THESIS

CUTICULAR HYDROCARBONS AS MODULATORS OF SOCIAL INTERACTIONS IN HONEYBEE COLONIES

Submitted by

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In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Fall 2011

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ABSTRACT

CUTICULAR HYDROCARBONS AS MODULATORS OF SOCIAL INTERACTIONS IN HONEYBEE COLONIES

Honeybees are known for their highly complex social organization with individuals of different ages working in a coordinated manner to ensure colony functionality. While local-level inter-individual interactions are critical in transferring global-level information about colony needs, these same interactions are also exploited by various pathogens to spread themselves within the colony. It is therefore important to understand the proximate mechanisms that generate the exact structure of the interaction network within the colony. While bees of different ages possess unique cuticular hydrocarbon (CHC) profiles providing a potential basis for mediating these interactions, it is not entirely clear whether these odor cues in fact play a role in organizing the interaction network among them.

The first part of my thesis examines the CHC profiles of bees of different ages and how their neuronal sensitivity to these odors enable them to discriminate each other and generate the observed interaction network in the colony. Using behavioral observations to quantify the interaction frequencies between different age groups and using electroantennograms to determine the olfactory sensitivity of each age to the odor

of every other age, I determined the correlation between the two. The results show that young bees are indiscriminant in their interactions, which matches their lack of olfactory bias toward any age-specific odor, while old bees interact mostly with bees of a similar age, which corresponds with their higher olfactory sensitivity to the odor of such bees. Age-based differences in both cuticular hydrocarbons and the olfactory sensitivity to them thus provide a mechanistic basis to the observed interaction structure in the colony and suggests that an active behavioral segregation is the primary mechanisms that generates the organizational immunity in the colony, shielding the younger bees from interacting with older bees who are also more likely to be infected with pathogens.

The second part of my thesis examines if the energetic stress related to a pathogenic infection can alter the hydrocarbon profiles of individuals and lead to changes in the interaction network within the colony. Using gas chromatography, I was able to show that energetic state of an individual has a significant influence on its CHC profile. Following this, using a choice test where subjects at different energetic states were made to choose between chemical mimics of starved and satiated bees in a y-maze, I demonstrated that both fed and starved bees preferred to interact with recipients that are at similar energetic states. While this is somewhat surprising, a cost-benefit analysis showed how the decision to donate food is a function of both the energetic state of the receiver as well as the donor. While the benefit to cost ratio is positive for a depleted donor to donate to a starved recipient, this ratio is not positive for a fed donor to donate to the same starved recipient. This suggests that energetic stress, by changing the CHC profiles of individuals, can lead to social interactions being restricted between individuals of similar energetic states. Since the energetic state of an individual is likely to be

correlated with its infection status, this has the potential to generate a behavioral segregation between uninfected and infected individuals and help maintain the organizational immunity of the colony. My thesis research therefore establishes the role of age- and condition-dependent olfactory cues in organizing the interaction network within the colony and its implications for disease dynamics.

TABLE OF CONTENTS

CHAPTER 1:

OLFACTORY DISCRIMINATION OF AGE-SPECIFIC HYDROCARBONS GENERATES BEHAVIORAL SEGREGATION IN A HONEYBEE COLONY

SUMMARY

The functioning of a honeybee colony relies on the coordination of colony activities *via* inter-individual interactions. While the structure of this interaction network keeps the young individuals relatively isolated from the rest of the colony, there are two possible mechanisms that can generate this organizational immunity. A spatial segregation that restricts the young bees to the center of the colony can shield them with equal effectiveness as a behavioral segregation in which old bees choose to interact with young bees less frequently. We test the role of these two mechanisms by determining the interaction frequency between different age groups and testing their correlation with the olfactory sensitivity of different age groups to the cuticular odor of each other. Young bees were found to interact with bees of all age groups with equal frequency which correlates with their lack of olfactory bias for any specific age, while old bees interacted more with other old bees which correlates with their higher olfactory sensitivity toward the cuticular odor of old bees. The distribution of olfactory responsiveness was found to be positively skewed for old bees, which provides a mechanistic basis for the heterogeneous connectivity of the interaction network observed in an earlier study. As old bees are more likely to be responsible for introducing a potential disease into the colony from the outside and spreading it *via* the interaction network, these results suggest that behavioral segregation mediated by olfactory discrimination plays an important role in generating the organizational immunity within the honeybee colony.

INTRODUCTION

Integration of worker activities is critical in a social insect colony for keeping pace with the constantly changing colony demands and the external environment. Inter-individual interactions at the local level relay information about the state of the colony regarding nutrition (Seeley 1989; Camazine 1993; Schulz et al. 2002), hygiene (Arathi et al. 2000), and defense (Couvillon et al. 2008) at the global level. It has been argued more recently that the same interactions that are critical for colony functioning also form the pathways through which a pathogen can spread through the colony (Schmid-Hempel 1998; Naug and Camazine 2002). However, the organization of the interaction network is such that the younger individuals are structurally most isolated from the densely connected parts. This allows them to remain relatively insulated from the centripetal transmission force of any potential disease that might be spreading through the colony, a feature that has been termed organizational immunity (Naug and Smith 2007, Naug 2008). Such an interaction structure could arise *via* two alternative, although not entirely independent, mechanisms. Younger individuals could receive fewer interactions either as an indirect result of a spatial segregation that keeps them away from the rest of the individuals or as a result of behavioral segregation whereby other individuals actively direct interactions away from them.

As interactions among the various individuals in the colony are likely to be largely a by-product of their labor profiles, mechanisms which organize the interaction structure are inevitably linked to the forces which organize the division of labor in the colony. In most social insects including honeybees, this mainly consists of young individuals

performing nursing duties at the center of the colony, old individuals foraging outside, and a number of other somewhat overlapping task roles being performed by individuals of intermediate ages (Seeley 1982; Johnson 2008a). If individuals simply interact with their nearest neighbors, this spatial segregation is sufficient to result in young individuals interacting the least with old individuals. The spatial segregation hypothesis therefore does not require that individuals discriminate among different ages. In contrast, interactions between young and old individuals could also be low if each age actively seeks and interacts with only individuals of the same age. The behavioral segregation hypothesis therefore requires that individuals can discriminate among the different ages and the extent of this discrimination is correlated to the frequency with which they interact with these ages. One should however note that both these mechanisms can work independently of one another at the same time and lead to an even higher effect in terms of an organizational immunity.

Cuticular hydrocarbons (CHCs) are the primary candidates that could mediate discrimination among individuals of different ages in the colony because they have been shown to play a major role in nest-mate recognition (Breed 1998; Howard and Blomquist 2005). CHCs have also been shown to act as task-specific cues that regulate task allocation in ant colonies (Wagner et al. 1998; Greene and Gordon 2003) and Kather et al. (2011) have recently shown similar differences in the CHC makeup among honeybee individuals of different task groups. However, such differences alone cannot translate into active discrimination patterns and individuals of different ages or task groups must also have different levels of olfactory sensitivity for the cuticular odors of each other.

Using behavioral observations and electroantennogram (EAG) recordings in this study, we examine the correlation between the interaction pattern among the different task groups and their olfactory sensitivity toward the CHC profile of each other to identify the role played by behavioral and spatial segregation in organizing the interaction network in a honeybee colony.

MATERIALS AND METHODS

Observation Hive Setup

We set up a colony of approximately 1,500 bees in a two-frame observation hive with the bottom frame containing on average 75% brood and the top frame containing pollen and honey stores as well as empty space. Five hundred one-day old bees were introduced each week from a source colony so that the colony contained three age cohorts spaced 1-week apart. We sequestered the source queen to lay eggs on an empty frame and after about 18 days we removed the frame, now containing mature pupae, from the source colony and placed it in an incubator set at 32 °C, 50% RH. The newly hatched bees were marked on the thorax with a cohort-specific color and placed in the observation hive after removing the oldest cohort and any other bees that emerged in the observation hive, which preserved the demographic structure of the colony throughout the experiment. In order to focus solely on the contribution of age-based differences in cuticular hydrocarbon profiles to the interaction structure and control for any such differences due to the genetic variation among individuals (Breed 1998), we used singly mated sister queens inseminated by brother drones in both the source colony and the observation hive.

Behavioral Assay

Each week, 2-4 days after the youngest cohort was introduced, we recorded the behavioral activities on the top and the bottom frame of the hive with a video camera for one hour each, for a total of 6 hours over a three week span. Therefore, the typical ages were 3-5 days for young bees, 10-12 days for middle-aged bees, and 17-19 days for old bees on the day of recording. We then divided the entire view of each frame into 28 squares (5 cm x 5 cm each) and sampled random squares in 2-minute all-occurrence bouts, with a total of 30 bouts viewed for each frame each week for a total of 180 bouts over the entire experiment, to quantify the performance of three tasks: nursing (head inside cell in brood area), storing (head inside cell in storage area), and foraging (exiting the colony). We also quantified all interactions consisting of trophallaxis and antennation that lasted for at least two seconds in the same bouts, noting down who initiated each interaction. From this data, we calculated the relative proportion of a task performed by a specific age group (modified from Wilson 1976) as

$$RP_{ij} = \frac{N_{ij}}{\sum_{j=1}^{k} N_{ij}}$$

where

RP_{ij} = Relative proportion of task i performed by age j

N_{ij} = Number of performances of task i by age j

k = Total number of age groups

We also estimated the total number of bees of each age group present in the colony by counting their numbers in each of the 28 squares in 10 random scan samples in each of the three weeks of recordings.

