Ovarian Regulation of Honey Bee (*Apis mellifera*) Foraging Division of Labor

by

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ABSTRACT

There is increasing evidence that ovarian status influcences behavioral phenotype in workers of the honey bee *Apis mellifera*. Honey bee workers demonstrate a complex division of labor. Young workers perform in-hive tasks (e.g. brood care), while older bees perform outside tasks (e.g. foraging for food). This age correlated division of labor is known as temporal polyethism. Foragers demonstrate further division of labor with some bees biasing collection towards protein (pollen) and others towards carbohydrates (nectar). The Reproductive Ground-plan Hypothesis proposes that the ovary plays a regulatory role in foraging division of labor. European honey bee workers that have been selectively bred to store larger amounts of pollen (High strain) also have a higher number of ovarioles per ovary than workers from strains bred to store less pollen (Low strain). High strain bees also initiate foraging earlier than Low strain bees. The relationship between ovariole number and foraging behavior is also observed in wild-type *Apis mellifera* and *Apis cerana*: pollen-biased foragers have more ovarioles than nectar-biased foragers.

In my first study, I investigated the pre-foraging behavioral patterns of the High and Low strain bees. I found that High strain bees progress through the temporal polyethism at a faster rate than Low strain bees. To ensure that the observed relationship between the ovary and foraging bias is not due to associated separate genes for ovary size and foraging behavior, I investigated foraging

behavior of African-European backcross bees. The backcross breeding program was designed to break potential gene associations. The results from this study demonstrated the relationship between the ovary and foraging behavior, supporting the proposed causal linkage between reproductive development and behavioral phenotype. The final study was designed to elucidate a regulatory mechanism that links ovariole number with sucrose sensitivity, and loading decisions. I measured ovariole number, sucrose sensitivity and sucrose solution load size using a rate-controlled sucrose delivery system. I found an interaction effect between ovariole number and sucrose sensitivity for sucrose solution load size. This suggests that the ovary impacts carbohydrate collection through modulation of sucrose sensitivity. Because nectar and pollen collection are not independent, this would also impact protein collection.

DEDICATION

For my mother, Carole Jean Siegel, who has always been there for me. Your encouragement has been instrumental and I love you.

And for my cousin Ralph M. Siegel, Ph.D., who's continued pursuit of knowledge in the face of adversity, inspires me.

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My apologies to anyone left out by mistake.

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CHAPTER 1

INTRODUCTION TO OVARIAN CONTROL OF FORAGING BEHAVIOR

The mechanisms of evolution have lead to a remarkable diversity of life on our planet (Sadava 2008). One such mechanism is the co-option and reshaping of a trait to serve a novel function in a new context. Many scientists have studied the co-option and reshaping of anatomical traits. A classic example is the vertebrate forelimb which has been shaped through evolution to function as a walking, flying, swimming, and grasping limb (Krogh 2000). Few behavioral traits have been explored in this fashion.

Eusocial insects demonstrate an extreme form of behavioral task specialization, division of labor (DOL), which is believed to be a prime enabler for this group's ecological success (Oster and Wilson 1978). Honey bees are an often studied model eusocial system because of their highly organized division of labor and economic importance (Winston 1987, Graham et al. 1992, Seeley 1995, Page et al. 2006). This thesis explores the co-option and reshaping of a solitary insect foraging behavioral control mechanism to serve a new function controlling foraging task specialization in a social context.

The Reproductive Ground-plan Hypothesis (RGPH) provides an evolutionary framework for mechanisms controlling aspects of honey bee foraging division of labor between protein and carbohydrate collection. The RGPH

suggests that reproductively associated mechanisms that controlled foraging behavior during the life cycle of solitary honey bee ancestors have been co-opted and reshaped to control foraging DOL in facultatively sterile honey bee workers. Specifically, the ovary has been proposed as an organ with regulatory effects on behavior. There is much empirical evidence demonstrating a relationship between ovary status and foraging division of labor (Amdam et al. 2004, Amdam et al. 2006, Page et al. 2006), however; questions remain unanswered regarding regulation of foraging behavior.

This dissertation uses comprehensive behavioral and anatomical investigations to address several components of reproductively associated foraging behavioral control. First, the relationship between pre-foraging behavior and foraging behavior was studied in an observational study. Next, a behavioral and anatomical study of bees from a backcross breeding program was used to rule out the possibility that associated separate genes for reproductive anatomy and behavior could explain the observed relationship between the ovary and foraging bias. Finally, a proposed mechanism for ovarian control of foraging behavior through sucrose sensitivity modulation was tested using a rate-controlled artificial sucrose feeder. These investigations lead to a greater understanding of the transition from solitary to social bee as well as the control mechanisms of honey bee foraging division of labor.

IMPORTANCE OF HONEY BEES

Rock art depicts human honey collection as early as 6000 B.C.E. Humans have been actively cultivating bees in artificial hives since at least 5000 B.C.E. Today honey bees are kept in every region of the world excluding the extreme poles. In industrialized countries, the main focus of beekeeping is pollination (Graham et al. 1992). In the United States alone, bee pollination adds $15 billion in value to agricultural products on an annual basis (USDA 2010). Honey bee societies are also the most frequently used and comprehensively studied system for investigations on insect division of labor (DOL) and task specialization (Winston 1987, Seeley 1995, Page et al. 2006).

HONEY BEE LIFE HISTORY AND DIVISION OF LABOR

Honey bees demonstrate a complex and highly organized DOL. Honey bee reproductive DOL is facilitated by anatomically distinct female castes. A colony's single queen is the only reproductive female under normal colony conditions. A young queen will typically mate with many males (drones) and then spend the remainder of her life laying between 1000-2000 eggs daily. All other tasks are performed by facultatively sterile female workers. Within the worker caste, there is a temporally-associated task division of labor known as temporal polyethism. Young workers generally perform in-hive tasks such as brood care and nest construction. These are typically followed by transitional tasks such as nest entrance guarding. Finally, workers transition to outside tasks such as food

collection. Honey bee food foragers demonstrate additional task specialization. Individual foragers tend to bias their foraging efforts towards either nectar (carbohydrate) or pollen (protein) collection (Lindauer 1952, Winston 1987). The foraging efforts of the worker population result in carbohydrate and protein storage in the nest and are a major mechanism for plant pollination.

THE REPRODUCTIVE GROUND-PLAN HYPOTHEIS

The Reproductive Ground-plan Hypothesis (RGPH) is an evolutionary framework for the control of foraging DOL. This hypothesis is based on the earlier Ovarian Ground-plan Hypothesis of Mary Jane West-Eberhard (OGPH; 1987, 1996). The OGPH states that mechanisms controlling the behavioral life cycle of solitary ancestors of eusocial insects have been co-opted and selected upon, resulting in the distinct queen and worker female castes of extant eusocial insects. The RGPH extends this concept, suggesting that mechanisms controlling foraging DOL in honey bee workers are derived from mechanisms controlling food collection during the reproductive life cycle of solitary honey bee ancestors (Amdam et al. 2004, Amdam et al. 2006). Solitary insects go though a reproductive life cycle including both non-reproductive and reproductive life stages. The non-reproductive stage is characterized by inactive ovaries and carbohydrate collection. The reproductive stage is characterized by activated ovaries and protein collection (Chapman 1998, Clemets 2000). The RGPH proposes a relationship where the ovary has a regulatory effect on foraging bias

with bees with larger ovaries (more ovarioles) more likely to demonstrate a protein foraging bias, and bees with smaller ovaries (fewer ovarioles) more likely to demonstrate a carbohydrate foraging bias (Amdam et al. 2004, Amdam et al. 2006). Empirical evidence supports this hypothesis. Honey bees selected for high (High strain) and low (Low strain) pollen storage (Page and Fondrk 1995) have larger and smaller ovaries respectively. This relationship is also observed in wild type honey bee foragers (Amdam et al. 2006). Despite the support for the RGPH, an alternate explanation exists for these observed relationships. Separate but associated genes for ovary size and foraging bias would also explain the observed link between ovary size and foraging behavior. This dissertation addresses this issue.

Sucrose Sensitivity and Foraging

The work presented here suggests a foraging control mechanism involving ovarian regulation of sucrose sensitivity. Reproductive status, sucrose sensitivity, and sugar feeding are linked in many animal systems (Than et al. 1994, Curtis et al. 2005). A similar relationship is observed within non-reproductive worker honey bees; workers with more ovarioles are more sensitive to sucrose stimulation than those with fewer ovarioles (Tsuruda et al. 2008). Based on this relationshiop as well as relationships between ovary size, collected nectar sugar concentration and foraging bias presented in this dissertation, I hypothesized that the ovary

regulates sensory sensitivity which in turn impacts foraging decisions. This dissertation tests this hypothesis.

INVESTIGATIONS ON HONEY BEE DIVISOIN OF LABOR

In Chapter 2, I investigate the relationship between pre-foraging behavior and foraging initiation age. High strain bees store larger amounts of pollen, have larger ovaries (more ovarioles) and initiate foraging earlier than Low strain bees. To determine how pre-foraging behavior relates to the differences in foraging bias and foraging initiation age between the High and Low strain bees, I conducted a comprehensive observation-hive study of the pre-foraging behavior of the two strains as well as wild-type bees, and constructed age-based behavioral ethograms. High strain bees initiated and terminated different tasks significantly earlier than low strain bees, but did not disproportionately perform any tasks. Pollen consumption terminated earlier in the High strain bees, which may impact protein dynamics that have been shown to impact the timing of foraging initiation (Amdam et al. 2003, Nelson et al. 2007).

In Chapter 3, I investigated the possibility of associated separate genes for ovary anatomy and behavior as a potential explanation for the observed relationship between ovary size and foraging bias, the Associated Separate Gene Hypothesis (ASGH). To rule out the ASGH, the relationship between the ovary and foraging bias was investigated in bees where potential gene associations had

been broken using a backcross breeding design and within the High and Low strain populations. An interaction effect between ovariole number and nectar sugar concentration on foraging bias supported the RGPH, and refuted the ASGH. This relationship also suggested a potential mechanism where the ovary impacts foraging decisions through a modulation of sucrose sensitivity.

In Chapter 4, I investigated the proposed mechanism where the ovary impacts foraging decisions through a modulation of sucrose sensitivity. Bees were trained to collect from rate-controlled artificial sucrose feeders. Collected sucrose solution volume, sucrose sensitivity and ovariole number were measured for each experimental bee. An interaction effect between ovariole number and sucrose sensitivity on collected sucrose volume supports a mechanism where the ovary impacts nectar collection by impacting sucrose sensitivity. This in turn impacts pollen collection as nectar and pollen collection are not independent due to physical collection limitations (Page et al. 2000).

These results demonstrate a causal link between reproductively associated phenotypes and foraging division of labor in non-reproductive female honey bee workers. This supports the RGPH, an example of the cooption and reshaping of a behavioral regulatory mechanism to serve a new function in a novel context. This work sheds light on the transition from solitary to social insect. Finally, these studies elucidate a behavioral control mechanism for honey bee foraging, a scientifically interesting and economically important insect behavior.

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CHAPTER 2

CONSERVED PATTERNS OF TEMPORAL POLYETHISM IN HONEY BEES

Abstract: Honey bee workers exhibit an age-based division of labor (temporal polyethism), with younger workers specializing on within-nest tasks and older workers foraging outside the nest. Bees performing tasks within the nest transition through sets of tasks performed in different regions of the nest, while foragers specialize by biasing their foraging efforts toward pollen or nectar. The degree to which pre-foraging schedules of task performance can be altered by selection or environment is largely unknown. Additionally, it is unknown how variation in pre-foraging behavior may impact the transition from within-nest tasks to foraging. Honey bees selected for differences in stored pollen demonstrate consistent differences in the age at which they initiate foraging. Those selected for increased pollen storage (High pollen hoarding strain) initiate foraging earlier in life than those selected for decreased pollen storage (Low pollen hoarding strain). The selected strains have been used in numerous experiments on foraging behavior. Here, we investigate the timing and pattern of pre-foraging behavior to determine if a conserved pattern of temporal division of labor exists in honey bees and to further elucidate the mechanisms controlling foraging initiation. We found that High strain bees both initiate and terminate individual pre-foraging tasks earlier

than Low strain bees. Unselected commercial bees (wild type) generally demonstrated intermediate behavioral timing. There were few differences between genotypes for the proportion of pre-foraging effort dedicated to individual tasks though total pre-foraging effort differences differed dramatically. This demonstrates that behavioral pacing can be accelerated or slowed, but that the pattern of behavior is not fundamentally altered, suggesting a general pattern of temporal behavior in honey bees. Additionally, High strain bees terminated protein (pollen) consumption earlier in life than Low strain bees, perhaps contributing to an early decline in hemolymph (blood) vitellogenin (Vg) protein titers that can explain their early onset of foraging.

