VARIATION IN AND RESPONSES TO BROOD PHEROMONE OF THE HONEY BEE (*Apis mellifera*)

A Dissertation

by

BRADLEY NORMAN METZ

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2009

Major Subject: Entomology

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Approved by:

Chair of Committee, Tanya Pankiw
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ABSTRACT

Variation in and Responses to Brood Pheromone of the Honey Bee (*Apis mellifera*).

(December 2009)

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Chair of Advisory Committee: Dr. Tanya Pankiw

Brood pheromone of the honey bee, (*Apis mellifera*) has been shown to elicit a wide array of primer and releaser effects on non-foragers and foragers leading to the regulation of nursing, pollen foraging, and behavioral development such that the behavior of the colony may be regulated by the amount and condition of the larvae. To date, all studies into the effects of brood pheromone have either used uncharacterized whole extracts or a single blend of brood pheromone characterized from a population of honey bees in France. The variation in the relative proportions of the ten fatty-acid ester components that characterize brood pheromone and some effects of this variation on pollen foraging and sucrose response thresholds were therefore observed. The objectives met in this dissertation were to determine whether changes in brood pheromone component proportions (blend) or amount communicates larval nutritional status and reports the results of observations of nurses and foragers in response to blends of brood pheromone from deprived and-non deprived larvae, to measure how

changes in brood pheromone blend changed pollen foraging behavior and if such changes could account for the pollen foraging differences between Africanized and European bees, and finally to observe the effects of exposure time on brood pheromone blend and to observe whether non-foragers made contact with the pheromone.

Brood pheromone was found to vary by larval rearing environment, but did not elicit the expected behaviors that would support a cue of nutritional status. Brood pheromone also varied significantly by mitochondrial lineage/population source and, responses to brood pheromone appeared to be coadapted to blend, suggesting that brood pheromone may be important in race recognition. Finally, brood pheromone varied significantly over time and was found to be removed from sources by bees, suggesting possible mechanisms for loss of effect. Combined the results of this research indicate that brood pheromone blend differences lead to profound changes in colony behavior related to pollen foraging and food provisioning, providing novel tools for colony manipulation and mechanisms for understanding brood rearing division of labor and chemical communication.

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TABLE OF CONTENTS

Page

Page

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

Page

CHAPTER I

INTRODUCTION

The unit of selection, that is, the minimum unit that will survive and reproduce, of the honey bee is the colony made up of approximately 10,000 individual organisms (Winston, 1987). These individuals are differentiated by reproductive capacity, with the queen being the sole layer of fertile eggs, but unable to build comb in which to lay them, or forage to collect resources to feed larvae once they hatch. The bulk of individuals are workers, which are sterile, but capable of building comb, defending the nest, feeding themselves, larvae, and the queen, as well forage outside the nest for resources. Workers are further subdivided into groups that specialize in repeatedly performing certain tasks over a period of time. This role is not static, rather, an individual progresses stereotypically from performing tasks near her emergence site, such as cleaning cells, sealing brood cells, and attending the queen or larvae, to more peripheral tasks such as collecting nectar from foragers, ripening it and processing it, to outside tasks such as ventilating the nest, guarding, and finally foraging (Winston, 1987). In general, workers can be separated into two broad groups: in-hive bees that

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care for the larvae, the queen, and the immediate internal needs of the colony, and foragers, that procure resources from the environment required for future survival, growth, and reproduction.

Of particular importance to this study are nurses and pollen foragers. Pollen is the principle protein resource of the honey bee and is essential to brood rearing (Haydak, 1935). Pollen foraging is usually initiated during the third week of adult life (Lindauer, 1953); they collect pollen from flowers onto specialized structures of the hind leg, termed corbiculae and then return to the nest where they seek out an empty or partially filled cell and pack the pollen into it (Calderone and Johnson, 2002). Foraging ontogeny is affected by genotype. This is especially apparent in the choice of whether to forage for pollen or non-pollen resources as there is a wealth of research on artificially selected strains of bees that forage preferentially for pollen or nectar (Hellmich et al., 1985; Page et al., 1998; Page et al., 2000; Pankiw and Page, 1999; Pankiw et al., 2002; Pankiw et al., 2001). The high pollen foraging strain of bees also develops more quickly than the low pollen foraging strain, initiating foraging at an earlier age (Pankiw and Page, 2001b). Additionally, Africanized bees, a naturally selected tropically adapted strain, are more likely to collect pollen and forage at a younger age than European bees, a temperately evolved strain (Danka et al., 1986; Guzmán-Novoa and Page, 2000; Pesante et al., 1987; Winston and Katz, 1982; Winston et al., 1983). Environmental factors such as amount of stored pollen and empty comb space, amount of honey, quantity and quality of resources in the environment also affect foraging

choice (Dreller et al., 1999; Dreller and Tarpy, 2000; Free, 1967; Page and Erber, 2002; Seeley, 1989; Winston, 1987). Further, amount of larvae also affects the choice to collect pollen (Al-Tikrity et al., 1972; Dreller et al., 1999; Eckert et al., 1994; Free, 1967; Jaycox, 1970; Winston and Fergusson, 1986).

Once pollen has been collected and returned to the nest, nurse bees, which are approximately 7-14 days old (Lindauer, 1953), consume and process pollen into proteinaceous secretions of the hypopharyngeal and mandibular glands that constitute brood food, which is then progressively provisioned to larvae over the course of their development (Haydak, 1957). Hypopharyngeal gland development does not occur in the absence of brood (Brouwers, 1982, 1983; Free, 1961; Huang and Otis, 1989). Nursing behavior can be altered by changing the amount of available pollen and larval demographics. Consumption of pollen in nurse-aged bees decreases as colonies are deprived of new larvae, but is restored a few days after addition of larvae to colonies previously deprived (Hrassnigg and Crailsheim, 1998). In colonies experimentally deprived of pollen, nurse bees adjust feeding towards older instar larvae, eventually cannibalizing young larvae in order to recycle the protein (Schmickl et al., 2003; Schmickl and Crailsheim, 2001, 2002).

Social life stabilized the rearing environment such that protection against predation, disease, resource dearths, and environmental instability allows approximately 85-95% of honey bee larvae to survive to adulthood (Fukuda and Sakagami, 1968). Stabilizing the feeding environment is especially important as larvae

grow very rapidly over the course of their 5-6 day development and even a short period of nutritional deprivation may result in developmental abnormalities and dwarfism in adults (Jay, 1964; Nelson and Sturtevant, 1924). Larvae isolated from contact with nurse bees are deprived of their sole source of nutrition and display unusual behaviors, such as crawling to the top of the cell (~ 12 hr after removal from the colony), eventually leaving the cell (~24 hr), and often dropping to the bottom of the hive where they die or are cannibalized by adults (*personal observations*). It is therefore crucial that nurse and forager feeding efforts are coordinated to the needs of the larvae to allow maximum growth and survival. All individuals of the colony do not contact the larvae directly, and a single nurse certainly doesn't contact each individual larva. Instead information about the larvae comes from chemical cues, or pheromones.

Pheromones are chemicals that are used for intraspecific communication and cause changes in behavior and physiology (Wyatt, 2003). Functionally, pheromones can be categorized into two broad categories, based on the elicited effects: releasers and primers. Releasers are behaviorally mediated, induce immediate effects that terminate after the pheromone is no longer present. Primers take effect only after an extended exposure and often result in physiological changes, such as modification of the glandular or neurosensory system. Primer effects are usually irreversible, even in the absence of a continued pheromone stimulus (Pankiw, 2004b; Pankiw and Page, 2003). Brood pheromone, a blend of the methyl and ethyl esters of palmitic (16:0), stearic (18:0), oleic (18:1 Δ^{9}), linoleic (18:2 $\Delta^{9, 12}$), and linolenic (18:3 $\Delta^{9, 12, 15}$) acid extractable from

the larval cuticle, is one of two characterized pheromones that elicit both primer and releaser effects, the other being queen mandibular pheromone (LeConte et al., 1990; Winston, 1987).

Brood pheromone generally communicates the presence of larvae in the colony and elicits a number of effects on both foragers and non-foragers. Brood pheromone treatment increases the proportion of returning pollen foragers, pollen load weights, and forage trip frequency (Pankiw, 2004a, 2007; Pankiw et al., 1998). Components of brood pheromone may induce cell capping by nurses (LeConte et al., 1990), increase the acceptance of queen cells placed into the colony (LeConte et al., 1994a), , and pollen consumption (Pankiw et al., 2008), all releaser effects.

Brood pheromone modulates age of foraging onset in a dose dependant manner (LeConte et al., 2001; Sagili, 2007) as well as the sucrose response thresholds of non-foraging bees (Pankiw and Page, 2001a). Brood pheromone may also increase the amount of protein extractable from the hypopharyngeal glands in the presence of queen mandibular pheromone (Mohammedi et al., 1996), inhibit worker ovary development (Mohammedi et al., 1998), and affect the amount of royal jelly provisioned (LeConte et al., 1995), all primer effects.

Prior to the initiation of this dissertation research, studies into the effects of brood pheromone either used uncharacterized larval non-polar extracts or a single blend of brood pheromone formulated from an indeterminate number of larvae and colony sources characterized from *A. m. ligustica* in France (LeConte et al., 1990;

LeConte et al., 2001; LeConte et al., 1995; Mohammedi et al., 1996; Pankiw, 2004a, 2007; Pankiw and Garza, 2007; Pankiw and Page, 2001a; Pankiw et al., 1998; Pankiw and Rubink, 2002; Pankiw et al., 2008; Sagili and Pankiw, 2009). Current understanding of the effects and context of brood pheromone is limited by a lack of knowledge of the variation of the relative proportion of the components (blend), the total amount and the relationship this variation on behavior. The total amount of extractable esters may increase with larval age (LeConte et al., 1994b; Trouiller et al., 1994; Trouiller et al., 1991). Also, changes in the proportions of methyl to ethyl esters may occur as larvae molt into prepuae (LeConte et al., 1994b). Variation in the relative proportions of individual components has also been shown among the different castes (Trouiller et al., 1994). However, published analyses of brood pheromone components are based on a single sample of worker larvae (LeConte et al., 1994b; Trouiller et al., 1994; Trouiller et al., 1991, 1992). There appears to be no sample replication and virtually no understanding of variation within or between colonies. The goal of this dissertation was to characterize the variation in brood pheromone and understand the social context and behavioral effect of this variation.

In chapter II, it was hypothesized that changes in brood pheromone communicated larval nutritional status. Larvae were deprived of nurse contact for four hours and the response of nurses to side-by-side patches of deprived and non-deprived larvae was observed. Non-volatile compounds from the larval cuticle were extracted and brood pheromone was characterized; testing the hypothesis that blend or amount

changed with nurse deprivation. The effect of nurse-deprived versus non-deprived larval chemicals was tested in a nursing bioassay, which tested the hypothesis that nurses aggregate over areas treated with larval chemicals or brood pheromone from deprived larvae relative to that from non-deprived larvae. The effect of deprived versus non-deprived brood pheromone blend was also measured in a pollen foraging bioassay.

In chapter III, it was hypothesized that brood pheromone varies with larval colony source. Brood pheromone was characterized from larvae from four different sources characterized by geographic location and mitochondrial DNA. The effects of brood pheromone source on foraging behavior was tested in two populations of bees using a pollen foraging bioassay; the first compared the pollen foraging response of European bees in Texas to brood pheromone characterized from the same population and the originally published blend of brood pheromone characterized from a population in France (LeConte et al., 1990; Pankiw and Page, 2001a), which tested the hypothesis that different blends from different honey bee sources do not differentially affect pollen foraging. The second compared the response of Texas-Africanized and Georgia-European colonies to brood pheromone characterized from Texas-Africanized and Georgia-European populations. The hypothesis tested was that Texas-Africanized brood pheromone elicits a higher proportion of returning pollen to non-pollen foragers than Georgia-European brood pheromone. It was also hypothesized that Texas-Africanized brood pheromone increases sucrose sensitivity of Texas-Africanized and

Georgia-European honey bees when compared to the Georgia-European blend of brood pheromone.

In chapter IV, it was hypothesized that foragers are relatively insensitive to sucrose response threshold priming by brood pheromone, compared to non-foragers. This may be due to behavioral avoidance of the pheromone, or due to physiological insensitivity. Therefore, brood pheromone was recovered from plates exposed to both foragers and non-foragers to observe whether the components were less recoverable over time in the presence of bees. It was also hypothesized that pheromone recovery changes over time exposed to the hive-like environment using a model system of glass plates, to observe whether exposure time altered the recovered blend such that it may be expected to elicit a different foraging response.

Honey bee larvae rely on foragers and nurses to collect food from outside the nest and feed them. The organization and optimization of the complex series of behaviors needed to accomplish these two goals are mediated in part by brood pheromone, a multi-component pheromone with multiple primer and releaser effects that are context specific. Results presented in this dissertation show variation of brood pheromone blend due to a number of different factors and suggest novel mechanisms for the regulation of brood rearing division of labor, the recognition of larvae, and larval nutritional status.

CHAPTER II

COMMUNICATION OF LARVAL NUTRITIONAL STATUS

INTRODUCTION

Considerable effort has been directed toward understanding when and how offspring should signal need for food and how parents should respond (Wright and Leonard, 2002). With a few exceptions, studies on food solicitation have focused primarily on vertebrates, with the majority of attention directed to bird family systems (Kilner and Johnstone, 1997; Royle et al., 2002; Wells, 2003). Parental care is not limited to birds and mammals or even vertebrates. Many insects, including those from the Hymenoptera, Isoptera, Coleoptera, Hemiptera, and Dermaptera, continuously provision their immature offspring with food (Clutton-Brock, 1991; Kölliker et al., 2006; Mas and Kölliker, 2008; Smiseth et al., 2003; Wilson, 1976). The larvae of social Hymenoptera are legless and depend on adult nurturing; because they undergo rapid growth in a short period of time (Jay, 1963; Thrasyvoulou and Benton, 1982), even a short period of deprivation can result in death of the larvae or dwarfism upon emergence (Haydak, 1970; Jay, 1964; Nelson and Sturtevant, 1924). Traditionally, larvae have been viewed as passive receptacles of adult provisioning, but a wealth of recent studies has provided

evidence that larvae play an active role in regulating their own development and the behavior of the colony as a whole.

Some ant larvae appear to vary in their nutritional need depending on size, caste, stages of development, as well as how long ago and how well they were last fed. For example, *Myrmica* workers preferentially feed deprived larvae with upward bending heads (Creemers et al., 2003). Kaptein et al. (2005) showed that *Gnamptogenys striatula* workers preferentially fed larvae that were near food and performing a swaying behavior. Food deprived larvae swayed more than well-fed larvae and, swaying did not move larvae closer to food sources (Kaptein et al., 2005). *Acromyrmex* leaf-cutting ant larvae isolated from food provisioning adults from 0 hr to 48 hr and returned to the colony at 12 hr intervals are more frequently fed than non-deprived larvae (Lopes et al., 2005). Longer isolation was positively correlated with more grooming behavior by workers (Lopes et al., 2005). Fire ant (*Solenopsis invicta*) larva feeding rate depends on size and hunger level of larva (Cassill and Tschinkel, 1995). Cassill and Tschinkel (1999) suggested that nonvolatile chemical cues signal hunger in fire ant larvae. Bumblebee (*Bombus terrestris*) workers provision deprived larvae earlier and more often than controls or experimentally fed larvae (Pereboom et al., 2003) and feed larvae treated with pentane extracts of deprived larvae more than those treated with non-deprived extract or control (den Boer and Duchateau, 2006). Signals communicating hunger can be found in various species of vespine wasps which scrape their mandibles against cell walls to attract the attention of workers with food and, non-

scraping larvae often reject food (Ross and Matthews, 1991). Salivary drops or anal secretions of some Hymenoptera larvae appear to contain valuable nutrients and have been implicated in attracting worker attention (Ross and Matthews, 1991; Wilson, 1976). Whether these substances induce feeding has not been demonstrated. The combined results suggest that some highly social insect larvae are, to some extent, able to produce signals of nutritional need and adults are able receive these signals and behave accordingly.

The chief difference between the offspring of most birds or mammals and those of the honey bee is that the immatures are reared in a highly social context with multiple generations displaying alloparental, cooperative brood care and adults are divided into reproductive and non-reproductive (or less reproductive) castes (Crespi and Yanega, 1995; Wilson, 1976). In a eusocial insect such as the honey bee, the collective effort of workers can stabilize the colony nutritional environment (Wheeler and Wheeler, 1979). This allows for rapid mass production of brood to achieve large colony populations of over 150,000 per year (Seeley, 1985). The work of colony growth and maintenance is divided and serialized among many specialized groups of individuals, particularly between foragers, that procure resources from outside the nest, and nurses which consume these resources and provision the brood (Winston, 1987). Many studies have shown that the presence of more larvae or adding larval cuticular chemicals known as brood pheromone stimulates increased pollen foraging (Al-Tikrity et al., 1972; Barker, 1971; Eckert et al., 1994; Hellmich and Rothenbuhler, 1986; Pankiw,

2004a, b, 2007; Pankiw and Page, 2001a; Pankiw et al., 1998; Pankiw and Rubink, 2002), a greater attraction to pollen storage areas in the colony, and greater consumption of pollen by nurse bees (Doull, 1974; Dreller et al., 1999; Dreller and Tarpy, 2000; Hrassnigg and Crailsheim, 1998; Taber, 1973; Winston and Fergusson, 1986). Also, nurses have been shown to alter feeding behavior in an age-dependent manner under pollen limiting conditions; decreasing provisioning to young larvae while maintaining rates of provisioning to old larvae (Schmickl et al., 2003).

In contrast to colony-level studies there is a paucity of studies on individual variation of larva feeding by nurse bees. Often cited statistics are that a larva may be inspected up to 30-40 times per day, and fed as much as 140 times during her tenure as a larva (Lindauer, 1953). Because honey bees store pollen and honey buffering the colony against changes in the foraging environment it is a common assumption that the nutritional environment for larvae is uniform. Due to age-related division of labor in the honey bee, the nursing of brood and the allocation of nutrients among them are performed by thousands of bees in parallel. But many of the tasks are also serialized among different workers in a production-line process. The collective behavior in total can be seen as a set of parallel or serially linked processes, leading to a network of linked tasks. The nursing subtasks are: locating brood, determining the status of the brood (=cell inspection), feeding larvae, locating pollen cells, eating and digesting pollen to produce brood food, and capping pupal cells (Schmickl et al., 2003). It is reasonable to hypothesize that in such a system there is 1) natural variation in nursing

attendance on individual larvae leading to feeding deficits, and if so, 2) larvae may communicate their nutritional status such that, 3) nurses respond accordingly, as well as 4) pollen foragers. These predictions were tested by observing nursing variation in un-manipulated observation hives, and further observed nurse response to side-by-side patches of deprived and non-deprived larvae. It was hypothesized that larval chemical cues, and specifically changes in brood pheromone blend, communicate nutritional status. Brood pheromone from deprived and non-deprived larvae was characterized and a nursing bioassay and pollen foraging bioassay was performed to test this.

MATERIALS AND METHODS

Nursing time variation

Five observation hives containing a queen, approximately 2000 bees, and equal amounts of brood, honey pollen, and empty space were installed and five, five-day-old larvae were randomly chosen from each hive for observation. The larvae were recorded with a digital camera (Sony, New York, NY, USA) continuously for 1 hr. Nurse feeding bouts defined after Schmickl et al., (2003) as head and thorax insertion into the larval cell for ≥3 sec were observed and timed. The time nurses spent feeding was summed for each larva to estimate total time fed in 1 hr. This was repeated 5 times within each colony for a total of 25 larvae observed. I hypothesized that if nurses were

equally provisioning larvae, then the frequency distribution of total feeding time would be uniform. Alternatively, the distribution could show variation, and therefore, suggest that same-staged larvae may be reared under differing short term nutritional conditions. Specifically, it was hypothesized that the variance of the observation times was greater than zero with Student's T-test (Sokal and Rohlf, 1995; SPSS, 2007).

Effect of deprivation on survival and weight

The queen of a single colony was caged on one side of a frame for approximately 24 hrs, after which she was removed from the frame and the cage replaced to isolate her from laying further eggs on the frame. Four days after the eggs hatched, half of the frame was deprived of contact with adults for 4 hrs using a 3 mm wire mesh cage pushed into the wax. The other half was not manipulated. Frame halves were photographed with a digital camera (Sony, New York, NY, USA) and larvae were counted. The frame was returned to the colony and larvae were allowed to develop for 2 wks. The frame was then removed from the colony and cut in half. The halves were stored in an incubator at 33° C, 55% RH for 2 days until bees emerged. Bees emerged over 24 hrs and were collected and freeze-killed at -20° C. Counts of larvae and emerged bees were analyzed by 2x2 chi-square contingency table analysis to test for a difference in emergence by deprived vs. non-deprived treatment (Sokal and Rohlf, 1995; SPSS, 2007). A subsample of 49 bees from each treatment was selected for measurement of emergence weights.

Bees were immediately weighed and then placed into a drying oven at 47° C for 12 days and subsequently weighed again. Wet and dry weights were analyzed by Mann-Whitney U to test for differences by larval treatment.

