Decoding Brood Pheromone:

The Releaser and Primer Effects of Young and Old Larvae

on Honey Bee (*Apis mellifera*) Workers

by

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ABSTRACT

How a colony regulates the division of labor to forage for nutritional resources while accommodating for changes in colony demography is a fundamental question in the sociobiology of social insects. In honey bee, *Apis mellifera*, brood composition impacts the division of labor, but it is unknown if colonies adjust the allocation of foragers to carbohydrate and protein resources based on changes in the age demography of larvae and the pheromones they produce. Young and old larvae produce pheromones that differ in composition and volatility. In turn, nurses differentially provision larvae, feeding developing young worker larvae a surplus diet that is more queen-like in protein composition and food availability, while old larvae receive a diet that mimics the sugar composition of the queen larval diet but is restrictively fed instead of provided *ad lib*. This research investigated how larval age and the larval pheromone e-beta ocimene (eβ) impact foraging activity and foraging load. Additional cage studies were conducted to determine if eβ interacts synergistically with queen mandibular pheromone (QMP) to suppress ovary activation and prime worker physiology for nursing behavior. Lastly, the priming effects of larval age and eβ on worker physiology and the transition from in-hive nursing tasks to outside foraging were examined. Results indicate that workers differentially respond to larvae of different ages, likely by detecting changes in the composition of the pheromones they emit. This resulted in adjustments to the foraging division of labor (pollen vs. nectar) to ensure that the nutritional needs of the colony's brood were met. For younger larvae and eβ, this resulted in a bias favoring pollen collection. The cage studies reveal that both eβ and QMP suppressed ovary activation, but the larval pheromone was more effective. Maturing in an environment of young or

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old larvae primed bees for nursing and impacted important endocrine titers involved in the transition to foraging, so bees maturing in the presence of larvae foraged earlier than control bees reared with no brood.

DEDICATION

To my husband and the bees,

who both bring sweetness and light to my life.

Thank you for the incredible journey

across continents, through valleys, and up mountains,

always in search of two million blossoms to call our own.

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and conjures up equipment, bees and queens whenever they're needed. Your tantalizing array of Turkish treats and generosity always picked me up during long hours at the lab.

A big hug to my parents, who instilled me with a love of traveling, encouraged my linguistic abilities, and have succumbed to my madcap adventures with bees and honey. I love you both dearly and thank you for everything.

Thank you, Michael, for always believing in me. I couldn't do it without your love and support. Thank you for always being ready to travel, don a bee suit, and jump right in to help. You keep me grounded and make the ordinary extraordinary. Exciting escapades lie ahead, so fasten your seatbelt and let's enjoy the ride.

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PREFACE

This dissertation is an original intellectual product of the author, Kirsten S. Traynor, who

was responsible for all major areas of experimental design, data collection and analysis,

as well as manuscript composition. None of the text of the dissertation is taken directly

from previously published or collaborative articles. The experiments reported in Chapters

2-4 were conducted at the bee lab research facility of Arizona State University, with input

and suggestions from my advisor Dr. Robert E. Page and my Fulbright host in France Dr.

Yves Le Conte. The analysis of vitellogenin in Chapter 4 was conducted by Dr. Ying

Wang, research associate at ASU. Research funding was provided by an Advanced

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CHAPTER 1

INTRODUCTION

Chemical communication, believed to be the most ancient form of signaling, mediates social interactions in a wide range of organisms from single celled bacteria to complex vertebrates (Wyatt 2003). Pheromones are chemicals released by individuals into the environment that trigger a behavioral response in members of the same species (Karlson and Butenandt 1959) and play central regulatory roles in many animal societies (Wilson 1971). Pheromones were originally known by the self-contradicting term "ectohormone", until Karlson and Butenandt's landmark Nature coined the new term from *pherein* "to carry" and *hormon* "to excite or stimulate." Among social insects pheromones are the primary mode of communication (Blum 1974), widely used because they convey the following benefits (Wilson 1975):

1) long range (i.e. sex pheromones)

2) slow transmission rate (i.e. marking pheromones)

3) low energetic cost of sending

4) last for variable lengths of time, depending on molecular weight

5) unlike visual cues, can be used in the dark

6) flow around barriers

7) can have high specificity

Within social insects the chemical communication system has proven to be highly diversified and richly complex, enhanced by synergistic interactions and context-

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dependent messaging (Slessor et al. 2005). For example, at least 50 substances derived from queens, workers and immatures are expressed within the colonies of honey bees (*Apis mellifera*) (Pankiw 2004b). Honey bees are small chemical factories, producing pheromones from a wide variety of glands including alarm pheromones produced in the Koschevnikov gland, footprint pheromones of the pretarsal glands, secretions that change in response to queen loss from the Dufour's gland, and the citral/geraniol odors of the Nasanov gland that help orient bees to the hive or swarm location.

Despite being chemically identified, many of these secretions have unknown effects on behavior, and their specific modes of action are even less well understood. A number of pheromones act as releasers, causing rapid but short-lived responses (Wilson and Bossert 1963), such as the clustering/orienting response caused by workers exposing their dorsal Nasanov gland (Pickett et al. 1980). Because releasers permit simple behavioral assays, their function can be readily assessed. However, other pheromones act as primers, which slowly influence behavior through long-term physiological effects, thereby influencing broad aspects of colony organization, caste structure and the division of labor. (Wilson and Bossert 1963; Winston and Slessor 1998; Le Conte and Hefetz 2008). Many multi-component pheromones in honey bees such as queen mandibular pheromone (QMP) and the mixture of fatty acid brood ester pheromone (BEP) emitted by older larvae act as both releasers and primers. Increasing evidence suggests that these primer and multifunctional pheromones have profound effects in shaping honey bee colony dynamics (Le Conte and Hefetz 2008; Alaux et al. 2010; Kocher and Grozinger 2011).

PHEROMONE DETECTION

Pheromones and other odorants are detected by specialized olfactory sensilla on the honey bee antennae. The most frequent are poreplate sensilla innervated by 5 to 35 co-localized olfactory receptor neurons which express specific odorant receptors. (Leal 2005; Leal 2013; Bortolotti and Costa 2014). Pheromones are shuttled by odorant binding proteins through the sensillar lymph to the dendrites of these olfactory receptor neurons (Pelosi and Maida 1995). These neurons converge on individual glomeruli that relate the signal onto projection neurons, which in turn send the signal to higher brain regions, where the perceived odors are integrated with other stimuli and the motor system is activated in response (Leal 2005; Leal 2013).

LARVAL DEVELOPMENT, NUTRITION, AND PHEROMONE EMISSIONS

A key aspect of chemical communication that contributes to honey bee colony organization and survival occurs between developing larvae and the adult workers that care for them. The larvae, confined to a cell and completely dependent on their caregivers for survival, must signal their needs to the adult workforce. Larvae hatch from eggs three days post-oviposition and subsequently molt through five larval instars during six days of worker development (Winston 1987). During this relatively short time of development, the pheromone profiles of larvae change in composition and volatility (Fig. 1.1). Young larvae produce the volatile pheromone e-beta ocimene (eβ), which peaks in production between the 2nd and 3rd larval instar and then rapidly diminishes (Maisonnasse et al. 2009; Maisonnasse et al. 2010). Beginning in the third larval instar, larvae start producing

the non-volatile mixture of ten ethyl and methyl fatty acid esters, known as brood ester pheromone (BEP) (Le Conte et al. 1990; Trouiller et al. 1991; Trouiller 1993), while continuing to produce minute amounts of eβ. The ethyl esters predominate during the third larval instar, while the methyl esters dominate just prior to cell capping.

Nurse bees tightly regulate larval growth by adjusting the larval feeding regime according to larval age (Linksvayer et al. 2011; Wang et al. 2014), indicating that nurse bees use larval pheromones to regulate larval diet (Le Conte et al. 1994; Le Conte et al. 1995). Young larvae are bipotent and can develop into workers or reproductive queens through the third larval instar, after which point hive reared larvae begin to lose their reproductive potential unless supplemented with treatments of juvenile hormone (Asencot and Lensky 1976; Asencot and Lensky 1984). Larval pheromone emissions map almost directly onto the hormone profiles of developing workers (Leimar et al. 2012), with eβ following the curve of juvenile hormone titers, while BEP production precedes a spike in ecdysteroids (Fig. 1.2). These co-varying curves suggest an interdependence of pheromones and hormones: the pheromones may be indicators of nutritional needs for larval development and those nutritional inputs from caregivers in turn regulate titers of key endocrine regulators linked to caste development.

DECODING BROOD PHEROMONE

By emitting brood pheromones, the larvae are able to influence the behavior and physiology of their caregivers, stimulating them to provide appropriate nutritional resources (Arnold et al. 1994; Mohammedi et al. 1996; Mohammedi et al. 1998; Pankiw

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et al. 1998; Le Conte et al. 2001; Pankiw 2004a; Sagili and Pankiw 2009; Maisonnasse et al. 2010). The nutritional needs and pheromone profiles of young versus old larvae differ dramatically, but the releaser and priming effects of different age larvae on their caregivers have never been compared. Towards that end, I examined the influence of larval age on the foraging division of labor for nutritional resources (pollen, nectar and water), assessing the releaser effects of young larvae, old larvae, capped brood, and eβ in contrast to a control of no brood (Chapter 2). Next, I studied caregiver physiology in laboratory controlled cage studies, examining the impact of eβ on nursing and reproductive physiology, both alone and in concert with QMP (Chapter 3). I complemented these cage studies with field trials, where I examined the early priming effects of young and old larvae, and eβ compared to a control of no brood on worker physiology and subsequent age of first foraging, an important marker of developmental maturation (Chapter 4).

These experiments help elucidate the complex chemical communication system that permits eusocial insects to rapidly adjust to changing internal and external environments, and adapt successfully to shifting resources and colony demography. The results detailed in the following chapters (Table 1.1) begin to decode the complex signal of brood pheromones, answering many unknowns and raising further questions in need of exploration in future studies.
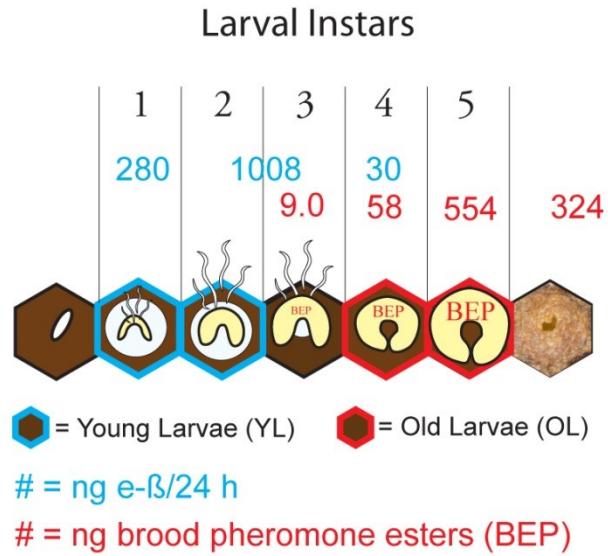
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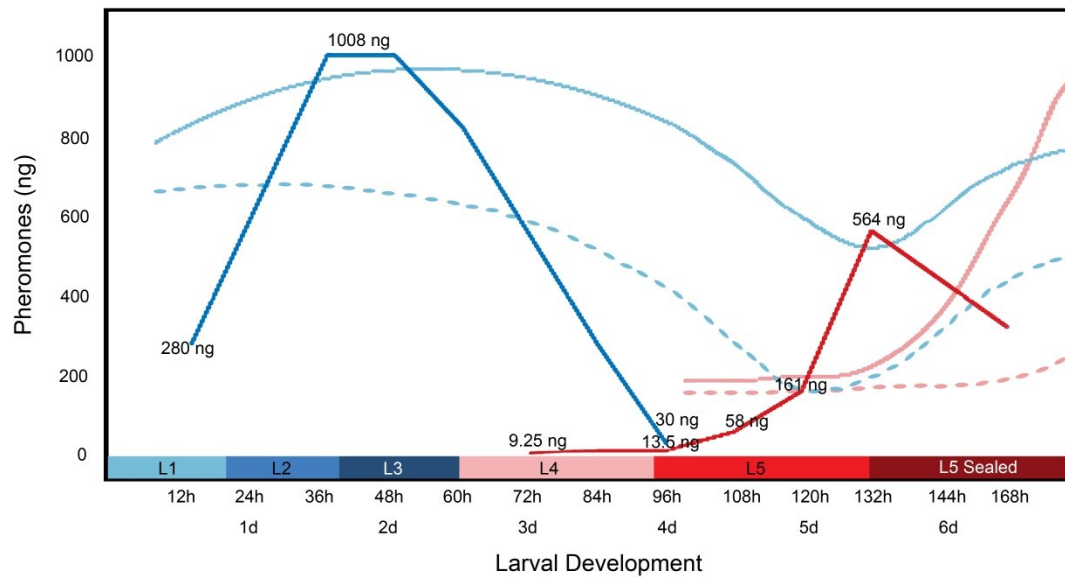
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with the hemolymph JH titers (pmol/mL) of queens (solid light blue) and workers

(dashed light blue). Emissions of eβ dominate during the first three instars (L1-L3) when

worker larval food is unrestricted, but contains only around 4% sugar. Feeding is

restricted in the fourth and fifth instar, when BEP production commences. BEP peaks

prior to cell capping, preceding the peak of ecdysteroid titers (solid pink = queens, dashed

pink = workers). Nurses seal the worker cells (L5 Sealed) and workers starve through to

the prepupal stage, whereas queens continue feeding until pupation. Through late L3,

nurses can reroute worker-destined larvae into queens via changes in larval nutrition,

while supplemental application of juvenile hormone can reroute larvae through L4.

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Table 1.1. Main effects of larval age and larval pheromones on honey bee behavior. Main effects of young larvae (YL), e-beta ocimene (eβ), old larvae (OL), and brood ester pheromones (BEP) on key characteristics that underlie developmental maturation, nursing, and foraging behavior. Results shaded in light grey were unknown prior to the experiments outlined in this thesis. Results shaded in dark grey indicate experiments previously conducted by others that were repeated to confirm results. Y = yes; N = no; Y & N = yes and no, depending on the experimental conditions; for pollen foraging both YL and eβ released significantly more pollen foraging than OL (represented by the + and -). OL was still significantly higher than the control of no brood (Chapter 2). The effects of eβ on ovary activation and HPG development were contradictory depending on whether studies were conducted in a cage or a colony, highlighting the importance of context for pheromone experiments. In cages eβ suppresses ovary activation and did not stimulate HPG development, except in bees with more ovarioles (Chapter 3). In field trials, eβ does not suppress ovary activation, but stimulates HPG development (see Chapter 4 for explanation).

Table 1.1 MAIN EFFECTS OF LARVAL AGE AND LARVAL PHEROMONES ON HONEY BEE BEHAVIOR				
Effect	**YL**	**eβ**	**OL**	**BEP**
Releases increased pollen foraging	Y +	Y +	Y -	Y
Reduces age of first foraging	Y	Y	Y	Y & N
Increases juvenile hormone (JH), regulator of maturation	Y	N	Y	N
Decreases vitellogenin (VG), regulator of JH	N	N	Y	Y
Inhibits ovary activation	Y	Y & N	Y	Y
Increases hypopharyngeal gland (HPG) development	Y	Y & N	Y	Y

CHAPTER 2

YOUNG AND OLD LARVAE RELEASE DIFFERENTIAL FORAGING BEHAVIOR

Abstract: How a colony regulates the division of labor to forage for nutritional resources while accommodating for size and demographic composition is a fundamental question in the sociobiology of social insects. In honey bees, *Apis mellifera,* young and old larvae produce pheromones that differ in composition and volatility. Nurses differentially regulate larval nutrition, feeding young worker larvae a surplus diet that parallels queen larvae in protein composition and food availability, while old larvae are restrictively fed a diet with similar sugar content as queens. The presence of brood impacts the division of labor, but it is unknown if foragers regulate resource collection based on the larval age and pheromone production in the nest. We studied how larval age demography and the larval pheromone e-beta ocimene impacts foraging activity and foraging load. Our results indicate that workers recognize larval age, likely by detecting changes in the pheromones emitted by larvae as they mature, and adjust the foraging division of labor (pollen vs. nectar) and meet the nutritional needs of the colony's brood. For younger larvae, this results in a bias toward pollen collection.

INTRODUCTION

Honey bees, *Apis mellifera*, exhibit two discrete divisions of labor (DOL): 1) a reproductive DOL distributed between the fertile queen and facultatively sterile worker castes and 2) a temporal DOL partitioned between young bees (nurses), that care for and feed immature nestmates (eggs, larvae and pupae) inside the hive, and older workers (foragers), that collect pollen and nectar outside the hive (Winston 1987; Robinson 1992; Seeley 1995). Queen-laid fertilized eggs are bipotent and can develop into either a queen or worker, depending on the quantity and quality of food provided by nurses. Recent evidence suggests that workers and queens begin to diverge in gene and protein expression almost immediately after hatching into a larva (Li et al. 2010; Schwander et al. 2010; Cameron et al. 2013). The queen–worker dimorphism is the result of discrete feeding regimes imposed by nurses. Queens develop when food is unrestricted and contains about 12% sugar throughout larval development (Shuel and Dixon 1968). In contrast, the production of a worker is more complex requiring a multi-stage feeding program. Artificial feeding regimes can result in a range of phenotypes including traits that are intermediate in expression between queens and workers (Leimar et al. 2012). Chemical signals produced by larvae change during larval development (Le Conte et al. 1994) and can orchestrate the feeding behavior of adult workers that feed the larvae (Le Conte et al. 1995).

During the six day period of the five-instar larval stage, the brood is confined to a cell and remains dependent on the care of nurse bees. The larvae emit pheromones that

influence worker physiology and behavior (Arnold et al. 1994; Mohammedi et al. 1996; Mohammedi et al. 1998; Pankiw et al. 1998; Le Conte et al. 2001; Pankiw 2004a; Sagili and Pankiw 2009; Maisonnasse et al. 2010). During the 1st and 2nd instars, a period of developmental totipotency (Rhein 1933; Jung-Hoffmann 1966), larvae produce volatile e-beta ocimene (eβ) (Maisonnasse et al. 2009; Maisonnasse et al. 2010). Larval food remains unrestricted in quantity from the 1st through early 3rd instar (Haydak 1943), but the sugar content of the diet for worker-destined larvae is 4% versus 12% fed to larvae in queen cells (Shuel and Dixon 1968). During the late 3rd and early 4th instars, these worker larvae diminish eβ production and start emitting small amounts of non-volatile ethyl and methyl fatty acid esters, collectively known as brood ester pheromone (BEP) (Trouiller et al. 1991; Trouiller 1993). This change in pheromone profile (Trouiller 1993; Maisonnasse et al. 2009; Maisonnasse et al. 2010) corresponds with a reduced capacity for larvae to develop into queens (Woyke 1971), and a shift in the diet provided by nurses. Although sugar content is quadrupled, overall food availability is reduced (Rhein 1956; Shuel and Dixon 1968; Asencot and Lensky 1988), including protein content (fresh weight) decreasing from 19% to 9% (Kunert and Crailsheim 1987). While the sugar increase is required for successful pupation (Shuel and Dixon 1968), the restricted diet slows the growth rate (Stabe 1930; Wang 1965) and may promote the loss of ovarioles that secures the caste of the larvae (Woyke 1971; Hartfelder et al. 1995; Hartfelder and Steinbrück 1997; Capella and Hartfelder 1998). After nurses seal the cells, the worker larvae starve through to the prepupal stage, unlike the mass provisioned queen larvae

(Linksvayer et al. 2011; Leimar et al. 2012; Wang et al. 2014), but they continue to produce minute amounts of both eβ and BEP (Maisonnasse et al. 2010).

Because the changes to brood pheromone emissions are stage specific, nurses probably use them to guide how they care for the larvae (Le Conte et al. 1995). Development is thus likely to be orchestrated by the interplay of larval signals and nurse responses. It has been widely established that immature honey bees directly impact the division of labor among workers, modulating pollen and nectar foraging (Free 1967; Fewell and Winston 1992; Camazine 1993; Pankiw et al. 1998; Dreller et al. 1999; Dreller and Tarpy 2000; Pankiw 2004a; Amdam et al. 2009; Tsuruda and Page 2009a). However, because previous research on the effect of brood on worker foraging behavior generalized larvae into a single category of larvae/open brood (Pankiw et al. 1998; Dreller et al. 1999; Dreller and Tarpy 2000; Pankiw and Page 2001), it was not possible to determine the relationship between brood pheromone release at different life stages and worker food collection. We hypothesized that young larvae would stimulate increased pollen foraging compared with old larvae, as younger larvae require more protein to complete development. To determine the influence of larval age on the division of foraging labor, we compared the releaser effects of pheromones from young larvae, old larvae, capped pupae and the absence of brood. We also tested if eβ by itself could produce the same foraging behavior in workers as the presence of young larvae, hypothesizing that the presence of concentrated eβ was sufficient to determine the foraging behavior of workers.

Experiment 1: Releaser Effects of Young Larvae, Old Larvae, and Capped Brood

To determine if workers perceive the different nutritional needs of immature honey bees and adjust their foraging accordingly, we compared the foraging releaser effects of three different immature stages against a control of no brood.

Bees. All colonies used in this experiment contained naturally mated *Apis mellifera* L. queens purchased from commercial beekeepers in California. Six kg of mixed age worker honey bees were collected from 10 colonies by shaking frames of bees into a ventilated box. The bees were placed in a cool room (35-40° C) for 4 h, then were equally divided into four 1.5 kg units and placed in small "nucleus" hives (nucs) in the local apiary adjacent to our research facilities in Mesa, Arizona. Each nuc received a mated queen, 1 synthetic queen pheromone strip (PseudoQueen; ConTech, Victoria, BC), 1 comb of honey, 1 empty comb, and 1 treatment comb. Synthetic queen pheromone is often used in experimental hives to control for possible differential queen pheromone effects across individual colonies, as pheromone quality is influenced by numerous physiological factors.

Treatments. Workers were exposed to a comb containing one of four stimulus treatments: 1) young larvae (YL); 2) old larvae (OL); 3) pupae (capped brood, CB); or 4) no brood (NB). To generate these treatment combs, queens from additional colonies were caged on empty combs for 18 h. Each brood treatment consisted of ~ 2000 cells containing larvae or pupae. When foraging activity was measured, YL were 5 d post-oviposition, predominantly 2nd instar larvae that emit up to 1008 ng of volatile e-beta

ocimene (eβ) as their pheromone in 24 h (Maisonnasse et al. 2009; Maisonnasse et al. 2010). OL were 8 d post-oviposition, predominantly 5th instar larvae that emit up to 564 ng of non-volatile brood ester pheromone (BEP) and minute amounts of eβ (up to 30 ng) (Trouiller et al. 1991; Trouiller 1993). The CB were pupae at 13 d post egg laying that emit small amounts of both eβ and BEP underneath a semi-permeable wax cap (Trouiller 1993; Maisonnasse et al. 2010). The experiment was replicated four times over a 10 d interval. New nucs were established for each replicate.

Foraging activity. Full size colonies, maintained in the same apiary as the experimental colonies, collected abundant pollen and nectar resources from fall blooming plants, indicating sufficient resource availability. Hive entrances were partially blocked for 5 min with a wire mesh screen that allowed colony odors to pass through, thus helping foragers orient to the entrance, but restricted returning foragers to re-enter via a small opening. During this time, an observer sat at the side of the entrance with hand held counters and recorded all returning foragers as either pollen foragers or non-pollen foragers. Pollen foragers had visible pollen loads on their hind legs; non-pollen foragers had no noticeable loads. Pollen foragers included those that collected both pollen and nectar, while non-pollen included nectar, water and empty bees. Total foraging activity was determined by summing the foragers for each 5 min interval (4-5 intervals per hive) and comparing these foraging intervals across treatments. Pollen foraging activity was determined by calculating the proportion of total foragers that collected pollen (pollen foragers/total foragers).

Foraging loads. After foraging activity was recorded, entrances were completely

blocked with mesh screens so that returning foragers landed at the entrance but could not

enter the hive. From each of the four test colonies, 50 random foragers were collected.

