via changes in juvenile hormone (JH) levels during specific JH-sensitive periods in development. The timing of these periods, however, can vary and may relate to phenotypic differences observed among species. In order to gain insight into the evolution of caste determining systems in eusocial insects, we investigated the presence of a JH-sensitive period for queen determination in the ant *Harpegnathos saltator*. This species displays a number of ancestral characteristics, including low queen–worker dimorphism, and should allow insight into the early evolution of caste determining systems in ants. We identified four larval instars in *H. saltator*, and we found that the application of a JH analog (JHA) to third and fourth instar larvae induced queen development while treatment of early instars did not. This indicated the presence of a JH-sensitive period for queen determination at the end of the larval stage. These results contrast with what has been found in other ant species, where queen determination occurs much earlier in development. Therefore, our results suggest that caste determination originally occurred late in the larval stage in the ancestral condition but has shifted earlier in development in species that began to acquire advanced characteristics. This shift may have facilitated the development of greater queen–worker dimorphism as well as multiple worker castes.

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1. Introduction

Many insect species display a form of developmental plasticity termed polyphenism, where a single genotype can give rise to two or more distinct phenotypes (Nijhout, 2003; Simpson et al., 2011). Within the eusocial insects, caste polyphenisms define a reproductive division of labor between the queen and worker castes (Wilson, 1971). Species in this group display an incredible diversity in the degree of dimorphism between queens and workers as well as variation in the presence of additional worker castes. Underlying this phenotypic diversity there must also have been changes to the regulatory systems that control caste differentiation. Identifying how these systems vary across species is a central component for understanding the evolution of morphological castes.

Evidence from a wide range of species indicates the importance of hormones in caste determination, particularly juvenile hormone

(JH) (Hartfelder and Emlen, 2005; Nijhout and Wheeler, 1982). JH has been shown to affect caste determination during critical periods in development that are characterized by an increase in hormone sensitivity (Wheeler, 1986). If JH passes a specific threshold during one of these periods it can redirect development towards an alternative trajectory. This has been clearly demonstrated in the honey bee, *Apis mellifera*, where an increase in larval nutrition triggers a rise in JH levels in fourth and fifth instar larvae that induces queen development (Rachinsky and Hartfelder, 1990; Rachinsky et al., 1990; Rembold, 1987). Application of JH to worker larvae during this period can also induce queen development (Wirtz and Beetsma, 1972). JH has been found to affect caste determination in stingless bees (Hartfelder et al., 2006), bumble bees (Cnaani et al., 2000; Röseler, 1976) as well as ants, but the timing of the JH-sensitive period may vary among species (e.g. Röseler, 1970). While the effect of JH on caste determination appears to be highly conserved, this variation in timing of the JH-sensitive period raises questions about the evolution of caste determining systems in different lineages.

Despite a single origin of distinct queen and worker castes in ants, the factors affecting queen determination in this group vary widely (Nijhout and Wheeler, 1982). In some cases, caste

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determination is under genetic control (Anderson et al., 2008; Cahhan et al., 2002; Schwander et al., 2010; Smith et al., 2008), but caste in most species is thought to be environmentally determined. Larvae of temperate species may need to overwinter before they become bipotential, and treatment of overwintered larvae of *Myrmica rubra* with a JH analog induced a greater portion of these larvae to develop as queens (Brian, 1974). In other species, a JH-sensitive period occurs earlier in development, even inside the embryo. Treatment of eggs and first instar larvae of the fire ant *Solenopsis invicta* induced a portion of larvae to develop as queen-like workers (Vinson and Robeau, 1974). In *Pheidole*, treatment of the queen or her eggs induced queen development (Abouheif and Wray, 2002; Passera and Suzzoni, 1979). Species with polyphenic worker castes display additional JH-sensitive periods that control worker phenotype. In *Pheidole*, treatment of worker larvae during the last instar can trigger soldier development (Wheeler and Nijhout, 1981), and a second sensitive period controls the development of “super-soldiers” in *Pheidole rhea* (Rajakumar et al., 2012).

While techniques for investigating the role of JH in larval development have been available for some time, there have been relatively few studies on ants with respect to queen development. Past studies have focussed on species within the single subfamily Myrmicinae, where most species display relatively advanced characteristics. There is a lack of information about how JH may affect larval development in species that display ancestral characteristics, which could provide information about the evolution of caste determining systems in ants. Because ants display a high diversity of alternative morphologies, studies on caste development in this group should shed light on the general mechanisms involved in developmental plasticity.