Preparation of CHC odors and Gas Chromatography

After the completion of behavioral recordings each week, we collected 12 bees from each of the three age groups from the observation hive and freeze-killed them. We soaked each bee in 2 ml of 100% pentane for 10 minutes and eluted the CHCs by pouring the pentane extract through a silica gel column followed by a 1 ml wash with 100% pentane (Greene and Gordon 2007). In order to reduce the inter-individual variation in CHCs, we pooled the extracts from all bees of a given age group. We prepared age-specific odor cartridges by soaking a strip of filter paper in 50 µl of 1 bee-equivalent extract and placing it in a glass syringe. Using the queen from the source colony at the end of the experiment, we also prepared odor extract corresponding to the queen. In order to confirm if there were differences among the CHC profiles of the three age groups, we subjected the extracts from each age group to gas chromatography analysis by injecting 7-8 µl of 1 bee-equivalent sample into the column and comparing the relative abundance of specific hydrocarbons based on the elution patterns and retention times.

Electroantennograms

We made EAG recordings 2-3 days each week, with 3-5 bees from each of the three cohorts recorded per day. The subjects were removed from the observation hive, chilled and harnessed in plastic straws. For making a recording, we placed a bee in front of a continuous air stream, excised the distal tip of one antenna and inserted the remaining portion into an electrode filled with conductive gel. We then inserted a ground electrode into the posterior region of the head. Placing an odor cartridge 1 cm from the antennae and pulsing it with air, we passed odors corresponding to each age group over the

antennae of the subject for 2 seconds and recorded the signals generated by the antenna with the IDAC-2 acquisition system (Syntech, Germany). We exposed each subject first to a pentane control and then to the odor of each age group in a random sequence with a two minute interval between two successive odors. We replaced each odor cartridge after three uses. We extracted the queen from the observation hive on the final day of the experiment and recorded her response to the odor extracts of all the age groups. We subtracted the EAG amplitude for the control pentane stimulus from that obtained with each odor before further analysis.

RESULTS

Behavioral and CHC profiles of different age groups

Old, middle-aged, and young bees performed behaviors at levels that matched their known task profiles. Old bees foraged significantly more than the other age groups (G test of proportions: $G=727.50$, $N=682$, $d.f.=2$, $P<0.0001$, Fig. 1.1), while young bees nursed significantly more than the other age groups ($G=172.97$, $N=161$, $d.f.=2$, $P<0.0001$). The task profile for middle-aged bees was somewhat less clear as they showed only a slight preference for storing over the other age groups ($G=5.99$, $N=291$, $d.f.=2$, $P=0.05$). Based on the elution patterns and the retention times of desorbed components, the more abundant (>5%) long chain CHCs were identified and compared among the three age groups (Table 1.1), confirming the known differences in the hydrocarbon profiles across ages.

Interaction frequencies across different age groups

While all the three age groups seemed to interact more with bees of the same age group, only the old bees interacted significantly more than expected with bees of their own age group, when corrected for the number of bees in each age group (G=6.68, d.f.=2, N=38, P=0.03, Fig. 1.2). Young bees interacted with all the three age groups at proportions expected by their respective numbers in the colony (G=1.29, d.f.=2, N=45, P=0.53) as did the middle-aged bees (G=4.8, d.f.=2, N=38, P=0.09). When involved in interactions with bees of the other age groups, both old and middle-aged bees interacted less than expected with young bees. If the old and the middle-aged bees are pooled into a single age class, the combined group shows a significantly higher proportion of interactions directed toward their own kind compared to the young bees (G=8.45, d.f.=1, N=76, P=0.003) while young bees still do not show any such bias in directing interactions toward a specific age group (G=1.19, d.f.=1, N=45, P=0.27).

EAG responses of different age groups

Frequency distributions of EAG responses were calculated by pooling all the responses of all the individuals for each age group. It fit a normal distribution for both young and middle-aged bees (Kolmogorov-Smirnov test for goodness of fit: Young: g_{max}=0.08, N=107, P=0.36, Fig. 1.3a; Middle: g_{max}=0.08, N=110, P=0.45, Fig. 1.3b). However, the frequency distribution of EAG responses for old bees did not fit a normal distribution (g_{max}=0.15, N=100, P=0.01) but fit an exponential distribution (g_{max}=0.10, P=0.22, Fig 1.3c). Both the old and middle-aged bees showed significantly higher olfactory sensitivity for the odors of the same age group in comparison to young bees

(Mann-Whitney U-test: O-O vs. O-Y: U=731.5, N=67, P=0.03, M-M vs. M-Y: U=819, N=72, P=0.05; Fig. 1.4) but not the other age group (O-O vs. O-M: U=677.0, N=68, P=0.22; M-M vs. M-O: U=755.5, N=75, P=0.58). In contrast, the olfactory sensitivity of young bees was not significantly different for the odors of any of the three age groups (Y-Y vs. Y-M: U=685.5, N=71, P=0.52; Y-Y vs. Y-O: U=740, N=64, P=0.3; Y-M vs. Y-O: U=657.5, N=71, P=0.75). Young bees however seemed to show higher sensitivity to the cuticular odor of the queen compared to middle-aged and old bees (Fig. 1.5) although a statistical comparison was not possible due to a single queen being available for odor extraction. The queen herself did not show an EAG response to the odors of the workers.

DISCUSSION

Our results suggest that cuticular hydrocarbons play an important role in generating the interaction network of a honeybee colony. By examining the more abundant long chain CHCs, we were able to confirm the existence of age-based differences in surface hydrocarbon components (Blomquist et al. 1980; Kather et al. 2011). The result that old and to a lesser extent middle-aged bees have a preference to interact with bees of the same age that is independent of their respective numbers, suggests that they are not merely interacting randomly with any bees they come across but are actively choosing their interaction partners. This trend in interaction frequencies also matches the olfactory responsiveness of these age groups toward the other age groups, suggesting active behavioral segregation by older individuals. Younger bees on the other hand appear to be less specific in their choice of interaction partners and this corresponds with their lack of olfactory bias toward any particular age group. However, we did not have sufficient

interaction data to specifically determine whether young bees merely interact with whoever is in their neighborhood. Young bees themselves are known to contain fewer hydrocarbons in their cuticle (Francis et al. 1989) and this could be a reason why odor does not play an important role in driving their interaction profile. The known increase in olfactory sensitivity with age is probably correlated with the need to assimilate a wider range of sensory information as a bee transitions from being a within-nest nurse to a forager searching for food outside the colony (Masson and Arnold 1984; Withers et al. 1993).

The positive correlation between interaction frequency and olfactory sensitivity in the old bees indicates that their olfactory discrimination for different age groups is based on a model of label-acceptance rather than label-rejection (Getz 1982). Associative learning (Châline et al. 2005) as well as non-associative learning mechanisms such as sensitization could also play an important role in the ability of older bees to discriminate among cuticular odors. While being sensitive to an odor may not necessarily translate to a behavioral response (Allan et al. 1987), a number of studies have demonstrated such correlations between antennal sensitivity to specific odors and the performance of specific behaviors in social insects (Masterman et al. 2001; Gramacho and Spivak 2003; Lopez-Riquelme et al. 2006). In fact, it has been shown that even a physical contact between the CHC molecules and the contact chemosensilla in the antennae is not necessary to generate a behavioral response to CHC (Brandstaetter et al. 2008; Brockmann et al. 2003).

The observed structure of the interaction network makes adaptive sense in terms of worker integration and division of labor. The tasks associated with old and middle-aged bees are tightly linked, with the old bees acting as foragers and the middle-aged bees acting as storers (Seeley 1989). These two tasks performed by the bees of the older two age groups are however only weakly linked to the nursing duties performed by the young bees. The observed interaction structure is also adaptive from an epidemiological viewpoint as it ensures that the old and middle-aged bees, who are more likely to come in direct contact with materials brought into the nest, interact less as a group with the nurses and minimize the latter's exposure to potential pathogens. The major point to note is that the connectivity imposed by the older age groups is not incidental but guided by an active olfactory discrimination mechanism. This has important implications for transmission dynamics within the colony because it means that behavioral segregation can supplement or even transcend spatial segregation in generating the organizational immunity observed in the colony. This is supported by the earlier result that the young bees remain relatively unexposed to a pulse of food entering the colony and spreading through it even when all the spatial regions show similar exposure to the pulse (Feigenbaum and Naug 2010). Behavioral segregation is therefore likely to be the primary mechanism that generates the organizational immunity in the context of a centripetal wave of food-borne infection that is introduced into the colony by the older foraging bees acting as primary infectives (Naug and Smith 2007). However, our results also suggest that a transmission wave emanating from the brood source in the center of the colony may not be as well contained due to the lack of behavioral segregation displayed by the young bees. One might

therefore speculate whether this plays any role in brood diseases being some of the most virulent diseases in a honeybee colony.

