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Workers Make the Queens in *Melipona* Bees: Identification of Geraniol as a Caste Determining Compound from Labial Glands of Nurse Bees

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Abstract Reproductive division of labor in advanced eusocial honey bees and stingless bees is based on the ability of totipotent female larvae to develop into either workers or queens. In nearly all species, caste is determined by larval nutrition. However, the mechanism that triggers queen development in *Melipona* bees is still unresolved. Several hypotheses have been proposed, ranging from the proximate (a genetic determination of caste development) to the ultimate (a model in which larvae have complete control over their own caste fate). Here, we showed that the addition of geraniol, the main compound in labial gland secretions of nurse workers, to the larval food significantly increases the number of larvae that develop into queens. Interestingly, the proportion of queens in treated brood exactly matched the value (25%) predicted by the two-locus, two-allele model of genetic queen determination, in which only females that are heterozygous at both loci are capable of developing into queens. We conclude that labial gland secretions, added to the food of some cells by nurse bees, trigger queen development, provided that the larvae are genetically predisposed towards this developmental pathway. In *Melipona beecheii,* geraniol acts as a primer pheromone representing the first caste determination substance identified to date.

Key Words Social insects · Stingless bees · *Melipona* · Caste determination · Larval provision · Labial gland secretions · Primer pheromone · Geraniol · Apidae · Hymenoptera

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Introduction

Female larvae of the eusocial honey bees (Hymenoptera: Apidae: Apini) and stingless bees (Hymenoptera: Apidae: Meliponini) may develop into either an adult queen or a worker. In the vast majority of species, queens are reared in special cells, which are larger than worker cells, and receive more food, which is sometimes of special quality (de Wilde and Beetsma 1982; Wheeler 1986; Hartfelder et al. 2006). Thus, the caste fate of the larvae in these species is determined trophically, which means that an individual's nutritional history triggers endocrine signals, which are caste-specific modulations of juvenile hormone and ecdysteroid titers (Hartfelder and Emlen 2005; Hartfelder et al. 2006) that mediate the subsequent patterns of developmental differentiation (Wheeler 1986). However, queen production in bees of the genus *Melipona* is unique among social bees because brood cells that produce queens and workers are indistinguishable, and all larvae feed on similar amounts of food (Sakagami 1982). Furthermore, *Melipona* queens are reared in significant numbers all year round (Kerr 1948; Darchen and Delage-Darchen 1975; Moo-Valle et al. 2001; Sommeijer et al. 2003; Wenseleers et al. 2004; Morais et al.

2006). To explain the observation that up to 25% of female larvae in *Melipona* nests develop into queens, Kerr proposed a mechanistic, proximate model of genetic caste determination, based on two unlinked loci, each with two alleles, in which only double heterozygous females could develop into queens (Kerr 1948, 1950a, b) (the original assumption of 3 loci for some species was later dismissed; Kerr 1969). Since male bees are haploid, their gametes must have an equal set of alleles at each locus. Therefore, the female progeny of a single mated queen, which are produced from fertilized diploid eggs, separate into 25% queens that are heterozygous at both loci, and 75% workers that are homozygous at one or both loci (Kerr 1948, 1950a, b). However, usually the proportion of queens in *Melipona* broods is lower than 25% (Kerr 1948; Darchen and Delage-Darchen 1975; Moo-Valle et al. 2001; Sommeijer et al. 2003; Wenseleers et al. 2004; Morais et al. 2006). Thus, it was proposed that female larvae that are genetically queens also can develop into workers under sub-optimal food conditions (Kerr et al. 1966; Kerr 1969; Maciel-Silva and Kerr 1991; Velthuis and Sommeijer 1991). A different set of studies, based on theoretical considerations on caste conflict and colony kin structure, viewed the large numbers of queens in *Melipona* as support for the model of self determination of caste fate in social insects (Bourke and Ratnieks 1999; Ratnieks 2001; Wenseleers et al. 2003, 2004; Wenseleers and Ratnieks 2004). According to this evolutionary or ultimate model, a female larva that develops in a normal sized, mass provisioned brood cell has the ability to control her own caste fate (Bourke and Ratnieks 1999; Ratnieks and Helanterä 2009). Thus, when resources are equally available, she could become a queen instead of a worker, thus becoming the reproductive dominant rather than an altruistic worker rearing sisters. One of the assumptions of this model is that all larvae not only feed on the same amount of food, but also on food of the same quality. However, this assumption overlooks existing experimental evidence, which demonstrates that caste fate can be influenced by quantitative, and possibly qualitative, differences in nutrition (Kerr et al. 1966; Kerr 1969; Darchen and Delage-Darchen 1975; Maciel-Silva and Kerr 1991). Nevertheless, specific nutrients that influence the developmental pathway of a female *Melipona* larva have not been described.

