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A larval 'princess pheromone' identifies future ant queens based on their juvenile hormone content

Clint A. Penick [a, b, *], Jürgen Liebig [c]

[a] North Carolina Museum of Natural History, Raleigh, NC, U.S.A.
[b] Department of Applied Ecology and Keck Center for Behavioral Biology, North Carolina State University, Raleigh, NC, U.S.A.
[c] School of Life Sciences, Arizona State University, Tempe, AZ, U.S.A.

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Numerous studies have identified cuticular compounds that distinguish adult queens from workers in social insect colonies, but how future queens are identified at the larval stage is poorly understood. Nevertheless, the ability of workers to discriminate queen and worker larvae is necessary for them to regulate caste determination and queen production. In the ant *Harpegnathos saltator*, workers bite larvae to inhibit queen development, and we used biting as an assay to test how workers identify queens at the larval stage. The transfer of cuticular compounds from queen to worker larvae through direct physical contact (rubbing) or using a hexane extract both elicited biting. Gas chromatography revealed significant differences in cuticular hydrocarbon profiles of queen and worker larvae that could be induced by treatment with a juvenile hormone (JH) analogue. Finally, treatment of male larvae with a JH analogue also elicited worker biting, which suggests a direct connection between JH levels and the production of a larval queen signal. These results demonstrate that workers identify larval caste using a chemical signal present on the cuticle, a 'princess pheromone', that reflects endocrine changes associated with queen development. Based on the connection between JH levels and the production of a larval queen signal, we developed a model for caste determination in *H. saltator* that incorporates endocrine, pheromonal and behavioural control of caste development.

The transition from solitary to social living in insects required new forms of communication (Blum, Kerr, & Fales, 1970). In social insects, communication is primarily chemical. Members of a colony identify each other based on a blend of chemical compounds present in a wax layer on their cuticle (van Zweden & d'Ettorre, 2010), and these waxes can also help colony members differentiate reproductive castes from sterile workers (Liebig, 2010). Despite a vast literature on chemical communication among adults in social insect colonies (Keller & Nonacs, 1993; Kocher & Grozinger, 2011; Le Conte & Hefetz, 2008; Peeters & Liebig, 2009; Vander Meer, Breed, Winston, & Espelie, 1998), little is known about communication between adults and their brood (larvae and pupae). Nevertheless, proper identification of brood based on development stage, sex and caste is clearly important for adult workers rearing the next generation of a colony's offspring.

Interactions between adults and brood determine a reproductive division of labour in insect colonies through the production of distinct queen and worker castes (Wilson, 1971). Queens are specialized reproductives that leave the nest to mate and establish new colonies, while workers forgo reproduction and remain in the nest to help raise their parents' offspring. A colony's fitness depends on the production of new queens, but the overproduction of queens or the production of queens during the wrong season could drain colony resources and negatively affect colony growth (Oster & Wilson, 1978). To prevent overproduction of queens, adult workers must inhibit larval queen development through control of larval diet (Masuko, 1986) or using other behaviours, such as larval biting, that inhibit queen determination (Brian, 1973; Penick & Liebig, 2012; Suryanarayanan, Hermanson, & Jeanne, 2011; Villalta, Amor, Cerdá, & Boulay, 2016). For behavioural regulation of caste to be successful, however, adult workers must have a reliable mechanism to identify larvae developing as queens.

Workers can distinguish adult reproductive castes based on differences in cuticular compounds (Liebig, 2010), but research on brood-specific pheromones has proven difficult, especially outside of honeybees. In honeybees, a blend of 10 fatty acid esters distinguishes brood stages from adults (Le Conte, Arnold, Trouiller, Masson, & Chappe, 1990; Le Conte et al., 1989; Slessor, Winston,

* Correspondence: C.A. Penick, Department of Applied Ecology, North Carolina State University, Raleigh, NC 27695-7617, U.S.A.
E-mail address: capenick@ncsu.edu (C. A. Penick).

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& Le Conte, 2005; Traynor, Le Conte, & Page, 2015), and a difference in the relative proportion of these 10 compounds provides further information about the age and caste of larvae (Le Conte, Sreng, & Poitout, 1995; Le Conte et al., 1994; Le Conte et al., 2006). For other social insects, evidence for brood pheromones is scant. Male and female larvae of the wasp *Polistes dominulus* display differences in their cuticular compounds, but there is no evidence that adult workers actually use this information (Cotoneschi et al., 2009). Similarly, workers of the ant *Camponotus floridanus* seem unable to distinguish between male and female larvae (Nonacs & Carlin, 1990), and queen larvae of the ant *Aphaenogaster senilis* do not differ from worker larvae based on their cuticular hydrocarbon profiles (Villalta et al., 2016). Conflicting information about brood pheromones in ants has caused some authors to question whether ant brood pheromones even exist (Morel & Vander Meer, 1988). Nevertheless, behavioural evidence suggests that workers do use chemical signals to distinguish brood from adults (Brian, 1975; Tschinkel, 2006; Walsh & Tschinkel, 1974), to identify larval sex (Passera & Aron, 1996) and to identify larval caste (Brian, 1973; Penick & Liebig, 2012; Villalta et al., 2016).

