EVALUATION OF PHYSIOLOGICAL AND PHEROMONAL FACTORS REGULATING HONEY BEE, *Apis mellifera* L. (HYMENOPTERA: APIDAE) FORAGING AND COLONY GROWTH

A Dissertation

by

RAMESH REDDY SAGILI

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

May 2007

Major Subject: Entomology

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Approved by:

Chair of Committee,	Tanya Pankiw
Committee members,	Keyan Zhu-Salzman
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May 2007

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ABSTRACT

Evaluation of Physiological and Pheromonal Factors Regulating Honey Bee, *Apis mellifera* L. (Hymenoptera: Apidae) Foraging and Colony Growth. (May 2007)

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Chair of Advisory Committee: Dr. Tanya Pankiw

This dissertation examines some important physiological and pheromonal factors regulating foraging and colony growth in honey bee colonies. The first study analyzed effects of soybean trypsin inhibitor (SBTI) on the development of hypopharyngeal gland, midgut enzyme activity and survival of the honey bee. In this study newly emerged caged bees were fed pollen diets containing three different concentrations of SBTI. Bees fed 1% SBTI had significantly reduced hypopharyngeal gland protein content. This study indicated that nurse bees fed a pollen diet containing at least 1% SBTI would be poor producers of larval food.

In the second study nurse bee biosynthesis of brood food was manipulated using SBTI, and the resulting effects on pollen foraging were measured. Experimental colonies were given equal amounts of SBTI treated and untreated pollen. SBTI treatments had significantly lower hypopharyngeal gland protein content than controls. There was no significant difference in the ratio of pollen to non-pollen foragers and

pollen load weights collected between the treatments. These results supported the pollen foraging effort predictions generated from the direct independent effects hypothesis.

In the third study we tested whether brood pheromone (BP) regulated queen egg laying via modulation of worker-queen interactions and nurse bee rearing behaviors. This experiment had BP and control treatments. Queens in the BP treatment laid greater number of eggs, were fed for a greater amount of time and were less idle. Significantly more time was spent in cell cleaning by the bees in BP treatments. The results suggest that brood pheromone regulated queen egg-laying rate by modulating worker-queen interactions and nurse bee rearing behavior.

The final study of this dissertation focused on how dose-dependent BP-mediated division of labor affected the partitioning of non-foraging and foraging work forces and the amount of brood reared. Triple cohort colonies were used and there were three treatments, Low BP, High BP and Control. Low BP treatments had significantly higher ratio of pollen to non-pollen foragers and greater pollen load weights. Low BP treatment bees foraged at a significantly younger age. This study has shown that BP elicits dose-dependent modulation of foraging and brood rearing behaviors.

ACKNOWLEDGEMENTS

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I would like to thank Stephanie Walkup-Birkhead, Robert Vaughn, Swaroop, Haribhaskar, GW Burlin and Roman Roman for providing technical assistance during my research. I am thankful to my colleagues Brad Metz, Lizette Peters and Stephen Bahr for their support and friendship. I acknowledge the moral support and patience of my wife Suma, who has been a constant source of encouragement. Also, my two little boys Sai and Srikar have been a source of joy and inspiration for me. I also extend my sincere thanks to my parents and in-laws for their understanding, support and encouragement. I further express my gratitude to all my friends and relatives for their constant support and encouragement.

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CHAPTER I

INTRODUCTION

Honey bees are important both economically and ecologically, and also serve as a model organism for studying social organization and behavior. It has been estimated that honey bee pollination contributes approximately $15 billion in value annually to U.S agriculture (Morse and Calderone 2000). An ordered caste system exists in honey bees and thus a typical honey bee colony consists of a single fertile queen, semi-sterile female workers ranging between ten to thirty thousand and few thousand males called drones, depending on time of the year. Egg laying is the primary function of the queen and she is the mother of all other colony members. Workers perform all the hive tasks both inside and outside the hive. The queen and worker bees are diploid in origin, whereas drones are haploid and develop from unfertilized eggs by parthenogenesis. Eggs, larvae, and pupae present in a colony are collectively referred to as brood. Honey bee colonies reproduce by process of colony budding, commonly referred to as swarming (Winston 1987).

Colony growth is an important fitness trait in honey bees and many other social insects. Behavioral change is considered to favor colony fitness, and thus the individual

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This dissertation follows the style and format of Behavioral Ecology and Sociobiology.

fitness in social insects (Page and Erber 2002). Colonies with appropriate behavior survive and reproduce (Page and Erber 2002). In general, a larger population increases colony survival and reproduction; fitness traits (Cole 1984; Lee and Winston 1987; Little 1979; Michener 1964; Pomeroy 1979; Richards and Richards 1951; Seeley 1985; Seeley and Visscher 1985; Winston 1987). Adult population size at swarming is highly variable and not the singular determinant for swarming (Winston 1987; Winston 1993). Other factors such as time of year, adult age distribution, and foraging environment also affect the timing of swarming (Winston 1987).

Colony growth rates and trajectories are critical to colony reproductive rate, the size of swarms and the timing of swarming (Lee and Winston 1985a; Lee and Winston 1985b; Lee and Winston 1987). Survival of swarms is likewise dependant on parental colony size and the timing of issue (Seeley 1978; Lee and Winston 1985a; Lee and Winston 1985b; Morales 1986; Lee and Winston 1987). Lee and Winston (1985b) found a positive correlation between swarm size and both brood production and emergent worker weight in newly founded colonies. Larger colonies invest more workers to swarms, which confers an increased probability of swarm survival (Lee and Winston 1987). Larger swarms also produce more total brood comb, that area in which brood are reared (Lee and Winston 1985a). The number of swarms that a colony produces is positively correlated with the amount of pupae at the time the first swarm issues (Winston 1979; Winston 1980). Collectively these studies demonstrate that colony growth rate affects colony fitness.

It is the intensity with which individuals collect resources that profoundly affects colony growth and development (Farrar 1944; Moeller 1958; Moeller 1961; Free and Racey 1968; Nelson and Jay 1972; Smirl and Jay 1972). It is generally assumed that various colony foraging strategies are adaptive (Robinson 1992). How foraging strategies contribute to colony fitness is usually not addressed. A principal focus of this thesis aims to addresses this gap in our knowledge by examining the relationship between protein content of nurse bee hypopharyngeal glands, brood pheromone regulated foraging behavior and their affects on the amount of colony brood rearing.

Division of labor

Division of labor is one of the striking features observed in social insects, where groups of individuals perform different tasks repeatedly to enhance individual and colony efficiency. Plasticity is a key characteristic of division of labor, where colonies respond to changes in the internal and external environments by making adjustments to worker ratios involved in different tasks (Robinson 1992). Individual worker plasticity also contributes to colony level behavior plasticity.

Temporal polytheism is an important phenomenon observed in honey bees where individuals perform different tasks as they age. Worker honey bees perform within colony tasks early in their lives such as cell cleaning, brood and queen tending, nectar receiving, pollen packing, and then progress to tasks like ventilating and guarding before foraging outside the nest; final task before they die (Winston 1987). Hence, the

adult work force can be categorized in to non-foraging hive bees that rear brood and maintain the nest and foraging bees that work outside collecting food for the colony.

Foraging and mechanisms regulating the quantity of food collected are essential attributes for all organisms. The honey bee scientific literature is dominated by studies of foraging division of labor (Calderone and Page 1991; Robinson 1992; Seeley 1995; Calderone 1998; Page and Mitchell 1998; Dreller and Page 1999; Pankiw and Page 2000; Beshers and Fewell 2001; Page and Erber 2002; Robinson 2002; and references therein). This is but a small sample of which there are so many, listing more is of no increased benefit to the reader. In the ontogenetic profile of worker bees, virtually every task may be shifted by a change in colony conditions (Seeley 1985; Winston 1987; Robinson 1992; Seeley 1995; Page and Erber 2002; Robinson 2002). Briefly, factors demonstrated to affect age of first foraging include genotype, hemolymph titers of juvenile hormone, perturbed demographic distributions of young and old adult bees, and primer pheromones. The foraging behavior of social insects is especially interesting because individuals do not forage to meet their own nutritional needs; rather they forage to meet the needs of the colony. Foraging labor is also divided such that some individuals forage for nectar, some for pollen, some individuals return to the nest carrying both nectar and pollen, and a small proportion of the foraging force return with water.

Equally robust is the literature demonstrating a division of foraging labor for pollen and non-pollen resources. Briefly, factors that affect forage choice behavior include genotype, responsiveness to sucrose, amount of larvae and their pheromones called brood pheromone, amount of stored pollen, amount of honey and available empty

comb space, as well as quality and quantity of resources in the foraging environment (Frisch 1967; Seeley 1982; Winston 1987; Seeley 1989; Hunt et al. 1995; Seeley 1995; Dreller and Page 1999; Page and Erber 2002). In stark contrast to the number of empirical studies on division of labor and individual foraging effort there is a paucity of studies demonstrating how foraging strategies affect an important life history trait of colonies; amount of brood rearing. To place colony foraging strategies in both an evolutionary and apicultural context it is important to increase our understanding of how different foraging strategies affect colony growth.

Division of brood rearing labor

Pollen foragers collect pollen from available plant sources then return to the nest and deposit their loads of pollen directly into cells. Stored pollen is consumed by nurse bees that use the proteins derived from the pollen to produce proteinaceous hypopharyngeal gland secretions that are fed to developing larvae (Crailsheim et al. 1992). Brood food is composed of a clear secretion from hypopharyngeal gland that is presumably mixed with honey, digestive enzymes and water, and is fed to the developing larvae by the nurse bees (Winston 1987). Nurse bees, aged about 7 to 14 days consume pollen and convert it to proteinaceous secretions provisioned to larvae. In this way larvae consume pollen via nurse bees.

Larval cues and pollen are necessary for hypopharyngeal gland development, activity, and protein production (Brouwers 1982; Brouwers 1983; Huang and Otis 1989; Huang et al. 1989; Mohammedi et al. 1996; Hrassnigg and Crailsheim 1998). Larvae or

their fatty acid esters stimulate hypopharyngeal gland development even in the absence of a pollen diet. However, a protein source is necessary for glandular protein biosynthesis resulting in greater amounts of extractable protein (Brouwers 1983; Huang and Otis 1989; Huang et al. 1989; Mohammedi et al. 1996). Larvae and larval esters have clearly been demonstrated to prime hypopharyngeal gland development and pollen provides the protein source fueling brood-food production. Addition of larval esters to average colonies also increases amount of protein extractable from hypopharyngeal glands (Pankiw et al. 2004). In second part of the dissertation research I manipulated protein biosynthesis of hypopharyngeal glands by using a protease inhibitor to examine the effects of protein constrained brood food on pollen foraging and colony growth.

Protease inhibitors

Protease inhibitors are compounds that inhibit digestion by preventing the break down of proteins. Insecticidal properties of protease inhibitors have been established in transgenic plants. Plant protease inhibitor genes encode proteins that can inhibit insect protein digestive enzymes, resulting in starvation and even death of the insect (Michaud 2000). Along with the targeted pest, honey bees will also be exposed to the protease inhibitors when they are foraging in the field for nectar or pollen. Hence it is important to assess possible effects of protease inhibitors on beneficial insects like the honey bee. Hence as a part of this dissertation research I studied the effects of SBTI, a serine protease inhibitor on hypopharyngeal gland development, midgut protease activity and

survival of the honey bee. This study constitutes the first part of this dissertation research.

Pheromones

Social organization in honey bees is regulated to a greater degree by the pheromones. Pheromones are chemicals that are primary source of intraspecific communication in many organisms (Wyatt 2003) and cause changes in behavior and physiology. Social insect pheromones are classified in to two categories, primer and releaser pheromones. Releaser pheromones elicit an immediate effect on the behavior of a receiver, and once the pheromone has dissipated or is removed individuals revert to their previous state (Pankiw 2004b). Primer pheromones physiologically alter reproductive, endocrine, and neurosensory systems (Pankiw 2004a). Change occurs over a time scale of days and only incomplete reversion occurs if the pheromone is removed. Primer pheromones are responsible for altering the behavioral state of an individual. This change results via putative shifts in response thresholds to different stimuli by altering reproductive, endocrine and neural systems (Pankiw and Page 2003; Pankiw 2004b).

Honey bees have an extraordinary and highly intricate chemical communication system (Winston 1987). Only two honey bee primer pheromones, queen mandibular pheromone (QMP) and brood pheromone (BP) have been isolated and chemically characterized. Brood pheromone is a 10-component mixture of methyl and ethyl fatty esters that can be extracted from the surface of honey bee larvae (Le Conte et al. 1990). Brood pheromone communicates the presence of larvae in the colony. Brood pheromone

is a tool that can be used to alter the foraging stimulus environment and change honey bee colony foraging strategies. In the third study of this dissertation research, I examined effects of brood pheromone regulated queen egg laying by measuring worker-queen interactions, the larval nutritional environment and nurse bee rearing behaviors. In the final study I examined how dose-dependent brood pheromone mediated division of labor affected the partitioning of foraging and non-foraging work force and the amount of brood reared.

The goal of this study was to investigate the effects of some important physiological and pheromonal factors that regulate colony growth and foraging in the honey bee. The following were the specific objectives:

1) Analyze effects of soybean trypsin inhibitor on hypopharyngeal gland protein content, total midgut protease activity and survival of the honey bee (*Apis mellifera* L.).

The hypothesis here was that SBTI would have deleterious effects on hypopharyngeal gland development, protein digestion, and length of life of adult bees.

2) Evaluate the effects of protease inhibitor induced constrained hypopharyngeal gland protein content on honey bee (*Apis mellifera* L.) pollen foraging and colony growth.

3) Examine how brood pheromone regulated queen egg-laying rate in the honey bee (*Apis mellifera* L.).

The hypothesis here was that brood pheromone regulates queen egg-laying by modulating worker-queen interactions, increases nutritional environment and nurse bee rearing behaviors.

4) Examine the brood pheromone regulated foraging ontogeny effects on brood rearing in the honey bee.

This dissertation is divided in to six chapters. Chapter I is an introduction on honey bee colony growth and foraging, and also lists the objectives of the dissertation. Chapter II reports the effects of soybean trypsin inhibitor on hypopharyngeal gland protein content, total midgut protease activity and survival of the honey bee. In chapter III effects of protein constrained brood food on honey bee pollen foraging and colony growth are reported. Chapter IV examines how brood pheromone regulates egg laying in honey bee queen. Chapter V focuses on brood pheromone regulated ontogeny effects on brood rearing. Chapter VI provides an overall summary of the conclusions pertaining to each chapter.

CHAPTER II

EFFECTS OF SOYBEAN TRYPSIN INHIBITOR ON HYPOPHARYNGEAL GLAND PROTEIN CONTENT, TOTAL MIDGUT PROTEASE ACTIVITY AND SURVIVAL OF THE HONEY BEE (*Apis mellifera* L.)*

Introduction

Plant protease inhibitor genes encode proteins that can inhibit insect protein digestive enzymes, resulting in starvation and even death of the insect (Michaud, 2000). Insect pests however, are capable of evolving biotypes with adaptations to protease inhibitors that overcome or bypass toxic effects of protease inhibitors (Roush and Mackenzie, 1987). Beneficial insects, that act as pollinators, are additional co-evolutionary members among many plant-insect interactors (Delaplane and Mayer, 2000). The advent of genetic engineering techniques allows the transfer of plant insecticidal genes from one species to another (Gatehouse and Gatehouse, 1998).

Pollen is the most likely channel through which the honey bee will be exposed to transgenic protease inhibitors (Malone and Pham-Delegue, 2001). The honey bee has serine proteinases as digestive enzymes (Moritz and Crailsheim, 1987). Two serine trypsin endopeptidase inhibitors, bovine pancreatic trypsin inhibitor (BPTI) and soybean

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*Reprinted with permission from "Effects of soybean trypsin inhibitor on hypopharyngeal gland protein content, total midgut protease activity and survival of the honey bee (*Apis mellifera* L.)" by Sagili, R.R., Pankiw, T., Zhu-Salzman, K., 2005. Journal of Insect Physiology, 51, 953-957, 2005 Elsevier Ltd.

trypsin inhibitor (SBTI), known to be effective against a range of insect pests, are also toxic to adult honey bees at 1% wt: vol in sugar solution (Malone et al., 1995). There are very few published measurements of transgene expression levels in pollen; hence this limits our ability to design toxicity tests that mimic expression levels expected in the field. Plants can be protected from pests when protease inhibitors are expressed at approximately 1% of total soluble leaf protein (Hilder et al., 1987; McManus et al., 1994). Protease inhibitor concentrations used in this study were estimates of the range of transgene product concentrations a bee is expected to encounter while foraging. The lower concentration of 0.1 % SBTI in pollen used in this study may represent a value closer to field relevance and the higher concentrations are unlikely to be encountered in the field and thus represent a worst-case scenario.

I hypothesized that SBTI would have deleterious effects on honey bee protein digestion. In this study, I evaluated effects of soybean trypsin inhibitor (SBTI) on hypopharyngeal gland protein content, total midgut proteolytic enzyme activity and survival of adult honey bees. This study is the first to measure the effects of a protease inhibitor on hypopharyngeal gland protein content of honey bees. Hypopharyngeal glands are the brood food or protein-producing glands located in the head of worker honey bees called nurses (Patel et al., 1960). The diameters of the acini of hypopharyngeal glands in hive bees are largest when the hive bees are 8 days old (Crailsheim and Stolberg, 1989). Protein synthesis rates in hypopharyngeal glands are highest when the bees are 8-16 days old (Knecht and Kaatz, 1990). Pollen is the only source of protein for adult honey bees and consumption is necessary for gland

development and protein production (Mohammedi et al., 1996). Insufficient pollen consumption early in life results in poor gland development and a shorter worker length of life (Maurizio, 1950; Haydak, 1970).

Protein digestion disruption affects hypopharyngeal gland protein production and consequently is expected to affect the ability of nurse bees to provision larvae with food. The combined effects of low larval food production and decrease in adult length of life could have serious consequences on colony population maintenance and growth. Hypopharyngeal glands in newly emerged bees treated with SBTI (0.1% and 1% w:v in sucrose solution) for 10 days have significantly reduced mean weights and acini diameter (Babendreier et al., 2005). Malone et al. (2004) reported no significant effects on survival and hypopharyngeal gland development of honey bees during evaluation of potential effects of a Bt toxin, a biotin binding protein and a protease inhibitor.

Methods

Combs containing pupae were placed in an incubator maintained at 33ºC and 50% RH. Six hours later, newly emerged adults were placed in plexiglass-wire mesh cages (15 cm x 11 cm x 8 cm) and provisioned with gravity feeders containing sugar solution (40% w:v). Powdered pollen and SBTI dissolved in a small volume of sugar solution were mixed thoroughly. This uniform pollen mixture was packed into inverted vial caps and provided to the caged bees. Cages were provisioned daily with fresh sucrose solution and pollen diet.