In recent chemical analyses involving the species *Melipona beecheii*, we found striking similarities between the pattern of volatiles from labial gland secretions of nurse workers and that of the rectal waste of emerging queens (Jarau, unpublished data). This suggests, that nurse workers may specifically add labial gland secretions to the food provision of certain brood cells, thus inducing the development of queens. In the present study, we tested this hypothesis through structure elucidation of volatiles of labial gland secretions, as well as bioassays, in which synthetic samples of the identified compound were added to the food provisions of developing larvae.

Methods and Material

Study Site and Bee Colonies We used two colonies of *Melipona beecheii* BENNETT, 1831 (Hymenoptera: Apidae: Meliponini) that were kept in a dark room at the Center for Tropical Bee Research (CINAT) of the National University of Costa Rica in Heredia, Costa Rica (9°58′22″N, 84°07′45″W). The nests were connected to the outside via plastic tubes leading through the wall of the building. Colony 1 was used for the collection of bees for chemical analyses (March and April 2007), whereas colony 2 was used for the food manipulation bioassays (March to May 2009). All manipulations in the nests were done under red light without disturbance of the bees.

Chemical Analyses We extracted labial glands of workers that were engaged in the construction and provisioning of brood cells (nurse bees) as well as the hind gut (rectum) of both newly emerged queens and workers. To prepare the extracts, an individual was killed by freezing, the respective body part dissected under a stereo microscope and left in 200 μl pentane for 24 hr at room temperature. The volume of each extract was reduced to 30 μl, and an internal standard (n-undecane) was added to allow quantification of the compounds.

For quantitative analyses, we used a Hewlett Packard HP 5890 gas chromatograph (Series II, Palo Alto, CA, USA) equipped with a DB-5MS capillary column (30 m× 0.25 mm i.d., 0.25 μm film thickness, J & W Scientific, Folsom, CA, USA) and a flame ionization detector (FID), with hydrogen as carrier gas (2 ml/min constant linear flow rate). Injection of 1 μl extract (per sample) was done in the splitless mode with an initial temperature of 50°C. After 1 min, the splitter was opened and the temperature increased by 10°C/min until the oven reached 310°C. The final temperature was held for another 23 min.

For qualitative analyses, the samples were analyzed by using a combined Fisons Instruments GC 8000 series / MD 800 mass spectrometer (carrier gas: helium; column: DB-5MS, 30 m×0.25 mm i.d., 0.25 μm film thickness, J & W Scientific; electron impact: 70 eV). The temperature was initially 60°C for 5 min, then increased by 10°C/min to 300°C and held at this temperature for 30 min. Identification of compounds was based on comparisons of mass spectra with literature data (McLafferty and Stauffer 1989), and with mass spectra and retention times of authentic reference substances. Non-commercial esters were synthesized from the corresponding acid chlorides and alcohols

according to standard procedures. Mass spectra and NMR-data were in accordance with expected data (Francke et al. 2000).

Bioassays To test the effect of geraniol on larval development, we added 10 μg of geraniol (Sigma-Aldrich®) to the larval food provision of recently sealed brood cells by puncturing each treatment cell with a fine needle and injecting 0.01 μl (=10 μg; equivalent to the labial gland contents of 3–4 nurse bees) of geraniol using a 0.5 μl gastight® syringe (Hamilton, Switzerland). The larvae in the treated brood cells were left to develop in their normal environment within the nest for 7–8 wk. We then opened the treatment cells (N=158) and untreated control cells (N=212) from the same colony in order to determine the caste of each pupa. We compared the observed distribution of queens and workers in treated and untreated cells using a 2×2 *chi-square* analysis of contingency table. A Yates correction was applied to adjust the *chi-square* value.