Nursing response to deprived and non-deprived larvae

The experiment was replicated 6 times consisting of a single observation colony prepared as above and allowed 2 wks to acclimate. Side-by-side areas containing approximately 500 4^{th} instar larvae were selected and wire mesh cages of 169 cm^2 were pushed into the wax surrounding the larvae. Two mesh sizes were utilized, 13 mm, which did not impede adult access to larvae and served as a control and 3 mm, which restricted adult bee access and served as a deprived treatment. Larval areas were caged for 4 hrs, after which the hives were briefly opened, treatment areas cleared of bees with light puffs of smoke, and the cages removed. Treatment areas were then recorded for 1 hr with a digital camera (Sony, New York, NY, USA).

Nurse bee observations

Screen captures of the digital video were created using Pinnacle Studio v. 12.0 (Avid Technology, Mountain View, CA). Initial captures were taken that did not contain bees and used to count the number of larvae in each area. Counts of bees present over

deprived and non-deprived areas were taken at 5 min intervals subsequent to cage removal. Counts were transformed to proportion of bees to larvae and analyzed by linear regression using the following exponential rise-to-maximum equation: $Y = a(1 - e^{-b(x)})$ where Y represented the transformed bee count, x represents time in minutes, a represents the maximum limit, and b represents the rate of increase. I then used Student's T-test to test the hypotheses that more bees arrived to the deprived treatment areas ($a_{deprived} > a_{non\text{-}deprived}$) and that bees arrived more rapidly to deprived treatment areas ($b_{deprivd} > b_{non\text{-}deprived}$; Sokal and Rohlf, 1995; SPSS, 2007).

Larva observations

Six larvae were randomly selected from each treatment area for further observation. Larvae were observed continuously for 30 min subsequent to cage removal for nurse feeding, defined as head and thorax insertion for ≥3 sec, and inspection, defined as head and thorax insertion of <3 sec. Further observations were also recorded: time to first feeding, time to first inspection, total number of feeding bouts, total number of inspections, duration of feeding bout and total time fed. Time to first feeding and time to first inspection were log-transformed and analyzed by ANOVA (Sokal and Rohlf, 1995; SPSS, 2007). Total number of feeding bouts and total number of inspections were analyzed by Chi-square (Sokal and Rohlf, 1995; SPSS, 2007). Mean feeding bout

duration was analyzed by Mann-Whitney U (Sokal and Rohlf, 1995; SPSS, 2007). Total time fed was analyzed using ANOVA.

Characterization of brood pheromone of deprived and non-deprived larvae

Larva collection and extraction

A single frame containing larvae was selected and bees were removed. One half of the frame was randomly selected to be deprived of adult contact as above with a 3 mm mesh cage, the other half was covered with at 13 mm mesh cage that did not restrict adult access. The frame was returned to the colony for 4h after which the frame was taken to the lab for processing. Each frame represented a single replication and the experiment was replicated 10 times. Larvae were extracted from frames with short pulses of room temperature water. Ten larvae were selected, staged by size and morphology (Dade, 1977; Thrasyvoulou and Benton, 1982), weighed and pooled for extraction. Larvae were immersed into 10 ml beakers containing 2 ml 95% n-hexane containing 1.0 μg of >99% purity methyl myristate (Sigma-Aldrich, St. Louis, MO, USA) as an internal standard for 1 min. Extracts were filtered through a Buchner funnel and the beaker was rinsed with a further 2 ml 95% n-hexane which was added to the extract. Extracts were then chilled at -20° C for 10 min to remove any water and water soluble

compounds. Extracts were collected into 15 ml conical vials (VWR, West Chester, PA, USA) and evaporated at 55° C under low nitrogen stream to 1 ml for fractionation.

Sample fractionation

Extracts were fractionated using columns constructed of 4 mL glass pipettes (Fisher Scientific, Waltham, MA, USA) plugged with a small piece of Kimwipe® (Kimberly-Clark, Neenah, WI, USA) and packed with 70-230 mesh Silica gel 60A (Sigma-Aldrich, St. Louis, MO, USA). Columns were rinsed with 10 mL each of ethyl acetate, dichloromethane, and n-hexane. Extracts were added and fractionated using 10 mL n-hexane, 99.9% dichloromethane (Sigma-Aldrich, St. Louis, MO, USA), and ethyl acetate (99.9% purity, Sigma, St. Louis, MO, USA). The dichloromethane fraction contained all brood pheromone esters. Fractioned extracts were then evaporated to dryness at 40° C under nitrogen stream and reconstituted in 1 ml hexane. Fractioned extracts were then vortexed for 15 sec at 2000 rpm and then dried at 55° C under nitrogen and reconstituted in 0.5 ml hexane. Samples were vortexed again 15 sec at 2000 rpm and dried at 55° C and reconstituted into 0.1 ml hexane and transferred into 300 μL chromatographic vials (Alltech, Deerfield, IL, USA). Conicals were rinsed twice with 0.1 ml hexane which was added to the sample. Samples were dried under nitrogen and reconstituted with 10 μL n-hexane containing 1.0 μg octadecane (Sigma-Aldrich, St. Louis, MO, USA) as a secondary standard.

Gas chromatography

An HP6890 gas chromatograph (Agilent, Santa Clara, CA) with splitless programmable temperature vaporization injection and flame ionization detection was used for sample analysis. 1.0 μL was injected onto an HP-88 60 m X .251 mm ID column ((88%-cyanopropyl)-methylarylpolysiloxane from Agilent, Santa Clara, CA). Inlet temperature was held at 60° C for 0.10 min and then increased to 250° C at 500 C/min. Oven temperature was held at 50° C for 2 min, then increased at 20° C/min to 170° C, held for 3 min, increased at 30° C/min to 230° C and held at the final temperature for 6 min. Carrier gas was pure hydrogen at 2 ml/min. Individual fatty acid ester retention times were identified using > 99% purity standards (Sigma-Aldrich, St. Louis, MO). To estimate quantities, the areas beneath the peaks of known amounts of each ester were calculated using GC Chemstation software version B.01.03.204 (Agilent, Santa Clara, CA). These data were used to generate a standard curve for each fatty acid ester expressed as a first order regression equation. Quantified esters were corrected by proportionate error of the octadecane amount to correct for machine error and evaporative concentration, and subsequently adjusted by proportionate error of methyl myristate amount to account for methodological loss.

Statistical analyses

Weight of larvae selected did not differ significantly by treatment ($F_{1,19}$= 0.818, p=0.378) with a mean larval weight of 0.107±0.005 g for non-deprived and 0.112±0.002 g for deprived larvae. However, because weights ranged from 0.085-0.140 g all ester amounts were transformed into nanograms per gram of larvae (ng/g). Transformed amounts were then summed. Although the total ester values represented proportions, each value represented a different larval pool. Therefore, each total ester proportion was independent of all others. Total esters were analyzed using Welch's test (Ruxton, 2006; Sokal and Rohlf, 1995; SPSS, 2007). Individual ester amounts were then transformed as a proportion of total esters. Proportions were independent between samples and treatments and were subsequently analyzed using Welch's test for differences by deprived vs. non-deprived treatment.

Blend formulation

Mean ester proportions were then used to formulate synthetic deprived and non-deprived blends of brood pheromone (Table 1). The amount of total esters differed between deprived and non-deprived larvae; therefore the average amount extractable from an average weight larva was calculated for each treatment. This amount is termed larval equivalent (LEq). 1 LEq for the non-deprived blend was 24.66 ng while 1 LEq for

the deprived blend was 8.37 ng. Synthetic esters were all >99% purity and were added by volume (for liquids) or mass (for solids). Combined esters were dissolved in 95% n-hexane for experimentation.

Table 1. Formulation of deprived and non-deprived brood pheromone blends

Ester	Percent	
	Non-deprived	Deprived
methyl palmitate	5.0	5.0
ethyl palmitate	3.0	2.0
methyl stearate	14.0	9.0
ethyl stearate	4.0	3.0
methyl oleate	19.0	17.0
ethyl oleate	15.0	33.0
methyl linoleate	3.0	3.0
ethyl linoleate	4.0	5.0
methyl linolenate	16.0	9.0
ethyl linolenate	18.0	13.0

Nurse aggregation bioassay

It was observed in previous experiments that nurses aggregated over deprived larval areas in greater numbers than over non-deprived areas (*personal observation*). It was unclear whether this was because nurses simply spent more time performing nursing behaviors in deprived areas or because the nurses were induced to aggregate by larval

cues, I constructed a chamber in which to measure nurse bee aggregation in response to deprived and non-deprived larval chemical cues.

Bioassay chamber construction and treatment

A 5-sided acrylic glass chamber was constructed to snugly fit a standard 96-well microtiter plate (VWR, West Chester, PA, USA) with a space of 9.5 mm above the wells. A fresh, sterile microtiter plate was treated with 2 μl concentrated extract as follows: 4-well columns of non-deprived and 4-well columns of separated by 4 columns of solvent (95% n-hexane) treated wells. Left-right orientation of treatments was randomized, but solvent treated wells always separated the treatments. Solvent was allowed to dry for at least 5 min while bees were placed into the bioassay chamber.

Fifty bees were collected from a single colony from frames containing larvae and returned to the lab, cold anesthetized for 5-10 min at -20° C. Anesthetized bees were counted into the bioassay chamber and the treated microtiter plate was then added. The bioassay chamber was placed in an incubator at 55% RH, 33° C and illuminated with red light. Bees were active in about 5 min and a digital camera was then started to record behaviors for 1 hr.

Response to deprived and non-deprived whole larval extract

This experiment was replicated 12 times. Side-by-side patches of 200 larvae were caged for 4 hrs as above. Larvae were collected and soaked in approximately 20 ml 95% n-hexane (Sigma-Aldrich, St. Louis, MO, USA) for 1 min. Extracts were filtered through a Buchner funnel (VWR, West Chester, PA, USA) then concentrated to 200 µl under nitrogen stream and stored in sealed chromatography vials at -20° C until use. A single, different colony source was used for each replication.

Response to deprived and non-deprived synthetic brood pheromone

The experiment was also replicated 12 times. Wells were treated with 4 LEq deprived or non-deprived blend brood pheromone formulated as in Table 1 or an equal volume no-pheromone control.

Statistical analyses

To eliminate zeros, 0.10 was added to count data to allow for analysis by Pearson's chi-square test used to test for differences nurse bee cluster counts over the three different treatment areas (Sokal and Rohlf, 1995; SPSS, 2007). To ensure that the total Type I error

of the experiment conformed to 0.05, the Dunn-Šidák method was used (Sokal and Rohlf, 1995).

Pollen forager bioassay

Response to deprived and non-deprived whole larval extract

Extract of deprived and non-deprived larvae was collected similarly to that in the laboratory nursing bioassay with the exception that extracts from multiple colonies were pooled. Six replications were performed each consisting of a recently installed, broodless colony of approximately 4,000 bees provided one of three treatments in a random order over the course of three days: extract of 500 deprived larvae, extract of 500 non-deprived larvae, or an equal volume of 95% n-hexane. Extracts or solvent were provided on glass plates measuring 14 cm x 7 cm and allowed at least 5 min to dry. A single plate was then hung on a wire between two frames in the center of each colony. One hour after inserting the plates, colony entrances were observed for 5 min for the number of returning pollen and non-pollen foragers (*as per* Pankiw et al., 1998). Pollen foragers return with distinct pellets of pollen on their hind legs in specialized structures, called corbiculae. All treatment and observations occurred in the morning between 8:00 and 11:00 hrs.

Response to deprived and non-deprived blends in extracted amounts

The experiment was performed as above with the following exceptions: nine replications were performed, 2000 LEq of deprived (16.74 μg) or non-deprived (49.32 μg) blend brood pheromone formulated as in Table 1 or an equal volume no-pheromone control was applied to colonies.

Forager response to deprived and non-deprived blends in equal amounts

This experiment proceeded identically to the previous with the exception that plates were treated with 49.32 μg of non-deprived or deprived brood pheromone, or an equal volume of control.

Statistical analyses

Effects of deprived or non-deprived extract were analyzed by 3x2 chi-square contingency table analysis (Sokal and Rohlf, 1995; SPSS, 2007). Pairwise comparisons were performed using 2x2 chi-square contingency table analysis with the Dunn-Šidák method as above.

RESULTS

Nursing time variation

Distribution of feeding times differed from uniform over 1 hr of observation (Z=-4.73, 124 df, $p<0.0001$). Mean feeding time was 6.82±0.68 min with a range of 36.57 min and a median of 3.86 min (Fig. 1).

Effect of deprivation on survival and weight

Adult emergence proportion was not significantly affected by deprived vs. non-deprived larval treatment (χ^2= 1.299, 2 df, p=0.254; SPSS, 2007). Deprivation did not significantly affect bee emergence wet weight (Z= -0.911, 98 df, p=0.362; SPSS, 2007) with a mean of 98.31±1.35 mg for deprived and 100.82±1.120 mg for non-deprived larvae. Dry weights did not differ by treatment (Z= -1.639, 98 df, p=0.101).

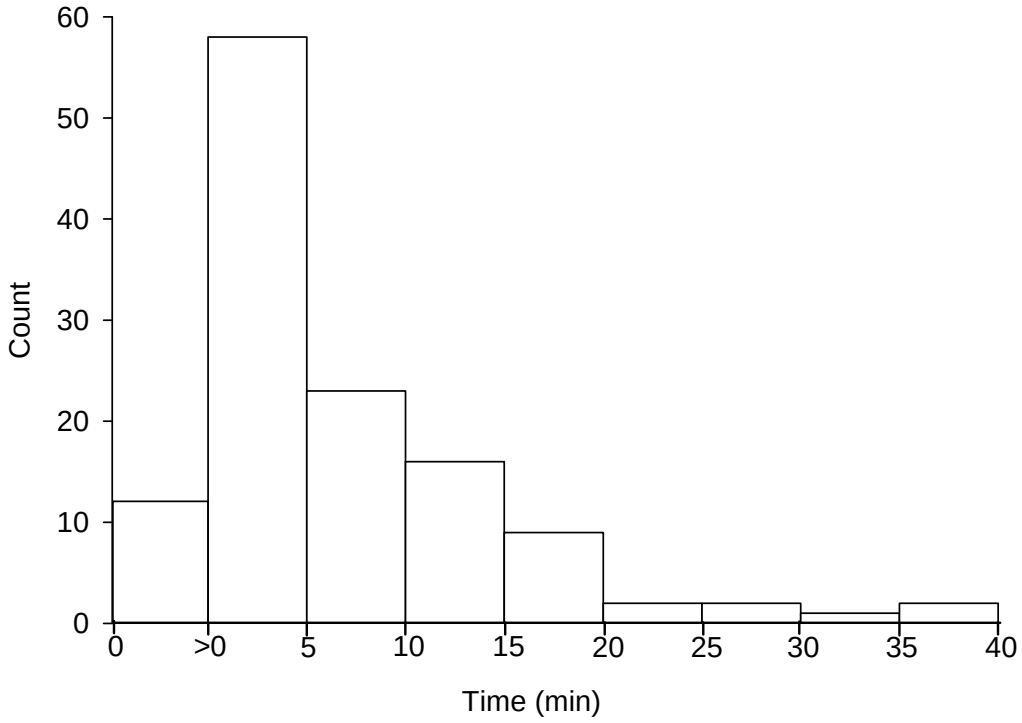

Fig. 1 Distribution of feeding times of 125 larvae in 1 hr. Standard error was significantly different from zero ($p<0.05$) and 10% of larvae were not fed at all, over 40% were fed for under 5 min, and 10% fed for 30 min or more

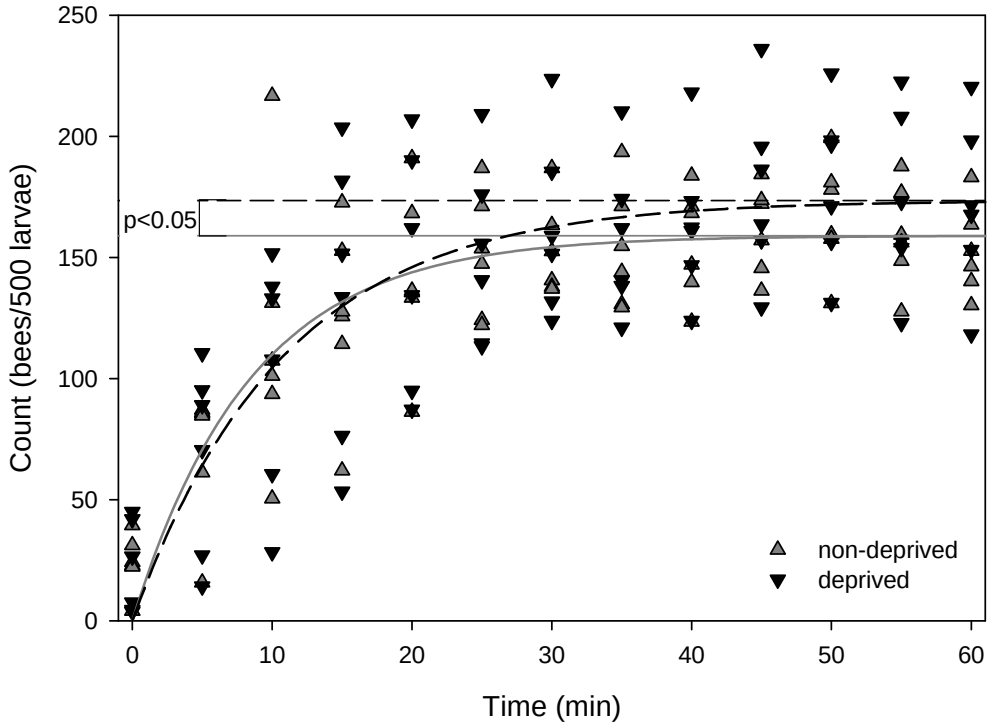

Fig. 2 Accumulation of bees over deprived and non-deprived larval treatment areas over 1 hr. The solid lines represent the regression of non-deprived (R^2= 0.679) and the dotted line, deprived (R^2= 0.605). Regression followed the equation $Count = a(1 - e^{-b(time)})$: *a,* the maximum proportion of bees/500 larvae differed significantly by treatment ($p<0.05$), however *b,* the rate of accumulation did not differ among the treatments

Nursing response to deprived and non-deprived larvae

Nurse bee behavior

The non-linear regression model described the non-deprived data set with an R^2= 0.679 ($F_{1,76}$=159.2, p<0.0001; SPSS, 2007) and the deprived data set with an R^2= 0.605 ($F_{1,76}$=116.2, p<0.0001). The *a* maximum limit parameter was significantly different among the treatments with a mean of 158.91±5.37 for non-deprived and 173.54±6.19 for deprived (Z=-2.513, 10 df, p=0.031; SPSS, 2007; Fig. 2). The *b* rate of increase parameter was not significantly different among the treatments with a mean of 0.118±0.019 for non-deprived and 0.092±0.014 for deprived (Z=1.534, 10 df, p=0.156; Fig. 2).

Time to first feeding and inspection

Time to first feeding differed significantly by replicate ($F_{5,5}$= 5.282, p= 0.046; SPSS, 2007) however there was no significant replicate by treatment interaction ($F_{5,60}$= 0.971, p= 0.443; SPSS, 2007), therefore pooling the replicates for analysis was justified. Time to first feeding differed significantly by treatment ($F_{1,5}$= 6.853, p= 0.047; SPSS, 2007; Fig. 3) with a mean of 12.62±1.6 min for non-deprived and 7.78±0.88 min for deprived larvae.

Replications were not significantly different ($F_{5,5}$= 4.458, p= 0.063) among time to first inspection data therefore all replicates were pooled for further. Time to first

inspection differed significantly by deprived versus non-deprived treatment ($F_{1,70}$= 4.988, p= 0.029) with a shorter time to first inspection for deprived (2.94±0.45 min) compared to non-deprived larvae (4.09±0.60 min; Fig. 3).