While the younger bees do not seem to show any behavioral segregation toward bees of other age groups, they surprisingly have a higher olfactory responsiveness to the queen than the other two age groups. This makes it interesting to ask if CHCs are involved in generating the retinue behavior of the young bees as otherwise no differences have been found in the olfactory responsiveness of different age groups to the queen mandibular pheromone (Pham-Delegue et al. 1993). The lower olfactory sensitivity of the older bees to the queen is probably responsible for their lower propensity to interact with her even though she travels relatively widely across the entire colony. There is in fact potential for a large amount of mixing among bees of all age groups since even middle aged bees have been shown to move throughout the colony (Johnson 2008b). However, the lower olfactory sensitivity of the older bees to the nurses and the queen is strong evidence that behavioral segregation could be an important force for generating an organizational immunity that keeps these valuable individuals relatively isolated and safe.

The shapes of the EAG response distributions for the different age groups are especially revealing in terms of the structure of the interaction network within the colony. It suggests that while young bees are likely to be more similar in terms of their propensity to initiate interactions, older bees in contrast are going to be highly heterogeneous in this regard. A few of the old bees with hypersensitive olfaction are likely to engage in a large number of interactions and play a disproportionate role in driving the overall interaction

network of the colony. Thus, olfactory sensitivity likely provides the mechanistic basis to the heterogeneous connectivity distributions observed in the interaction network of the honeybee colony (Naug 2008). It is also interesting to note the gradual transition in the shape of the EAG profiles with age and it opens up an interesting line of inquiry regarding the role of intrinsic and social influences on the ontogeny of olfactory sensitivity and the consequent interaction network.

Given the fundamental role of the interaction network in transmitting food, information, and even pathogens within the colony, it is important to understand the proximate basis underlying its structure. Our results show that olfactory sensitivity to age-specific cuticular hydrocarbons plays an important role in structuring the interaction network in the colony. The colony organization observed in a natural colony is obviously much more complex than what was observed under our experimental simplification and other factors such as the more continuous age distribution, and the inherent genetic variation would play additional roles in determining the interaction structure and testing their impacts would add to our findings. While response threshold models predict the behavioral profile of each individual as an outcome of its thresholds for responding to different stimuli (Page & Robinson 1991; Gordon 1992), these models have been empirically investigated only in terms of a few tasks (Page et al. 1998; Masterman et al. 2001) and for one task at a time. In this paper, we concurrently determine the different thresholds of each task group for interacting with every other group. This sets the stage for inquiries into the mechanisms by which the interaction network in the colony could be

possibly modified under different colony situations, especially those related to the pathophysiology of an infectious disease which influence its spread within the colony.

FIGURES

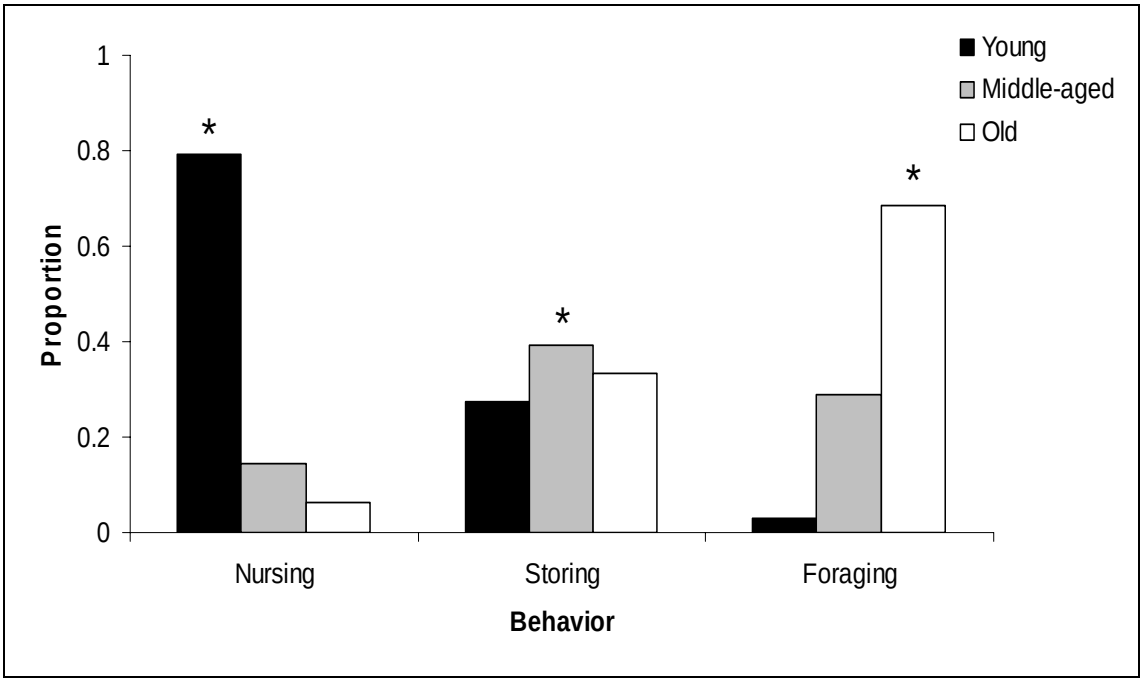


Figure 1.1. Proportion of nursing, storing, and foraging performed by young, middle-aged, and old bees with asterisks denoting significant differences in performance for a task among the three age groups.

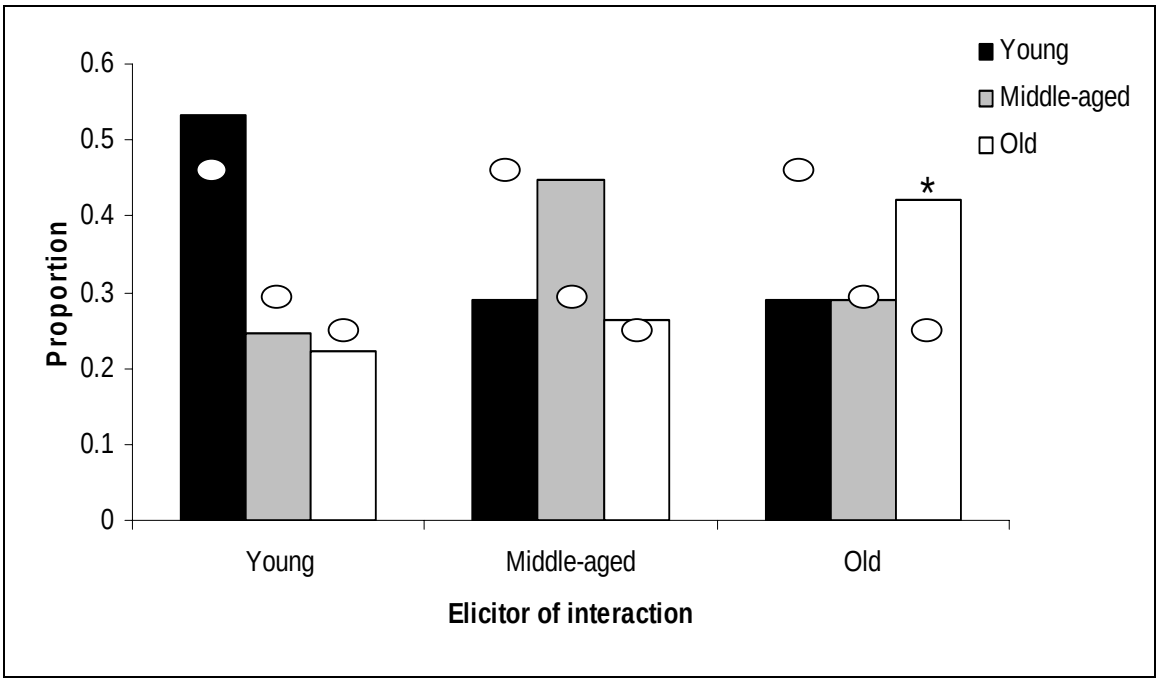
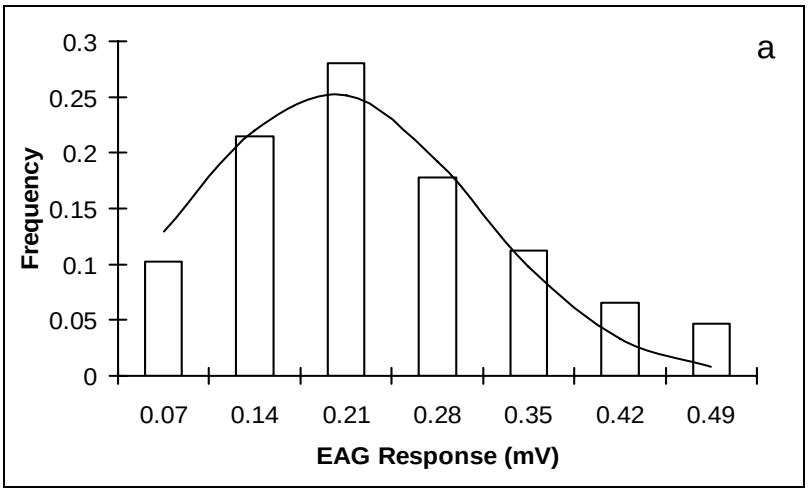
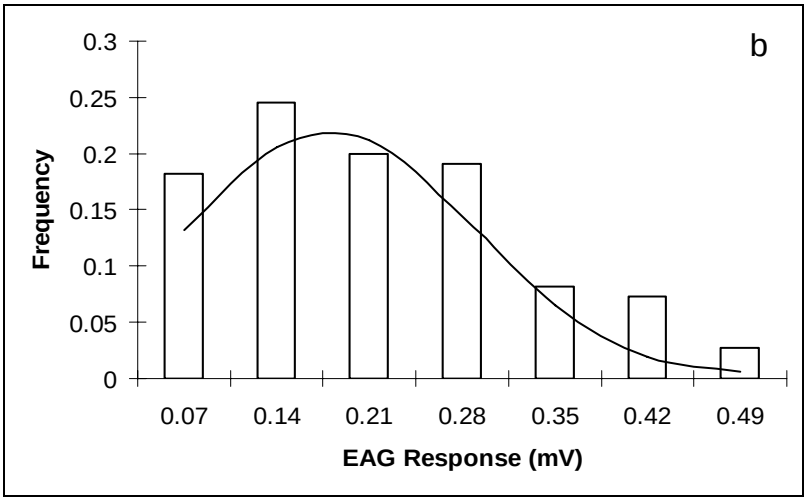