Hypopharyngeal gland protein quantification

The caged bees were fed 3 different concentrations (0.1%, 0.5% and 1 % w: w) of SBTI (Sigma Aldrich product T-9003, St. Louis, MO, USA). Controls were handled in the same way but without the inhibitor. A randomized complete block design was used for this experiment. Eighty bees were randomly assigned to each cage and the cages were randomly assigned to treatments. The experiment was replicated four times for a total of 16 cages (4 treatments × 4 replications). On day 7, ten bees were removed from each cage. Bees were cold anaesthetized, their hypopharyngeal glands removed and stored in Tris buffer at -80ºC prior to analysis. Frozen HP glands were homogenized and centrifuged at 10,000 rpm for 5 min. The supernatant was used to determine the protein concentration after Bradford (1976), described below.

Bradford assay

Both hypopharyngeal glands from each bee were stored in 20 µl Tris Buffer pH 7.9 in 1.5 ml microcentrifuge tubes. Glands were homogenized using a homogenizer that tightly fitted onto each tube. Subsequently, tubes were centrifuged at 1,000 rpm for 2 min. Supernatant from each tube was used for analysis. I used the 500-0202 Quick Start Bradford Protein Assay Kit 2, containing all reagents and dyes (Bio-Rad Laboratories, Hercules, CA, USA). Dye reagent was prepared by diluting 1 part Dye Reagent concentrate (Coomassie Brilliant Blue G-250) with 4 parts distilled water. I added 2 µl

or 5 µl quantities of each sample to be analyzed to microcentrifuge tubes with 1 ml Bradford reagent. Tubes were vortexed to homogenize the contents, then incubated for 5 min at room temperature. Standard-curves were prepared using bovine serum albumin (BSA). Protein absorbance was measured at 595 nm against blank reagent using a Beckman Spectrophotometer (Model #D4-640, Beckman Instruments, Inc., Columbia, MD, USA). Weight of protein (BSA) was plotted against the corresponding absorbance value to generate a linear regression equation (SAS PROC REG; SAS 2000). To calculate micrograms of protein extracted from hypopharyngeal glands from measured absorbance values, we applied the linear regression equation generated from the BSA standard curve above.

Protein quantity was further analyzed using analysis of variance (ANOVA) (Sokal and Rohlf, 1995; SPSS, 2000). The data were log transformed prior to analysis to normalize the distribution (Sokal and Rohlf, 1995). Least Significant Difference (LSD) was used to signify between treatment differences. Beta or Type II error is more important in case of risk assessment studies. Hence LSD which is a less conservative test for finding differences among treatments was used for multiple comparisons of treatments.

Total midgut proteolytic enzyme activity

The midguts of the same 7-day-old bees were excised from which the hypopharyngeal glands were removed above. Midguts were placed in centrifuge tubes containing 100 µl

Tris-HCl buffer (pH 7.9) each and stored at -80ºC prior to further processing. Frozen guts were crushed, homogenized in Tris-HCl buffer (pH 7.9) and, centrifuged at 10,000 rpm for 5 min to remove particulate matter. The supernatant was analyzed for total midgut proteolytic enzyme activity (casenolytic activity) as described by Michaud et al (1995).

Five microlitres of supernatant was used for each reaction. Twenty microlitres of assay buffer (0.1 M Tris-HCl, pH 7.9) and 60 µl of 2% (w/v) azocasein diluted in assay buffer were added respectively to the supernatant and incubated for 6 h at 37ºC. To remove the residual azocasein after proteolysis, 300 µl of 10 %(w: v) TCA was added to each mixture and centrifuged for 5 min at 10,000 rpm. 350µl of supernatant was added to 200 µl of 50% ethanol in water, and the absorbance of this mixture was measured at 440nm using a Beckman DU 64 spectrophotometer. Absorbance of the sample without proteolysis (no incubation) was used to zero the machine. Total midgut proteolytic activity was expressed in terms of OD_{440}. Data were log transformed prior to analysis to meet assumptions of ANOVA (Sokal and Rohlf, 1995). Mean total midgut proteolytic enzyme activities from each treatment were analysed using ANOVA and LSD (Sokal and Rohlf, 1995; SPSS, 2000). Correlation analysis (parametric) using SPSS was performed to measure the strength of linear association between midgut enzyme activity and hypopharyngeal gland protein quantity.

Survival analysis

Daily, for 30 days, the number of dead bees per cage was recorded and bodies removed. Survival curves were generated by plotting the number of surviving bees against days from initiation of the experiment. Kaplan-Meier survival curves were used to plot and interpret the survival data (Le, 1997). Survival curves were compared using Log rank tests (Allison, 1998; SAS, 2000). A Cox proportional hazard (PH) model was used to model the survival data using SPSS. Bees that survived up to the termination of the experiment (day 30) and those that were removed from the cages for the Bradford Assay and midgut enzyme activity analysis were treated as censored cases.

Results

Hypopharyngeal gland development

Dose-dependent effects of SBTI on amount of extractable protein from adult hypopharyngeal glands are summarized in Fig. 1. Replicates were not significantly different and therefore pooled for subsequent analysis ($F_{3,156}$=0.976, P=0.42).

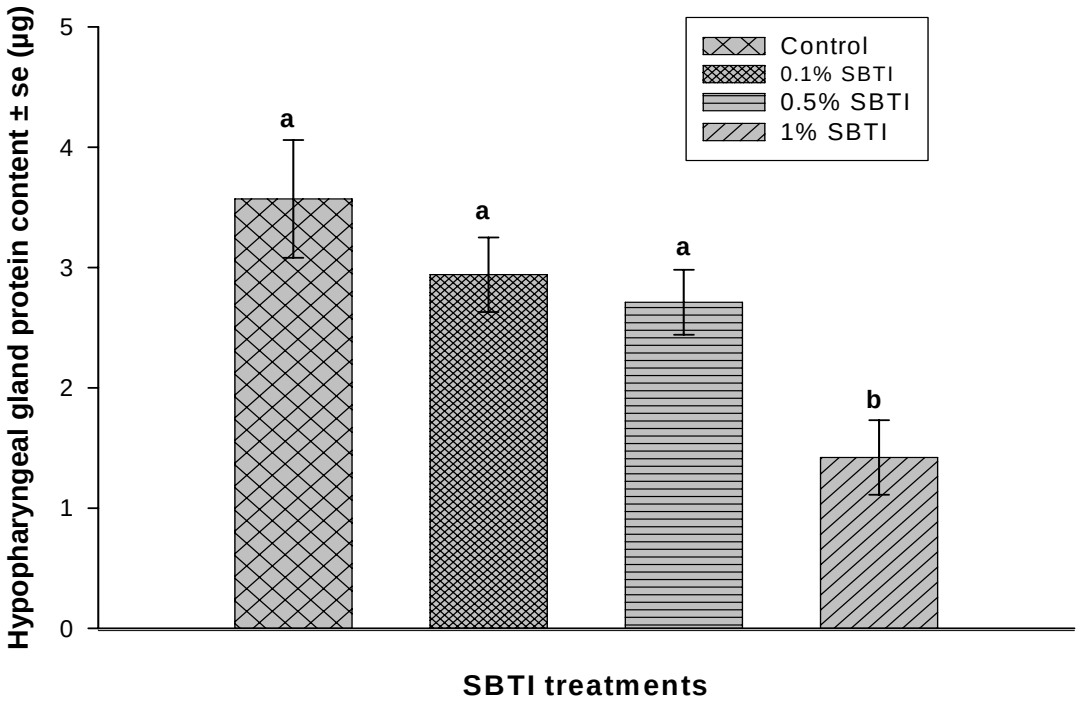

Fig. 1 Mean hypopharyngeal gland protein quantities of bees (±SE) fed with different concentrations of soybean trypsin inhibitor in pollen. Different letters indicate significant differences among the treatments ($P < 0.0001$)

Significant differences were observed between 1% SBTI and remaining diets i.e. 0.1%, 0.5% and control (ANOVA, $F_{3,156}$=6.4, P<0.003). 1% SBTI diet had significantly lower hypopharyngeal gland protein quantity than all other diets (P<0.003).

Total midgut protease activity

Overall 1% SBTI resulted in significantly lower midgut protease activity compared to all the other doses. Effects of SBTI on the total midgut protease activity of adult bees fed different doses of SBTI in a pollen diet are summarized in Fig. 2. The four replicates were pooled because there were no significant differences between them ($F_{3,156}$=0.654, P=0.582). Pairwise comparisons of the SBTI treatments showed that there were significant differences among treatments (ANOVA, $F_{3,156}$=237.5, P<0.0001). 1% SBTI treatment had significantly lower midgut protease activity (P<0.0001). There were no significant differences between control, 0.1% and 0.5% SBTI. Midgut enzyme activity explained 31.1% of the variation in hypopharyngeal gland protein quantity (correlation analysis *rho* = 0.311, P = 0.01).

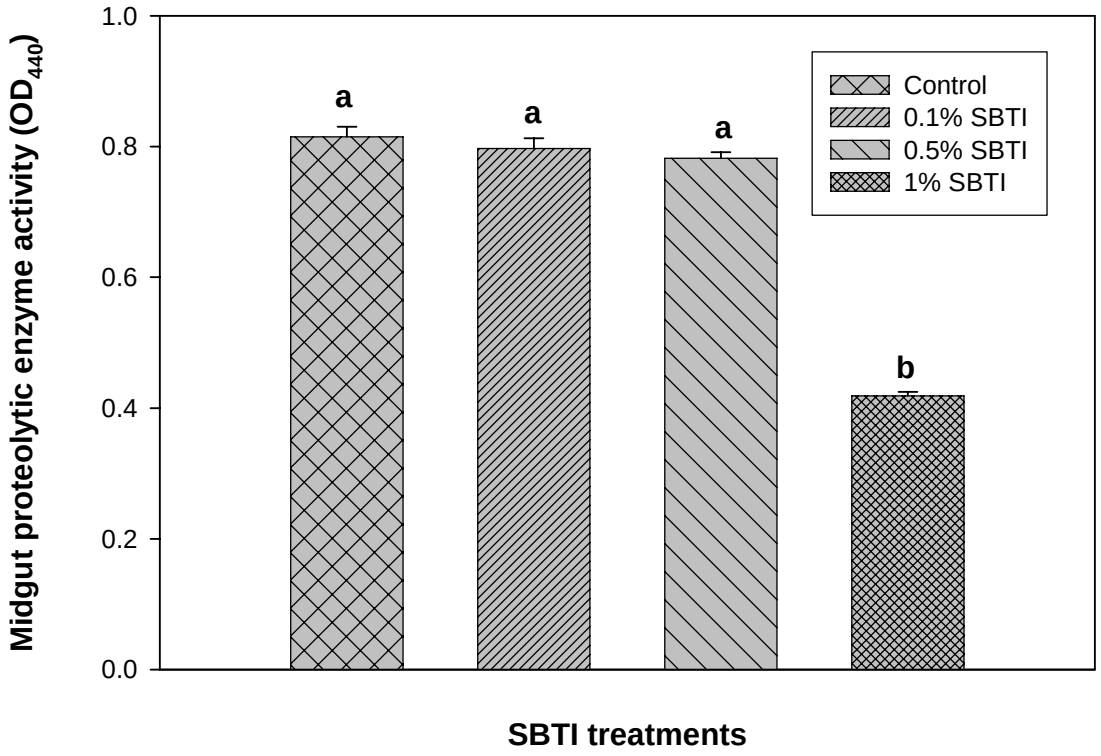

Fig. 2 Mean midgut proteolytic enzyme activities of bees (+SE) fed with different concentrations of soybean trypsin inhibitor in pollen. Different letters indicate significant differences among the treatments ($P < 0.0001$)

Survival

Kaplan-Meier survival curves (SAS) were used to plot survival data. Log-Rank tests indicated that there were significant differences in survival among bees that were fed different doses of SBTI (χ^2=87.27, df =3 and P<0.0001). Kaplan-Meier curves showed that bees fed with 1% SBTI concentration had lowest survival, followed by 0.5% and 0.1% SBTI (Fig. 3). Control had the highest survival. Cox proportional hazard (PH) model was used to model the survival data using SPSS. Cox regression is a method for modeling time-to-event data in the presence of censored cases. SBTI treatment had a significant effect on survival (χ^2=81.75, df =3 and P<0.0001). Hazard ratio (e^{β}) was 1.667. The hazard ratio statistic e^{β}, was transformed to a more meaningful statistic indicating that with each dosage increase of SBTI used in this study, mortality increased by 66.7% over the 30-day experiment period. This meaningful statistic was obtained by subtracting 1.0 from the risk ratio and multiplying by 100.

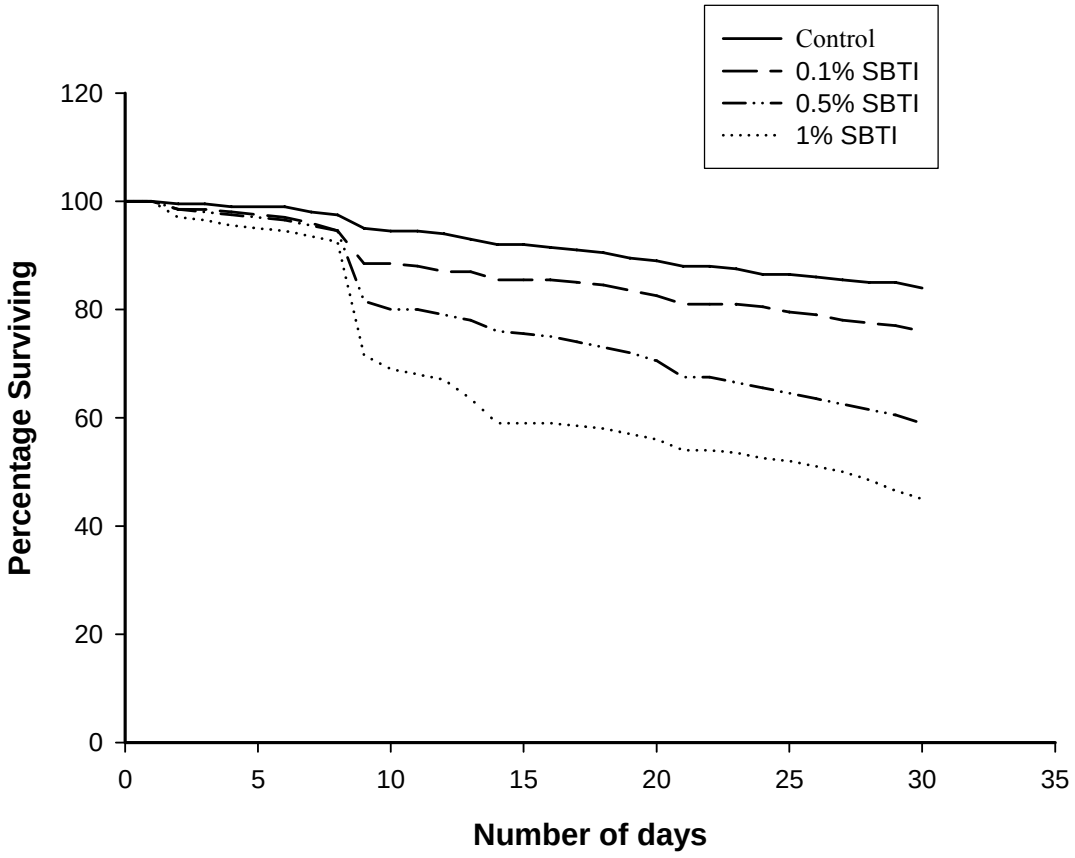

Fig. 3 Survival of bees fed with different concentrations of soybean trypsin inhibitor in pollen

Discussion

In this study, hypopharyngeal gland protein quantity, midgut protease activity and survival were significantly lower when bees were fed 1% SBTI in pollen, strongly suggesting a dose dependent effect. Nurse bees ingesting SBTI at higher concentrations may be poor producers of brood food as a result of poor development of hypopharyngeal glands. Babendreier et al. (2005) reported that bees fed with 1% SBTI (w:v in sucrose solution) treatment did not rear any brood while there was brood rearing in other treatments.

Hypopharyngeal glands in bees fed with 0.1% or 1% SBTI (w:v in sucrose solution) are lighter in weight and have smaller acini when compared to controls (Babendreier et al., 2005). These results can not be directly compared to our results as the bees in the above study were fed SBTI in sucrose solution instead of pollen as in our study. Malone (2004) reported that there was no effect of three transgene products a Bt toxin, a biotin-binding protein (avidin) and a protease inhibitor (aprotinin) on the hypopharyngeal gland development of bees.

Bees fed 1% SBTI had significantly lower levels of midgut protease activity compared to controls. Similar results were reported by Burgess et al (1996), where bees fed with a highest dose of 1% SBTI had significantly lower levels of three endopeptidases, chymotrypsin, elastase and trypsin. Effects on survival may be attributed to a certain extent to lowered midgut protease activity levels. Apart from reduced midgut proteolytic enzyme activity there may be some other factors which are

responsible for the reduced hypopharyngeal gland protein biosynthesis and bee survival, because midgut proteolytic enzyme activity accounted for only 31% of hypopharyngeal gland extractable protein. Burgess et al. (1996) reported that in addition to lowered endopeptidase levels, decreased bee longevity may be also explained by additional metabolic cost incurred because of compensatory hyperproduction of proteolytic enzymes, to compensate for deactivation of enzymes by the protease inhibitor. Hence we may infer that, additional metabolic cost incurred as a result of compensatory hyper production is the other major factor responsible for decrease in hypopharyngeal gland protein quantity apart from reduced midgut enzyme activity.

The higher concentration of SBTI used in this study or in other similar studies is unlikely to be encountered by the bees in the field and hence represent a worst-case scenario. The lower concentration of SBTI (0.1%) used represents a value closer to field relevance if it is expressed in the pollen. Expression levels of protease inhibitors like SBTI also depend upon the type of promoter used. In our experimental design the bees didn't rear any brood which is considered to be a factor stimulating hypopharyngeal gland development. But this criterion doesn't affect our study as we are comparing the hypopharyngeal gland development between treatments receiving different concentrations of SBTI, keeping all other factors constant. Also Malone et al. (2004) observed measurable hypopharyngeal gland development in caged bees that were used in a study to evaluate potential impacts of transgene products on hypopharyngeal gland development.

In conclusion, this study has revealed that SBTI at 1% of pollen diet can negatively impact the hypopharyngeal gland development, midgut protease activity and survival of honey bees. In contrast it also showed that lower doses of SBTI were not deleterious to adult bees. Because honey bee larvae are completely dependent on the hypopharyngeal gland secretions of nurse bees for their nutritional needs, the deleterious effects of SBTI on hypopharyngeal glands could negatively impact colony growth and maintenance. However, the threshold response shown in this study strongly suggests that pollen diets containing less than 1% SBTI are tolerated and unlikely to adversely affect colonies.