INTRODUCTION

Division of labor has been a central theme in evolutionary studies of social insects since Darwin (Darwin 1859). Honey bees demonstrate a complex division of labor. Reproductive division of labor in honey bees is demonstrated by anatomically distinct reproductive queens, and facultatively sterile female workers (Winston 1987). Among honey bee workers, there is an age-correlated behavioral division of labor, referred to as temporal polyethism. Young workers perform within-nest tasks including cell cleaning, brood care, food processing, and nest construction. As bees age, they transition to tasks such as nest entrance ventilation and entrance guarding. Finally, older bees progress to foraging outside

the nest for food (Lindauer 1952, Seeley 1982, Winston 1987, Seeley and Kolmes 1991). The final shift from within-nest tasks to foraging is one of the most easily recognized and commonly studied transitions, and is often used as a benchmark measure for the pacing of temporal polyethism. However, prior behavioral transitions are also essential to colony function and may have an impact on the transition to foraging (Seeley 1982, Calderone and Page 1991, Seeley and Kolmes 1991, Pankiw and Page 2001).

There is variation in the pacing of the hive to forager transition between individual bees, colonies, and genotype (Calderone and Page 1988, 1991). Variation in behavioral pacing between colonies and genotypes may be limited to shortening or extending of time spent performing within-nest tasks proportionally. Alternatively, some tasks could be skipped, disproportionately truncated or extended, or the order of task performance could be fundamentally different between populations of honey bees.

The first aim of this study was to determine if there is a fundamental pattern to temporal polyethism across honey bee populations. This information will lead to a better understanding of the constraints on temporal division of labor. To address this aim, we investigated the order of, total time spent on, and proportion of time spent on within-nest tasks in distinct honey bee populations. Populations investigated were wild type (unselected commercial bees), and two strains of artificially selected honey bees that demonstrate predictable differences

in foraging-onset. If the foraging onset differences between these populations result from proportional differences in pre-foraging task performance, this would suggest a general temporal behavioral program that can be accelerated or slowed down but not profoundly altered. Alternatively, disproportionate pre-foraging task performance extension, truncation, or task skipping between these groups would demonstrate that temporal polyethism is highly flexible and can be fundamentally different between isolated honey bee populations. This would suggest no general temporal pattern of behavior in honey bees.

Page and Fondrk (1995) selected for the amount of surplus pollen stored, in combs by colonies, creating the High and Low pollen hoarding strains. Selection was based on the methods developed by Hellmich et al. (1985). At the time of this study, High and Low pollen hoarding strains had undergone selection for 26 generations over 14 years with out-crossing every third generation. Both selection programs, Hellmich et al. (1985) and Page and Fondrk (1995), resulted in behavioral syndromes related to foraging. High strain bees from both selection programs collected and stored more pollen, and foraged earlier in life than Low strain bees (Calderone and Page 1988, Pankiw and Page 2001). In addition, Page and Fondrk (1995) High strain bees are more sensitive to sucrose than Low strain bees, and are willing to accept nectar of a lower sugar concentration (Pankiw and Page 1999). It is important to note that in both selection programs, founding queens originated from commercially available stocks and that these observed

behavioral relationships are present in non-selected, commercially available wild-type bees.

High strain bees from generation 7 of Hellmich et al. (1985) foraged approximately 1 day earlier than Low strain bees (Calderone and Page 1988). Calderone and Page (1991) conducted a study of pre-foraging behavior of bees from generation 8 of the Hellmich et al. (1985) strains and found few behavioral differences between them, consistent with the small difference in foraging onset. By generation 11, High strain bees of Page and Fondrk (1995) were foraging as many as 12 days earlier in life than Low strain bees (Pankiw and Page, 2001). Amdam et al. (Amdam et al. 2006, Amdam et al. 2010) have demonstrated that differences in the foraging behavioral syndrome are controlled by developmental processes that begin prior to the onset of adult life.

The second aim of this study was to bridge the gap between development and foraging onset. We attempted to meet this aim by comparing the protein feeding dynamics between the High and Low strain bees. Vitellogenin (Vg), a behavioral affecter protein, interacts with Juvenile Hormone to play a regulatory role on foraging initiation (Amdam et al. 2003, Amdam et al. 2007, Nelson et al. 2007). Significant differences in Juvenile Hormone levels between High strain (early foraging onset) and Low strain (late foraging onset) bees begin during larval development (Amdam et al. 2010). Vg titers are high in young workers, and decrease as they age (Rutz and Luscher 1974). Nelson et al. (2007) demonstrated

that elevated Vg inhibits the onset of foraging. High strain bees demonstrate both an earlier drop in Vg titers and a correspondingly earlier foraging onset compared to Low strain bees (Amdam et al. 2007). In young nurse bees, some of the protein (in the form of pollen) consumed (Crailsheim et al. 1992, Hrassnigg and Crailsheim 1998), is converted to Vg and incorporated into the brood food (proteinous glandular secretions produced by the hypopharyngeal glands). The brood food is then fed to developing larvae (Amdam et al. 2003). The earlier drop in Vg and initiation of foraging observed in High strain bees could be facilitated by either increased brood feeding, thereby depleting circulating Vg, or earlier termination of pollen consumption by High strain bees compared to Low strain bees.

Here, we describe the pre-foraging dynamics of the Page and Fondrk (1995) High and Low strain bees. We conducted an observation hive study comparing the age of transition through a series of tasks between High strain, Low strain, and unselected commercial bees. We then constructed temporal polyethism schedules of the High strain, Low strain, and unselected bees, with particular attention paid to the analysis of behavior that could impact Vg titers including pollen consumption and brood feeding. If one of these pre-foraging tasks impacts Vg titers, then this would be an example of an early life behavior having a pacing role for a later behavior, in this case foraging onset. Comparing the differences in the appearance and duration of the pre-foraging behavior of these strains will shed light on the fundamental nature of behavioral pacing in honey bees. Additionally,

investigating the protein feeding dynamics of the High and Low strains will help bridge the gap between development and foraging onset.

MATERIALS AND METHODS

This research was conducted in June and July of 2005 at the University of California at Davis Bee laboratory. The daily activities of individual High strain, Low strain, and unselected bees were observed over a 29-day period in a common hive environment.

Source of Bees

Focal bees were derived from the 26th generation of bees selected for area of pollen stored in the combs (Page and Fondrk 1995). Measurements of stored pollen revealed that Low and High pollen strain colonies stored 298 cm^2 of pollen [n=21] and 1049.2 cm^2 [n=14] respectively in the same comb area (Student's t-test, $p < 0.0005$). Commercial bees that were located near UC Davis were used for controls. Bees from three source colonies of each strain were used in this study. Two additional colonies of commercial origin served as the source of background bees in the experiment.

Bee Preparation

Combs of mature pupae from the source colonies were placed in an incubator (34ºC, 50% RH) overnight. Three hundred newly-emerged workers of each selected strain and the commercial controls were uniquely tagged with plastic numbered tags (Honig Müngersdorff) glued to the thorax. A paint mark (Testors Enamel) was placed on the abdomens to differentiate bees from two experimental replicates and facilitate identification of tagged bees when their thoraces were obscured in a comb cell (Seeley 1982, Seeley and Kolmes 1991). Tagged workers were introduced to a four-frame observation-hive (hereafter referred to as Hive 1, see below) within 12 hours of emergence. The marking procedure was repeated 24 hours later using 900 additional unique tags and abdomen marks, and the second group was introduced to a second four-frame observation-hive (hereafter referred to as Hive 2).

Observation-Hive Colony Setup

Two commercial colonies (not of the High or Low selected strains) were transferred to two four-frame observation hives and placed in an observation-hive shelter 6-7 days before the introduction of the tagged experimental bees. As has been used in previous studies, the unselected observation-hive colonies had adult workers covering both sides of 3 combs, approximately 2.25-2.50 combs of brood in all stages of development, 0.50-0.75 combs of pollen, and 1.0 comb of honey

(Calderone and Page 1988). A runway, with a glass top to allow for observations of exiting and returning bees, connected the observation hive with the outside of the observation structure. Petroleum jelly was applied on both ends on the inside of the glass bridge cover to minimize the number of bees walking upside-down.

Nest Activity Observations

Daily observations of distinct honeybee behavioral task categories were recorded on 26 days over a 29-day period beginning on the third day of adult life for the bees in Hive 1 and the second day of adult life in Hive 2. A behavioral catalog, derived from multiple sources (Seeley 1982, Winston and Punnett 1982, Kolmes 1985, Robinson 1987, Calderone and Page 1991, Seeley and Kolmes 1991, Seeley 1995, Calderone and Page 1996, Fondrk Personal Communication, Page Personal Communication), was used to categorize observed hive behavior (Table 2.1).

Each side of the observation-hive colony was overlaid with a transparent plexi-glass grid of 128 squares that were approximately 60 x 60 mm. Each runway was overlaid with a transparent plexi-glass grid of 20 squares of the same dimensions. Each square was assigned a unique number, and observation recordings were based on randomized lists of these numbers created using a randomized sequence generator (www.random.org). Half of the squares on both sides of each hive and the bridge were observed each day. For each observation,

the bee identification code, behavior code from Table 2.1, and in-hive location of the tagged bee nearest to the center of the observed square was recorded. No square was observed twice on a given day. Observations were performed on one comb and one side at a time for convenience and to allow for more data to be collected in a given period of time. The daily order of hive, hive-side, and comb was determined by a coin toss. To avoid bias, the unique tag identification codes assigned to each genetic strain were not revealed to the observer until after all data were recorded.

Brood Care (BC) and Inspecting Brood (IB) were combined for statistical analysis, as were Nest Care (NC) and Construction (CT). The initiation and termination age for each task category was compared for the two strains and commercial control bees using a log-rank test. The proportion of total pre-foraging effort that was dedicated to each individual task by each strain was calculated using the following procedure: first, the total number of times each individual was observed performing an individual task was summed. This sum was then divided by the total number of times the individual bee was observed performing all tasks. A Kruskal-Wallis Test was then used to compare the proportions of the individuals for each task across the three strains. Mann-Whitney U tests were used to make comparisons between the two selected strains. Non-parametric analyses were performed because the data were not normally distributed.

Foraging Activity Observations

Foraging behavior of marked bees was observed to determine at what age bees of each strain initiated foraging. Observations took place at the glass-topped bridge that connected the hive to the outside of the observation shelter. Prior to bridge observations, the outside of the hive was observed daily for five minutes to determine if marked bees were leaving the hive vicinity, or performing pre-foraging orientation flights in front of the hive. No data were collected when bees were observed performing orientation flights.

Foraging data collection began when tagged bees were observed leaving the immediate vicinity of the hive. Twenty-minute observations of bees leaving and returning were conducted on each hive every second day beginning on the 9th day of adult life and continuing to the end of the experiment. The following information was collected for each bee: the bee identification code, whether it was leaving or returning to the hive, whether it was returning with or without pollen. If a bee returned carrying a pollen load it was classified as a pollen forager. If a bee returned without a pollen load it was classified as a non-pollen forager. It is not possible to differentiate between nectar, water, or empty returning foragers without using destructive sampling. Workers that left and returned within five minutes were excluded from the forager category as a round trip of less than 5 minutes suggests an orientation flight (Sekiguchi and Sakagami 1966, Winston and

Katz 1982, Robinson 1985). A contingency table G-test was used to compare strains for the proportion of bees returning with pollen. A Student's t-test was used to compare strains for the mean foraging initiation age.

RESULTS

As has been previously demonstrated, High strain bees were more likely to return to the hive with pollen loads than were Low strain bees (Contingency Table G-test, Figure 2.1; (Page et al. 1998, Amdam et al. 2004, Amdam et al. 2006, Page et al. 2006). High strain bees also initiated foraging at a significantly younger age (5.3-5.5 days depending on replicate) than Low strain bees (Student's t-test, Figure 2.2(Page et al. 1998, Page et al. 2006). In contrast to findings by Calderone and Page (1991), we found several significant strain differences in pre-foraging behavior in addition to the expected differences in pollen collection and foraging initiation age. Self grooming, nest care, food care, manipulating brood comb, manipulating honey comb, brood care tasks, head insertion into pollen cells, and standing in the nest were frequently observed (refer to Table 2.1 for task descriptions). High strain bees initiated and terminated several of these tasks earlier than Low strain bees, and wild type bees generally demonstrated intermediate initiation and termination ages (Log-rank test, N_{min}=11, N_{max}=113, N_{median}=56.5, Figure 2.3). In both hive replicates, there were significant differences among the three groups tested for median initiation age for self

grooming, patrolling, food care tasks, manipulating brood comb, and brood care tasks. In a single replicate there were significant differences in initiation age for nest care tasks, manipulating honey comb, and standing (Log-rank test, Figure 2.3). In both replicates, there were significant differences among the three groups tested for median termination age for self grooming, nest care, patrolling, food care, and brood tasks. In a single replicate there were significant differences in termination age for manipulating brood comb, and manipulating honey comb. A two-way comparison of task groups between the High and Low strain shows additional significant differences in nest care initiation age (Replicate 2: Mann-Whitney U test, $Z= -2.19$, $P<0.05$), and manipulation of brood comb termination age (Replicate 1: Mann-Whitney U test, $Z= -2.19$, $p<0.05$). These trends demonstrate a faster rate of transition between tasks in the High strain bees and are consistent with their earlier foraging age.