Each bee was placed in an individual cage so that it could not exchange food via

trophallaxis. Bees were anesthetized with CO_2 and the nectar load from their honey

stomachs expressed into a 50 µl capillary tube (Kimble, Vineland, NJ) by gently

squeezing the abdomen (Gary and Lorenzen 1976). The volume of nectar was measured

using a millimeter-scale ruler. The sucrose concentration was then determined using an

electronic Brix refractometer (MISCO Palm Abbe, Cleveland, OH). The pollen load from

one leg of each pollen forager was removed and weighed. Because bees carry a balanced

pollen load (Winston 1987), the weight of the single pellet was doubled to represent total

pollen load collected. Foraging loads were classified as: empty, water, nectar, or pollen.

Empty foragers had no visible pollen load and < 3 µl in their crop; water foragers had

crop contents with < 5% sucrose. Bees that had collected both pollen and nectar were

counted as pollen foragers, as there were too few to include an additional foraging

category (2-7/treatment).

Experiment 2: Releaser Effects of Young Larvae and E-Beta Ocimene

To determine if the pheromone signal alone can stimulate increased pollen

foraging or if workers must interact with live larvae, we compared the releaser effects of

young larvae and e-beta ocimene on foraging behavior to a no brood control.

Bees. Twelve colonies were established from 1.5 kg packages purchased from a commercial beekeeper. Each colony contained one honey comb and two empty combs on which the queens laid eggs. Colonies developed for 3 d prior to beginning the experiment, after which the queen was confined in a small cage. All combs were removed from each colony and replaced with 1 comb of honey, 1 empty comb, and 1 treatment comb.

Treatments. Workers were exposed to one of three stimulus treatments 1) young larvae (YL); 2) a mixture of ocimene isomers including eβ (Sigma-Aldrich, St. Louis, MO) or 3) no brood (NB). Since eβ and NB treatments don't require live brood, workers were exposed to an empty comb. YL and NB received a 1 ml paraffin oil control treatment, while the treatment eβ received 10,000 larval equivalents of ocimene in 1ml of paraffin oil (Sigma-Aldrich). Larval pheromones are described in terms of larval equivalents (Leq), which indicate the known amount of pheromone emitted by one developing larva over 24 h (volatile pheromones) or rinsed off the cuticle (non-volatile) of one larva (Le Conte et al. 1990; Maisonnasse et al. 2009; Maisonnasse et al. 2010). Pheromone treatments were presented in a mesh screened glass petri dish below the brood nest area 2 h prior to measuring foraging activity. Bees could not contact the pheromone directly (Maisonnasse et al. 2010).

Foraging activity. Four hives per treatment group were monitored for foraging activity in 5-min intervals. Foraging activity changes throughout the day as temperatures increase and impact pollen and nectar availability. To compensate for fluctuations in resource availability, foraging activity was measured in 5 rounds. A round consisted of

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one 5 min foraging interval for each of the 12 experimental hives; subsequent rounds were spaced by approximately 30 min. Foraging activity was determined as described in Experiment 1. One hive was discarded from the analysis, because it had fewer than 60 total foragers during the entire experiment, while all other colonies had between 177 and 680 foragers during the same time frame.

Statistics

To account for factors of replicate/round and treatment, two-way ANOVA was used to analyze total foraging activity, pollen foraging activity and individual foraging loads; LSD Student's t-test post-hoc analyses were conducted on significant results (Sokal and Rohlf 1995) using JMP Pro v. 10 (SAS, Cary, NC). Because foraging load data is categorical, distributions of foraging load collected were analyzed with 3-way and 2-way contingency tables using custom Chi-Square Contingency Table program available at http://vassarstats.net/newcs.html. Replicates were conducted on different days over a 10 d interval and thus encompass variance across days and individual nucleus colony differences. Significant differences in foraging activity across replicates may be due to differences in forage availability.

RESULTS

Foraging Activity

Experiment 1: Releaser effects of young larvae, old larvae, and capped brood.
We monitored foraging activity in 5 min intervals, counting every bee that returned to the

hive from a foraging trip. Total foraging activity differed by replicate ($F_{3,67} = 18.72$, $P <$ 0.001), but not by treatment (Fig. 2.1a; $F_{3,67} = 2.61$, $P = 0.062$). If a returning forager carried a visible pollen load, it was classified as a pollen forager. Pollen foraging activity differed significantly by replicate ($F_{3,52} = 17.16$, $P < 0.001$) and by brood treatment (Fig. 1.2a; $F_{3,52} = 9.35$, $P < 0.001$). YL had twice as many pollen foragers than CB ($t_{52} = 4.84$, $P < 0.001$) or NB ($t_{52} = 4.27$, $P < 0.001$) and 1.5-fold more than OL ($t_{52} = 2.83$, $P = 0.007$).

Experiment 2: Releaser effects of young larvae and e-beta ocimene. Total foraging activity, measured as in Experiment 1, differed by round ($F_{4,40} = 9.65$, $P < 0.001$) and treatment (Fig. 2.1b; $F_{2,40} = 17.16$, $P = 0.023$). Differences across rounds were expected, as resource availability and hive needs fluctuate throughout the day. Hives treated with eβ exhibited 1.5-fold more total foraging than either YL ($t_{40} = 2.37$, $P = 0.023$) or NB ($t_{40} = 2.69$, $P = 0.010$), which did not differ from each other ($t_{52} = 0.35$, $P = 0.727$). Pollen foraging activity differed significantly by treatment (Fig. 2.2b; $F_{2,40} = 3.79$, $P = 0.031$), but not by round ($F_{4,40} = 2.14$, $P = 0.094$). Foragers exposed to eβ and YL had twice as many pollen foragers compared to NB exposure ($t_{40} = 2.54$, $P = 0.015$; YL: $t_{40} = 2.11$, $P = 0.041$), comparable to the results of the first experiment. The two brood treatments, YL and eβ, did not differ from each other ($t_{40} = 0.59$, $P = 0.562$).

Foraging Loads

Experiment 1: Releaser effects of young larvae, old larvae, and capped brood. Entrance counts do not provide details on non-pollen foraging loads of nectar, water or a

forager returning empty. We collected foragers and the contents of their crop were expressed to determine the effects of different aged brood on foraging load. Weather conditions were warm when Experiment 1 was conducted during a 10 d interval in mid-October 2012 in Mesa, AZ, with highs around 35°C and lows around 15°C. The frequency distribution of foraging load sucrose concentrations was strongly bimodal. Peaks occurred at 0% and around 50%. The peak at 0% was likely a consequence of water foraging activity to cool the hive.

Each bee was classified as one of four types of foragers, based on their foraging load (pollen, nectar, water, or empty). To determine interaction effects of foraging load with treatment and replicate, a three-way contingency table analysis (Sokal and Rohlf 1995) was conducted (Table 2.1). It showed that there was no significant interaction of treatment and replicate ($G_9 = 0.02$, $P = 1.00$). There was a significant interaction of treatment and type of foraging load collected ($G_9 = 43.40$, $P < 0.001$). There was also a highly significant interaction of treatment, foraging load and replicate ($G_{54} = 158.36$, $P < 0.001$). Because there was no significant interaction of treatment and replicate, the count data was pooled across replicates and a 4x4 contingency table analysis conducted (Table 2.2), which showed a highly significant effect of treatment on the type of load collected ($X_9 = 44.20$, $P < 0.001$). The individual foraging loads of nectar and pollen were also quantified (nectar load volume and sucrose concentration; pollen load: mass). Individual foraging loads classified as the same type did not differ between treatments for nectar volume ($F_{3,148} = 0.63$, $P = 0.600$), nectar concentration ($F_{3,148} = 1.80$, $P = 0.150$), or pollen mass ($F_{3,226} = 1.42$, $P = 0.240$). However, the number of foragers characterized as

empty returning to the hive varied significantly by treatment (Table 2.3; $X_3 = 20.55$, $P < 0.001$). NB had the greatest number of empty foragers, YL the fewest, and CB and OL were intermediate. The number of pollen foragers also varied significantly (Table 2.3; $X_3 = 35.10$, $P < 0.001$); NB and CB had the fewest pollen foragers, YL the most, and OL was intermediate.

Comparing pollen loads across all foragers provides a more accurate representation of total pollen intake per colony, as nectar, water and empty foragers returned with zero pollen loads. There was a significant effect of replicate on pollen load (Fig. 2.3; $F_{3,784} = 11.31$, $P < 0.001$). Replicates were conducted on 4 different days over a 10 d interval. Depending on numerous environmental influences, pollen availability can fluctuate widely, which in turn influences pollen load size. In three of four replicates, YL collected significantly more pollen than NB ($P < 0.001$); in two of four replicates, YL collected significantly more pollen than OL or CB ($P < 0.01$). There was a highly significant effect of brood treatment on the pollen load when measured across all foragers (Fig. 2.3b; $F_{3,784} = 11.72$, $P < 0.001$). Workers in hives treated with YL collected significantly more pollen than those exposed to the other treatments (NB: $t_{784} = 5.42$, $P < 0.001$; CB: $t_{784} = 4.72$, $P < 0.001$; OL: $t_{784} = 2.85$, $P = 0.005$). Workers exposed to OL collected significantly more pollen than NB ($t_{784} = 2.57$, $P = 0.010$). A significant interaction occurred between treatment and replicate ($F_{3,784} = 3.50$, $P < 0.001$). In contrast to the differences seen with pollen load, there were no significant treatment effects for nectar volume or nectar concentration when calculated across all foragers, as seen in previous experiments (Dreller et al. 1999).

DISCUSSION

Pheromones regulate complex interactions in insect societies and enable the colony to adapt to changing environments. Our experimental results demonstrate that honey bee colonies actively regulate their foraging allocation efforts for pollen and nectar in response to the signals produced by different aged larvae and pupae. The data we present provide additional evidence that total foraging (Fig. 2.1), pollen foraging (Fig. 2.2) and foraging load (Table 2.2, Fig. 2.3) are regulated by integrating in-hive stimuli of the brood nest with the availability of foragers to collect resources of nectar, pollen and water.

Young larvae are the principal sink for pollen-derived protein resources within the colony (Sagili and Pankiw 2007); accordingly we found that they stimulated foragers to collect more pollen (Fig. 2.2). Current experimental results demonstrated that eβ ocimene, the young larval pheromone, is also sufficient in itself to induce increased pollen foraging (Fig. 2.2b). This pheromone may also serve as a signal to stimulate provisioning behavior by nurse bees, although this remains to be tested. The resultant increase in demand by nurses for pollen may further enhance the response of foragers. In contrast, nurses feed old larvae a diet with reduced protein content and a higher proportion of carbohydrate rich nectar (Jung-Hoffmann 1966; Haydak 1970; Huang and Otis 1991). This restrictive feeding decreases the larval growth rate and results in the development of the worker phenotype. Correspondingly, we found that old larvae stimulated intermediate levels of pollen foraging and slightly increased nectar foraging in comparison to young larvae (Fig. 2.2, Table 2.2). This change in proportion may result

directly from the reduced release of eβ in the older larvae or may be due to the increase in BEP, stimulating foragers to respond to the new nutritional demands of older larvae.

Worker larvae increase their weight up to 1,500-fold during the six days of larval development (Snodgrass 1925), due in large part to protein synthesis in the fat body. The larval fat body undergoes two phases, beginning with a high rate of protein synthesis in early development (Chan and Foster 2008). Through the first 48 h of larval development, when larvae emit only eβ, worker- and queen-destined larvae grow at similar rates, with worker larvae slightly outpacing queen larvae in weight gain (Wang 1965) (cf. (Stabe 1930; Rembold and Kremer 1980). By 72 h, just as eβ production decreases and larvae start to emit BEP, worker-destined larvae substantially outweigh queen larvae, weighing 1.5 to 1.75-fold as much (Stabe 1930; Wang 1965). However, their weight gain slows to approximately ½ to ¼ the growth rate that occurs at 48 h (Himmer 1927; Wang 1965). Larvae remain bipotent through the 3rd larval instar under normal conditions, although gene and protein expression of worker- and queen-bound larvae begin to diverge almost immediately after hatching (Cameron et al. 2013).

The growth rate of worker larvae slows after the 3rd larval instar and the developmental pathways of hive reared worker and queen destined larvae diverge, with queen larvae continuing to gain weight at a faster rate and achieving a substantially larger body size by the time their cells are capped (Wang 1965). The third instar thus marks a shift from a stage of rapid and generalized growth to a stage during which development becomes canalized into a specific adult phenotype. A concomitant shift in pheromones

from volatile eβ to non-volatile BEP as nurses start reducing larval food suggests that eβ may be a "feed me protein" signal.

During the late 4th instar of larval worker development, the larval fat body undergoes the second phase and proteins are exported and stored in the hemolymph (Chan and Foster 2008), potentially a response to the change in food abundance. As the fat body starts to export its products, there is a coinciding decrease in the rate at which body mass is gained (Wang 1965), and an exponential rise in the emission of BEP (Trouiller 1993). There is a subsequent and considerable expansion of neuroblast cells during the 4th and 5th instar (Farris et al. 1999), and apoptosis of the larval ovary germ cells during the 5th instar of worker development (Hartfelder and Steinbrück 1997; Capella and Hartfelder 1998). However, through the late 4th instar the worker-destined larvae can be shunted into the queen phenotype with application of exogenous juvenile hormone (Asencot and Lensky 1976; Dietz et al. 1979; Asencot and Lensky 1984).

The changing pheromone signal of larvae as they mature may have evolved concurrently with the regulated feeding regime imposed by nurse bees on worker larvae. A highly proteinaceous diet has a benefit for larvae during a limited developmental window that rapidly closes after the 3rd instar (Haydak 1943; Haydak 1970; Woyke 1971; Rangel et al. 2013). After this time point rerouting the phenotypic trajectory from worker to queen is correlated with reduced ovariole number, queen weight and semen storage capacity, all signs of reduced queen fecundity (Woyke 1971; Rangel et al. 2013). This larvae-nurse bee signaling thus enables workers to rear bees of the worker phenotype without queen characteristics through restrictive feeding during late larval development

(Linksvayer et al. 2011; Page 2013). Even when artificially fed a proteinaceous larval diet *in vitro* that would turn younger larvae into queens, 5th instar larvae that were previously worker-destined develop into workers or intercastes that lack the full suite of anatomical characteristics that define a natural queen (Kaftanoglu, unpublished data).

Young larvae aren't the only emitters of eβ; well-mated queens that are exclusively fed a diet of proteinaceous royal jelly also emit eβ (Gilley et al. 2006). Virgin queens and queens that are rejected after introduction into a hive (superseded), perhaps due to insufficient mating, lack this pheromone signal (DeGrandi-Hoffman et al. 2007; Huang et al. 2009). Fecund queens and young larvae that maintain the ability to develop into reproductives thus share the same pheromone signature, suggesting, perhaps, that one of the two co-opted the "feed me protein" signal from the other and gained access to more food.

The shifting pheromone profiles of larvae may also provide a colony level cue about seasonality, in addition to informing workers about the age and reproductive potential of developing larvae. Brood nests in colonies typically consist of a mixture of eggs, young and old larvae, and capped brood so that workers are exposed to a complex chemical bouquet of pheromones. Although a mixture of different aged brood exists in the hive environment, the age distribution changes with the season (Bodenheimer 1937; McLellan 1978; Winston 1987) and can thus inform and co-ordinate the division of labor to ensure foragers return with the nutritional resources required for sustaining healthy and environmentally appropriate hive development. Young larvae dominate in the late winter and early spring; this is a climatically unpredictable time in temperate climates when it is

crucial that the hive collects protein-rich pollen (Farrar 1934; Seeley and Visscher 1985; Dustmann and von der Ohe 1988; Mattila and Otis 2006). Old larvae and capped brood dominate the hive environment in the late spring and summer (Bodenheimer 1937; Seeley and Visscher 1985), when the colony has its most substantial weight gain in honey (Seeley and Visscher 1985) amassing a surplus of food stores in preparation for winter to minimize winter mortality (Seeley 1978). The queen's egg-laying rate decreases and the brood nest where larvae were previously raised is filled with nectar during the summer; with reduced larvae there is a diminishing drive for pollen collection. This seasonal cycle in broodnest composition may enable the colony to integrate the changing pheromone signal into successful foraging decisions and exploit environmental resources in a seasonally appropriate manner, potentially an emergent property of normal colony development.

The chemical communication system of the complex social environment in a honey bee colony is in need of further decoding, but current results demonstrate that young and old larvae release very different chemical signals that strongly influence the feeding and foraging behavior of the colony. The immediate impact is a shift in the number of successful foragers and appropriate adjustments in foraging loads that provide for developing young. The "road to insect sociality was paved with pheromones," (Blum 1974) and honey bee colonies offer a plethora to study, many of which have both releaser and priming properties. We have demonstrated the releaser effects of young and old larvae and the young larval pheromone eβ on the division of foraging labor of adult workers. Current models predict additional priming effects, proposing that young larvae

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and their pheromones accelerate behavioral maturation of workers so that they transition to outside foraging precociously, while old larvae prolong nursing and thus delay maturation (Maisonnasse et al. 2010). Additional studies are still needed to investigate the priming influence of young and old larvae on the physiology of the caregiving nurses and their ensuing developmental maturation from in hive tasks to outside foraging.
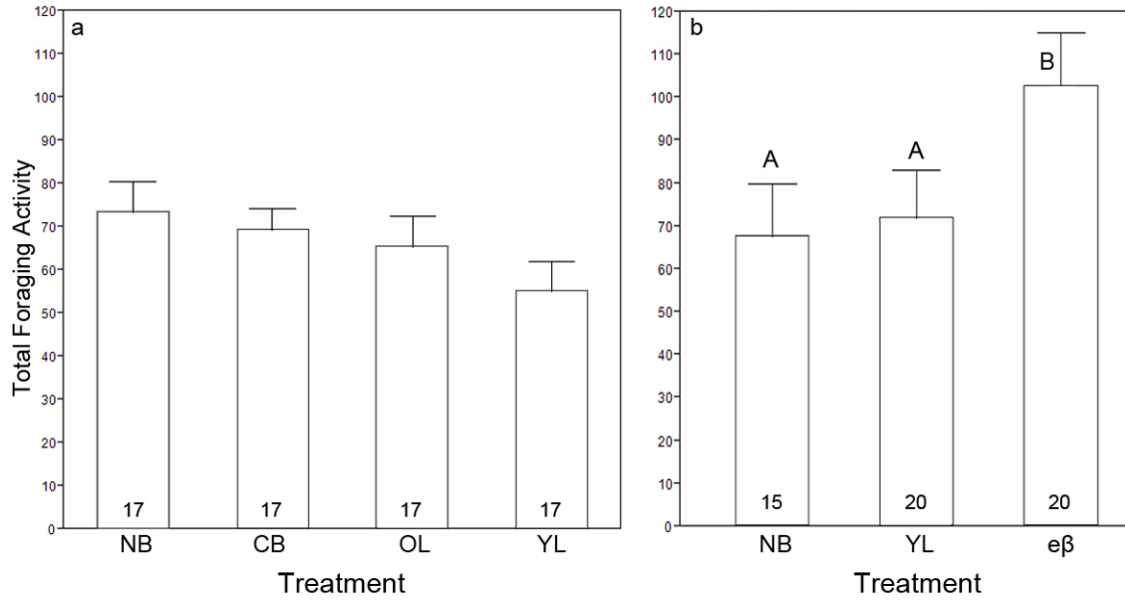
Figure 2.1. Total foraging activity.

Mean total foraging activity (returning pollen & non-pollen foragers) + S.E., calculated per 5 min interval and measured 4-5 times per colony, did not differ by treatment after exposure to brood of different ages (1a), but differed significantly when comparing foragers exposed to young larvae versus the predominant pheromone of young larvae, e-beta ocimene (eβ) (1b). Significant differences ($\alpha < 0.05$) are indicated by different letters. Treatments: no brood (NB); capped brood (CB); old larvae (OL); young larvae (YL); e-beta ocimene (eβ). Sample sizes are indicated.
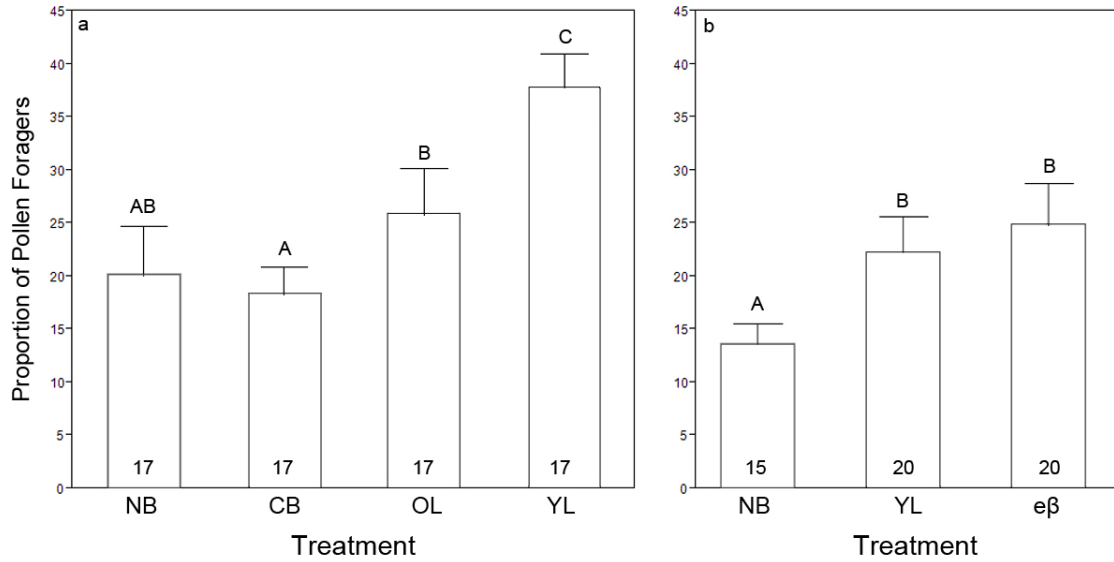
Figure 2.2. Pollen foraging activity.

Mean pollen foraging activity + S.E., calculated as the proportion of pollen foragers to the total number of returning foragers per 5 minute interval and measured 4-5 times per colony, varied significantly by brood treatment. Significant differences ($\alpha < 0.05$) are indicated by different letters. Treatments: no brood (NB); capped brood (CB); old larvae (OL); young larvae (YL); e-beta ocimene (eβ). Sample sizes are indicated.
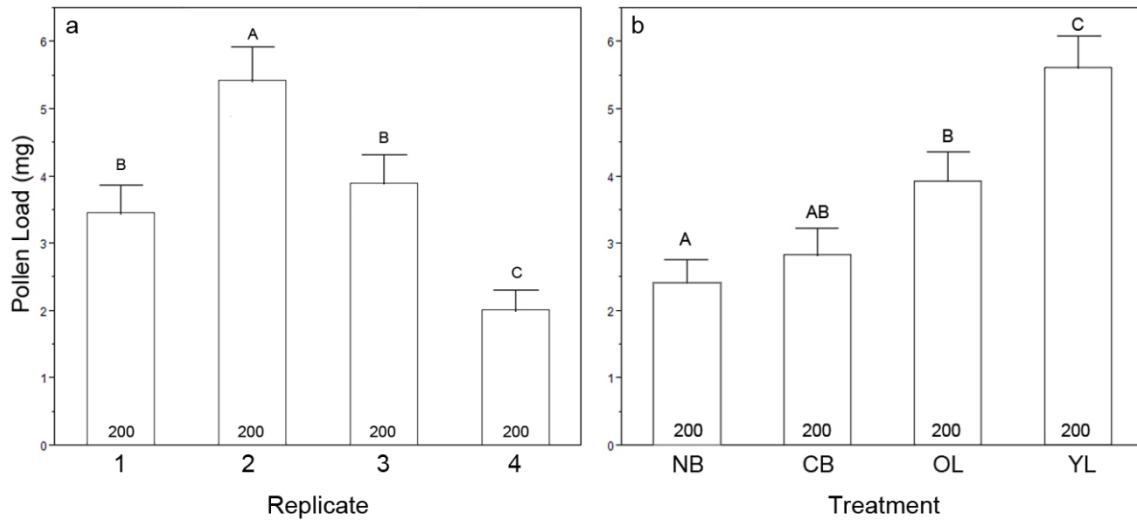
Figure 2.3. Pollen loads by replicate and treatment.