CHAPTER III

EFFECTS OF PROTEIN CONSTRAINED BROOD FOOD ON HONEY BEE (*Apis mellifera* L.) POLLEN FORAGING AND COLONY GROWTH[*]

Introduction

For the non-reproducing worker caste of social insect colonies, colony growth and reproduction are the principal sources of fitness. Honey bee colonies reproduce through a process of colony budding, commonly referred to as swarming (Winston 1987). In general, a larger adult population results in increased probabilities for colonies to reproduce and for swarms to survive; fitness traits (Cole 1984; Lee and Winston 1987; Little 1979; Michener 1964; Pomeroy 1979; Richards and Richards 1951; Seeley 1985; Seeley and Visscher 1985; Winston 1987). The mechanism for colony growth is increased brood rearing. The honey bee (*Apis mellifera* L.), like most social insects, have a division of labor whereby individuals perform different tasks as they age. Brood rearing labor is divided among nurse bees and foragers. Ordinarily, younger nurse bees work within the nest directly tending larvae. Older bees are more probably found foraging for nectar or pollen outside the nest.

Nurse bees consume forager collected pollen to biosynthesize a proteinacious hypopharyngeal gland secretion called brood food that is progressively provisioned to larvae. Pollen is the only source of protein available to bees and it is through nurse bees

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[*]This chapter has been recently accepted in the journal 'Behavioral Ecology and Sociobiology' and proofs are pending.

that larvae are the principal consumers of protein in a colony. Pollen foragers collect pollen from flowers, carry it back to the colony on the outside of the body packed onto special structures of their hind legs called corbiculae, and directly deposit their loads into wax cells usually situated around brood rearing areas of the nest (Camazine 1991). The allocation of the foraging force profoundly affects colony growth and development (Farrar 1944; Moeller 1958; Moeller 1961; Free and Racey 1968; Nelson and Jay 1972; Smirl and Jay 1972; Pankiw et al. 2004). As such the mechanisms that regulate the allocation of the pollen foraging force are integral to an understanding of colony fitness.

Two hypothetical mechanisms dominate studies of pollen foraging regulation. The first is an "information center" model and subsequent modifications (Seeley 1985; Seeley et al. 1991; Seeley 1995), here named the brood food hypothesis. The second is a "stimulus-response threshold model", here referred to as the direct independent effects of stored pollen and brood (Page and Mitchell 1998; Page and Erber 2002; Scheiner et al. 2004).

The brood food hypothesis predicts that brood and stored pollen indirectly affect the behavior of pollen foragers through a single inhibitory signal (Camazine 1993; Seeley 1995). Bees are activated to collect pollen, thus regulation occurs through inhibition. With excess pollen stored in a colony there is also an excess of inhibitor that is presumably distributed to foragers by trophallaxis with nurse bees. If pollen is in surplus, it is hypothesized that nurse bees transfer more protein to foragers and inhibit pollen foraging. Brood food is the most likely inhibitor, thus the brood food hypothesis for the regulation of pollen foraging. Some information center based studies have

focused on trophallaxis as mechanism for the transmission of information that may regulate pollen foraging (Camazine et al. 1998; Weidenmuller and Tautz 2002).

A competing hypothesis is that stored pollen and brood have direct, independent effects on pollen foraging. Many studies have demonstrated the effects of quantities of brood and stored pollen. Increasing the amount of larvae in colonies, or the chemical cues derived from larvae called brood pheromone, increases the number of pollen foragers and pollen load weights returned (Filmer 1932; Al-Tikrity et al. 1972; Free 1979; Eckert et al. 1994; Pankiw et al. 1998b; Fewell and Bertram 1999; Pankiw and Page 2001a; Pankiw and Rubink 2002; Pankiw 2004a; Pankiw 2004b; Pankiw 2004c; Pankiw et al. 2004). Pollen foraging activity level decreases in response to the addition of stored pollen (Free 1967; Barker 1971; Moeller 1972; Danka et al. 1987; Fewell and Winston 1992; Camazine 1993; Fewell and Bertram 1999) and increases in response to the removal of stored pollen (Free 1967; Fewell and Winston 1992; Camazine 1993). Increasing the amount of stored pollen in colonies concurrently increases brood rearing and decreases pollen foraging activity to a homeostatic set point (Fewell and Winston 1992). Empty comb space near the brood also stimulates pollen foraging behavior while stored pollen clearly inhibits. Dreller et al. (1999) demonstrated that pollen foraging decreases only when foragers have direct access to stored pollen, and direct access to brood is necessary for an increase in pollen foraging response to an increase in amount of brood.

Using conventional colony-level manipulations is problematic because both the direct and indirect hypotheses predict the same pollen foraging outcomes. But for the

different mechanistic reasons both hypotheses predict pollen foraging decreases with additional quantities of stored pollen and pollen foraging increases with additional amounts of brood. Given a fixed amount of available comb area, there is an interaction between amount of stored pollen, number of larvae and empty space. Changing one necessarily changes the others. However, manipulating amount of brood pheromone, allows for a change in the perceived number of larvae without changing the allocation of comb area for larvae, pollen and empty storage space (Pankiw et al. 1998; Le Conte et al. 2001; Pankiw and Rubink 2002; Pankiw et al. 2004; Pankiw 2004b; Pankiw 2004c). With brood pheromone added to colonies, the brood food hypothesis predicts no change in pollen foraging due to no change in demand for brood food. The direct independent effect of amount of brood and pollen hypothesis predicts an increase in amount of pollen foraging as a consequence of the increased pollen foraging stimulus of brood pheromone. Colonies treated with supplemental amounts of brood pheromone foraged more for pollen than did control colonies containing the same amount of brood and stored pollen (Pankiw et al. 1998; Pankiw and Rubink 2002; Schulz and Robinson 2002; Pankiw 2004a; Pankiw 2004b; Pankiw 2004c; Pankiw et al. 2004). Results of these studies support the direct, independent effects hypothesis, such that colonies approximated amount of larvae from their chemicals and foraged for pollen accordingly.

Direct experimental evidence of brood food protein as a feedback mechanism inhibiting pollen foraging has yet to be demonstrated. In this study we manipulated nurse bee biosynthesis of brood food using a protease inhibitor that interferes with midgut protein digestion in adults, significantly decreasing the amount of protein

extractable from hypopharyngeal glands (Sagili et al. 2005). Manipulating amount of hypopharyngeal gland protein and controlling for amount of stored pollen resulted in the following predictions tested here; 1) the direct, independent effects hypothesis predicts no difference in pollen foraging effort because amount of stored pollen is the same in treated and control colonies versus, 2) the brood food hypothesis predicts that protease inhibited colonies should allocate a greater pollen foraging effort due to a decreased amount of nurse produced protein. The primary objective in this experiment was to measure the effects of manipulating hypopharyngeal gland protein (brood food) content in nurse bees on pollen foraging.

Methods

This experiment was replicated four times and had two treatments, 1% SBTI (soybean trypsin inhibitor) (Sigma Aldrich product T-9003, St. Louis, MO, USA) and control. Micro-nucleus hives made of styrofoam (25x19x14 cm) were used for this experiment and the experiment was conducted for a 30-day period. Each hive consisted of 5 frames; 2 frames pollen (476 cm^2), 1 frame honey (238 cm^2), ½ frame (119 cm^2) with brood and, 1½ empty frames empty space (357 cm^2). Fifteen hundred newly emerged worker bees were introduced in to each hive and colonies were allowed to establish for 7 days. All the bees used in the experiment were obtained from a single colony source.

Each experimental colony was headed by an unrelated queen. Control colonies received powdered pollen without SBTI packed into 2 frames, whereas SBTI treated

colonies received powdered pollen mixed with 1% SBTI (wt:wt) packed into 2 frames. After packing the pollen into the cells, the surface was sprayed with 50 % sugar syrup (Dreller and Tarpy 2000). Each week for a period of 4 weeks, 100 newly emerged bees from a common source were individually identified with a number tag glued to the thorax and released into each colony starting from initiation of the experiment. The brood consisted of 2-day-old eggs at the beginning of the experiment and abundant pollen was available in the environment during the entire experimental period.

Hypopharyngeal gland protein analysis

From each colony on days 7 and 14, fifteen tagged bees from the brood nest area were collected for estimating hypopharyngeal gland protein content. Bees were cold euthanized, their hypopharyngeal glands dissected and stored in Tris buffer at -80°C for further analysis. Protein content of hypopharyngeal glands was determined using Bradford assay as per Sagili et al. (2005). Briefly, hypopharyngeal glands were homogenized using a homogenizer that tightly fits in microcentrifuge tubes used to store the glands. Subsequently, tubes were centrifuged at 1000 rpm for 2 min. Supernatant from each tube was used for analysis. We used the 500-0202 Quick Start Bradford Protein Assay Kit 2 (Bio-Rad Laboratories, CA, and U.S.A.). We added 2 µl or 5 µl from each sample to be analyzed to microcentrifuge tubes containing 1 ml Bradford reagent. Tubes were vortexed to homogenize the contents, then incubated for 5 min at room temperature. Standard-curves were prepared using bovine serum albumin (BSA).

Protein absorbance was measured at 595 nm against blank reagent using a Beckman Spectrophotometer (Model #D4-640, Beckman Instruments, Inc., Columbia, MD, USA). Weight of protein (BSA) was plotted against the corresponding absorbance value to generate a linear regression equation (SAS PROC REG; SAS 2000). Protein extracted from hypopharyngeal glands was calculated using the linear regression equation generated above. Protein quantity was further analyzed using analysis of variance (ANOVA) (Sokal and Rohlf 1995; SPSS 2000).

Midgut proteolytic enzyme activity

The midguts were also excised from the same bees from which the hypopharyngeal glands were removed and midgut proteolytic enzyme activity was measured as per Sagili et al. (2005) briefly described below. Frozen guts were crushed, homogenized in Tris-HCl buffer (pH 7.9) and, centrifuged at 10,000 rpm for 5 min. The supernatant was analyzed for total gut proteolytic enzyme activity (casenolytic activity) as described by Michaud et al (1995). Five microlitres of supernatant was used for each reaction. Twenty microlitres of assay buffer (0.1 M Tris-HCl, pH 7.9) and 60 µl of 2% (w/v) azocasein diluted in assay buffer were added respectively to the supernatant and incubated for 6 h at 37ºC. To remove the residual azocasein after proteolysis, 300 µl of 10 %(w: v) TCA (Trichloro acetic acid) was added to each mixture and centrifuged for 5 min at 10,000 rpm. 350µl of supernatant was added to 200 µl of 50% ethanol in water, and the absorbance of this mixture was measured at 440nm using a Beckman DU 64

spectrophotometer. Total gut proteolytic activity was expressed in terms of OD_{440}. Mean total gut proteolytic enzyme activities from each treatment were analyzed using ANOVA (Sokal and Rohlf 1995; SPSS 2000).

Foraging behavior measurements

The number of foragers returning with visible pollen loads (pollen foragers) and those returning with no visible pollen load (non-pollen foragers) was counted for a 5 minute period twice daily in the morning and afternoon beginning 24 hours after pollen treatments were applied. Beginning on fifth day, to the termination of the experiment, colony entrances were blocked with wire mesh for 15 min intervals between 0900 h to 1600 h for a total period of 2 h per day. Wire mesh was removed for a minimum of 30 minutes between each blocked interval.

Foragers with tags were captured individually in small cylindrical wire cages and their identity recorded. Pollen load weights of pollen foragers were measured by removing the pollen pellets from the corbicula of both the hind legs and weighing them. Age of first foraging was estimated by calculating the difference between the day of first observation as a returning forager and the day of emergence. Each week the comb area occupied by eggs, larvae, pupae, pollen and honey was measured using a metered grid (Pankiw et al. 2004).

Statistical analyses

Analysis of variance was used to analyze hypopharyngeal gland protein content, midgut enzyme activity, and pollen load weight. Correlation analysis (parametric) using SPSS was performed to measure the strength of linear association between midgut enzyme activity and hypopharyngeal gland protein quantity. Contingency table analysis was used to analyze the ratio of pollen to non-pollen foragers observed (Sokal and Rohlf 1995). Cox proportional hazards regression was used to analyze treatment effects on age of first foraging (PROC PHREG in Allison 1998; SAS 2000). Brood, pollen and honey areas were analyzed using repeated-measures ANOVA.

Results

Hypopharyngeal gland protein content and midgut proteolytic enzyme activity

Hypopharyngeal gland protein content was significantly lower in bees treated with 1% SBTI versus the control for both 7 and 14 day old bees (7 days, ANOVA $F_{1,118}$=14.6, P<0.001; 14 days, ANOVA $F_{1,118}$=12.2, P<0.001 respectively; Fig. 4). Midgut proteolytic enzyme activity was significantly lower in 1% SBTI treated bees than control bees that were 7 and 14 days old (7 days, ANOVA $F_{1,118}$=167.5, P<0.0001; 14 days, ANOVA $F_{1,118}$=139.5, P<0.0001; Fig. 5). Midgut enzyme activity explained 29.3% of the variation in hypopharyngeal gland protein quantity (correlation analysis ρ = 0.293, P = 0.01).

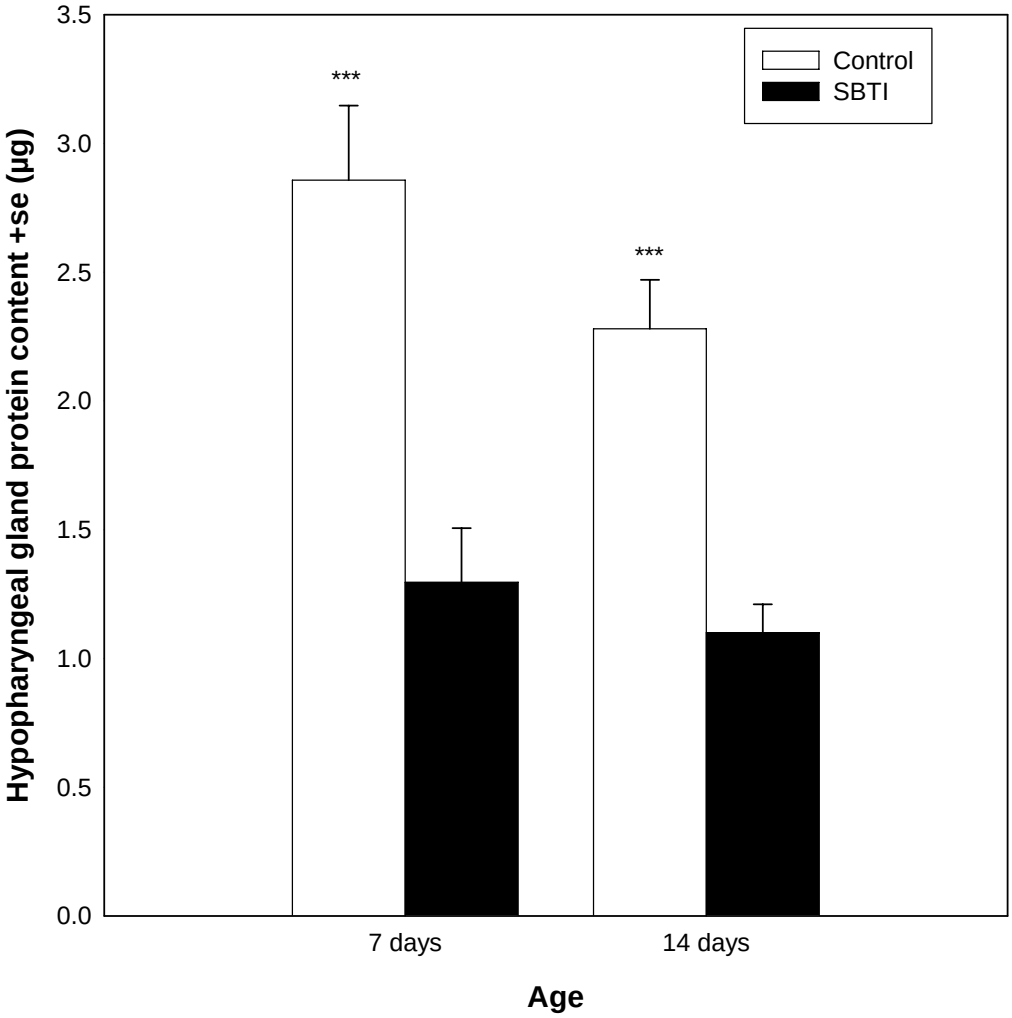

Fig. 4 Mean hypopharyngeal gland protein quantities of bees (+SE) that received control and SBTI treatments. Asterisk indicates significant difference (P<0.001)

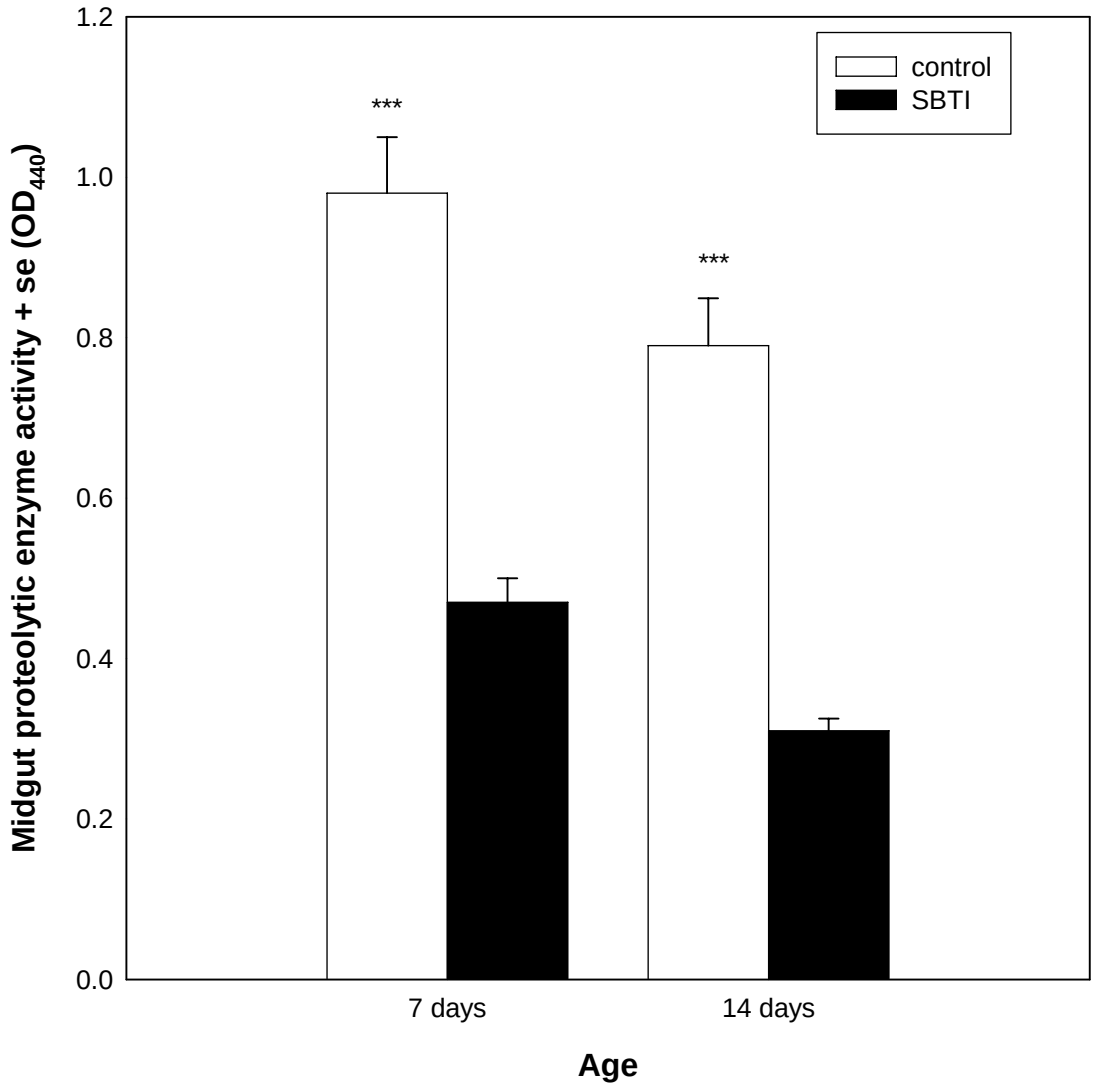

Fig. 5 Mean midgut proteolytic enzyme activities (+SE) of bees that received control and SBTI treatments. Asterisk indicates significant difference (P<0.0001)

Foraging behavior

There was no significant difference in the ratio of pollen to non-pollen foragers entering the colonies both in the morning and afternoon (morning: χ^2= 18.3, 1 *df*, *P*>0.05; afternoon: χ^2= 16.9, 1 *df*, *P*>0.05; Fig. 6) between SBTI treatments and controls. Number of pollen foragers evaluated for control and SBTI treatments were 1765 and 1708 respectively and the number of non-pollen foragers evaluated were 6070 and 5594 respectively for control and SBTI treatments. Pollen load weight was not significantly different between the 1% SBTI treatment and control ($F_{1,6}$=1.9, *P*>0.05; Fig. 7). Number of pollen foragers evaluated for pollen load weight analysis were 600 each for the control and SBTI treatments.