Here we investigated how workers of the ant *Harpegnathos saltator* discriminate between queen and worker larvae to regulate caste determination. Workers of *H. saltator* regulate caste determination by biting larvae that begin to develop as queens outside the normal queen-rearing season (Penick & Liebig, 2012). Larvae can be induced to develop as queens by treatment with a juvenile hormone (JH) analogue, and workers begin to bite larvae approximately 12 h after JH treatment to inhibit queen determination (Penick, Prager, & Liebig, 2012). Using larval biting as an indication that workers perceive larvae as queen-destined, we tested whether cuticular compounds of natural queen larvae elicited biting when applied to worker larvae. We then compared cuticular profiles of queen and worker larvae to determine whether chemical differences in cuticular compounds could provide caste information. Finally, we tested whether chemical compounds used for caste identification were related to JH levels by measuring cuticular profiles of female larvae treated with a JH analogue and testing whether workers would also bite male larvae treated with a JH analogue. Because JH does not trigger caste changes in male larvae, biting of male larvae would indicate that queen-specific compounds are directly related to JH rather than downstream changes associated with queen development. Based on the results of these tests, we create a model that links endocrine, pheromonal and behavioural components of caste regulation.

METHODS

Study Species and Laboratory Conditions

Colonies of *H. saltator* were collected from the Western Ghats in southern India between 1994 and 1999 (described in Peeters, Liebig, & Hölldobler, 2000) and have been continuously bred in the laboratory since that time. Our stock colonies were each housed in plastic nestboxes with a plaster floor and a glass-covered nest chamber. Colonies were kept on a 12:12 h light:dark cycle, fed live crickets (*Acheta domesticus*) twice per week, and the plaster inside each nest was moistened regularly to maintain humidity. *Harpegnathos saltator* is unusual among ants in that adult workers maintain the ability to mate and reproduce (Peeters & Hölldobler, 1995). After a colony's founding queen is lost, workers compete in an elaborate dominance tournament to establish a reproductive hierarchy (Penick, Brent, Dolezal, & Liebig, 2014; Sasaki et al., 2016). Once established, worker-led colonies function similarly to queen-led colonies, and both colony types were used in this study.

In the wild, colonies of *H. saltator* produce new dispersing queens and males during late spring and early summer, and they produce workers throughout the rest of the year (Peeters et al., 2000). Pre-monsoon rains trigger mating flights, and new queens found colonies independently after mating outside the nest. Queens are larger than workers and possess two pairs of wings that they use during their mating flight. Laboratory colonies of *H. saltator* rarely produce new queens but do so intermittently. Therefore, we checked stock colonies regularly over 2 years to acquire queen larvae for our experiments. We identified colonies producing queens by searching for larvae that were unusually large, which is characteristic of queen larvae in this species (Penick et al., 2012). Once identified, these larvae were transferred to a colony that was not rearing queens to observe whether they were bitten by workers, a clear indication that they were queen-destined (Penick & Liebig, 2012). Larval-directed biting was used in subsequent experiments as an indicator that workers perceived a larva as queen-destined. Larvae used in all experiments were fourth instar, which is the primary instar where caste determination occurs in *H. saltator* (Penick et al., 2012).

In concern for animal welfare, we monitored larval biting trials closely and removed larvae if we believed they were in danger of physical damage or death from adult workers. We ended trials prematurely in several cases, in which observation of biting was sufficiently clear without exposing larvae to additional stress. Furthermore, we opted for nonlethal methods to extract cuticular compounds (e.g. solid-phase microextraction, SPME) whenever possible.

