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

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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,

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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