We studied the role of JH in queen development of the ant *Harpegnathos saltator*, a member of the morphologically primitive subfamily Ponerinae, in order to gain further understanding of caste determination in ants. This species lives in small colonies (~50–300 workers) in southwestern India (Peeters et al., 2000) and produces winged sexuals during early spring prior to the monsoon rains. For the present study, we conducted an analysis of the developmental stages of this species to identify larval instars, and using this information we tested the effect of a JH analog (JHA) on queen determination in each instar. Because *H. saltator* retains a number of ancestral characteristics (e.g. low queen–worker dimorphism (Peeters, 1997)), our results provide information about the early control mechanisms for caste determination in ants.

2. Methods

2.1. Focal species and lab conditions

Stock colonies of *H. saltator* were originally collected in southwestern India as described in Peeters et al. (2000). In the laboratory, colonies were housed in plastic boxes (19 × 27 cm) with a dental plaster floor that featured a preformed nest chamber covered by a glass plate (12 × 15 cm). For all experiments, colonies were held at a constant temperature (25 °C) and 12:12 light/dark cycle. They were fed live crickets (*Acheta domesticus*) *ad libitum*, which the workers paralyze and bring into the nest chamber.

2.2. Larval instar measurements

In order to identify larval instars of *H. saltator*, we developed a new methodology for this species. We isolated 10–20 workers in a satellite nest with eggs from their parent colony. These groups were established from 10 independent stock colonies. Each nest was checked daily, and when new larvae eclosed they were photographed under a dissecting microscope (Leica MZ9.5) and moved into a new nest box with additional workers from their parent colony. All larvae were checked daily for signs that they had molted into a new instar. Unlike other ant species (Baratte et al., 2005; Penick et al., 2012; Petralia and Vinson, 1979), the larval instars of *H. saltator* cannot be easily distinguished by differences in hair morphology or other conspicuous morphological features. However, at the beginning of each instar the larvae of *H. saltator* appear to have a higher density of tubercles covering their body (Fig. 1). As a larva grows, these tubercles spread out as the body expands, and the larva appears shiny. This feature allowed us to clearly identify larvae that had recently molted. Each time a larva molted it was photographed under a dissecting scope and moved into a new nest box with larvae of the same instar.

We tracked larval development until the beginning of the pupal stage. For the purposes of this study we considered the beginning of the pupal stage to occur when larvae began to spin a pupal cocoon (larvae do not molt into true pupae until several days after they have completed their cocoon). The largest larvae in the nests were photographed approximately 1 day before they pupated, and this was used to define the maximum larval length reached before pupation. Colonies were checked daily after larvae pupated until the first adult workers eclosed, which marked the end of the pupal stage. To determine the duration of the egg stage, we removed all

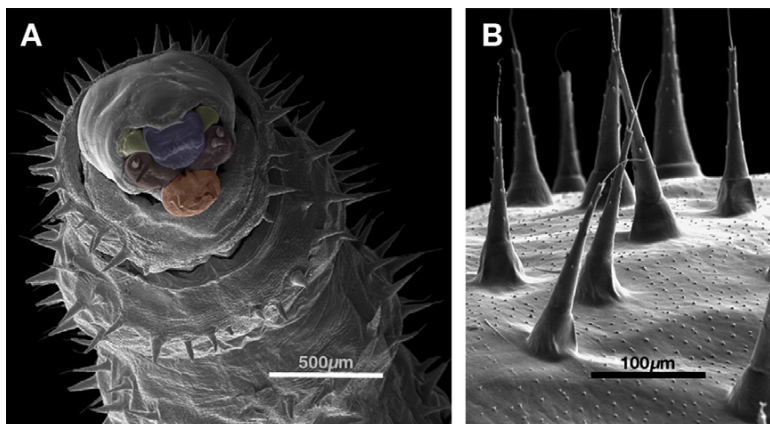


Fig. 1. Larval morphology of *H. saltator*. (A) SEM of a fourth instar larva showing the head with complete mouthparts (mandibles-yellow; maxillae-red; labrum-purple; labium-orange). (B) SEM of larval tubercles, which are present on all instars and aid in identification of instar number. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

eggs and young larvae from 10 colonies and monitored these nests until the first new larvae eclosed.