Figure 1.2. Proportion of interactions initiated by young, middle-aged, and old bees toward the three age groups. Expected numbers of interactions, given by the number of individuals that made up each age cohort, are represented by circles with asterisks denoting significant differences from expected values.





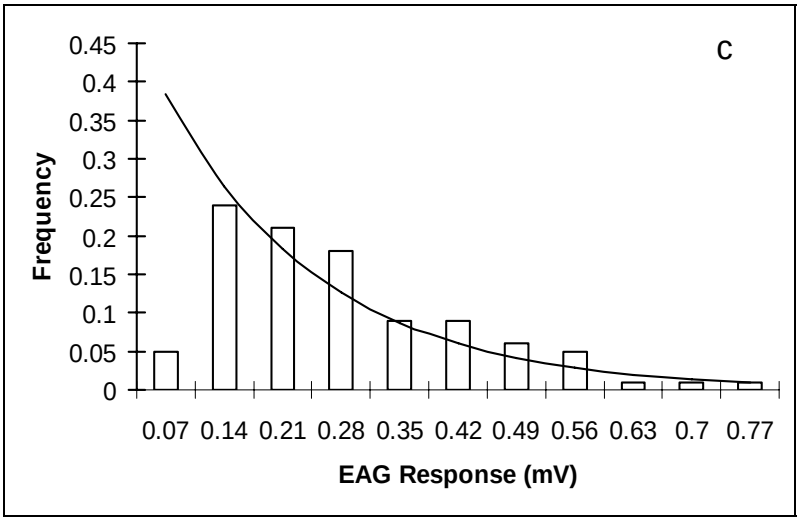


Figure 1.3. Frequency distributions of pooled EAG responses to odors of all age groups by (a) young, (b) middle-aged, and (c) old bees, with observed distributions given by bars and fitted distributions given by lines.

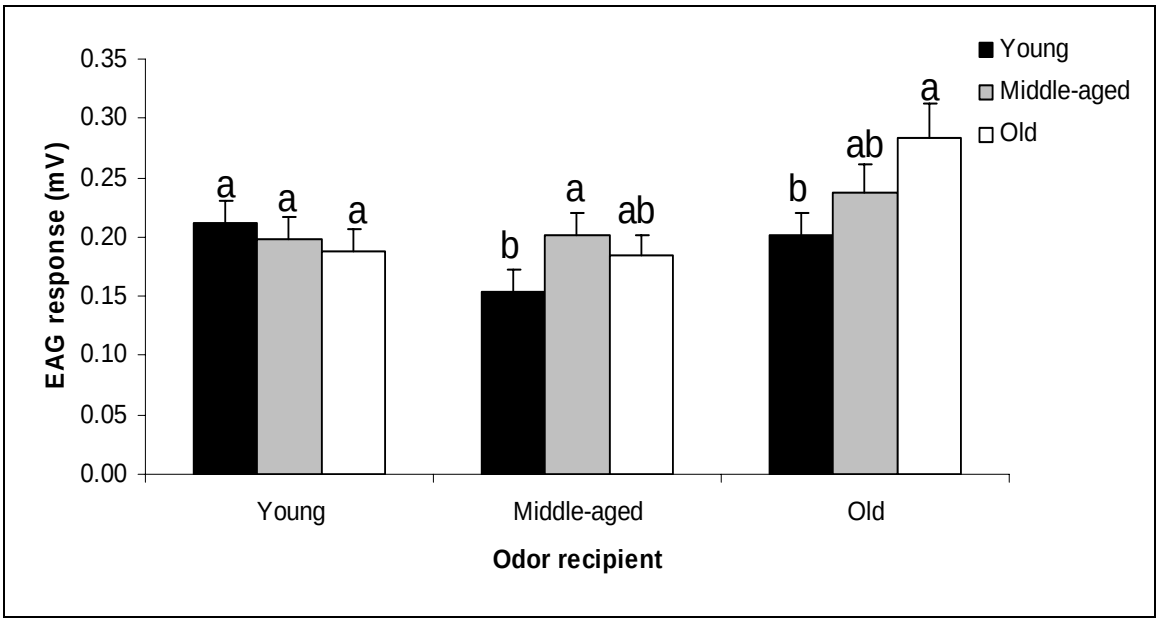


Figure 1.4. Mean EAG responses (± S.E.) of young, middle-aged, and old bees to the odors of the three age groups in the colony. Comparison is within each group and bars with different letters are significantly different.

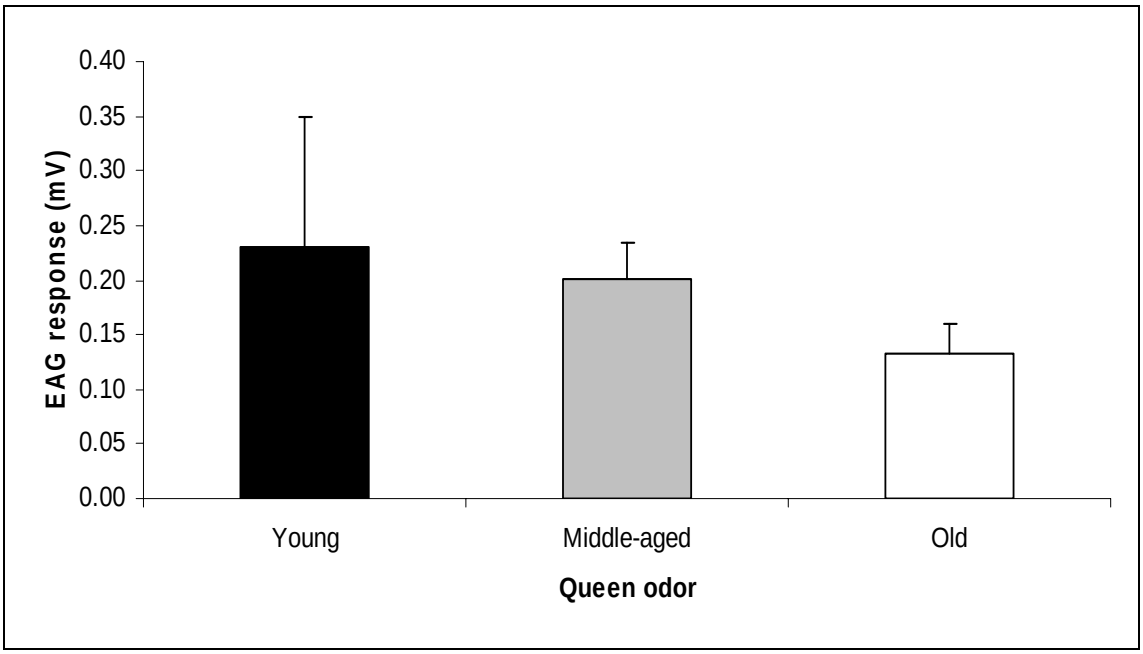


Figure 1.5 Mean EAG responses (± S.E.) of young (N = 3), middle-aged (N = 4), and old bees (N = 3) to the odor of the queen

TABLES

Table 1.1 Relative percent composition (mean ± S.D.) of some of the major cuticular hydrocarbons found on young, middle-aged, and old bees. The values were derived from relative peak abundance area corresponding to the 6 most prevalent peaks.