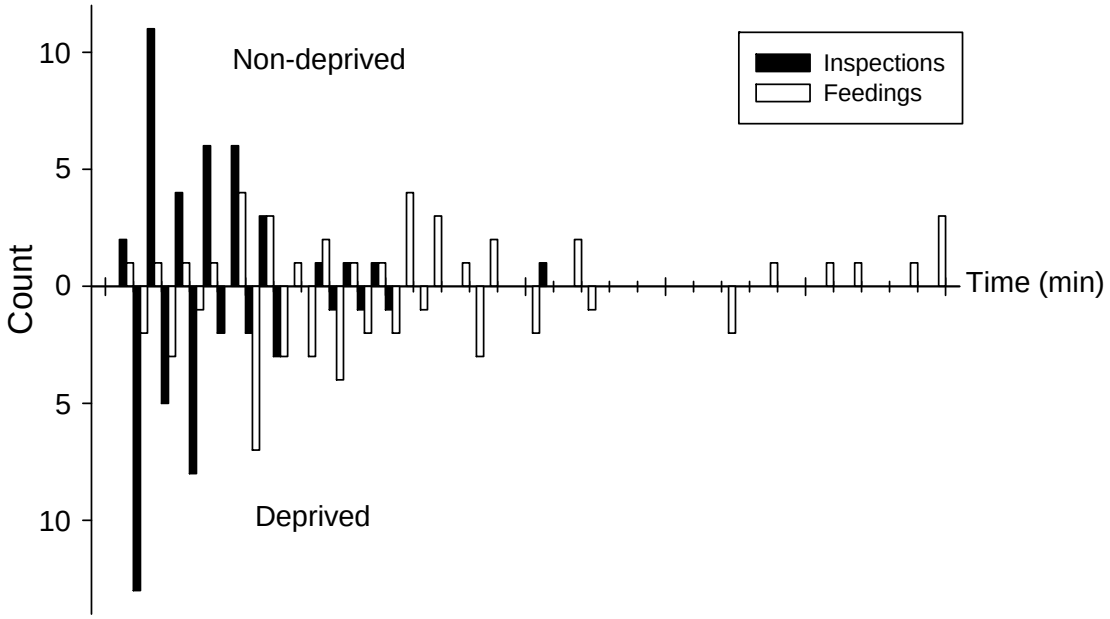

Fig. 3 Distribution in time to first inspection and the time to first feeding. Non-deprived larvae occupy the positive Y-axis and the deprived larvae the negative Y-axis. Both time to first inspection and time to first feeding were significantly shorter for the deprived larvae compared to the non-deprived larvae ($p<0.05$)

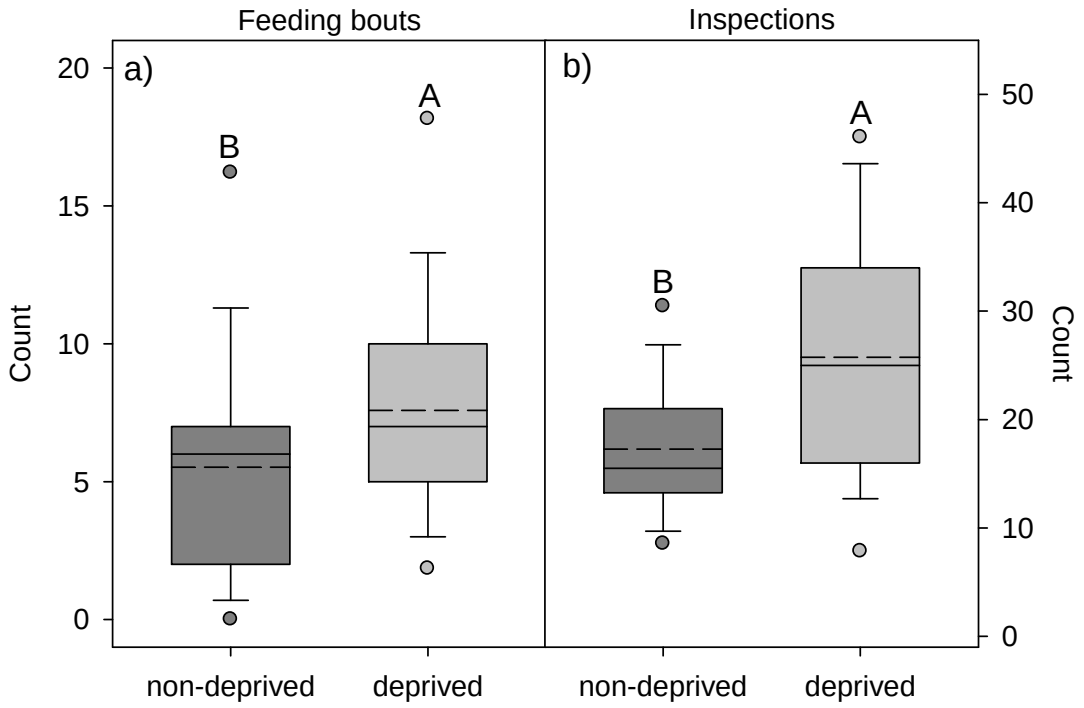

Fig. 4 Total number of feeding bouts (4a) and inspections (4b) observed in 30 min. Both feeding bouts and inspections were significantly different by treatment ($p<0.05$).Boxes enclose the 25th and 75th percentile while the whiskers represent the 10th and 90th percentile and outlier points indicate the 5th and 95th percentile. Solid lines represent the median, while dotted lines represent the mean. Capital letters denote statistical significance ($p<0.05$) separately for each figure part

Total number of feeding bouts and inspections

Feeding bouts differed significantly by treatment (χ^2= 11.602, 1 df, p= 0.001), with mean number of feeding bouts greater for deprived (7.58±0.68) as compared to non-deprived larvae (5.53±0.79; Fig. 4a). Inspection counts differed significantly by treatment (χ^2=

60.055, 1 df, $p < 0.001$), with mean number of inspections greater for deprived larvae (25.75±1.9) as compared to non-deprived (17.28±1.0; Fig. 4b).

Duration of feeding bout

Feeding bout durations differed significantly from a normal distribution (Kolmogrov-Smirnov, $Z_{470} = 6.194$, $p<0.0001$; SPSS, 2007). However, homogeneity of variance of the ranked values were not significantly different (Levene's, $F_{1,469}=2.893$, $p=0.090$; SPSS, 2007). Therefore, Mann-Whitney U was used to test for homogeneity of distribution in duration of feeding bout between deprived and non-deprived larvae (SPSS, 2007). Distributions were not significantly heterogeneous among the treatments ($Z_{470} = -0.150$, $p=0.881$; SPSS, 2007) suggesting that duration of feeding did not differ between the two treatments with a mean duration of 16.74±1.5 sec for non-deprived and 18.94±1.6 sec for deprived larvae (Fig 5a).

Total time fed

There was no significant effect of replication on total time fed ($F_{5,5} = 2.757$, $p = 0.145$), consequentially replicates were pooled for further analysis. There was a significant effect of deprived versus non-deprived treatment on total time fed ($F_{1,70}$=

6.892, p= 0.011), such that total time fed was greater for deprived (2.39±0.25 min) versus non-deprived larvae (1.52±0.22 min; Fig. 5b).

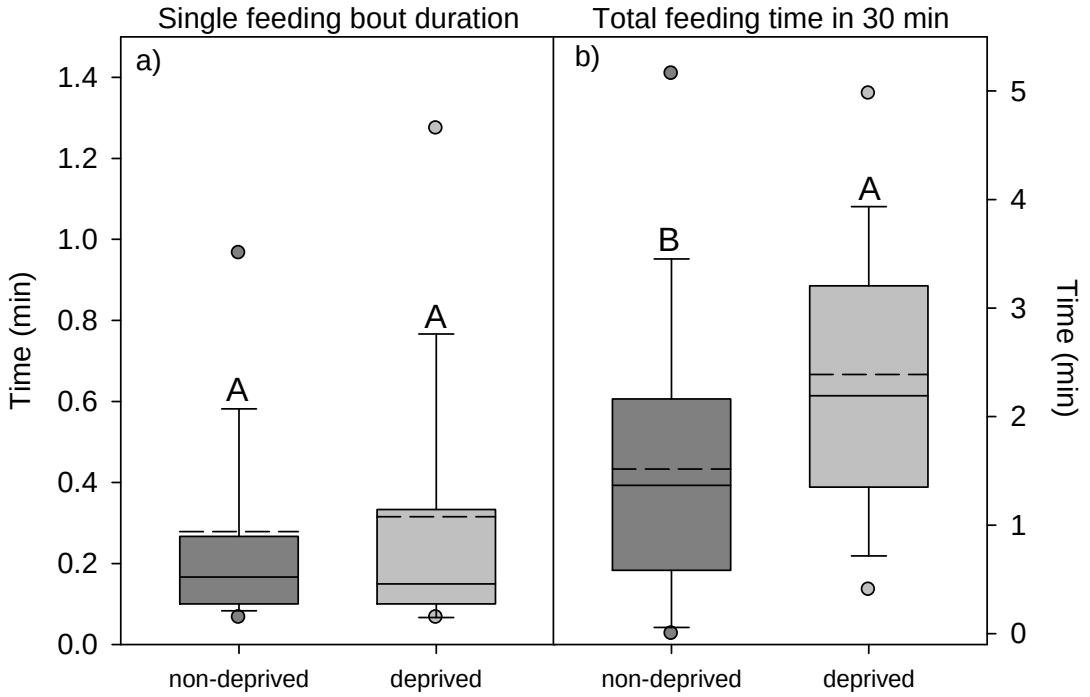

Fig. 5 Duration of a single feeding bout (5a) and the total cumulative feeding time in 30 min (5b). Single feeding bout duration and total feeding time were both significantly different ($p<0.05$). Boxes enclose the 25th and 75th percentile while the whiskers represent the 10th and 90th percentile and outlier points indicate the 5th and 95th percentile. Solid lines represent the median, while dashed lines represent the mean. Capital letters denote statistical significance ($p<0.05$) separately for each figure part

Characterization of brood pheromone of deprived and non-deprived larvae

Total amount of esters was normally distributed (Kolmogorov-Smirnov; Z= 0.677, N=20, p=0.748; SPSS, 2007) and variances were homogenous (Levene's; $F_{1,18}$ = 2.476, p=0.133; SPSS, 2007). Significantly greater amounts of brood pheromone was extractable from non-deprived larvae (246.6±36.5 ng) compared to deprived larvae (83.7±17.0 ng; Welch's, $F_{1,12.73}$= 16.362, p=0.001; SPSS, 2007).

Proportions of each ester were normally distributed (Kolmogorov-Smirnov; p>0.05) except for ethyl linolenate (Kolmogorov-Smirnov; Z= 1.463,N=20, p=0.028) which was natural log transformed to conform to normality (Kolmogorov-Smirnov; Z= 0.981,N=20, p=0.218) and variances were homogenous between treatments (Levene's; p>0.05) excepting methyl oleate (Levene's; $F_{1,18}$= 7.262, p=0.015) and ethyl oleate (Levene's; $F_{1,18}$= 13.979; p=0.002) . The proportions of methyl stearate (Welch's; $F_{1,16.282}$= 5.629, p=0.030) and ethyl oleate (Welch's; $F_{1,12.749}$= 4.769, p=0.048) differed significantly between deprived and non-deprived larvae. All other ester proportions were not significantly different (Fig. 6).

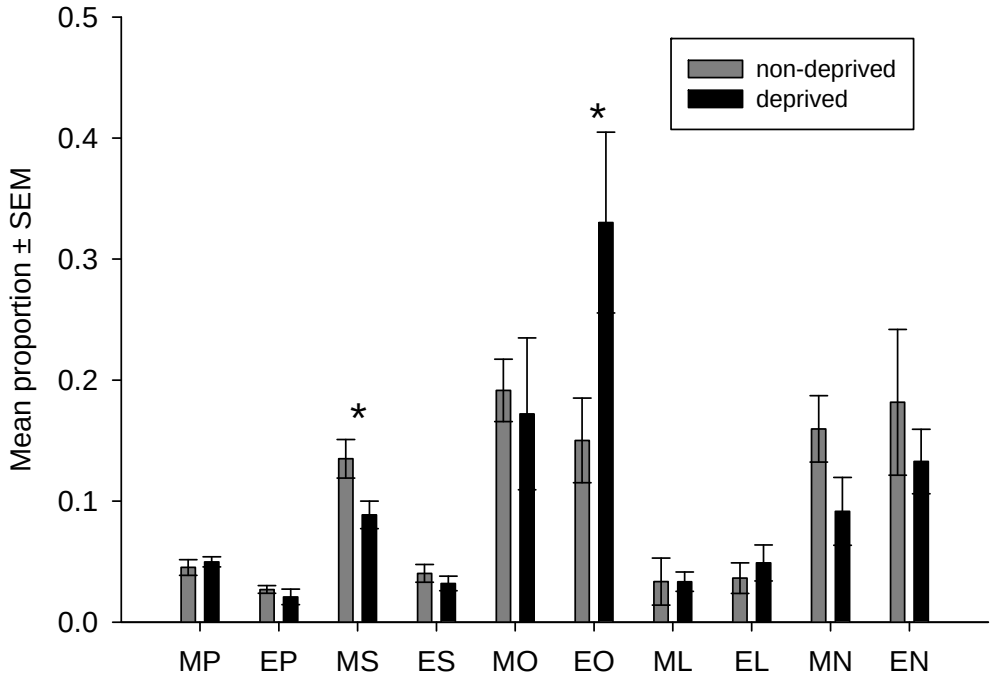

Fig. 6 Proportion of fatty acid esters of brood pheromone extractable from the cuticle of non-deprived or deprived larvae. Bars are labeled with the acronyms for the (M)ethyl and (E)thyl esters of (P)almitic, (S)tearic, (O)leic, (L)inoleic, and (L)inolenic acids. Asterisks denote statistically different ($p<0.05$) proportions among non-deprived and deprived larval extracts

Nurse aggregation bioassay

Response to deprived and non-deprived whole larval extract

Nurse bee counts were highest over wells treated with deprived extract compared to wells treated with non-deprived extract ($\chi^2= 22.387$, 1 df, $p<0.001$; SPSS,

2007; Fig. 7a) and solvent control (χ^2= 206.9, 1 df, p<0.001; Fig. 7a). Nurse counts were higher for non-deprived extract treated wells compared to solvent control (χ^2=94.165, 1df, p<0.001; Fig. 7a).

Response to deprived and non-deprived brood pheromone blends

Nurses did not aggregate preferentially over areas treated with deprived or non-deprived brood pheromone blends (χ^2= 1.698, 1 df, p=0.193; Fig. 7b), however nurses preferentially aggregated over wells treated with either deprived or non-deprived brood pheromone blend when compared to solvent control (χ^2=92.2, 1 df, p<0.0001 and χ^2=118.745, 1 df, p<0.0001, respectively).

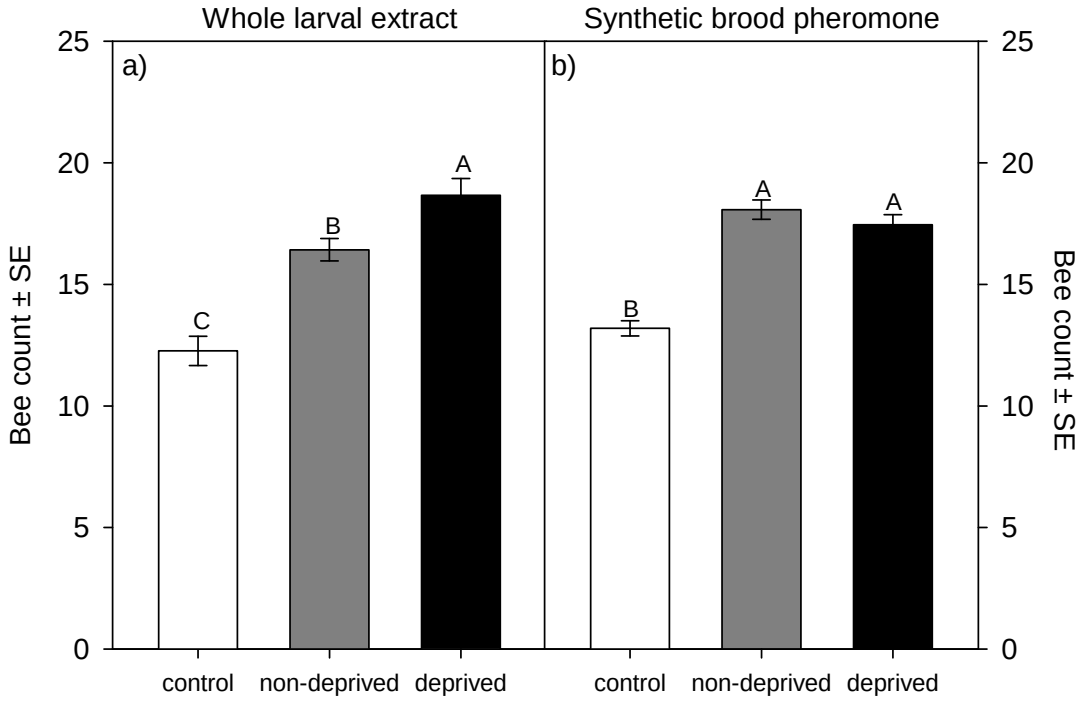

Fig. 7 Bee counts over wells treated with no-pheromone control, non-deprived, or deprived whole larval hexane extract (7a) or synthetic brood pheromone as formulated in Table 1 (7b). Capital letters denote statistically significant ($p<0.05$) subgroups separately for each figure part

Pollen foraging bioassay

Response to deprived and non-deprived whole larval extracts

Pollen to non-pollen forager proportions differed significantly by treatment (χ^2= 358.098, 2 df, $p< 0.001$; Fig. 8a). Pairwise comparisons showed that all treatments were

significantly different from each other (deprived vs. non-deprived χ^2= 22.803, 1 df, p<0.0001; control vs. non-deprived χ^2= 188.283, 1 df, p<0.0001; control vs. deprived χ^2=350.241, 1 df, p<0.0001; Fig. 8a).

Response to deprived and non-deprived brood pheromone blend in extractable amounts

Proportion of pollen to non-pollen foragers was significantly different by treatment (χ^2= 33.890, 2 df p<0.0001, Fig. 8b). Pairwise comparisons showed all treatments were significantly different from each other (deprived vs. non-deprived χ^2=33.883, 1 df, p<0.0001; control vs. non-deprived χ^2= 8.620, 1 df, p=0.003; control vs. deprived χ^2= 8.915, 1 df, p=0.003; Fig. 8b).

Response to deprived and non-deprived brood pheromone blend in equal amounts

Proportion of pollen to non-pollen foragers was significantly different by treatment (χ^2= 24.719, 2 df, p<0.0001, Fig. 8c). Pairwise comparisons showed that deprived and non-deprived treatments resulted in significantly different pollen to non-pollen forager ratios (χ^2=9.014, 1 df, p=0.003) and non-deprived treatment was significantly different from control (χ^2= 24.137, 1 df, p<0.0001), however using the Dunn -Šidák calculated significance value of p=0.017, colonies treated with deprived blend brood pheromone did not differ from those treated with control (χ^2= 4.444, 1 df, p=0.035; Fig. 8c).

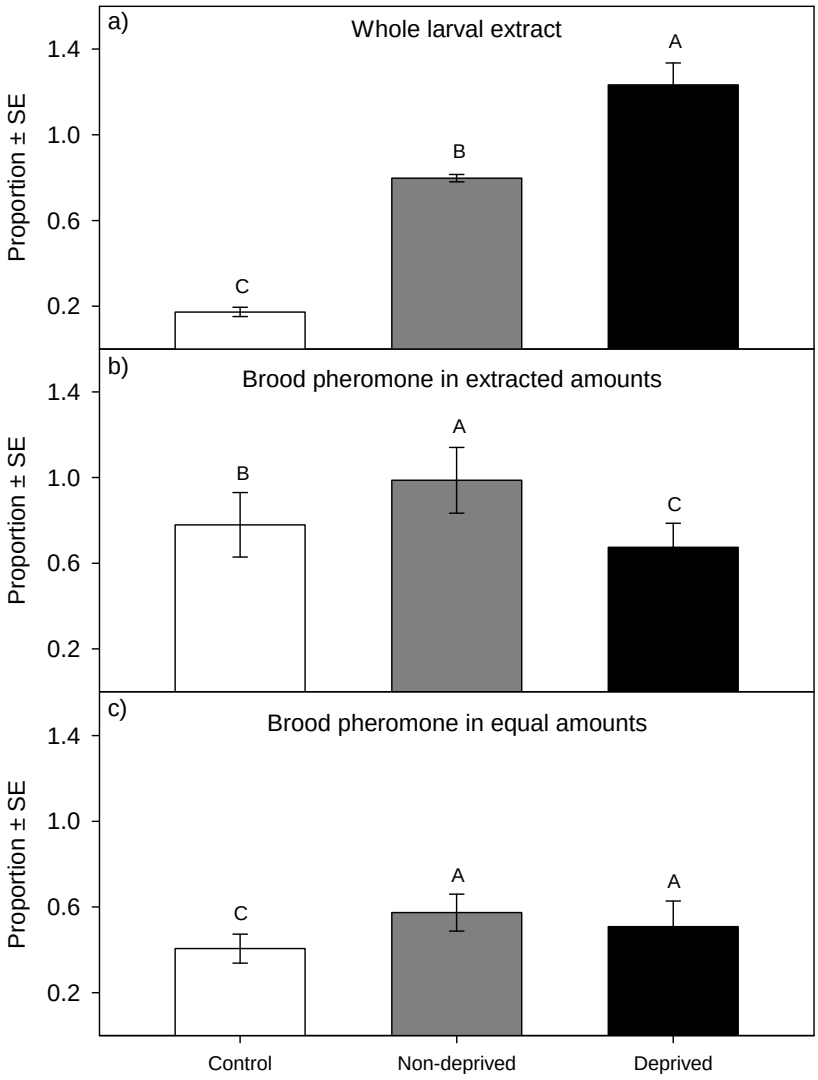

Fig. 8 Proportion of pollen to non-pollen forager returning to colony entrance over a 5 min observation period 1 hr after treatment with a no-pheromone control, deprived, or non-deprived whole larva hexane extract (8a) synthetic brood pheromone (Table 1) in extractable amounts (8b) or in equal amounts (8c). Capital letters denote statistically significant ($p<0.05$) subgroups separately for each figure part

DISCUSSION

This is the first study to demonstrate that honey bees use chemical cues to communicate nutritional status of larvae. Both provisioning task groups responded to these chemical cues, with nurses and foragers being more responsive to deprived larval extracts compared to non-deprived. Changes to synthetic brood pheromone mimicking extracts of deprived or non-deprived larvae however, did not appear to affect nursing behaviors but did change pollen foraging behavior.