Of particular note, when a comparison was performed between only High and Low strain bees, the High strain bees were shown to terminate the behavioral category 'observed head in pollen cell' (HP) significantly earlier than the Low strain bees in one of the replicates, as would be predicted if the earlier drop in Vg observed in High strain bees was a direct result of earlier termination of protein consumption (Replicate 1: One-tailed Mann-Whitney U-test, $Z= -1.73$, $p<0.05$; Replicate 2: One-tailed Mann-Whitney U-test, $Z=-0.078$, $p >0.05$).

While there were differences between the strains in initiation and termination age, there were few significant differences for the proportion of pre-foraging effort dedicated to task groups between the strains (Kruskal-Wallis Test, Figure 2.4, refer to methods for full calculation procedure). Only one rarely observed task category, 'manipulating honey comb' (TH) demonstrated a consistent difference in proportion across replicates. Of particular note is the lack of inter-strain differences for brood care (BC, Figure 2.4).

DISCUSSION

High strain bees demonstrated a strong tendency to initiate and terminate tasks earlier than Low strain bees. This suggests a constant, faster rate of transition between tasks in the High strain bees. This is consistent with the previous studies of behavioral transition rates of bees independently selected for pollen storage (Calderone and Page 1988, 1991), as well as the earlier foraging age demonstrated by High strain bees compared to Low strain bees (Page et al. 1998, Page et al. 2006, Amdam et al. 2007). In contrast to these findings, there were few significant differences for observed behavioral performance as a proportion of total within-nest activity. Additionally, all observed tasks were performed by both strains and the controls. This suggests that the task performance distribution requirements are similar across the strains, and that observed differences are due to

variation in the rate of task transition, rather than to changes in the order or proportion of effort associated with each task.

The results presented here, strongly support temporal polyethism as an organizational principal for task performance. Genetically differentiated groups made transitions at different times even though they shared a common hive environment, demonstrating task transition rates are intrinsically controlled (Calderone and Page 1988, 1991, Pankiw and Page 2001). This view was challenged by the "Foraging for Work" hypothesis of Tofts and Franks (Tofts and Franks 1992, Johnson 2010). Tofts and Franks proposed that the apparent pattern of temporal polyethism was an artifact of young bees moving out of the central brood nest towards the periphery of the hive in search of tasks to perform, rather than the consequence of an intrinsic behavioral pacer as is demonstrated by the data presented here.

Our results further suggest a mechanism through which protein consumption dynamics may have a regulatory affect on foraging initiation age. An earlier decrease in Vg in High strain bees compared to Low strain bees has been demonstrated and shown to be associated with earlier foraging initiation (Amdam et al. 2007, Nelson et al. 2007). Two non-mutually exclusive hypotheses can explain the faster rate of Vg decline in High strain bees. First, the faster decrease in Vg titers observed in High strain bees could be due to a higher proportion of their pre-foraging time spent feeding brood food to larvae compared to the Low strain

bees. Second, the faster decrease in Vg titers might be a result of earlier termination of pollen consumption in High strain bees compared to the Low strain bees. Pollen consumption is a primary source for protein in young workers (Crailsheim et al. 1992, Hrassnigg and Crailsheim 1998). Much of the consumed protein is converted to Vg, which is a major component of the protein rich jelly used to feed larvae (Amdam et al. 2003); therefore feeding larvae depletes circulating Vg.

There was no difference in the proportion of time spent on brood care tasks between the High and Low strains, showing that the earlier decrease in Vg in the High strain bees is unlikely to be due to increased larval feeding. A two-way comparison between the High strain and Low strain bees for the behavioral category 'observed head in pollen cell' (HP) demonstrated that the High strain bees terminated pollen consumption earlier in both of the replicates, though statistically significant in only one replicate. This result suggests that earlier termination of pollen consumption is likely a contributing factor to the earlier decrease in Vg titers observed in High strain bees and their subsequent earlier foraging.

CONCLUSIONS

Studies of the transitional differences between High and Low strain honey bees are central to current research on the evolution of division of labor in social

insects (Page and Fondrk 1995, Pankiw and Page 1999, Page et al. 2000, Pankiw and Page 2000, 2001, Amdam et al. 2004, Amdam et al. 2006, Page et al. 2006, Amdam et al. 2007). This study demonstrates that differences in the worker age of transition to foraging are a consequence of the time spent performing each task being shortened or lengthened proportionally. From this, we conclude that task performance requirements across genetically distinct strains of honey bees under similar environments are similar and task performance effort is distributed accordingly. High and low strain bees, as well as wild type controls, have different intrinsic rates of behavioral maturation reflected in changes in the tasks they perform, but no major differences in the pattern of temporal changes. This suggests that within nest task transitions are linked and cannot be readily disassociated. Finally the data suggest that likely a mechanism for the observed differences in timing of the onset of foraging involves the timing of cessation of pollen consumption, thereby reducing circulating titers of vitellogenin.

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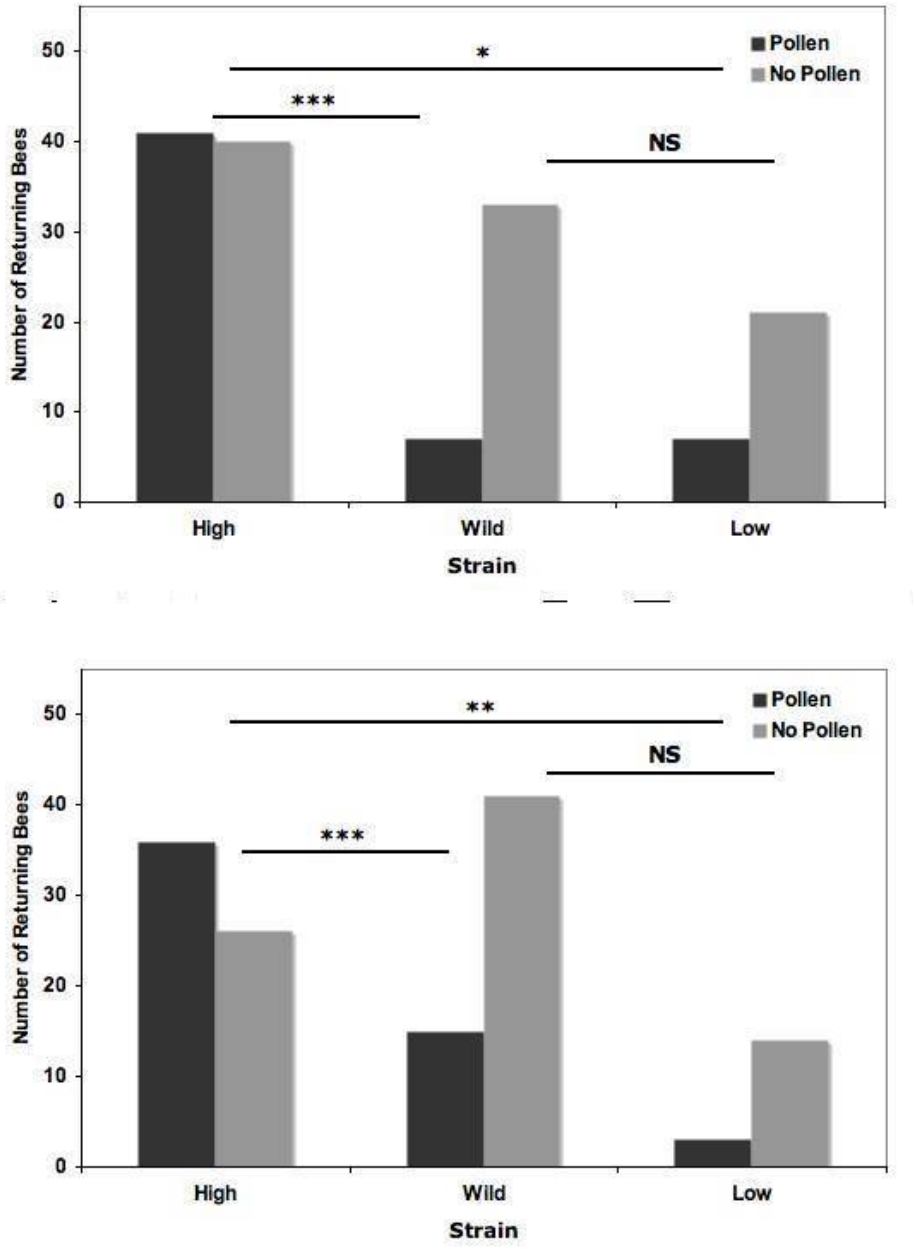

Figure 2.1. Number of foragers of each strain returning with and without pollen. Replicate 1 (top); Replicate 2 (bottom). High strain bees are more likely to collect pollen than Low strain bees (Contingency Table G-test, *p<0.05, **p<0.01, ***p<0.001, NS=Not Significant).

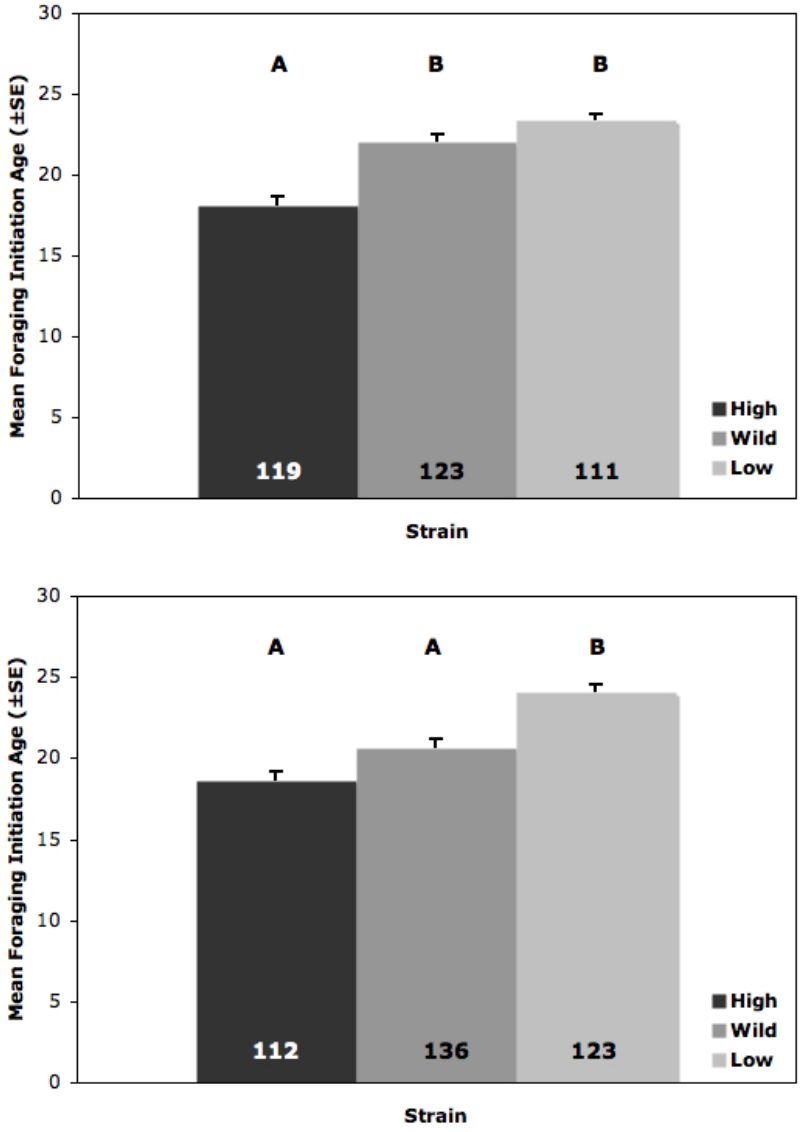

Figure 2.2. Mean (+SE) foraging initiation age for bees of each strain. Replicate 1 (top); Replicate 2 (bottom) 2. High strain bees forage earlier than Low strain bees in both replicates (Student's t-test, Letters represent significant difference $p < 0.0001$).

Figure 2.3. Median task initiation and termination age for bees from each strain. Replicate 1 (top); Replicate 2 (bottom). Left hand stars represent significant difference in initiation age. Right hand stars represent significant difference in termination age. Refer to Table 2.1 for task codes (Log-rank test; N_{min}=11, N_{max}=113, N_{median}=56.5; *p<0.05, **p<0.01, ***p<0.005, ****p<0.001, *****p<0.0001).