Larval measurements were taken from photographs using ImageJ software (version 1.44, 2010). Larval length was measured across the long axis of the body (the neck and head were excluded). In addition to length, we also measured the mass of each instar. This was conducted as a separate study, and larvae were taken from 10 stock colonies and sorted by instar. In order to get the full spectrum of larval sizes for each instar we chose larvae from 3 size classes within each instar (small, medium, and large larvae). These were then weighed using a Mettler Toledo XS105 DualRange analytical balance (sensitive to 0.1 mg). We also determined the dry mass of worker and male pupae by removing individuals from their pupal cocoons and drying them in an oven at 50 °C for 48 h.

2.3. Queen induction using JHA

To determine the presence of a larval critical period for JH sensitivity, we tested the effect of the JH analog (JHA) methoprene (Chem Service, West Chester, PA) on each individual instar. Methoprene degrades at a slower rate compared to natural JH-III and can remain biologically active over a longer duration (Bigley and Vinson, 1979; Quistad et al., 1975). We established 2 satellite nests for each of 10 stock colonies. Satellite nests consisted of 20–40 workers, and these nests were established at least 3 months prior to the start of the experiment to ensure that the reproductive hierarchy in these colonies was stable. We conducted three separate trials using either fourth instar larvae, third instar larvae, or first and second instar larvae combined. For each trial, all larvae from the instar designated were removed from their parent colony and randomly divided into two groups. One group was treated topically with 0.15 µg JHA per mg larval weight dissolved in acetone (0.1 µg, 0.5 µg, and 5 µg concentrations were used depending on instar, and the relative dose could be adjusted for larval size by controlling the volume administered). Larvae in the control group were treated with acetone only. After treatment, each group of larvae was separated into separate satellite nests, and these nests were checked after 2 days to determine whether workers were observed biting larvae. Workers bite larvae as a mechanism to inhibit queen development (Penick and Liebig, 2012), and biting can be used as an early indicator that queen development has been induced. We also noted when at least three larvae had pupated in order to determine if JHA treatment delayed pupation. After all larvae had pupated, we opened pupal cocoons to determine the caste and sex of larvae. Queens were identified based on the clear presence of wing buds.

We conducted a second experiment to determine if JHA treatment had a dose dependent effect on the induction of queen development. Satellite nests were established as described above, and fourth instar larvae were treated with one of four doses of JHA dissolved in 1 µl of acetone (0.1 µg, 1 µg, 5 µg, or 10 µg JHA). Control larvae were treated with an equivalent volume of the acetone solvent. Again, these colonies were observed 2 days after treatment to determine if workers were biting larvae, and caste and sex were determined at the pupal stage.

2.4. Statistical analysis

Statistical analyses were conducted using Statistica version 7 (StatSoft, Tulsa, OK, USA). Due to a lack of normality we used non-parametric analyses as a conservative choice over parametric tests for all comparisons. Our experiments on queen development used a paired design, and comparisons were made between treatment and control groups derived from the same individual colony. To determine the effect of JHA treatment on queen development, we analyzed our data using two metrics: (1) the percentage of

female brood in each colony that developed into queens, and (2) the total proportion of colonies that produced queens. For paired comparisons we used the Wilcoxon signed rank test, and for analyses of binomial data we used the McNemar's test. Because we had predicted that JH would induce queen development *a priori*, we used a one-tailed analysis. For unpaired analyses we used the Mann–Whitney *U* test.

3. Results

3.1. Developmental features

Observations of larval development in *H. saltator* indicated that larvae progress through four instars. Larvae of all instars possessed a mobile neck and a head with complete mouthparts (Fig. 1A) as well as numerous subconical tubercles (terminology from Wheeler and Wheeler (1976); Fig. 1B). These tubercles were present on all thoracic and abdominal segments in rings that wrapped the dorsal, ventral, and lateral sides. The absolute density of these tubercles was similar across instars, but the total number of tubercles increased with instar number. At the beginning of an instar these hairs were close together and appeared relatively dense; however, as a larva grew within an instar these tubercles gradually moved apart as the larva expanded in volume. This feature allowed instars of similar size to be distinguished based on relative tubercle density.