Feeding distribution was sufficiently variable to suggest that in a colony some larvae may be deprived for an extended period of time due to stochastic factors and as such they would benefit from a signal communicating nutritional status (Fig. 1). Signals of nutritional status have been cited to either signal short-term need, hunger, or long-term need, condition, or perhaps both (Price et al., 1996; Royle et al., 2002; Villaseñor and Drummond, 2007). I therefore attempted to avoid changing condition of the larvae to focus exclusively on short-term effects by using side-by-side patches of similarly aged larvae. Larval survival and emergence weight was not negatively impacted by four hour deprivation treatment, suggesting that larvae did not suffer long-term deleterious effects or changes in their overall condition. Changes in the larvae such as emergence lipid content or behavioral ontogeny of the adults were not observed; therefore it remains possible that the deprived adults were somehow different.

Observations of nurses showed that a greater proportion of adults aggregated over deprived patches of larvae (Fig. 2). Proportion of adults to larvae has been shown to affect amount of provisioning and larval behavior in ants (Cassill and Tschinkel, 1999) with lower ratios correlated with decreased feeding frequency. Interestingly, the rate at which nurse proportion increased over deprived larvae over time was not significantly different from that of non-deprived larvae (Fig. 2). This means that it was unlikely that deprived larvae were emitting any long-range signal recruiting bees to the area. In the close quarters and dense conditions of a social insect colony, such a signal would almost assuredly be lost in noise and the source would not be identifiable (Greenfield, 2002). This may also mean that the signal I observed elicited an arresting rather than an attracting response.

Deprived larvae were inspected and fed earlier than non-deprived (Fig. 3) which may seem contradictory with the result that bees did not accumulate over deprived larval patches more quickly than non-deprived, however, nurses have been observed to appear to sample the area above a cell prior to performing an inspection and it has been proposed that a hunger signal may operate over this short distance (Huang and Otis, 1991; Lindauer, 1953).

Deprived larvae were both fed and inspected more than non-deprived (Fig. 4a, b). A function of the increased number of feeding bouts is increased total feeding time as feeding durations were not different between treatments (Fig. 5a, b); feeding bout duration is likely to be regulated by factors other than nutritional requirement such as

nurse capacity. As such individual nurses are not necessarily provisioning more, but rather the collective nursing effort towards deprived larvae was increased. This is consistent with the idea that larvae are producing some signal of hunger that elicits inspection and feeding.

Amount of brood pheromone extracted from deprived larvae was a third that from non-deprived larvae, contrary to expectation. Proportions of ethyl oleate increased resulting in it being the major component of brood pheromone in deprived larvae while methyl stearate significantly decreased in proportion (Fig. 6). Ethyl oleate has previously been shown to modulate hypopharyngeal gland development and protein synthesis (Mohammedi et al., 1996) and methyl stearate may increase queen cell acceptance (LeConte et al., 1995). Increased ethyl oleate proportion may stimulate protein synthesis for enhanced nursing, while a decrease in methyl stearate proportion may signal a decrease in quality. Because the components of brood pheromone together elicit synergistic effects (Pankiw, 2004b), this supposition should be considered highly provisional on further study of the individual and collective effects of brood pheromone components on nursing behavior. Bees have been shown to learn a complex mixture by the major components or chemically distinct components rather than learning the whole blend (Reinhard et al., 2008); it is possible that the meaning of the brood pheromone signal changes with proportional changes in just two components.

In the laboratory bioassay, nurses aggregated over wells treated with deprived whole hexane extract preferentially compared to non-deprived extract, suggesting that nurses are arrested over deprived-treated wells (Fig. 7a). This may mean that chemicals extractable from the larval cuticle at least partially cause the nurse behaviors observed above.

Nurses aggregated over brood pheromone preferentially to solvent controls, but they did not differentiate between deprived and non-deprived blends, suggesting that both are equally active in this context (Fig. 7b). I did not observe feeding or inspections in either experiment using this bioassay; therefore chemical cues alone or the bioassay context did not stimulate a complete nursing response. Additional missing larval cues could be chemical, but are also likely to be tactile, as often direct contact between nurses and larvae is made during feeding (Nelson and Sturtevant, 1924).

Proportion of pollen to non-pollen foragers was greatest in colonies treated with the extract of adult deprived larvae, followed by non-deprived and, no-pheromone control (Fig. 8a) suggesting that larvae use chemicals to communicate nutritional status and colony foragers respond accordingly. In contrast, deprived brood pheromone significantly decreased pollen to non-pollen forager proportion relative to control, while non-deprived brood pheromone elicited the expected response similar to previous brood pheromone studies when applied in extracted amounts (Fig. 8b; Pankiw, 2003, 2004a, 2007; Pankiw et al., 1998; Pankiw and Rubink, 2002; Pankiw et al., 2004b; Sagili, 2007). It was surprising to note that, when presented to colonies in equal

amounts, deprived brood pheromone also elicited a decreased proportion of pollen to non-pollen forager compared to non-deprived and not significantly different from control (Fig. 8c), suggesting that the effect of a deprived blend brood pheromone is distinct from that of a non-deprived blend. Sagili (2007) showed that brood pheromone elicited different effects on foraging at different doses with a high dose not affecting the pollen to non-pollen forager ratio, but rather decreasing the forager to non-forager ratio in same aged bees. Metz el al., *submitted* showed that different blends of brood pheromone elicited differential pollen foraging response based on colony phenotype. Further, brood pheromone has been shown to increase nursing behaviors such as cell cleaning and brood tending (Sagili, 2007). More detailed behavioral analyses of the effects of deprived and non-deprived brood pheromone will be necessary to confirm this interpretation. Further chemical analyses will also be necessary to elucidate the blend of compounds that increases pollen to non-pollen forager ratios and results in nurse aggregation like the whole extract. Combined, the behavioral and chemical observations suggest that components extractable from the cuticle but not found in the 10 fatty acids of brood pheromone may account for the observed behaviors of increased pollen foraging and increased nursing in response to adult deprived larvae.

Brood pheromone could instead signal long-term quality or physiological state. Although I did not observe any gross changes in larval survival or emergence weight, the deprived larvae could still have been of a different quality than their non-deprived counterparts. Non-deprived larvae may therefore have represented a better investment

for a pollen foraging effort. It has been previously shown that under conditions of colony deprivation of pollen, adults will eventually cannibalize younger larvae to reprocess their protein and provision it to older instars (Schmickl et al., 2003; Schmickl and Crailsheim, 2002, 2004), suggesting that the colony adjusts nursing investment to maximize surviving brood at the expense of individuals. Although individual larval nutritional status was not manipulated in these experiments, this is somewhat similar to the practice of brood reduction, in which, under conditions of limited resources, parents will allow weaker, sickly, or smaller juveniles to starve or be killed by their siblings in order to increase the amount of food provisioned to the survivors (Mock and Parker, 1997). However it is unclear whether this is due to offspring competitive ability or parental choice (Godfray, 1995; Rauter and Moore, 1999). The fact that brood pheromone was extractable at lower amounts in the deprived larvae suggests that they may be unable to devote resources to brood pheromone synthesis. This is consistent with the hypotheses of honest signaling models (*reviewed in* Kilner and Johnstone, 1997). It is possible that brood pheromone represents a signal of quality to foragers, and a separate, as yet unidentified signal of hunger is produced eliciting the foraging behaviors observed in response to whole extracts. While much of the research into the communication of hunger has occurred in birds (*reviewed in* Clutton-Brock, 1991) some recent studies have begun exploring this in detail in insects (Agrawal et al., 2005; Kölliker et al., 2005; Kölliker et al., 2006; Mas et al., 2009; Mas and Kölliker, 2008). These studies limit themselves, similarly to those in birds, to the classic single parent, or

paired parenting systems where the same individual procures resources from the environment and provisions young. In the honey bee, these tasks are uncoupled by division of labor and are therefore free to respond to different signals produced by young. Honey bees therefore represent a unique model system in which to test hypotheses about signals of offspring need and condition.

CHAPTER III

VARIATION IN AND RESPONSES TO BROOD PHEROMONE AMONG DIFFERENT SOURCES OF HONEY BEES

INTRODUCTION

Honey bees are naturally distributed throughout Africa, Asia, Europe, and the Middle East (Ruttner, 1988). Within this range, about 24 subspecies have been recognized and grouped into four evolutionarily distinct branches that were first based on morphology, and later on allozymes, microsatellites, and mitochondrial DNA data (Pinto et al., 2003; Ruttner, 1988; Sheppard and Smith, 2000). Honey bees are not native to the Americas, having been imported in the late nineteenth and early twentieth centuries by bee breeders importing primarily European sub-species (Sheppard, 1989). The gene pool reflects these introductions in both the commercial and feral populations (Schiff and Sheppard, 1995, 1996; Schiff et al., 1994). In 1956 a South African sub-species, *A. m. scutellata*, was introduced to Brazil (Kerr, 1967) and queens mated with European males producing the Africanized honey bee, a genetic admixture of *A. m. scutellata* and European honey bees (Pinto et al., 2005). The first Africanized colony was discovered in the USA in 1990 (Pinto et al., 2005; Sugden and Williams, 1990). As a consequence, there are two behaviorally different types of honey bees found in the Americas referred

to as Africanized and European honey bees. Africanized and European honey bees are most notoriously different in their responses to defensive behavior stimuli as well as in their foraging behaviors, the latter being one focus of this study (Collins et al., 1982; Guzmán-Novoa et al., 2002; Uribe-Rubio et al., 2003).

Adaptations to tropical and temperate climates have shaped significant differences in the foraging behaviors among these two types of honey bees (Winston and Katz, 1982; Winston et al., 1983). When fostered at the same time in the same European colony, Africanized bees are more likely to forage for pollen and water than Europeans (Danka et al., 1987; Fewell and Bertram, 2002; Pankiw, 2003; Pesante et al., 1987). Among other factors (*reviewed in* Page and Erber, 2002), amount of larvae and larval chemicals called brood pheromone affect pollen foraging behaviors. Colony treatment with a relatively low dose of brood pheromone increases the proportion of pollen foragers, pollen load weight, and pollen foraging trip frequency (Pankiw, 2004a, 2007; Pankiw and Page, 2001a; Sagili, 2007). These foraging behaviors manifest shortly after pheromone application and terminate shortly after the pheromone stimulus is removed, signifying a pheromone releaser response (Pankiw, 2004b).

Africanized bees also forage at younger ages than European bees in common colony rearing studies (Pankiw, 2003; Winston and Katz, 1982). Younger ages of first foraging and a greater probability to forage for pollen is associated with greater sensitivity to sucrose as measured in the proboscis extension reflex assay (Pankiw, 2003; Pankiw and Page, 2000; Pankiw et al., 2002). Honey bees reflexively respond to a

sufficiently concentrated solution of sucrose when applied to the antennae by extending the proboscis (Bitterman et al., 1983; Page et al., 1998). The response threshold of an individual may be estimated by the lowest concentration that elicits proboscis extension when bees are presented with an ascending concentration series (Page et al., 1998; Pankiw, 2003; Pankiw and Page, 1999, 2000, 2001a, b, 2003; Pankiw and Rubink, 2002; Pankiw et al., 2002). Africanized bees have significantly lower response thresholds compared to European bees when newly emerged and prior to any feeding experience (Pankiw, 2003). Sucrose response thresholds are modulated by brood pheromone such that a relatively low amount of pheromone increases sucrose sensitivity and a relatively high amount decreases sensitivity (Pankiw and Page, 2001a, 2003; Pankiw et al., 2004b). Brood pheromone also affects age of first foraging such that bees in colonies treated with a relatively low amount of brood pheromone forage at significantly younger ages than bees in colonies treated with a relatively high amount of brood pheromone (LeConte et al., 2001; Sagili, 2007). These effects take days to manifest and continue long after the pheromone treatment is discontinued or are permanent, indicative of pheromonal primer responses (Pankiw, 2004b). Treatment with a low dose of brood pheromone for 30 days has been shown to increase the amount of brood area reared by colonies versus controls probably as a combined result of the suite of primer and releaser effects outlined above (Pankiw et al., 2008; Pankiw et al., 2004b; Sagili, 2007).

Brood pheromone is composed of the methyl and ethyl esters of palmitic (16:0), stearic (18:0), oleic (18:1 Δ^{9}), linoleic (18:2 $\Delta^{9, 12}$), and linolenic (18:3 $\Delta^{9, 12, 15}$) acids present

in different relative proportions, or blends (LeConte et al., 1990). Synthetic brood pheromone has dose-dependent effects on honey bee colony and individual bee foraging behaviors (Sagili, 2007). The pheromone blend and extractable amounts vary with larval age and caste (LeConte et al., 1994b; Trouiller et al., 1994; Trouiller et al., 1991). Treatment of colonies with a "young" blend of brood pheromone increased pollen to non-pollen forager ratios over control to a lesser degree than treatment with an "old" blend (Pankiw and Page, 2001a), however because different amounts and blends of brood pheromone were varied simultaneously, it is unclear whether this effect was due to dose or blend effects. In the present study, the dose of brood pheromone was controlled to test only the effect of blend. To date, all published studies on the effects of synthetic brood pheromone have been performed using the blend characterized from larvae of France (LeConte et al., 1994b; Trouiller et al., 1994; Trouiller et al., 1991, 1992) and many have used colonies of mixed European lineage in the USA (LeConte et al., 2001; Pankiw, 2004a, b, 2007; Pankiw and Page, 2001a, 2003; Pankiw et al., 2008; Pankiw et al., 2004b). The behavioral effect of introducing a potentially foreign brood pheromone into a colony has been previously explored only using whole hexane extracts (Pankiw and Garza, 2007; Pankiw and Rubink, 2002).

There is no known publication of variation in blends of brood pheromone among different populations characterized by mitochondrial lineage. The goal of this study was to explore this variation and behavioral and physiological responses to variant blends. Brood pheromone was characterized from honey bees having European

mitochondrial DNA in Texas and Georgia, USA and bees having African mitochondrial DNA from Texas, USA and Pretoria, South Africa. Subsequently, differences in blend were analyzed using a discriminant analysis model. Because releaser and primer responses are modulated by different physiological systems (Pankiw, 2004b), the results from one cannot inform on the results of the other. Therefore effect of blend was tested in both a pollen foraging bioassay (releaser response) and a sucrose response threshold bioassay (a primer response) with three populations of bees characterized by geographical location and mitochondrial lineage: Texas-European, Georgia-European, and Texas-Africanized honey bees.

MATERIALS AND METHODS

Classification of honey bee sources

Bee sources

In the course of this study four bee sources were used. Texas-European honey bees were procured from Navasota, Texas, USA and had European mitochondrial DNA. The prevalence of Africanized bees in Texas suggested that introgression of African nuclear DNA in colonies with European mitochondrial DNA was likely (Pinto et al., 2004). Therefore European queens and package bees were purchased from a breeder in

Moultrie, Georgia, USA where currently no record of the African mitotype has been reported (USDA-ARS, 2008). Africanized colonies were collected from Mission, Texas, USA. European and African mitochondrial lineage was confirmed. South African (*A. m scutellata*) sources were maintained in an apiary at the University of Pretoria, Pretoria, South Africa (25° 45′ S; 28° 14′ E). Texas-Africanized, Texas-European, and Georgia-European colonies were all maintained in a College Station, Texas apiary (30° 6′ N; 96° 32′ W). All the queens in this study were unrelated to each other and naturally mated. Honey bee queens mate with multiple males at one time in her life and each male mates with one queen and then dies (Winston, 1987). As a consequence, worker bees within a single colony share a mother, but not necessarily the same father; multiple patrilines (Tarpy and Nielsen, 2002; Tarpy et al., 2004).

Mitochondrial DNA analysis

The populations in this study were categorized by geographic origin and mitochondrial lineage. The cytochrome *b*/ *Bgl*II mtDNA bioassay was used to identify maternally Africanized bees. The *Bgl*II polymorphism discriminates the African mitochondrial haplotype (mitotype) of *A. m. scutellata* L. from that of European honey bees: *A. m. mellifera, A. m. caucasia, A. m. ligustica, A. m. carnica, A. m. lamarcki, A. m. cypria, A. m. syriaca,* and some *A. m. iberiensis* (Pinto et al., 2003). Mitochondrial DNA, which is shared by all individuals in a colony, was extracted from the thorax of a single worker

pupa per colony using a QIAamp® DNA Mini Kit (Qiagen Inc., Valencia, CA, USA). After extraction, template DNA was stored at -20° C prior to analysis. I used a primer that amplified a 485-bp section of the cytochrome *b* gene (Pinto et al., 2003). Single PCR amplifications were performed in 5 μl total volume containing 0.5 x *Taq* DNA polymerase buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 2 pM of each primer, 1 μl of template DNA, and 0.25 U of *Taq* DNA polymerase (Promega, Madison, WI, USA). The PCR temperature profile was 94° C for 3 min followed by 30 cycles of 94° C for 15 sec, 50° C for 15 sec, and 68° C for 5 sec. After the final cycle, an additional 10 min at 72° C was performed. Following PCR, samples amplified with cytochrome *b* were digested with the restriction enzyme *Bgl*II (Promega, Madison, WI, USA) using the temperature and buffer conditions recommended by the supplier. The total digestion volume was then electrophoresed on a 2% agarose/TBE gel stained with ethidium bromide and visualized under UV light. Although mitochondrial DNA type represents an unbroken maternal lineage, Africanized honey bee nuclear DNA is introgressed with an unknown amount of European nuclear DNA (Pinto et al., 2003).

Brood pheromone characterization

Larval collections

Larvae were collected from 8 Texas-Africanized, 8 Georgia-European colonies, 11 Texas-European colonies, and 7 South African colonies. South African extracts shipped by air-freight from Pretoria to College Station, but in other respects were treated identically. Single frames containing larvae were removed from their colonies and brought to the laboratory within 30 min where larvae were removed from their cells with a gentle stream of water and collected into 1 mm mesh cloth. Larvae were lightly shaken dry and ten fifth instar larvae were selected by weight and morphology (Dade, 1977; de F Michelette and Soares, 1993; Rembold et al., 1980; Thrasyvoulou and Benton, 1982).

Extraction and fractionation

Larvae were pooled into a small beaker (VWR, West Chester, PA, USA) and soaked for 1 min in 2 ml of 95% n-hexane containing 1 μg methyl myristate as an internal standard and 0.05% (w/w) t-butyl hydroquinone as an antioxidant. Extracts were then filtered through a Buchner funnel to remove particulate matter; larvae and glassware were rinsed with 2 ml of 95% n-hexane, which was added to the extract. Extracts were stored

in 7 ml glass, screw-top vials (VWR, West Chester, PA, USA) sealed with aluminum foil and paraffin and stored for up to 2 weeks at -20°C before further processing.

Whole extracts were then concentrated by evaporation to 1 ml under a nitrogen stream in a water bath at 55° C and vortexed for 30 sec at 2000 rpm to reconstitute any analyte on the glass. The extracts were fractionated by liquid chromatography with a column constructed from 0.5 g of 70-230 mesh/60Å silica gel (Sigma-Aldrich, St. Louis, MO, USA) packed into a 2 ml Pasteur pipette (VWR, West Chester, PA, USA) plugged with a piece of dust-free paper cloth (Kimberly-Clark, Neenah, WI, USA). Before use, columns were rinsed with 10 ml each of 99% dichloromethane (Sigma-Aldrich, St. Louis, MO, USA) and 95% n-hexane to remove soluble contaminants. Concentrated extracts were injected onto freshly prepared columns and 2 fractions were eluted into 15 ml conical vials (VWR, West Chester, PA, USA). The first fraction was eluted using 10 ml of 95% n-hexane; this fraction contained long and short-chain hydrocarbons, but did not contain esters. The second fraction, containing the esters, was eluted with 10 ml of 99% dichloromethane. Dichloromethane fractions were then evaporated to dryness under a nitrogen stream in a water bath at 50° C. Dried fractions were immediately reconstituted in 1 ml of 95% n-hexane and vortexed for 30 sec at 2000 rpm. The fractions were then concentrated to approximately 100 µl and transferred to 12 x 32 mm chromatographic vials (Grace-Davison, Columbia, MD, USA) with 250 µl glass inserts (Grace-Davison, Columbia, MD, USA) and the conical vials previously containing the liquid were rinsed 3 times with 50 µl hexane, which was added to the chromatographic

vials. Fractions were again evaporated to dryness at 50° C and reconstituted with 20 µl hexane containing 2 µg of n-octadecane (Sigma-Aldrich, St. Louis, MO, USA) as a reference. Fractionated extracts were stored at -20° C for up to 7 days until analysis by gas chromatography used flame ionization or mass spectroscopic detection. The mass spectroscopy method was utilized to confirm ester identity and the reliability of the flame ionization detection method.