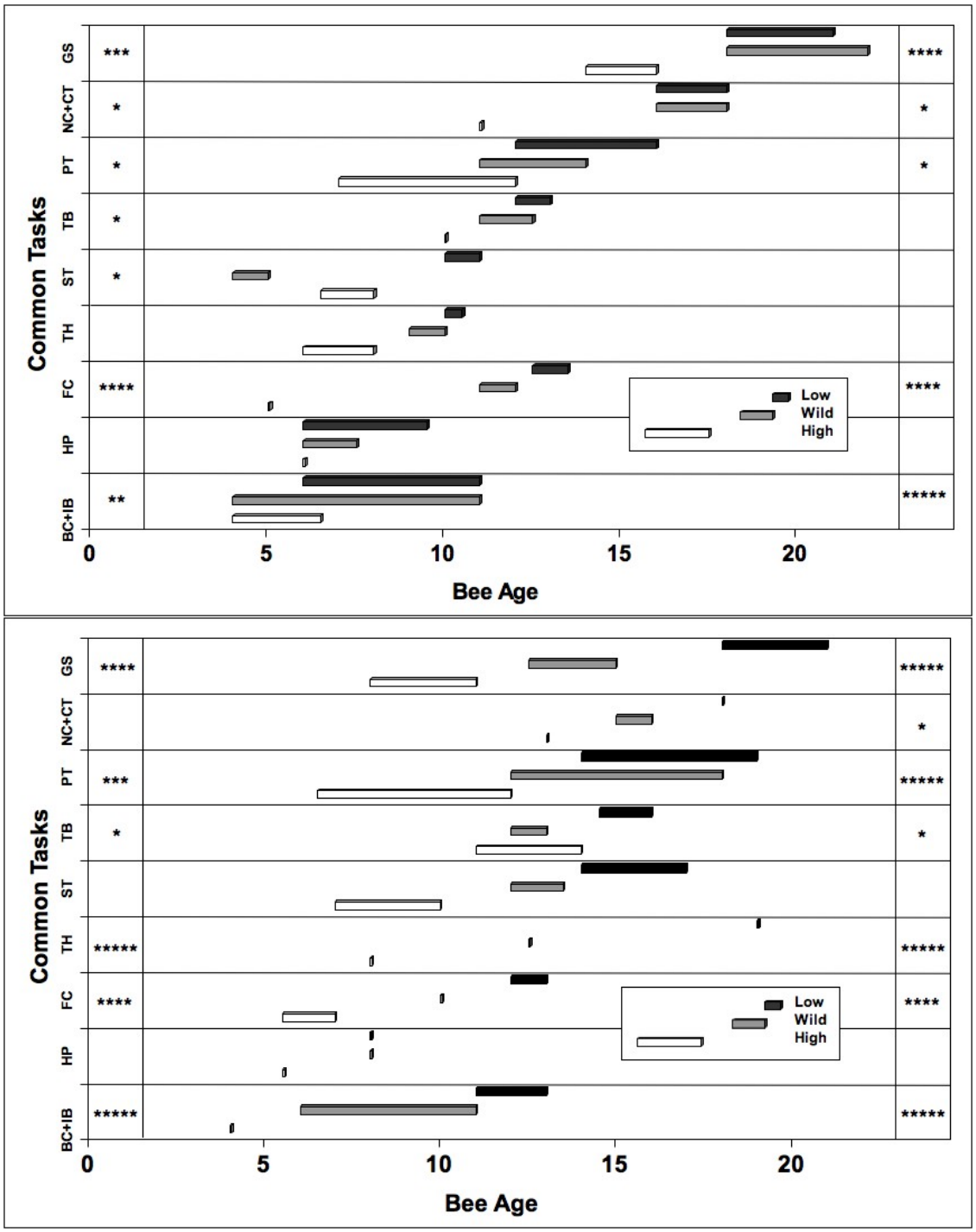

Figure 2.3, continued

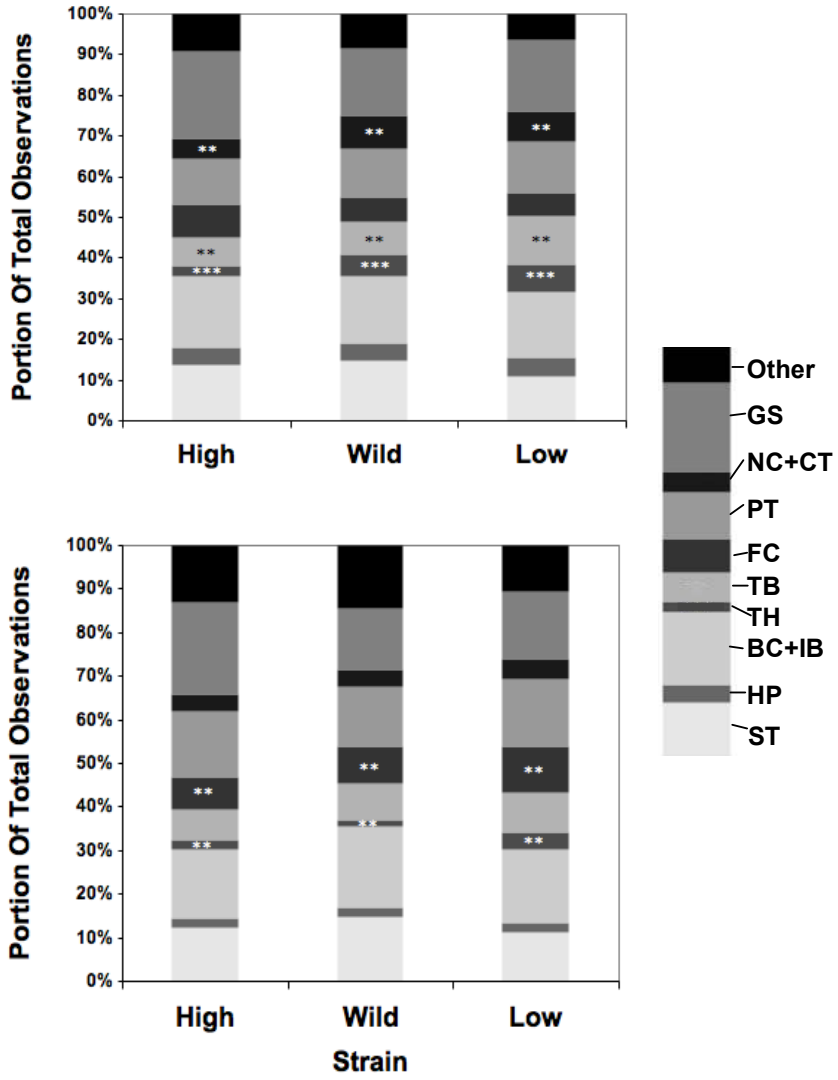

Figure 2.4. Proportion of observations bees of each strain were observed performing most common tasks. Replicate 1 (top); Replicate 2 (bottom). Most tasks show no difference in proportion of times individuals were observed performing a task. Refer to Table 2.1 for task codes (Kruskal-Wallis Test; N_{min}=11, N_{max}=113, N_{median}=56.5; *p<0.05, **p<0.01, ***p<0.001).

Table 2.1. Behavioral catalog including task codes.

Table 2.1

BEHAVIORAL CATALOG WITH TASK CODE DESCRIPTIONS

TASK	CODE	DESCRIPTION
Cell Cleaning	CC	Removing debris from used brood cells (cocoons, larvae excretion), cleaning cell walls. Takes place in a cell not currently being used.
General Nest Sanitation	NS	Removing debris from nest (moldy pollen, old cappings, dead brood, and dead adults).
Brood Care	BC	Feeding larvae (head in brood cell >1.3min), attending queen.
Construction	CT	Smoothing wooden hive parts with mandibles and manipulating wax and propolis in cracks and corners of the hive.
Fanning Wings	FA/FAe	Flapping wings while standing in hive/at entrance.
Food Care	FC	Insertion of head into a cell containing nectar, receiving nectar-on bridge.
Grooming a Nestmate	GG	Running nestmate body parts through mandibles.
Grooming Self	GS	Running own body parts through mandibles
Inspecting a Cell	IC	The momentary insertion of the anterior portion of the head into an empty cell.

Table 2.1, continued

TASK	CODE	DESCRIPTION
Nest Care	NC	Manipulating wax of cells (not cappings), building new empty cells.
Patrolling	PT	Walking around nest.
Standing and Chaining	ST	Standing stationary or hanging while stationary on nestmates.
Brood Cap Manipulation	TB	Trimming or smoothing wax cappings on brood cells and capping brood with wax.
Honey Cap Manipulation	TH	Trimming or smoothing wax cappings on cells of honey and capping honey with wax.
Trophallaxis	TR	Nestmate exchange of food (not near entrance), receiver thrusts tongue at donators mouthpart, donator opens mouthparts pushes tongue forward, and regurgitates a drop which is lapped up.
Vibrating	VB	Fast rhythmic body vibrations (non-dance).
Head in Pollen	HP	Insertion of head into a cell containing pollen.
Inspecting Brood	IB	Head in brood cell, < 1.3 min.
Dancing	DA/DA+	Dancing without/with pollen.
Washboarding/ Plaining	WA	Standing and rocking back and forth with mouthparts open.

Table 2.1, continued

TASK	CODE	DESCRIPTION
Attending Dance	AD/AD+	Dance attendance without/with pollen.

CHAPTER 3

CONFIRMATION OF OVARIAN REGULATION OF FORAGING DIVISION OF LABOR IN BACKCROSS HONEY BEES

Abstract: Division of labor (DOL) is the hallmark of social insects and has fascinated natural historians since Aristotle (Aristotle 350 B.C.E.). Honey bees have played a central role in scientific investigations of insect sociality because they demonstrate a highly organized DOL. One of the most studied components of honey bee DOL is foraging specialization through collection of protein (pollen) or carbohydrates (nectar). The Reproductive Ground-plan Hypothesis (RGPH) of Amdam et al. (2004, 2006) proposes that this foraging DOL is regulated by the same networks that controlled foraging behavior during the reproductive life cycle of the solitary ancestors of honey bees. Based on observed differences in foraging behavior and reproductive anatomy between bees selected for storing high and low quantities of pollen (the High and Low pollen hoarding strains of (Page and Fondrk 1995), and observed differences between wild-type pollen and nectar foragers, the RGPH suggests that ovary size is causally linked to variation in foraging behavior. An alternative explanation for an observed link between ovary size and foraging behavior is that genes for ovarian development and foraging are inherited together due to genetic linkage or chance gene associations in the selected and natural populations. To address this alternative explanation, we investigated

the relationship between ovary size and foraging bias in honey bees where genetic linkage was broken using a backcross breeding design. We also studied High and Low strain bees where ovary size effects were partitioned from other potential linkage effects. We found that ovariole number was related to foraging bias in the backcross bees and the selected strains as would be predicted by the RGPH. An interaction effect between nectar sugar concentration and ovariole number was observed in the backcross and High strain bees. This result suggests a mechanism by which the ovary affects sucrose perception, which in turn influences foraging behavior.

INTRODUCTION

Eusocial insects demonstrate a highly derived form of differential task performance referred to as division of labor (DOL) between nestmates (Wilson 1975, Oster and Wilson 1978, Pankiw and Page 2000). Honey bees are a model system because they demonstrate several distinct kinds of division of labor (Winston 1987, Seeley 1995, Page et al. 2006). The most easily recognized is the reproductive division of labor between castes of anatomically distinct females. A colony typically contains an egg-laying queen and several thousand facultatively sterile female workers. Workers demonstrate an age-correlated behavioral division of labor referred to as temporal polyethism. Young workers generally perform in-hive tasks (e.g. brood care) and older workers perform outside tasks (e.g. foraging

for food). During the late-life task of food collection, foragers demonstrate an additional level of differential task performance. Individual food foragers often specialize by biasing their foraging efforts toward pollen (protein) or nectar (carbohydrate) collection (Winston 1987). Variation in foraging decisions among nestmates contributes to a balance of protein and carbohydrate food storage in the hive.

Recent studies have described a regulatory mechanism of foraging DOL (Amdam et al. 2004, Amdam et al. 2006). This mechanism fits well into an evolutionary paradigm suggesting that mechanisms regulating honey bee foraging DOL are derived from the mechanisms controlling foraging during the reproductive life cycle of solitary ancestors. In solitary insects, the non-reproductive life stage is characterized by inactive ovaries and carbohydrate feeding. Alternatively, the reproductive life stage is characterized by activated ovaries and protein collection for egg production and provisioning of larvae (Chapman 1998, Clemets 2000). The hypothesis, known as the Reproductive Ground-plan Hypothesis (RGPH), proposes that the ovary of the facultatively sterile worker has a regulatory affect on foraging bias where bees with larger ovaries are more likely to bias foraging towards protein collection (Amdam et al. 2004, Amdam et al. 2006).