The larval period lasted approximately 18 days at 25 °C, and egg and pupal stages lasted 29 and 32 days respectively (Table 1). Total development time was approximately 79 days, which is relatively long compared to other ant species (Porter, 1988). Incidentally, 3 colonies in our study produced only males while another 4 colonies produced only females. A comparison of development times between these groups showed that the duration of larval and pupal stages did not clearly differ between sexes ([male larval period: median 17 days, range 16–18; female larval period: median 18 days, range 17–18 period]; [male pupal period: median 32 days, range 31–33; female pupal period: median 32 days, range 32–35]). We also measured the dry mass of male and worker pupae to determine whether there were differences in size associated with sex. The dry mass of male pupae was significantly higher than the dry mass of worker pupae (Mann–Whitney *U* test, male $N = 31$, worker $N = 29$, $p < 0.0001$). Males gained approximately 25% more weight during the larval stage compared to worker larvae (males: median, 9.25 mg; range, 6.21–11.63 mg; females: median, 7.29 mg; range, 5.37–9.57 mg). Based on these results, male larvae exhibited a higher growth rate and gained 25% more mass over the same period (~18 days) as worker larvae.

Table 1
Growth parameters of *H. saltator* (males and workers combined, held at 25 °C).

	Duration median/range	Length	Mass mean ± SD/range
Egg	29 days 27–31	–	–
Instar I	3 days 2–5	1.3–2.0 mm	1.01 ± 0.42 mg 0.31–1.56
Instar II	3 days 2–5	2.0–2.9 mm	3.05 ± 1.12 mg 0.77–4.89
Instar III	4 days 3–5	2.9–4.1 mm	7.59 ± 3.25 mg 2.09–14.97
Instar IV	7.5 days 6–9	4.1–6.5 mm	28.38 ± 15.26 mg 7.72–53.03
Pupa	32 days 31–35	–	–
Total	78.5 days		

3.2. Effects of JHA treatment on queen determination

We tested the effect of JHA treatment on separate instars to identify the presence of a JH-sensitive period for queen determination. Treatment of third and fourth instar larvae with JHA increased the percentage of female larvae that developed into queens but did not significantly affect first and second instar larvae (Wilcoxon signed rank test, $N = 10$: [5 μg fourth instar, $p = 0.028$; 0.5 μg third instar, $p = 0.043$; 0.1 μg first and second instar, $p = 0.12$]; Fig. 2). Likewise, JHA increased the proportion of colonies that produced queens when third and fourth instars were treated but not first and second instars (McNemar's test, one-tailed, $N = 10$: [5 μg fourth instar, $p = 0.021$; 0.5 μg third instar, $p = 0.037$; 0.1 μg first and second instar, $p = 0.24$]; Table 2). Observations of larval-directed biting showed that biting was significantly higher in JHA-treated third and fourth instar larvae 2 days after treatment (a sign that larvae were developing into queens), but no biting was observed in first and second instar larvae 2 days after treatment (McNemar's test, one-tailed, $N = 10$: [5 μg fourth instar, $p = 0.004$; 0.5 μg third instar, $p = 0.037$; 0.1 μg first and second instar, $p = 0.5$]; Table 2). We checked nests with first and second instar larvae again 1 week after treatment when larvae had molted into the third instar, and we did observe biting in 3 JHA-treated colonies and 2 control colonies; however, biting was still not significantly higher in JHA-treated first and second instar larval groups compared to controls (McNemar's test, one-tailed, $N = 10$, $p = 0.5$).

JHA treatment displayed a dose dependent trend increasing from 5% to 30% of female larvae that developed as queens, and the lowest and highest doses of JHA did not significantly induce queen development (Wilcoxon signed rank test, $N = 10$: [10 μg fourth instar, $p = 0.080$; 5 μg fourth instar, $p = 0.028$; 1 μg fourth

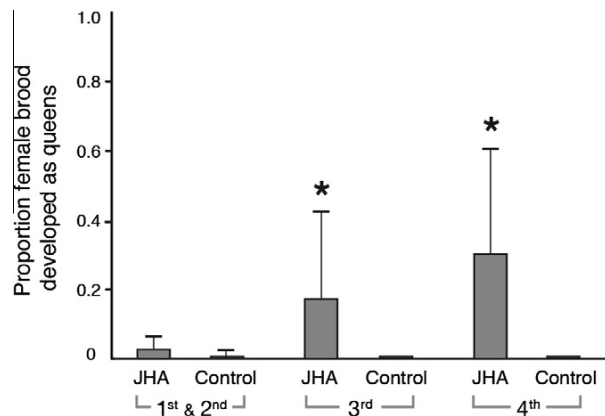


Fig. 2. Effect of JHA on queen development in each larval instar. Mean \pm SD of the percent of female brood that developed as queens in each colony. JHA significantly increased queen development in third and fourth instar larvae but not in early instars. * $p < 0.05$ between treatment and control groups.