Behavioural Response to Larval Cuticular Compounds

We used two methods to test whether workers distinguished queen and worker larvae based on differences in their cuticular compounds. First, we transferred cuticular compounds from queen larvae to worker larvae through direct physical contact. Either the anterior (head and neck) or posterior region of a queen larva was rubbed against a worker larva for 2 min to transfer compounds. The anterior and posterior regions of each larva were tested separately to determine whether the putative queen signal was localized to a specific body region, which could indicate a glandular source, or whether the signal was generally distributed over the body. As a control, a foreign worker larva was rubbed against a test larva using identical methods. We then reintroduced test larvae to their original colonies and quantified larval-directed biting over 5 min. We quantified larval biting by counting biting bouts, which were defined as observations of uninterrupted biting between one worker and one larva. If three workers were observed biting the same larva, this would be counted as three biting bouts; if a worker that was previously observed biting a larva stopped biting, walked more than 1 cm away and later resumed biting, this would be counted as a second biting bout (uninterrupted biting by the same worker was counted as a single biting bout). We tested the response of 10 colonies to larvae rubbed against a queen larva and the response of nine colonies to larvae rubbed against a foreign worker larva. The 10 queen larvae used in this experiment came from three independent colonies, and the nine foreign worker larvae came from nine independent colonies. All observations of biting were conducted blind to treatment for this and subsequent experiments.

For the second method to test how workers identified queen larvae, we transferred cuticular compounds from queen larvae to worker larvae using a chemical extract. Preliminary trials suggested that extracts using a polar solvent (methanol) did not elicit biting, so we used a solvent that primarily extracts nonpolar compounds (hexane) for subsequent trials. Queen-destined and worker larvae were soaked in hexane (Sigma–Aldrich, St Louis, MO, U.S.A.) for

15 min to extract their cuticular compounds. Hexane extracts were then concentrated to 1–2 μl by drying with nitrogen gas. The total extract was then applied to a worker larva from a colony not producing queens and allowed to evaporate for 2 min before larvae were returned to their nests. The extract was applied topically and spread over the body surface as evenly as possible using a syringe tip. Each trial was videorecorded, and we quantified larval biting in videos during the first 5 min after larvae were reintroduced by counting the number of biting bouts (as described above). We tested the response of seven colonies to larvae treated with extracts of a queen larva and the response of seven colonies to larvae treated with extracts of a foreign worker larva. Queen larvae used in this experiment came from seven independent colonies, and foreign worker larvae came from seven independent colonies.

Identification of Larval Cuticular Compounds

We compared cuticular compounds between queen and worker larvae using gas chromatography–mass spectrometry (GC–MS). First, we sampled cuticular compounds of naturally produced queen larvae from six colonies. Each larva was brushed for 2 min with a solid-phase microextraction (SPME) fibre coated with a 30 μm polydimethylsiloxane film (Supelco Inc., PA, U.S.A.). Next, we sampled cuticular compounds from two worker-destined larvae from each of 10 colonies. One larva from each colony was treated topically with 5 μg of the JH analogue methoprene (Chem Service, West Chester, PA, U.S.A.) dissolved in 1 μl of acetone to induce queen development (Penick et al., 2012), while the other larva was treated with an equivalent volume of acetone as a control. Larvae were then placed into small nests with six workers from their parent colony and observed 12 h after treatment to confirm that larvae treated with the JH analogue received biting and that control larvae did not. We sampled cuticular compounds from larvae treated with JH analogue and from control larvae 24 h after treatment using SPME. Larvae were rubbed with the SPME fibre 50 times while stabilized with clean metal forceps.

To identify cuticular compounds after extraction, we inserted the SPME fibre into the injection port of an Agilent 6890N gas-chromatograph (GC) (Agilent, Santa Clara, CA, U.S.A.) coupled with an Agilent 5975 mass selective detector operated in the electron impact ionization mode. The GC was run in the splitless injection mode with helium as the carrier gas at 1 ml/min flow rate. It was fitted with a 30 m × 0.25 mm × 0.25 μm DB-1MS nonpolar column (Agilent). The oven temperature was programmed to rise from 60 °C to 200 °C at a rate of 40 °C/min after an initial delay of 2 min, including a splitless time of 2 min. Subsequently, the temperature rose from 200 °C to 320 °C at 5 °C/min. Injector temperature was 260 °C, MS quad 150 °C, MS source 230 °C, and transfer line 300 °C. The relative abundance of each compound was taken as a percentage of peak area. We limited our analyses to the most abundant compounds (31 total) in profiles of larvae treated with the JH analogue and in profiles of control larvae that accounted for 80% of total peak area. We subsequently focused on four n-alkanes (C23, C25, C27, C29) that showed the most consistent differences between treatment groups (see Supplementary Fig. S1).