Bees that received 1% SBTI treatment foraged at a significantly younger mean age than the control bees (χ^2= 9.3, *P*<0.01, e^{β} = 0.67). The hazard ratio statistic e^{β}, was transformed to a more meaningful statistic indicating that bees ingesting 1% SBTI were 33 % more at risk to forage than control bees over the 30-day experimental period (Allison 1998). Mean age of first foraging in SBTI treatments and controls was 11.3±0.4 (SE) days and16.2±0.7 (SE) days respectively.

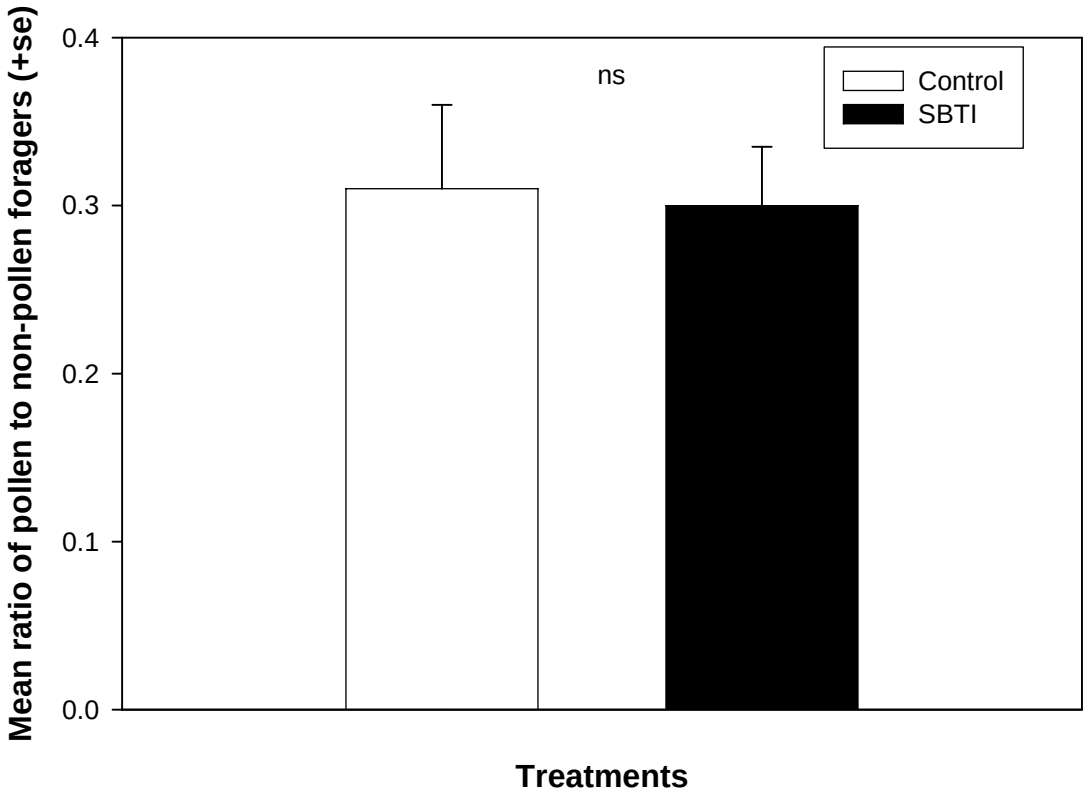

Fig. 6 Mean ratio of pollen to non-pollen foragers (+SE) entering the colonies (P>0.05). 'ns' indicates no significant difference. Pollen foragers: n= 1765 (control) and n= 1708 (SBTI). Non-pollen foragers: n=6070 (control) and n= 5594 (SBTI)

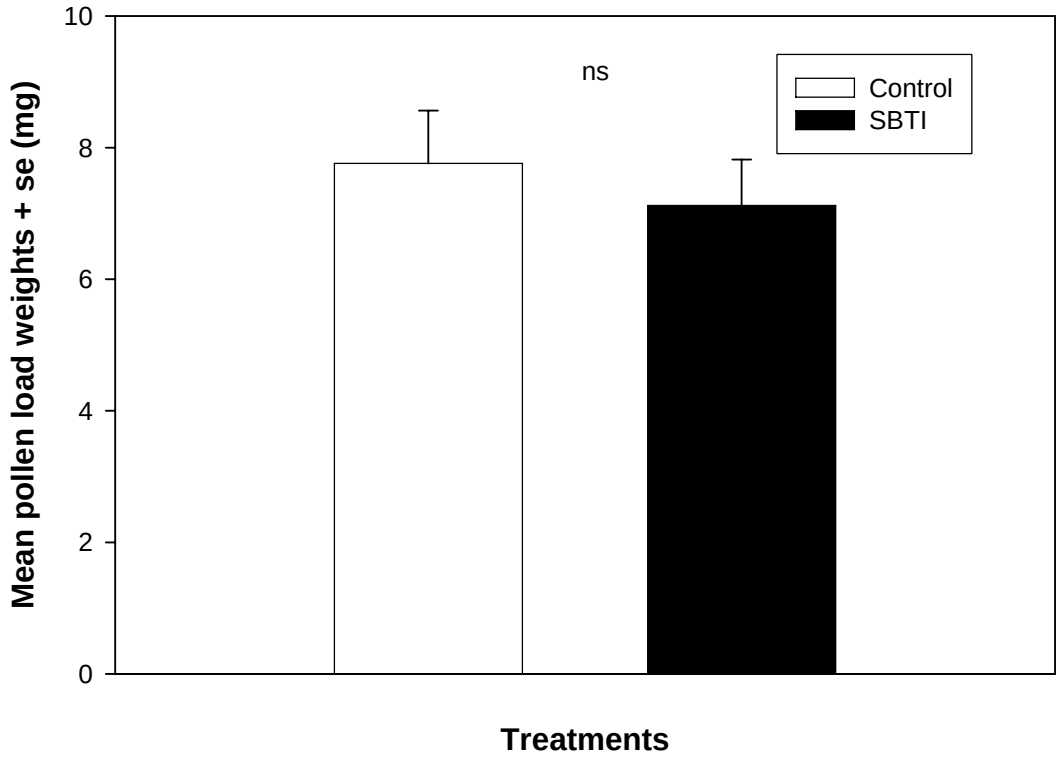

Fig. 7 Mean pollen load weights (+SE) collected by control and SBTI fed colonies (P>0.05). 'ns' indicates no significant difference

Colonies that received SBTI treatment reared significantly less brood area than control colonies (repeated measures $F_{1,6}$= 14, P <0.003; Fig. 8). Pollen and honey areas were not significantly different between the SBTI treatment and control colonies (repeated measures $F_{1,6}$=1.4, P= 0.1 and $F_{1,6}$=0.9, P=0.3 respectively). There was no significant difference in the mortality between 1% SBTI treatments and controls

($F_{1,6}$=4.8, P>0.05). The mean number of adult bees surviving at the termination of the experiment in SBTI treatments and controls were 1585 ± 8.89 (SE) and 1634.5 ± 20.66 (SE) respectively.

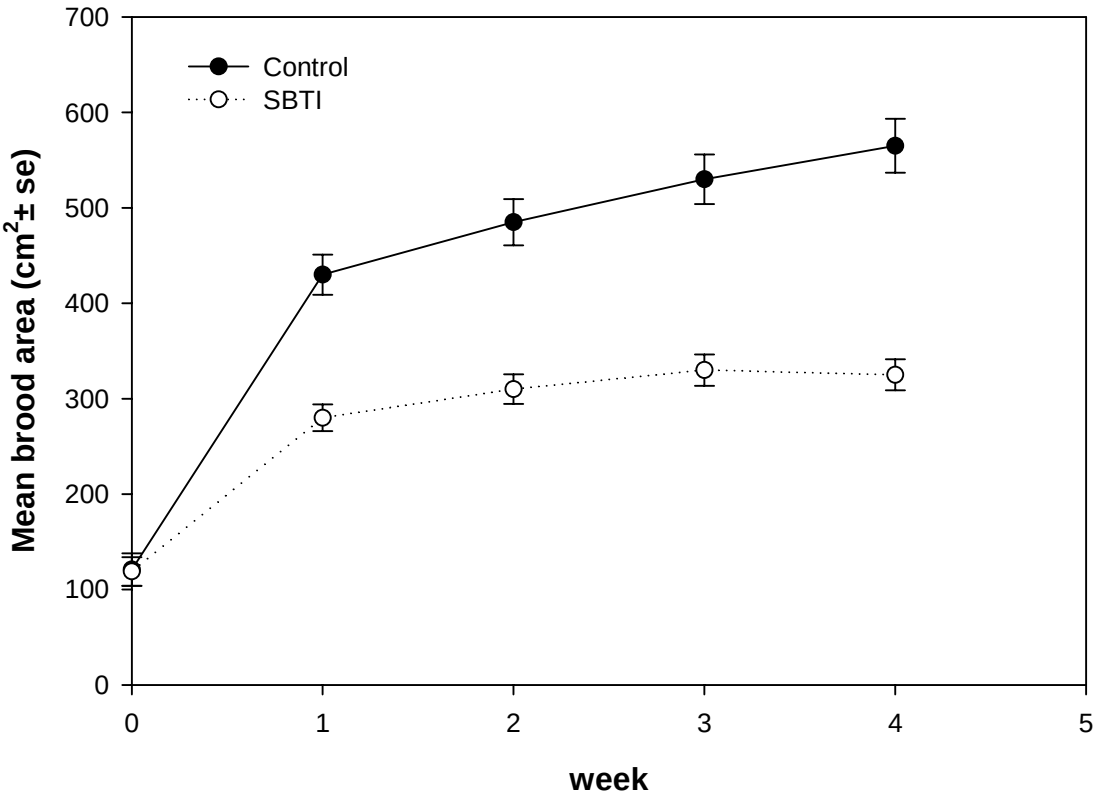

Fig. 8 Mean brood area reared (±SE) in control and SBTI treated colonies over a period of 4 weeks.

Discussion

The results of this study supported the prediction of no difference in pollen foraging effort between SBTI treated versus control colonies generated by the direct, independent effects hypothesis. Equivalent amounts of stored pollen in SBTI and control colonies were maintained throughout the course of the experiment. The ratio of pollen foragers and weight of pollen loads returned were similar between treatments. Amount of protein extractable from the hypopharyngeal glands of bees reared in SBTI treated colonies was significantly lower than those reared in control colonies. Hypopharyngeal glands in bees fed 0.1% or 1% SBTI (w:v in sucrose solution) are lighter in weight and have smaller acini when compared to controls (Babendreier et al. 2005). In this experimental paradigm, foragers appeared to be assessing need for pollen based on amount of stored pollen in colonies rather than by amount of hypopharyngeal gland protein extractable from workers.

The protease inhibitor used here inhibited midgut proteolytic enzyme activity of workers. Interference with midgut protein digestion was strongly associated with decreased amounts of protein extractable from the hypopharyngeal glands of colony-reared workers feeding on a pollen diet containing SBTI as well as caged-workers reared in an incubator (Sagili et al. 2005). Similar results were reported by Burgess et al (1996), where bees fed the highest dose of SBTI (1%) had significantly lower levels of three endopeptidases, chymotrypsin, elastase and trypsin. An inference of these physiological results is that bees ingesting SBTI were poor producers of brood food. This conclusion is supported by the significantly lower amount of brood area reared by

SBTI versus control colonies. It is important to note that despite the greater amount of brood area reared by control colonies, measures of pollen foraging remained statistically similar between treatments. This is further support for the direct, independent effects hypothesis such that there is a response threshold for amount of brood area at or beyond which increased pollen foraging is released (Page and Mitchell 1998). Although there was more brood in control colonies, it was not sufficiently great to induce more pollen foraging. This is consistent with results where brood pheromone amount is increased incrementally to a point where increased pollen foraging is observed (Pankiw et al. 1998; Pankiw and Page 2001).

SBTI interference of protein digestion in adults was associated with decreased age of first foraging. This could be interpreted as a 'stressor" effect of SBTI on adult length of life. In general foraging behavior is the terminus on the honey bee behavioral ontogenetic pathway (Winston 1987). Some factors associated with decreased age of first foraging may be viewed as stressors, for example, the handling of newly emerged adults (Pankiw 2003), removal of the foraging caste from colonies (Huang and Robinson 1992), exposure to primer pheromones (Le Conte et al. 2001; Pankiw 2004b; Pankiw 2004a), and mite infection (Korpela et al. 1992). Schulz et al. (1998) reported that shortage of food in honey bee colonies accelerated behavioral development, and starved colonies had significantly greater proportions of precocious foragers. Malone et al. (2001) demonstrated that bees fed aprotinin, a serine protease inhibitor, started flying 2.8 days earlier than control bees. Pollen and honey areas of control and SBTI treatments were identical which suggests that pollen and nectar foraging were similar in both the

treatments. At the termination of the experiment SBTI treatments had fewer bees compared to the controls, but this difference was not significant. This suggests that the SBTI treatment didn't adversely effect the survival of the bees. It is possible larvae were directly affected by the protease inhibitor, but only in the later stages, when they are fed small quantities of pollen along with the hypopharyngeal gland and mandibular gland secretions. However, this effect is not expected to be significant. In honey bee larvae pollen constitutes only a minor part of the protein supply (Babendreier et al 2004).

Multiple methods have now been utilized to attempt to generate predictions that clearly point to specific mechanisms for the regulation of pollen foraging. To date the majority of studies support the direct, independent effects hypothesis. However, despite all efforts, the question of how honey bee colonies regulate pollen foraging remains controversial. Models of behavioral organization in social insects all predict that workers will vary task performance in response to common environmental cues (Seeley 1985; Tofts and Franks 1992). The specific hypotheses addressed in these studies were developed to address different foraging behaviors; the indirect hypothesis was originally developed to address nectar foraging and the direct hypothesis addressed pollen foraging. They sometimes make different and competing assumptions about what produces variation in individual responses to stimuli and how individuals receive information about the colony environment that changes foraging responses.

Pitting one hypothesis against the other has been largely intractable, making hypothesis falsification impossible or unresolved, because they generate the same predictions but for different reasons, or a model is modified to address an unexpected

result. A philosophical resolution may be to adopt "integrative pluralism" (Mitchell 2002). Integrative pluralism recognizes that complex systems may comprise multiple causes. Theories and explanations are not always competing (Sherman 1988). Integrative pluralism allows for models working at the same level of analysis to be combined for a more complete synthesis. Fewell and Bertram (1999) generated predictions from central information and threshold models for honey bee foraging behavior responses to gradual increases in amount of stored pollen. Although not demonstrated directly, their results suggested that the regulation mechanisms forwarded by both hypotheses may be operating concurrently and they proposed a model that integrated the two mechanisms. Thus, there is recognition that factors identified through tests of models of colony organization may be at work concurrently or hierarchically. Integrative pluralism may be the next more fruitful direction to pursue insights to what is clearly a complex system.

CHAPTER IV

BROOD PHEROMONE REGULATION OF QUEEN EGG-LAYING IN THE HONEY BEE (*Apis mellifera* L.)

Introduction

Colony growth rates and trajectories are critical to colony reproductive rate, the size of swarms and the timing of swarming (Lee and Winston 1985a; Lee and Winston 1985b; Lee and Winston 1987). Survival of swarms is likewise dependant on parental colony size and the timing of issue (Seeley 1978; Lee and Winston 1985a; Lee and Winston 1985b; Morales 1986; Lee and Winston 1987). Lee and Winston (1985b) found a positive correlation between swarm size and both brood production and emergent worker weight in newly founded colonies. Larger colonies invest more workers in swarms, which confers an increased probability of swarm survival (Lee and Winston 1987). Larger swarms also produce more total brood comb, that area in which brood are reared (Lee and Winston 1985a). The number of swarms that a colony produces is positively correlated with the amount of pupae at the time the first swarm issues (Winston 1979; Winston 1980). It is the intensity with which individuals collect resources that profoundly affects colony growth and development (Farrar 1944; Moeller 1958; Moeller 1961; Free and Racey 1968; Nelson and Jay 1972; Smirl and Jay 1972). It is generally assumed that various colony foraging strategies are adaptive (Robinson 1992). To place foraging strategies within an evolutionary context it is important to understand the

interaction between foraging strategies and colony growth that leads to increased colony growth.

Pheromones are chemicals that are the primary source of intraspecific communication in many organisms. Brood pheromone is a 10-component mixture of methyl and ethyl fatty esters that can be extracted from the surface of honey bee larvae (Le Conte et al. 1990). Brood pheromone is a tool that can be used to alter the foraging stimulus environment and thus change honey bee foraging strategies (Pankiw et al. 1998; Pankiw and Page 2001; Pankiw and Rubink 2002).

Significantly greater brood area was reared by brood pheromone treated colonies compared to controls and increase in brood area was preceded by an increase in queen egg-laying rate (Pankiw et al. 2004). Queen egg laying rate depends on the quantity and quality of the food donated by the workers to the queen (Chauvin 1956; Allen 1960). Queen feeding and cell preparation rate to facilitate egg laying are the two mechanisms that enable workers to regulate queen egg-laying rate (Free and Williams 1972). Hence it is reasonable to hypothesize that one mechanism of colony growth is regulated by worker-queen interactions that affects the egg-laying rate of the queen. In this study we test whether brood pheromone regulates queen egg laying via modulation of worker-queen interactions, increased nutritional environment and nurse bee rearing behaviors.