Gas chromatography

Extracts were analyzed with a Hewlett Packard 6890 gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) with splitless programmable temperature vaporization injection and flame ionization detection. An aliquot of 1 µl of extract was injected onto an HP-88, 60 m X .251 mm ID column ([88%-cyanopropyl]-methylarylpolysiloxane, Agilent, Santa Clara, CA, USA). The inlet temperature was held at 60° C for 0.10 min and then increased to 250° C at 500 C/min. The oven temperature was held at 50° C for 2 min, then increased at 20° C/min to 170° C, held for 3 min, increased at 30° C/min to 230° C and held at the final temperature for 6 min. The carrier gas was pure hydrogen held at a flow of 2 ml/min. Individual fatty acid ester retention times were identified by referencing > 99% purity esters as standards (Sigma-Aldrich, St. Louis, MO, USA). To estimate quantities, the areas beneath the peaks of known concentrations of each ester were calculated with GC Chemstation software version B.01.03.204 (Agilent, Santa

Clara, CA, USA). These data were used to generate a standard curve for each fatty acid ester expressed as a first order regression equation. The quantified extract ester concentrations were then corrected for methodological error by reference to the internal standards.

GC/MS

Gas chromatographic analyses were performed on a Hewlett Packard 6890 gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) employing a HP- 5MS ((5%-phenyl)-methylpolysiloxane), 30 m x 0.25 mm fused silica capillary column (Agilent, Santa Clara, CA, USA). An aliquot of 1 μL of each extract was injected in the splitless (1 min)/split mode using helium as carrier gas at a constant flow of 1.0 ml/min and inlet temperature of 300° C. The column temperature was programmed at an initial temperature 150° C, ramped 20° C/min to 320° C, and held isothermally for 5 min.

The mass spectrometric analyses were performed using an Agilent MSD 5975C Inert XL quadrupole mass spectrometer (Agilent, Santa Clara, CA, USA), which was operated in electron ionization mode (EI) at 70-eV electron energy. Data acquisition was performed in selected ion monitoring (SIM) mode for two ions characteristic of each component of the following mass/charge (*m*/*z*) ratios: methyl myristate (*m*/*z* 74, 242), methyl palmitate (*m*/*z* 74, 270), ethyl palmitate (*m*/*z* 88, 284), methyl linoleate (*m*/*z* 81, 294), methyl oleate (*m*/*z* 84, 264), methyl linolenate (*m*/*z* 79, 292), methyl stearate (*m*/*z* 74,

298), ethyl linoleate (*m*/*z* 81, 308), ethyl oleate (*m*/*z* 98, 310), ethyl linolenate (*m*/*z* 79, 306), ethyl stearate (*m*/*z* 88, 312), and octadecane (*m*/*z* 85, 254). The temperature of the transfer line was 320° C; temperature of 350° C. Data were acquired using ChemStation E.02.00.493 (Agilent, Santa Clara, CA, USA).

Synthetic pheromone preparation

The synthetic blends of Texas-European, Texas-Africanized, and Georgia-European derived brood pheromone was formulated from mean ester percentages obtained using GC and GC/MS, while percentages for the French blend were personally communicated by Y. LeConte (Unit de Zoologia, Laboratoire de Biologie de L'Abeille; Domain St. Paul, France; March, 1998; Table 2). Synthetic esters were added by mass into an amber glass vial (Grace-Davison, Columbia, MD, USA) with 0.05% of t-butyl hydroquinone (Sigma-Aldrich, St. Louis, MO, USA) added as an antioxidant. The synthetic blends of brood pheromone were diluted in 95% n-hexane to a concentration of 0.5 μg/μl for application in the bioassays described below.

Table 2. Formulations of blends of brood pheromone from different geographic and genetic sources (%)

Fatty acid ester	European mtDNA			African mtDNA	
	France	Texas, USA	Georgia, USA	Texas, USA	South Africa
methyl palmitate	3.0	6.9	2.9	4.1	6.0
ethyl palmitate	3.0	3.9	3.9	4.7	8.8
methyl stearate	17.0	15.6	6.5	14.9	25.6
ethyl stearate	7.0	6.4	8.1	10.3	14.5
methyl oleate	25.0	16.8	20.6	9.0	11.5
ethyl oleate	8.0	15.4	13.8	15.9	12.9
methyl linoleate	2.0	2.9	4.2	9.3	4.5
ethyl linoleate	1.0	5.4	12.1	6.4	4.9
methyl linolenate	21.0	13.8	16.9	11.7	2.8
ethyl linolenate	13.0	12.9	11.0	13.7	8.5

Experiment 1. Comparison of brood pheromone blends among four larval sources

Amounts of individual components were transformed to proportion of total esters. Proportions were natural log-transformed and classified by predictive discriminant analysis (Huberty, 1994; SPSS, 2007). The natural log-transformed ester proportions were normally distributed, meaning that the assumption of multivariate normality was likely met (Huberty, 1994; Klecka, 1980). Group covariance matrices were not homogenous as tested by Box's M (M=486.06; $F_{110,15764}$= 3.64, $p<0.0001$; Klecka, 1980; SPSS, 2007). A linear model was applied despite the potential differences in covariance

matrices between sources for two reasons: first, Box's M is known to be overly sensitive, and second, the ratio of number of groups (n_g= 3) to total samples (p= 88) was low (n_g/p= 0.03) (*as per* Huberty, 1994). Under these conditions and using external classification through the leave-one-out method, linear discriminant functions perform as well or better than their quadratic counterparts (Huberty and Curry, 1978). A total of 34 extracts from Texas-Africanized colonies, 31 extracts from Georgia-European colonies and 23 extracts from South African colonies were analyzed. Because colonies are comprised of several patrilines (*reviewed in* Laidlaw and Page, 1996), it was reasonable to include colony-level subsamples to better represent the genetic diversity of the colony. The leave-one-out classification scheme was utilized for external validation, meaning that each data point was categorized based on classification functions generated from all other points (Huberty, 1994). Having no *a priori* hypothesis for unequal probabilities of group membership, equal probabilities were used (Huberty, 1994). One condition of the discriminant analysis is that members of a group be conclusively assigned to a single category (Klecka, 1980). Because a possibility existed for Africanized nuclear DNA introgression in the Texas-European population (Pinto et al., 2005; Pinto et al., 2004), I did not consider this condition true for this population. Therefore, brood pheromone blends from the Texas-European larvae were not categorized by the analysis, and were rather classified as ungrouped samples.

Experiment 2. Pollen foraging response of Texas-European colonies to France and Texas-European derived brood pheromone

Because there was no known study demonstrating different blends of brood pheromone releasing differential pollen foraging behaviors, here I hypothesized that there would be no difference in the proportion of pollen to non-pollen foragers returning to colonies treated with France or Texas-European derived synthetic brood pheromone.

Pollen forager bioassay

The bioassay was performed in twelve similarly-sized colonies, installed one week earlier. Each colony was comprised of approximately 1 kg of bees, 1 queen, and few very young larvae (< 2 cm^2). Colonies received 0.6 mg of France or Texas-European derived brood pheromone characterized as above or an equal volume of solvent as blank control. This dose was selected through a series of dose-response trials performed prior to experimentation (*as per* Pankiw and Page, 2001a). Pheromone was applied on glass plates measuring 14 cm x 7 cm. The solvent was allowed to evaporate prior to placement in colonies. A single plate was then hung on metal wire between two frames in the middle of the colony. One hour after placement of glass plates the number of pollen and non-pollen foragers entering colonies was counted for a 5 min

period (*as per* Pankiw et al., 1998). Pollen foragers are visually distinguishable from non-pollen foragers by the pollen clumps on their hind legs in specialized structures, called corbiculae. Counts were performed between 08:00-10:00 hrs. All colonies received all treatments on subsequent days in a randomized manner. Counts were analyzed by 3 x 2 chi-square contingency table analysis for the effect of pheromone treatment on pollen to non-pollen forager ratio (Sokal and Rohlf, 1995; SPSS, 2007). Pairwise comparisons were performed using 2x2 chi-square contingency table analysis with the Dunn-Šidák method of correcting for possible Type I errors (Sokal and Rohlf, 1995).

Experiment 3. Pollen foraging responses to Texas-Africanized and Georgia-European derived brood pheromone by Texas-Africanized and Georgia-European colonies

Here I tested the hypothesis that the pollen foraging differences among Africanized and European honey bees were a consequence of responses to different blends of brood pheromone.

Pollen forager bioassay

The pollen foraging bioassay was performed as in Experiment 1 with six Texas-Africanized and six Georgia-European colonies each treated with 1.0 mg of the

synthetic blend of Texas-Africanized or Georgia-European brood pheromone, or an equal volume of solvent control. Texas-Africanized and Georgia-European colony responses to pheromone treatments were statistically analyzed separately.

Experiment 4. Modulation of sucrose response thresholds by Texas-Africanized and Georgia-European derived brood pheromone

Brood pheromone has been shown to modulate sucrose response thresholds (Pankiw and Page, 2001a, 2003; Pankiw et al., 2004a) and Africanized and European honey bees exhibit differential sucrose sensitivities (Pankiw, 2003). I therefore tested the hypothesis that different pheromone blends delivered at the same total amount would differentially modulate sucrose response thresholds in newly emerged Africanized and European bees.

Experimental design and treatment

Frames of pupae collected from six Texas-Africanized and eight Georgia-European colony sources were taken to the lab and adults were allowed to emerge for 24 hrs in an incubator at 30° C, 55% RH. Three acrylic glass/wire-mesh cages (1400 cm^3) each received 300 newly emerged bees. Cages were provisioned with 30 ml of 30% sucrose solution and 30 ml of water. Pheromone was applied on 7 cm x 7 cm glass plates hung

by metal wire in the center of each cage. Each cage received one of three treatments: 1.8 mg of either Texas-Africanized or Georgia-European derived brood pheromone (Table 2), or an equivalent volume of solvent control. Solvent was allowed to evaporate prior to inserting glass plates into cages. Cages were stored in a dark incubator at 30° C, 55% RH. Treatment plates, sucrose, and water were changed daily. Bees were reared thus for five days so that they were tested within the first week of life (*as per* Pankiw and Page, 1999, 2000, 2001a, 2003). On the sixth day, a subset of 60 bees was collected from each cage and their sucrose response thresholds were measured using the proboscis extension reflex assay.

Proboscis extension reflex bioassay

The proboscis extension reflex bioassay followed previously reported methodologies (Pankiw and Page, 2001a, 2003; Pankiw et al., 2004a). Briefly, bees were restrained by thin strips of tape such that their heads and mouthparts moved freely. Bees were allowed 1 hr to acclimate to restraint before testing (Pankiw and Page, 2003). All bees were tested for proboscis extension to antennal stimulation with water; those responding were allowed to drink water to satiation. Bees were then presented with logarithmically ascending concentrations of sucrose (0.1, 0.3, 1.0, 3.0, 10 and 30%) by touching a single droplet to each antenna. A positive response was recorded if the bee extended her proboscis. Positive responses to sucrose were summed to yield scores that

were analyzed by Kruskal-Wallis (Sokal and Rohlf, 1995; SPSS, 2007). A high score corresponded to high sensitivity to sucrose as an individual who extended her proboscis to a low sucrose concentration typically responded positively to all more concentration solutions as well. Mann-Whitney U was performed to assess pairwise differences in sucrose response threshold by treatment using the Dunn-Šidák method as above (Sokal and Rohlf, 1995; SPSS, 2007).

RESULTS

Experiment 1. Comparison of brood pheromone blends among four larval sources

Two discriminant functions were generated. Means of the functions were significantly different between sources (χ^2= 117.50, 20 df, $p<0.0001$; SPSS, 2007). Squared canonical correlation coefficients showed that function 1 explained 57.2% of the variation among the groups, while function 2 explained 37.2%. Overall, 77% of the samples were correctly classified. That is, 66.7% of the Georgia-European blends categorized correctly, 30% of this group were categorized as Texas-Africanized, and 3.3% as South African. Among the Texas-Africanized blends 76.5% were correctly categorized, 11.8% were categorized as Georgia-European and 11.8% as South African. The categorization of South African blends was 91.3% correct, while 4.3% were categorized as Georgia-European and 4.3% as Texas-Africanized.

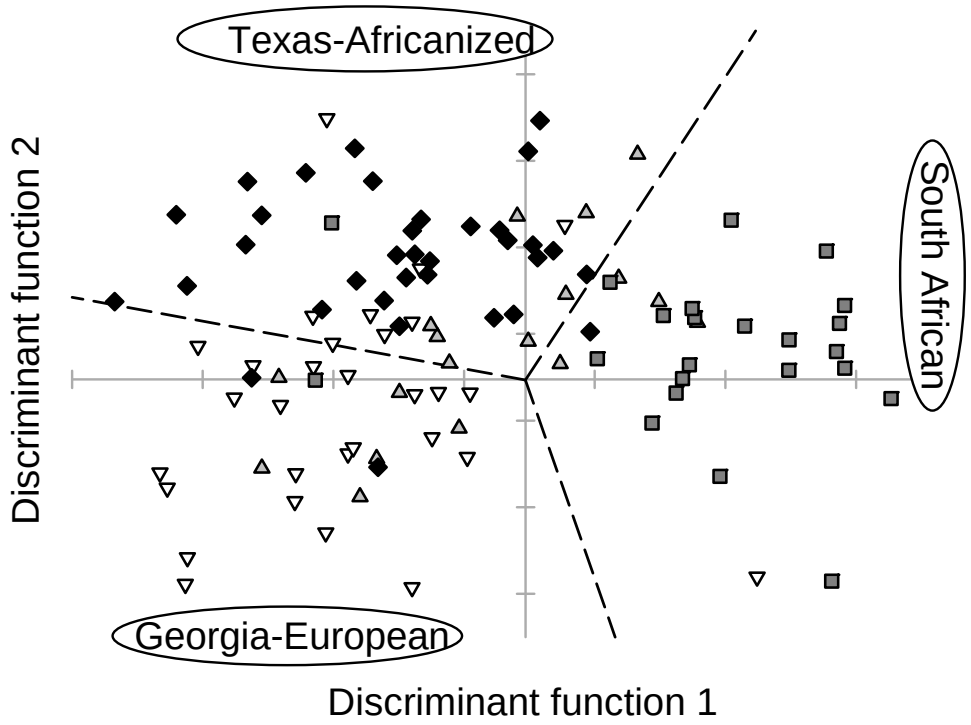

Fig. 9 Discriminant scores for brood pheromone ester proportions extracted from larvae of colonies of Texas-Africanized and Georgia-European and South African honey bees. Each point represents a single sub-sample. Placement is determined from the canonical coefficients of the two functions of the discriminant analysis. Circled labels indicate the classification of all sub-samples within the territories demarcated by the dashed lines. Georgia-European samples are represented by open, upside-down triangles, Texas-European by grey triangles, Texas-Africanized by black diamonds, and South African samples are represented by dark grey squares. Means of the groups were significantly different ($p<0.05$)

Of the 18 Texas-European blends, 39% were classified as Georgia-European, 33% as Texas-Africanized, and 28% as South African (Fig. 9). Function 1 was the primary discriminator between Texas-Africanized and Georgia-European blends and the South

African blends, while function 2 was the primary discriminator between the Georgia-European and Texas-Africanized blends (Fig. 9).

Plotting the structure matrix coefficients of the transformed ester proportions to the discriminant functions revealed the relative importance of each ester to the classification of the blends (Fig. 10; Klecka, 1980). Vectors were plotted at an angle: the tangent of which is the ratio of function 2 to function 1. The method of calculating the magnitude of the vectors was adapted from Overall and Klett (1972) and was the ratio of the standard error of the group means for each ester proportion to the mean standard error of the ester proportions within each group. The ethyl and methyl esters of palmitic (16:0) and stearic (18:0) acids were highly positively correlated to function 1, while methyl linolenate (Me-18:3 $\Delta^{9, 12, 15}$) was highly negatively correlated (Fig. 10). Palmitic and stearic acids are both saturated, while linolenic acid is triple-unsaturated. This means that the South African blends were more likely to exhibit a high proportion of unsaturated fatty acid esters and a relatively low proportion of poly-unsaturated fatty acid esters, particularly methyl linolenate. Both methyl and ethyl linolenate were highly positively correlated with function 2, while methyl oleate (Me-18:1 Δ^{9}) and ethyl linoleate (Et-18:2 $\Delta^{9, 12}$) were highly negatively correlated. Georgia-European blends were more likely to exhibit a higher proportion of methyl oleate and ethyl linoleate, and a lower proportion of the esters of linolenic acid.

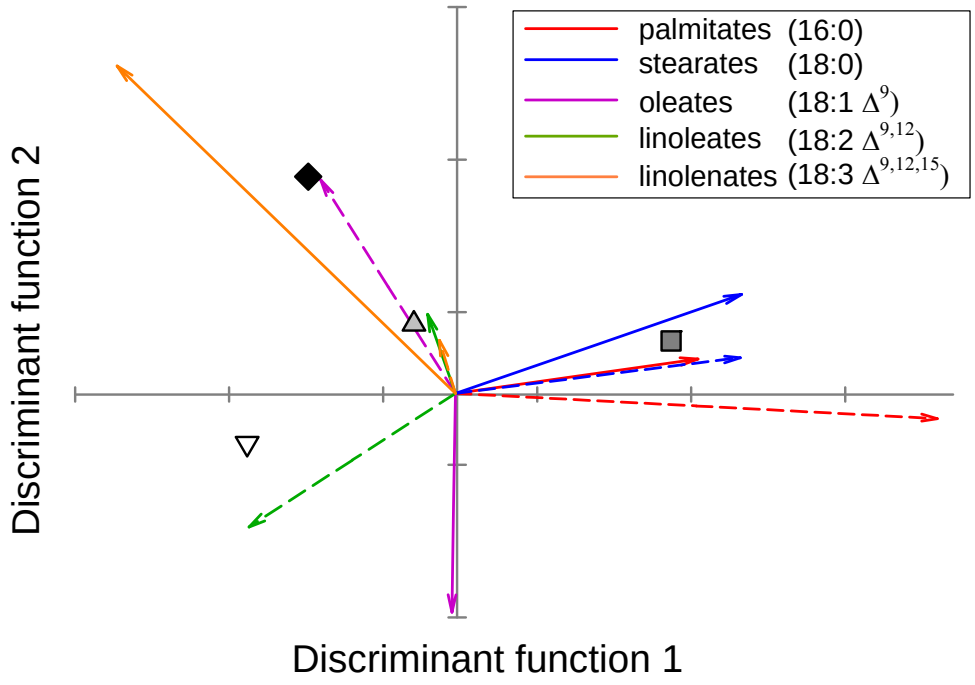

Fig. 10 Strength of the relationship of the proportions of brood pheromone esters to the discriminant functions. Solid lines represent methyl esters and dashed lines ethyl esters of palmitic, stearic, oleic, linoleic, and linolenic acids. Vector angles represent the ratio of the correlation of the ester proportion to the discriminant function and magnitudes represent the ratio of between- to within-groups standard error. Points represent the discriminant score of the group mean (or group centroid): Georgia-European by an open, upside-down triangle, Texas-European by a grey triangle, Texas-Africanized by a black diamond, and South African by a dark grey square

Experiment 2. Pollen foraging response of Texas-European colonies to France and Texas-European derived brood pheromone

Proportion of pollen to non-pollen foragers differed significantly by pheromone treatment (χ^2= 63.95, 2 df, p<0.0001; Fig. 11; SPSS, 2007). Pairwise comparisons indicated that all treatments were different from each other, with the greatest ratio of pollen to non-pollen foragers in response to the Texas-European derived brood pheromone compared to the French blend (χ^2= 15.08, 1 df, p<0.0001) or the solvent control (χ^2= 64.198, 1 df, p<0.0001). Pollen to non-pollen forager ratios in response to the French blend was significantly greater than control (χ^2= 22.11, 1 df, p<0.0001), which was consistent with previous studies (Pankiw, 2004a; Pankiw et al., 1998; Pankiw and Page, 2001a).