Table 2
Induction of queen development in colonies of *H. saltator*.

Instar	JHA treatment	Colonies producing queens (JHA/control)	Colonies with biting observed (JHA/control)
4th	10 μg	4/1	10/1*
4th	5 μg	6/0*	10/0*
4th	1 μg	7/2*	9/0*
4th	0.1 μg	4/0	6/0*
3rd	0.5 μg	5/0*	5/1*
1st/ 0/0	2nd 0.2 μg		3/1

* $p < 0.05$.

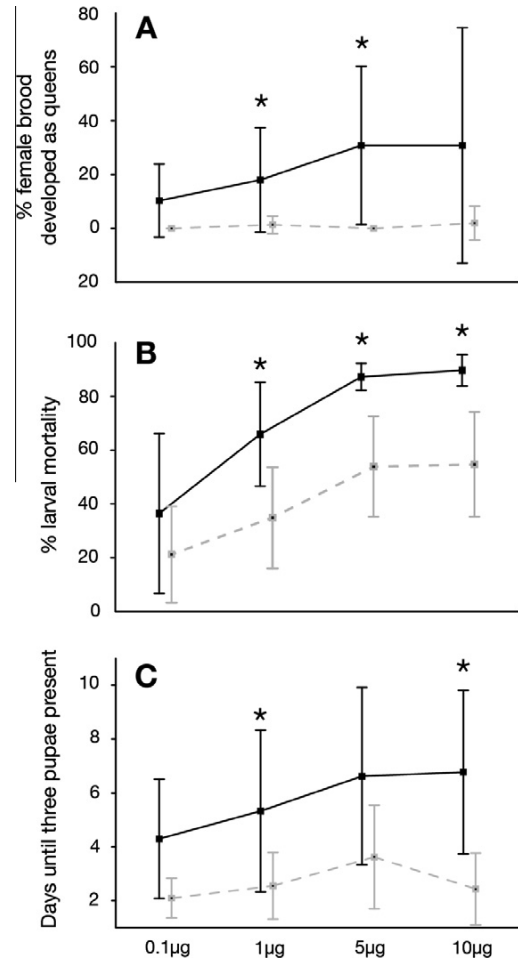


Fig. 3. Dose dependent effect of JHA on fourth instar larvae. Mean \pm SD of (A) the percentage of female brood that developed into queens, (B) larval mortality, and (C) the length of time required until three pupae were present (higher values indicate delayed pupation). (JHA treatment—solid; control—dashed). * $p < 0.05$ between treatment and control groups.

instar, $p = 0.028$; 0.1 μg fourth instar, $p = 0.068$]; Fig. 3A). The proportion of colonies that produced queens showed a similar pattern, where the lowest and highest doses of JHA did not induce a significant portion of colonies to produce queens (McNemar's test, one-tailed, $N = 10$: [10 μg fourth instar, $p = 0.37$; 5 μg fourth instar, $p = 0.021$; 1 μg fourth instar, $p = 0.037$; 0.1 μg fourth instar, $p = 0.065$]; Table 2). With respect to larval-directed biting, there was a slight decrease in biting observed in colonies treated with the lowest dose of JHA, but the proportion of colonies where biting was observed was higher in all JHA-treated colonies compared to controls (McNemar's test, one-tailed, $N = 10$: [10 μg fourth instar, $p = 0.008$; 5 μg fourth instar, $p = 0.004$; 1 μg fourth instar, $p = 0.008$; 0.1 μg fourth instar, $p = 0.041$]; Table 2). We also observed an increase in larval mortality due to JHA treatment (Wilcoxon signed rank test, $N = 10$: [10 μg fourth instar, $p = 0.005$; 5 μg fourth instar, $p = 0.005$; 1 μg fourth instar, $p = 0.014$; 0.1 μg fourth instar, $p = 0.12$]), and larvae treated with higher concentrations of JHA had the highest levels of mortality (Fig. 3B). Some ant species possess a supernumerary instar associated with queen development (Nijhout and Wheeler, 1982), but we did not observe this in *H. saltator*. However, we did observe a delay in pupation (2–5 days) associated with JHA treatment at some concentrations,

which may have allowed larvae to feed longer (Wilcoxon signed rank test: [10 μ g fourth instar, $N = 9$, $p = 0.018$; 5 μ g fourth instar, $N = 8$, $p = 0.091$; 1 μ g fourth instar, $N = 9$, $p = 0.018$; 0.1 μ g fourth instar, $N = 10$, $p = 0.12$; Fig. 3C).