Compound	Young	Middle-aged	Old
C_{23}	5.3 ± 0.0084	3.0 ± 0.0067	4.8 ± 0.0150
C_{25}	5.1 ± 0.0086	6.7 ± 0.0063	12.2 ± 0.0573
C_{27}	15.1 ± 0.0184	18.6 ± 0.0116	30.3 ± 0.0486
C_{29}	14.0 ± 0.0216	18.6 ± 0.0154	16.2 ± 0.0162
C_{31}	9.0 ± 0.0096	14.5 ± 0.0099	7.6 ± 0.0207
$C_{31:1}$	12.4 ± 0.0073	16.0 ± 0.0177	9.9 ± 0.0296

REFERENCES

Allan SA, Slessor KN, Winston ML, King GGS (1987) The influence of age and task specialization on the production and perception of honey bee pheromones. Journal of Insect Physiology 33:917-922

Arathi HS, Burns I, Spivak M (2000) Ethology of hygienic behaviour in the honey bee *Apis mellifera* L. (Hymenoptera: Apidae): Behavioural repertoire of hygienic bees. Ethology 106:365-379

Blomquist GJ, Chu AJ, Remaley S (1980) Biosynthesis of Wax in the Honeybee, *Apis-Mellifera* L. Insect Biochemistry 10:313-321

Brandstaetter AS, Endler A, Kleineidam CJ (2008) Nestmate recognition in ants is possible without tactile interaction. Naturwissenschaften 95:601-608

Breed MD (1998) Chemical cues in kin recognition: Criteria for identification, experimental approaches, and the honey bee as an example. In Chemical communication in social insects, R. K. Vander Meer, M. L. Winston, K. E. Espelie and M. D. Breed, eds. Westview Press: Boulder, pp. 57-78

Brockmann A, Groh C, Fröhlich B (2003) Wax perception in honeybees: contact is not necessary. Naturwissenschaften 90:424-427

Camazine S (1993) The regulation of pollen foraging by honey bees: how foragers assess the colony's need for pollen. Behavioral Ecology and Sociobiology 32:265-272

Châline N, Sandoz J, Martin SJ, Ratnieks FLW, Jones GJ (2005) Learning and discrimination of individual cuticular hydrocarbons by honeybees (*Apis mellifera*). Chemical Senses 30:327-335

Couvillon MJ, Robinson EJH, Atkinson B, Child L, Dent KR, Ratnieks FLW (2008) En garde: rapid shifts in honeybee, Apis mellifera, guarding behaviour are triggered by onslaught of conspecific intruders. Animal Behaviour 76:1653-1658

Feigenbaum C, Naug D (2010) The influence of social hunger on food distribution and its implications for disease transmission in a honeybee colony. Insectes Sociaux 57: 217-222

Francis BR, Blanton WE, Littlefield JL, Nunamaker RA (1989) Hydrocarbons of the cuticle and hemolymph of the adult honey bee (Hymenoptera, Apidae). Annals of the Entomological Society of America 82:486-494

Getz WM (1982) An analysis of learned kin recognition in hymenoptera. Journal of Theoretical Biology 99:585-597

Gordon DM (1992) A parallel distributed model of the behaviour of ant colonies. Journal of Theoretical Biology 156:293-307

Gramacho KP, Spivak M (2003) Differences in olfactory sensitivity and behavioral responses among honey bees bred for hygienic behavior. Behavioral Ecology and Sociobiology 54:472-479

Greene MJ, Gordon DM (2003) Cuticular hydrocarbons inform task decisions. Nature 423:32

Greene MJ, Gordon DM (2007) Interaction rate informs harvester ant task decisions. Behavioral Ecology 18:451-455

Howard RW, Blomquist GJ (2005) Ecological, behavioral, and biochemical aspects of insect hydrocarbons. Annual Review of Entomology 50:371-393

Johnson BR (2008a) Within-nest temporal polyethism in the honey bee. Behavioral Ecology and Sociobiology 62:777-784

Johnson BR (2008b). Global information sampling in the honey bee. N*aturwissenschaften* 95: 523-530

Kather R, Drijfhout F, Martin S (2011) Task Group Differences in Cuticular Lipids in the Honey Bee *Apis mellifera*. Journal of Chemical Ecology 37:205-212

López-Riquelme GO, Malo EA, Cruz-López L, Fanjul-Moles ML (2006) Antennal olfactory sensitivity in response to task-related odours of three castes of the ant *Atta mexicana* (hymenoptera: formicidae). Physiological Entomology 31:353-360

Masson C, Arnold G (1984) Ontogeny, maturation and plasticity of the olfactory system in the worker bee. Journal of Insect Physiology 30:7-14

Masterman R, Ross R, Mesce K, Spivak M (2001) Olfactory and behavioral response thresholds to odors of diseased brood differ between hygienic and non-hygienic honey bees (*Apis Mellifera* L.). Journal of Comparative Physiology A 187:441-452

Naug D (2008) Structure of the social network and its influence on transmission dynamics in a honeybee colony. Behavioral Ecology and Sociobiology 62:1719-1725

Naug D, Camazine S (2002) The role of colony organization on pathogen transmission in social insects. Journal of Theoretical Biology 215:427-439.

Naug D, Smith B (2007) Experimentally induced change in infectious period affects transmission dynamics in a social group. Proceedings of the Royal Society B-Biological Sciences 274:61-65

Page Jr. RE, Robinson GE (1991) The genetics of division-of-labor in honey-bee colonies. Advances in Insect Physiology 23:117-169

Page Jr. RE, Erber J, Fondrk MK (1998) The effect of genotype on response thresholds to sucrose and foraging behavior of honey bees (*Apis mellifera* L.). Journal of Comparative Physiology A 182:489-500

Pham-Delegue MH, Trouiller J, Caillaud CM, Roger B, Masson C (1993) Effect of queen pheromone on worker bees of different ages - behavioral and electrophysiological responses. Apidologie 24:267-281

Schmid-Hempel, P (1998) Parasites in social insects. Princeton, NJ: Princeton University Press.

Schulz DJ, Vermiglio MJ, Huang ZY, Robinson GE (2002) Effects of colony food shortage on social interactions in honey bee colonies. Insectes Sociaux 49:50-55

Seeley TD (1982) Adaptive significance of the age polyethism schedule in honeybee colonies. Behavioral Ecology and Sociobiology 11:287-293

Seeley TD (1989) Social foraging in honey bees: how nectar foragers assess their colony nutritional status. Behavioral Ecology and Sociobiology 24:181-199

Wagner D, Brown MJF, Broun P, Cuevas W, Moses LE, Chao DL, Gordon DM (1998) Task-related differences in the cuticular hydrocarbon compositions of harvester ants, *Pogonomyrmex barbatus*. Journal of Chemical Ecology 24:2021-2037

Wilson EO (1976) Behavioral discretization and the number of castes in an ant species. Behavioral Ecology and Sociobiology 1:141-154

Withers GS, Fahrbach SE, Robinson GE (1993) Selective neuroanatomical plasticity and division-of-labor in the honeybee. Nature 364:238-240

CHAPTER 2:

CUTICULAR HYDROCARBONS INFORM HONEYBEE WORKERS ABOUT THE COSTS AND BENEFITS OF FOOD-SHARING INTERACTIONS

SUMMARY

Food sharing is a critical feature of social insect colonies but the mechanisms which regulate sharing between specific individuals are not clear. In this study, we test whether the cuticular hydrocarbon makeup of a honeybee worker is a function of its hunger level and can be used by other workers to inform their food sharing decisions. Our gas-chromatography results show that short-term changes in hunger level can lead to significant differences in the cuticular hydrocarbon profile of individuals. Choice tests using fed and depleted donors choosing between odor mimics of satiated and starved bees showed that cuticular hydrocarbons can modulate the behavior of potential donors. While fed bees surprisingly chose to interact more with the mimics of satiated bees and depleted bees interacted more with the mimics of starved bees, we use a cost-benefit analysis to explain these food sharing patterns in terms of recipient need and quality. We also discuss the possible implications of these patterns and processes on the dynamics of food distribution within the colony and its consequent impact on disease transmission.

INTRODUCTION

Food-sharing is a vital component of success in group-living animals and involves extensive communication between donors and recipients regarding who should be allocated food. This communication consists of solicitation signals of various types from the recipients (reviewed in Kilner and Johnstone 1997; Mock et al. 2011) and the reaction to these by the donors. Social insects, which are characterized by extensive food sharing networks within the colony, are known to use inter-individual interactions for organizing colony activities (Gordon 1996; Wilson and Hölldobler 2005). This suggests that such interactions are likely to be important in transferring information about the nutritional state of individual workers as well as the colony. However, the exact mechanisms by which the nutritional state of workers modulates food sharing interactions in order to adjust food distribution within the colony is not entirely clear (Howard 1980; Schulz et al. 2002).

Olfactory discrimination mediated by cuticular hydrocarbons (CHCs) is known to play a key role in modulating inter-individual interactions within the dark and crowded confines of the colony (Breed et al. 1985; Francis et al. 1989; Wagner et al. 1998; Greene and Gordon 2003; Howard and Blomquist 2005; Richard et al. 2008; Scholl and Naug 2011). For instance, odor cues are used by guard bees to discriminate between nestmates and non-nestmates (reviewed in Breed 1998), by healthy bees to identify immunocompromised nestmates (Richard et al. 2008), by hygienic bees to discriminate between healthy and infected brood (Masterman et al. 2001), and by nurse bees to convey information about pollen needs of the colony (Dreller and Tarpy 2000). However, it is not

known whether olfaction plays any role in the identification of starved individuals and the subsequent decision by a forager to transfer food to a particular receiver among a number of potential recipients.