Mean pollen load + S.E. Replicate (3a) had a significant effect on pollen load. Replicates were conducted on 4 different days over a 10 d interval and pollen availability can fluctuate widely. Brood treatment had a significant effect on mean pollen load + S.E. Significant differences ($\alpha < 0.05$) are indicated by different letters. Sample sizes are indicated. Treatments: no brood (NB); capped brood (CB); old larvae (OL); young larvae (YL); e-beta ocimene (eβ). Sample sizes are indicated.

Table 2.1. Three-way contingency table comparing foraging loads.

Foraging loads from 50 foragers were measured for each treatment and replicate. Bees were classified as "empty" if they carried no foraging load; "pollen" if a visible pollen pellet was found on their hind leg; "nectar" if they had no pollen load and their crop contained at least 3 µl with > 5% sucrose solution; and "water" if their crop contained a solution with < 5% sucrose. The last three rows of the analysis results represent the 2-way interactions for each pair of variables when controlling for the effects of the third variable (parantheses).

Table 2.1

THREE-WAY CONTINGENCY TABLE COMPARING FORAGING LOADS

	Empty	Nectar	Pollen	Water
	Replicate 1			
	Empty	Nectar	Pollen	Water
NB	28	9	3	10
CB	25	4	10	11
OL	15	8	22	5
YL	13	6	20	11
	Replicate 2			
NB	25	8	16	1
CB	15	7	20	8
OL	20	8	16	6
YL	13	6	28	2
	Replicate 3			
NB	18	8	21	3
CB	19	18	8	5
OL	18	7	18	7
YL	15	11	20	4
	Replicate 4			
NB	29	17	4	0
CB	23	18	6	3
OL	19	20	7	4
YL	16	9	23	2
	Empty	Nectar	Pollen	Water

Table 2.1, *continued*

Results:

Source	G^2	df	p
Treatment x Load x Replicate	158.36	54	<0.001
Treatment x Load	43.36	9	<0.001
Treatment x Replicate	0.02	9	1
Load x Replicate	56.96	9	<0.001
Treatment x Load (Replicate)	101.38	36	<0.001
Treatment x Replicate (Load)	58.04	36	0.011
Load x Treatment (Replicate)	114.98	36	<0.001

Table 2.2. 4x4 contingency table comparing foraging loads.

Foraging loads were pooled across replicates so that foraging loads from 200 individuals
were measured for each treatment. Foraging loads were classified as in Table 1. Values
given in parentheses are percentage deviations from expected if foraging load is
independent of treatment.

Table 2.2				
4X4 CONTINGENCY TABLE COMPARING FORAGING LOADS				
Treatment	**Empty**	**Nectar**	**Pollen**	**Water**
No Brood	100 (+28.6)	42 (+2.4)	44 (-27.3)	14 (-32.5)
Capped Brood	82 (+5.5)	47 (+14.6)	44 (-27.3)	27 (+30.1)
Old Larvae	72 (-7.4)	43 (+4.9)	63 (+4.1)	22 (+6.0)
Young Larvae	57 (-26.7)	32 (-22.0)	91 (+50.4)	20 (-3.6)

$$X_9 = 44.20, P < 0.001$$

Table 2.3. 4x2 contingency tables comparing foraging loads.

Foraging loads were pooled across replicates so that foraging loads from 200 individuals were measured for each treatment. Foraging loads were classified as in Table 1and compared for each type of load. Values given in parentheses are percentage deviations from expected.

Table 2.3					
4X2 CONTINGENCY TABLES COMPARING FORAGING LOADS					
EMPTY			**POLLEN**		
Treatment	**Empty**	**Not Empty**	**Treatment**	**Pollen**	**Not Pollen**
No Brood	100 (+28.6)	100 (-18.2)	No Brood	44 (-27.3)	156 (+11.8)
Capped Brood	82 (+5.5)	118 (-3.5)	Capped Brood	44 (-27.3)	156 (+11.8)
Old Larvae	72 (-7.4)	128 (+4.7)	Old Larvae	63 (+4.1)	137 (-1.8)
Young Larvae	57 (-26.7)	143 (+17.0)	Young Larvae	91 (+50.4)	109 (-21.9)
$X_3 = 20.55, P < 0.001$			$X_3 = 35.10, P < 0.001$		
NECTAR			**WATER**		
Treatment	**Nectar**	**Not Nectar**	**Treatment**	**Water**	**Not Water**
No Brood	42 (+2.4)	159 (-0.6)	No Brood	14 (-31.7)	159 (+3.6)
Capped Brood	47 (+14.6)	153 (-3.8)	Capped Brood	27 (+31.7)	153 (-3.6)
Old Larvae	43 (+4.9)	157 (-1.3)	Old Larvae	21 (+2.4)	157 (-0.3)
Young Larvae	32 (-22.0)	168 (+5.7)	Young Larvae	20 (-2.4)	168 (+0.3)
$X_3 = 3.72, P = 0.291$			$X_3 = 4.62, P = 0.202$		

CHAPTER 3

QUEEN AND YOUNG LARVAL PHEROMONES IMPACT NURSING AND

REPRODUCTIVE PHYSIOLOGY OF WORKERS

Abstract: Several insect pheromones are multifunctional and have both releaser and primer effects. In honey bees (*Apis mellifera*) the queen mandibular pheromone (QMP) and e-beta-ocimene (eβ), emitted by young larval workers, have such dual effects. There is increasing evidence that these multifunctional pheromones profoundly shape honey bee colony dynamics by influencing cooperative brood care, a fundamental aspect of eusocial insect behavior. Both QMP and eβ have been shown to regulate worker physiology and behavior, but it has not yet been determined if these two key pheromones have additive or synergistic effects on nurse bee physiology by promoting hypopharyngeal gland (HPG) development or suppressing ovary activation. Experimental results demonstrate that both QMP and eβ significantly suppress ovary activation compared to controls, but that the larval pheromone is more effective than QMP. The underlying reproductive physiology (total ovarioles) of workers influenced the effect of eβ on HPG development and ovary activation, so that worker bees with more ovarioles were more likely to develop their HPG and have activated ovaries.

Pheromones are chemicals released by individuals into the environment that trigger a behavioral response in members of the same species (Karlson and Butenandt 1959) and play central regulatory roles in many animal societies (Wilson 1971). Within social insects the chemical communication system has proven to be highly diversified and richly complex, enhanced by synergistic interactions and context-dependent messaging (Slessor et al. 2005). For example, at least 50 substances derived from queens, workers and immatures are expressed within the colonies of honey bees (*Apis mellifera*) (Pankiw 2004b). Despite being chemically identified, many have unknown effects on behavior, and their specific modes of action are even less well understood. A number of pheromones act as releasers, causing rapid but short-lived responses, such as the attraction/orienting behavior in response to the seven-component emission from the dorsal Nasanov gland (Pickett et al. 1980; Free 1987). Because releasers permit simple behavioral assays, their function can be readily assessed. However, other pheromones act as primers, which slowly influence behavior through long term physiological effects, thereby influencing broad aspects of colony organization, caste structure and the division of labor. (Wilson and Bossert 1963; Winston and Slessor 1998; Le Conte and Hefetz 2008). Because of the relative difficulty of identifying the function of putative primer pheromones, they have received less attention than releasers. Several pheromones are multifunctional and have both releaser and primer effects, such as queen mandibular pheromone (QMP) and brood ester pheromones (BEP) produced by larvae. There is

increasing evidence that these primer and multifunctional pheromones may have profound effects in shaping honey bee colony dynamics.

One of the primary effects elicited by honey bee pheromones is the organization of care received by immature bees. Larvae, which are confined to a cell and cannot fend for themselves, must signal their needs to adult caregivers. By emitting pheromones, the larvae are able to influence the behavior and physiology of their caregivers, stimulating them to provide appropriate nutritional resources (Arnold et al. 1994; Mohammedi et al. 1996; Mohammedi et al. 1998; Pankiw et al. 1998; Le Conte et al. 2001; Pankiw 2004a; Sagili and Pankiw 2009; Maisonnasse et al. 2010). However, the nutritional needs of the larvae change over time as they develop through five larval instars. It appears that to signal these changing needs, they change the composition and quantity of larval pheromones emitted (Le Conte et al. 1990; Trouiller 1993; Maisonnasse et al. 2010). The volatile pheromone e-beta-ocimene (eβ) is produced predominantly by young larvae in the first through third instar (Maisonnasse et al. 2009; Maisonnasse et al. 2010), while the non-volatile brood ester pheromone (BEP), a mixture of ten fatty acid ethyl and methyl esters, is emitted by larvae from the third through fifth larval instar (Le Conte et al. 1990; Trouiller et al. 1991; Trouiller 1993). Nurse bees tightly regulate larval growth by adjusting the larval feeding regime according to larval age (Linksvayer et al. 2011; Wang et al. 2014), indicating that nurse bees use larval pheromones to regulate larval diet (Le Conte et al. 1994; Le Conte et al. 1995).

Primer pheromones, which can strongly impact cooperative brood care, are emitted by both queens and brood. This kind of redundancy in control mechanisms

appears to be a common feature of pheromone-based signaling systems in eusocial insects (Hoover et al. 2003). Queens produce queen mandibular pheromone (QMP), a blend of five different components. In addition to releasing a worker retinue response, these pheromones also impact worker behavior through induced changes to their endocrine and reproductive physiology (De Groot and Voogd 1954; Jay 1970; Jay 1972; Jay and Jay 1976; Kaatz et al. 1992; Hoover et al. 2003). Both QMP and BEP of older larvae suppress ovary activation and stimulate hypopharyngeal gland (HPG) development of facultatively sterile workers, priming them to forego reproduction and activate both HPG and mandibular glands for brood care (Mohammedi et al. 1996; Mohammedi et al. 1998; Hoover et al. 2003; Peters et al. 2010). The paired HPGs of nurse-aged bees produce the protein-rich food fed to developing larvae (Snodgrass 1925). Normally to activate their HPG, bees must consume protein and have contact with larvae for 3 days (Huang et al. 1989; Huang and Otis 1989). Young adult bees receive proportionally more brood food from nurse aged bees than older bees (Crailsheim 1991; Crailsheim 1992). This protein-rich diet can trigger young workers to activate their HPGs, while poor worker nutrition negatively impacts HPG development (Peters et al. 2010). A restricted diet also suppresses ovary activation, as bees do not have the protein resources to develop oocytes (Lin and Winston 1998; Hoover et al. 2006). Recent research has shown simultaneous exposure to QMP and BEP, even in the absence of a protein resource, can increase protein production in HPGs (Peters et al. 2010), suggesting that under the queenright like conditions of a hive environment—(i.e. presence of queen and brood pheromones)—workers can catabolize bodily proteins for larval food production.

The effects of BEP on honey bee physiology have been well-investigated, but less is known about the priming effects of the volatile young larval pheromone eβ and its synergistic impacts with QMP have not yet been probed. Pheromones are often context specific and may require the natural conditions of the hive to trigger physiological responses, however, studying the effects of pheromones on the physiology of workers in the context of the hive creates unique obstacles due to trophallactic transmission of pheromone signals among nestmates (Korst and Velthuis 1982; Leoncini et al. 2004), the impact of feeding larvae on worker physiology (Amdam et al. 2009), and the impact of the external environment on developmental maturation and resource foraging (Dreller et al. 1999). We thus resolved to study the effects of eβ on the physiology of larval caregivers in the laboratory, while mimicking the conditions of a natural hive in a controlled cage setting.

In order to test the synergistic effects of eβ and QMP in a tightly controlled environment, we first needed to determine if royal jelly could substitute for access to nurse bees and if synthetic QMP results in the same suppression of ovary activation as a live queen. To determine if newly emerged bees need to be fed by nurses to activate HPG development we compared access to nurse bees through a single mesh screen to protein-rich royal jelly incorporated directly into the diet (Experiment 1). Having established that access to royal jelly was sufficient to activate HPG development, we compared synthetic QMP to the effects of live queens. Due to earlier controversy on the ability of QMP to suppress ovary activation (Willis et al. 1990; Winston and Slessor 1998), we compared the effects of live mated queens, virgin queens, and synthetic queen mandibular

pheromone (QMP) on ovary activation (Experiment 2). Virgin queens do not emit the full suite of pheromones of a mated queen (Richard et al. 2007). Since brood pheromones have often produced dose-dependent results (Mohammedi et al. 1998; Sagili et al. 2011), we investigated the effects of high versus low eβ dose on HPG development, protein (pollen) and carbohydrate consumption and ovary activation. Having established that QMP significantly reduced ovary activation, we tested the effects of eβ and QMP in combination, to see if the queen and young larval brood pheromones had additive or synergistic effects on HPG development and ovary activation.

MATERIALS & METHODS

Bees

For each experiment, combs of capped honey bee mature pupae were removed from 5-7 wildtype colonies and placed in an incubator at 34°C in cages. The following morning newly emerged bees less than 18 hours old were collected. To minimize genetic variance across treatments within a single replicate, bees were randomly selected from only 2-3 colonies. Each subsequent replicate (6-7 per experiment) used a different random selection of bees from 2-3 of the 5-7 wildtype colonies removed so that replicates were each composed of different genotype combinations. Thus replicate encompasses genetic variance between colonies. 100 newly emerged bees were paint marked on the thorax according to treatment and placed in an acrylic cage similar in design to the Pain cage (Pain 1966) with the addition of a divider that split the cage in half. The cages ensured that the pheromones and diet were distributed among all members via

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trophallaxis and removed additional pheromone exposure from other colony sources. The cages were maintained at 30°C±3°C and 35%±4% humidity in individual, disposable incubators assembled from wax coated cardboard with individual radiant heat sources stored in a vented fume hood for each treatment group. The bees were fed *ad libitum* with water, queen candy and pollen paste, replaced every 1-2 days as necessary. Queen candy was made from 80% powdered sugar and 20% honey. Pollen paste was made from frozen pollen pellets ground and mixed with distilled water until it had the consistency of dough.

Data Collection

Bee mortality was recorded daily. Candy and pollen consumption were recorded every 1-2 days, when food was replaced in all cages by subtracting remaining food from initial weight. After 10 days, the cages of bees were frozen and for each cage 6-10 bees were randomly selected, dissected and evaluated for hypopharyngeal gland (HPG) development, total number of ovarioles comprising each ovary and ovary activation.

Dissections

Both HPGs were dissected from the head capsule and placed into a drop of saline (0.25 mol/l NaCl) on a microscope slide. A representative section was examined at 100x. The activity of HPGs are positively correlated with size (Knecht and Kaatz 1990; Deseyn and Billen 2005). Numerous globular acini attach to the long, slender main channel of the HPG, and these acini increase in diameter until 6 days of age, when they begin to shrink. The gland continues to diminish, so that by 15 days of age, when bees typically transition

to foraging, their size corresponds to the still undeveloped gland of newly emerged bees. HPG development was thus rated using an established scale (Hess 1942), which uses the shape and density of the acini as the main criterion for classification and ranks them from atrophied (1) to fully developed (4). Glands were additionally assigned to one of three classes according to lobe morphology (Wegener et al. 2009). Class one, typical of young broodless workers, consists of glands with small acini showing an uneven surface. Class two, representative of active nurse bees, are comprised of medium-sized to large acini with a smooth surface and numerous secretory vesicles, giving them a yellowish color. Class three glands, representative of older foragers, consist of large, but slightly pale and translucent lobes. Class three was not found among our samples.

Both ovaries were removed from the bees and placed in a drop of saline. The number of ovary filaments (ovarioles) was counted using a 100x dissecting microscope (Zeiss, Jena, Germany). The stage of ovary activation was classified using an established scale (Pernal and Currie 2000): 0 - no follicle development, 1 - slight enlargement, 2 - presence of distinct cells leading to swellings and constrictions, 3 - egg volume exceeding that of the nutritive follicle, 4 - presence of fully formed eggs. For both HPG development and ovary activation, the mean score of the pair of organs was used for statistical analyses, as occasionally there were disparities within a bee.

Treatments

Experiment 1: Royal jelly compared to nurse bee environment. To determine if newly emerged bees must be exposed to nurse bees to activate their HPG, we compared

the effects of contact with nurse bees compared to direct access to just royal jelly. Each cage was subjected to one of three treatments: 1) royal jelly (RJ); 2) nurse bees (N) or 3) control (C) without royal jelly, nurse bees or QMP. For the first treatment, royal jelly (RJ) constituted 10% of the queen candy. For the second treatment, newly emerged bees had access to 100 bees that were collected from a comb of open larvae in wildtype colonies, where they were actively engaged in nursing behavior. Access was through a single mesh screen through which the nurses could feed younger workers, who receive substantial amounts of brood food from them (Crailsheim 1991; Crailsheim 1992). Bees in the first two treatments received 1 slow release strip of synthetic queen mandibular pheromone (QMP) (PseudoQueen, Contech Industries, Victoria, British Columbia) attached near the top of the cage using a plastic zip tie to simulate queenright conditions of a hive to enhance social order among the nurse bees and newly emerged bees. The control group did not receive QMP, as QMP inhibits ovary activation. We wanted the control to represent a queenless colony with higher levels of ovary activation for comparison, as a diet incorporating royal jelly can stimulate ovarian activation (Lin and Winston 1998).

Experiment 2: Queen comparison. To determine if synthetic queen mandibular pheromone (QMP) was as effective as a live queen in suppressing ovary activation, we compared cages subjected to one of five treatments: 1) mated queen; 2) virgin queen; 3) virgin queen subjected to two successive CO_2 treatments, which results in oviposition within a few days despite the lack of mating flight (Mackensen 1947); 4) synthetic QMP as used in Experiment 1; or 5) control which received no queen or synthetic QMP. The

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live queens in the first three treatment groups were unconfined and free interact with the workers as in a natural colony. We compared three different live queens, because the pheromone profile of queens changes after mating and oviposition; less fecund queens are quickly replaced by a hive (Gilley et al. 2006; DeGrandi-Hoffman et al. 2007; Richard et al. 2007; Huang et al. 2009). Each cage received RJ candy as their carbohydrate source, prepared as in Experiment 1.

Experiment 3: High vs. low e-beta ocimene (eβ) dose. Live larvae suppress ovary activation in attending worker bees via larval pheromones (Mohammedi et al. 1998; Maisonnasse et al. 2009), though the effectiveness of pheromones is often dose dependent. To confirm that eβ can suppress ovary activation, we subjected each cage to one of three treatments: 1) low eβ dose of 1 larval equivalents (Leq)/bee; 2) high eβ dose of 10 Leq/bee; 3) carrier control. Due to the high volatility of eβ and in order to avoid pheromone saturation in the cages, the molecule was mixed with 1 ml paraffin oil and a similar droplet was used as the control (Maisonnasse et al. 2009). Treatments were supplied in a mesh screened glass petri dish below the cage, so bees could not contact the chemicals directly (Maisonnasse et al. 2010). Treatments were replaced daily. Each cage received RJ candy as their carbohydrate source, prepared as in Experiment 1.

Experiment 4: eβ & QMP synergy. Pheromones are often context specific and interact with other pheromone components. To determine if eβ and QMP have additive or synergistic effects, each cage was subjected to one of four treatments: 1) eβ- / QMP-, 2) eβ- / QMP+, 3) eβ+ / QMP-; and 4) eβ+ / QMP+. The eβ was supplied at 10 Leq/bee in 1 ml paraffin oil as in Experiment 3. The QMP was supplied in a slow release strip of

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synthetic QMP (PseudoQueen, Contech Industries), as in Experiments 1 & 2. Each cage received RJ candy as their carbohydrate source, prepared as in Experiment 1.

Statistics

Daily mortality was compared using two-way ANOVA with replicate and treatment as factors. Significant effects were compared using LSD Student t-tests. Daily candy and pollen consumption per cage were converted into cumulative consumption for each day over the entire 10 d period and compared across treatments using one-way ANOVA by age. Significant differences between multiple treatment groups were established using Tukey-Kramer HSD tests. Total ovarioles, mean ovary activation and HPG development were compared using two-way ANOVA with replicate and treatment as factors. Bivariate correlations for total ovarioles, mean ovary activation and HPG development were calculated using nonparametric Spearman's rank correlations. All calculations were performed using JMP Pro 10.0.0 (SAS, Cary, NC).

RESULTS

Experiment 1: Royal Jelly Compared to Nurse Bee Environment

Young bees are fed protein rich royal jelly from nurse bees (Crailsheim 1991; Crailsheim 1992), which may help enhance survivorship and promote development of both the ovaries and HPGs. We investigated the effects of access to nurse bees versus direct access to royal jelly. Although daily mortality remained below 1 bee per day for all cages, there were significant differences by treatment (Fig. 3.1a; $F_{2,144} = 4.13$, $P = 0.018$).

Bees in the control group without synthetic QMP had significantly higher mortality than bees with access to nurses (N) or royal jelly (RJ), both of which also contained synthetic QMP to simulate a natural hive environment. There was no effect of replicate ($F_{5,144} = 0.50$, $P = 0.779$), nor was there an interaction of treatment and replicate ($F_{10,144} = 1.07$, $P = 0.385$). Candy consumption and pollen consumption did not differ across treatments for any given age (Candy: $F_{2,15} \leq 2.07$, $P \geq 0.161$; Pollen: $F_{2,15} \leq 2.29$, $P \geq 0.136$).

Ovariole number becomes set during larval development, and as expected total ovarioles per bee were evenly distributed across treatment groups and replicates ($F_{2,162} = 2.01$, $P = 0.137$ and $F_{5,162} = 0.94$, $P = 0.458$ respectively). In contrast, ovary activation occurs during the adult worker stage, and here differed significantly by treatment (Fig 3.2a; $F_{2,162} = 23.64$, $P < 0.001$) and by replicate ($F_{5,162} = 4.04$, $P = 0.002$). Bees in the control treatment were not exposed to QMP and had significantly more active ovaries than the groups exposed to QMP. Bees in Replicate 1 had the least active ovaries and bees in Replicate 4 had the most active. The trend in ovary activation was consistent across replicates, but it was significant only in R4-6, leading to a significant interaction of treatment and replicate (Fig. 3.2b; $F_{10,162} = 1.99$, $P = 0.038$).

Nurse aged bees typically have well-developed HPGs, needed to produce the protein rich food they feed to larvae. HPG development differed significantly by treatment (Fig. 3.3a; $F_{2,162} = 6.97$, $P = 0.001$) and replicate ($F_{5,162} = 6.26$, $P < 0.001$). There was also an interaction effect of treatment and replicate (Fig. 3.3b; $F_{10,162} = 1.94$, $P = 0.044$). RJ significantly increased HPG development compared to N and the control ($t \geq 2.88$, $P < 0.005$). HPG development and ovary activation were significantly correlated for bees

reared in the control (Spearman $\rho = 0.40$, n $= 60$, $P = 0.002$), but not in the bees reared

with RJ ($\rho = 0.25$, n $= 60$, $P = 0.057$) or access to nurse bees ($\rho = 0.06$, n $= 60$, $P = 0.679$).

However, total ovarioles and ovary activation were correlated in the bees with access to

nurse bees ($\rho = 0.32$, n $= 60$, $P = 0.013$).

Experiment 2: Queen Comparison

We compared the effects of synthetic QMP and live queens on mortality and

ovarian status in caged worker bees. The mated queen in Replicate 2 died on day 6 of the

experiment and the cage was excluded from analysis. Daily mortality was significantly

affected by treatment (Fig 3.1b; $F_{4,200} = 6.14$, $P < 0.001$). Control and QMP cages had

significantly higher mortality than the treatments that received a live queen ($t \geq 2.51$, $P <$

0.013), though mean mortality never exceeded 1 bee/day for any of the treatment groups.

There was no effect of replicate ($F_{4, 200} = 0.63$, $P = 0.640$) or interaction between

treatment and replicate ($F_{16, 200} = 0.81$, $P = 0.671$). While total ovarioles per bee did not

differ by treatment ($F_{4, 164} = 0.21$, $P = 0.930$) or replicate ($F_{5, 164} = 1.25$, $P = 0.289$), ovary

activation differed significantly by both treatment (Fig. 3.4a; $F_{4, 164} = 12.46$, $P < 0.001$)

and replicate ($F_{5, 164} = 3.77$, $P < 0.003$). Bees in the control group had significantly

greater ovary activation compared to the four other queen treatments ($t \geq 3.48$, $P <$

0.001). QMP was not significantly different from the virgin queen treated with CO_2 ($t =$

1.37, $P = 0.172$), but the ovaries of bees reared with QMP were significantly more

developed than those of bees reared with a mated queen ($t = 2.05$, $P < 0.042$) and virgin

queen ($t = 2.32$, $P < 0.022$). No worker bees reared with a live queen had ovary activation

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above stage 1, which corresponds to oogenesis starting with a slight swelling at the top of the ovariole. In the QMP cages, 8% of bees had at least one of the paired ovaries at stage 2 of activation, which corresponds to slight egg development, while 25% of the control bees had at least slight egg development and 8% had full egg development in one of the paired ovaries. There was a significant interaction of treatment and replicate for ovary activation (Fig 3.4b; replicate 2 excluded; $F_{16, 125} = 3.03$, $P < 0.001$). Total ovarioles and ovary activation are significantly correlated in bees reared in the control (Spearman $\rho = 0.37$, n = 36, $P = 0.025$) or QMP ($\rho = 0.37$, n = 36, $P = 0.027$) environment, but not in any of the treatments with a live queen.