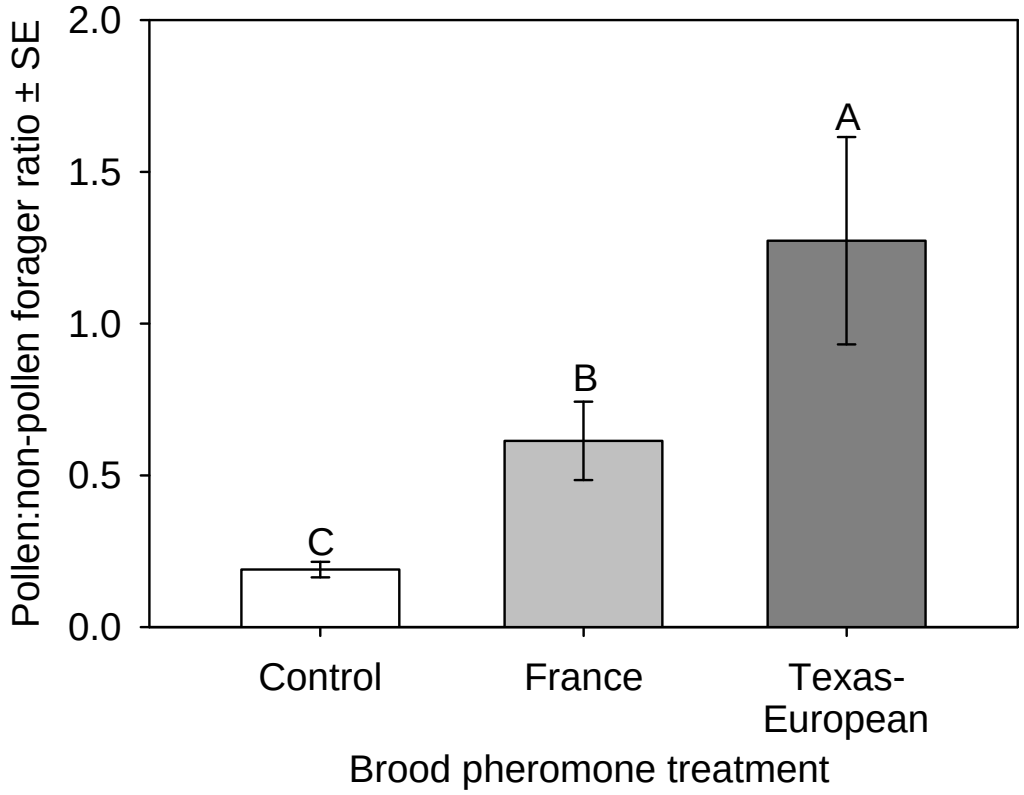

Fig. 11 Pollen to non-pollen forager ratio in Texas-European colonies treated with French or Texas-European derived synthetic brood pheromone or solvent control. Each letter denotes a statistically different subset ($p<0.017$)

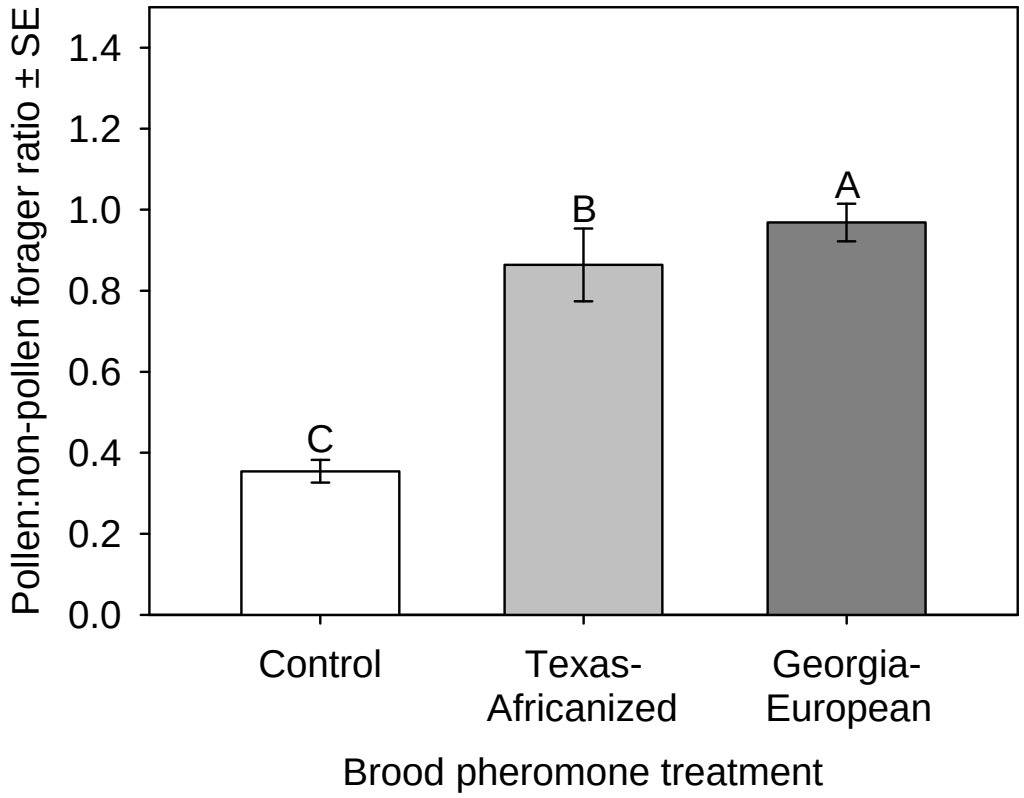

Fig. 12 Pollen to non-pollen forager ratio of Georgia-European honey bee colonies in response to synthetic brood pheromone derived from Texas-Africanized and Georgia-European larval sources. Each letter denotes a statistically different subset ($p<0.017$)

Experiment 3. Pollen foraging responses to Texas-Africanized and Georgia-European derived brood pheromone by Texas-Africanized and Georgia-European colonies

Proportions of pollen to non-pollen foragers were also significantly different by pheromone treatment among Georgia-European colonies (χ^2= 121.4, 2 df, p<0.0001; Fig. 12). Pollen to non-pollen forager ratios were greatest in response to treatment with Georgia-European derived brood pheromone compared to either Texas-Africanized blend (χ^2= 3.33, 1 df, p=0.038) or solvent control (χ^2= 109.06, 1 df, p<0.0001). Pollen to non-pollen forager ratios in response to the Texas-Africanized blend was significantly greater than control (χ^2= 72.56, 1 df, p<0.0001).

Proportions of pollen to non-pollen foragers differed significantly by treatment among Texas-Africanized colonies (χ^2_2= 59.75, 2 df, p<0.0001; Fig. 13). Pollen to non-pollen forager ratios were highest in response to Texas-Africanized derived brood pheromone when compared to the Georgia-European blend (χ^2= 58.80, 1 df, p<0.0001) or solvent control (χ^2= 26.01, 1 df, p<0.0001). Pollen to non-pollen forager ratios in response to the Georgia-European blend were significantly greater than control (χ^2= 7.13, 1 df, p=0.004).

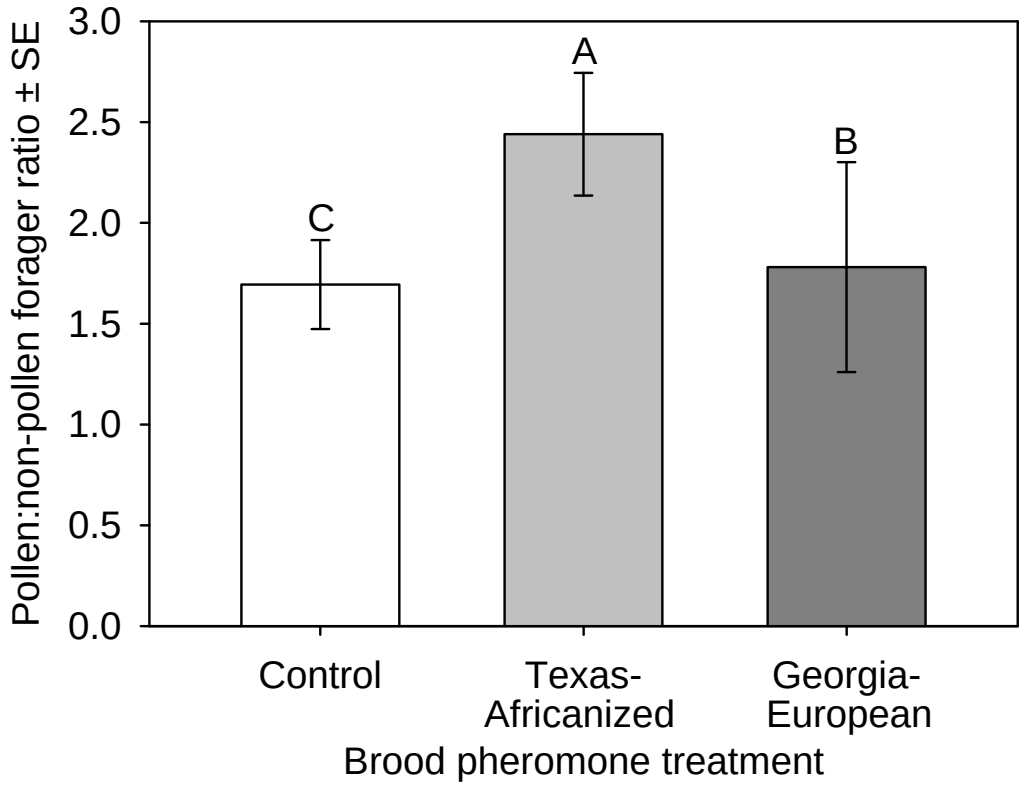

Fig. 13 Pollen to non-pollen forager ratio of Texas-Africanized honey bee colonies in response to synthetic brood pheromone derived from Texas-Africanized and Georgia-European larval sources. Each letter denotes a statistically different subset ($p<0.017$)

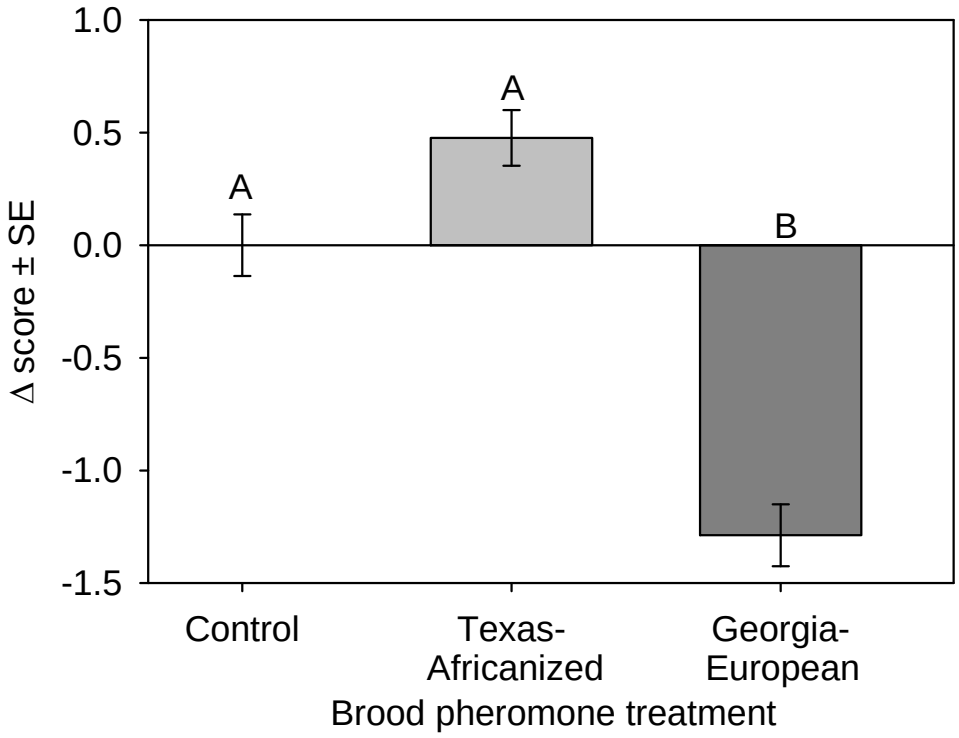

Fig. 14 Proboscis extension reflex scores of Georgia-European honey bees in cages treated with synthetic brood pheromone derived from Georgia-European or Texas-Africanized sources compared as a mean difference from solvent control treated bees. Letters denote statistically different Δ scores ($p<0.017$)

Experiment 4. Modulation of sucrose response thresholds by Texas-Africanized and Georgia-European derived brood pheromone

Georgia-European bees exhibited significantly different proboscis extension reflex score distributions between pheromone treatments (χ^2= 77.24, 2 df, p<0.0001: Fig. 14). In Mann-Whitney comparisons, scores were higher when bees were treated with Texas-Africanized blend compared to control, but non-significantly (Z =-2.27, p=0.023). Scores were significantly lower when bees were treated with Georgia-European blend when compared to control (Z = -6.45, p<0.0001). Georgia-European bee scores were significantly higher when treated with Texas-Africanized derived brood pheromone versus the Georgia-European blend (Z = -8.31, p<0.0001). Proboscis extension reflex scores were not significantly different by pheromone treatment in Texas-Africanized bees (χ^2= 1.36, 2 df, p=0.51; Fig. 15).

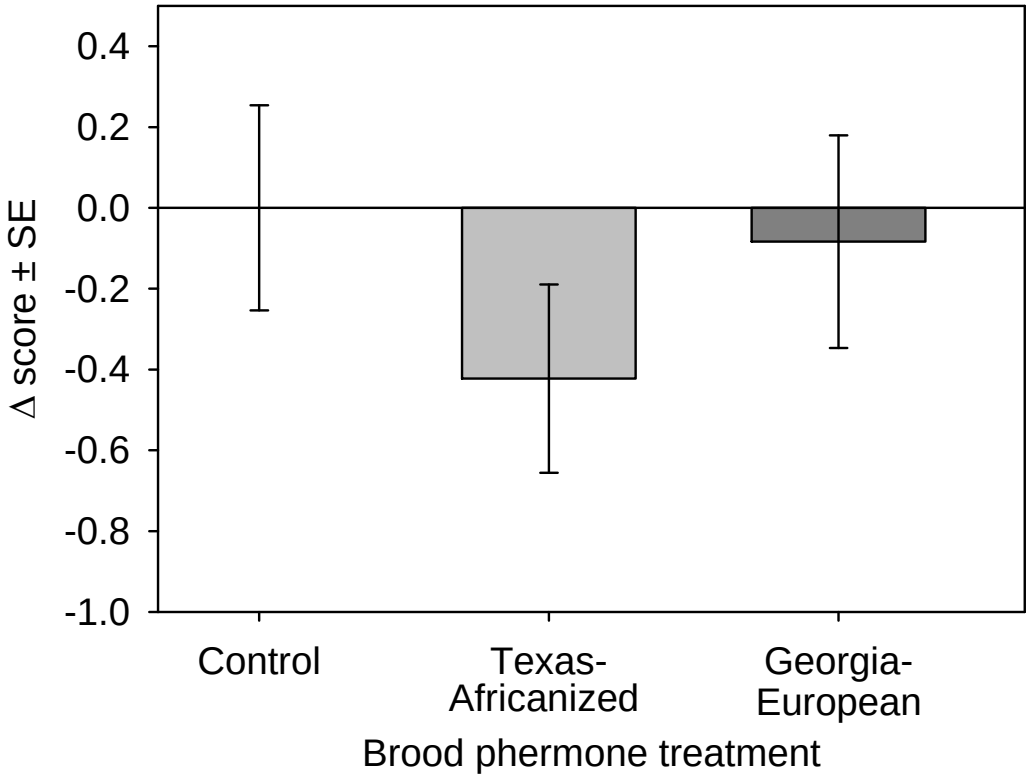

Fig. 15 Proboscis extension reflex scores of Texas-Africanized honey bees in cages treated with synthetic brood pheromone derived from Georgia-European or Texas-Africanized sources compared as a mean difference from solvent control treated bees. None of the treatments were significantly different from each other ($p>0.017$)

DISCUSSION

Brood pheromone blend clearly varied with source of honey bee larva. Overall, blends were significantly and sufficiently different to correctly classify the source of 77% of those used in this study (Fig. 9). Discriminant analysis strongly suggested the ratio of saturated to unsaturated compounds were separating the South African blends from those derived from European or partly European origins. The South African derived brood pheromone was characterized by higher proportion of esters of the saturated palmitic and stearic acids (Fig. 10). In contrast, the European and Africanized blends were characterized by increased proportion of esters of unsaturated fatty acids, particularly ethyl linoleate, ethyl oleate, and methyl linolenate. Unsaturated fatty acid esters oxidize more readily and are therefore more labile than saturated fatty acid esters (Kotz et al., 2006). It is reasonable to hypothesize that the South African blend may be more persistent over time because it is less labile. At this time it can only be speculated that tropical bees may benefit from a more stable blend of brood pheromone as pollen foraging stimulus as an adaptation to a comparatively faster rate of reproduction fueled by high pollen foraging resulting in rapid brood rearing as adaptations to surviving in tropical climates where predators limit survival rather than extended periods of cold (Winston et al., 1983). Conversely, temperate evolved honey bees tend to grow larger colonies that reproduce at a slower rate and more nectar resulting in greater amounts of stored honey, as adaptations for surviving long winters (Winston et al., 1983). This may

explain why a less persistent brood pheromone pollen foraging stimulus may be found in European bees. A more comprehensive comparative analysis of brood pheromone blends and foraging responses among tropical and temperate evolved honey bees would be necessary in conjunction with comparative time to oxidation assays to test this hypothesis.

Proportions of methyl oleate were highest in the European populations compared to those populations with African maternal lineage and suspected Africanized DNA introgression (Fig. 10). Methyl oleate has been demonstrated as an attractant for young adult bees in queen retinue bioassays (Keeling et al., 2003). Because the bioassays in this study did not measure attraction, the effect of this variation is unknown. It may be that different populations of bees are differently attracted to various pheromone components. If this is the case, changes in the proportions of individual components may disrupt an important communication function that could be identified and easily manipulated through changes made to synthetic blends.

Texas-Africanized brood pheromone may be viewed as a blend that is intermediate between the Georgia-European and South African. This may not seem surprising, except that it has been at least 56 years since *A. m. scutellata* were released in Brazil and all subsequent crosses and backcrosses have been uncontrolled mixes of Africanized and European queens and drones. This particular blend may imply that this intermediate trait is adaptive in a subtropical environment (Pankiw et al., 2008).

Interestingly, the Texas brood pheromone profiles derived from larvae with European *mt*DNA was evenly categorized among Texas-Africanized, Georgia-European, and South African suggesting the introgression of African genes in this Texas population of honey bees. It also suggests Mendelian independent assortment inheritance for alleles regulating brood pheromone component proportions; however this is highly speculative. It could also mean that the Texas-European population was by chance alone a nearly perfect mixture of sources that were one third European, one third European-African hybrids, and one third *A. m. scutellata.* Further studies into brood pheromone variation while strictly controlling the genetic relationships will be necessary to better understand the phenomenon observed in this study of Texas-European brood pheromone samples.

In population-based pollen foraging responses to blends of brood pheromone, colonies were most responsive to a synthetic blend of brood pheromone that was formulated from the component proportions detected from larvae that were from a similar population but unrelated colony sources. The Texas-Africanized and Georgia-European colonies treated with blends of brood pheromone derived from Texas-Africanized or Georgia-European larvae also responded with the greatest proportion of pollen foragers when treated with their own blend of pheromone. If the hypothesis that increased pollen foraging behaviors observed in Africanized honey bees were a consequence of a different blend of brood pheromone was correct, I would expect the blend derived from Texas-Africanized larvae to release greater proportions of pollen

foraging regardless of whether the treated colony was of Africanized or European maternal lineage. This means that variation in brood pheromone blend was not sufficient to explain variation in pollen foraging among Africanized and European colonies. Pankiw and Rubink (2002) found no race-by-pheromone interaction when testing pollen foraging response to whole hexane extracts of larvae. Use of whole extracts could have confounded their results by containing an unknown and uncontrolled amount of brood pheromone. This study did not address dose of pheromone as a contributory factor to behavioral variation, but rather controlled dose to study blend effects. Brood pheromone dose affects the magnitude of response in pollen foraging bioassays (Sagili, 2007). Whole hexane extracts may contain in excess of 100 components (*unpublished observations*), with fatty acid esters representing only a small proportion of the total amount of compounds. This condition may lead to a decreased signal to noise ratio in whole extracts, modulating specific responses (Greenfield, 2002). These experiments showed that colonies were stimulated to forage for pollen most strongly in response to a synthetic blend simulating that detectable from their own population, suggesting that colonies may differentiate larval source in a pollen foraging context.

Co-adaptation of the brood pheromone signal to the response of the receiver may predate the eusocial condition and served originally as a method of distinguishing one's own young from nearby unrelated larvae. Geographic isolation and subsequent selection of naturally and human distributed honey bee races are potential contributory

factors to pheromone variation. An additional factor is differential apicultural selection pressures on colonies for pollination or honey production in the USA, France, and South Africa, that may also change brood pheromone. Colony usurpation and social parasitism as in the case of Africanized bees in Arizona and *A. m. capensis* in Africa (Neumann and Moritz, 2002; Neumann et al., 2001; Schneider et al., 2004) may also act on the maintenance of brood pheromone as a recognition cue of larval source.

Modulation of sucrose response thresholds, a primer response, also showed a pheromone blend by population -dependent response interaction. Brood pheromone derived from Georgia-European origin significantly decreased sucrose responsiveness of newly emerged bees of the same source by nearly 2 times (on a $\log_{10}$ scale) versus the control response. However, Texas-Africanized bees appeared to be insensitive to the modulating effects of brood pheromone on sucrose sensitivity regardless of the brood pheromone blend environment in which they were reared. The high baseline sucrose sensitivity (control response) of Texas-Africanized bees was consistent with that previously observed by Pankiw and Rubink (2002).