In social insects, food transfer among adults, or trophallaxis, is generally accompanied by antennal contact (Free 1956; Lenoir 1982; Mc Cabe et al. 2006), suggesting that chemical cues probably play a role in determining the suitability of an individual to receive food from a donor. This in turn points toward a role of CHCs in such discrimination, as diet is known to influence the CHC profile of individuals (Liang and Silverman 2000). A number of recent studies have shown the importance of chemical cues in modulating the food provisioning behavior of parents toward their offspring (Kolliker et al. 2005; Mas et al. 2009). These studies show that the cuticular hydrocarbon signatures of offspring can convey information about their hunger level to the parents, who in turn can use this signal to choose which one to feed based on either offspring need or quality (Haig 1990, Godfray 1991). While brood pheromone is known to perform a similar function in honeybees, conveying information about hunger from the brood to the nurses (Pankiw et al. 1998), in this study we examine if similar signaling occurs between adult bees such that differences in cuticular hydrocarbons act as a signal of either need or quality to drive food transfer between a donor and specific recipients.

MATERIALS AND METHODS

Hunger Treatment setup

We extracted a brood frame containing mature pupae from a colony headed by a singly mated queen and placed it overnight in an incubator set at 32°C, 50% RH for hatching. Next day, we placed approximately 800 newly emerged one-day old bees on a frame full of nectar and pollen enclosed in a mesh cage (0.25 x 0.25 in. mesh) and placed this cage back in the same colony. This setup allowed the bees to age while being exposed to the social milieu of the colony, including interactions with other members, while making it easy to extract them for the next step of the experiment. After ten days, we removed the cage from the colony, placing half the bees in three 12 x 12 x 12 cm cages and feeding them *ad libitum* with a 30% sucrose solution (Satiated Treatment) and placing the other half in three cages and giving them only water (Starved Treatment). After 24 hours, we collected the living bees from the two treatments and freeze-killed them for chemical extraction of surface lipids.

Extraction of surface lipids and preparation of chemical mimics

We extracted surface hydrocarbons by thawing the frozen bees and soaking each of them in about 2 ml of 100% pentane (HPLC grade) for 10 minutes and eluting the extract through a chromatography column with a silica gel solid phase (60-75 mesh; Sigma Aldrich). We allowed the solvent to evaporate and added 20 µL of 100% pentane to each sample, thus resulting in each tube containing the hydrocarbon-pentane extract of one bee from one of the two respective groups. We used 15 Satiated and 15 Starved bee extracts for chemical analysis and used the remaining extracts to prepare the mimics.

We made chemical mimics of Satiated and Starved bees by placing a 5-mm diameter glass bead in a tube of extract from one of the two respective groups and allowed the pentane to evaporate so that each bead was coated with approximately one bee-equivalent of hydrocarbons (Greene and Gordon 2003). We stored the beads at -20 °C until they were used in the behavioral assay.

Chemical analysis of surface hydrocarbons

We analyzed the cuticular hydrocarbons from the Satiated and the Starved bees by injecting 10 μL of a sample extract into a Varian 3900 gas chromatograph with a DB-5 fused silica capillary column (30 m, 0.25 um ID, 0.25 um film thickness; J&W Scientific). We held the oven temperature at 170°C for 5 min during injection, raising it to 220°C at a rate of 25°C per min, and then to 310°C at 3°C per min with a 5 min hold. We measured the peak areas by integrating peaks and calculated the relative abundance of each peak by dividing its area by the total area of 8 peaks, each of which had an abundance of over 1%.

Behavioral Assay

We used a Y-maze (with arms measuring 5 cm each) to test the choice of potential donors between the mimics of Satiated and Starved bees by placing a bead corresponding to each type placed at the end of each arm. In order to reduce any confounding effects due to genetic variance, our test subjects consisted of returning foragers from the same colony that provided the bees for the hydrocarbon extracts. About 10 foragers were collected every 1-2 hours, fed with 5-10 μL of 30% sucrose solution

and held in a small wire cage. Individuals that were not active, that did not feed, and those that had pollen loads were not used in the assays. In order to assess if the energy level of the donor played any role in the food sharing process, one choice test was conducted with a forager immediately after feeding (Fed donor) while a second choice test with a forager that was sequestered for about an hour after feeding (Depleted donor). Note that the energetic state of the donors and the mimics are named differently to point out that the exact nature of these treatments differs as well as to avoid any potential confusion. These behavioral assays took place between 9am and 4pm in a dark room with diffuse light and the maze was cleaned with ethanol and air-dried after each trial.

For each trial, we placed a forager in the maze and observed it for 10 minutes, noting its location with respect to the two arms every 15 seconds. In addition, every occurrence of antennation (the bee touching the mimic with its antennae) and trophallaxis (the bee extending its proboscis to the mimic) on the two mimics was recorded. However, trophallaxis occurred so infrequently that it was later excluded from the data. The observer was blind to the identity of the mimics and the two different types of mimics were placed in opposite arms for each subsequent trial to eliminate any side bias. From these data, we calculated the proportion of time each individual spent near each mimic and its frequency of antennation with each mimic. Individuals that did not enter both arms of the maze or that did not perform antennation at all were excluded from the dataset.

Statistical Analysis.

Linear discriminant analysis was used to determine if cuticular hydrocarbon profiles of Satiated and Starved bees have different relative abundances of the various compounds. The relative abundance data were transformed (Aitchison 1986) using the equation $Z_{ij} = \ln(Y_{ij}/g(Y_j))$, where Z_{ij} is the relative peak area i for individual j, Y_{ij} is the observed peak area i for bee j, and $g(Y_j)$ is the geometric mean of all peak areas used in the analysis for bee j. We limited the number of peaks used in the analysis to the eight most abundant hydrocarbons in order to avoid significant discrimination where none exists, since false discrimination may occur when there are large numbers of independent variables relative to the sample size (Panel on Discriminant Analysis and Clustering 1989). Discriminant scores were calculated for each sample as the position along a new axis that represents the linear combination of variables providing the best discrimination among groups. Overall effectiveness of the discrimination was tested by using a Wilks' lambda test. For the behavioral data, a G-test of proportions was used to compare the proportion of time spent by each donor (Fed or Depleted) near each of the mimics (Satiated or Starved) and a Kruskal-Wallis test was used to compare the number of antennations on each mimic.

RESULTS

CHC composition of Satiated and Starved bees

Satiated and Starved bees differed in the relative abundance of compounds in their cuticular hydrocarbon profiles (Fig. 2.1a). The relative abundances of the eight most abundant cuticular hydrocarbons allowed for discrimination of samples from Satiated and

Starved bees (one discriminant function: canonical correlation = 0.596; Wilks' $\lambda = 0.645$, Chi-squared = 11.415, d.f. = 4, p = 0.022; Fig. 2.1b). Twelve out of 15 Satiated bee samples were correctly classified and 13 out of 15 Starved bee samples were correctly classified.

Donor behavior toward Satiated and Starved mimics

The type of mimic (Satiated vs. Starved) did not influence donor choice in terms of proportion of time spent in the two arms of the y-maze near each mimic (G test of proportions: G = 0.052, d.f. = 1, N = 47, P = 0.819). However, Fed donors performed a significantly higher number of antennations on the Satiated mimics (Kruskal-Wallis H = 8.207, d.f. = 1, N = 18, P = 0.004; Fig. 2.2) while the Depleted donors antennated the Starved mimics significantly more (Kruskal-Wallis H = 13.024, d.f. = 1, N =18, P < 0.001).

DISCUSSION

Our results suggest that CHCs play an important role as modulatory cues that influence food-sharing interactions among honeybees. While the makeup of an individual's long-term diet has been shown to alter the hydrocarbon profile of some social insects (Francis et al. 1989; Liang and Silverman 2000), we show for the first time that short-term changes in the nutritional state of an individual can alter its CHC profile. Such short-term alterations in CHC expression have been also shown to occur in the context of dominance interactions in *Drosophila* (Petfield et al. 2005; Kent et al. 2008; Thomas and Simmons 2011). Our results show that Fed donors interacted more with the

mimics that represented Satiated recipients while Depleted donors interacted more with the mimics representing Starved recipients. This suggests that the energetic state of both parties play a role in modulating food exchange interactions between potential donors and recipients. However, the direction of the interactions is somewhat surprising at first because it would seem more natural for individuals at a higher energetic state to direct their food transfer toward individuals with higher need and *vice versa*, the exact opposite of what we found.