Experiment 3: High vs. Low E-Beta Ocimene (eβ) Dose

After determining that royal jelly incorporated into the diet can substitute for access to nurse bees, we tested if eβ can suppress ovary activation by comparing high and low doses of this volatile pheromone of young larvae. Daily mortality differed significantly by treatment (Fig 3.1c; $F_{2, 144} = 3.54$, $P = 0.032$) and replicate ($F_{5, 144} = 3.81$, $P < 0.003$), but mean mortality remained below 1 bee/day for all treatments. There were no significant differences in candy consumption for any given age ($F_{2,15} \leq 1.14$; $P \geq 0.347$). Pollen consumption differed across treatments at ages 5-8 and 10d (Fig. 3.5, $F_{2,15} \geq 4.03$, $P \leq 0.040$). Post-hoc Tukey's HSD tests showed that bees exposed to the low dose of eβ (1 Leq/bee) consumed significantly more pollen than bees from controls at 6d, 7d, and 8d of age and more than bees exposed to the high dose of eβ at 5d, 6d, 7d and 10d. All other comparisons were not significant.

Total ovariole number per bee did not differ by treatment ($F_{2, 162} = 1.31$, $P = 0.274$) or by replicate ($F_{5, 162} = 0.56$, $P = 0.729$). Treatment significantly impacted ovary activation (Fig. 3.6, $F_{2, 162} = 26.46$, $P < 0.001$), but replicate had no effect ($F_{5, 162} = 0.70$, $P = 0.628$) and there was no interaction ($F_{10, 162} = 1.44$, $P = 0.168$). Bees that received the high eβ dose of 10 Leq/bee had significantly fewer developing oocytes than bees in the control group or those receiving the low dose of 1 Leq/bee ($t \geq 5.19$, $P < 0.001$). In bees exposed to the high dose, only 10% had mean ovary activation at or above stage 1 (slight ovariole swelling) compared to 70% in the low dose and 58% in the control. There was no significant effect of treatment on HPG development, indicating that eβ did not increase HPG development compared to controls ($F_{2, 162} = 0.42$, $P = 0.658$); HPG development differed across replicates ($F_{5, 162} = 3.05$, $P = 0.012$). Both total ovarioles and HPG development were significantly correlated with ovary activation for bees reared in the low eβ (Total ovarioles: Spearman $\rho = 0.34$, n = 60, $P = 0.008$; HPG: $\rho = 0.38$, n = 60, $P = 0.003$) and high eβ environment (Total ovarioles: $\rho = 0.35$, n = 60, $P = 0.006$; HPG: $\rho = 0.31$, n = 60, $P = 0.017$), but not in the control group (Total ovarioles: $\rho = 0.15$, n = 60, $P = 0.243$; HPG: $\rho = 0.19$, n = 60, $P = 0.143$).

Experiment 4: Eβ & QMP Synergy

Having established that QMP can substitute effectively for a live queen and that the high dose of eβ significantly suppresses ovary activation, we tested the synergistic effects of eβ and QMP. Because the effects of pheromones are often context dependent, we examined if there were an additive or synergistic effects of eβ and QMP on worker

physiology. Mortality did not differ significantly by treatment (Fig. 3.1d; $F_{3, 168} = 1.96$, $P = 0.122$), but differed significantly by replicate ($F_{5, 168} = 7.16$, $P < 0.001$). For 5 of 6 replicates the mean daily mortality was below 0.8, while one replicate had a mean daily mortality twice that. There were no significant differences in candy consumption for any given age. Pollen consumption did not differ significantly between treatment groups for any given age, except at 9d ($F_{3,20} = 3.69$, $P = 0.030$), when the group exposed to eβ-/QMP- consumed significantly more pollen than the group eβ+/QMP+. All other comparisons were not significant.

Total ovariole number per bee did not differ by treatment ($F_{3, 216} = 0.30$, $P = 0.822$), but varied significantly by replicate because each replicate was composed of a different subset of genotypes ($F_{5, 216} = 2.53$, $P = 0.030$). There was no interaction effect ($F_{15, 216} = 1.13$, $P = 0.329$). Treatment significantly impacted ovary activation (Fig. 3.7a; $F_{3, 216} = 26.61$, $P < 0.001$), a prerequisite for egg-laying. Bees reared with eβ had significantly less developed ovaries than bees reared without eβ ($t_{216} \geq 3.91$, $P < 0.001$). The bees reared with QMP and no eβ had significantly less developed ovaries than control bees reared without either pheromone ($t_{216} = 3.24$, $P = 0.001$). However, bees reared with only QMP had significantly more developed ovaries than bees exposed to eβ ($t_{216} \geq 3.91$, $P < 0.001$), indicating that eβ is more effective at suppressing ovary activation than QMP. In the control group, 48% of bees had at least stage 1 ovary activation compared to 30% of the bees exposed only to QMP, 10% of the bees exposed to only eβ, and 8% of the bees exposed to both eβ and QMP. Ovary activation also differed by replicate ($F_{5, 216} = 17.02$, $P < 0.001$), seemingly a consequence of differences

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in total ovarioles as Replicates 2 and 3 had the most total ovarioles combined with the most activated ovaries. There was a significant interaction of treatment and replicate (Fig. 3.7b; $F_{15, 216} = 1.80$, $P = 0.036$).

In contrast, HPG stage development did not differ significantly by treatment ($F_{3, 216} = 1.32$, $P = 0.269$) or replicate ($F_{5, 216} = 1.37$, $P = 0.237$). HPG development and ovary activation were significantly correlated for bees reared in the eβ+/QMP- (Spearman $\rho = 0.35$, n = 60, $P = 0.006$), but not in the any of the other groups. Total ovarioles and ovary activation also correlated significantly in the eβ+/QMP- ($\rho = 0.34$, n = 60, $P = 0.009$) and the eβ-/QMP+ ($\rho = 0.43$, n = 60, $P < 0.001$) environments, but not in the other treatment groups.

DISCUSSION

Our results demonstrate how social insect pheromone communication is defined by complexity, synergy, context, and dose (Slessor et al. 2005; Alaux et al. 2010). Throughout our experiment QMP significantly suppressed ovary activation in worker bees (Fig. 3.4. & 3.7), as did the eβ pheromone of young larvae (Fig. 3.6 & 3.7). Our results also show that eβ had significant interactive effects on the reproductive physiology of worker bees, resulting in bees with more ovarioles having increased HPG development and ovary activation (Table 3.1). Both the high and the low dose of eβ showed similar correlations (Exp. 3), but the correlations disappear in the presence of QMP (Exp 4).

Egg laying in insects involves two distinct processes: the production of the egg yolk proteins from the egg yolk precursor vitellogenin (VG), and the incorporation of these proteins into eggs, followed by the physical oviposition of developed eggs. QMP and eβ appear to act on different components of the reproductive physiology in honey bee workers, with the former suppressing physical egg-laying, while the later suppresses ovary activation at the higher dose of 10 larval equivalents per bees (Exp. 3 & 4). When queens are present in a colony, there are very low incidences of worker egg-laying (Page and Erickson 1988). In queenless colonies, some workers become the dominant egg-layers and act as false queens (Sakagami 1958) that attract a queen retinue and suppress egg-laying in other workers by emitting a queen-like mandibular pheromone (Crewe and Velthuis 1980). When these false queens are removed, the other workers immediately begin laying eggs (Robinson et al. 1990; Page and Robinson 1994), illustrating that queen pheromones suppress egg-laying, but not ovary activation (Jay and Nelson 1973). Workers with activated ovaries are often found in queenright colonies that lack brood (Jay 1972) or when the broodnest is diminished just prior to swarming (Kropacova and Haslbachova 1970). Thus QMP inhibits egg-laying behavior. Other behavioral effects of QMP are produced through the pheromone's ability to suppress juvenile hormone (JH) biosynthesis (Robinson et al. 1992).

JH and VG are co-regulated in a double-repressor relationship (Amdam and Omholt 2003); high circulating titers of JH suppress production of VG and conversely high titers of VG suppress JH. Since QMP suppresses JH production, these low JH titers in turn augment VG titers, stimulating production of the egg-yolk precursor required for

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ovary activation. Previous research has shown that ovary activation in bees with more ovarioles in turn influences the division of labor. Workers engaged in nursing typically have high titers of VG (Amdam et al. 2003a), although the amount circulating will gradually diminish due to the energetic cost of feeding larvae (Amdam et al. 2009). As titers of VG decrease, there is a corresponding increase in JH production and bees transition from in-hive tasks to foraging. Having more ovarioles is linked with an early increase and subsequent decrease in VG, precocious foraging and a bias toward protein resources that benefit young larval nutrition (Pankiw and Page 2001; Amdam et al. 2006; Tsuruda et al. 2008; Ihle et al. 2010). Therefore, the early environment of a developing worker larva dictates the adult physiology, and that in turn influences the response of the workers to the current nest conditions.

Our cage studies determined conditions for lab-testing the synergistic effects of QMP and eβ by first establishing that incorporating royal jelly into the diet at 10% was more effective than access to nurse bees in stimulating HPG development, resulting in almost twice as many bees with well-developed HPGs, classified as stage 3 or 4. Adequate nutrition is essential for both HPG development and ovary activation (Haydak 1970; Hrassnigg and Crailsheim 1998; Hoover et al. 2006). Our queen-comparison experiment showed that synthetic QMP functions as an effective substitute for a live queen for suppression of ovary activation, though live queens are more effective than QMP alone in suppressing ovary activation and reducing worker mortality (Fig. 3.4 and 3.1b, respectively). Bees had continual access to QMP, frequently clustering over the synthetic strip. The inability of QMP to suppress ovary activation as strongly as a live

queen suggests more factors are involved in reproductive suppression. This difference between QMP and live queens has been postulated to be a sign of a queen 'control' and a continuing evolutionary arms race over male reproduction, with queens evolving additional pheromones to suppress worker reproduction as workers evolve to escape that suppression (Katzav-Gozansky 2006). Alternatively, the multi-component pheromone could represent an honest signal of queen fecundity linked to reproductive state that encourages worker 'cooperation' and informs the colony when the queen starts to fail (Kocher and Grozinger 2011).

The bees in the queen-comparison experiment with more ovarioles were most likely to activate their ovaries in the absence of a live queen (Table 3.1), as has been shown previously (Amdam et al. 2006; Page et al. 2006; Page and Amdam 2007; Tsuruda et al. 2008; Linksvayer et al. 2009; Wang et al. 2010; Graham et al. 2011; Page et al. 2012). Ovariole number is a recognized marker of reproductive potential in honey bees (Tanaka and Hartfelder 2004; Makert et al. 2006) demonstrating that workers with the most ovarioles and thus greatest reproductive potential are most likely to escape ovary suppression. Only live queens, who emit multiple pheromones (QMP, Dufour's gland and tergal pheromones), fully suppressed ovary activation in workers, disassociating total ovarioles from ovary activation (Table 3.1).

Our replicates set up on the same day were designed to be as similar as possible in all regards, except that each one was composed of a subset of different genotypes. Replicate thus encompasses both cage and genotypic differences. Replicate frequently proved a significant factor in the experiments, suggesting that genotype may influence

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individual response thresholds to pheromones, as has been demonstrated in other experiments (Pankiw and Page 1999; Pankiw et al. 2001; Amdam et al. 2009).

In the absence of QMP, the high dose of 10 Leq/bee significantly suppressed ovary activation (Fig. 3.6) as seen in previous experiments (Maisonnasse et al. 2009), paralleling the effects of live larvae, which inhibit worker ovary activation (Jay 1972; Jay and Jay 1976). A queenless hive can survive by rearing a replacement queen from larvae present in the colony (Hatch et al. 1999). However, workers made queenless refrain from rearing an emergency queen for 24 h in the presence of eggs and young larvae, but start rearing queens immediately when only older larvae (3rd-5th larval instar) are available (Pettis et al. 1997), indicating that the eggs and/or young larvae provide a fecundity signal that gradually declines. The low dose of 1 Leq/bee of eβ had no effect on ovary suppression, but stimulated greater pollen consumption (Fig. 3.5), suggesting that a fading young larval stimulus indicates a failing queen, perhaps driving bees to gorge on pollen and activate their own ovaries (Fig. 3.6).

Bees reared with either eβ treatment demonstrated a significant correlation of total ovarioles with ovary activation (see Table 3.1), confirming that bees with larger ovaries are more likely to activate them, as seen in the queen comparison of experiment 2. We also saw that ovary activation and HPG development were correlated in bees exposed to eβ, indicating that reproductive physiology is linked with nursing physiology, but only in the presence of the young larval pheromone. This suggests worker bees may be more strongly influenced to activate their HPG for larval feeding if they are predisposed to caregiving by possessing more ovariole filaments. Additionally they may be more prone

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to activate their ovaries if they have no larvae to receive the brood food, thus repurposing the VG from their HPG (Amdam et al. 2003a; Seehuus et al. 2007) into their ovaries to produce eggs. Early ovary activation in bees with more ovarioles is correlated with higher hemolymph VG titers during early adult life stages that subsequently drop. It is hypothesized that the dynamics of VG expression influences the onset of foraging and foraging behavior (Nelson et al. 2007; Ihle et al. 2010; Page 2013). Thus eβ appears to have greater effects on bees with more ovarioles, priming them for both larval care and protein rich pollen foraging, behavior that supports the nutritional development of the young larvae emitting the pheromone.

Our current results reinforce the reproductive groundplan hypothesis that postulates ancestral reproductive physiology was coopted and used to regulate foraging behavior (Amdam et al. 2004a; Amdam et al. 2006; Page et al. 2006; Page and Amdam 2007). Early nutritional differences in larval development lead to variation in adult worker ovariole number (Leimar et al. 2012; Wang et al. 2014) and thus may contribute to differential response thresholds to eβ priming. In field trials eβ both releases and primes bees toward pollen collection (Chapter 2 & 4), a pollen-foraging bias predicted by the reproductive groundplan hypothesis (Page et al. 2006; Page and Amdam 2007; Page 2013) Our results thus suggest that eβ impacts worker physiology tied to maternal traits in predisposed bees that possess more ovariole filaments at both life stages of worker development: during early adult life eβ improves nursing physiology by stimulating HPG development. After the transition to foraging, eβ biases bees toward pollen collection to provide protein for the developing brood nest.

Because live brood suppresses ovary activation in a hive (De Groot and Voogd 1954; Jay 1970), we used the higher dose of 10 Leq/bee for all subsequent experiments. In a colony, queens and brood pheromones interact to suppress worker reproduction, with QMP inhibiting the physical laying of worker eggs and brood pheromones suppressing the production of the egg-yolk precursor VG necessary for egg production (Jay 1970; Winston and Slessor 1998; Smedal et al. 2009). Our eβ and QMP synergy experiment support this division of pheromone effects on worker reproduction suppression, demonstrating that eβ is more effective than synthetic QMP at suppressing ovary activation, and there is no apparent synergy between the two pheromones on ovary activation (Fig. 3.7), at least not at 10 d of age. Brood pheromones of both young (current results) and old larvae (Arnold et al. 1994; Mohammedi et al. 1998; Maisonnasse et al. 2009) are very effective in suppressing ovary activation and worker reproduction.

Just as live queens resulted in a disassociation between total ovarioles and ovary activation, suggesting suppression of ovary activation regardless of the underlying reproductive physiology, a similar disassociation occurred in our eβ and QMP synergy experiment in bees exposed to both brood and queen pheromones. Throughout all of our experiments we saw very low levels of ovary activation at 10 d of age, with mean ovary activation never exceeding Stage 1, classified as slight swelling at the top of the ovariole. Bees typically transition out of the broodnest and into other in-hive tasks at 10-12 d of age (Seeley 1982; Seeley and Kolmes 1991; Seeley 1995). Worker HPG reach peak development at 6 d, then typically diminish in size by 15 d of age and atrophy as bees transition to foraging (Deseyn and Billen 2005). As we were interested in the impacts of

eβ on nurse bee physiology, we limited the duration of our cage trials to 10 d. Thus the possibility remains that synergy between QMP and eβ on suppression of worker reproduction could occur in more prolonged experiments, with eβ suppressing ovary activation and QMP stopping egg-laying, although no significant differences or trends were evident between eβ+/QMP- and eβ+/QMP+ at 10 d.

In contrast, ovary activation and total ovarioles correlated in bees reared with eβ alone or QMP alone, replicating the results seen under the same treatment conditions in experiment 2 (QMP) and 3 (eβ High). Although eβ had no direct treatment effect on HPG development in the synergy experiment, bees exposed to eβ alone had significantly correlated ovary activation and HPG development and significantly correlated total ovarioles and ovary activation, confirming the findings of experiment 3. Young adult bees actively tending the brood nest typically have the most developed HPG in a colony. The queen spends the majority of her time in the brood nest laying eggs in the vicinity of these nurse bees, thus the nurse bees have the greatest opportunity for interaction with the queen. When the queen is absent, QMP is not present, and when her reproductive potential starts to fail there will be a reduction of brood and thus a diminishing eβ signal. At this point the nurse bees may detect the changes and reroute VG from their HPG to their own ovaries for activation and an opportunity for reproduction, as seen in the control bees raised without eβ or QMP (Bier 1954; Bier 1958).

Our experimental results illustrate that pheromones in social insects provide complex signals that must be interpreted in context dependent circumstances and are strongly impacted by individual worker physiology. Honey bee chemical communication

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has dynamic properties and functions as a property of a complex system (Pankiw 2004b). QMP and eβ play important roles in honey bee society as both primer and releaser pheromones that change putative response thresholds to different stimuli by altering reproductive physiology and interacting with innate response thresholds of different genotypes. The young larval pheromone eβ acts predominantly on bees that have become tuned to caregiving because of their heightened number of ovarioles. Larval eβ primes these responsive workers to enhance larval provisioning by increasing HPG development to produce more brood food, and by activating their ovaries, tuning those workers to bias later foraging toward pollen collection (Amdam et al. 2004a; Amdam et al. 2006; Nelson et al. 2007; Page 2013). Additional field trials that examine the role of eβ on honey bee physiology in the context of the hive are needed to complement our current results.
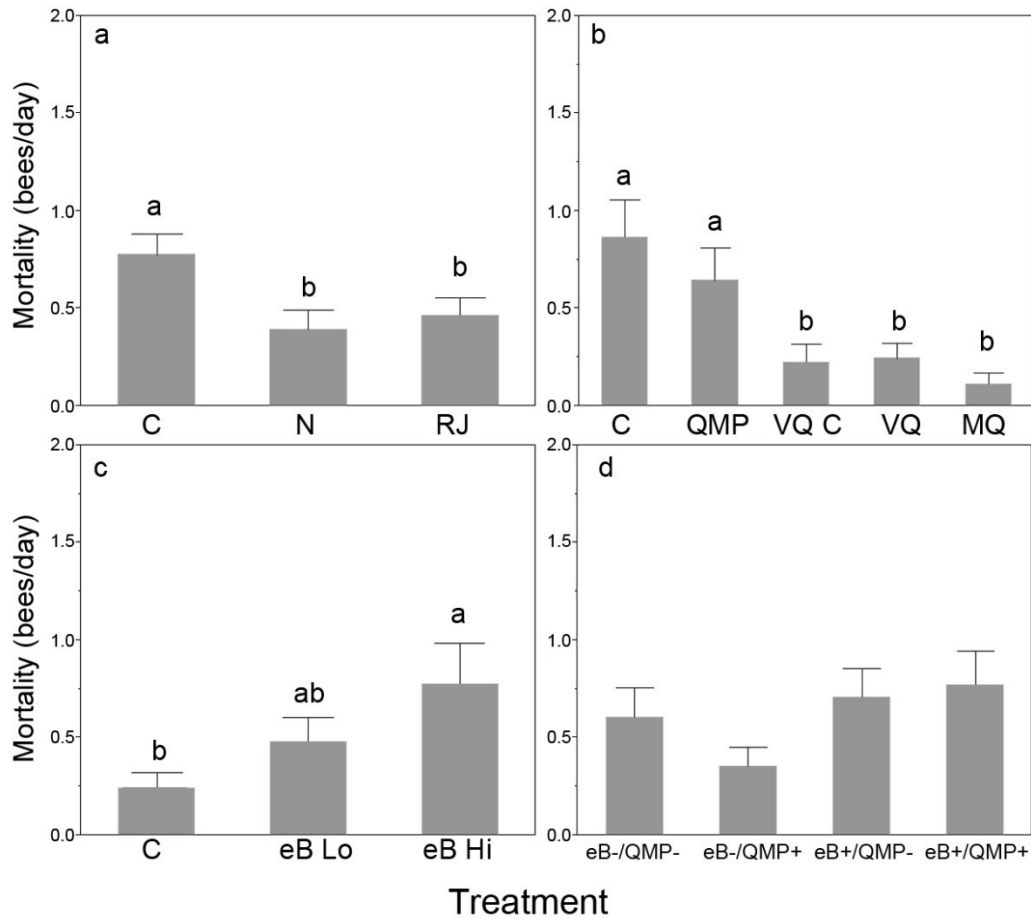
Figure 3.1. Daily bee mortality.

Mean (+ S.E.) daily bee mortality per cage. Different letters indicate significant differences. a) Exp. 1, *Royal Jelly compared to Nurse Bee Environment.* C = control; N = access to 100 Nurse Bees; RJ = 10 % royal jelly incorporated into candy diet; b) Exp. 2, *Queen Comparison.* QMP = synthetic strip of queen mandibular pheromone; VQ C = Virgin queen exposed to 2 treatments of CO_2; VQ = virgin queen; MQ = mated queen; c) Exp. 3, *High vs. Low e-beta ocimene (eβ) dose.* eB Lo = 1 Leq of eβ/bee; eB Hi = 1 Leq of eβ/bee; d) Exp. 4 *eβ & QMP synergy*
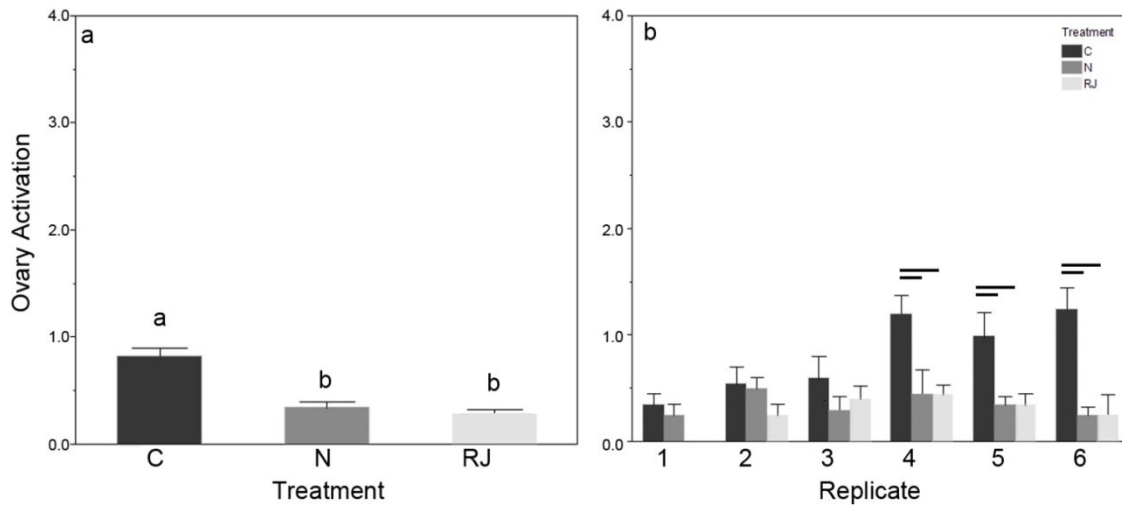
Figure 3.2. Ovary activation: access to nurse bees versus royal jelly.