Empirical evidence gathered to date supports the RGPH. Honey bees can be selectively bred to exhibit skewed foraging patterns. Page and Fondrk (1995) selected for two extremes of colony pollen storage (hereafter referred to as High

and Low strains). Compared to Low strain workers, those of the High strain store more pollen in the nest. A suite of additional behavioral and physiological changes that result from such selection suggest a possible control mechanism regulating components of honey bee foraging decisions. Compared to Low strain workers, those of the High strain collect more pollen and less nectar, are more responsive to low concentrations of sugars, are more likely to lay eggs in the absence of a queen, and have ovaries composed of more ovarioles (filaments where eggs develop in a reproductively active female; (Amdam et al. 2004, Amdam et al. 2006, Page et al. 2006). A similar relationship between ovariole number and foraging behavior is observed in wild-type *Apis mellifera* and *Apis cerana*; pollen-biased foragers have more ovarioles than nectar-biased foragers (Amdam et al. 2006, Rueppell et al. 2008). Africanized honey bees (AHB) also demonstrate a higher pollen bias and higher average ovariole numbers than European honey bees (EHB; (Pankiw and Page 2003).

This commonality suggests a general physiological link between ovarian development and foraging division of labor. However, there are alternative explanations. Independent genes for variation in the ovary and behavior may be genetically linked and/or associated by chance and inherited together. In the haplodiploid sex-determination system of honey bees, workers receive all of their father's genes, increasing the chance of allelic co-inheritance (Page and Laidlaw 1988). The High and Low strain bees were initially founded with distinct,

relatively small populations. This could have lead to a decrease in allelic diversity in the two populations and reduced variation due to genetic drift. If there are separate genes for foraging bias and ovary size, High strain bees could have been selected from an initial population that by chance had many individuals with alleles for more ovarioles. Low strain bees could have been developed from an initial population that by chance had many individuals with alleles for fewer ovarioles. Over time there could have been fixation through genetic drift of the more common alleles for ovariole number in the two populations (Crow and Kimura 1970). Hereafter, this will be referred to as the Associated Separate Genes Hypothesis (ASGH). AHB and EHB are also derived from separate populations and their ancestral populations were likely exposed to very different environments. There could have been genetic drift and/or differential selection in these populations impacting foraging behavior and ovariole number.

To test the ASGH, we performed a backcross of hybrid (AHBxEHB) queens to AHB drones of the same parental line (Figure 3.1). The backcross design facilitated the reshuffling of genes due to recombination during prophase I of meiosis in the hybrid queens, thus decreasing the probability of chance associations between genes affecting ovariole number and foraging traits. In addition, the breeding program resulted in backcross workers derived from different queen sources that were highly related (G=0.5625; (Pamilo and Crozier 1982), but varied greatly in mean ovariole number. We then investigated the

foraging behavior and ovariole number of bees from two highly related backcross colonies that demonstrated high variation in ovariole number. If the observed relationship between ovariole number and foraging behavior is causal (the RGPH), we expected to see a relationship between ovariole number and foraging bias in the backcross bees. If the observed relationship is not causal (the ASGH), we expected to see no relationship.

In addition, we investigated the relationship between foraging behavior and ovariole number within the High and Low strains. Selection for pollen hoarding in closed populations of High and Low strain bees for more than 26 generations should have reduced allelic variation for genes influencing ovariole number (Laidlaw and Page 1997). Variation in ovariole number within strains should be primarily due to environmental effects on developing larvae, not differences in ovary genotype. If there is a causal relationship between ovariole number and foraging bias (the RGPH), ovariole number should still correlate with foraging bias within the selected strains. If the relationship is not causal (the ASGH), there is no expectation that environmental variation in ovariole number will correlate with foraging bias.

MATERIALS AND METHODS

The relationship between ovariole number and foraging bias was investigated using (EHBxAHB)xAHB backcross bees and bees from the High and

Low pollen hoarding strains. The backcross breeding program was designed to re-assort chance associations between genes. EHB were *Apis mellifera L.* commercial colonies. AHBs were from feral colonies captured in Mesa, AZ. The observations were conducted March-May 2007 at Arizona State University in Tempe, AZ. Two non-simultaneous replicates were performed.

Backcross Preparation

To develop the backcross bees, workers from twelve EHB and twelve AHB colonies maintained at the ASU Bee Laboratory in Mesa, AZ were screened for the average number of ovarioles. An EHB colony (worker ovariole mean= 6) and an AHB colony (worker ovariole mean=8) were chosen to produce an EHB x AHB hybrid cross. Hybrid queens were raised and each backcrossed to an AHB male derived from the original drone mother (Figure 3.1). Two of the resulting colonies (designated Y75 and Y84) were chosen as experimental sources of workers. Workers from these two colonies were selected because they consistently differed in ovariole number for their workers (Y75 mean= 12.46, Y84 mean= 16.43, n=343, 193; Mann-Whitney U test, $Z= -6.43$, $p < 0.0001$). Colonies from the backcross population had workers with more ovarioles on average than either of the original parental colonies and had higher variance in ovariole number within and between colonies (Linksvayer et al. 2009).

Combs from each source (backcross, High, Low) were transferred to a common incubator (35ºC, 50% RH) one day before emergence of adult workers. Newly emerged bees were marked with paint on the thorax (Testors Enamel); a unique color was used for each source. Marked workers were introduced to a Langstroth nucleus hive containing an unrelated wild-type colony from a commercial source. Over two-day periods, newly-emerged workers from each source were introduced to the first (Y75 = 285, Y84 = 105, High = 200, Low = 200) and second (210 for each source) replicates. Differences in introduction number were due to varied worker emergence rates between the sources.

Background colony maintenance

Background colonies consisted of open-mated, queen-right commercial colonies in standard 5-frame Langstroth nucleus hives. The hives contained approximately 2.25-2.50 combs of brood in all stages of development, 0.25-0.50 combs of pollen, 1.0 comb of honey, and one empty comb. Hives were managed to maintain empty space in the colony for egg laying and food storage.

Foraging behavior

The entrances of the experimental hives were observed daily over a one month period for at least 2 hours. Paint-marked foragers were collected in wire cages at the hive entrance on the first day they were observed foraging. Collection began in

the morning when foraging flights were regularly observed. Collection was discontinued 30 minutes prior to the estimated initiation time of orientation flights. The previous day's orientation flight initiation time was used as the estimate. Foragers were narcotized using carbon dioxide within 30 minutes of capture. All foragers were kept in the shade between capture and narcotization. After narcotization, pollen loads were collected from one leg and weighed. The right leg load was used unless it was missing. Weights were doubled to determine an estimated total pollen load. After pollen load weight was determined, the nectar loads were expressed from the crop into pre-weighed glass capillary tubes by gently squeezing the bee from the tip of the abdomen to the base of the thorax. Nectar loads were weighed and the sugar content was estimated using a digital refractometer (Misco) to determine a BRIX (percent solids) score.

Ovariole counts

After measuring foraging loads, bees were further anesthetized by placing them in a refrigerator (~4ºC). Bees were then pinned though the thorax on a wax plate. Ovaries were removed under magnification and, the individual ovariole filaments were counted.

Analysis

An index of foraging bias was created by determining the proportion of pollen collected relative to the entire foraging load of a worker (hereafter refereed to as pollen proportion). This was calculated by dividing the collected pollen load weight by the total foraging load weight (nectar and pollen). A generalized linear model was constructed to analyze the impact of various factors on the proportion pollen in the foraging load. The full factorial model included ovariole number, genotype (colony source), sugar concentration of collected nectar, and observation replicate. With ovariole number in the model, genotype (colony source) tested for potential genetic effects on foraging bias that were additional to ovary size. Observation replicate tested for environmental conditions that may have changed between the two replicates. Interaction effects were also included, as ovary effects and nectar concentration effects would not be expected to be independent if the ovaries are influencing foraging behavior through modulation of the response to sugar concentration of nectar. A revised model included ovariole number, nectar sugar concentration, replicate, and interaction effects.

RESULTS

Backcross Bees

In the Africanized backcross bees, the full regression model showed no genotype effects that were additional to ovary size on the pollen proportion (Table 3.1), therefore, genotype was dropped from the model. The revised model demonstrated significant interaction effects between ovariole number and nectar sugar concentration, and between ovariole number, nectar sugar concentration, and replicate on the pollen proportion (Table 3.2). Because there was no significant impact of genotype on foraging bias, bees from the different sources were pooled and split into two groups based on median ovariole number for further analysis. There was a significant difference in the loading of nectar in response to sucrose concentration by bees in the higher and lower ovariole number groups (ANCOVA, $F_{98,95} = 14.79$, $p < 0.001$, Figure 3.2), with those having fewer ovarioles being more likely to collect nectar at lower concentrations.

High and Low strain bees

High strain bees exhibited significant interaction effects between ovariole number and nectar sugar concentration as well as between ovariole number, nectar sugar concentration, and replicate on pollen proportion (Linear Regression Analysis, N = 63, Table 3.3). Low strain bees demonstrated a significant

interaction effect between ovariole number and replicate on foraging bias (Linear Regression Analysis, N = 119, Table 3.4).

DISCUSSION

Our results support the RGPH by supporting a mechanism where the overy impacts foraging bias. In the Africanized backcross bees, ovariole number and nectar sugar concentration interact in their effects on the pollen proportion (an index of foraging bias – see methods) in Africanized backcross bees. In addition, ovariole number impacted foraging behavior within the selected High and Low strains.

Within the context of this reproductive ground-plan framework, the ovary-foraging bias relationship in the backcross bees adds strong support for a causal link between ovary and foraging behavior. The backcross breeding design facilitates re-assortment of genes during meiosis. If separate genes controlled ovary and foraging phenotypes, no ovary-behavior relationship would be expected after meiotic gene recombination. However, the ovary-behavior relationship can still be observed (Table 3.2 and Figure 3.2). Further, the lack of genotypic effects in the backcross system excludes genetic effects on foraging bias that were additional to ovary size (Table 3.2). This was not unexpected due to the high relatedness across the singly-mated backcross colonies (G = 0.56). Replicate effects are likely an artifact of seasonal change in nectar sugar concentration (Table

3.2). Replicates were not conducted concurrently, and nectar sugar concentration increases during warmer months.

The ovary-foraging behavior relationships within the High and Low strains add additional support for a causal link between ovary and behavior. Genetic variation in these small breeding populations should be greatly reduced due to selection and genetic drift. Instead, variation in ovary size within these strains is likely the result of environmental effects during development. If separate genes control ovary size and foraging behavior, environmental impacts on ovary size should have no effect on foraging bias. In fact, we can observe ovary-foraging bias relationships within these selected strains.

In addition to supporting a causal relationship between the ovary and behavior, results of this study suggest a mechanism through which the ovary could be acting. The interaction effect between ovariole number and nectar sugar concentration on foraging bias observed in the Africanized backcross and High Strain bees as well as the difference in nectar loading between bees with higher and lower ovariole counts suggest that bees with different ovariole numbers are demonstrating divergent nectar and pollen loading responses to varying nectar sugar concentrations (Tables 3.2 and 3.3, Figure 3.2). Reproductive status has been shown to correlate with sugar response in many animal systems (Than et al. 1994, Curtis et al. 2005). Tsuruda et al. (2008) demonstrated a positive correlation between ovariole number and sucrose responsiveness in honey bees using

naturally mated wild-type bees, although at the time, they could not rule out the possible joint effects of different patrilines on ovariole number and behavior. Additionally, a negative correlation has been established between sucrose responsiveness and collected nectar sugar concentration (Pankiw and Page 2000). Finally, sucrose concentration is positively correlated with crop load size (Núñez and Giurfa 1996).

These patterns suggest a potential mechanism by which the ovary impacts nectar collection through a modulation of sucrose perception. If the ovary is modulating sucrose perception, then, hypothetically, workers with smaller ovaries are more likely to collect nectar with more concentrated sugars, and this higher concentration would induce them to collect a larger crop load. This in turn would diminish their ability to carry pollen due to physical limitations in loading capacity (Page et al. 2000).

Recent Quantitative Trait Loci (QTL) mapping has revealed four QTL associated with foraging bias (*pln1-4*; (Hunt et al. 1995, Page et al. 2000, Rüppell et al. 2004). Graham et al. (submitted) have mapped QTL in the same (EHBxAHB) x AHB backcrosses used in this study, and found that the behavioral QTL *pln1* and *pln2*, originally mapped in the High and Low strains, also have significant effects on ovariole number, thus confirming the effects of these QTL on ovariole in bees other than the High and Low strains. Our study shows in the same bees, the connection between ovariole number and foraging behavior. Our

study along with those of Graham et al., therefore, demonstrate the connections between gene, ovary, and foraging bias in the backcross bees, thus supporting the central components of the RGPH in a population that is independent of the selected pollen hoarding strains.