Methods

This experiment was replicated 4 times and had two treatments, brood pheromone and control. A pair of colonies was derived by dividing a single colony. Each colony pair

consisted of approximately 4,000 workers headed by naturally mated sister queens. Colonies were installed in 4-frame observation hives (Gary and Lorenzen 1976). Five days prior to the onset of the experiment 200 newly emerged bees derived from the parental source were added to each pair. Each individual bee was uniquely identified with a plastic colored number tag (BioQuip Products Inc. 1172, CA, USA) glued to the thorax and a color mark on the abdomen (Seeley and Kolmes 1991). At the onset of the experiment each colony contained 1 frame of honey, ½ frame of pollen, and the remaining area comprised empty cell space. The queen was confined to a single frame for a period of 3 days using queen excluding material. This provided the queen with nearly 5,000 cells to deposit individual eggs. On average, in larger colonies, queens lay fewer than 1500 eggs per day (Winston 1987), therefore we did not limit egg laying space in a 3-day period. Worker bees were able to pass through the queen excluder material and freely move throughout the colony. After the 3-day period the queen was switched to another empty frame for 3 more days.

One colony of a pair received 336 µg of brood pheromone daily for 9 days (Pankiw and Page 2001). The other colony received iso propanol (EMD Chemicals Inc. PX1835-5, Gibbstown, NJ, USA) rinsed glass plate. The treatments were delivered on a glass plate (9.5 cm x 5 cm). The glass plates were inserted through a port installed in a wall of each hive. The glass plate was positioned against the hive wall in such a way that it didn't interfere with bee activities or observations.

Queen observations

Each day we digitally recorded (Sony DCR-TRV70) the queen for 2 hrs in the morning and 2 hrs in the afternoon for 1hr intervals alternating between treatments. The following behavioral categories were recorded: 1) *idle*: the queen was motionless on the comb and moved only her antennae or individual legs. Retinue bees groomed or licked her, but no food was given 2) *Patrolling*: the queen walked across the comb at a speed greater than 5 mm per sec and (often) inspected cells. 3) *Receiving food*: the queen extended her proboscis between the mandibles of a worker for more than 5 sec (Allen 1960). 4) *Egg laying*: after a brief inspection of an empty cell, the queen inserted her abdomen into the cell to lay an egg. Each day a map tracing the area of eggs, larvae and pupae was drawn on a transparent sheet for each hive. Daily mapping continued for about 9 days when the last larvae of the first frame pupated and were sealed over with wax. Data from the maps was used to calculate egg laying rate, numbers of larvae, larvae surviving to pupation, and total brood area.

Protein content of hypopharyngeal glands was measured using Bradford assay as per Sagili et al. (2005). Briefly, hypopharyngeal glands were homogenized using a homogenizer that tightly fitted in microcentrifuge tubes used to store the glands. Subsequently, tubes were centrifuged at 1000 rpm for 2 min. Supernatant from each tube was used for analysis. We used the 500-0202 Quick Start Bradford Protein Assay Kit 2 (Bio-Rad Laboratories, CA, and U.S.A.). We added 2 µl or 5 µl from each sample to be analyzed to microcentrifuge tubes containing 1 ml Bradford reagent. Tubes were

vortexed to homogenize the contents, then incubated for 5 min at room temperature. Standard-curves were prepared using bovine serum albumin (BSA). Protein absorbance was measured at 595 nm against blank reagent using a Beckman Spectrophotometer (Model #D4-640, Beckman Instruments, Inc., Columbia, MD, USA). Weight of protein (BSA) was plotted against the corresponding absorbance value to generate a linear regression equation (SAS PROC REG; SAS 2000). Protein extracted from hypopharyngeal glands was estimated using the linear regression equation generated from the BSA standard curve.

Observing larvae and nurse bees

Twice daily, once in the morning and once in the afternoon, we digitally recorded 4 selected larvae for 30 minutes each and recorded all nursing acts, for a total of 8 larvae per day. We observed larvae that were two and five days old to create two age classes for observation. The reason for choosing these two ages was that 2-day old larvae are in an early stage of development but large enough to be seen and 5-day larvae are near the end of larval development and are the greatest food consumers. Young larvae exclusively receive brood food, while older larvae receive some pollen and honey along with brood food (Winston 1987). The map tracings were used to locate such larvae on a daily basis. A bee was defined as nursing if she inserted her head and part of her thorax inside a cell containing a larva. Additionally, a nursing bee was distinguished from one that was inspecting or cleaning an empty cell by duration of the act. A nursing act is

defined as lasting between 3 seconds and 3 minutes (Lindauer 1952; Huang and Otis 1991; Schmickl et al. 2003).

The following variables were measured after Schmickl et al. (2003). 1) *nursing time,* calculated as the ratio of nursing time over total time observed. Each day we randomly selected 5 number tagged workers per colony and observed each for a total of 15 min, recording nursing bouts, cell inspections, and cell cleaning. A bee was defined as 2) *inspecting a cell* if she inserted her head and part of her thorax in an empty cell for ≤ 3 sec or less (Lindauer 1952). Cell inspection may lead to cell cleaning behavior, an act that prepares a cell to accept an egg (Winston 1987). 3) *Cell cleaning* was defined as a bee entering an empty cell, as above, for a duration > 3 sec. Ten nurse bees from the brood nest area were selected randomly from each colony on days 3, 6 and 9 for analyzing protein content of hypopharyngeal glands.

Statistical analyses

Frequency data was analyzed using the Kruskal-Wallis test, timed variables were analyzed using analysis of variance (Sokal and Rohlf 1995). Protein quantity of hypopharyngeal glands was analyzed using analysis of variance (ANOVA) and repeated-measures ANOVA was used to analyze brood area (Sokal and Rohlf 1995).

Results

In 3 out of 4 colony-level replications, queen egg laying rate per hour was significantly higher in brood pheromone treatments than control (Fig. 9)(Table 1).

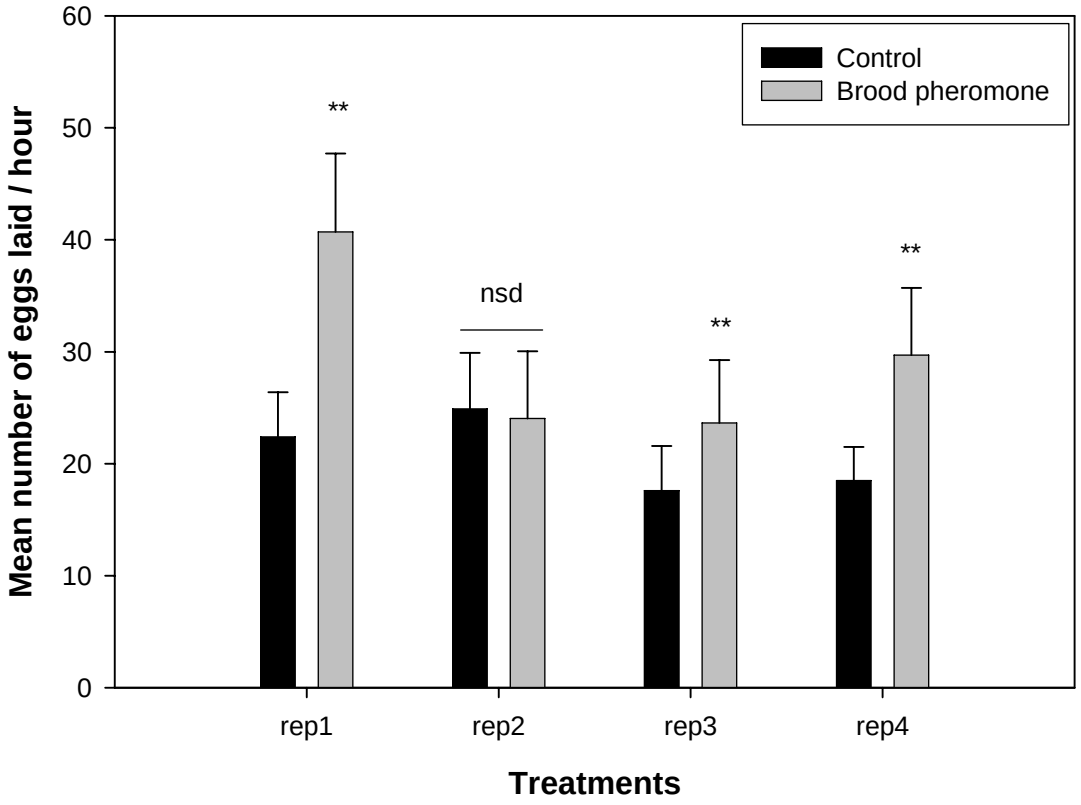

Fig. 9 Mean number of eggs laid by the queen in an hour (+SE). Asterisks indicate significant difference and 'nsd' denotes no significant difference

Table 1 ANOVA pertaining to number of eggs laid by the queen in an hour showing degrees of freedom, F-statistics and P-value for the four replications

	Rep1	Rep2	Rep3	Rep4
df	1, 38	1, 38	1, 38	1, 38
F	30.56	1.43	4.81	10.32
P	0.0001	0.83	0.01	0.001

In all the 4 replications total number of eggs laid by the queen over the 9-day experimental period was significantly greater in brood pheromone treated colonies ($p<0.01$) (Fig. 10)(Table 2).

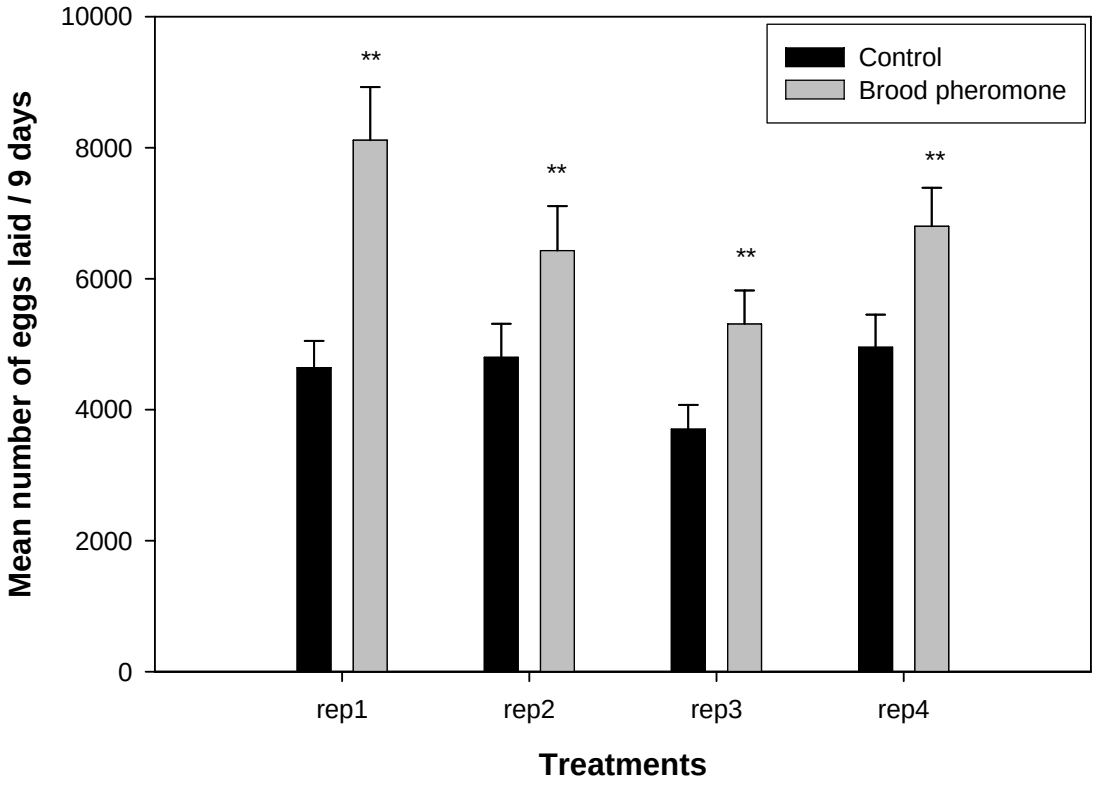

Fig. 10 Mean number of eggs laid by the queen in a period of 9 days (+SE). Asterisks indicate significant difference

Table 2 ANOVA of eggs laid by the queen in 9 days showing degrees of freedom, F-statistics and p-value for the four replications

	Rep1	Rep2	Rep3	Rep4
df	1, 38	1, 38	1, 38	1, 38
F	46.37	9.47	21.16	28.58
P	0.0001	0.01	0.001	0.001

Frequency of feeding bouts was not significantly different between treatments in all the 4 replications (Fig. 11)(Table 3).

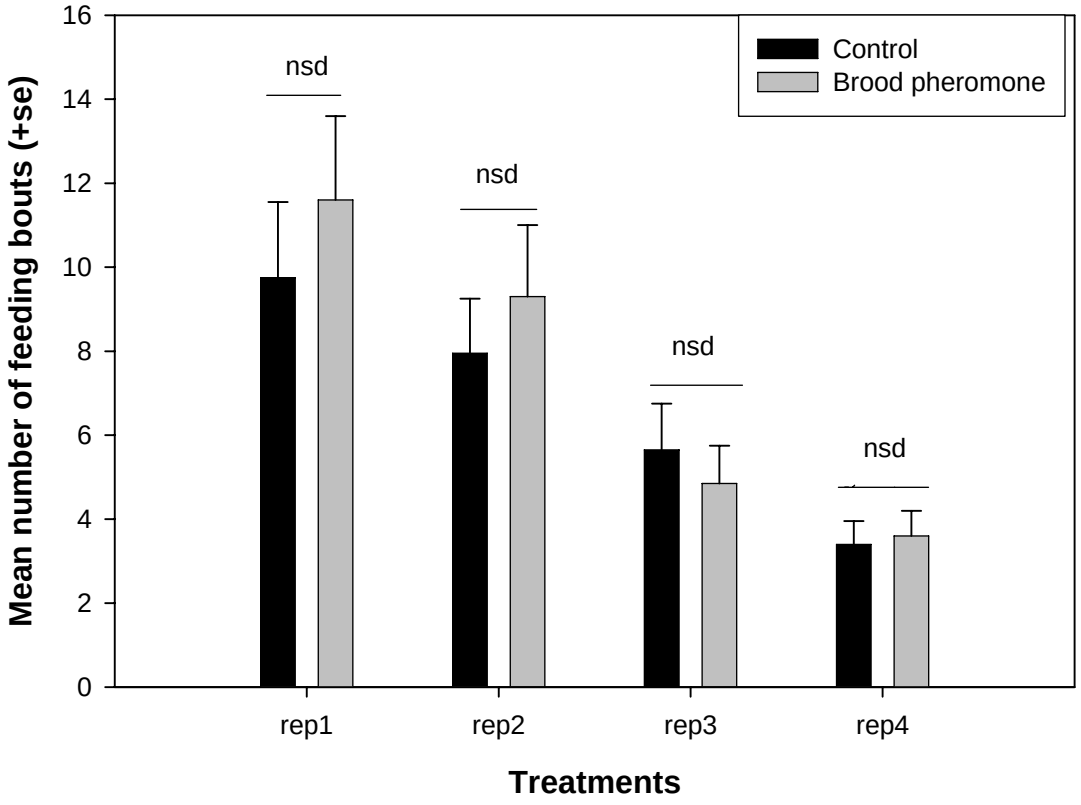

Fig. 11 Mean number of feeding bouts received by the queen (+SE). Asterisks indicate significant difference. No significant difference is denoted by 'nsd'.

Table 3 Kruskal-Wallis test statistics pertaining to feeding frequency analysis of queen for the four replications ('df' denotes degrees of freedom, χ^2 is the Chi-Square value and 'P' is the probability value)

	Rep1	Rep2	Rep3	Rep4
df	1	1	1	1
χ^2	31.83	4.66	9.43	7.36
P	0.12	0.22	0.51	0.63

Total amount of time spent feeding the queen was significantly greater in brood pheromone treated colonies ($P<0.01$) (Fig. 12)(Table 4).

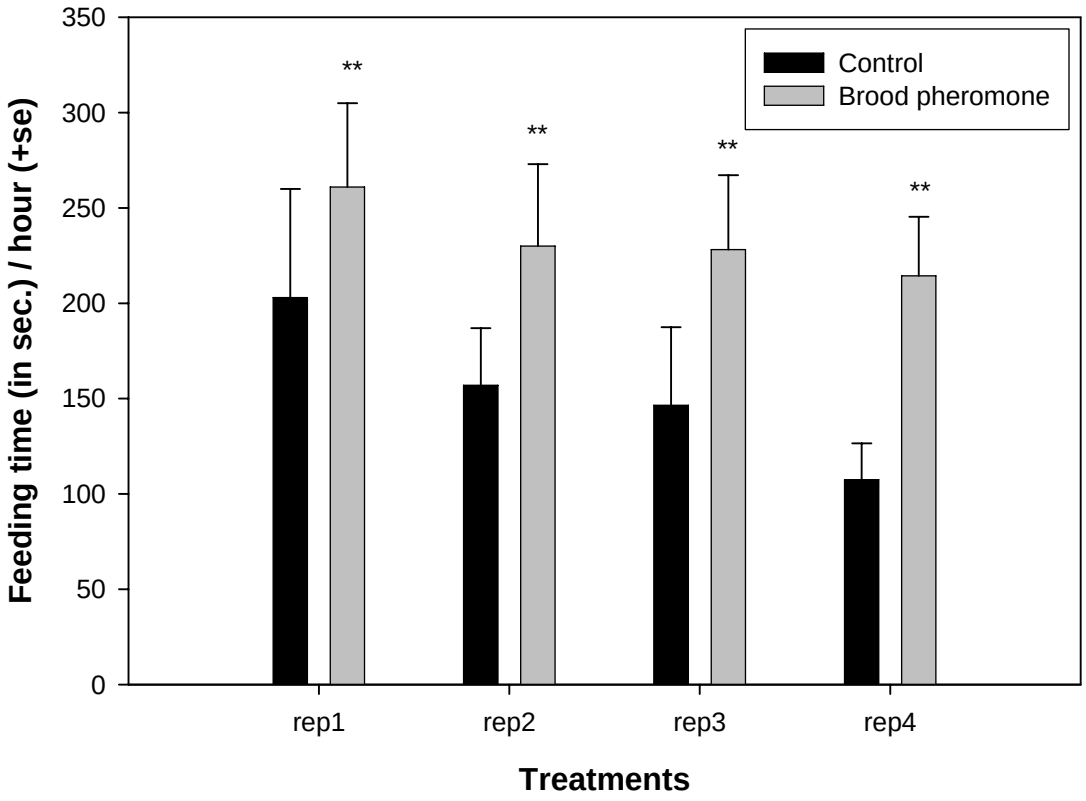

Fig. 12 Mean feeding time of queen per hour (+SE) (in seconds). Asterisk indicates significant difference

Table 4 ANOVA of queen feeding time analysis showing degrees of freedom, F-statistics and p-value for the four replications

	Rep1	Rep2	Rep3	Rep4
df	1, 38	1, 38	1, 38	1, 38
F	5.14	7.74	7.16	15.46
P	0.01	0.008	0.01	0.0001

Queen idle time was significantly lower in the brood pheromone treatments than the controls ($P<0.01$) (Fig. 13)(Table 5).