Worker Response to Male Larvae Treated with JH Analogue

Because queen development in *H. saltator* can be induced by treating female worker larvae with a JH analogue (Penick et al., 2012), we hypothesized that JH may be directly linked to the production of a queen-specific larval signal. To test whether JH plays a direct role in the production of a larval queen signal, we treated male larvae with a JH analogue to determine whether they would be bitten by adult workers similar to female larvae treated with a JH analogue. Male and female larvae were identified from 20 colonies based on sex-specific differences in genital disc morphology (Penick, Ebie, & Moore, 2014). We then set up 10 nests (each contained six workers and three larvae) for each treatment group: (1) acetone-treated male larvae (control); (2) JH analogue-treated male larvae; (3) JH analogue-treated female larvae. JH analogue-treated larvae were treated topically with 5 μg of methoprene dissolved in 1 μl of acetone, and control-treated larvae received an equivalent volume of acetone without methoprene. Biting was quantified by counting the number of biting bouts (as described above) 24 h after treatment during three observation sessions that were separated by at least 1 h. Each observation session lasted 5 min. Observations were conducted blind so that observers were unaware of the larval treatment for each group. As above, observation of biting was used to indicate that workers perceived larvae as queen-destined.

Statistical Analyses

We used nonparametric tests to analyse results from all experiments because of a general lack of normality and relatively small sample sizes. We used the Mann–Whitney *U* test without a Bonferroni correction for pairwise comparisons if a Kruskal–Wallis test indicated there were significant differences between groups. These analyses were performed using GraphPad Prism (v6.0h, Graph Pad Software, Inc., San Diego, CA, U.S.A.) statistical software. Additionally, we performed a cluster analysis on cuticular hydrocarbon data using Statistica 7.1 (Stat Soft Inc., Tulsa, OK, U.S.A.). We transformed cuticular hydrocarbon data following Aitchison (1986) using the following formula:

$$Z_{ij} = \ln\left[\frac{Y_{ij}}{g(Y_j)}\right]$$

Here, Z_{ij} is the standardized peak area i for individual j, Y_{ij} is the peak area i for individual j, and $g(Y_j)$ is the geometric mean of all peaks for individual j. The transformed data were subject to cluster analyses using unweighted pair group averages with Euclidean distances, and zero values were replaced with 10^{-6}.

RESULTS

Worker Response to Queen Larval Compounds

Workers of *H. saltator* bit worker larvae that were treated with cuticular compounds from queen larvae, which is an indication that workers perceived these larvae as queen-destined. This occurred when worker larvae were rubbed against either the anterior or posterior region of a queen larva to transfer cuticular compounds through direct physical contact, but biting did not occur when worker larvae were rubbed against another foreign worker larva (Kruskal–Wallis test: $H_2 = 18.24$, $N_{\text{worker}} = 9$, $N_{\text{anterior}} = 10$, $N_{\text{posterior}} = 10$, $P = 0.0001$; Mann–Whitney *U* test: worker versus anterior: $U = 0.50$, $P < 0.0001$; worker versus posterior: $U = 0.50$, $P < 0.0001$; anterior versus posterior: $U = 43.5$ $P = 0.64$; Fig. 1a). Additionally, workers bit test larvae that were treated with hexane extracts of queen-destined larvae but not when test larvae were treated with extracts from other worker larvae (Mann–Whitney *U* test: $U = 0$, $N_{\text{queen}} = 7$, $N_{\text{worker}} = 7$, $P = 0.0006$; Fig. 1b).

Differences in Queen and Worker Larval Compounds

Natural queen larvae exhibited a shift towards shorter-chained hydrocarbons compared to worker-destined larvae (Fig. 2). When worker larvae were treated with a JH analogue to induce queen development, a similar shift in profile was detectable within 24 h

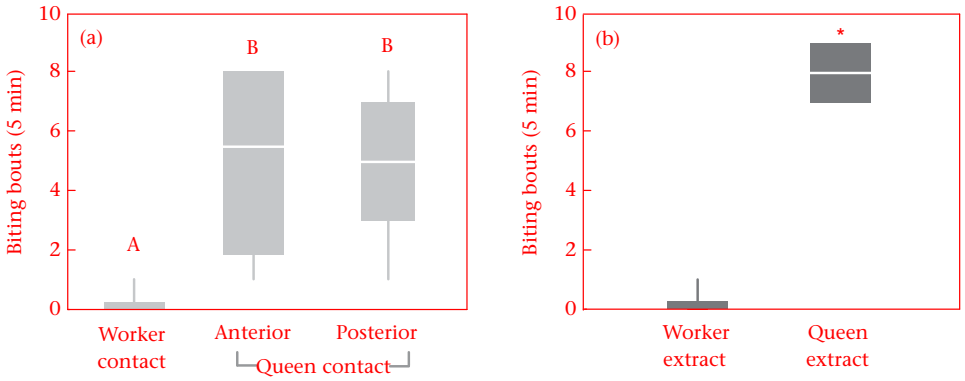

Figure 1. The frequency of biting bouts (over 5 min) received by worker larvae treated with queen larval compounds (median, 25–75%, range). (a) Biting received by worker larvae that were rubbed against a foreign worker larva ($N = 9$) or a queen larva (anterior and posterior portion of queen larvae tested separately, $N = 10$). (b) Biting received by worker larvae that were treated with a hexane extract from a foreign worker larva ($N = 7$) or a queen larva ($N = 7$). Letters and asterisks indicate significant differences ($P < 0.001$).