Results

Chemical Analyses Chemical analyses revealed that the main component in labial gland secretions of nurse workers of *Melipona beecheii* was (*E*)-3,7-dimethyl-2,6-octadien-1-ol, also known as geraniol (2.7±0.9 μg, N=6). Geraniol also was found in rectal extracts collected from newly emerged queens (3.8±1.2 μg, N=6), but was absent from extracts collected from newly emerged workers (N=12; Fig. 1).

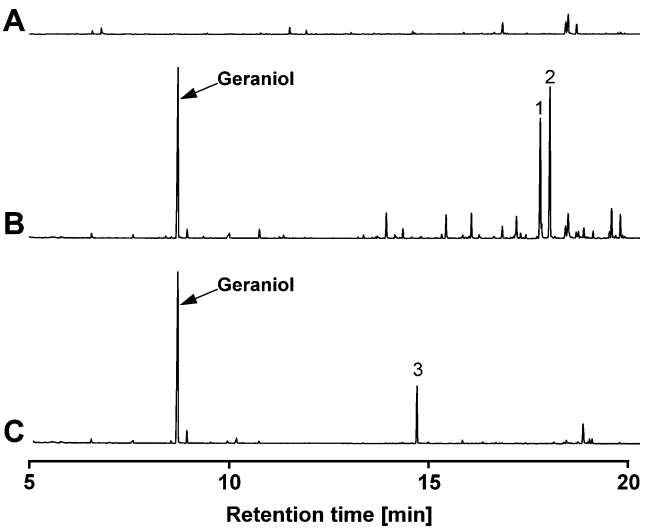

Fig. 1 Gas chromatograms of extracts from the rectum of a newly emerged *Melipona* worker (**A**) or a newly emerged virgin queen (**B**) and from the labial glands of a nurse bee (**C**). Peak height is a measure of abundance (flame ionization detector, A–C same scale). Additional compounds: *1*, (*Z*)-9-octadecenol; *2*, 1-octadecanol; *3*, geranyl hexanoate

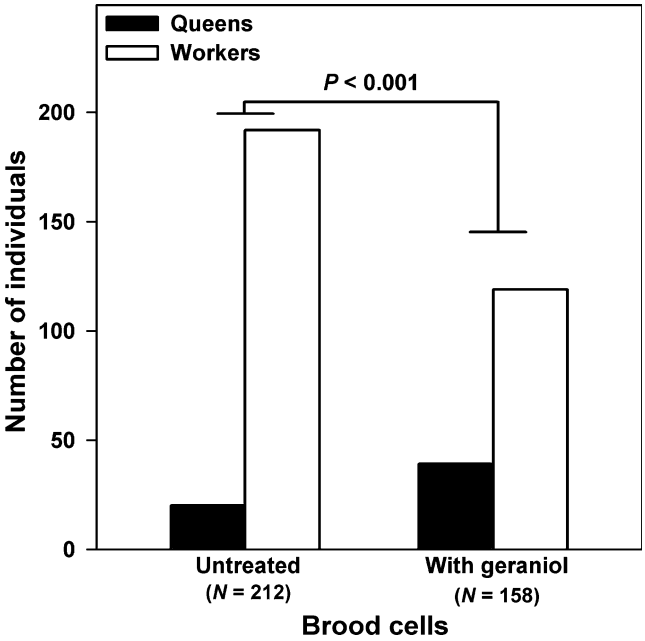

Fig. 2 Distribution of *Melipona* queens and workers among untreated brood and among brood that developed in cells to which 10 μg geraniol were added to the larval provision. 2×2 *Chi-square* analysis of contingency table, Yates correction, df=1, χ^2=14.590

Bioassays The holes in the cells made by injection of geraniol were closed by workers after the treatment, which demonstrates that they were accepted as normal brood cells. Naturally provisioned brood cells contained 116.6±9.4 mg (mean ± SD; N=14) of food. Thus, the addition of 10 μg of geraniol is far below the naturally occurring variation in food amount. We, therefore, assume that any observed effect of geraniol on larval development was caused by the qualitative rather than the quantitative alteration of the food. To test the effect of geraniol on larval development we compared the numbers of queens and workers in brood cells in which 10 μg of synthetic geraniol were added to the larval food provision, with the respective numbers in untreated brood cells from the same colony. The addition of geraniol to the larval food caused significantly more larvae to develop into queens as compared to untreated brood (geraniol: 39 queens, 119 workers; untreated: 20 queens, 192 workers; 2×2 *chi-square* analysis of contingency table, Yates corrected χ^2=14.590, df=1, P<0.001; Fig. 2). Furthermore, the proportion of larvae that developed into queens in geraniol treated brood cells perfectly matched the value (25%) predicted by Kerr's two-locus, two-allele model of genetic caste determination, whereas in untreated cells only 9% of the larvae developed into queens.