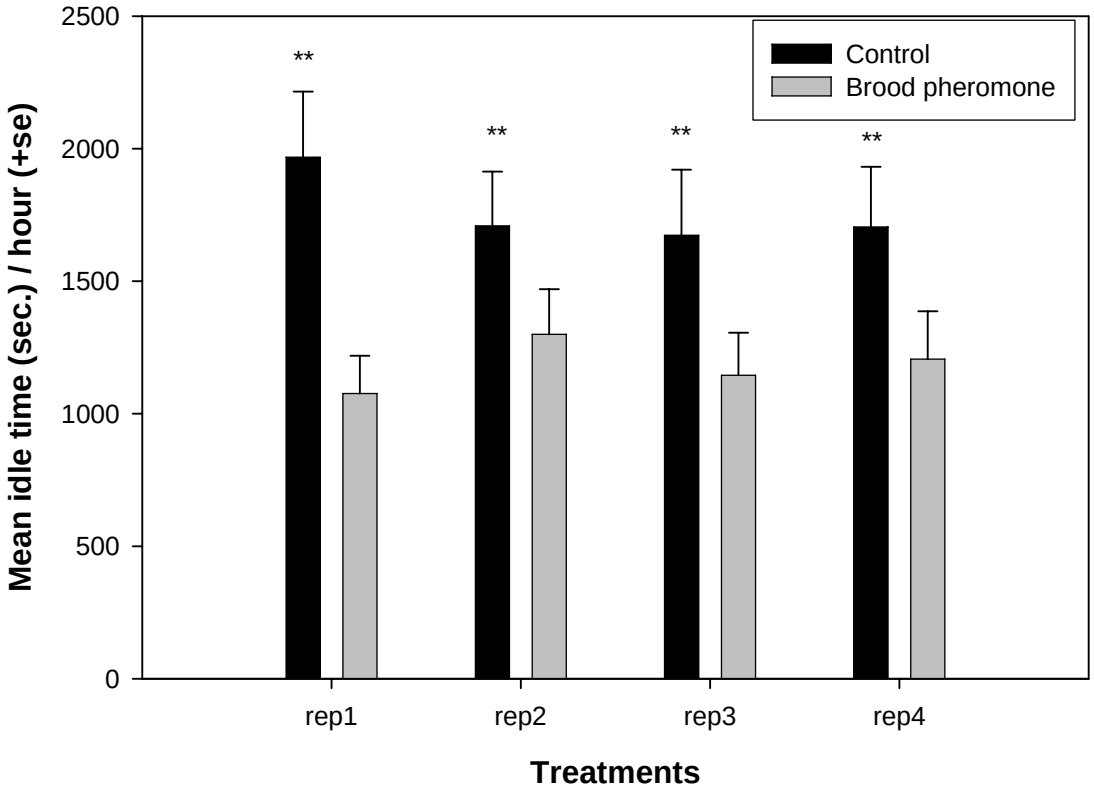

Fig. 13 Mean idle time of queen per hour (+SE) (in seconds). Asterisks indicate significant difference

Table 5 ANOVA of queen idle time analysis showing degrees of freedom, F-statistics and p-value for the four replications

	Rep1	Rep2	Rep3	Rep4
df	1, 38	1, 38	1, 38	1, 38
F	25.93	6.32	10.12	10.4
P	0.0001	0.01	0.003	0.003

Queen patrolling time, presumably seeking a cell to lay an egg was significantly greater in the brood pheromone treatments ($P<0.01$)(Fig. 14)(Table 6).

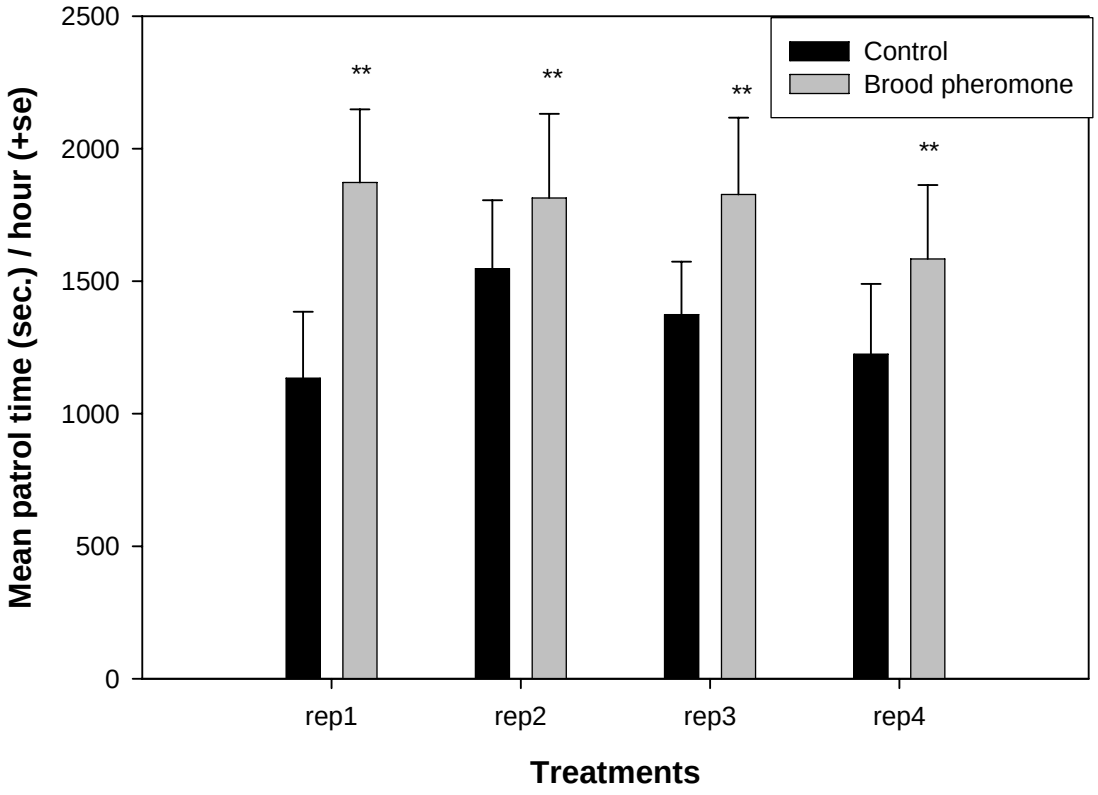

Fig. 14 Mean patrol time of the queen per hour (+SE) (in seconds). Asterisks indicate significant differences

Table 6 ANOVA of queen patrol time analysis showing degrees of freedom, F-statistics and p-value for the four replications

	Rep1	Rep2	Rep3	Rep4
df	1, 38	1, 38	1, 38	1, 38
F	31.83	4.66	9.43	7.36
P	0.0001	0.01	0.004	0.01

Amount of time spent by nurse bees on inspecting and nursing larvae was not significantly different between the brood pheromone and control treatments (P>0.05)(Fig.15 & 16).

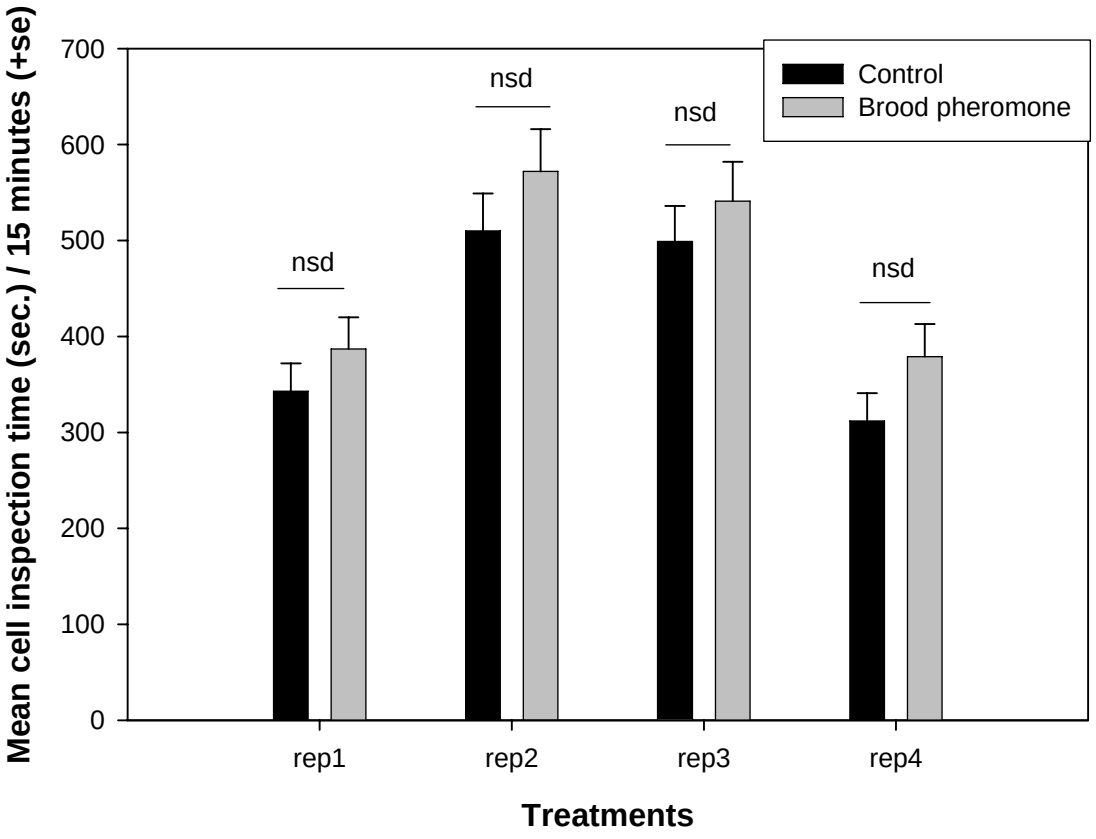

Fig. 15 Mean cell inspection time in an observation period of 15 minutes (+SE). Asterisks indicate significant differences

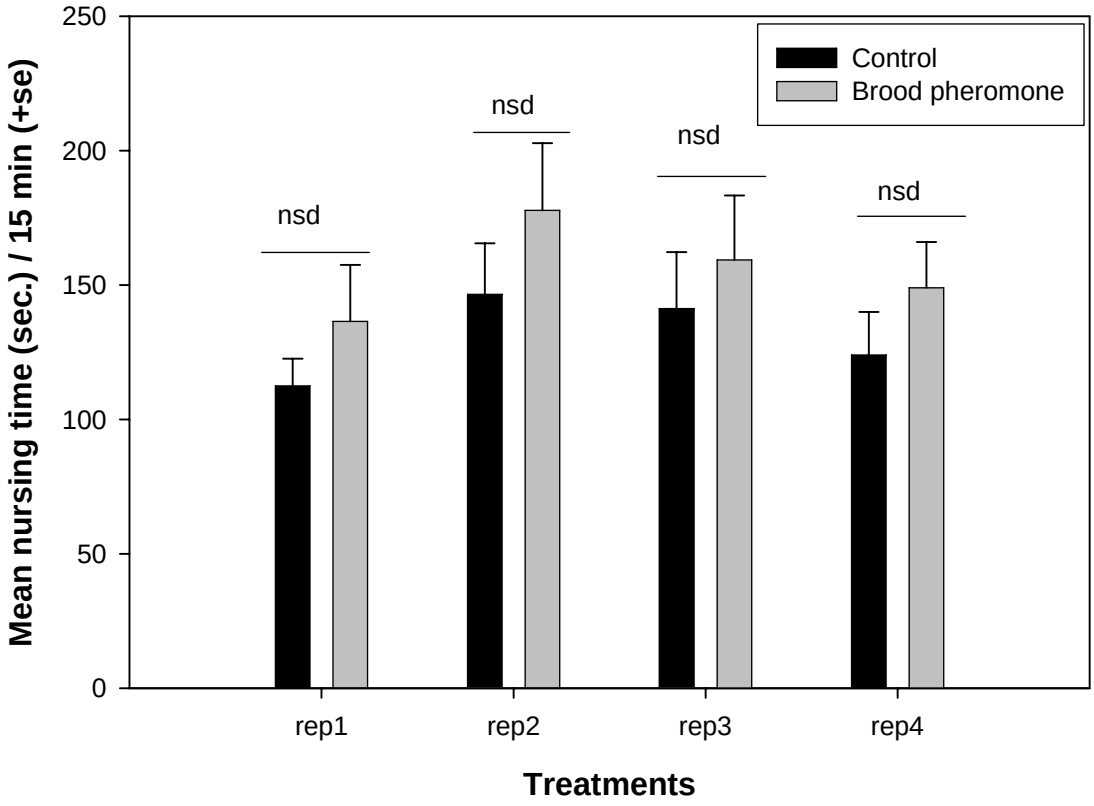

Fig. 16 Mean nursing time of the nurse bees in an observation period of 15 minutes (+SE). Asterisks indicate significant differences

Bees in the brood pheromone treated colonies spent significantly more time cleaning cells, presumably meeting a demand due to the greater egg-laying rate ($P<0.01$) (Fig. 17)(Table 7).

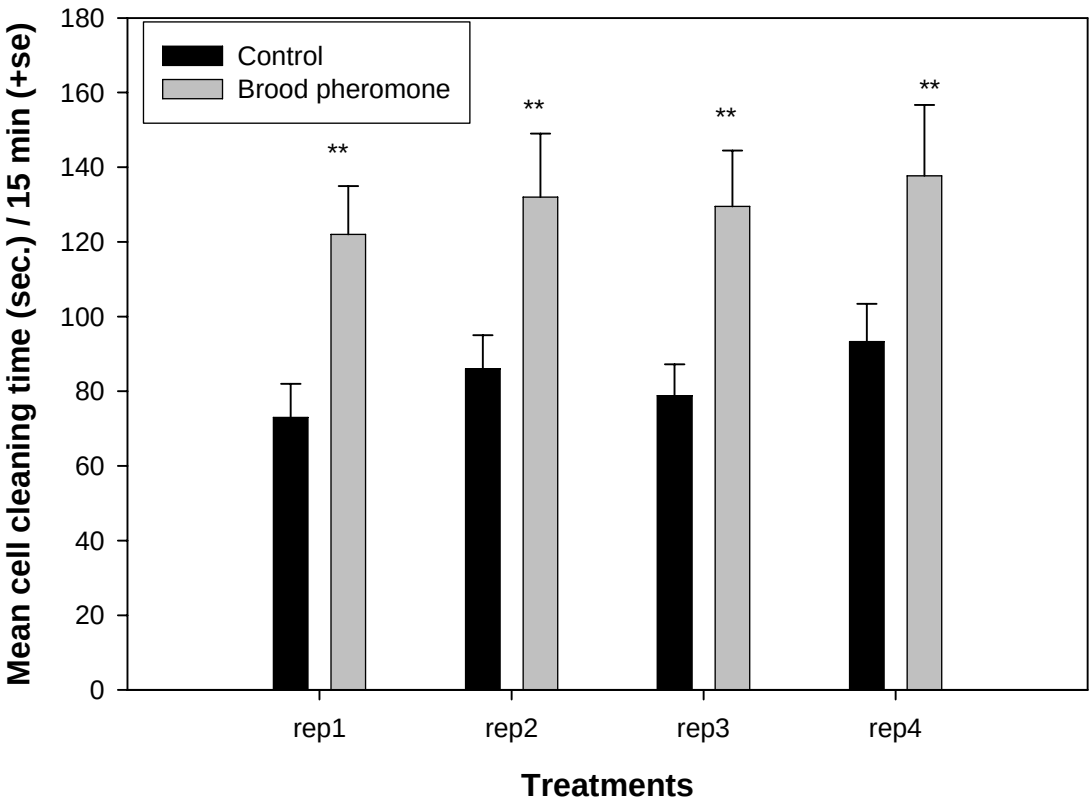

Fig. 17 Mean cell-cleaning time in an observation period of 15 minutes (+SE). Asterisks indicate significant difference

Table 7 ANOVA of nurse bee cell cleaning time showing degrees of freedom, F-statistics and p-value for the four replications

	Rep1	Rep2	Rep3	Rep4
df	1, 70	1, 70	1, 70	1, 70
F	12.1	24.09	12.89	28.34
P	0.001	0.0001	0.001	0.0001

Hypopharyngeal gland protein content was significantly higher in bees treated with brood pheromone compared to controls (ANOVA $F_{1,238}$=139.6, P<0.001). Brood pheromone treated colonies reared significantly more brood than the controls ($F_{1,6}$= 17, P=0.006). In the BP treatments significant correlation was observed between queen feeding time and the total number of eggs laid by the queen (ρ= 0.61, P=0.001). There was also a significant correlation between time spent cleaning cells and total number of eggs laid (ρ= 0.56, P=0.001).

Discussion

The results of this study support our hypothesis that brood pheromone regulates queen egg laying by modulating worker-queen interactions, increased queen nutritional environment, and multiple worker bee behaviors. Brood pheromone treated colonies contained significantly greater number of eggs compared to controls. Queens in the brood pheromone treatment were fed for a greater amount of time compared to controls. In this study it appeared that BP modulated worker behaviors, such that workers fed the queen for a longer amount of time, possibly transferring greater amounts of food. We may infer from this result that queens rapidly respond to a higher nutritional environment by greater rates of egg production. Total duration of feeds per hour is a better approximation of the amount of food received by the queen (Allen 1960). Though there was significant difference in total feeding time of the queens between the two treatments, there was no significant difference in the frequency of feeding. This further

suggests that total duration of feeding is a reliable index of amount of food transferred rather than total number of feeding bouts.

Queens in the brood pheromone treatments were less idle compared to controls. This might be a result of queens spending more time seeking cells in which to lay eggs and in egg laying. Queens in BP treatments patrolled for longer durations than controls, possibly searching for prepared cells to lay eggs. Significantly more time was spent in cell cleaning by the bees in brood pheromone treated colonies. This strongly suggests that BP plays a role in the division of worker labor associated with brood rearing. BP increases the brood rearing stimulus environment across a wide spectrum of workers; increases number of pollen foragers, increases pollen load weights returned, increases number of pollen grains extractable from non-pollen foragers, increases number of pollen foraging trips per unit time (Pankiw and Page 2001;Pankiw and Rubink 2002; Pankiw 2004c; Pankiw et al. 2004; Pankiw 2007). To this list we may add a long list of brood rearing behaviors by bees working in the nest such as cleaning cells, inspection of cells, nursing larvae, feeding the queen etc. In conclusion, BP has far reaching effects on a colony that profoundly affects the course of colony development.