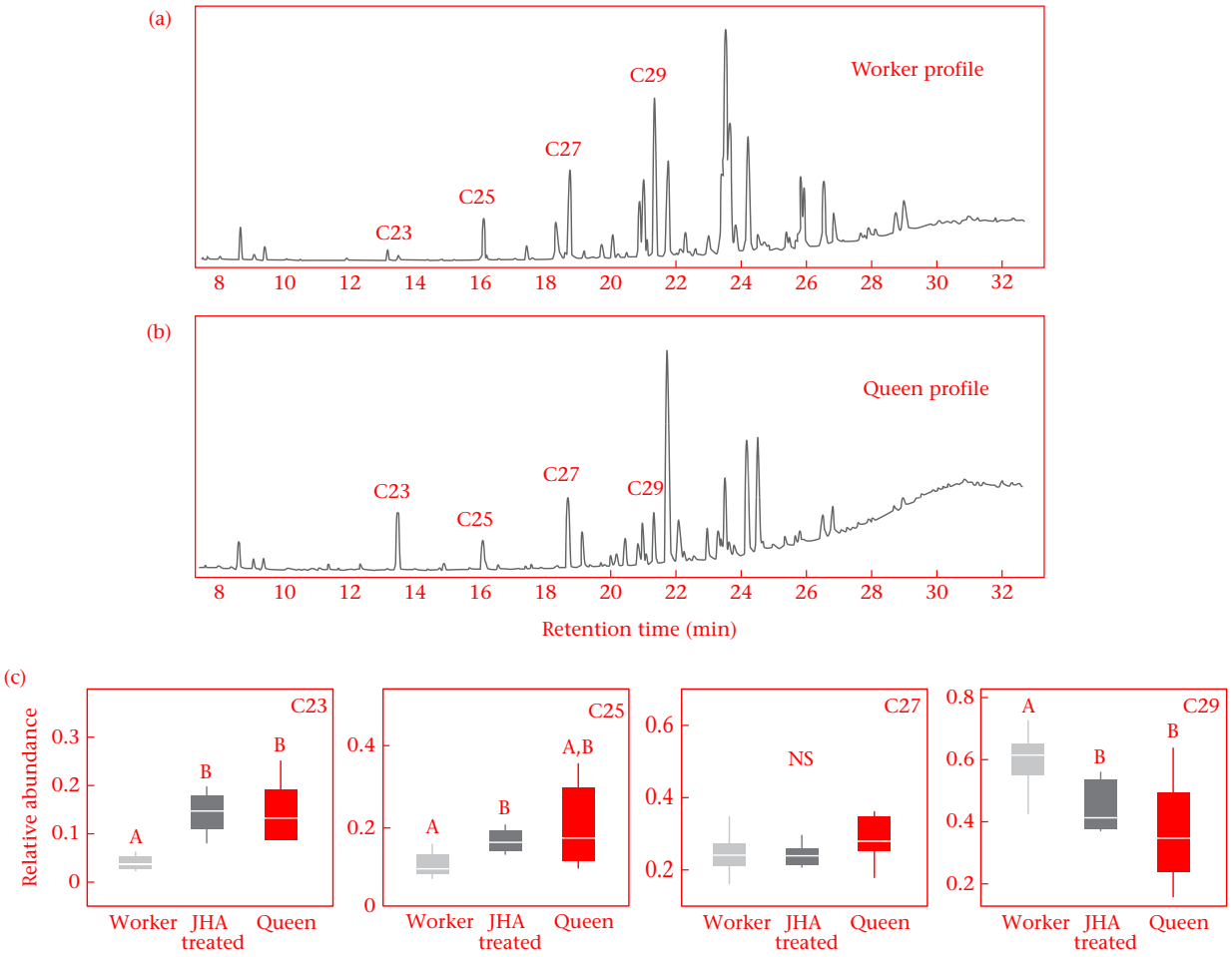

Figure 2. Comparison of queen and worker larval compounds. Gas chromatograph showing the relative abundance of cuticular hydrocarbon compounds on (a) a worker larva and (b) a queen larva. (c) Comparison of relative proportions of n-alkanes on the cuticle of worker larvae ($N = 10$), JH analogue-treated larvae ($N = 10$), and natural queen larvae ($N = 6$). Box plots show median, 25–75% and range. Letters indicate significant differences between groups ($P < 0.05$).