Discussion

The mechanism that triggers queen development in bees of the genus *Melipona* has remained unresolved to this day despite more than half a century of research and debate. In the present study, we identified geraniol, the main compound in labial gland secretions of nurse workers, as an exogenous caste determination factor in *M. beecheii*. The finding that in our bioassays 25% of the female brood in geraniol treated cells developed into queens corroborates the two-locus, two-allele model of genetic caste determination in *Melipona* proposed by Kerr (see Introduction). We, therefore, conclude that caste fate in *Melipona* is controlled both genetically and by a factor associated with larval provision: Female larvae that are genetically predisposed towards being queens only will follow this developmental pathway if they received sufficient amounts of a caste determining compound, which, in the case of *M. beecheii*, is geraniol. From the observation that the proportion of queens among larvae in *Melipona* nests is typically less than 25% (Kerr 1948; Darchen and Delage-Darchen 1975; Moo-Valle et al. 2001; Sommeijer et al. 2003; Wenseleers et al. 2004; Morais et al. 2006), we conclude that the concentration of geraniol usually is below this critical threshold in the majority of cells in nature. This indicates that nurse bees only add labial gland secretions to the normal provision (pollen, carbohydrates, hypopharyngeal gland secretions; Velthuis and Sommeijer 1991) in selected brood cells (we are currently investigating the distribution of geraniol among naturally provisioned brood cells). Thus, adult *Melipona* workers, which have little power to control the caste fate of developing larvae by limiting the *quantity* of their food, may exert suppression of queen development by limiting the access to a specific *qualitative* food factor—the primer pheromone geraniol. As a consequence, our results refute the assumption that *Melipona* larvae completely control their own caste fate (Bourke and Ratnieks 1999; Ratnieks 2001; Wenseleers et al. 2003; Wenseleers and Ratnieks 2004; Ratnieks and Helanterä 2009).

The high abundance of young queens in *Melipona* and, thus, the colony-level costs that are associated with the production of excess queens (Wenseleers and Ratnieks 2004), may well be a constraint imposed by its unique mechanism of caste determination. To answer this, detailed knowledge about the exact proximate mechanism by which geraniol acts at the molecular level is needed. It may function directly on gene regulation by triggering the expression of new genes or the repression of others (e.g., Schlichting and Pigliucci 1995; Nijhout 1999; West-Eberhard 2003). Geraniol may represent an essential precursor of a terpenoid compound exhibiting hormonal activity, such as juvenile hormone (JH), or, possibly, geranyl esters. Since queen development in eusocial bees is linked to higher JH titers during particular developmental stages as compared to worker larvae (de Wilde and Beetsma 1982; Hartfelder and Emlen 2005; Hartfelder et al. 2006), it is tempting to regard geraniol as an essential compound that triggers directly or indirectly the production or the physiological activity of JH.

The impact of exogenous agents, including nutrition, on the development of an organism is well documented for a large variety of species (Gilbert and Epel 2009). However, to the best of our knowledge, geraniol is the first caste determination substance identified as a physiologically active signal for queen development from the larval food of a social insect. Moreover, geraniol is the only primer pheromone, as defined by Nordlund (1981), identified from an insect other than the honey bee (Le Conte and Hefetz 2008)—almost 50 years after the identification of 9-oxo-(*E*)-2-decenoic acid, the queen substance primer pheromone of honey bees (Butler et al. 1962). The impact of geraniol on an individual's physiology sheds new light on the versatility of this compound and its derivatives, which are generally regarded as typical releaser pheromones. It should be noted that a second major compound in the labial gland secretions of nurse bees of *Melipona beecheii* is geranyl hexanoate (Fig. 1), which also is widespread among solitary and other eusocial bees (Bergström 2008). Whether geranyl hexanoate represents a lipophilic storage form of geraniol, or shows physiological activities on its own, awaits further investigation.

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