Hypopharyngeal gland protein content was significantly greater in nurse bees sampled from brood pheromone treatments, indicating an increased nutritional environment. This enhanced nutritional status might facilitate provisioning of larger quantities of brood food to the developing larvae. Significantly higher brood area in brood pheromone treated colonies appears to be a consequence of higher egg laying and increased nutritional environment. Pankiw et al. (2004) reported similar results, where

BP-treated colonies reared significantly more brood area, and also hypopharyngeal gland protein content of nurse bees was higher in BP treatments than controls. Results here point to increases in multiple worker-queen interactions induced by brood pheromone as a proximate mechanism with domino effects leading to a steeper colony growth trajectory. Significant positive correlations observed in queen feeding times and nurse bee cell cleaning times with respect to total number of eggs laid by the queen further suggest that queen egg laying is regulated by worker-queen interactions and nurse bee rearing behaviors.

In conclusion, this study suggests that brood pheromone regulates queen egg-laying rate by modulating worker-queen interactions and nurse bee rearing behavior. Larvae are principal organizers of colony life. For example, BP has profound effects on age of first foraging either increasing or decreasing age of first foraging, depending on the amount. Amount of larvae or BP rapidly rallies colonies to collect more pollen through multiple mechanisms. Now we found that BP is organizing activities taking place in the center of the nest, including the queen. Effects on pre-foragers are physiological and behavioral. Organization is centered on brood rearing and brood regulate virtually every aspect of colony life. Everything that happens can be traced back to larvae. Colonies are organized by larvae found in the center of the nest, with profound affects radiating outward.

CHAPTER V

BROOD PHEROMONE REGULATED FORAGING ONTOGENY EFFECTS ON BROOD REARING IN THE HONEY BEE (*Apis mellifera* L.)

Introduction

Honey bee workers perform different tasks as they age and this phenomenon is referred to as temporal polyethism or division of labor (Robinson 1992; Beshers and Fewell 2001; Anderson and Franks 2001). After emergence as adults, usually the worker bees first clean cells, and then as they age they feed the larvae and queen, process and store food, secrete wax and construct comb, and guard the entrance. The most prominent behavioral change is observed when the bees are about three weeks old, the age when they start foraging (Lindauer 1952; Seeley and Kolmes 1991). Plasticity is an important attribute of division of labor and colonies respond to changes in the internal and external environment by manipulating the ratios of individual workers involved in different tasks (Robinson 1992). Such plasticity in division of labor can be partially attributed to the behavioral flexibility of the individual workers (Robinson 1992).

Brood rearing in honeybees is accomplished by the combined labor of nurse and forager bees who directly or indirectly provision larvae, respectively. Pollen and nectar are the two primary resources for which bees forage. Nectar serves as a carbohydrate source for both adults and larvae, whereas pollen is the primary source of protein. Pollen is consumed by nurse bees to biosynthesize proteinaceous glandular secretions

that are progressively provisioned to larvae (Winston 1987). Studies with brood pheromone have indicated that brood pheromone influenced suites of foraging and brood rearing behaviors.

Pheromones are chemicals that are the primary source of intraspecific communication in many organisms (Wyatt 2003). Social insect pheromones are broadly classified as primer and releaser pheromones. Primer pheromones change individuals to an altered behavioral state. Change occurs through putative response threshold shifts to different stimuli, altering reproductive, endocrine, and neurosensory systems (Höllbobler and Wilson 1990; Pankiw and Page 2003). Releaser pheromones elicit an immediate effect on the behavior of a receiver, and once the pheromone has dissipated or removed, individuals revert to their previous state (Pankiw 2004b).

Brood pheromone (BP) is a 10-component mixture of fatty acid esters that can be extracted from the surface of honey bee larvae (Le Conte et al. 1990). One larval equivalent (LEq) of the BP blend contains; 5.6ng ethyl linoleate, 72.8ng ethyl linolenate, 44.8ng ethyl oleate, 16.8ng ethyl palmitate, 39.2ng ethyl stearate, 11.2ng methyl linoleate, 117.6ng methyl linolenate, 140.0ng methyl oleate, 16.8ng methyl palmitate, and 95.2ng methyl stearate (Trouiller 1993; Pankiw and Page 2001). Brood pheromone is a tool that can be used to alter the foraging stimulus environment and thus change honey bee foraging strategies (Pankiw et al 1998; Pankiw and Page 2001; Pankiw and Rubink 2002).

Brood pheromone has dose-dependent effects on foraging ontogeny (LeConte et al. 2001). A relatively high amount of brood pheromone increases age of first foraging, whereas a relatively low amount of brood pheromone decreases age of first foraging

(LeConte et al. 2001). Exposure of bees to relatively low amounts of brood pheromone for 2-4 hours resulted in increased number of pollen foragers and heavier pollen loads (Pankiw and Page 2001; Pankiw and Rubink 2002). Brood pheromone treated colonies rear significantly greater amounts of brood, have significantly higher ratios of pollen to non-pollen foragers, foragers return with heavier pollen loads and take more foraging trips per unit time, and age of first foraging is significantly lower (Pankiw et al. 2004; Pankiw 2004a; Pankiw 2004b; Pankiw 2007). In this study I focused on how dose-dependent BP-mediated division of labor affected the partitioning of non-foraging and foraging work forces and the amount of brood reared.

Methods

This experiment was replicated 5 times using triple-cohort colonies (Giray and Robinson 1994; Le Conte et al. 2001). A triple-cohort colony was comprised of three cohorts of 700 bees per cohort in their first, second and third week of adult life, respectively and a naturally mated queen. Beginning four weeks prior to establishing the triple cohort colony 2500 newly emerged bees were paint marked a unique color for each week and placed in a common foster colony for aging. A total of 2500 bees per target cohort ensured that at least 700 bees for the combined age and behavioral classes were easily found and collected. Cohort 1 comprised of 700 newly emerged adult bees less than 24 hours after emergence. Newly emerged bees were derived from combs of pupae placed in an incubator maintained at 32º C and 55% RH for 6 hours. Cohort 1 received a colored plastic number tag glued (BioQuip Products Inc. 1172, CA, USA) to the thorax

and was the focal cohort for age of first foraging. Cohort 2 consisted of 700 nurse bees ranging in age from 8 to 11 days and selected from the brood nest area. Cohort 3 consisted of 700 foragers in their third week of adult life. Nurses and foragers were collected from their foster colony using a portable insect vacuum device (Gary and Lorenzen 1987).

On a weekly basis 50 newly emerged bees were added to the triple cohort colonies to simulate natural emergence of an established colony. Triple-cohort colonies have been recorded to demonstrate normal rates of behavioral development, with the benefit of a controlled adult demographic distribution (Giray and Robinson 1994; Le Conte et al. 2001). At the onset of the experiment each colony was provided with 1 frame of honey (1600 cm^2), ¼ frame of pollen (400 cm^2), and 2 frames of empty comb space (4800 cm^2). There were three treatments as follows for 30 days: 1) BP dose of 336 µg per day 2) BP dose of 168µg per day, and 3) blank control. Treatments 1 and 2 represent high and low doses of brood pheromone, respectively. Empty comb space was added as necessary and equally to all treatments.

Measurements

The ratio of pollen to non-pollen foragers was measured by daily counting the number of foragers of each type that enter colonies in a 5-minute period once in the morning and once in the afternoon. Daily observations of foraging activity began 24 hours after onset of the experiment. Every third day the comb area occupied by eggs, larvae, pupae, pollen, honey and empty space was measured with a metered grid (Pankiw et al. 2004).

Beginning on the third day, to the termination of the experiment, colony entrances were blocked with wire-mesh for 15 min intervals separated by at least 30 min to enable the capture of returning focal foragers. The entrances were blocked from 0800 h to 1700 h for a total of 4 h per day. Foragers were individually captured in small cylindrical wire cages. The identity of the captured foragers was recorded and the individuals released. Foragers were also classified as pollen or non-pollen foragers. At the termination of the experiment all number tagged bees were collected. Number of days from emergence to date of observation was used to estimate age of first foraging. Those that were not observed as foragers were categorized as censored cases in subsequent survival analysis.

Every week 10 bees from each cohort were collected for hypopharyngeal gland protein analysis. The Bradford assay was used to estimate the hypopharyngeal gland protein content (Sagili et al. 2005). Bees that were sampled for hypopharyngeal gland protein analysis were also included as censored cases in the survival analysis data set.

Statistical analyses

Contingency table analysis was used to analyze the ratio of pollen to non-pollen foragers observed and also to analyze proportion of foragers to non-foragers (Sokal and Rohlf 1995). ANOVA was used to analyze pollen load weights and protein extractable from hypopharyngeal glands (Sokal & Rohlf 1995). Brood and pollen areas were analyzed

using repeated measures ANOVA. Survival analysis was used to analyze age of first foraging data (Allison 1998).

Results

The ratio of pollen to non-pollen foragers entering the colonies in an interval of 5 minutes was significantly higher in Low BP treated colonies during the experimental period (3x2 contingency table analysis $\chi^2 = 81.5$, 2df, $P<0.001$) (Fig. 18). There was no significant difference in the ratio of pollen to non-pollen foragers entering the colonies between controls and High BP treatments ($P>0.05$). The proportion of foragers and non-foragers were significantly different between the treatments (3x2 contingency table analysis $\chi^2 = 29.3$, 2df, $P<0.01$). Low BP treatments had higher percentage of foragers followed by control and High BP treatments (Fig. 19).

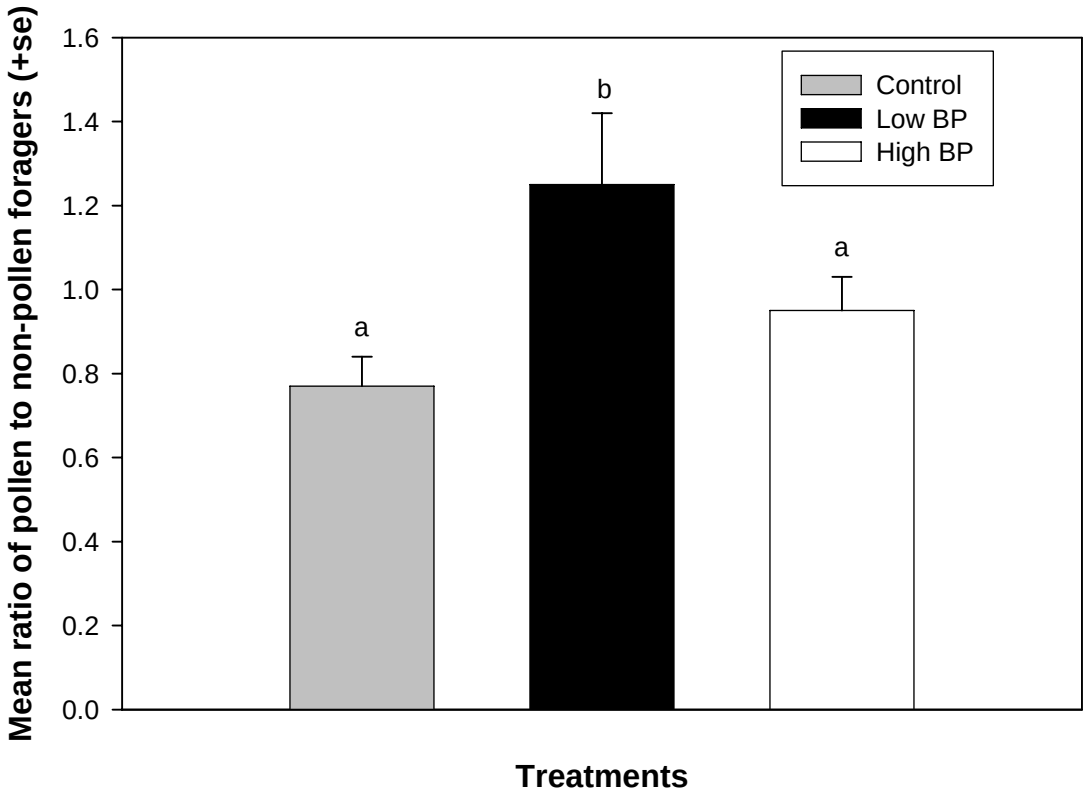

Fig. 18 Mean ratio of pollen to non-pollen foragers (+SE). Different letters indicate significant differences

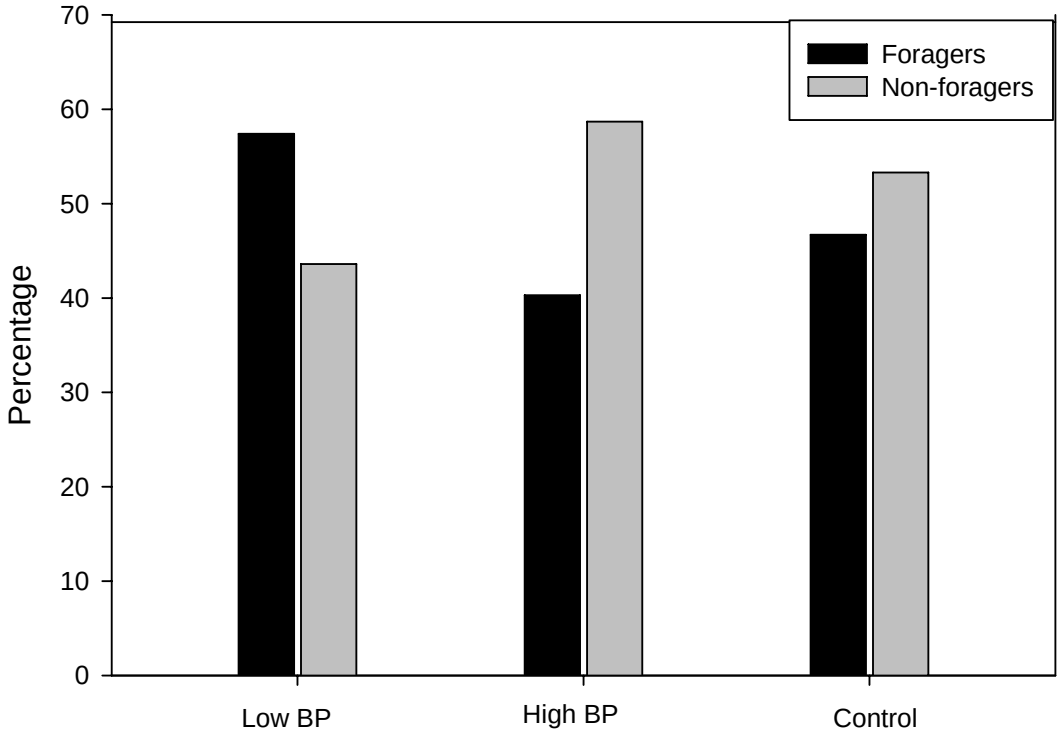

Fig. 19 Mean percentage of foragers and non-foragers in each of the three treatments

Bees in Low BP treated colonies returned with significantly heavier pollen loads than control and High BP treated colonies ($F_{2,12} = 14.3$, $P < 0.001$) (Fig. 20), and there was no significant difference in the pollen loads returned between High BP treatment colonies and controls.

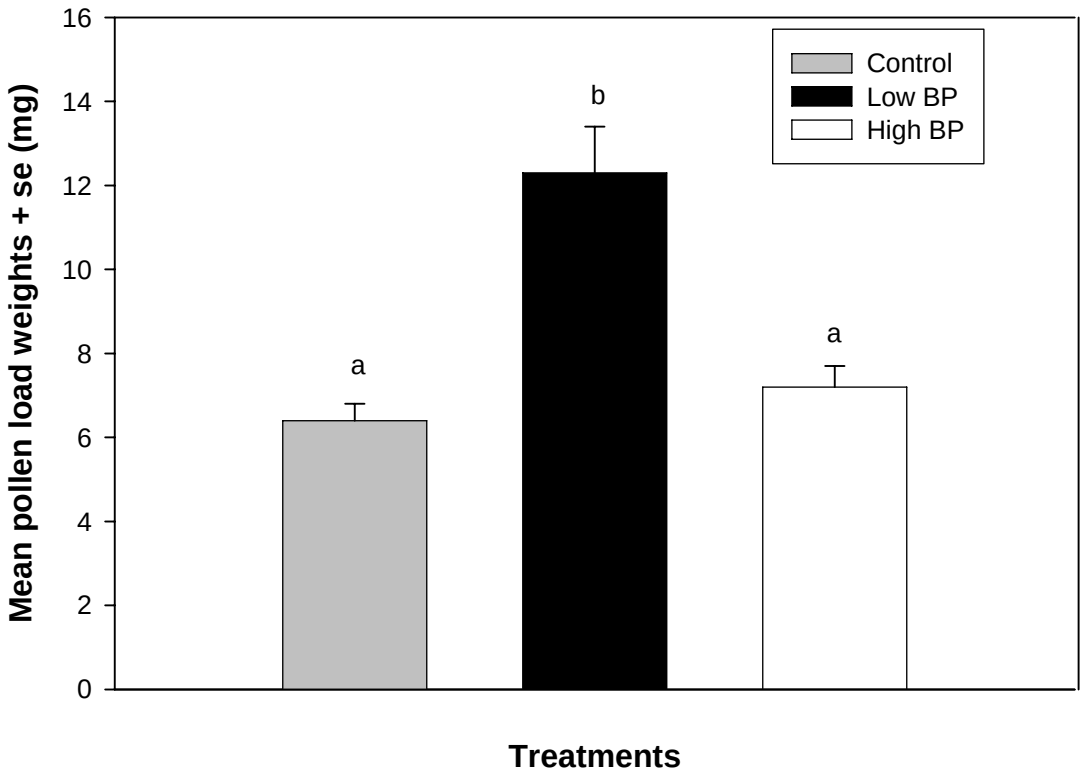

Fig. 20 Mean pollen load weights collected by the foragers (+SE). Different letters indicate significant differences

Low BP treated colonies reared significantly more brood area than High BP treatment colonies and controls (repeated measures $F_{2,12}$ =19, P<0.001)(Fig. 21). There was no significant difference between the brood areas reared by High BP and control colonies (P>0.05).