Mean (+ S.E.) ovary activation (0 = lowest, 4 = highest) in bees given access to queen candy and pollen only (C), candy, pollen, QMP and 100 nurses (N), or candy, pollen, QMP and royal jelly (RJ). Results are presented collectively by treatment (a) and by replicate (b); N = 180 bees, 60 per treatment, 30 per replicate. Different letters (a) and black bars (b) indicate significant differences.
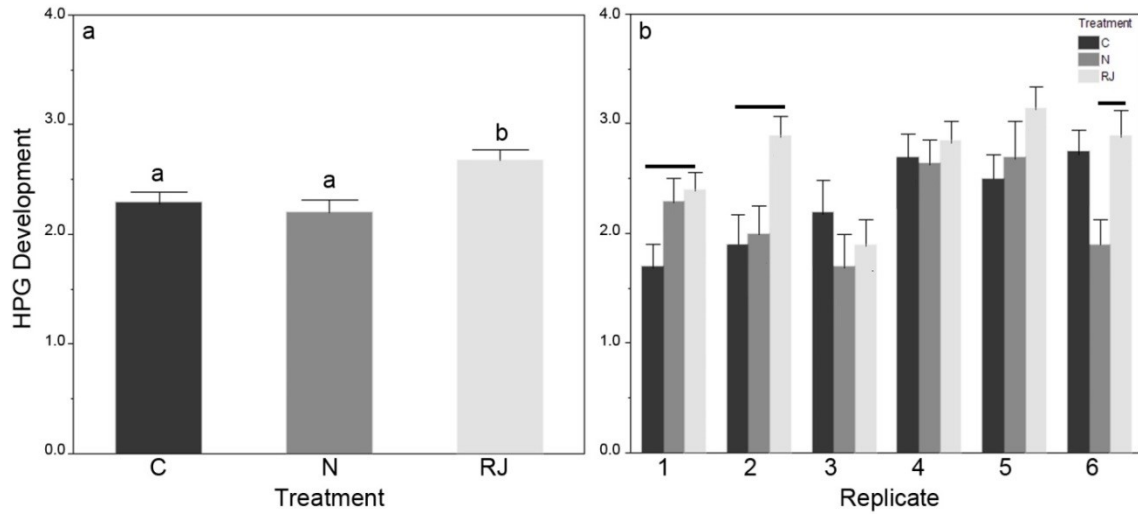
Figure 3.3. HPG development: access to nurse bees versus royal jelly.

Mean (+ S.E.) HPG development (0 = lowest, 4 = highest) in bees exposed to queen

candy and pollen only (C), candy, pollen, QMP and 100 nurses (N), or candy, pollen,

QMP and royal jelly (RJ). Results are presented collectively by treatment (a) and by

replicate (b); N = 180 bees, 60 per treatment, 30 per replicate. Different letters (a) and

black bars (b) indicate significant differences.
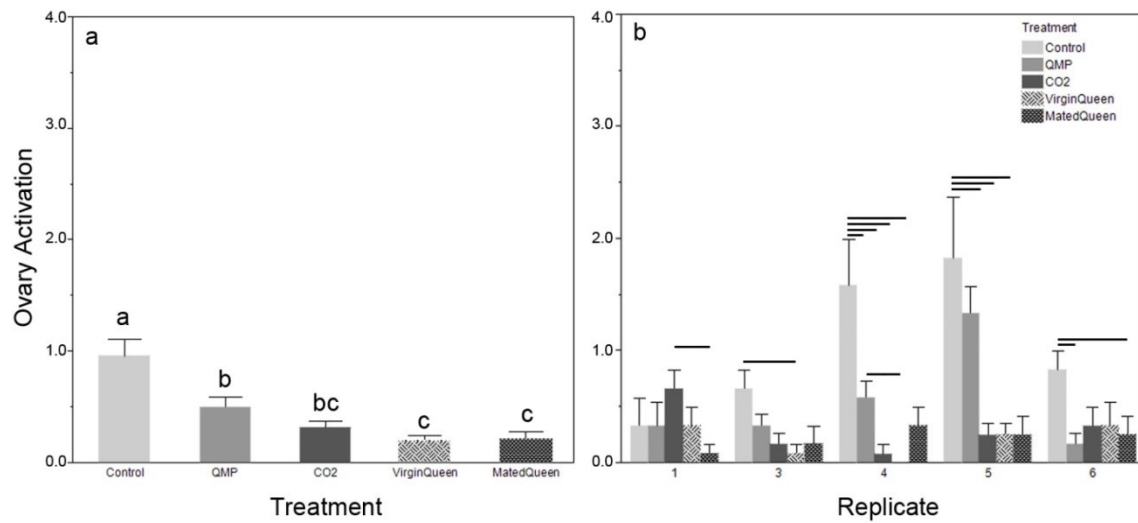
Figure 3.4. Ovary activation: queen comparison.

Mean (+ S.E.) ovary activation in bees exposed to synthetic QMP, virgin queen induced

to lay eggs by exposure to CO_2 (CO2), untreated virgin queen, mated queen, or control

conditions. Results are presented collectively by treatment (a) and by replicate (b); N =

180 bees, 36 per treatment, 30 per replicate. Different letters (a) and black bars (b)

indicate significant differences.
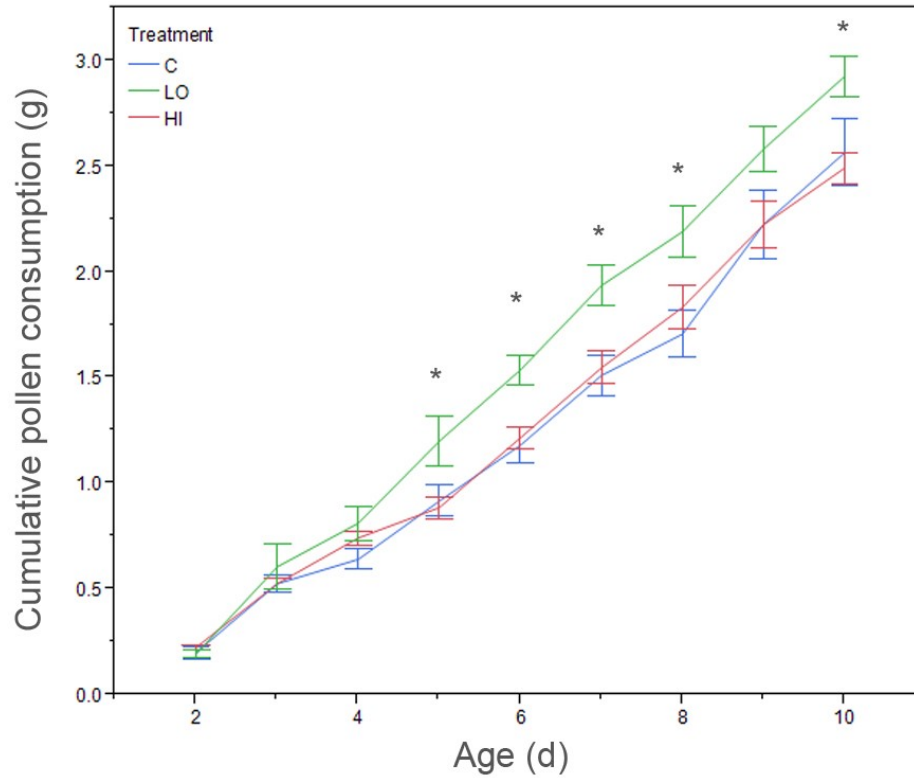
Figure 3.5. Pollen consumption: eβ dose.

Mean (±S.E.) cumulative pollen consumption over ten days in groups of bees exposed to high (10 Leq) or low (1 Leq) doses of the larval pheromone eβ ocimene, or the control. For each treatment there were 6 replicates. Significant differences between groups indicated by an asterisk.
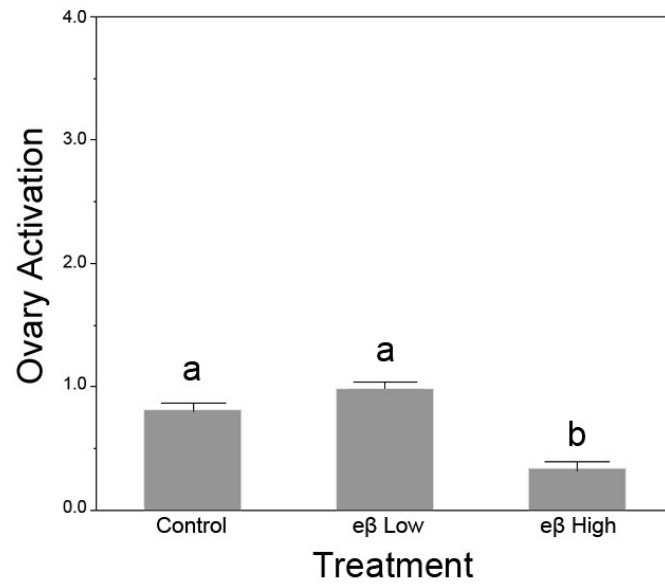
Figure 3.6. Ovary activation: eβ dose.

Mean (+S.E.) ovary activation in bees exposed to high (10 Leq) or low (1 Leq) doses of the larval pheromone eβ ocimene, or the control. For each treatment there were 60 bees.
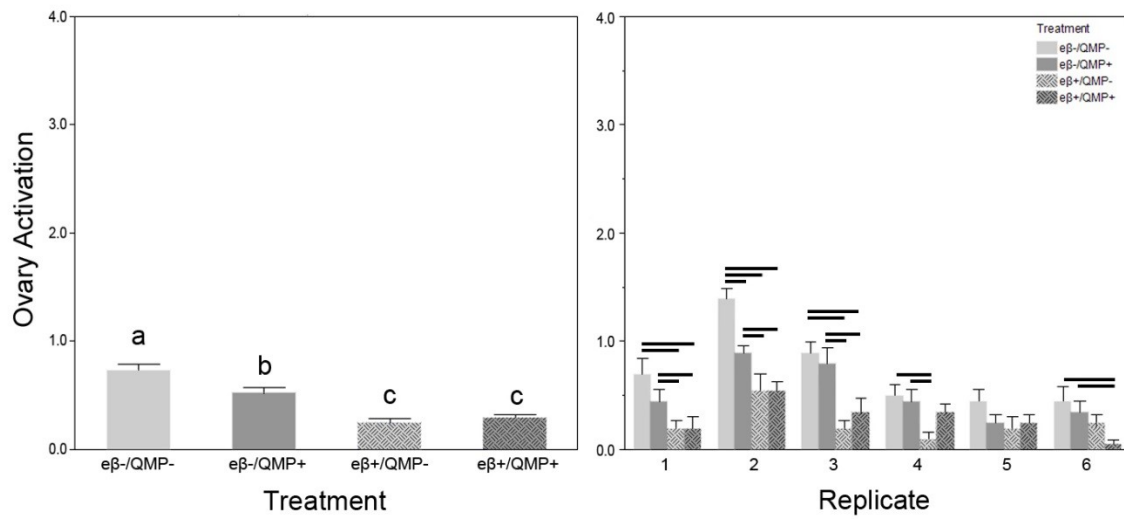
Figure 3.7. Ovary activation: QMP and eβ synergy.

Mean (+S.E.) ovary activation in bees exposed to treatments with or without eβ and

QMP. Results are presented collectively by treatment (a) and by replicate (b); N = 240

bees, 60 per treatment, 40 per replicate. Different letters (a) and black bars (b) indicate

significant differences.

Table 3.1. Significant correlations by experiment.

Significant correlations between total ovarioles (Total Ovl), ovary activation, (Ov Act) and HPG development (HPG Dev) are given for each of the five experiments are indicated. Significant correlations indicated by + or -, depending on relationship. Untested correlations because the HPG were not dissected indicated by n/a.

| Table 3.1 | | | | |
| SIGNIFICANT CORRELATIONS BY EXPERIMENT | | | | |
Experiment	Treatments	Ov Act & Total Ovl	Ovy Act & HPG Dev	Total Ovl. & HPG Dev
1	C	NS	+	NS
	RJ	NS	NS	NS
	N	+	NS	NS
2	C	+	n/a	n/a
	QMP	+	n/a	n/a
	CO2	NS	n/a	n/a
	VQ	NS	n/a	n/a
	MQ	NS	n/a	n/a
3	C	NS	NS	NS
	Low eβ	+	+	NS
	High eβ	+	+	NS
4	eβ - / QMP -	NS	NS	NS
	eβ - / QMP +	+	NS	NS
	eβ + / QMP -	+	+	NS
	eβ + / QMP +	NS	NS	NS

CHAPTER 4

YOUNG AND OLD LARVAE PRIME THE

DEVELOPMENTAL MATURATION OF THEIR CAREGIVERS

Abstract: In eusocial insects daughters rear the offspring of the queen to adulthood. In honey bees, *Apis mellifera,* nurses differentially regulate larval nutrition, so that young worker larvae receive a diet that parallels queen larvae in protein composition and food availability, while old larvae are restrictively fed a sugar rich diet. This differential feeding behavior may result from the pheromones of young and old larvae that differ in composition and volatility. To determine if these pheromones have a priming effect on nurse behavior, we examined whether young workers exposed for the first ten days of their adult development were influenced by the presence of young larvae, old larvae, or the young larval pheromone e-beta ocimene (eβ) compared to a control of no brood. Our results demonstrate that the early brood-priming environment of young larvae, old larvae and eβ alters caregiver physiology, endocrine titers of the egg-yolk precursor vitellogenin, and juvenile hormone, and thus the timing of the transition to foraging. All three brood environments significantly reduced the age of first foraging (AFF), while priming with young larvae and eβ increased pollen collection. Genotypic differences interacted with the early brood priming environment to regulate individual response thresholds and AFF.

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Honey bees exhibit a division of labor characterized by temporal polyethism (Wilson 1971; Seeley 1982; Calderone and Page 1988; Seeley 1995) where young bees (nurses) care for and feed immatures (brood) inside the hive while older workers (foragers) collect resources outside the hive (Winston 1987). This behavioral transition is a key component of the colony's plasticity in responding adaptively to fluctuating internal and external environmental conditions (Robinson 1992). Substantial physiological and behavioral changes occur during the shift from in-hive bee to forager (Robinson 1992; Nelson et al. 2007). Workers pass through this key transition at different rates with some individuals showing precocious behavioral development, while others mature more slowly (Ribbands 1952; Calderone and Page 1988; Siegel et al. 2013).

The transition to foraging is regulated by a number of both intrinsic and extrinsic factors. Among the primary endogenous influences are juvenile hormone (JH) and vitellogenin (VG). JH is produced in the corpora allata of bees and mediates honey bee development, maturation and social behavior (Wirtz and Beetsma 1972; Rachinsky and Hartfelder 1990; Sullivan et al. 2000; Amdam and Omholt 2003). VG, an egg-yolk precursor, is produced by the fat body (Excels 1974) and in honey bees increases immunity (Amdam et al. 2004b) and oxidative stress resistance (Seehuus et al. 2006), and is used to produce larval food in nurses (Amdam et al. 2003a; Seehuus et al. 2007). JH and VG co-regulate each other in most honey bees in a double-repressor feedback system (Amdam and Omholt 2003), however, genetic variation exists in populations for the

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coupling of JH and VG (Ihle et al. 2010). JH hemolymph titers and rates of biosynthesis are low in nurses and high in foragers, while VG titers are correspondingly high in nurses and low in foragers (Amdam et al. 2003a). An earlier onset of foraging can be induced by either inhibition of *vg* mRNA production through RNAi (Ihle et al. 2010) or artificial increase of JH through injection or topical application (Robinson 1992; Robinson and Vargo 1997). However, removal of the corpora allata, the glands that produce JH (Tobe and Stay 1985), delays but does not eliminate the transition to foraging (Sullivan et al. 2000), suggesting that factors outside the VG/JH feedback loop may also influence the transition to foraging.

An extrinsic factor that influences the timing of transition is the presence of immature honey bee brood (eggs, larvae and pupae) (Dreller et al. 1999; Pankiw and Page 2001; Amdam et al. 2009). Larvae, confined in a cell of the wax comb, are completely dependent on the care provided by the nurses and must communicate their nutritional needs via pheromones (Le Conte et al. 1994; Le Conte et al. 1995; Sagili and Pankiw 2009). Pheromones play central regulatory roles in many animal societies (Wilson 1971), and induce rapid but short-lived behavioral responses, or exert long term physiological effects in the recipient that promote a delayed behavioral response (primers; (Wilson and Bossert 1963; Le Conte and Hefetz 2008)). The signals given off by developing larvae change as they age (Trouiller et al. 1991; Trouiller 1993; Maisonnasse et al. 2009; Maisonnasse et al. 2010), reflecting their changing nutritional needs (Le Conte et al. 1994), and correspondingly releasing selective foraging in their caregivers for appropriate nutritional resources (Chapter 2). Young worker larvae, which

require protein-rich brood food produced by nurse bees, emit the volatile pheromone e-beta-ocimene (eβ). Old larvae destined to become workers are restrictively fed diets with quadruple the sugar content (Rhein 1956; Shuel and Dixon 1968; Asencot and Lensky 1988) and half the protein content (Kunert and Crailsheim 1987) of their younger sisters, and they emit predominantly non-volatile ethyl and methyl fatty acid esters, collectively known as brood ester pheromones (BEP), along with much smaller amounts of eβ (Trouiller et al. 1991; Trouiller 1993). Larval development is thus orchestrated by the interplay of larval signals and nurse feeding responses.

Nurse bees produce the protein rich food fed to developing larvae from paired hypopharyngeal glands (HPG) (Snodgrass 1925). To activate their HPG, bees must consume protein and have contact with larvae for 3 days (Huang et al. 1989; Huang and Otis 1989). The activity of the gland is positively correlated with its size (Knecht and Kaatz 1990; Deseyn and Billen 2005). Numerous globular acini attach to the long, slender main channel of the HPG and these acini increase in diameter until 6 days after adult emergence, and then begin to shrink. The gland continues to atrophy with increased age. By 15 days of age, when bees typically transition to foraging, the HPG corresponds in size to the still undeveloped glands of newly emerged bees.

Larvae and their pheromones stimulate increased nursing behavior, longer larval feeding visits, increased HPG protein content, and the preparation of new cells for an expanded brood nest (Sagili 2007; Sagili and Pankiw 2009; Sagili et al. 2011), and may prime their caregiver physiology, thus influencing the timing of the transition to foraging and the proportion of nectar to pollen that they collect. Maisonnasse et al. (Maisonnasse

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et al. 2010) showed that continuous exposure to eβ caused a more rapid onset of foraging in workers and from this result they suggest that nurse bees caring for young larvae transition to foraging earlier than those caring for older larvae (Maisonnasse et al. 2010).

We have found that eβ releases increased pollen foraging (Chapter 2). Additionally there is evidence that the early development of workers may shape their sensitivity and foraging responses to such stimuli. In previous work on pollen foraging behavior, we found that in bees bi-directionally selected for pollen hoarding behavior (Page and Fondrk 1995), those that bias their foraging toward pollen collection (high pollen hoarding strain) initiate foraging earlier (Calderone and Page 1988; Page et al. 1998; Pankiw and Page 2001), and have lower response thresholds to sucrose (Page et al. 1998; Pankiw and Page 1999; Pankiw and Page 2000) when compared to those with a bias towards collecting less pollen and more nectar (low pollen hoarding strain). Even in unselected strains, a reduced sucrose response threshold in nurses has been linked with a precocious transition to foraging and a bias toward pollen collection (Pankiw and Page 2000; Pankiw et al. 2001; Rueppell et al. 2006). Bees exposed to eβ also exhibit reduced sucrose response thresholds (Traynor, unpublished). Thus the eβ induced earlier age of first foraging seen previously (Maisonnasse et al. 2010) may have been due to the releaser effects of an increased stimuli for supplying pollen, the priming effects of an altered physiology and a reduced sucrose response threshold, or both. The responses to brood stimuli may be further modulated by individual response thresholds, which are strongly influenced by genotype.

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To determine how larvae of different ages affect the development and later behavior of young workers, through both their different nutritional demands and pheromonal signals, we measured several physiological characteristics involved in the transition from nursing to foraging, the rate of behavioral maturation determined via the age of first foraging, and the type of foraging load collected. Based on earlier model predictions (Maisonnasse et al. 2010), we hypothesized that maturing in an environment of young larvae for the first 10 days of adult life would accelerate maturation by priming individuals to seek out protein rich pollen sources required for early larval development and thus transitioning out of the hive more rapidly to forage for critical protein resources. The caregivers of young larvae would thus have precociously elevated circulating JH titers and suppressed *vg* mRNA and VG protein titers. Young larvae float in a pool of nutritious food and thus need infrequent feeding. The reduced demand for feeding from a high proportion of young larvae could allow nurses to accelerate their transition to foraging to accommodate for the high protein demand. Old larvae, in contrast, require continual, but restricted feeding and thus should prolong the nursing phase of their caregivers. The old larvae should stimulate in nurses high titers of circulating VG that are required for hypopharyngeal gland development, which in turn suppresses JH, thus leading to a delayed onset of first foraging. In order to disentangle the releaser and primer effects of brood pheromones on age of first foraging and bias towards pollen collection, bees were primed for only the first 10 days of adult life with different brood treatments or received a control of no brood. We also tested whether the volatile eβ pheromone of

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young larvae produced similar physiological and endocrine changes as exposure to live larvae.

Bees

Three genetic sources were used; a commercial "wildtype" stock (WT) and two sources derived from the discontinued high and low pollen hoarding strains of Page and Fondrk (1995). Continuous selection over 40+ generations for a single trait difference between the strains influenced an entire suit of behavioral and physiological characteristics (Page and Fondrk 1995; Amdam and Page 2010; Page 2013). These strains underwent a dramatic decline in the phenotypic differences, demonstrated by a substantial increase in stored pollen and ovariole number in workers of the low strain. However, significant 4-fold differences in pollen storage still remain between genetic sources, which we have designated the high (HDB) and low derived bee (LDB) strains and thus we expect differences is age of first foraging and sensitivity to the brood stimulus. For 24 h, 5-7 queens from each of the genetic sources were caged on empty combs to oviposit. The combs of eggs were transferred into full size WT colonies and the larvae raised to adulthood in a brood box placed above a queen excluder, which prevented the queen from laying additional eggs. These combs were removed and placed in an incubator set at 34° C and 50% humidity the day prior to bee emergence. The following morning newly emerged (NE) bees were collected and paint marked on the thorax to indicate genetic source (3 colors; Sharpie Paint Markers, Oak Brook, IL), and on the abdomen to indicate treatment (4 colors). This focal cohort of NE marked bees

was added to 4 small "nucleus" colonies (nuc) containing about 1.5 kg of WT bees. Each

nuc was provided with one caged, mated queen and 1 comb of honey, 1 empty comb, 1

comb of wax foundation and 1 comb containing the treatment. The experiment was

replicated 3 times over consecutive days, with the relative positioning of the treatment

nests changed each day.

Treatments

Each nuc received 1 of 4 possible early environment brood treatments: 1) young

larvae (YL); 2) old larvae (OL); 3) e-beta ocimene (eβ); or a control 4) the absence of

brood (NB). Since eβ and NB treatments don't require live brood, they received an empty

comb. Appropriately aged larvae were provided by caging queens in additional colonies

on empty combs for 18 h. Each brood treatment consisted of ~ 2000 cells of honey bee

larvae. Brood/empty combs were replaced daily. At the time of insertion, YL were early

2nd instar larvae (4 d post egg laying). At the time of removal, 24 h later, larvae were

predominantly late 2nd instar, a stage that emits volatile e-beta ocimene (eβ) as their

pheromone (Maisonnasse et al. 2009; Maisonnasse et al. 2010). OL were early 5th instar

larvae (7 d post egg laying) at insertion. At removal, OL were predominantly late 5th

instar with a few cells already capped. OL primarily emit non-volatile brood ester

pheromones (BEP) (Trouiller et al. 1991; Trouiller 1993). Two hours after brood comb

replacements each nuc received an eβ pheromone/control treatment. This was contained

within a glass petri dish placed below the brood nest area and covered with mesh to

prevent direct contact (Maisonnasse et al. 2010). YL, OL and NB received 1 ml of

paraffin oil as a control; eβ received 10,000 larval equivalents of ocimene (mixture of isomers including eβ; Sigma-Aldrich, St. Louis, MO, USA) dissolved in 1 ml of paraffin oil. Daily brood and pheromone treatments were continued for 10 days.