There has been considerable debate over whether food-sharing interactions are guided by need (Godfray 1991) or quality (Haig 1990; Mock et al. 2011) when parents provision their offspring. Rather than considering these as two different alternatives, we suggest that food sharing occurs in the direction of need or quality depending upon the relative levels of satiety of the donor and the recipients. In this framework, there is a non-linear relationship between resource level and fitness and the relative positions of a donor and a recipient on this fitness function dictate the cost-benefit ratio of and therefore the potential of sharing between the two (Wilkinson 1984, Whitlock et al. 2007). In our case, considering that the survival of individual honeybees as a function of their satiety has been shown to follow a non-linear relationship (Mayack and Naug 2009; Fig. 2.3), for a Fed donor (D_1), the cost (C_{D1}) of donating food to a Starved recipient (R_2) far outweighs the benefit that the recipient gains (B_{R2}) from this transfer, thus not favoring such a transfer. In contrast, for a Depleted donor (D_2), its cost (C_{D2}) of transferring food to the Starved recipient is less than the benefit the recipient would gain from it (B_{R2}), leading to such a transfer being favored. This could potentially explain why Depleted donors were

seen interacting with the mimics corresponding to Starved individuals. However, the reason Fed donors (D_2) were seen interacting with the mimics representing Satiated recipients (R_1) seems more complicated since the latter stand to gain little (B_{R1}) from receiving any food while the cost to the donors for sharing any food is high (C_{D1}). We speculate that these interactions probably represent the food transfer between foragers and storers in the colony when food is shared with individuals of quality for the purpose of storage and colony benefit rather than individual benefit.

While most experimental work involving food sharing has focused on the benefits gained by the recipient, taking the energetic state of the donor into account can explain the apparent discrepancies in the direction of sharing that are seen sometimes (Grodzinski and Johnstone 2011). Studies about food sharing however rarely consider the fitness consequences of sharing for the donor, perhaps because recipient solicitation signals are usually regarded as stronger modulators of these interactions. A few studies involving birds have shown that parents adjust the amount of food provisioned to young based not only on the need of the young but also based on their own state (Tveraa et al. 1998; Thorogood et al. 2011). Here we provide evidence that a potential donor in a honeybee colony chooses a specific recipient for food sharing by concurrently assessing its own energetic state and the energetic state of the recipient that is communicated by its cuticular hydrocarbons.

Such a mechanism of food transfer has the potential to lead to a type of behavioral segregation in the colony where food sharing is restricted to individuals of similar

energetic states. Previous research has suggested that individuals infected with a pathogen suffer from energetic stress (Mayack and Naug 2009) and this could lead to infected individuals interacting more amongst themselves, which in turn could reduce the transmission of the pathogen to uninfected individuals. While it is important to note that information from other sensory modalities probably play additional roles in modulating food-sharing interactions (Goyret and Farina 2003; Mc Cabe et al. 2006), the use of chemical mimics allowed us to isolate the role of cuticular hydrocarbons and olfaction in modulating food sharing in the absence of all other stimuli.

FIGURES

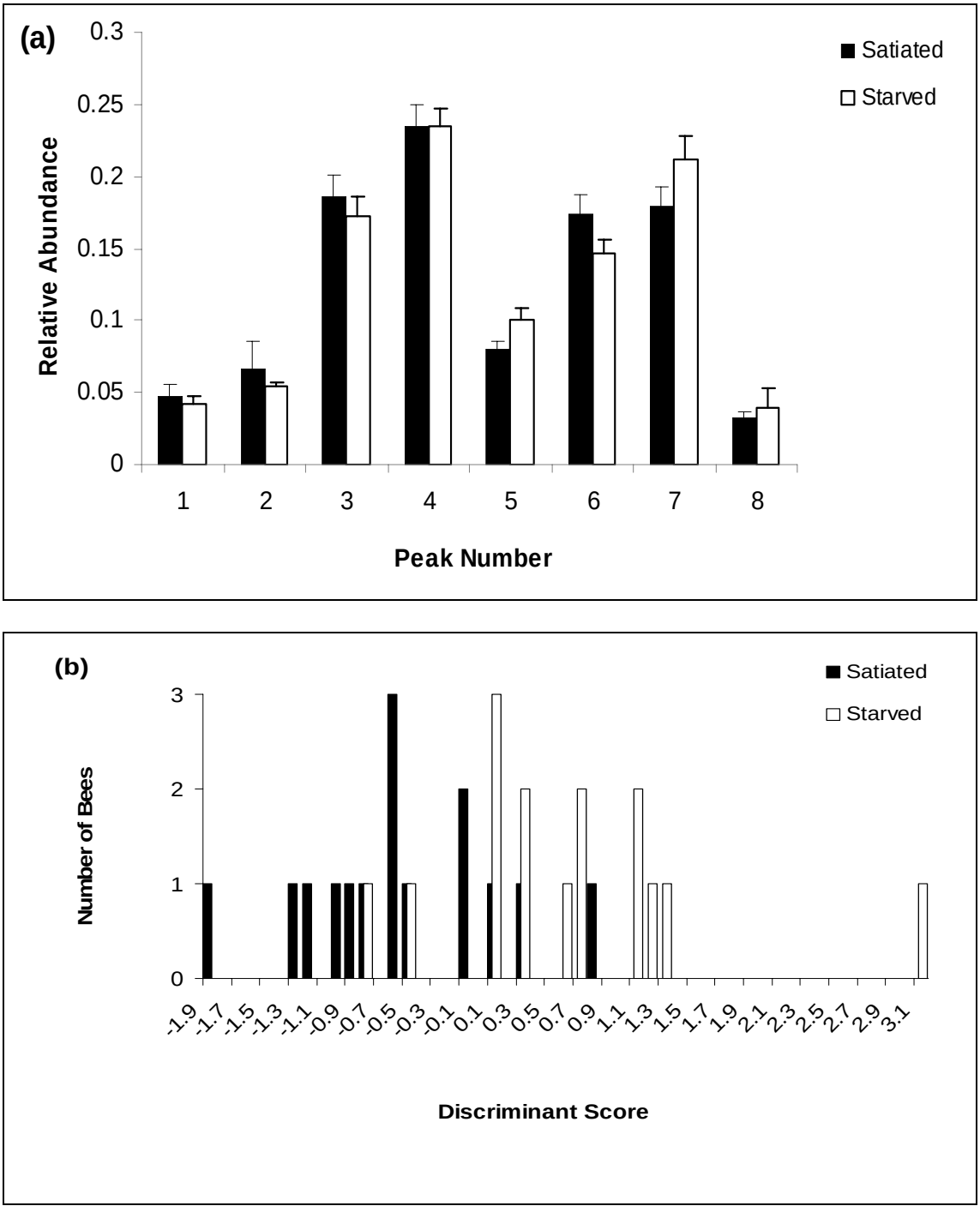


Figure 2.1. Cuticular hydrocarbon profiles for Satiated and Starved bees in terms of (a) Relative abundances of the eight most abundant cuticular hydrocarbons with data consisting of mean ± S.E., and (b) Frequency distribution of discriminant scores.

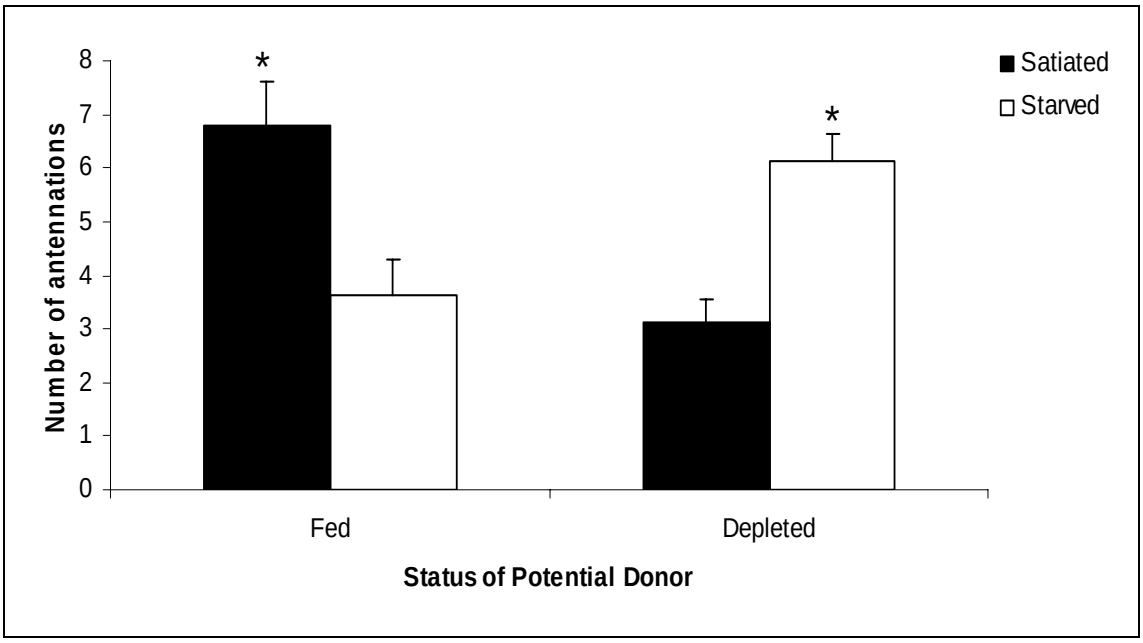


Figure 2.2. Behavior of donors toward Satiated and Starved mimics in terms of number of antennations performed by Fed (N = 18) and Depleted (N = 18) donors, with data consisting of mean (± S.E.) and asterisks denoting significant differences within each group.