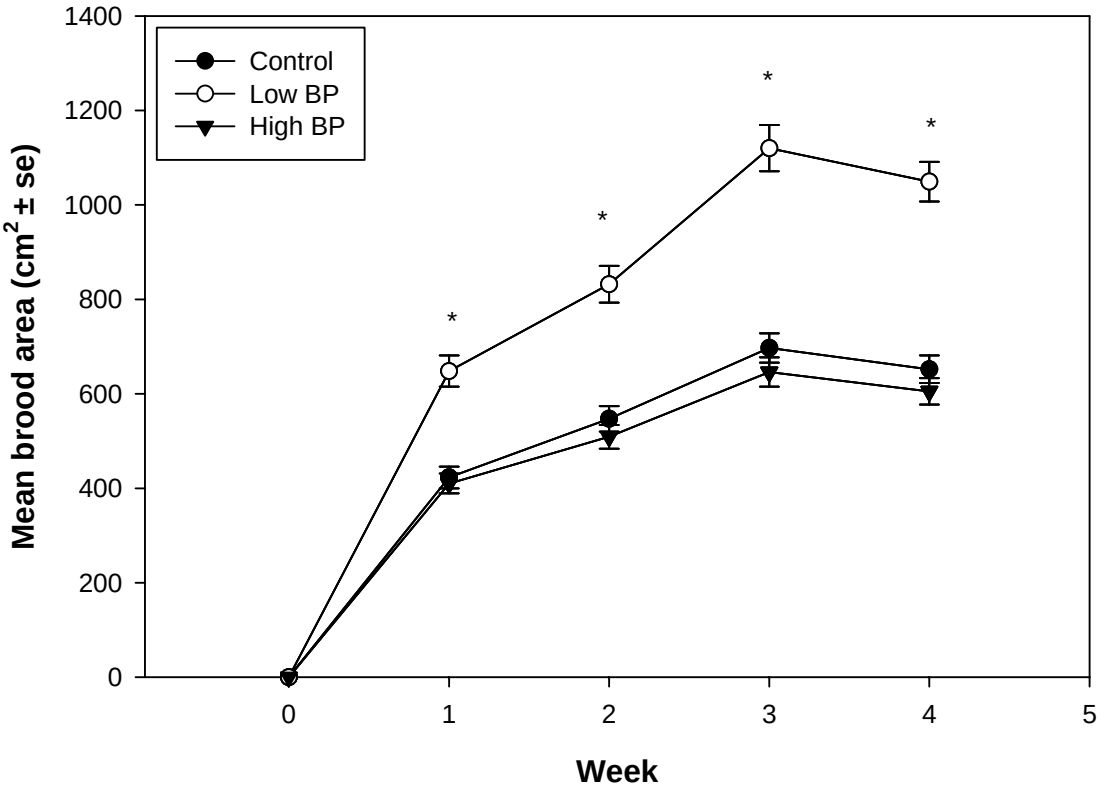

Fig. 21 Mean brood area in cm^2 (+SE). Asterisks indicate significant difference

Amount of stored pollen was not significantly different between treatments during all the four weeks (repeated measures $F_{2,12}$ =1.3, P=0.3)(Fig. 22).

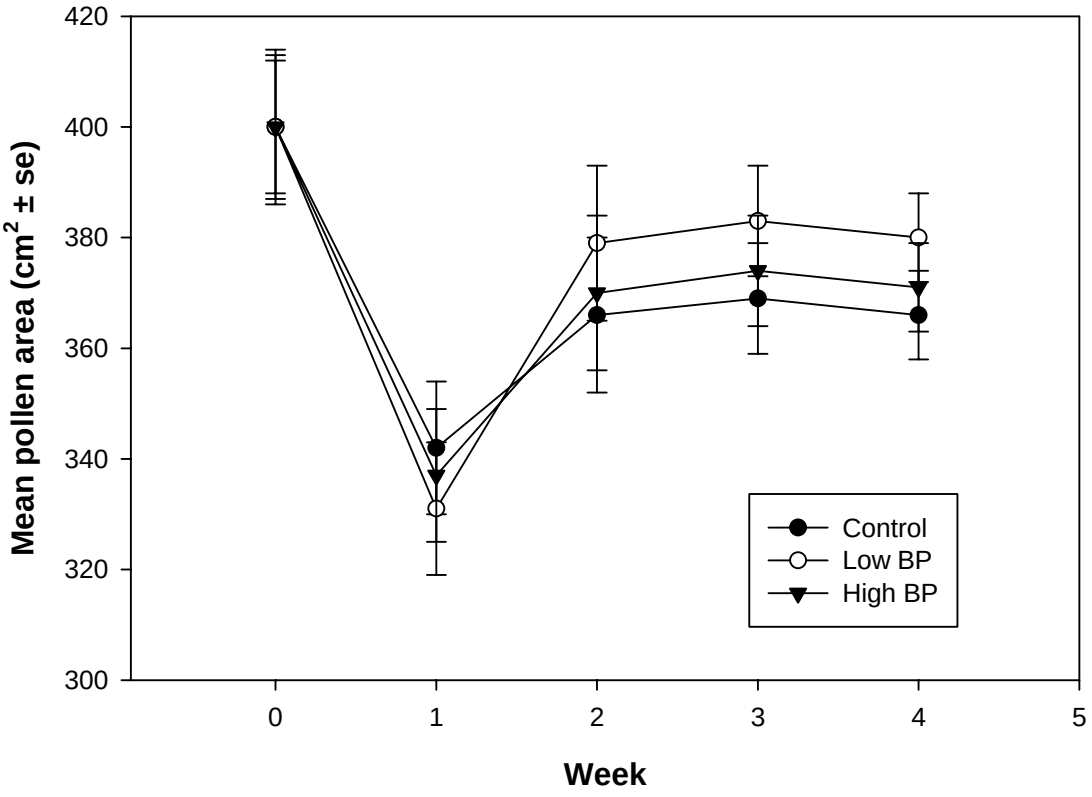

Fig. 22 Mean pollen area in cm^2 (+SE) for the three treatments

Hypopharyngeal gland protein content of bees analyzed from cohort 1 was significantly lower in the control treatments compared to High BP and Low BP treatments (Fig 23) (Table 8), and there was no significant difference between the High and Low BP treatments ($P>0.05$). Similar results were obtained for bees obtained from cohort 2 with respect to hypopharyngeal gland protein content. In bees analyzed from Cohort 3, hypopharyngeal gland protein content was significantly different between the three treatments with Low BP treatments having highest protein content followed by High BP and controls respectively ($P<0.001$) (Table 8).

Table 8 ANOVA pertaining to hypopharyngeal gland protein analysis showing degrees of freedom, F-statistic and p-value for each of the three cohorts

	Cohort 1	Cohort 2	Cohort 3
df	2	2	2
F	26.7	39.5	24.8
P	0.001	0.001	0.001

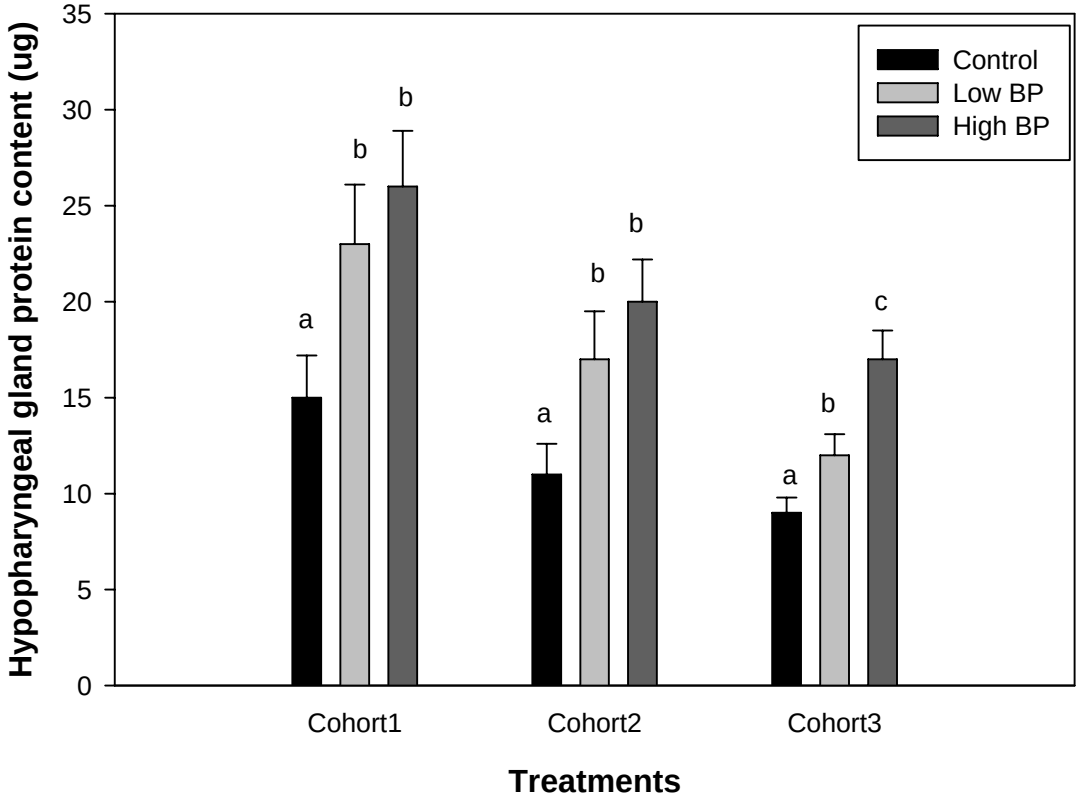

Fig. 23 Mean hypopharyngeal gland protein content in micro grams (+SE). Different letters indicate significant difference

There were significant differences in the age of first foraging. In 4 out of 5 replications, bees in the Low BP treatments foraged at significantly younger age compared to controls and High BP treatments (Fig. 24) (Table 9). In all the five replications, bees from High BP treatment colonies foraged at a significantly older age than controls and Low BP treated colonies. Overall, Low BP treatments foraged at a significantly younger age followed by controls and High BP treatments respectively.

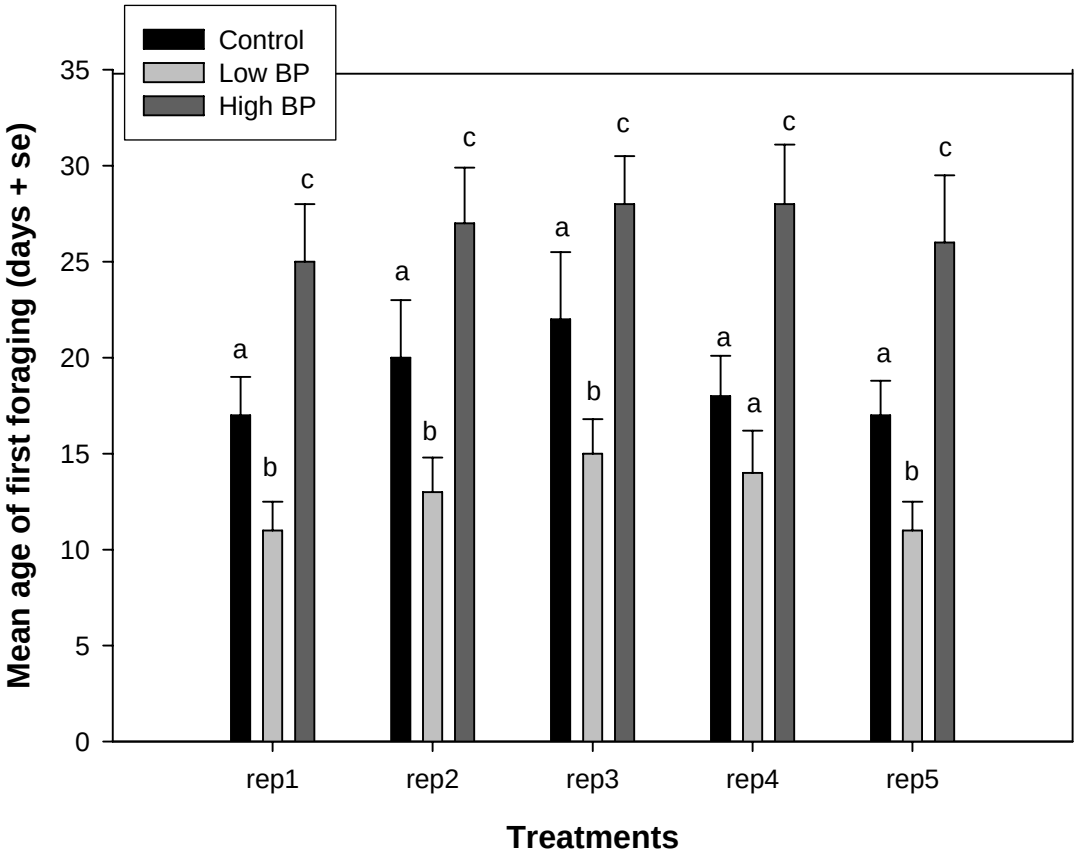

Fig. 24 Mean age of first foraging in days (+SE). Different letters indicate significant difference

Table 9 Cox regression statistics pertaining to age of first foraging showing degrees of freedom, Chi-Square value and p-value for each of the five replications

	Rep1	Rep2	Rep3	Rep4	Rep5
df	2	2	2	2	2
χ^2	26.7	39.5	24.8	19.2	31.5
P	0.001	0.001	0.001	0.01	0.001

Discussion

The results of this study suggest that brood pheromone at different dose levels differentially modulates the foraging division and brood rearing division of labor in the honey bee. Colonies receiving Low BP treatments had significantly higher ratio of pollen to non-pollen foragers and greater pollen load weights than controls and High BP treatments, indicating greater pollen collection by Low BP colonies. There was no significant difference in the amounts of stored pollen between the three treatments in spite of significant differences in pollen collection, as previously observed (Jeffree and Allen 1957; Fewell and Winston 1992; Camazine 1993; Eckert et al. 1994; Pankiw et al. 2004). Proportion of foragers was significantly high in Low BP treatments when compared to controls and High BP treatments. This suggests that Low BP induced the colonies to field greater number of foragers and thus increased colony growth.

Brood rearing was significantly higher in the Low BP treated colonies. The increased brood rearing appeared to be a result of greater pollen intake and presumably consumption given that amount stored was not different between treatments. Pankiw et al. (2004) reported similar findings, where increased brood rearing in brood pheromone treatments was attributed in part to an increased pollen intake rather than increased consumption of stored pollen. They concluded that increase in pollen intake induced by brood pheromone was directly utilized for raising a greater number of bees.

Hypopharyngeal gland protein content was significantly greater in Low BP and high BP treated bees compared to controls in both cohort 1 and cohort 2 indicating an increased nutritional environment. In cohort 3 significant differences in the

hypopharyngeal gland protein content were observed between all the three treatments with High BP treatment having the highest protein content followed by Low BP and control treatments. LeConte et al. (2001) speculated that exposure to high BP dose delayed the behavioral development in bees, thus resulting in a lengthened nursing phase. The results from cohort 3 appear to support the above speculation of extended nursing phase as a result of exposure to higher dose of BP. The presence of greater number of non-foragers than foragers in the High BP treatment indicated that High BP dose extended the nursing phase in the bees such that these colonies fielded less number of foragers.

Bees from Low BP treatment foraged at a significantly younger age, whereas the bees from High BP treatments foraged at significantly older age. Foraging age of the bees from control colonies was in-between the low and high BP treatments. These results are in agreement with findings of Le Conte et al. (2001), where they found that a relatively high amount of brood pheromone increased age of first foraging and a relatively low amount of brood pheromone decreased age of first foraging. Brood pheromone exerts dose dependent effects on sucrose response threshold modulation and regulation of foraging ontogeny (Pankiw and Page 2001).

In conclusion, this study has shown that brood pheromone elicits dose-dependent modulation of foraging and brood rearing behaviors. This clearly shows that colonies manipulated their work force extensively depending on the amount of BP. Hence the amount of BP or the number of larvae present in the colony at a point of time appears to be the driving force in organizing activities in the colony, either directly or indirectly. It

is known that BP is multifunctional, as it brings about many physiological and behavioral changes such as increase in hypopharyngeal gland protein in nurse bees, increase in number of pollen foragers, increase in pollen load weights, increase in pollen foraging trips, increase or decrease in age of first foraging, increase in cell cleaning behavior, increase in queen feeding etc., and now this study further suggests that BP is not only multifunctional, but also elicits dose dependent effects.

CHAPTER VI

CONCLUSIONS

The goal of this dissertation was to examine some important physiological and pheromonal factors regulating foraging and colony growth in honey bee colonies. The results shown in the preceding chapters provide new insights into the less known mechanisms that pheromonal and physiological factors employ to regulate foraging and colony growth in honey bee colonies. In the first study of this dissertation I studied the effects of soybean trypsin inhibitor (SBTI) on the development of hypopharyngeal gland, midgut enzyme activity and survival of the honey bee. In this study newly emerged caged bees were fed pollen diets containing three different concentrations (0.1%, 0.5% and 1% w: w) of soybean trypsin inhibitor (SBTI). Hypopharyngeal gland protein content, total midgut proteolytic enzyme activity of these bees, and survival were measured. Bees fed 1% SBTI had significantly reduced hypopharyngeal gland protein content and midgut proteolytic enzyme activity. There were no significant differences between control, 0.1% and 0.5% SBTI treatments. I concluded that nurse bees fed a pollen diet containing at least 1% SBTI would be poor producers of larval food.

The primary objective of the second study was to measure the effects of manipulating hypopharyngeal gland protein (brood food) content in nurse bees on pollen foraging. In this study nurse bee biosynthesis of brood food was manipulated using SBTI, significantly decreasing the amount of protein extractable from hypopharyngeal glands. Experimental colonies were given equal amounts of SBTI treated and untreated pollen. Colonies receiving protease inhibitor treatment had significantly lower

hypopharyngeal gland protein content than controls ($P<0.001$). There was no significant difference in the ratio of pollen to non-pollen foragers between the treatments ($P>0.05$). Pollen load weights were also not significantly different between treatments ($P>0.05$). The results supported the pollen foraging effort predictions generated from the direct independent effects of pollen on the regulation of pollen foraging and did not support the prediction that nurse bees regulate pollen foraging through amount of hypopharyngeal gland protein biosynthesis.

The third study tested whether brood pheromone (BP) regulated queen egg laying via modulation of worker-queen interactions and nurse bee rearing behaviors. This experiment had two treatments, BP and control. Brood pheromone treated colonies contained significantly greater number of eggs compared to controls. Queens in the brood pheromone treatment were fed for a greater amount of time and were less idle compared to controls. Queens in BP treatments patrolled for longer durations than controls, possibly searching for prepared cells to lay eggs. Significantly more time was spent in cell cleaning by the bees in brood pheromone treated colonies. The results suggested that BP played a role in the division of worker labor associated with brood rearing. Hypopharyngeal gland protein content was significantly greater in nurse bees sampled from brood pheromone treatments, indicating an increased nutritional environment. This study suggested that brood pheromone regulated queen egg-laying rate by modulating worker-queen interactions and nurse bee rearing behavior.

The final study focused on how dose-dependent BP-mediated division of labor affected the partitioning of non-foraging and foraging work forces and the amount of

brood reared. Triple cohort colonies were used in this study, and there were three treatments, Low BP, High BP and Control. Colonies receiving Low BP treatments had significantly higher ratio of pollen to non-pollen foragers and greater pollen load weights than controls and High BP treatments, indicating greater pollen collection by Low BP colonies. There was no significant difference in the amounts of stored pollen between the three treatments in spite of significant differences in pollen collection. Bees from Low BP treatment foraged at a significantly younger age, whereas the bees from High BP treatments foraged at significantly older age. Foraging age of the bees from control colonies was in-between the low and high BP treatments. This study has shown that brood pheromone elicits dose-dependent modulation of foraging and brood rearing behaviors.

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