(Fig. 2c). For comparisons between worker larvae and larvae treated with a JH analogue, we focused specifically on n-alkanes C23, C25, C27 and C29 because these showed the most consistent difference between groups (Supplementary Fig. S1). Larvae treated with a JH analogue and natural queen larvae had a higher proportion of the short-chained n-alkane tricosane (C23: Kruskal–Wallis test: $H_2 = 17.86$, $N_{worker} = 10$, $N_{JH} = 10$, $N_{queen} = 6$, $P = 0.0001$; Mann–Whitney U test: worker versus JH analogue-treated: $U = 0$,

$P < 0.0001$; worker versus natural queen: $U = 0$, $P = 0.0002$; JH analogue-treated versus natural queen: $U = 26$, $P = 0.69$) and a lower proportion of the long-chained n-alkane nonacosane (C29: Kruskal–Wallis test: $H_2 = 12.34$, $N_{worker} = 10$, $N_{JH} = 10$, $N_{queen} = 6$, $P = 0.0021$; Mann–Whitney U test: worker versus JH analogue-treated: $U = 7$, $P = 0.0005$; worker versus natural queen: $U = 7$, $P = 0.011$; JH analogue-treated versus natural queen: $U = 21$, $P = 0.37$). Larvae treated with a JH analogue also had a relative increase in pentacosane, although the increase was not significantly different from worker larvae or natural queen larvae (C25: Kruskal–Wallis test: $H_2 = 10.76$, $N_{worker} = 10$, $N_{JH} = 10$, $N_{queen} = 6$, $P = 0.0046$; Mann–Whitney U test: worker versus JH analogue-treated: $U = 6$, $P = 0.0003$; worker versus natural queen: $U = 12$, $P < 0.056$; JH analogue-treated versus natural queen: $U = 29$, $P = 0.96$). Heptacosane did not differ between groups (C27: Kruskal–Wallis test: $H_2 = 4.04$, $N_{worker} = 10$, $N_{JH} = 10$, $N_{queen} = 6$, $P = 0.13$).

Worker Response to Male Larvae Treated with JH Analogue

Workers of *H. saltator* bite female larvae treated with JH analogue to inhibit queen development at times when colonies are not producing new queens (Penick & Liebig, 2012), and we found that workers also bite male larvae treated with a JH analogue (Fig. 3). Male larvae treated with a JH analogue received similar levels of biting as female larvae treated with a JH analogue, while control-treated male larvae received little to no biting (Kruskal–Wallis test: $H_2 = 20.08$, $N = 11$, $P < 0.0001$; multiple comparisons using Mann–Whitney U test: male control versus male JH analogue: $U = 0.00$, $P < 0.0001$; male control versus female JH analogue: $U = 7.50$, $P = 0.0001$; male JH analogue versus female JH analogue: $U = 47.50$, $P = 0.41$). Male larvae are incapable of developing into queens, but the biting they received from adult workers indicates that they smelled like queens after treatment with a JH analogue.

DISCUSSION

The ability of social insects to distinguish reproductive from nonreproductive individuals in their colonies is necessary to maintain a reproductive division of labour. Numerous studies have identified cuticular compounds that distinguish adult queens from their workers (e.g. Holman, Jørgensen, Nielsen, & d'Ettorre, 2010; Liebig, Eliyahu, & Brent, 2009; Slessor et al., 2005; Smith, Hölldobler, & Liebig, 2009; Van Oystaeyen et al., 2014), and here we show that similar compounds also distinguish queens from workers at the larval stage. Transferring queen compounds to worker larvae through direct physical contact (rubbing) or using a hexane extract elicited biting from adult workers, a clear indication that workers perceived larvae as queen-destined. Queen larvae also differed from worker larvae based on their cuticular hydrocarbon profile, and similar changes could be induced by application of a juvenile hormone (JH) analogue. Taken together, these results show that workers identify larval caste based on a chemical signal, a 'princess pheromone', that reflects endocrine changes associated with queen development.