CONCLUSIONS

This study independently supports mechanisms proposed by the RGPH, whereby ovarian status impacts foraging bias for pollen or nectar, by demonstrating the relationship in a system where potentially linked genes have been re-assorted during meiosis though a backcross breeding program. We additionally demonstrated the association of ovary size and foraging behavior within worker populations from the High and Low strains where genetic variation for ovary size is reduced due to selection and genetic drift in a small breeding population. The interaction effect observed between ovariole number and nectar sugar concentration on foraging load bias in the backcross and High strain bees and the difference in nectar loading by backcross bees with different ovariole number provides evidence for a mechanism by which the ovary has a regulatory effect on sucrose perception. Sucrose perception, in turn, impacts nectar load volume decisions. Nectar loading decisions would necessarily impact pollen load size due to physical loading constraints.

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Figure 3.1. Pedigree demonstrating the breeding program that resulted in (EHBxAHB) x AHB backcross workers. Solid lines represent egg gametes. Dashed lines represent sperm. Relatedness of backcross worker offspring of super-sister queens is 0.5625.

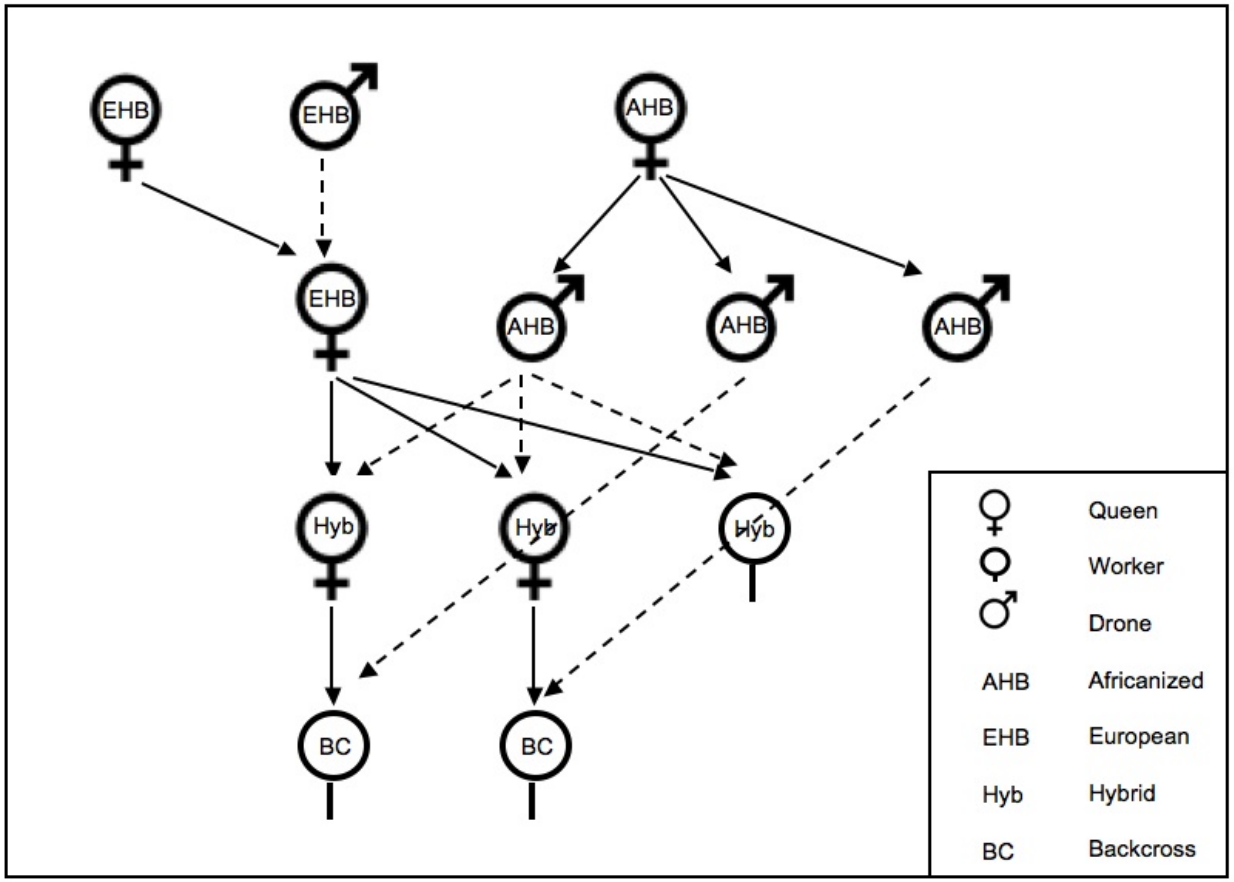

Figure 3.1, continued

Table 3.1. Original model of all factors potentially impacting the proportion of the foraging load that is pollen including interaction effects in the backcross bees. Note that Genotype has no main or interaction effect on the proportion of the foraging load that is pollen and was dropped from the final model (Linear regression analysis, N = 197,* = $p < 0.05$, ** = $p < 0.01$).

Table 3.1

ORIGINAL MODEL OF ALL FACTORS POTENTIALLY IMPACTING THE PROPORTION OF THE FORAGING LOAD THAT IS POLLEN IN THE BACKCROSS BEES

SOURCE OF EFFECT	DF	F RATIO	STATISTICAL SIGNIFICANCE
Genotype	1	3.70	-
× Nectar Sugar Concentration	1	1.94	-
× Total Ovariole Number	1	0.00	-
× Replicate	1	1.00	-
× Nectar Sugar Concentration × Total Ovariole Number	1	0.89	-
× Nectar Sugar Concentration × Replicate	1	0.11	-
× Total Ovariole Number × Replicate	1	1.00	-
× Nectar Sugar Conc. × Total Ovariole Number × Replicate	1	3.41	-
Nectar Sugar Concentration	1	0.85	-
× Total Ovariole Number	1	2.14	-
× Replicate	1	4.40	*
× Total Ovariole Number × Replicate	1	7.33	**
Total Ovariole Number	1	0.89	-
× Replicate	1	0.40	-
Replicate	1	0.04	-
Error	181		

Table 3.2. Revised model of factors potentially impacting the proportion of the foraging load that is pollen including interaction effects in the backcross bees. Note the interaction effect between collected nectar sugar concentration and total number of ovarioles as well as the interaction between these factors and replicate (Linear regression analysis, N = 197, ** = $p < 0.01$, *** = $p < 0.005$).

Table 3.2

REVISED MODEL OF FACTORS POTENTIALLY IMPACTING THE PROPORTION OF THE FORAGING LOAD THAT IS POLLEN INCLUDING INTERACTION EFFECTS IN THE BACKCROSS BEES

SOURCE OF EFFECT	DF	F RATIO	STATISTICAL SIGNIFICANCE
Nectar Sugar Concentration	1	0.50	-
× Total Ovariole Number	1	7.13	**
× Replicate	1	0.12	-
× Total Ovariole Number × Replicate	1	8.77	***
Total Ovariole Number	1	0.02	-
× Replicate	1	0.28	-
Replicate	1	0.12	-
Error	189		

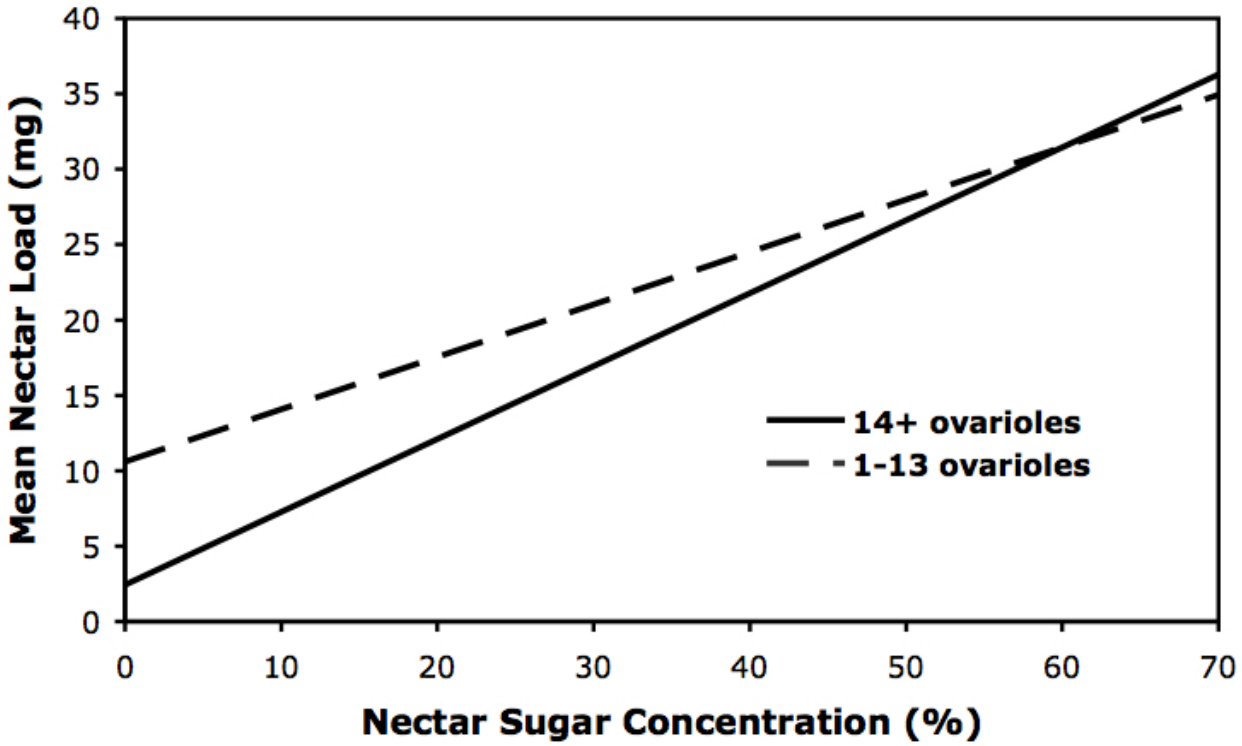

Figure 3.2. Mean nectar load response to nectar sugar concentration of bees with 14+ ovarioles compared to bees with 1-13 ovarioles. Median ovariole number was used to split the bees into two groups. The slopes are significantly different (ANCOVA, $F_{98,95} = 14.79$, $p < 0.001$)

Table 3.3. Model of factors potentially impacting the proportion of the foraging load that is pollen including interaction effects in the High strain bees. Note the interaction effect between collected nectar sugar concentration and total number of ovarioles as well as the interaction between these factors and replicate (Linear regression analysis, N = 63, * = $p < 0.05$).

Table 3.3

MODEL OF FACTORS POTENTIALLY IMPACTING THE PROPORTION OF THE FORAGING LOAD THAT IS POLLEN INCLUDING INTERACTION EFFECTS IN THE HIGH STRAIN BEES

SOURCE OF EFFECT	DF	F RATIO	STATISTICAL SIGNIFICANCE
Nectar Sugar Concentration	1	2.97	-
× Total Ovariole Number	1	6.18	*
× Replicate	1	0.16	-
× Total Ovariole Number × Replicate	1	6.85	*
Total Ovariole Number	1	1.76	-
× Replicate	1	0.19	-
Replicate	1	0.68	-
Error	55		

Table 3.4. Model of factors potentially impacting the proportion of the foraging load that is pollen including interaction effects in the Low strain bees. Note the interaction effect between total number of ovarioles and replicate (Linear regression analysis, N = 119, * = $p < 0.05$).

Table 3.4

MODEL OF FACTORS POTENTIALLY IMPACTING THE PROPORTION OF THE FORAGING LOAD THAT IS POLLEN INCLUDING INTERACTION EFFECTS IN THE LOW STRAIN BEES

SOURCE OF EFFECT	DF	F RATIO	STATISTICAL SIGNIFICANCE
Nectar Sugar Concentration	1	1.033	-
× Total Ovariole Number	1	1.169	-
× Replicate	1	0.334	-
× Total Ovariole Number × Replicate	1	0.006	-
Total Ovariole Number	1	1.17	-
× Replicate	1	4.51	*
Replicate	1	0.01	-
Error	111		

CHAPTER 4

THE LINK BETWEEN THE OVARY, SUCROSE SENSITIVITY, AND SUCROSE COLLECTION IN HONEY BEES

Abstract: Honey bees are a model system for the study of division of labor (DOL). Worker bees demonstrate a foraging DOL by biasing collection towards carbohydrates (nectar) or protein (pollen). The Reproductive ground-plan hypothesis of Amdam et al. (2004, 2006) proposes that foraging DOL is regulated by the networks that controlled foraging behavior during the reproductive life cycle of honey bee ancestors. Here we test a proposed mechanism through which the ovary of the facultatively sterile worker impacts foraging bias. The proposed mechanism suggests that the ovary has a regulatory effect on sucrose sensitivity, and sucrose sensitivity impacts nectar loading. We tested this mechanism by measuring worker ovary size (ovariole number), sucrose sensitivity, and sucrose solution load size collected from a rate-controlled artificial feeder. We found a significant interaction between ovariole number and sucrose sensitivity on sucrose solution load size when using low concentration nectar. This supports our proposed mechanism. As nectar and pollen loading are not independent, a mechanism impacting nectar load size would also impact pollen load size.