4. Discussion

We examined the role of JH in queen determination in *H. saltator* and identified a JH-sensitive period during the larval stage where queen development can be induced. This sensitive period occurs during the last two larval instars (Fig. 4), which is relatively late compared to ants previously studied. *H. saltator* is from the morphologically primitive subfamily Ponerinae and retains a number of ancestral characteristics, so it is likely that this late switch represents an ancestral condition in ants. These results shed light on the evolution of caste development and raise further questions about how the switch controlling queen development has changed within the social Hymenoptera.

In order to investigate the role of JH in queen development in *H. saltator*, we first described the developmental stages of this species. We developed a method to quickly identify larval instars whereby larvae could be separated based on size and then compared under a microscope to distinguish among similar instars. Using this technique, we identified four larval instars in *H. saltator*, which is consistent with instar number in other ponerine species investigated (see references in Solis et al. (2010)).

The JH-sensitive period for caste determination occurred during the last two instars, where application of JHA induced queen development in third and fourth instar larvae but did not significantly influence development of early instars. Workers of *H. saltator* can successfully inhibit queen determination by biting JHA-treated larvae (Penick and Liebig, 2012), so it is possible that workers were able to inhibit queen development when early instars were treated. This was unlikely, however, because workers were rarely observed biting JHA-treated first and second instar larvae while biting was observed in almost all cases when later instars were treated with JHA. It is likely that even lower doses of JHA would have induced queen development in third and fourth instar larvae if biting were absent, but we could not dissociate biting from our treatments since workers were needed to care for developing larvae. A small portion of first and second instar larvae did develop as queens in our trials, but this may be due to the fact that JHA is more stable than natural JH-III and can persist inside the body for an extended period of time (Bigley and Vinson, 1979; Quistad et al., 1975). It is likely that residual JHA may have affected queen development of first and second instar larvae if it was still present in later instars. Overall, our results provide strong evidence that the switch between queen and worker development occurs in late instar larvae,

and even larvae near the end of larval development remained bipotential.

The response to JHA treatment was dose dependent, which suggests that there is a threshold level that JH must pass in order to trigger queen development. It is likely that nutritional factors earlier in larval life are translated into increased JH levels during this instar (Mutti et al., 2011). If larvae gain sufficient resources prior to this period then JH levels may surpass the threshold needed to induce queen development, and larvae will molt into queens during the pupal stage. The strongest effect of JHA treatment on queen determination in *H. saltator* occurred in the last instar, and it is during this instar that larvae experienced the greatest period of growth. In *Pheidole bicarinata*, high doses of JHA extended the larval period when larvae were induced to develop into soldiers and may have allowed them to feed longer (Wheeler and Nijhout, 1983). We found evidence that JHA delayed pupation by several days in *H. saltator*, which may have allowed larvae to gain the excess resources needed for developing into the larger queen morph.

In the honey bee, *A. mellifera*, a longer feeding period is not required for the increased larval growth of queens. In fact, queens develop slightly faster than workers in *A. mellifera* and grow nearly twice the size (Winston, 1991). Interestingly, we did not observe a difference in development times between workers and males in *H. saltator* despite finding evidence that males gain close to 25% more mass than workers. Similar to honey bee queens, it appears that males experience a faster growth rate than workers, and this means queens of *H. saltator* could exhibit an increased growth rate as well. Further investigation of development times under natural conditions in *H. saltator* could provide information into how larval metabolism, growth, and caste determination are related.