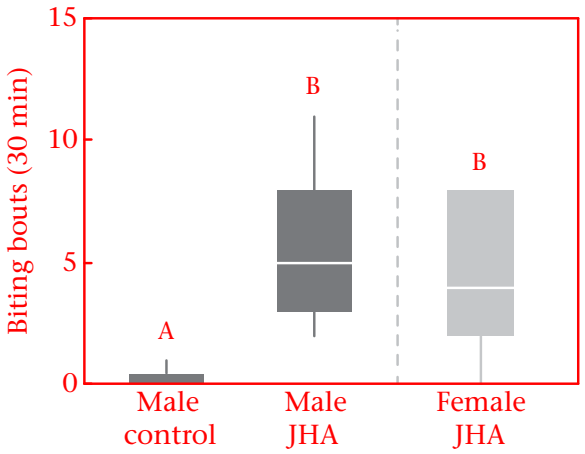

Figure 3. The frequency of biting bouts (over 30 min) received by male larvae treated with a JH analogue (or acetone as a control) and female larvae treated with a JH analogue ($N = 11$ for each group). Box plots show median, 25–75% and range. Letters indicate significant differences between groups ($P < 0.001$).

Because workers responded similarly to larvae that were rubbed against either the anterior or posterior region of a queen larva, the princess pheromone is most likely distributed generally over the body and not localized to a specific gland or body region. Insects are coated with a wax layer composed of cuticular hydrocarbons that provide desiccation resistance (Gibbs, 1998), and the complex blend of compounds in this wax layer has been shown to carry information related to fertility, caste, nestmate recognition and species recognition among adult social insects (Howard & Blomquist, 2004; Singer, 1998). While few studies have investigated the communicative function of cuticular hydrocarbons in larvae, studies on social parasites have found evidence that nest parasites mimic larval hydrocarbon profiles of their hosts to avoid detection (Blomquist & Bagnères, 2010; Dettner & Liepert, 1994; Howard & Blomquist, 2004). In *H. saltator*, cuticular hydrocarbon profiles of queen and worker larvae were qualitatively similar but queen profiles exhibited a shift towards shorter-chained compounds. Similar shifts in hydrocarbon profiles are associated with reproductive status in adult social insects (Liebig, 2010), including *H. saltator* (Liebig, Peeters, Oldham, Markstadter, & Hölldobler, 2000).

In adult insects, ovarian activity is under the influence of JH (Hartfelder, 2000; Penick, Liebig, & Brent, 2011), and JH levels, in turn, influence the production of adult fertility compounds (Brent, Penick, Trobaugh, Moore, & Liebig, 2016; Cuvillier-Hot, Gadagkar, Peeters, & Cobb, 2002; Kelstrup, Hartfelder, Nascimento, & Riddiford, 2014). Similarly, treatment of *H. saltator* larvae with a JH analogue caused a shift in profile that resembled that of natural queens, and this shift occurred within 24 h of treatment. The rapid change in hydrocarbon profile observed in *H. saltator* is consistent with findings for hydrocarbon turnover in other insects (Böröczky, Wada-Katsumata, Batchelor, Zhukovskaya, & Schal, 2013; Krupp et al., 2008) and explains how workers are able to discriminate larvae treated with a JH analogue from worker larvae within 12 h of treatment (Penick & Liebig, 2012). Moreover, application of a JH analogue to male larvae was able to stimulate the same worker response despite the fact males are incapable of developing into queens. A similar case occurs in the house fly, *Musca domestica*, where treatment of male flies with the insect hormone ecdysone induces them to produce the female sex pheromone (Adams, Dillwith, & Blomquist, 1984). In both instances, pheromone production seems to be tied directly to hormone levels rather than downstream factors associated with female reproduction or development.

Despite finding differences in cuticular hydrocarbon profiles between queen and worker larvae, the exact chemical compounds that workers use to identify queen larvae is not known. Queen larvae exhibited a relative increase in tricosane and pentacosane, but treatment of worker larvae with synthetic versions of these compounds did not elicit biting when they were applied