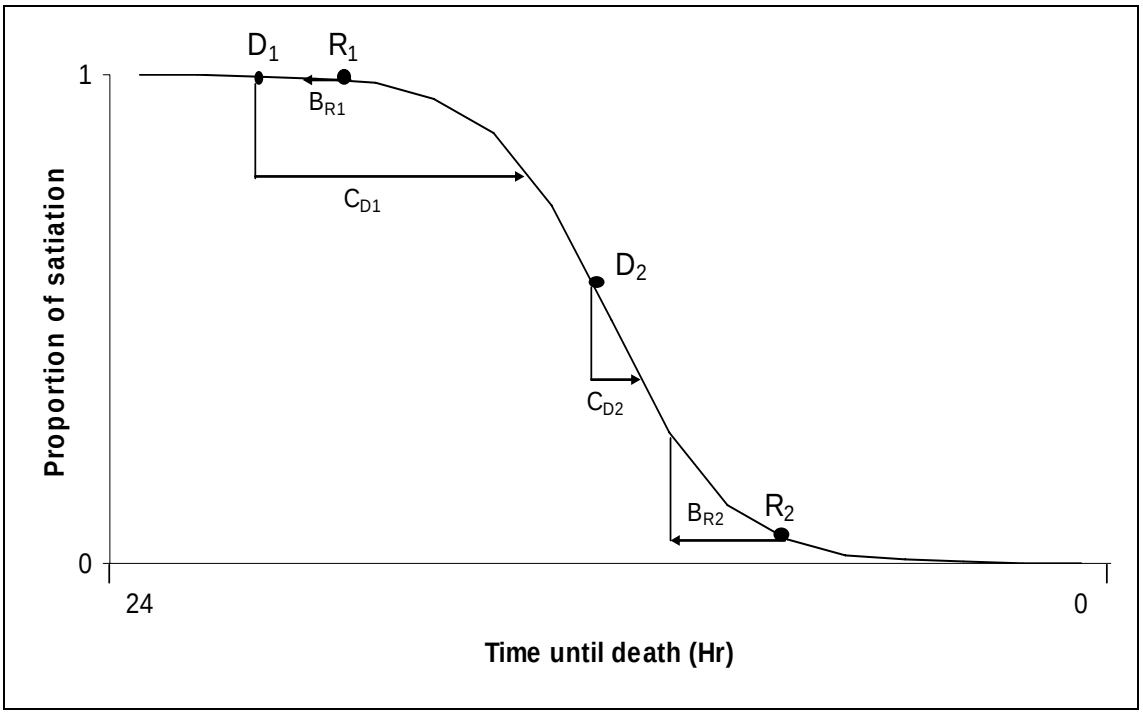


Figure 2.3. Theoretical function showing fitness of an individual bee as a function of its level of satiation over a period of 24 hours (derived from Mayack and Naug 2009) and the ensuing costs (C) and benefits (B) of food sharing interactions involving donors (D) and recipients (R) at different points on the function. The subscripts 1 and 2 denote donors and recipients at different points of the function, 1 corresponding to Fed donors and Satiated recipients and 2 corresponding to Depleted donors and Starved recipients. For an unbiased comparison between the two cases, the costs and benefits are calculated in each case assuming the same amount of food sharing.

REFERENCES

Aitchison, J (1986) The statistical analysis of compositional data. Chapman and Hall, London, England

Breed MD, Butler L, Stiller TM (1985) Kin discrimination by worker honey bees in genetically mixed groups. Proceedings of the National Academy of Sciences of the United States of America 82:3058-3061

Breed MD (1998) Recognition pheromones of the honey bee. Bioscience 48:463-470

Dreller C, Tarpy DE (2000) Perception of the pollen need by foragers in a honeybee colony. Animal Behaviour 59:91-96

Francis BR, Blanton WE, Littlefield JL, Nunamaker RA (1989) Hydrocarbons of the cuticle and hemolymph of the adult honey bee (Hymenoptera, Apidae). Annals of the Entomological Society of America 82:486-494

Free JB (1956) A study of the stimuli which release the food begging and offering responses of worker honeybees. British Journal of Animal Behaviour 4:94-101

Gnanadesikan R et al. (1989) Panel on discriminant analysis, classification, and clustering. Statistical Science 4:34-69

Godfray HCJ (1991) Signalling of need by offspring to their parents. Nature 352:328-330

Gordon DM (1996) The organization of work in social insect colonies. Nature 380:121-124

Goyret J, Farina WM (2003) Descriptive study of antennation during trophallactic unloading contacts in honeybees *Apis mellifera carnica*. Insectes Sociaux 50:274-276

Greene MJ, Gordon DM (2003) Cuticular hydrocarbons inform task decisions. Nature 423:32

Grodzinski U, Johnstone RA (2011) Parents and offspring in an evolutionary game: the effect of supply on demand when costs of care vary. Proceedings of the Royal Society B: Biological Sciences

Haig D (1990) Brood reduction and optimal parental investment when offspring differ in quality. American Naturalist 136:550-556

Howard DF, Tschinkel WR (1980) The effects of colony size and starvation on food flow in the fire ant, *Solenopsis invicta* (Hymenoptera: Formicidae). Behavioral Ecology and Sociobiology 7:293-300

Howard RW, Blomquist GJ (2005) Ecological, behavioral, and biochemical aspects of insect hydrocarbons. Annual Review of Entomology 50:371-393

Kent C, Azanchi R, Smith B, Formosa A, Levine JD (2008) Social context influences chemical communication in *D. melanogaster* males. Current Biology 18:1384-1389

Kolliker M, Chuckalovcak JP, Brodie Iii ED (2005) Offspring chemical cues affect maternal food provisioning in burrower bugs, *Sehirus cinctus*. Animal Behaviour 69:959-966

Lenoir A (1982) An informational analysis of antennal communication during trophallaxis in the ant *Myrmica-rubra* L. Behavioural Processes 7:27-35

Liang D, Silverman J (2000) "You are what you eat": Diet modifies cuticular hydrocarbons and nestmate recognition in the Argentine ant, *Linepithema humile*. Naturwissenschaften 87:412-416

Mas F, Haynes KF, Kolliker M (2009) A chemical signal of offspring quality affects maternal care in a social insect. Proceedings of the Royal Society B-Biological Sciences 276:2847-2853

Masterman R, Ross R, Mesce K, Spivak M (2001) Olfactory and behavioral response thresholds to odors of diseased brood differ between hygienic and non-hygienic honey bees (*Apis mellifera* L.). Journal of Comparative Physiology A 187:441-452

Mayack C, Naug D (2009) Energetic stress in the honeybee *Apis mellifera* from *Nosema ceranae* infection. Journal of Invertebrate Pathology 100:185-188

Mc Cabe S, Farina WM, Josens RB (2006) Antennation of nectar-receivers encodes colony needs and food-source profitability in the ant *Camponotus mus*. Insectes Sociaux 53:356-361

Mock DW, Dugas MB, Strickler SA (2011) Honest begging: expanding from signal of need. Behavioral Ecology 22:909-917

Pankiw T, Page RE, Fondrk MK (1998) Brood pheromone stimulates pollen foraging in honey bees (*Apis mellifera*). Behavioral Ecology and Sociobiology 44:193-198

Petfield D, Chenoweth SF, Rundle HD, Blows MW (2005) Genetic variance in female condition predicts indirect genetic variance in male sexual display traits. Proceedings of the National Academy of Sciences of the United States of America 102:6045-6050

Richard F-J, Aubert A, Grozinger CM (2008) Modulation of social interactions by immune stimulation in honey bee, *Apis mellifera*, workers. BMC Biology 6:50

Scholl J, Naug D (2011) Olfactory discrimination of age-specific hydrocarbons generates behavioral segregation in a honeybee colony. Behavioral Ecology and Sociobiology 65: 1967-1973

Schulz DJ, Vermiglio MJ, Huang ZY, Robinson GE (2002) Effects of colony food shortage on social interactions in honey bee colonies. Insectes Sociaux 49:50-55

Thomas ML, Simmons LW (2011) Short-term phenotypic plasticity in long-chain cuticular hydrocarbons. Proceedings of the Royal Society B: Biological Sciences 278:3123-3128

Thorogood R, Ewen JG, Kilner RM (2011) Sense and sensitivity: responsiveness to offspring signals varies with the parents' potential to breed again. Proceedings of the Royal Society B: Biological Sciences 278:2638-2645

Tveraa T, Sether B-E, Aanes R, Erikstad KE (1998) Regulation of food provisioning in the Antarctic petrel; the importance of parental body condition and chick body mass. Journal of Animal Ecology 67:699-704

Wagner D, Brown MJF, Broun P, Cuevas W, Moses LE, Chao DL, Gordon DM (1998) Task-related differences in the cuticular hydrocarbon compositions of harvester ants, *Pogonomyrmex barbatus*. Journal of Chemical Ecology 24:2021-2037

Whitlock MC, Davis BH, Yeaman S (2007) The costs and benefits of resource sharing: reciprocity requires resource heterogeneity. Journal of Evolutionary Biology 20:1772-1782

Wilkinson GS (1984) Reciprocal food sharing in vampire bat. Nature 308:181-184

Wilson EO, Hölldobler B (2005) Eusociality: Origin and consequences. Proceedings of the National Academy of Sciences USA 102:13367-13371