Sample Collection

After being exposed for 10 d, 7-12 bees of each genetic source were collected from each treatment colony for hemolymph extraction. Bees were anesthetized by chilling. From each bee, one µl of hemolymph was extracted into a glass capillary tube after piercing the cuticle above the second tergite. For each JH sample the hemolymph from three randomly selected individuals of the same treatment group and genetic source were pooled and stored at −80°C in 1 ml 50% acetonitrile (EMD Chemicals, Gibbstown, NJ) until analysis. An additional 1 µl hemolymph per bee was extracted for VG protein samples, evacuated into an Eppendorf tube and stored at −80°C until analysis. Subsequently the gut was removed and the individual abdominal cuticle sample with its adherent layer of fat body was dissected into 500 µl of TRIzol (Invitrogen, Carlsbad, CA) and stored at -80°C until *vg* mRNA expression could be analyzed. For ovary and hypopharyngeal gland assessment, an additional 25 bees per group were collected and frozen at -80°C until dissection.

RNA Extraction and cDNA Synthesis of Fat Body

After thawing and homogenization in TRIzol, RNA was extracted by following Invitrogen's instructions. The quality and quantity of RNA were determined via

spectrophotometry (Nanovue, GE Healthcare, Piscataway, NJ). DNase (RNase-free, DNase kit, Applied Biosystems, Bedford, MA, USA) was added to the total RNA extract to remove trace DNA contaminants. One μg of such treated RNA was used for reverse transcription following an established method (Wang et al. 2012) using TaqMan® Reverse Transcription Reagents (Applied Biosystems).

Quantitative Real-time PCR Analysis

First-strand cDNA was used for real-time quantitative PCR (RT-qPCR) assays. Sixteen samples were randomly picked from each treatment group of each genetic source for *vg* gene (AJ517411) expression analysis (Wang et al. 2012). The biological samples from the four treatment groups of each genetic source were run together on every PCR plate, enabling *vg* expression comparisons between the four treatment groups. Each sample had three technical replicates run on an ABI Prism 7500 Real-Time PCR system (Applied Biosystems) for measuring *vg* transcript levels in comparison with those of the reference gene *actin* by means of the Delta-Delta Ct method (Wang et al. 2012). Studies have shown that *actin* is stably expressed throughout all stages in honey bees (Lourenço et al. 2008; Reim et al. 2013). By monitoring negative control samples (without reverse transcriptase) and melting curve analysis, we verified that the RT-qPCR assays were not confounded by DNA contamination or primer dimers (Vandesompele et al. 2002).

Hemolymph VG Protein Analysis

For each of the three genetic sources individual hemolymph samples for all four treatment groups were subjected to one-dimensional SDS-electrophoresis using 4-10 % polyacrylamide gels. Each gel included two samples from each treatment group and three β-galactosidase standard samples (1.25, 2.5 and 3.75 µg) of *Esherichia coli* (Sigma), randomly distributed. Sample aliquots containing 0.5 µl hemolymph were mixed with loading buffer (Laemmli 2×, Sigma) and boiled for 2 min. Electrophoresis on 4–10% polyacrylamide gels (Bio-Rad, Hercules, CA) was performed according to published protocols (Amdam et al. 2003b; Seehuus et al. 2006). Electrophoresis was carried out at constant current of 15mA for 1 h at 20°C. Gels were stained with 0.025% coomassie blue dissolved in a solution of acetic acid (7%), methanol (40%) and purified water that was also used for gel destaining. VG was identified by molecular mass (180-190 KD) in comparison with known protein markers (Bio-Rad).

Hemolymph JH Titer

JH was titered using the gas chromatography/mass spectrometry (GC-MS) method of Bergot et al. (1981) (Bergot et al. 1981) as modified by Shu et al. (1997) (Shu et al. 1997) and detailed in Amdam et al. (2010) (Amdam et al. 2010). Samples were eluted through aluminum oxide columns successively with hexane, 10% ethyl ether–hexane and 30% ethyl ether–hexane. Samples were derivatized with methyl-d alcohol (Sigma-Aldrich, St Louis, MO, USA) and trifluoroacetic acid (Sigma-Aldrich) and then subjected to a second series of aluminum oxide elutions (30% ethyl ether-hexane, then

50% ethyl-acetate–hexane). Purified samples were analyzed on an HP 7890A Series GC (Agilent Technologies, Santa Clara, CA, USA) equipped with a 30 m × 0.25 mm Zebron ZB-WAX column (Phenomenex, Torrence, CA, USA) and coupled to an HP 5975C inert mass selective detector (Agilent Technologies). Helium was the carrier gas. MS analysis occurred in the SIM mode, monitoring at m/z 76 and 225 to ensure specificity for the d_3-methoxyhydrin derivative of JHIII. Farnesol (Sigma-Aldrich) was used as an internal standard in each sample to ensure consistent peak calibration. Total abundance was quantified against a standard curve of derivatized JHIII (Sigma-Aldrich). The assay's detection limit is 1 pg.

Foraging Activity, Age of First Foraging and Pollen Foraging Load

When the focal cohorts reached 10 d of age, and sample collection had ceased, the combs and the queen were removed from each of the four colonies and the bees were left to form a cluster inside the empty hive. After dark, when all foragers had returned to the hive, the entrance was screened and the four hives moved into a cool, dark location overnight. In the morning the bees from the four different hives were misted with water so they could not fly, then shaken into a ventilated wooden box. They formed one large 6 kg cluster and were allowed to sit overnight to lose their individual colony odors. The following morning, when the marked cohort of bees was 12 days old, this combined colony was introduced into a full size hive, provided with a different mated queen, 2 combs of larvae, 2 combs of pupae, 3 empty combs, and 2 honey combs. The hive entrance was opened and the bees were allowed to start foraging.

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Foraging activity was monitored daily in four 20 min intervals, twice in the morning and twice in the afternoon. The hive entrance was closed with mesh screening so that returning foragers could still orient to the hive odors and land on the entrance board, but were barred from entering the hive. All marked foragers from the focal cohort were collected into a cage and cold anesthetized. The collected bees were sorted by paint marks into genetic source and treatment, then the number of pollen and non-pollen foragers for each group was tallied. Pollen foragers had visible pollen loads on the hind legs; non-pollen foragers had no noticeable loads. Since the hive was monitored daily, the age of the focal cohort at time of capture represents the individual bee's age of first foraging (AFF). Pollen foraging activity was determined by comparing the proportion of pollen foragers for each genetic source and treatment group. For a subset of pollen foragers one pollen pellet was removed from the hind leg and weighed. Because bees carry a balanced pollen load (Winston 1987), the weight of the single pellet was doubled to represent total pollen load collected. Pollen load mass was calculated across all collected foragers to provide a better representation of pollen intake per colony. All NE marked bees were counted at time of introduction. When > 50% of each treatment/genetic source had foraged, the experiment was terminated. A census was conducted for all focal cohort bees that remained in the hive; these bees were recorded as non-foragers. Foraging activity was determined by comparing the number of foragers to non-foragers.

Ovariole Number, Ovary Development & HPG Development

Reproductive physiology influences individual behavioral response thresholds, the age of first foraging and foraging preference (Page and Amdam 2007; Page et al. 2012; Page 2013), so we assessed ovary condition in bees of each strain and treatment. The paired ovaries were dissected into saline (0.25 mol/l NaCl) and the ovariole number for each was determined under a microscope. Additionally, the state of oocyte development within each ovary was scored using a 5 point scale (Pernal and Currie 2000): stage 0 = no follicle development, stage 1 = slight enlargement, stage 2 = presence of distinct cells leading to swellings and constrictions, stage 3 = egg volume exceeding that of the nutritive follicle, stage 4 = presence of fully formed eggs. The mean scores of the ovary pairs were used for analysis.

Both HPG glands were dissected from the head capsule into saline. Under a microscope at 100x magnification they were scored on a scale from stage 1 to 4 based on acini size and globe density (Hess 1942): Stage 1) atrophied; 2) slightly swollen with noticeable spacing between acini; 3) swollen with small spacing between acini, capable of producing brood food; and 4) fully developed and tightly clustered, channel obscured by acini. Glands were additionally assigned to one of three classes according to lobe morphology (Wegener et al. 2009). Class one, typical of young, broodless workers, consisted of glands with small acini showing an uneven surface. Class two, characteristic of active nurse bees, have medium-sized to large acini with a smooth surface and numerous secretory vesicles, giving them a yellowish color. Class three, common in older foragers, have glands with large, but slightly pale and translucent lobes. Class three was

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not observed in our samples. For statistical analysis, only bees ranked stage 4 and class 2 were considered the fully developed glands typical of nurse bees.

Statistical Analysis

Two-way ANOVA was used to analyze all multi-factorial data, followed by LSD Student's t-test post-hoc analyses when results were significant (Sokal and Rohlf 1995). We had *a priori* expectations that there would be significant differences between the brood and control treatments for *vg* mRNA expression, and so paired Student T-tests were performed. For *vg* mRNA and VG protein, we were more interested in treatment effects than strain effects, so data were normalized on a scale of 1 to 10 for each strain and then compared using a non-parametric Kruskal-Wallis ANOVA on ranks. Total ovarioles, ovary development and HPG development were compared using Spearman Rank correlations, to determine if physiological factors underlying nurse physiology were co-regulated. Pollen foraging activity was calculated from categorical data (pollen = 1; no pollen = 0) using the nominal logistic fit and Chi-square Likelihood Ratio tests. The impact of early priming environment and genetic source on foraging activity was calculated using 2x4 and 2x3 contingency tables using the online programs available at http://www.vassarstats.net. All other analyses were conducted using JMP Pro 10 (SAS, Cary, NC).

Age of First Foraging

The age at which bees transition from in-hive tasks to outside foraging is referred to as the age of first foraging (AFF). Genetic source significantly influenced the age of first foraging (Fig. 4.1; $F_{2, 6572} = 102.81$, $P < 0.001$) of individuals that made a successful transition to foraging. As expected, HDB bees foraged approximately 1.5 d earlier than WT and 3 d earlier than LDB bees (t = 17.09, $P < 0.001$; t = 9.84, $P < 0.001$). The WT foraged 1.5 d earlier than LDB (t = 8.29, $P < 0.001$). Exposure to brood primes bees to forage. The early priming environment of different brood treatments thus had a significant effect on age of first foraging (Fig 4.2; $F_{3, 6572} = 5.00$, $P = 0.002$). Bees that experienced the no brood environment foraged up to one day later than any of the other three brood treatments ($t = 4.84$, $P < 0.001$). Replicate significantly influenced the AFF ($F_{2, 6572} = 8.82$, $P < 0.001$); replicates 2 and 3 foraged one day later in life compared to Replicate 1.

Genotypes have different response thresholds and responded differentially to treatment. There was thus a significant interaction of genetic source with treatment for the age of first foraging (Fig. 4.3; $F_{6, 6572} = 3.17$, $P = 0.004$). Priming with a brood environment (pheromone or larvae) significantly reduced the AFF in HDB ($F_{3, 2366} = 9.46$, $P < 0.001$) and WT bees ($F_{3, 2497} = 7.08$, $P < 0.001$), but not in LDB bees ($F_{3, 1709} = 0.63$, $P = 0.596$) for which AFF did not differ significantly between treatment groups. Priming HDB bees with any brood treatment significantly decreased AFF compared to those

exposed to NB ($t \geq 2.14$, $P \leq 0.030$). HDB bees are more sensitive to brood priming environments and significant differences in AFF occurred between brood treatments, with YL significantly decreasing AFF compared to eβ ($t = 2.85$, $P = 0.005$), with OL falling in between. Like HDB bees, WT bees primed in brood environments had significantly earlier AFF's compared to those not exposed to brood ($t \geq 3.53$, $P < 0.001$), but there was no significant difference between YL, OL and eβ.

Pollen Foraging Activity and Pollen Load

Pollen foraging provides the colony with rich protein resources. The proportion of workers foraging for pollen was significantly influenced by age ($\chi^2_{1, 6608} = 220.00$, $P < 0.001$), genetic source (Fig. 4.4a; $\chi^2_{2, 6608} = 81.33$, $P < 0.001$), and treatment (Fig. 4.4b; $\chi^2_{3, 6608} = 8.16$, $P = 0.043$). As expected from previous experiments, HDB strain bees had the highest proportion of pollen foragers (17.3%), followed by WT (11.9%), with very few pollen foragers among the LDB strain bees (8.1%). In releaser experiments, young larvae and eβ each bias foraging toward pollen. Bees primed by the YL or eβ environments had significantly higher proportions of pollen foragers (13.8% and 14.1% respectively) than bees reared in the OL environment (11.4%; $\chi^2_{3, 6608} = 5.99$, $P \leq 0.014$).

There were significant effects of replicate ($F_{2, 6263} = 3.11$, $P = 0.045$) and genetic source on the pollen load collected by foragers (Fig. 4.5; $F_{2, 6263} = 7.66$, $P < 0.001$), but no effect of treatment ($F_{3, 6263} = 1.19$, $P = 0.311$). As seen previously, HDB bees collected

heavier pollen loads than WT ($t = 2.05$, $P = 0.040$) or LDB strain bees ($t = 4.83$, $P < 0.001$). WT bees collected heavier pollen loads than LDB bees ($t = 3.03$, $P = 0.003$).

Foraging Activity

Brood priming environment significantly influenced the number of bees that successfully transitioned to foraging in the HDB ($\chi^2 = 13.45$, df = 3, $P = 0.004$) and WT ($\chi^2 = 11.43$, df = 3, $P = 0.010$) strains, where more bees reared with OL or YL became foragers. This difference was not seen in the LDB strain ($\chi^2 = 5.14$, df = 3, $P = 0.162$) (Table 4.1). Genetic source also had a highly significant effect on the number of foraging bees for all early priming environments (Table 4.2). In all four priming environments, two to three times more LDB strain bees than WT or HI never transitioned to foraging.

Physiological Development: Ovaries and HPG

Both genetic source and early priming environment can influence worker reproductive physiology. There was a significant effect of genetic source on ovariole number (Fig. 4.6; $F_{2, 164} = 8.07$, $P < 0.001$), with the LDB having significantly more ovarioles than HDB or WT strains. There was no significant effect of treatment ($F_{3, 164} = 0.38$, $P = 0.770$). In contrast, ovary development, measured as stage of follicle development at 10 days of age, was significantly different for the brood treatment environments (Fig. 4.7; $F_{3, 164} = 6.17$, $P < 0.001$), but was not influenced by genetic source ($F_{2, 164} = 1.98$, $P = 0.141$). Compared to the ovaries of workers experiencing NB,

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the ovaries were significantly less developed in bees exposed to YL ($t = 2.60$, $P = 0.010$) or OL ($t = 3.78$, $P < 0.001$), while there was no difference with eβ treated bees ($t = 0.55$, $P = 0.586$).

HPG development significantly differed across replicates ($F_{2, 164} = 15.75$, $P < 0.001$) and treatments (Fig. 4.8; $F_{3, 164} = 9.73$, $P < 0.001$), but not across genetic sources ($F_{2, 164} = 0.57$, $P = 0.750$). There were no significant interactions across any factors. Less than 40% of bees reared in the NB environment had the fully developed HPG of nurse bees after 10 d, which is significantly fewer than the 75-88% found in those exposed to the three brood environments ($t \geq 3.86$, $P < 0.001$).

Total ovariole number and ovary development are significantly correlated across all bees (Spearman $\rho = 0.20$, n = 200, $P = 0.005$). When examined by individual strain, total ovariole number and ovary development are significantly correlated in the WT ($\rho = 0.35$, n = 64, $P < 0.005$), but not in the HDB ($\rho = 0.12$, n = 72, $P = 0.320$) or LDB ($\rho = -0.04$, n = 64, $P = 0.740$) strain bees. However, in the HDB, total ovariole number and HPG development are correlated ($\rho = -0.28$, n = 72, $P = 0.015$), but they are not correlated in the two other strains. In the YL treatment group, total ovariole number and ovary development are linked ($\rho = 0.29$, n = 51, $P = 0.041$), and there is a suggestive relationship between ovary and HPG development ($\rho = 0.27$, n = 51, $P = 0.053$).

Examining correlations between ovariole number, ovary development and HPG development by individual strain, reveals different strain responses to treatment effects. In the HDB, total ovariole and ovary development are only correlated in the NB

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treatment ($\rho = 0.51$, n = 18, $P = 0.037$). HPG development and ovary development are only correlated in the YL treatment ($\rho = 0.49$, n = 19, $P = 0.038$). In contrast, there were no correlations for any of the three factors examined in the LDB. In the WT, total ovarioles and ovary development correlated in both of the live larvae treatments (OL: $\rho = 0.54$, n = 17, $P = 0.031$; YL: $\rho = 0.60$, n = 17, $P = 0.014$) but no other correlations were observed.

vg mRNA in Fat Body

Vg mRNA expression levels influence JH titers and thus impact the transition to foraging. Because expression levels for each genetic source were run on individual plates, we ranked the data for each genetic source on a scale of 1 to 10 to compare the impact of early priming environment across all bees. The early brood environment bees experienced had a significant impact on *vg* mRNA (Fig. 4.9a; $\chi^2 = 11.38$, $P < 0.010$). *Vg* mRNA was significantly down-regulated in bees reared with old larvae ($z \geq 2.21$, $P \leq 0.027$) compared to the three other environments. For each individual genetic source there is a common trend for the *vg* mRNA to decrease in bees raised in a brood environment of old larvae, but the influence of early priming environment is not significant for any individual genotype in a factorial analysis (Fig. 4.10; HDB: $F_{3, 56} = 1.23$, $P = 0.306$; WT: $F_{3, 60} = 2.63$, $P = 0.058$; LDB: $F_{3, 60} = 2.62$, $P = 0.059$). We had an *a priori* expectation that bees raised with larvae would have significantly different *vg* expression compared to bees raised in no brood environments, so we conducted a one-

tailed t-test. This was significant for the WT bees ($t = 2.52$, $P = 0.010$) and LDB bees ($t = 1.78$, $P = 0.042$), but was only suggestive for HDB bees ($t = 1.56$, $P = 0.066$).

VG Hemolymph Protein

VG protein titers influence JH titers and thus impact the timing of the transition to foraging. Different genetic sources were run on separate gels, so to compare across all bees the data was ranked as above for *vg* mRNA. Early environment had a significant impact of the levels of circulating VG protein in the hemolymph (Fig. 4.9b; $\chi^2 = 30.68$, $P < 0.001$). Bees reared with OL had significantly less VG than all other groups ($z \geq 3.65$, $P < 0.001$). When analyzed by individual genetic source, there was no difference in VG protein levels across brood treatments in HDB (Fig. 4.11; $F_{3, 67} = 1.18$, $P = 0.323$), but VG titer was significantly reduced in workers exposed to old brood as compared to the no brood treatment for both WT (Fig. 4.11; $F_{3, 54} = 3.77$, $P = 0.016$) and LDB (Fig. 4.11; $F_{3, 61} = 4.13$, $P = 0.010$).

JH Titers

Juvenile hormone impacts the timing of the transition to foraging. Genetic source had no influence on the JH titers of 10-day old bees ($F_{2, 118} = 0.19$, $P = 0.831$), while the early brood priming environment significantly influenced JH titers (Fig. 4.12; $F_{2, 118} = 12.52$, $P < 0.001$). Bees reared with live larvae had approximately 50% higher JH titers than bees reared with NB or eβ.

Although it had been previously established that brood can impact the foraging behavior of nestmates (Hellmich and Rothenbuhler 1986; Dreller et al. 1999; Amdam et al. 2009), we now demonstrate that the age composition of the brood, and the associated pheromones emitted by the differently aged larvae, can influence the foraging response by priming the physiology associated with the transition from nursing to foraging (see Table 4.3). In addition, we confirmed that a worker's genotype can modulate their response to these brood stimuli.

Maturing in the environment of YL or OL impacts the development of the nurse-aged bees in fundamental ways and, as expected, stimulated greater HPG development than in bees reared with no brood. Despite having no access to live larvae, bees in the eβ environment also had better developed HPGs compared to those reared without brood (see Fig. 4.8). This finding seemingly contradicts previous studies showing that isolated young workers exposed to eβ do not develop their HPGs (Maisonnasse et al. 2010). However, this difference may imply that while the volatile brood pheromone can stimulate HPG development, it can only do so in the context of a colony, where a queen and honeycombs are present to provide synergistic stimuli. Additional stimuli may be a prerequisite given that activation of the HPGs and feeding larvae is an energetically expensive commitment, requiring bees to consume pollen and convert it into protein rich brood food (Brouwers 1983; Hrassnigg and Crailsheim 1998). A particular combination

of stimuli appears to be necessary even under normal colony conditions. Previous work has shown that nurse bees only activate their glands after three days of rearing brood (Huang and Otis 1989) and that they require direct contact with larvae (Huang et al. 1989); however, these trials used mixed age larvae, which accordingly produce lower levels of eβ than just young larvae, a concentration that may not have been adequate to stimulate HPG development.

Larvae typically suppress ovary development (Jay 1970); this was confirmed in our experiment as bees reared with YL or OL had significantly less developed ovaries than bees reared in the NB environment. Larval pheromones, both eβ and BEP, have been shown to suppress ovary development in cage trials; the latter also suppresses ovary development in small queenless field colonies of 1,500 bees (Arnold et al. 1994; Mohammedi et al. 1998; Maisonnasse et al. 2009). The results of our current experiment, which used colonies of ~15,000 bees as a priming environment contradicts the previous eβ cage trial results, as we found bees reared in the eβ environment, had comparable ovary development to the control (NB) bees, which was significantly greater than in bees reared with live larvae (Fig. 4.7). We measured both ovariole number and ovary activity, because they are typically positively correlated, as seen in this experiment. The reproductive physiology of a worker affects a suite of behaviors, including the age of first foraging, sucrose response thresholds and foraging bias (Pankiw et al. 2001; Amdam et al. 2004a; Amdam et al. 2006; Rueppell et al. 2006; Wang et al. 2010). A possible explanation for the increased ovarian development is that bees in the eβ environment

activated their HPG in preparation of feeding larvae, but with no larvae to receive the protein-rich food, the nurse aged bees stored the egg-yolk precursor protein into vitellogenic oocytes. Such a flexible repurposing of resources within workers has been postulated for social insects by Bier (Bier 1954; Bier 1958; Hölldobler and Wilson 1990).

The focal cohorts of bees were the predominant nurse aged bees in the colonies, as no other bees were allowed to emerge during their 10 days of priming. The bees reared with old larvae significantly depleted their own vitellogenin stores, both in circulating hemolymph VG protein and the transcription of new *vg* mRNA in fat body where VG is produced. Compared to the young larvae used in this experiment, old larvae are substantially larger (Wang 1965; Rembold and Kremer 1980), required more frequent feeding and consumed more food. Nurse aged bees convert VG into protein rich jelly in the HPG (Amdam et al. 2003a); the more intensive demands of caring for OL may deplete VG faster than it can be replenished. This in turn would cause a rise in JH titers that would induce the transition to foraging at an earlier age compared to workers reared without brood (Amdam and Omholt 2003; Nelson et al. 2007; Amdam et al. 2009). This early transition to foraging after rearing larvae occurred in all but the LDB workers (Fig. 4.3).

Our results contradict some of the model predictions by Maisonnasse *et al.* (2010), who postulated exposure to old larvae would delay the transition to foraging. Their model proposed delayed foraging of bees caring for old larvae, because exposure to high levels of BEP produced by old larvae have been linked to a delay in transition (Le

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Conte et al. 2001). However, the purported influence of BEP on age of first foraging has been varied, with some experiments showing an acceleration of maturation (Pankiw et al. 2004) and others a delay (Le Conte et al. 2001; Alaux et al. 2009). Both worker age and BEP dose impact the timing of the transition to foraging (Sagili 2007; Alaux et al. 2009; Sagili and Pankiw 2009), suggesting a multi-component regulatory system.