INTRODUCTION

Task specialization and division of labor are principal features of insect societies and are believed to be the prime enablers of their ecological and evolutionary success (Oster and Wilson 1978). Honey bees provide a model system for the study of task specialization and division of labor (Winston 1987, Seeley 1995, Page et al. 2006). Reproduction is normally restricted to the queen and her male mates (drones). Facultatively sterile female workers perform all of the tasks associated with nest construction and maintenance, care of young, resource exploitation, and colony defense. Task performance by workers is age correlated; young workers perform in-hive tasks while older workers perform outside tasks. Typically, foraging outside the nest is performed by the oldest workers. Most honey bees specialize on carbohydrate or protein foraging by respectively biasing food gathering towards nectar (carbohydrate) or pollen (protein) collection (Winston 1987). The foraging behavior of thousands of workers results in a surplus of pollen and honey in the nest.

The Reproductive Ground-plan hypothesis (RGPH) is a framework for explaining the control of foraging division of labor. The RGPH suggests that the regulatory mechanisms that controlled food collection during the reproductive life cycle of the solitary ancestor of the honey bee have been co-opted and modified to regulate foraging division of labor (Amdam et al. 2004, Amdam et al. 2006). Female solitary insects go through a reproductive life cycle, with a non-

reproductive stage characterized by inactive ovaries and carbohydrate feeding, and a reproductive stage characterized by activated ovaries and protein feeding. In honey bees, ovary size (measured by counting ovarioles, the egg producing filaments of the ovary) is determined during larval development. Honey bee foragers with larger ovaries (more ovarioles), a reproductively associated characteristic, are biased toward protein collection compared to those with smaller ovaries (fewer ovarioles). This relationship between ovariole number and foraging preference has been demonstrated in honey bees selected for pollen storage levels as well as unselected wild-type *Apis mellifera* and *Apis cerana* foragers (Amdam et al. 2004, Amdam et al. 2006, Page et al. 2006, Rueppell et al. 2008). According to the RGPH, there is a causal relationship between the worker ovary and foraging behavior.

Recent studies using workers derived from a backcross between European-Africanized Hybrid (EHB x AHB) queens and Africanized (AHB) drones further supported the RGPH by demonstrating that ovary size is associated with the individual foraging decisions of workers (Siegel et al. In Preparation). The (EHB x AHB) x AHB backcross studies demonstrated that ovary size and the sugar concentration of collected nectar have an impact on foraging bias. The impacts of these factors were not independent. Ovariole number and nectar concentration had an interaction effect on the proportion of the total foraging load that was pollen. This demonstrates that foragers with more ovarioles make different carbohydrate

and protein loading decisions in response to the sugar concentration of nectar than do foragers with fewer ovarioles (Siegel et al. In Preparation). In addition to impacting food collection decisions, reproductive status has been shown to correlate with sugar response in many animal systems (Than et al. 1994, Curtis et al. 2005). Non-reproductive honey bee workers exhibit a similar relationship. Worker bees with more ovarioles are more sensitive to sucrose stimulation than worker bees with fewer ovarioles (Tsuruda et al. 2008). We hypothesize that the ovary regulates sensory sensitivity, which in turn affects nectar volume foraging decisions. We tested the hypothesis by investigating the relationship between ovariole number, sucrose sensitivity, and the amount of sucrose solution collected by honey bee workers foraging at a flow-rate controlled feeder.

Materials and Methods

In this series of experiments, the relationship between ovariole number, sucrose sensitivity, and sucrose collection was investigated in wild-type bees. The experiment was designed to test the hypothesis that ovariole number has a modulating effect on sucrose perception, which in turn impacts nectar collection. The experiments were conducted October-November, 2009 at the Arizona State University Bee Facility in Mesa, AZ. Three non-simultaneous replicates were performed using 10% and 30% sucrose solutions. Prior to beginning the main experiment (Experiment 3), we confirmed that time on an artificial feeder was an

accurate method for estimating collected sucrose volume (Experiment 1). We also confirmed that it was possible to control for the effects of previous foraging experience on sucrose sensitivity (Experiment 2).

Experiment 1: Test of time spent on feeder as an estimate of crop load.

We used a method developed by Núñez (1971) to estimate crop load, where time spent imbibing from a sucrose solution delivery rate-controlled artificial feeder is multiplied by solution flow rate. The rate-controlled feeder has been suggested as a non-destructive method for measuring collected sucrose volume (Núñez 1971, Núñez 1982). Established methods of crop load estimation involve physically expressing crop contents, a technique that can damage or kill study animals. To test the accuracy of the proposed rate-controlled feeder method of crop load estimation, we timed a group of bees while they collected from the rate-controlled feeder and then expressed and weighed their crop loads using the traditional method. If time spent imbibing from the rate-controlled feeder multiplied by flow rate is an accurate index for measuring crop load size, there should be a significant linear relationship between crop load estimate based on time spent collecting and physically expressed crop load size.

A population of foragers was trained to forage at a rate-controlled feeder containing 30% sucrose solution. Twenty bees were timed while collecting solution, and then each bee was collected and narcotized using carbon dioxide. The

crop load was expressed into a capillary tube by manually squeezing the abdomen, then weighed. One bee ruptured during this process and was excluded from analysis. A regression analysis was used to compare estimated crop load volume (time spend imbibing from the feeder multiplied by flow rate) to crop load weight determined by manually expressing collected solution.

Experiment 2: Test of control for previous sucrose concentration exposure.

Honey bees demonstrate a baseline sucrose sensitivity that can be modulated by experience (Page et al. 1998, Pankiw and Page 1999, Pankiw et al. 2001). In experiment 3, bees were given access to feeders containing either a 10% sucrose solution feeder or a 30% sucrose solution feeder (only one feeder was present at a time). We wanted to determine the baseline sensitivity of bees captured on the two feeders, as baseline sucrose sensitivity is believed to affect the collection decisions of bees on the different feeders. However, experience at the feeders modulates the sucrose sensitivity response, which could mask our ability to measure the baseline sensitivity (Page et al. 1998). Therefore, we exposed collected bees to a common feeding environment prior to measuring sucrose sensitivity to control for experience on the feeder.

Three-hundred newly emerged wild-type honey bee workers from each of three wild-type sources (900 total) were paint marked (Testors Enamel) on the thorax and abdomen over a three day period and split evenly between two wild-

type background colonies. A unique color combination was used for each source on each day. After bees had been in the colonies for 10 days, all marked foragers were captured at the hive entrance and discarded to allow for complete control of food collection experience. The remaining marked bees were collected from the inside of the hives, randomly divided into groups of twenty and placed into small wire cages (~10x10x20cm). Half of the cages had 10% *ad libitum* sucrose solution feeders installed. The remainder had 30% *ad lib* sucrose solution feeders installed. The cages were kept in an incubator (35ºC, 50% RH) for 3 days, after which, a random subset of 30 bees of mixed origin was collected across cages for each concentration. Sucrose responsiveness was determined for the subset of bees exposed to 10% and 30% sucrose using a proboscis extension response (PER) assay to generate a gustatory response score (GRS; (Scheiner et al. 2001a, b, 2004).

Bees were cooled to 4ºC until immobile and then individually restrained in small tubes. Restrained bees were allowed to acclimate to the experimental conditions in an incubator (35ºC, 50% RH) for at least 60 minutes. After the acclimation period, bees were allowed to drink water *ad lib* to avoid false positive responses due to dehydration (Pankiw and Page 2000, Pankiw et al. 2001, Pankiw and Page 2003). Bees were then tested by stimulating both antennae with an ascending logarithmic sucrose concentration series (0, 0.1, 0.3, 1, 3, 10, 30% sucrose by weight) and honey. An inter-trial interval of at least 3 minutes was

maintained. The GRS was determined by counting the number of concentrations for which a bee extended her proboscis in response to the antennal stimulation. Bees that did not respond to honey were excluded from the experiment. GRS for the 10% and 30% exposed bees was compared using a one-tailed Student's t-test. A one-tailed test was used because of the *a priori* expectation that bees exposed to 10% sucrose would be more responsive than bees exposed to 30% sucrose. The tested subset of bees was then discarded.

To determine if honey bee sucrose responsiveness could be quickly reconditioned, all cages then had the *ad lib* feeders replaced with 30% sucrose *ad lib* feeders. After 24-29 hour exposure to the 30% sucrose feeders a GRS was determined for all remaining bees. The GRS of the bees that had been exposed to three days 10% sucrose solution followed by one day of 30% sucrose solution was then compared to the GRS of the bees that had been exposed to three days of 30% sucrose solution followed by an additional day of 30% sucrose solution, separately for bees from each original source.

Experiment 3:Relationship between ovariole number, sucrose sensitivity, and sucrose collection.

Several wild-type colonies were screened for ovariole number. Three source colonies were chosen that demonstrated high variation in ovariole number across workers. Colony strength was estimated at over 10,000 workers for all

chosen colonies. All experienced foragers were removed from the source colony prior to the initiation of data collection (Amdam et al. 2005). Colonies were placed in outdoor 6 x 12m screen flight cages 2-4 days prior to starting data collection. Using the flight cage allowed for complete control over available foraging resources.

Once a new foraging population of several hundred workers was re-established, foragers were trained over 1 day to collect either 10% or 30% sucrose solution from *ad lib* artificial flower feeders 6 m from the entrance of the hive (Figure 4.2a). Only one concentration was available at a time. When a population of foragers was established at the pre-established collection site, the feeder was replaced with a visually similar *ad lib* feeder that required the bees to crawl into a small tube to access the sucrose reward (Figure 4.2b). When bees had learned to navigate the tube feeder, the feeder was replaced again with a flow rate-controlled feeder set at a solution delivery rate of 3.73µl/min (Núñez 1971) Figure 4.2c-d).

Crop load size based on time at the feeder was estimated for 50-53 bees captured on the feeder for each concentration and replicate over a period of 4-6 days. Prior to testing, the feeder was allowed to run for 60 seconds to build up a small reservoir of sucrose solution to attract foragers. This volume was included in the collection volume estimate. When a single bee entered the feeder port, time collection was initiated and a small wire cage (3x3x12cm) was placed over the opening to exclude other bees from the port. The cage avoided competition effects.

As honey bees will often stop and start collection, the bee was allowed to continue collection until it had ceased collection for 60 continuous seconds. At this time, the focal bee was captured in the small wire cage. The time spent on the feeder plus the initial 60 second 'charge' was multiplied by the flow rate of 3.73µL/min to estimate crop load volume.

At the end of each day's collection period, all captured foragers were individually paint-marked (Testors Enamel) and split between two large wire cages with access to 30% sucrose *ad lib* feeders and kept for 26-29 hours in an incubator (35°C, 50% RH). This sequestration was performed to control for sucrose exposure experience so that we could compare sucrose sensitivity of bees collected on feeders containing different sucrose concentrations. Sucrose responsiveness was determined after 26-29 hours in the incubator by generating a GRS using the protocol outlined above. After the behavioral assays, the bees were dissected under magnification and ovarioles (egg producing filaments) were counted for both ovaries as an index of ovary size.

Student's t-tests were conducted to compare the sucrose solution volume collected at 10% vs. 30% sucrose and to compare the GRS of bees collected on the 10% feeders vs. the 30% feeders. Source colony replicates were pooled for the volume and GRS comparisons, as source colony had no effect on collection volume (see results). A Generalized Linear Mixed Model (GLMM; JMP) was constructed to determine which factors impacted the volume of collected sucrose.

Total Ovariole number and GRS were set as fixed factors. Hive ID (source colony) was set as a random factor. Bees for each concentration were analyzed separately. The model included ovariole number, GRS (sucrose sensitivity), ovariole number*GRS interaction and Hive ID as the error factor. Because the three replicates were conducted sequentially, Hive I.D. includes noise due to the temporal order of the replicates, colony source of the bees, or any additional potential replicate impact (i.e. genotype of the bees, quantity of brood in the hive, etc.)

RESULTS

Experiment 1: Test of time spent on feeder as an estimate of crop load.

There was a strong positive correlation between load size estimate based on time spent collecting from the rate-controlled feeder multiplied by solution flow rate and load size estimate based on manually expressing collected sucrose solution from the crop (Regression Analysis, F-ratio=122.44, N = 19, $P<0.0001$). This relationship was linear ($R^2=0.89$, Figure 4.3).

Experiment 2: Test of control for previous sucrose concentration exposure.