A significant implication of our results is that the timing of the JH-sensitive period for caste determination in ants shifted from late in the larval stage to early in development over evolutionary time. A late JH-sensitive period is common among bees (Copijn et al., 1977; Hartfelder et al., 2006; Röseler, 1976; Wirtz and Beetsma, 1972) but so far has not been clearly demonstrated in ants except in cases where larvae have already passed through an overwintering stage (Brian, 1974). With respect to size dimorphism between queens and workers, *H. saltator* may be more similar to bees than to other ant species. Queens of *H. saltator* are approximately 1.7 times the size of workers (Peeters et al., 2000), and queens of *A. mellifera* are 2 times the size of honey bee workers (Winston, 1991). In contrast, queens of other ant species can be 20 times the size of their average worker (Fjerdingstad and Crozier, 2006). In order to generate this large difference in size, it may be necessary for the period of caste determination to occur earlier in development in species with greater queen-worker dimorphism. Wheeler (1986) predicted that early caste determination may have also contributed to the evolution of additional worker castes, and a separate phylogenetic analysis found a positive correlation between levels of queen-worker dimorphism and the degree of worker polymorphisms among ants (Fjerdingstad and Crozier, 2006). Our results suggest that the timing of the JH-sensitive period for caste determination may be related to the degree of queen-worker dimorphism displayed by a species and to the potential for developing additional polymorphic worker castes.

Selection for larger queen size may have promoted the evolution of earlier queen determination. Larger queens generally have increased fecundity due to an increase in ovary size (Peeters, 1997). This translates into a higher egg-laying rate, which may also allow for the development of larger colony sizes. Another advantage of increased size is that larger queens may possess enough resources to found a colony without having to forage (Johnson, 2002; Peeters and Ito, 2001). Alternatively, early caste determination may have also evolved in order to reduce social conflict. Under some conditions a larva may benefit from developing into a queen

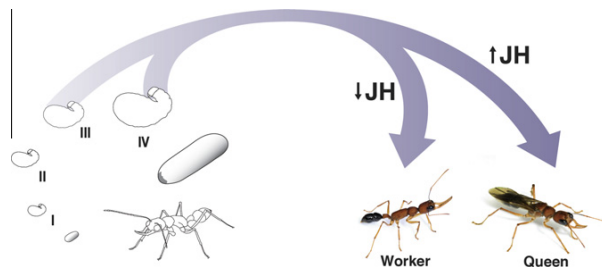


Fig. 4. Proposed mechanism of queen determination in *H. saltator*. Increased JH in third and fourth instar larvae leads to queen development (roman numerals indicate instars), while larvae with lower JH levels develop into workers. Queens are 1.7 times the size of workers by dry weight (Peeters et al., 2000) and possess functional wings until after mating flights (when the wings are shed).

rather than a worker even if excess queen production comes as a cost to the colony as a whole (Bourke and Ratnieks, 1999). Early caste determination may give workers more flexibility to control larval development and prevent selfish queen determination. Even in the absence of conflict it may benefit workers to prevent “mistakes” in development that could cause a larva to develop as a queen out of season (Penick and Liebig, 2012). Conflict may also occur between queens and workers over sex ratio (Mehdiabadi et al., 2003). If caste determination occurs inside the egg, then it may give the queen greater control over caste and allow her to bias sex ratios in her favor.

While it is clear that the switch for caste determination has shifted earlier in development, it is still not clear how this shift occurred during evolution. The most simple explanation is that the switch gradually shifted over time, and changes in the timing of JH release or tissue sensitivity could have facilitated this. Alternatively, some species are known to develop multiple critical points to control polyphenic development, such as the dung beetle *Onthophagus taurus*. In *O. taurus*, application of JH during the last larval instar can suppress horn development in male beetles but an increase of JH during the prepupal period induces horn development (Emlen and Nijhout, 2001; Hartfelder and Emlen, 2005). Ant species are already known to possess multiple JH-sensitive periods for caste determination, such as *Pheidole*, where early sensitive periods control queen determination and later periods control soldier development. Recently, two distinct critical periods were found within the same instar in *P. rhea*, and application of JH to soldier pupae induced the development of super-soldiers (Rajakumar et al., 2012). It is possible that multiple switch points originally evolved to regulate queen development in ants, but that late JH-sensitive periods were either lost or switched function to regulate worker caste polyphenisms.

Most studies on caste determination, including this study, have used JH treatment experiments to identify critical periods for caste determination, but few studies have measured actual hormone levels. Measurement of hormone levels throughout development may reveal additional sensitive periods for caste determination as well as possible interactions between JH and other hormones, such as ecdysteroids. While our results provide new information about the ancestral control of caste determination in ants, this work should be expanded by investigating additional species and using new methods to gather information about how this switch evolved.

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