Bees reared in the no brood environment transitioned to foraging later in life than other bees except the LDB (see Fig. 4.3). Bees reared without brood did not reduce their *vg* mRNA or VG protein titers, as they did not need to draw on internal protein resources to feed hungry larvae. Their high levels of circulating *vg* and VG likely suppressed the production of JH to cause a delayed transition to foraging. Surprisingly, when VG titers from hemolymph and *vg* from fat body were measured in bees reared in the YL and eβ environment, we found they did not experience the typical *vg* mRNA depletion caused by extensive nursing that precedes an early transition to foraging (Amdam et al. 2009; Smedal et al. 2009). Consumption of protein-rich pollen protects bees against VG depletion (Bitondi and Simoes 1996). In our laboratory studies bees exposed to eβ consumed more pollen than controls, indicating that the young larval pheromone may drive the bees to gorge on protein sources, which in turn elevates circulating *vg* mRNA titers so that they reach a point of equilibrium. This in turn would cause a delay in JH titers rising so that the transition to foraging is consequently delayed. Since bees reared in both eβ and YL environments transitioned to foraging earlier than bees reared without brood, despite elevated *vg* and VG titers in the HDB and WT bees, we postulate that

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exposure to eβ lowers the individual's response threshold to JH, so that the transition to foraging is induced by lower JH levels (see Fig. 4.13). Another possibility is that young larvae and eβ influence other redundant regulatory systems outside the VG/JH loop or a complimentary component of the feedback loop, such as the insulin signaling system which is sensitive to nutritional cues and is located upstream of JH production (Wang et al. 2012), thus influencing the transition to foraging. We know redundancy is built into the regulatory system as allectomized bees, rendered incapable of producing JH, can still transition to foraging successfully (Sullivan et al. 2000).

Although both YL and eβ reduced age of first foraging, there were significant differences by genetic source in their sensitivity to the brood stimulus. These genetic source effects are likely due to different innate response thresholds. Compared to wildtype workers, the original HI strain bees were typically very sensitive to gustatory and visual stimuli, and LO strain bees were less sensitive, impacting the timing of the transition to foraging and the foraging preference (Pankiw and Page 2000; Pankiw and Page 2001; Tsuruda and Page 2009a; Tsuruda and Page 2009b). Our results confirmed this anticipated pattern of strain-specific responsiveness: HDB bees foraged first, followed by WT and LDB (see Fig. 4.1). HDB and WT bees primed with YL or eβ transitioned to foraging earlier than those exposed to no brood (see Fig. 4.3) and biased their resource collection toward pollen (see Fig. 4.4b), while the early priming environment had no effect on age of first foraging in the insensitive LDB. Thus genotype and early priming environment interact to differentially influence the age of first foraging

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(Fig. 4.3). We purposely used three different strains that have a genetically-based variance in their behavioral response thresholds to brood to shed light on interindividual differences among nestmates with different fathers. Even though the phenotypic differences between the original strains have decreased 280% for ovarioles and 425% for stored pollen (Page, unpublished), the HDB and LDB still maintained significant differences in behavior that followed expectations for the HI and LO strains.

Because the HDB have lower response thresholds, the three early brood environments had significantly different impacts on age of first foraging. Although the bees reared with eβ mimicked the pattern of bees reared with YL, we believe the physical interaction with YL increased circulating titers of JH while eβ did not, so that bees reared with YL transitioned approximately 1 d earlier to foraging than those reared with just the pheromone. Thus, eβ primed bees toward pollen collection without impacting the endocrine regulators associated with reduced age of first foraging. Pollen foragers have lower response thresholds and forage earlier than bees that preferentially collect nectar (Page et al. 1998; Pankiw and Page 1999; Pankiw and Page 2000; Pankiw et al. 2001), suggesting that eβ influenced the individual response threshold so that bees transitioned to foraging despite lower levels of juvenile hormone (see Fig. 4.13). Our cage studies have demonstrated that eβ lowers sucrose response thresholds, a trait which has been repeatedly linked to an earlier transition to foraging and a bias toward pollen collection (Page et al. 1998; Pankiw and Page 1999; Pankiw and Page 2000; Pankiw et al. 2001; Pankiw and Rubink 2002; Pankiw 2003). In contrast, HDB bees reared with OL

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experienced a trend toward VG depletion typically seen in bees with heavy nurse loads. This loss of VG was coupled with an upregulation of JH and thus the OL-exposed HDB bees transitioned to foraging at a time between YL and eβ exposed bees. These results suggest that bees sensitive to brood stimuli respond to the physical feeding of larvae separately from larval pheromones, with corresponding individual impacts on endocrine titers and response thresholds, resulting in fine-tuning of the age of first foraging (see proposed model in Fig. 4.13).

In WT bees the priming influence of the three different brood environments had similar impacts on endocrine physiology as seen in the HDB, and likewise decreased age of first foraging compared to NB. However, there were no discernible differences in age of first foraging among the WT bees exposed to the three different brood environments. All three brood treated groups foraged approximately 1 day before the bees reared without brood despite three different early developmental endocrine trajectories: 1) the expected pattern of low VG and high JH of precocious foraging seen in bees reared with OL, 2) a previously un-encountered pattern of high VG and high JH in bees reared with YL; and 3) the pattern of high VG and low JH typically seen in bees that prolong the nursing phase after exposure to eβ. This suggests that after their introduction into the common garden environment these early endocrine differences were overcome, so that only NB bees delayed their transition to foraging. The NB-exposed bees most likely delayed their transition, because they experienced no VG depletion nor was their foraging initiation response modulated as it was by the YL and eβ environment (Fig. 4.13).

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While the results of our study still point to an incomplete understanding of the mechanism regulating the temporal polyethism of honey bees, they do highlight the overall complexity of the system. In order to eliminate unimportant cues and threats (noise) and still adapt to changing environmental conditions, robust communication systems require flexibility and redundancy. Polyandry in honey bee colonies results in numerous patrilines that add to the genetic diversity of colonies. The differences exhibited by the three genetic sources used in this experiment mimic interindividual differences seen among nestmates with different fathers and provide insight into varied individual response thresholds. Although pheromones play a key regulatory role within eusocial colonies, the chemical language is interpreted through the individual's response threshold, which in turn is influenced by the individual's genotype. The built-in redundancy permits flexibility, so that no single factor (genotype or early environment) dominates and fixes later behavior. This collective plasticity enhances a colony's capacity to have graded responses to endogenous and exogenous stimuli.
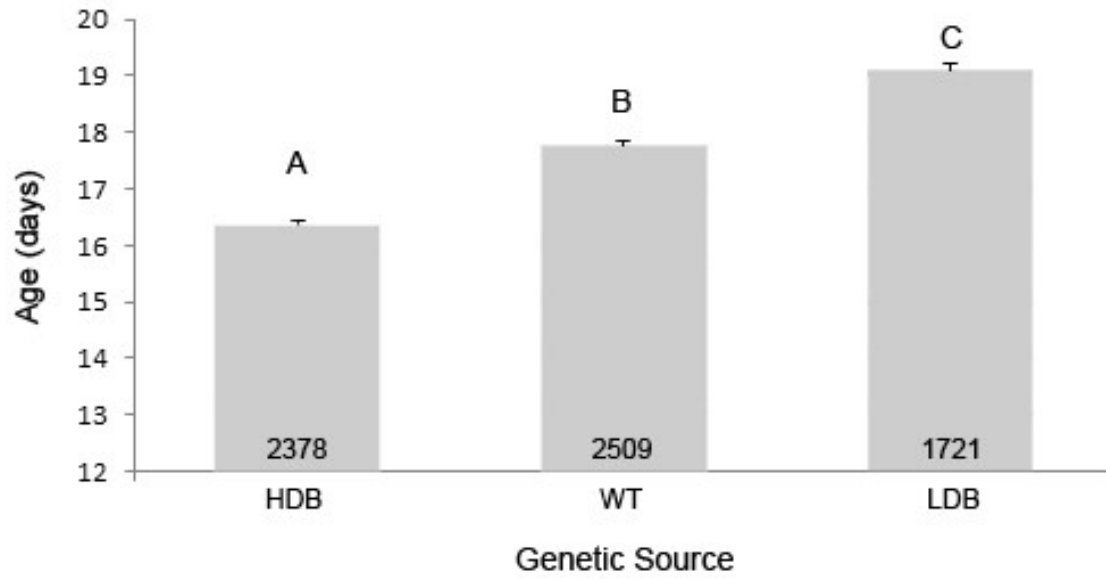
Figure 4.1. Age of first foraging by genetic source.

Mean age + S.E. of first foraging as a function of genetic source (High strain derived bees = HDB; Wildtype = WT; Low strain derived bees = LDB). Significant differences ($\alpha <$ 0.05) are indicated by different letters. Sample sizes are provided.
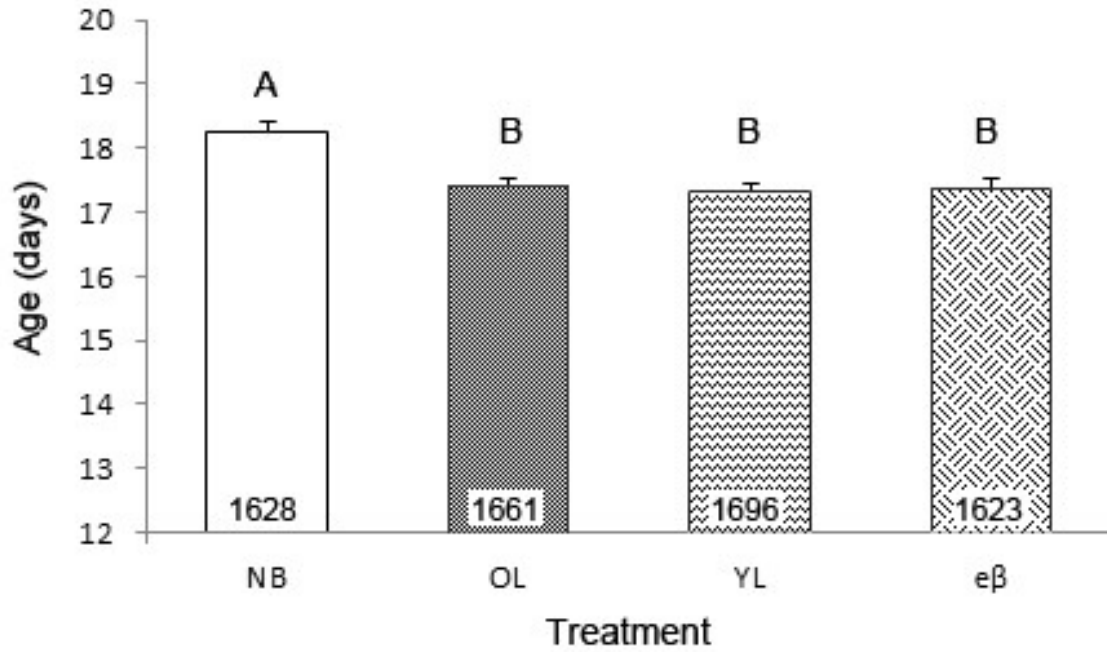
Figure 4.2. Age of first foraging by treatment.

Mean age + S.E. of first foraging as a function of the early priming environment the bees

experienced for the first 10 days of life (No Brood = NB, Old Larvae = OL; Young

Larvae = YL; e-beta ocimene = eβ) Significant differences ($\alpha < 0.05$) are indicated by

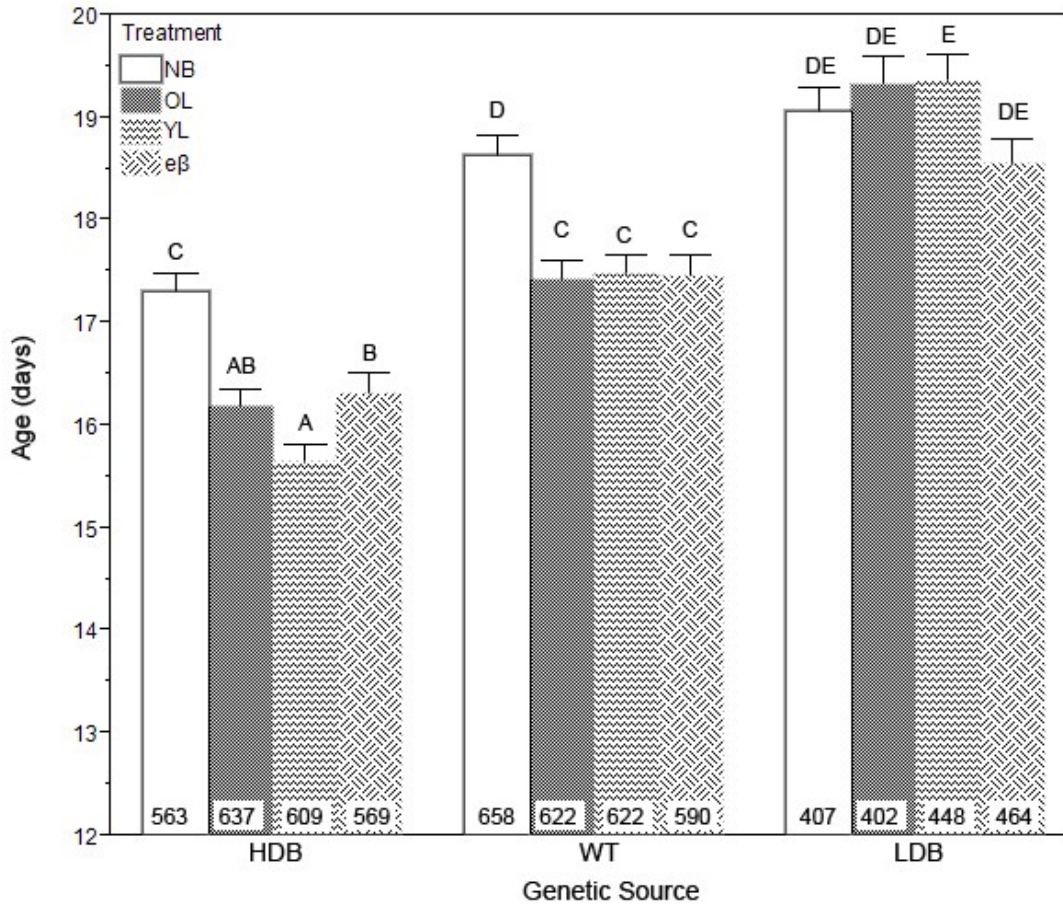different letters. Sample sizes are provided. .

Figure 4.3. Age of first foraging genetic source and treatment.

Mean age of first foraging as a function of genetic source and the early priming

environment (No Brood = NB, Old Larvae = OL; Young Larvae = YL; e-beta ocimene =

eβ). Significant differences (α < 0.05) are indicated by different letters. Sample size is
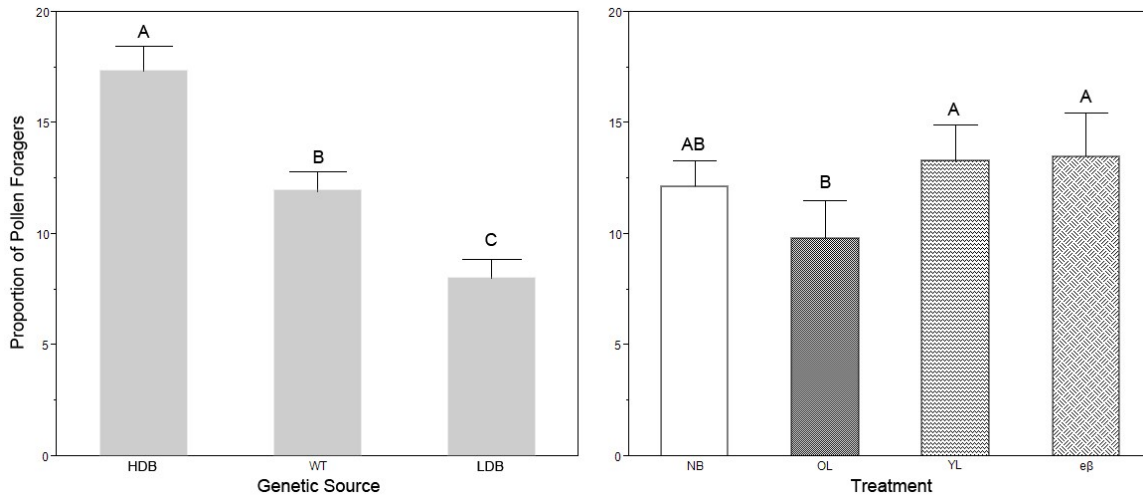
indicated.

Figure 4.4. Proportion of pollen foragers.

Foragers were defined categorically as either a pollen forager or non-pollen forager on their first foraging trip. Both (a) genetic source (High strain derived bees = HDB; Wildtype = WT; Low strain derived bees = LDB) and (b) early brood priming environment (No Brood = NB, Old Larvae = OL; Young Larvae = YL; e-beta ocimene = eβ) had a significant effect on the number of pollen foragers.
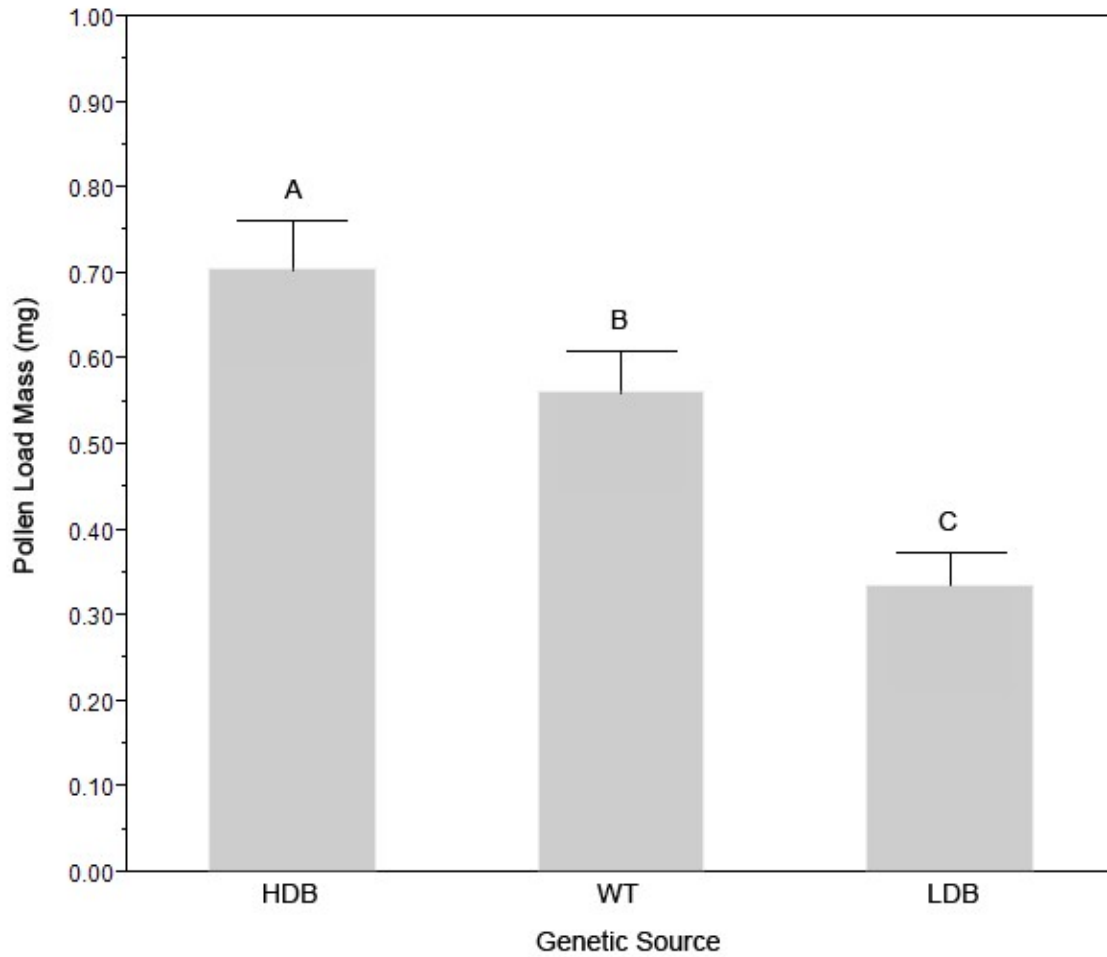
Figure 4.5. Pollen load mass.

Mean pollen load mass + S.E. calculated across all foragers (High strain derived bees = HDB; Wildtype = WT; Low strain derived bees = LDB). Significant differences (α < 0.05) are indicated by different letters. Sample sizes are provided
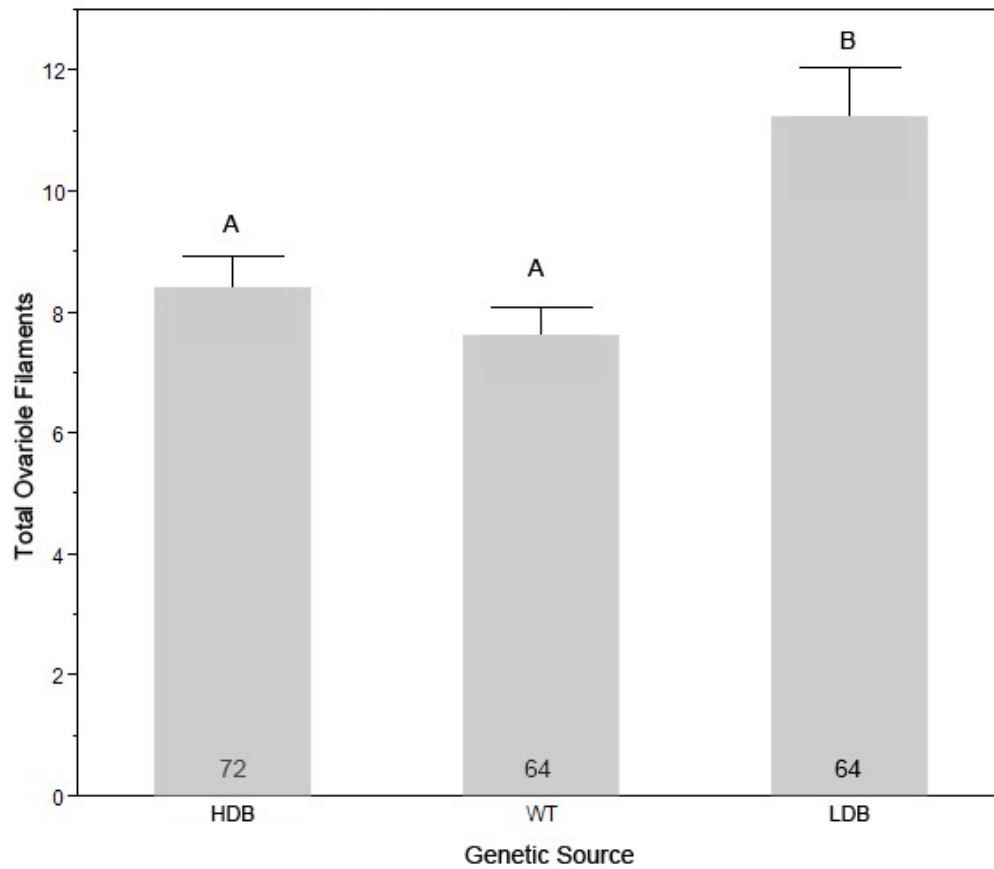
Figure 4.6. Ovariole filaments by genetic source.