All previous studies involving brood pheromone modulation of sucrose responsiveness used the French blend and USA bees having a European mitotype. Sucrose responsiveness increased and decreased with respectively low and high amounts of brood pheromone (Pankiw and Page, 2001a, 2003; Pankiw et al., 2004a). Low and high amounts of the French blend may also decrease and increase foraging age respectively (LeConte et al., 2001; Sagili 2007). Additionally, the effect of a low

amount of the French blend has varying effects on probability to forage depending on the age or maturity of pre-foragers (Pankiw, 2004b). For example, bees aged 5-8 days old were 31 time more likely to begin foraging due to the brood pheromone treatment effects than same source 9-12 day old bees reared in the same pheromone treated colony environment (Pankiw, 2004b). Honey bees of different artificially and naturally selected strains have different baseline sucrose responsiveness measured when newly emerged and prior to feeding experience (Pankiw, 2003; Pankiw and Page, 1999). Additionally, increasing sucrose responsiveness appears associated with increasing probability to forage and a higher probability to forage for pollen (Pankiw and Page, 1999; 2000; Pankiw et al., 2001). This study represents the first examination of sucrose response modulation by different blends of brood pheromone delivered at the same amount. The dose of brood pheromone used in this study was expected to decrease sucrose responsiveness based on past experience using the French blend and bees of USA origin having a European mitotype. Only the Georgia-European pheromone modulated sucrose responsiveness among bees of USA origin having a European mitotype in a manner predictable from previous studies. The highly responsive baseline of Africanized bees may be indicative of a more mature sucrose sensitivity status associated with a faster rate of foraging ontogeny (Pankiw, 2003) and overall greater sensitivity to environmental stimuli consistent with high responsiveness to defensive stimuli (Collins et al., 1982; Uribe-Rubio et al., 2007). If brood pheromone modulates sucrose responsiveness in pre-foraging Africanized bees, then I predict that

modulation would be measurable in bees that are less mature than those tested here, given that the French synthetic blend does not modulate USA European foragers (*unpublished observations*). Alternatively the amount of brood pheromone used may not have been appropriate to change sucrose responsiveness in Texas-Africanized bees. Brood pheromone modulated sucrose responsiveness is likely a complex interaction of baseline responsiveness, amount and, blend of brood pheromone.

This study has shown that the pheromone blend changes among same-stage larvae from different sources. A pheromone comprised of at least 10 compounds presents an enormous challenge to our understanding of how honey bees process mixtures of pheromone compounds. An additional complication in this challenge is that the principle role of the pheromone may change from primer to releaser depending on the state of the adult receiver (Pankiw & Page 2001a). Honey bee pheromone neurobiology is a nascent science that to date has primarily focused on male-female differences in processing queen produced sex pheromone (*reviewed in* Sandoz et al., 2007). Little to nothing is known of the olfactory processing of the larval fatty acid esters by adult workers. Relatively few genes coding for odorant-binding proteins have been found in the honey bee genome, so that the relative importance of this remains unclear (Forêt and Maleszka, 2007). In contrast, 170 odorant receptor genes were annotated in the honey bee (Honeybee Genome Sequencing Consortium, 2006; Robertson and Wanner, 2006). However, little is known of odorant receptor specificity in the honey bee (*reviewed in* Sandoz et al. 2007). Likewise the roles that the antennal

lobes and mushroom bodies may play in processing brood pheromone components is yet to be considered.

In conclusion, brood pheromone clearly varied by larval source and colonies responded to variation in brood pheromone for pollen foraging and modulation of sucrose responsiveness. The pattern observed for pollen foraging responsiveness to variation in blends of brood pheromone suggests brood pheromone is co-adapted as a recognition cue. That is, in each case there was a positive and significant response to a synthetic blend of brood pheromone that was characterized from a source of larvae most similar to the colony source. Pheromone modulated sucrose response threshold did not follow the same pattern, however primer and releaser responses are not necessarily coordinated but rather uncoupled generating a complex interaction to variation in brood pheromone at various levels within a colony.

CHAPTER IV

PHYSIOLOGICAL INSENSITIVITY OF FORAGERS TO SUCROSE RESPONSE THRESHOLD PRIMING BY BROOD PHEROMONE

INTRODUCTION

Sucrose response threshold is defined as the lowest sucrose concentration that elicits an extension of the bee's proboscis; this is estimated using the proboscis extension reflex bioassay (Page et al., 1998; Pankiw and Page, 1999, 2000, 2001a, 2003; Pankiw et al., 2001) and is related to sucrose sensitivity such that a high sucrose response threshold corresponds to low sucrose sensitivity and vice versa. Sucrose response threshold is constrained by genotype such that strains of honey bee selected to collect large amount of pollen are characteristically more responsive to sucrose than strains selected to collect a small amount of pollen (Page et al., 1998; Pankiw, 2003; Pankiw and Page, 1999). Sucrose response thresholds are also correlated with foraging behavior such that those measured in the first week of adult life predict the resources collected 2-3 weeks later, after foraging onset. Returning foragers caught collecting water were found to be most sensitive to sucrose (that is, having the lowest sucrose response threshold), followed by pollen and then nectar foragers (Pankiw and Page, 2000). Those pre-foragers that were most insensitive to sucrose were most likely to be caught later as foragers returning to the nest empty, presumably because they did not find a nectar

source concentrated enough to elicit collecting behavior (Pankiw and Page, 2000). Sucrose response thresholds are also correlated with age of foraging onset such that a low sucrose response threshold corresponds to a younger age of first foraging and a high response threshold to older age of foraging onset (Pankiw and Page, 2000). Because pollen and nectar foragers that are collected exhibit the same relationship in their sucrose responsiveness (pollen foragers are more sensitive to sucrose than nectar foragers; Pankiw and Page, 1999), it is assumed that sucrose sensitivity is relatively constant throughout life, although it is known that sucrose thresholds decrease as bees age (Pankiw and Page, 1999) and tend to change transiently throughout the season (Scheiner et al., 2003) and based on feeding or foraging environment (Pankiw et al., 2001).

Pre-forager sucrose response thresholds are modulated by brood pheromone, such that a 1:1 ratio of whole extract larval equivalents to bees increases sucrose sensitivity over controls (Pankiw and Page, 2001a). Further a 100:1 ratio of synthetic brood pheromone blend elicits the same response. Alternatively, a 1000:1 ratio of synthetic brood pheromone decreases sucrose sensitivity relative to controls (Pankiw and Page, 2001a). Pankiw and Page (2003) treated pre-foragers for 7 days in the presence or absence of brood pheromone, and then treatment was switched for half the bees for a further 24 hrs. It was found that control bees exposed to brood pheromone for 24 hrs prior to measurement and brood pheromone treated bees left in the absence of brood pheromone for 24 hrs prior to measurement both exhibited an intermediate

sucrose response threshold with controls being most sensitive to sucrose, brood pheromone treated bees the least sensitive to sucrose, and those bees in which treatments were swapped with sensitivity level in the middle (Pankiw and Page, 2003). This indicates that the modulation of sucrose response threshold of pre-foragers by brood pheromone is a primer response and suggests that the changes in sucrose sensitivity caused by brood pheromone are long lasting or permanent. Colony treatment with brood pheromone increases the number of pollen foragers returning to the nest, and also increases the load weights, number of foraging trips, and the number of pollen grains washed from non-pollen foragers, all indications of increased foraging effort (Pankiw, 2004a, 2007; Pankiw et al., 1998). However, as Pankiw and Page (2003) showed in a pollen forager bioassay, removing the brood pheromone treatment from a colony results in the number of returning foragers to decrease to the level of control colonies after approximately 1 hr, indicating a releaser response. Further, manipulating sucrose concentration of feeding and foraging environments modulates sucrose response thresholds in both foragers and non-foragers transiently over a period of minutes to hours (Pankiw et al., 2004a; Pankiw et al., 2001).

In pre-foragers, treatment with brood pheromone has been found to modulate age of foraging onset in a dose-dependent manner such that a low dose decreases age of first foraging and a high dose increases age of first foraging (LeConte et al., 2001; Sagili, 2007). This combined with the knowledge that age of first foraging and sucrose response thresholds are genetically correlated in both natural and artificially selected

strains of honey bee (Page et al., 1998; Pankiw, 2003; Pankiw and Page, 2001b; Pankiw et al., 2002; Pankiw et al., 2001; Winston and Katz, 1982; Winston et al., 1983) and the aforementioned dose-dependent effects of brood pheromone on sucrose sensitivity reviewed above (Pankiw and Page, 2001a), leads to the conclusion that behavioral development is closely tied to the amount of larvae and larval chemicals.

The above review highlights the importance of sucrose response threshold on foraging division of labor and the impact that brood pheromone has on both. Onset of foraging is accompanied by changes in glandular development (Winston, 1987) and hormone production (Amdam and Omholt, 2002; Huang and Robinson, 1996; Huang et al., 1991; Robinson, 2002), resulting in profound changes in the physiology of the individual. It is unclear whether brood pheromone affects foragers and pre-foragers in the same manner, that is, does brood pheromone act as a primer of sucrose response thresholds in both foragers and pre-foragers or does brood pheromone rather act as a primer on pre-foragers and as a releaser on foragers? No study has yet determined whether the physiological changes during foraging onset result in a change in responsiveness to brood pheromone modulation of sucrose response thresholds. A goal of the following experiments is to address this gap in our knowledge.

In studies on the effects of brood pheromone on honey bee pollen foraging, it has been observed that the effect of the synthetic pheromone decreases significantly after 3 hrs and is nearly indistinguishable from controls after more than 6 hrs (Pankiw et al., 1998). Although the pollen forager bioassay measures a releaser response to

brood pheromone, it seems reasonable to assume that synthetic brood pheromone may lose effect over time in a sucrose response threshold bioassay as well. Loss of function of brood pheromone over time could be due to a number of factors; the first is habituation to the pheromone, which seems unlikely because of evidence that colonies exposed to brood pheromone daily for 5 or more days continue to exhibit behavioral effects (Pankiw et al., 2008; Pankiw et al., 2004b). Secondly, the pheromone could be dissipated throughout the colony, removed from the source, and ultimately deactivated in some fashion by the bees themselves. In the second part of this study, this alternative was addressed by recovering brood pheromone from plates exposed to experimental colonies in the field, foragers and non-foragers in cages in a laboratory assay, and no-bee controls. The recovered proportions among the treatments were compared to test the hypothesis that the presence of bees negatively affected pheromone recovery, in effect, that bees remove brood pheromone.

In addition to removal of the pheromone by bees, it is also likely that exposure of brood pheromone to the environment results in decay and/or evaporation of the components. Because brood pheromone represents a mix of methyl and ethyl esters of saturated and unsaturated fatty acids, it is probable that some of the components are more labile, that is reactive to oxygen, and more volatile, that is, soluble in air, than others. As such, it was hypothesized that some components would be more likely to disappear from the pheromone blend earlier than others, thus the blend would change over time.

Pheromone primer effects require long-term exposure, on a time scale of hours to days (Wyatt, 2003). This could occur in two ways: through a single exposure to a persistent, stable pheromone, or through repeated exposure to a labile pheromone. This study tested the persistence of brood pheromone components over time, in different blends. It has been previously observed that natural variation in brood pheromone occurs among different populations of the honey bee and in larvae deprived of adult contact (*personal observations;* see chapters II and III). This variation in brood pheromone resulted in varying pollen foraging response. The effect of blend was tested shortly after application and as such, the persistence of the different blends of pheromone was never considered to be a contributory factor to the variation in response to the different blends. However, it remains possible that the specific proportions of the blends of brood pheromone change the elicited effects and therefore the regulation of colony and individual behaviors. A longer lasting brood pheromone would stimulate increased levels of pollen foraging, while a less stable cue would presumably have the opposite effect. I therefore tested the synthetic blend of brood pheromone against single esters and an equal proportions blend to test the hypothesis that brood pheromone components are differentially recoverable based on blend.

MATERIALS AND METHODS

Brood pheromone formulation and application

Formulations as proportion of components are presented in Table 3 (Pankiw and Page, 2001a). Brood pheromone was formulated from synthetic components of 99% purity (Sigma-Aldrich, St. Louis, MO) and diluted to 560 ng/µl with 95% n-hexane (Sigma-Aldrich, MO).

Table 3. Formulation of brood pheromone as communicated by Y. Le Conte

Ester	Percent (%)*
methyl palmitate	3.0
ethyl palmitate	3.0
methyl stearate	17.0
ethyl stearate	7.0
methyl oleate	25.0
ethyl oleate	8.0
methyl linoleate	2.0
ethyl linoleate	1.0
methyl linolenate	21.0
ethyl linolenate	13.0

*Corresponds to the France-European blend reported in Table 2

Modulation of sucrose response threshold of foraging and non-foraging bees

Forager collection

The entrance of a single colony was completely blocked with newspaper in the morning or early afternoon as determined by observed periods of highest forager activity for each colony. Two cages were stocked with pollen foragers and two cages were stocked with non-pollen foragers collected with soft forceps into acrylic glass/wire mesh cages from a colony entrance blocked with newspaper. Bees were identified as returning foragers by either having pollen-packed corbiculae (pollen foragers) or distended abdomens (non-pollen foragers). Six replications were performed. Two-hundred and fifty bees were collected into each cage for the first 5 replications. Because of difficulty collecting foragers, the sixth replication was performed with 3 cages of 100 bees each. In all other respects, this replication proceeded identically to the others. Cages were returned to the laboratory within 2 hr and incubated at 30° C and 55% RH and fed 30% sucrose and water *ad libitum.* For each pair of cages, one received 1.12 μg/bee of brood pheromone and the other received an equal volume no-pheromone control (n-hexane) applied on glass plates hung in the center of the cage on wire. Plates were allowed to dry at least 5 min before being exposed to the bees. Bees were reared thus for 5 days; treatment plates, sucrose, and water was changed daily.

Non-forager collection

Two to five frames containing emerging bees were removed from a single colony and taken to the lab where they were incubated overnight at 30° C and 55% RH. Two cages were each stocked with 300 bees and provided 30% sucrose and water as above. 6 pairs of cages were stocked with bees; each pair from a separate colony source. For each pair of cages, one was treated with 1.12 μg/bee brood pheromone, and the other an equal volume no-pheromone control. As above, bees were reared for 5 days.

Proboscis extension reflex bioassay and statistical analyses

On the sixth day, sixty bees, or the maximum surviving bees, were subjected to proboscis extension response assay as in Pankiw and Page (2001). Briefly, bees were anesthetized by chilling for 5 min at -20° until movement had nearly stopped. Anesthetized bees were then restrained in a testing apparatus by straps across the pronotum and petiole. A single drop of water was presented to bees through a light touch on both antennae. If an individual extended her proboscis, she was allowed to drink to satiation. Bees were then allowed to acclimate to restraint for 1 hr. Subsequently, positive and negative proboscis extension responses to 0.1, 0.3, 1.0, 3.0, 10, and 30 % sucrose were recorded. At least 3 min elapsed between presentations of ascending concentrations of sucrose. A high score corresponded to responses to a low

sucrose concentration and therefore a high sensitivity to sucrose. Alternatively, a low score corresponded to the first positive response to a high sucrose concentration and therefore a low sensitivity to sucrose. Statistical analyses were performed using pair-wise Mann-Whitney U tests (Sokal and Rohlf, 1995; SPSS, 2007).

Brood pheromone recovery

In the presence of bees in colonies

Glass plates measuring 18 x 15 cm were treated with 1.12 mg brood pheromone and hung between two frames in the center of the brood nest of colonies containing 4000-10000 bees. Plates were exposed to bees for 24 hrs and then removed from the colony and immediately returned to the lab for further processing. Fifty plates were collected.

In the presence of bees in cages

Frames of newly emerging bees were taken from a single colony for each replication as above. Bees were immediately returned to lab and placed in an incubator at 30° C 55% RH and were fed 30% sucrose and de-ionized water. Bees were allowed to emerge for 24 hr and 500 were counted and placed into 5 cages. Five cages were each stocked with 500 foragers collected from a single source colony as above. Five empty cages were

provided sucrose and water, and left empty in the incubator to serve as controls. Each cage received a single glass plate treated with 280 μg of brood pheromone or solvent control. Each plate measured 7 x 8 cm, which represented the approximate area occupied by 500 larvae and the amount used represented the published amount of brood pheromone extractable from 500 larvae (LeConte et al., 1990). One plate was removed at the each following time points: 1, 3, 6, 12, and 24 hr and stored at -20° C for up to 4 hr before further processing. Four replications of each time point were performed.

Recovery of brood pheromone components in different blends

The effect of blend on ester recovery was tested in the absence of bees. Glass plates measuring 9 x 7 cm were treated with 280 μg brood pheromone, 280 μg of all ten components in an equal proportion mixture, 280 μg of a single brood pheromone ester, or a solvent control consisting of 95% n-hexane. All plates were suspended with steel wire from a rack that separated the plates by one inch. Plates were hung in an incubator at 30° C 55% RH for 0, 24, and 48 hrs. Three replications were performed for each ester and blend.

Pheromone collection and processing

Each side of the large plates were washed with three sequential washes of 10 ml 95% n-hexane containing 1.0 μg methyl myristate (99% purity; Sigma-Aldrich, St. Louis, MO) as a standard into a 50 ml glass beaker. Small plates were washed with three sequential washes of 5 ml, but otherwise processed identically. Extracts were concentrated to 10 ml by nitrogen stream and transferred to 15 ml conicals; beakers were rinsed with 3 ml n-hexane and this was added to the extracts. Extracts were stored at -20 °C for up to twenty-four hours before further processing. Extracts were evaporated under nitrogen stream in a 55° C water bath until approximately 50 μl and reconstituted to 1ml with n-hexane, agitated 5 sec at 2500 rpm and concentrated again. This process was repeated twice, decreasing the reconstituted volume to 500 μl and then 100 μl for transfer into chromatographic vials. Conicals were rinsed three times with 50 μl n-hexane which was added to the sample. Samples were then evaporated to dryness and reconstituted in 10 μl of n-hexane containing 50 ng/μl octadecane (99% purity; Sigma-Aldrich, St. Louis, MO) as an internal standard and stored at -20° C until analyzed by gas chromatography.

Gas chromatography

An HP6890 gas chromatograph (Agilent, Santa Clara, CA) with splitless, programmable temperature variation injection and flame ionization detection was used for sample analysis. One microliter was injected onto a HP-88 30 m X .251 mm ID column (Agilent, Santa Clara, CA). Inlet temperature was held at 60° C for 0.10 min and then increased at 500° C/min to 250° C. Initial oven temperature was 50° C held for 2 min, followed by a rate of 20° C/min to 170° C, held for 3 min, 30° C/min to 230° C as the final temperature, held for 6 min. Carrier gas was pure hydrogen held at a constant flow of 2 ml/min. Peaks were identified using Agilent GC Chemstation software version B.01.03.204 (Agilent, Santa Clara, CA) and compared to retention times of fatty acid ester standards of 99% purity. Peak areas were standardized across runs by comparison to the octadecane peak area and corrected for methodological error by proportion to the methyl myristate peak.

Statistical analyses

Bee removal

Recovered amounts of brood pheromone components were standardized as described and were then summed to attain a total brood pheromone amount which was transformed to a proportion of the applied total amount. Proportions were normally distributed over time (Kolmogorov-Smirnov; Z=0.882, df=11, p=0.418) and therefore analysis of covariance was performed to test the hypothesis that recovered proportion of total esters varied by treatment. Pairwise comparisons were performed using the Dunn-Šidák correction for multiple comparisons (Sokal and Rohlf, 1995; SPSS, 2007).

Recovery over time

Recovered amounts of brood pheromone components were standardized as above. Corrected amounts of each ester were then calculated as a proportion of the applied amount of that ester. Proportions were log transformed to normalize them over time (Kolmogorov-Smirnov; Z=1.29; p=0.072). Analysis of covariance was performed to test the hypothesis that recovered proportions of brood pheromone esters over time varied when components were formulated differently.

To determine which ester qualities affected recovered proportion, I also performed an analysis of covariance using blend as an explanatory factor and time as the covariate. The residuals from this model were then used in Pearson's bivariate correlations with ester functional group length, number of double bonds, boiling point, and molecular weight.

RESULTS

Modulation of sucrose response threshold of foraging and non-foraging bees

Sucrose response thresholds were not significantly different by brood pheromone treatment among either non-pollen ($Z = -1.204$, $p=0.228$) or pollen ($Z = -1.129$, $p=0.259$) foragers (SPSS, 2007; Fig. 16). As expected, sucrose response thresholds of newly emerged bees were significantly modulated by BP treatment ($Z = -14.730$, $P<0.0001$) with PER scores significantly higher in BP treated bees (Fig. 16).Further, scores were significantly higher in pollen foragers compared to non-pollen foragers ($Z = -7.011$, $p<0.0001$; Fig. 16).