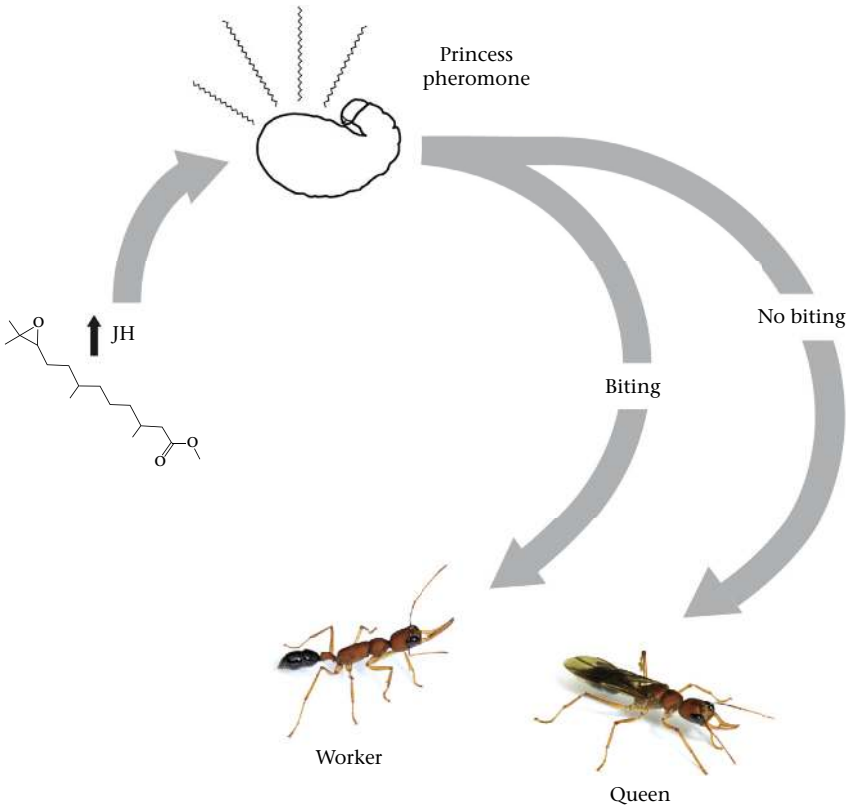

Figure 4. Model for caste determination in *H. saltator*. Increased juvenile hormone (JH) levels induce larvae to develop as queens and stimulate the production of queen-specific cuticular compounds. Depending on a colony's breeding status, workers will then either bite queen larvae to inhibit queen development or allow larvae to develop as queens.

individually or in combination (Penick, 2012). These compounds may be components of the princess pheromone, but they appear to be insufficient to provoke the full worker response on their own. Unlike many insect sex pheromones, hydrocarbon-based signals may be context specific and only elicited with a correct hydrocarbon background (Smith, Millar, & Suarez, 2015). While simple n-alkanes can serve as signalling compounds on their own (Smith et al., 2009) or in combination with alkenes or methyl-branched alkanes (Liebig, 2010), other classes of compounds may also serve as caste signals, including oxygenated compounds (Yew et al., 2009) or cuticular proteins (Hanus, Vrkoslav, Hrdý, Cvačka, & Šobotník, 2010). Our experiments using hexane extracts of queen larvae suggest that the queen signal in *H. saltator* is most likely a nonpolar compound, but we cannot rule out the possibility that polar compounds play a role. Additional work on the chemistry of larval compounds will be necessary to identify the complete set of compounds specifically involved in caste identification.

Based on the connection between JH and the production of a princess pheromone, we developed a model for how caste is regulated in *H. saltator* (Fig. 4). Queen development in *H. saltator* is triggered by a rise in JH, which causes a change in the cuticular profile that identifies larvae as queen-destined. Workers then respond by either allowing larvae to develop as queens or by biting larvae to suppress queen development. This decision is likely mediated by environmental or social cues that inform workers whether queens are developing out of season or if too many queens have already been produced. What is missing from this model is the initial stimulus that leads larvae to develop as queens in the first place. The initial stimulus for queen development could be related to nutrition, maternal effects, queen or worker pheromones and/or environmental cues, but more research is required to understand how these external factors could influence JH production to induce queen development.

Because *H. saltator* retains a number of ancestral traits, it provides a useful model to understand how caste-determining systems may have evolved in ants more generally. JH has a conserved role in caste determination in ants (Wheeler, 1986), but the timing of the JH-sensitive critical period for queen determination differs among species. Larvae of *H. saltator* remain bipotential until near the end of larval period (Penick et al., 2012), but the JH-sensitive period has shifted earlier in development in species that have more advanced traits (e.g. Passera & Suzzoni, 1979). Similarly, the mechanisms that workers use to regulate caste determination may also differ. Early ant lineages, including *H. saltator*, lack the ability to control larval nutrition through mouth-to-mouth food exchange (Peeters, 1997), so species like *H. saltator* must use alternative means to inhibit queen development, such as larval biting (Penick & Liebig, 2012). For species outside these lineages, control over larval nutrition is probably the primary means to regulate caste (Trible & Kronauer, 2017; Wheeler, 1986). Whether a species uses nutritional control to regulate caste or other behaviours, workers still need a reliable mechanism to discriminate between queen and worker larvae. Therefore, it is likely that princess pheromones are common in ants despite being difficult to identify.

Author Contributions

C.A.P. and J.L. both participated in the experimental design and writing the final manuscript. C.A.P. conducted laboratory experiments and data analyses.

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Supplementary Material

Supplementary Material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.anbehav.2017.03.029.

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