Mean ovary filaments + S.E.as a function of genetic source (High strain derived bees = HDB; Wildtype = WT; Low strain derived bees = LDB). Significant differences ($\alpha <$ 0.05) are indicated by different letters. Sample size is indicated.
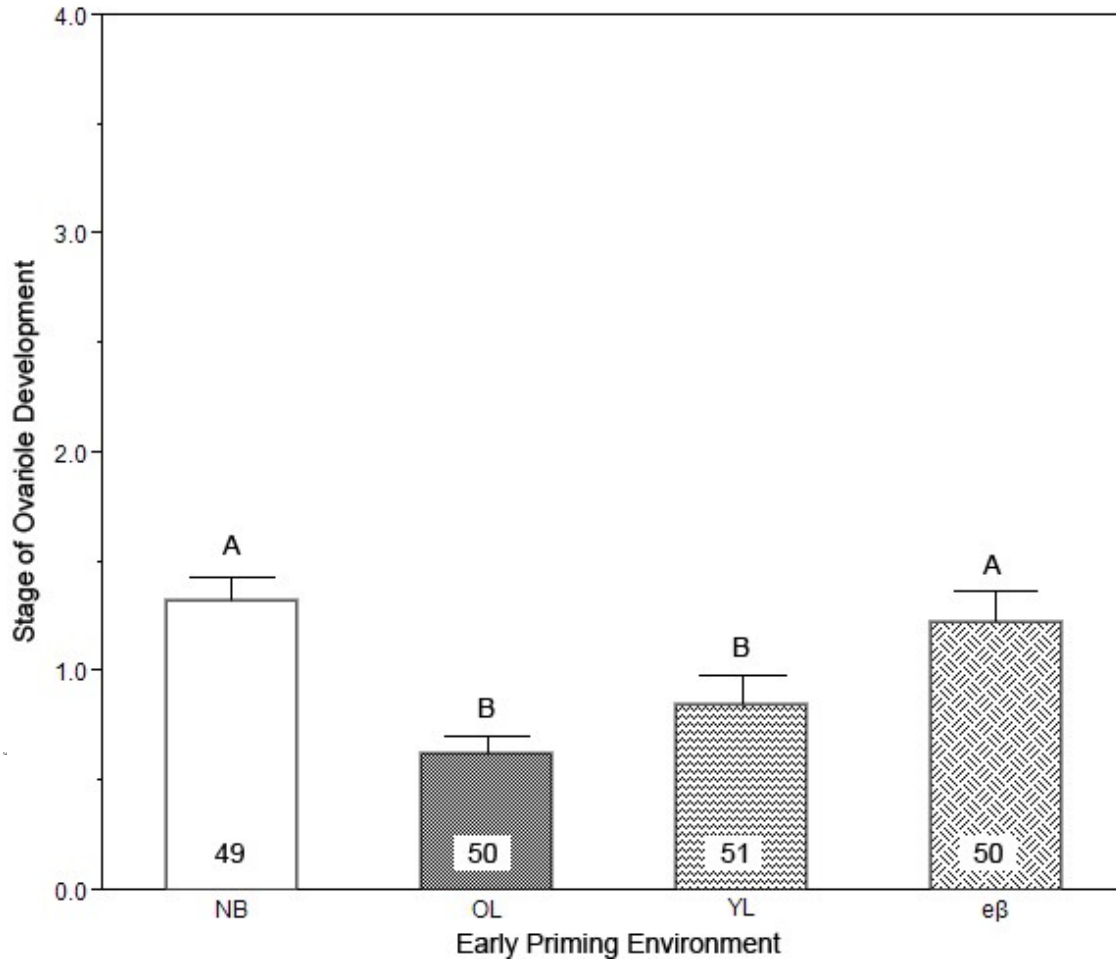
Figure 4.7. Ovary activation by treatment.

Mean ovary development + S.E. as a function of the early priming environment (No Brood = NB, Old Larvae = OL; Young Larvae = YL; e-beta ocimene = eβ). Significant differences ($\alpha < 0.05$) are indicated by different letters. Sample sizes are provided.

Figure 4.8. HPG by treatment.

Proportion of bees with fully developed HPG of nurse bees (see methods) as a function of early priming environment (No Brood = NB, Old Larvae = OL; Young Larvae = YL; e-beta ocimene = eβ). Significant differences (α < 0.05) are indicated by different letters. Sample sizes are provided.
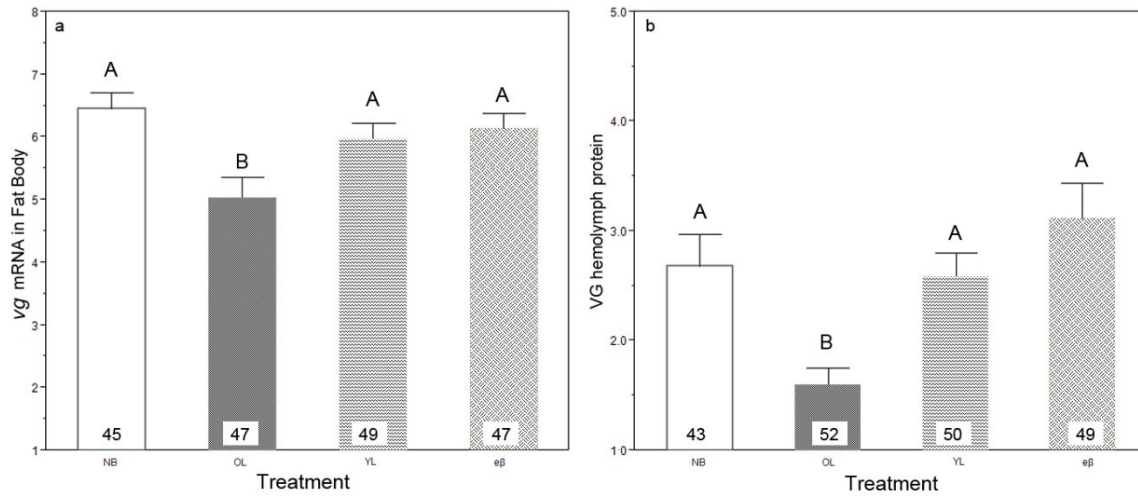
Figure 4.9. *vg* mRNA and VG protein by treatment.

Mean *vg* mRNA and VG protein titers + S.E. ranked across each genetic source (High

strain derived bees = HDB; Wildtype = WT; Low strain derived bees = LDB; No Brood

= NB, Old Larvae = OL; Young Larvae = YL; e-beta ocimene = eβ). Significant

differences (α < 0.05) are indicated by different letters.

Figure 4.10. *vg* mRNA by genetic source and treatment.

Mean *vg* mRNA titers in fat body as a function of early priming environment (No Brood = NB, Old Larvae = OL; Young Larvae = YL; e-beta ocimene = eβ). Significant differences ($\alpha < 0.05$) indicated by a connecting bracket.

Figure 4.11. VG protein by genetic source and treatment.

Mean VG protein levels in hemolymph as a function of early priming environment (No Brood = NB, Old Larvae = OL; Young Larvae = YL; e-beta ocimene = eβ). Significant differences ($\alpha < 0.05$) are indicated by different letters.
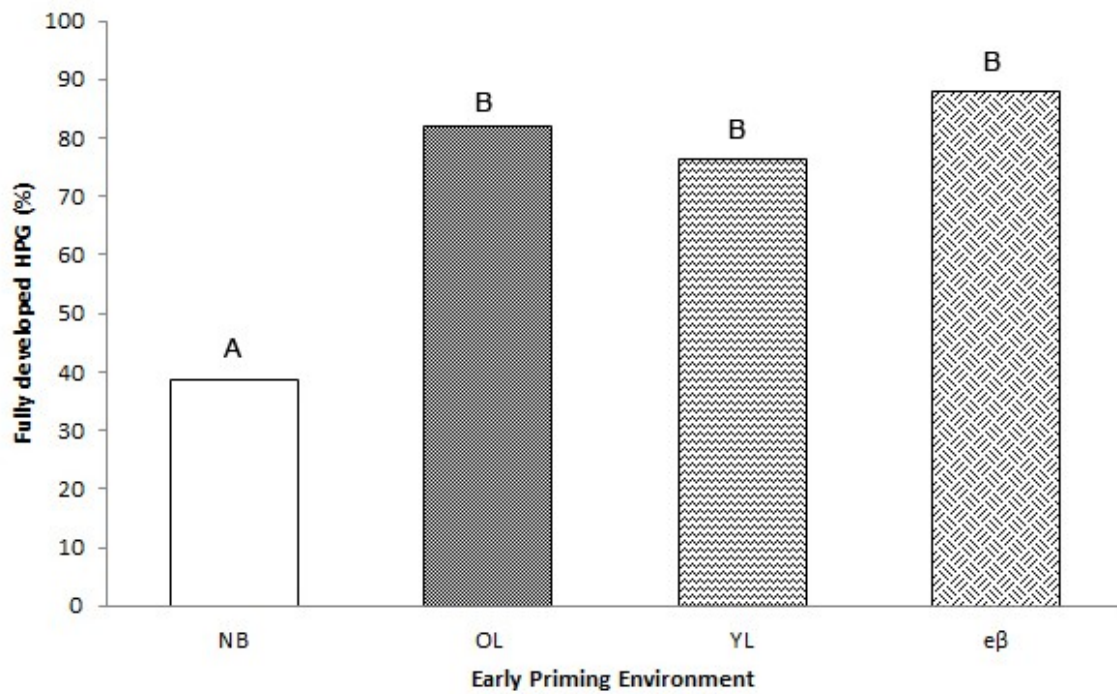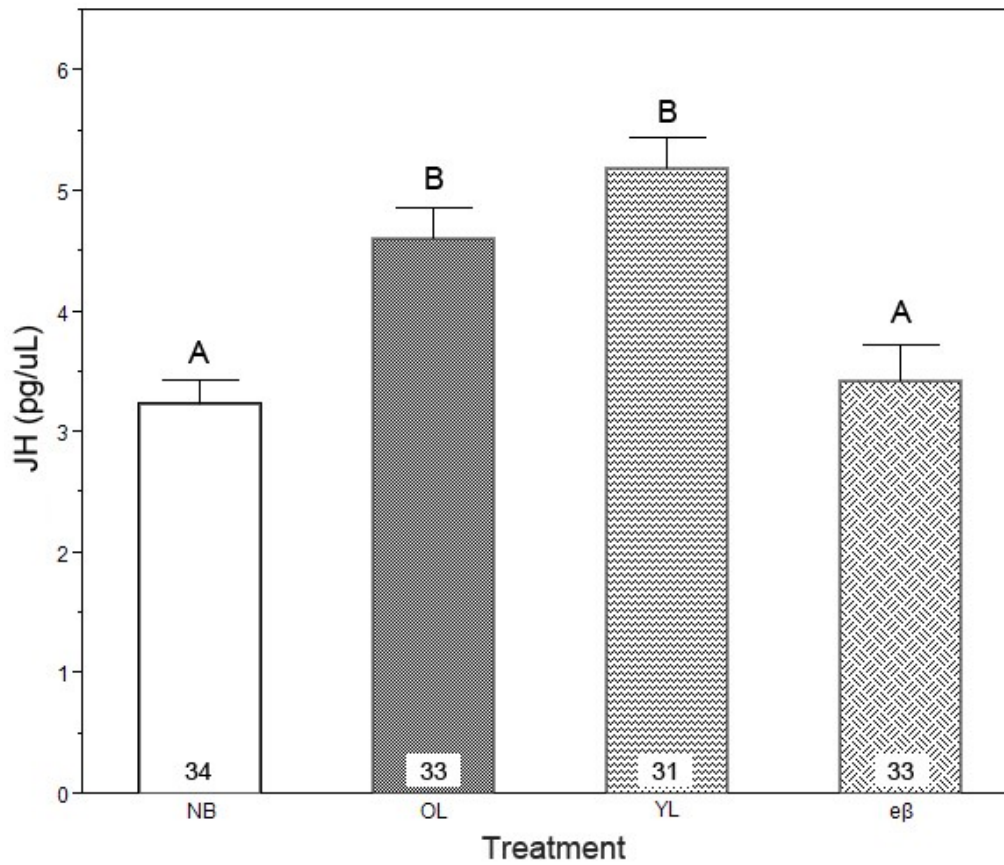
Figure 4.12. Juvenile hormone by treatment.

Mean Juvenile Hormone titers + S.E. as a function of early priming environment (No Brood = NB, Old Larvae = OL; Young Larvae = YL; e-beta ocimene = eβ). Significant differences ($\alpha < 0.05$) are indicated by different letters. Sample sizes are indicated.
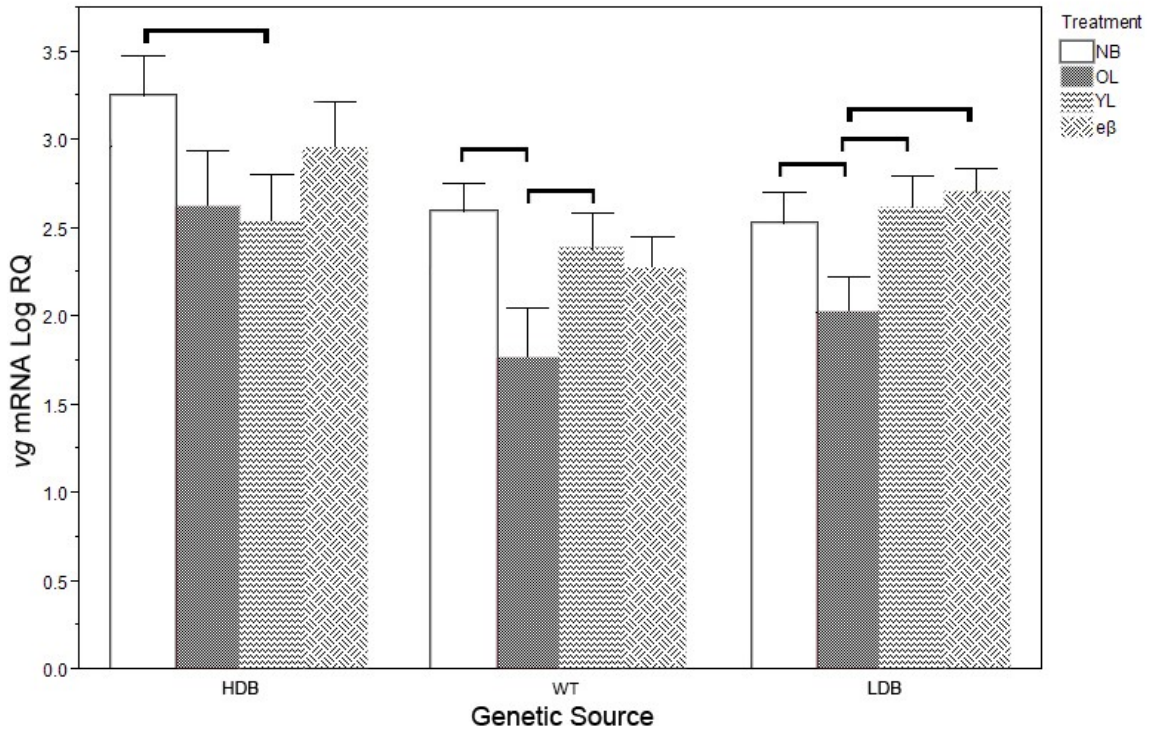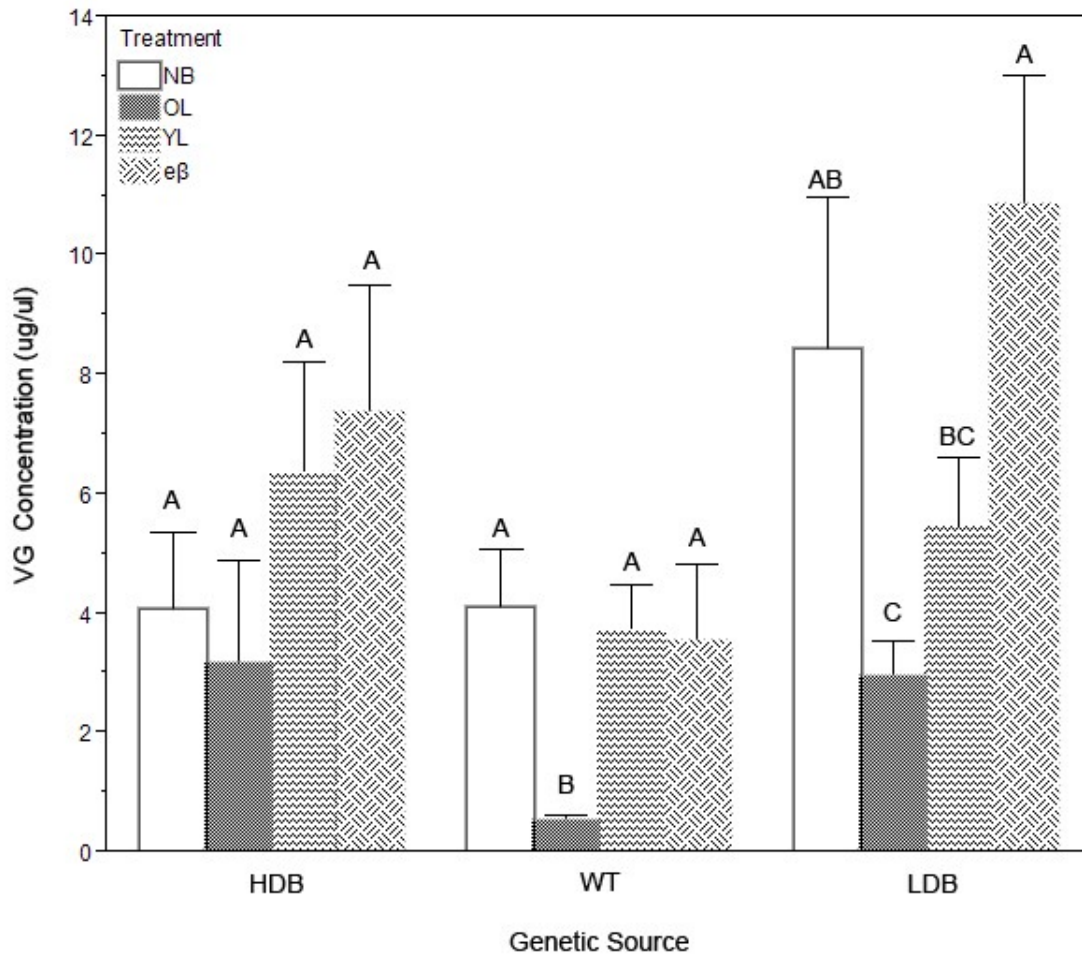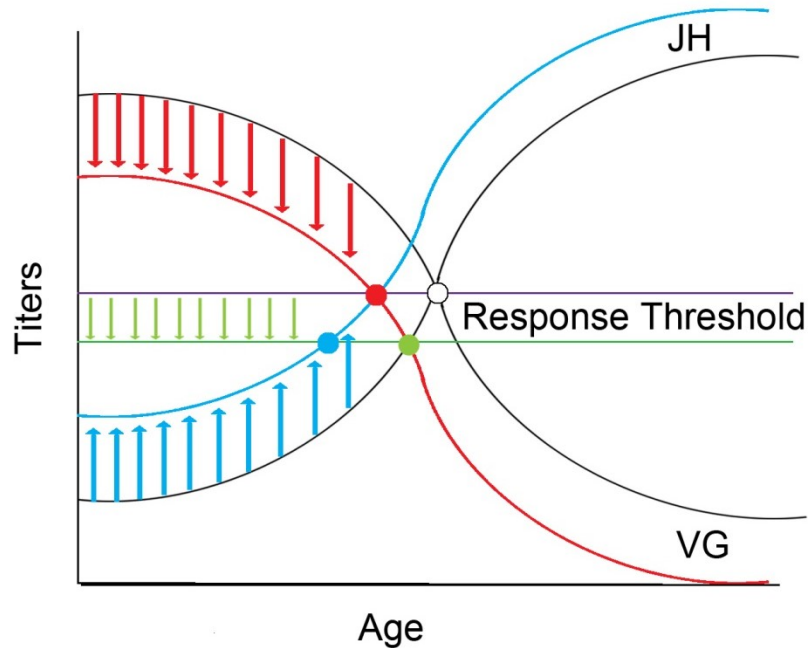
Figure 4.13. Model of early priming environment on age of first foraging.

Bees normally transition to foraging when their juvenile hormone (JH) titers rise above a

response threshold, as indicated by the white circle. However, the early environment

experienced by honey bees primes their development to ultimately influence their

foraging behavior, and bees that are sensitive to brood are more strongly influenced by

this early priming experience.

The presence of young larvae (YL) increases JH titers (blue arrows) in attending bees and

because YL simultaneously release e-beta ocimine (eβ), a pheromone that reduces the

response threshold (green line) in attending bees (green arrows), bees raised in a YL

environment are the first to transition from in-hive nursing tasks to outside foraging.

Bees that are raised in an old larvae (OL) environment have down-regulated vitellogenin (VG) titers (red arrows), which, through a negative feedback loop, induces an increase in JH titers (blue arrows). This again causes an earlier transition to foraging. However the OL environment does not impact the response threshold to rising JH titers. Thus they forage later than bees raised in a YL environment.

The eβ environment does not change the VG or JH titers of the bees, but it does modulate their response threshold downwards (green arrows), making them more sensitive to the same stimuli. Thus bees reared in an eβ environment transition to foraging earlier than bees raised in a broodless environment.

Table 4.1. 2x4 Contingency tables of foraging activity for each genetic source. Some bees never transitioned from in-hive tasks such as nursing to outside foraging during the 28-30 days of the experiment. The early priming environments had a significant effect on the foraging activity for both HDB and WT bees, but not for LDB bees. For both HDB and WT, fewer bees in the NB or eβ environment transitioned successful to foraging.

Table 4.1
2x4 CONTINGENCY TABLES OF FORAGING ACTIVITY BY GENETIC SOURCE

HDB	Foraged	Never Foraged	% Never Foraged
YL	607	49	7.5%
OL	635	28	4.2%
eβ	568	58	9.3%
NB	563	49	8.0%

$\chi^2 = 13.45$, df = 3, $P = 0.004$

LDB	Foraged	Never Foraged	% Never Foraged
YL	447	162	26.6%
OL	401	183	31.3%
eβ	464	191	29.2%
NB	406	191	32.0%

$\chi^2 = 5.14$, df = 3, $P = 0.162$

WT	Foraged	Never Foraged	% Never Foraged
YL	634	90	12.4%
OL	622	67	9.7%
eβ	587	106	15.3%
NB	656	111	14.5%

$\chi^2 = 11.43$, df = 3, $P = 0.010$

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Table 4.2. 2x3 Contingency tables of foraging activity for each priming environment. Regardless of early priming environment, genetic source had a highly significant effect on foraging activity. The LDB bees had significantly fewer bees transition successfully to foraging for all four priming environments.

Table 4.2
2X3 CONTINGENCY TABLES OF FORAGING ACTIVITY BY PRIMING ENVIRONMENT

YL	Foraged	Never Foraged	% Never Foraged
HDB	607	49	7.5%
LDB	447	162	26.6%
WT	634	90	12.4%

$X^2 = 96.48$, df = 2, $P < 0.001$

eβ	Foraged	Never Foraged	% Never Foraged
HDB	568	58	9.3%
LDB	464	191	29.2%
WT	587	106	15.3%

$\chi^2 = 91.13$, df = 2, $P < 0.001$

OL	Foraged	Never Foraged	% Never Foraged
HDB	635	28	4.2%
LDB	401	183	31.3%
WT	622	67	9.7%

$\chi^2 = 204.29$, df = 2, $P < 0.001$

NB	Foraged	Never Foraged	% Never Foraged
HDB	563	49	8.0%
LDB	406	191	32.0%
WT	656	111	14.5%

$\chi^2 = 128.33$, df = 2, $P < 0.001$

Table 4.3. Significant effects compared to the control of no brood.

For each characteristic measured, the three brood treatments (OL, YL, eβ) were compared against the control of NB. Only if there were significant differences by strain are the data provided for each genetic source. Significant differences (α < 0.05) are indicated by ↑ if upregulated and ↓ if downregulated in comparison to NB. For pollen foraging, YL and eβ had significantly more pollen foragers than OL, but none of the groups differed from the intermediate levels of pollen foraging seen in the control of NB. AFF = age of first foraging; Ovy # = total ovarioles; Ovy Dev = ovary development; HPG = hypopharyngeal gland development; VG = vitellogenin; JH = juvenile hormone, All = all strains; HDB = high strain derived bees; WT = wildtype; LDB = low strain derived bees.

Table 4.3 SIGNIFICANT EFFECTS COMPARED TO THE CONTROL OF NO BROOD											
	AFF				Proportion Foraged				Pollen Foraging	Ovy #	Ovy Dev	HPG
	All	HDB	WT	LDB	All	HDB	WT	LDB	All	All	All	All
OL	↓	↓	↓	NS	↑	↑	↑	NS	NS	NS	↓	↑
YL	↓	↓	↓	NS	↑	NS	NS	↑	NS	NS	↓	↑
eβ	↓	↓	↓	NS	NS	NS	NS	NS	NS	NS	NS	↑

	vg mRNA				VG protein				JH			
	All	HDB	WT	LDB	All	HDB	WT	LDB	All	HDB	WT	LDB
OL	↓	NS	↓	↓	↓	NS	↓	↓	↑	↑	↑	NS
YL	NS	↓	NS	NS	NS	NS	NS	NS	↑	↑	↑	↑
eβ	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

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