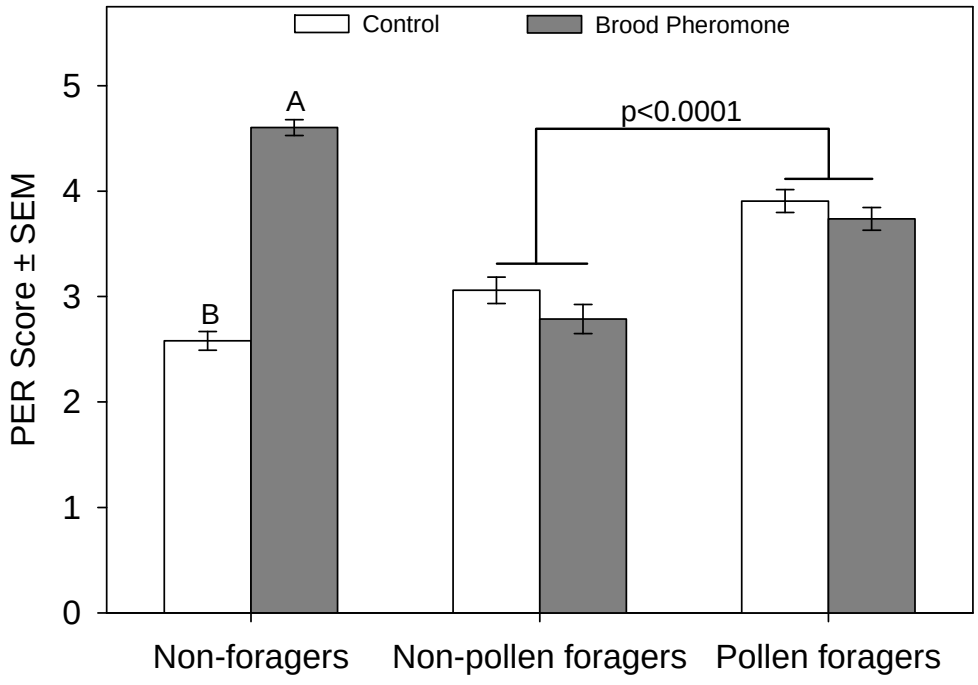

Fig. 16 Proboscis extension reflex scores of non-foragers, non-pollen foragers, and pollen foragers treated with brood pheromone in cages. Capital letters denote a statistically significant difference by treatment ($p<0.05$)

Recovery of brood pheromone exposed to bees

In the presence of bees in colonies

None of the analyzed glass plates placed into colonies contained identifiable brood pheromone components in detectable amounts (data not shown) after 24 hrs, although

several unidentified components were observed. Often, visible, waxy material was found on the plates after their removal from the colony (*personal observations*).

In the presence of bees in cages

Recovered total pheromone proportion significantly decreased by time ($F_{1,57}$= 24.6; p<0.0001). Recovery differed significantly by treatment ($F_{2,57}$= 13.7; p<0.0001; Fig. 17). Mean recovery of total proportion in the absence of bees was higher than either proportion in the presence of non-foragers (mean difference=1.35±0.26; p<0.001) or foragers (mean difference=0.81±0.26; p=0.008). Mean recovered proportion of brood pheromone did not differ between foragers and non-foragers (mean difference=0.55±0.26; p=0.12).

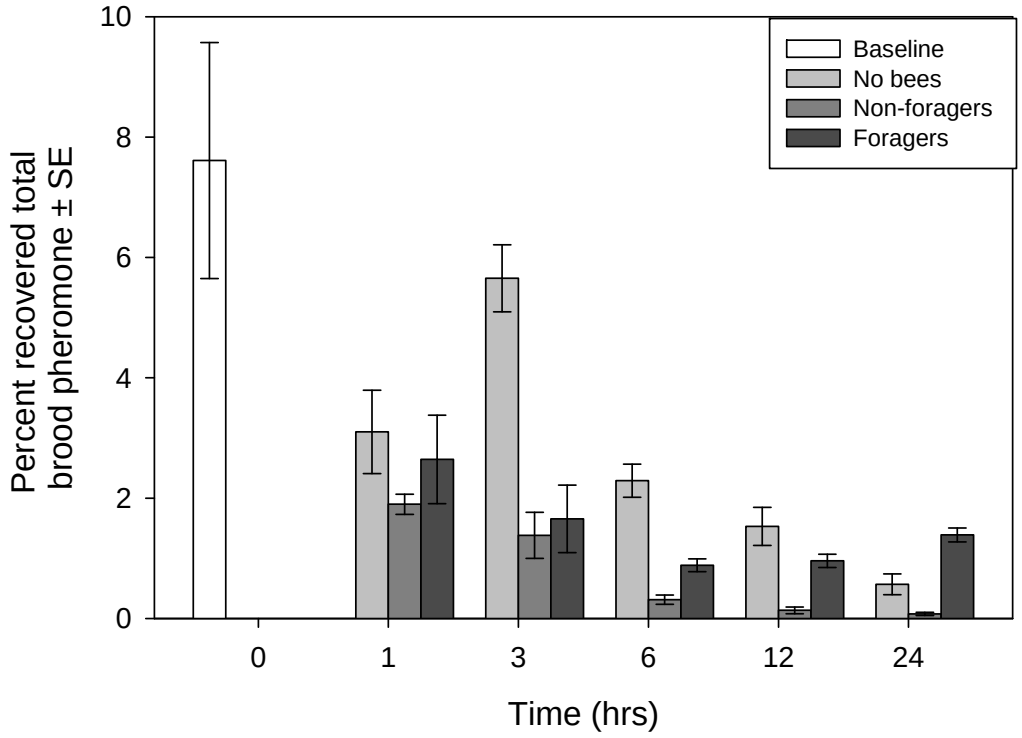

Fig. 17 Recovered proportion of total brood pheromone over time in the absence of bees, or exposed to either non-foragers or foragers. Time zero recovery is provided as an indicator of the base methodological loss. Recovered proportions differed significantly by treatment over time ($p<0.0001$). Recovered proportion was greatest from pheromone plates not exposed to bees. Generally, recovery was slightly higher from forager compared to non-forager exposed plates

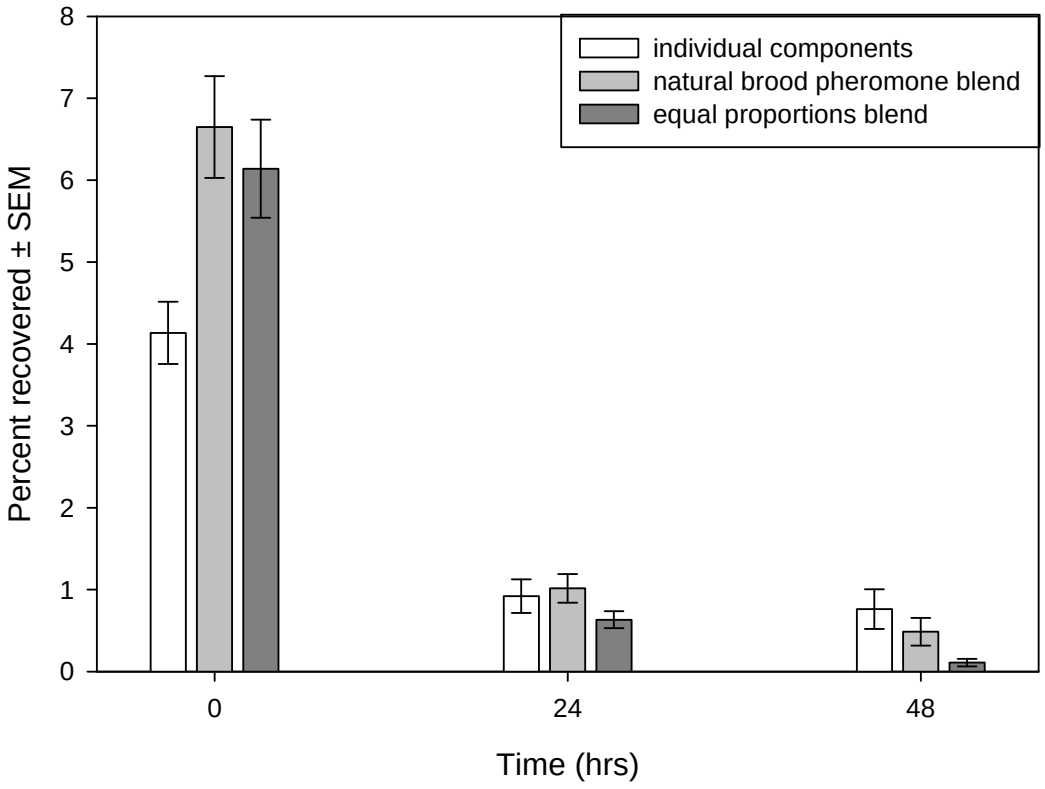

Fig. 18 Percent recovered of brood pheromone components on glass plates over time either alone, combined in natural brood pheromone proportions, or mixed in an equal proportions blend. Recovered proportion differed significantly by blend over time (ANCOVA; p=0.002) with components being generally more recoverable over time when formulated in extractable proportions relative to formulated individually or in equal proportions

Recovery of brood pheromone in the absence of bees

Brood pheromone component recovered proportion decreased significantly over time ($F_{1,239}$= 491.1; p<0.0001). Recovered proportion was also significantly different by ester ($F_{9,239}$= 8.9; p<0.0001) and blend formulation ($F_{2,239}$= 6.2; p=0.002). There was no significant interaction between ester and blend ($F_{18,239}$= 1.4; p=0.151). Residuals from the secondary model (not including ester as an explanatory factor) were significantly positively correlated with molecular weight, significantly negatively correlated with boiling point and weakly correlated with ester functional group length and number of double bonds (Table 4).

Table 4. Pearson's bivariate correlations of recovered proportion residuals to ester component characteristics

	residuals	Ester-group length	number of double bonds	boiling point	molecular weight
residuals		.058	-.090	-.151*	.272**
Ester-group length	.058		.000	-.009	.566**
number of double bonds	-.090	.000		.153*	.277**
boiling point	-.151*	-.009	.153*		-.130*
molecular weight	.272**	.566**	.277**	-.130*	

* = p<0.05; **= p<0.01

DISCUSSION

When reared in cages for 5 days in the presence of brood pheromone, newly emerged bees in their first week of life exhibited an increased sucrose response threshold relative to no-pheromone control (Fig. 16). This is similar to previously reported results (Pankiw and Page, 2000). Sucrose response thresholds of pollen and non-pollen foragers were not modulated when reared in cages for the same amount of time and exposed to the same proportion of brood pheromone (Fig. 16). The lack of response threshold modulation in foragers could have resulted from foragers avoiding brood pheromone, not contacting the plate and therefore limiting their exposure to brood pheromone-induced modulations. Alternatively, foragers may simply have been physiologically insensitive to sucrose modulation, despite contacting and removing it from the plate.

Preliminary analyses of plates placed inside colonies showed that no brood pheromone components were recoverable after 24 hrs. This is not surprising given the observation of bees clumping on, crawling, and licking the pheromone plate upon its insertion into the brood nest. This combined with the practice of inserting the pheromone-treated plate in the space between two frames, essentially forcing bees to come into contact with the plate (and therefore the pheromone) makes this result unsurprising. It is well established that bees must contact brood pheromone in order to be affected (Mohammedi et al., 1996), and previous research has shown at least one

component of BP (methyl oleate) to be attractive to non-foragers (Keeling et al., 2003). However, it is unclear whether all bees are contacting brood pheromone or whether a subset of bees, e.g. nurses, is responsible for the removal of BP from the plates and presumed dissemination throughout the colony. A second experiment was performed under a controlled setting in the lab, separating two main categories of honey bee workers, foragers, and non-foragers.

Recovered proportion of brood pheromone was lower when exposed to foragers or non-foragers compared to a no-bee control (Fig. 17). This suggests that both foragers and non-foragers were removing brood pheromone from the plates and are therefore exposed to potential behavioral and physiological effects. It is not certain whether incidental cuticular contact with brood pheromone was sufficient to induce a behavioral or physiological effect. Queen mandibular pheromone has been shown to be translocated from its source to workers through grooming and antennal contact (Naumann, 1991). It may be supposed that a similar mechanism occurs with brood pheromone and as such, the pheromone would be similarly transferred.

Although foragers removed pheromone from a glass plate when forced into close proximity, it is uncertain that they would do so in the colony where they are presumably able to choose to approach or not approach the plate. Generally, recovered proportions of brood pheromone components were somewhat higher for foragers compared to non-foragers, suggesting they may not have been as active in removing the pheromone as the non-foragers. Also, it should be noted that in the forager exposed

pheromone plate; a high amount of single components, mainly ethyl esters (*personal observation*) was observed in the later time points. The foragers in particular had a large amount of pollen in their setae (*personal observation*). They also occasionally defecated within the cage (*personal observation*). This tended to result in visible deposits of unknown material on the plates, some of which appeared to be hexane soluble. This may have confounded the results of the recovery of brood pheromone especially in the later time periods; while it cannot be ruled out that bees deposit single components of brood pheromone esters onto the plates, the more likely scenario is that the material on the plate coelutes with the brood pheromone esters. It would be necessary to perform further chemical analyses to identify putative coeluting compounds to rule out this possibility.

Given that foragers placed in direct proximity with brood pheromone make contact with brood pheromone, resulting in decreased recovery over time, did not exhibit modulated sucrose response thresholds, it may be that foragers are physiologically insensitive to sucrose response threshold priming. This is the first study to show this result. Because sucrose response threshold is correlated with forage choice behavior (Pankiw and Page, 2000), it may be supposed that brood pheromone did not induce a change in forage choice, although this study did not directly demonstrate this.

Different blends of brood pheromone have been observed (see chapters II and III) and, in addition to their differential biological effects, these blends may be

differentially recoverable over time, leading to more or less persistent brood cues and therefore affect colony level perception of brood. Recovered proportion of brood pheromone components differed by blend (Fig. 18). Interestingly, components in the brood pheromone blend were similarly recoverable as those exposed to hive conditions individually, while the equal proportions blend was less recoverable over time than the other formulations. Brood pheromone in a naturally occurring blend is generally more recoverable over time, suggesting that the signal may be stable for a longer period and therefore bioactive for longer as well. Further study into the effect of blend on the longevity of the signal and its behavioral effect is necessary to elucidate the impact changes in blend have on signal persistence and ultimately colony response to brood.

Unsurprisingly, esters differed in their recoverability over time; generally, ethyl esters were more recoverable than methyl esters, while higher molecular weight compounds were more recoverable than lower molecular weight, leading to the conclusion that the more saturated compounds are less recoverable over time compared to the less saturated fatty acid esters. Although there wasn't a significant correlation between recovered proportion and number of double bonds, other characteristics such as esterification and boiling point could have masked this effect. It should be noted that there was significant colinearity between number of double bonds and molecular weight, so the effect of variation of each single characteristic was difficult to separate. Because of the variation in ester recovery over time, the blend of brood pheromone necessarily changed as it was exposed to the incubator conditions. Different blends of

brood pheromone elicit different pollen foraging responses (see chapters II and III). Blends of brood pheromone missing components are often ineffective in releasing pollen foraging (Pankiw et al., *unpublished*). As such this may be the mechanism through which brood pheromone becomes ineffective at eliciting a pollen foraging response. The dynamic nature of the brood pheromone blend could mean that a subtle interplay of physical characteristics of brood pheromone, physiological sensitivity of the receiver, and variation in production by the larvae occurs in the hive to regulate individual and colony level foraging effort.

CHAPTER V

SUMMARY AND CONCLUSIONS

The goal of this dissertation was to characterize variation in brood pheromone and gain some understanding of the social context and behavioral effect of variation on individual bees and colonies. Results of this research showed that brood pheromone blend varied by honey bee population, larval rearing environment, and time exposed to a simulated hive environment.

In the second chapter, I tested the hypothesis that brood pheromone blend or amount changed as a consequence of adult contact with larvae and that the changes in brood pheromone communicated larval nutritional status to adults. While brood pheromone blend differed between deprived and non-deprived larvae, the blend did not result in differential aggregation in the nursing bioassay. It was therefore concluded that brood pheromone did not communicate short term larval nutritional status, but perhaps long term larval condition. Interestingly, the deprived blend of brood pheromone decreased the proportion of returning pollen foragers relative to control when provided in extractable amounts, suggesting that the nurses and foragers, two separate task groups devoted at least in part to brood rearing, responded differently to larval cues.

In the third chapter, I tested the hypothesis that brood pheromone blend changed based on honey bee population, in effect, that there was a genotypic component to brood pheromone variation. Blends were significantly different by source. Additionally, it was shown that Africanized and European colonies responded with a greater proportion of returning pollen foragers when treated with a brood pheromone blend derived from similar sources, rather than from sources from a different mitochondrial lineage. It was concluded from these results that the production of brood pheromone blend was co-adapted to the response, possibly as a mechanism of self-recognition. Interestingly, it was found that Africanized bees insensitive to sucrose response threshold priming by brood pheromone. Because Africanized bees are known to develop more quickly, as measured by their characteristically younger age of first foraging (Winston and Katz, 1982), this unexpected result led to the conclusion that there may be a certain period of time during which a young adult bee is sensitive to the primer effect of brood pheromone, after which she is no longer able to be modulated.

In the fourth chapter, I hypothesized that foragers would be physiologically insensitive to sucrose response threshold priming by brood pheromone. Forager sucrose response thresholds were not modulated by brood pheromone treatment, although recovery of the pheromone from treatment plates was significantly less than a no-bee control; results supported the hypothesis as foragers clearly removed brood pheromone but were unaffected by it, at least in the proboscis extension reflex bioassay.

Making some logical extrapolations and considering the whole of the results of this dissertation, it is possible to suggest some interesting grand conclusions. The component esters of brood pheromone are derived from fatty acids obtainable from the larval diet, some of which may be essential, that is, larvae cannot synthesize the acids *de novo*, it is possible that deprivation of adult contact, and therefore deprivation of feeding, leads to changes in the production, processing, or excretion of brood pheromone (Manning et al., 2007). Combined with the results in chapters II and IV, it may be supposed that deprived larvae may alter or stop production or secretion of brood pheromone in response to food deprivation, resulting in the altered blend observed. This change, however did not appear to communicate nutritional status *per se*. Instead, something in the whole extract, rather than the characterized compounds of brood pheromone attracted nurses and increased colony level pollen foraging. Further work with deprived and non-deprived brood pheromone and larval extracts, encompassing more in-depth observation hive studies will be necessary alongside an increased analytical capacity to better understand how adults assess larval nutritional state.

Brood pheromone blends have different rates of recovery and different blends may change more quickly than others. From the results in chapter III, it is clear that within the same population of bees, different blends of brood pheromone elicit different responses. It may be therefore supposed that over time, provided brood pheromone is not replenished, its effects may change or diminish. Different blends may be adapted to

different environmental or social conditions and therefore optimized to be stable for longer or shorter periods of time. This may represent one possible mechanism inducing differential foraging behaviors between Africanized and European bees. The study in chapter III only observed short-term foraging effects of brood pheromone; a study observing the time for proportion of pollen to non-pollen foragers to become statistically similar to a no-pheromone control would be necessary to test this.

The results of the sucrose response threshold bioassays of chapters III and IV suggest that different genotypes and ages of honey bee have different response thresholds to brood pheromone. Because blend of brood pheromone also affects this response, it may be supposed that this response threshold may be based on a comparison to either a learned or imprinted blend of brood pheromone. Based on observations with queen pheromone, the only other primer-releaser pheromone characterized in the honey bee (Keeling et al., 2003), it is reasonable to assume that brood pheromone is passed through contact transmission. Because there are multiple sources of brood pheromone (larvae) and intra-colony variation in brood pheromone blend is quite prevalent (chapter III), it may be supposed that there is a gestalt, colony blend of brood pheromone that differs somewhat from that produced by the individual. Understanding the effects that genotype and environment have on brood pheromone blend, and adult response to different blends is crucial to furthering our understanding of the social context of brood pheromone blend variation. This could be observed in cross-fostering experiments using related genotypes. Further, detailed study into the

relationship between brood pheromone amount, colony and environmental conditions, and response effects will be necessary to show that differential response thresholds to brood pheromone exist.

Variation in a social pheromone has never before been thoroughly explored and demonstrated to occur in such a variety of modes with clear behavioral effects. The methodology used in the course of this dissertation made it possible to characterize brood pheromone from a small number of larvae, but never from an individual. It should be clear that in the context of measuring how responses to individual larval need relates to colony level response to the collective needs of the larvae, we must be able to look at individual larval variation in relation to age, genotype, and rearing environment. It is also important to observe and quantify the method by which brood pheromone is transferred to bees that do not contact larvae. It is likely that brood pheromone is transferred through adult contact as for queen mandibular pheromone (Naumann, 1991; Naumann et al., 1992), however further study must be performed to confirm this supposition. Further studies of rearing a small cohort of larvae in a larger unrelated colony, using controlled genotypes such as sister, single drone inseminated queens, will allow separation of the genetic and environmental components of brood pheromone modulation and its effects on adult behavior. This dissertation has shed light on the basic variation of brood pheromone, yet has raised a number of questions and avenues of research that would present a fascinating challenge to understanding and managing brood rearing and colony foraging behavior in the future.

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