As expected, after three days exposure to differing concentrations of sucrose, the bees exposed to a 10% sucrose solution were significantly more responsive to sucrose than those exposed to the 30% sucrose solution (One-tailed

Student's t-test, t-ratio = -1.93, $N_{10\%} = 26$, $N_{30\%} = 23$, $p < 0.05$, Figure 4.4; (Pankiw et al. 2001). After all remaining bees had been given 24-29 access to an *ad lib* 30% sucrose feeder, there was no longer any difference in sucrose responsiveness between bees that had previously been exposed to 10% sucrose and those exposed to 30% sucrose for bees from any of the three sources, thus validating our methods (one-tailed Student's t-test, t-ratios: Source 1=0.44, Source 2=1.12, Source 3=2.43, N=32-45 for each group, $p > 0.05$ for all sources, Figure 4.5).

Experiment 3:Relationship between ovariole number, sucrose sensitivity, and sucrose collection.

Differences between bees captured on 10% sucrose feeder and 30% sucrose feeder

Honey bees captured on the 10% feeder collected significantly less sucrose solution than those captured on the 30% feeder (Student's t-test, t-ratio=7.70, $N_{10\%}$=155, $N_{30\%}$=158, $p<0.0001$, Figure 4.6). This is consistent with previous findings (Núñez and Giurfa 1996). In addition, honey bees that accepted the 10% feeder were more sensitive to sucrose in lab assays, even after controlling for experience by allowing bees to feed on 30% sucrose for 26-29 hours prior to GRS testing (Student's t-test, t-ratio=-2.32, $N_{10\%}$=131, $N_{30\%}$=142, $p<0.005$, Figure

4.7). This demonstrates that the bees accepted the feeders according to sucrose sensitivity.

Ovary size and sucrose sensitivity relationship with sucrose collection

Statistical analysis indicated a significant interaction effect between ovariole number and sucrose sensitivity on sucrose collection volume for bees foraging on 10% sucrose (GLMM, N = 131, Table 4.1). No other factors demonstrated an independent significant effect on sucrose collection volume, and there was no source colony effect. There were no significant effects on volume of 30% sucrose collected (Generalized Mixed Linear Model, N = 138, Table 4.2).

DISCUSSION

The results of this study demonstrate a link between ovariole number, sucrose sensitivity and nectar collection. These results support a proposed foraging division of labor control mechanism where the ovary impacts sucrose responsiveness in honey bees. Sucrose responsiveness, in turn, impacts the loading of sugar rich nectar. This mechanism fits well into the evolutionary RGPH that mechanisms controlling food collection during the life cycle of solitary ancestors of honey bees have been co-opted and remodeled to control foraging decisions in extant honey bees.

In this series of experiments, collected sucrose volume was estimated by multiplying the time foragers spent collecting sucrose solution from a delivery rate-controlled artificial feeder by the known solution flow rate (Núñez 1971). The rate-controlled feeder had several benefits over an *ad lib* feeder. First, it more closely resembles natural conditions, as many insect pollinated flowers deliver nectar at extremely restricted rates (Pacini et al. 2003). Second, when exposed to the unnatural conditions of an *ad lib* feeder, honey bees are much less likely to make a discriminating foraging decision (Mujagic and Erber 2009). This is possibly due to the minimal foraging costs under these conditions. A forager can completely fill its crop in under 60 seconds on an *ad lib* feeder, compared to 15-20 minutes on natural flowers or rate-controlled feeders (Núñez 1982, Núñez and Giurfa 1996).

We observed a strong linear relationship between the physically measured crop load size and the crop load estimate based on time spent on the rate-controlled feeder (Figure 4.3). This relationship validates the use of the time based estimate as a consistent non-destructive measure of foraging crop load size. As it is impossible to completely empty the crop of a forager by squeezing, the time based estimate may be a more accurate measure of crop load size than the standard squeezing technique. Additionally, bees imbibe all liquid in the feeder, further supporting the accuracy of this method.

We observed no difference in sucrose sensitivity between caged bees previously exposed to 10% sucrose and bees previously exposed to 30% sucrose,

after one day of exposure of all bees to 30% sucrose feeders. From this, we conclude that one-day exposure to a common sucrose solution is sufficient to negate sucrose sensitivity effects of previous sucrose solution experience. Therefore, differences in sucrose sensitivity observed between bees collected on field feeders of different sucrose concentration after the one day cage treatment were due to the sorting of bees between sucrose feeders of differing sucrose concentration according to individual gustatory sensitivity. Bees that were more sensitive to sucrose accepted the 10% solution and the 30% solution; those that were less sensitive accepted only the 30% solution.

Bees collected larger loads of 30% sucrose solution than 10% sucrose solution (Figure 4.6). This demonstrates that bees are able to assess the relative value of nectar. Recently, (Mujagic and Erber 2009) found no difference in time spent by foragers collecting sucrose solution (which can be used as a measure of collection volume- see methods) of different sucrose concentrations. However, the differences between their results and ours may be explained by their use of an *ad lib* feeder. Increased flow rate is positively correlated with crop load size (Núñez and Giurfa 1996). Honey bees are able to completely fill their crops in fewer than 60 seconds when exposed to an *ad lib* feeder. This removes much of the cost associated with increased time spent foraging, and likely masks effects of different concentrations of sucrose solutions.

Bees collected on the 10% feeder demonstrated higher average sucrose sensitivity than bees collected on the 30% feeder, even after one day exposure to 30% sucrose feeders (Figure 4.7). The results of this study again differ from those of Mujagic et al (2010). They failed to demonstrate a relationship between sucrose sensitivity and acceptance thresholds of free flying bees. However, again methodological differences probably explain the differences in results. Mujagic et al. (2010) used *ad lib* feeders to determine the field acceptance threshold of bees. Our study used flow-rate limited feeders. Because increased sugar concentration and increased solution flow rate both positively impact solution collection (Núñez and Giurfa 1996), it is likely that many bees in their study collected solutions in the field of a lower sucrose concentration than they would accept under the more natural conditions of restricted sucrose solution delivery, masking any effects of sucrose sensitivity on acceptance of sugar solution. Additionally, previous experience impacts sucrose sensitivity (Pankiw et al. 2001). Testing bees without a control for experience would also mask differences in sucrose response sensitivity.

The results of this study support our hypothesis that the ovary modulates sucrose perception, which in turn affects the volume of nectar collected. An interaction effect between ovariole number and sucrose sensitivity on volume of solution collected was observed within the 10% sucrose group (Table 4.1), as would be expected if ovary is affecting gustatory response to sugar and gustatory

sensitivity is impacting nectar collection. Bees with different numbers of ovarioles demonstrated different responses to sucrose concentration and this is impacting their foraging decisions regarding nectar loading. Nectar and pollen collection are not independent due to physical collection limitations (carrying more of one floral product necessitates carrying less of the other; (Page et al. 2000). Therefore, a nectar collection regulatory system should also indirectly impact pollen collection (Figure 4.1). The interaction between ovary and sucrose perception was not observed in the 30% sucrose group. Thirty percent sucrose is a highly valuable resource even in unrestricted environments. The majority of bees captured on the 30% sucrose feeder had near maximum foraging load sizes. We believe that the response to high sucrose concentration in a resource limited environment masked any potential foraging decisions due to ovary size.

CONCLUSIONS

This study elucidates a mechanism regulating foraging division of labor that links ovariole number with sucrose sensitivity, and nectar loading decisions. As nectar loading and pollen loading are coupled due to physical loading constraints, a mechanism impacting nectar loading would also impact pollen loading. The results of this study demonstrate a link between reproductively associated phenotypes and foraging behavior in non-reproductive honey bee workers. This supports the RGPH, that reproductively associated regulation has been co-opted and reshaped

to impact foraging division of labor. This sheds light on the transition from solitary to social behavior in Hymenoptera.

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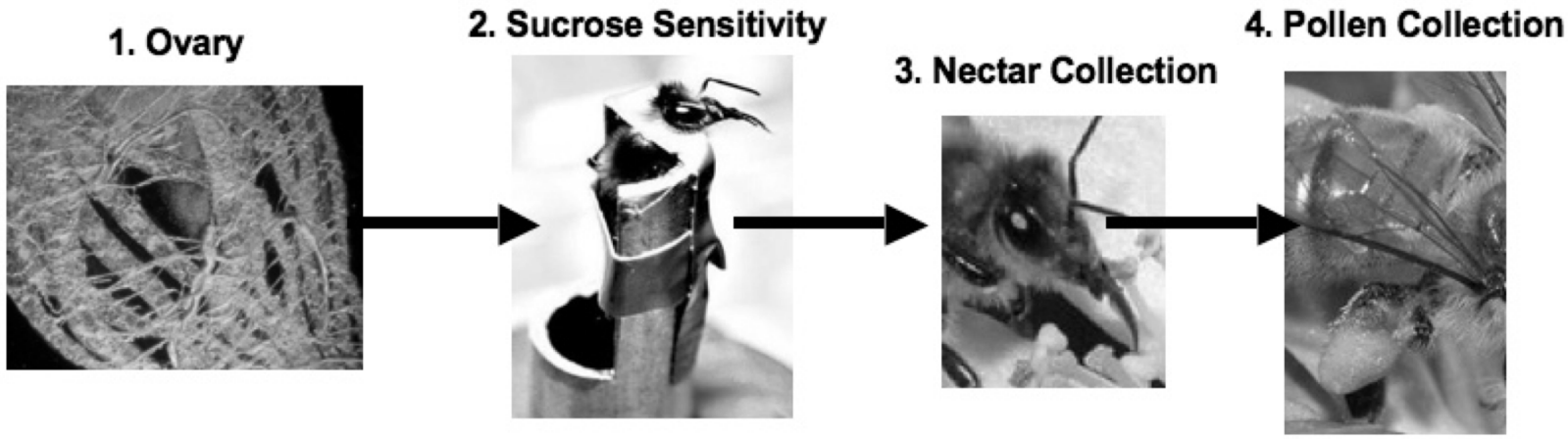

Figure 4.1. Proposed mechanism for ovary impact of foraging bias. The ovary (1) tunes sucrose sensitivity (2), which impacts nectar collection decisions (3). This would indirectly impact pollen collection (4) due to physical limitations on collection quantity (Photos: 1. O. Kaftanoflu; 2. J. S. Engen; 3. & 4. Z. Huang).

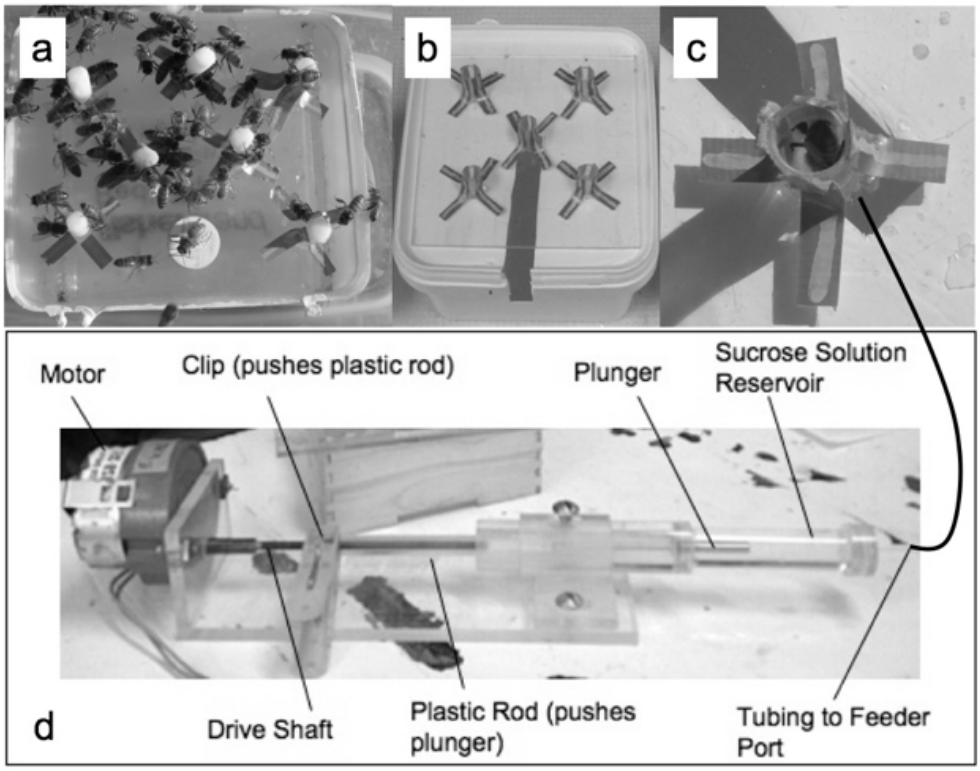

Figure 4.2. Sucrose feeders. (a) *Ad libitum filter* feeder. (b) Transitional *ad lib* tube feeder. (c) Honey bee forager inside rate restricted feeder port. (d) Rate restricted sucrose delivery device.

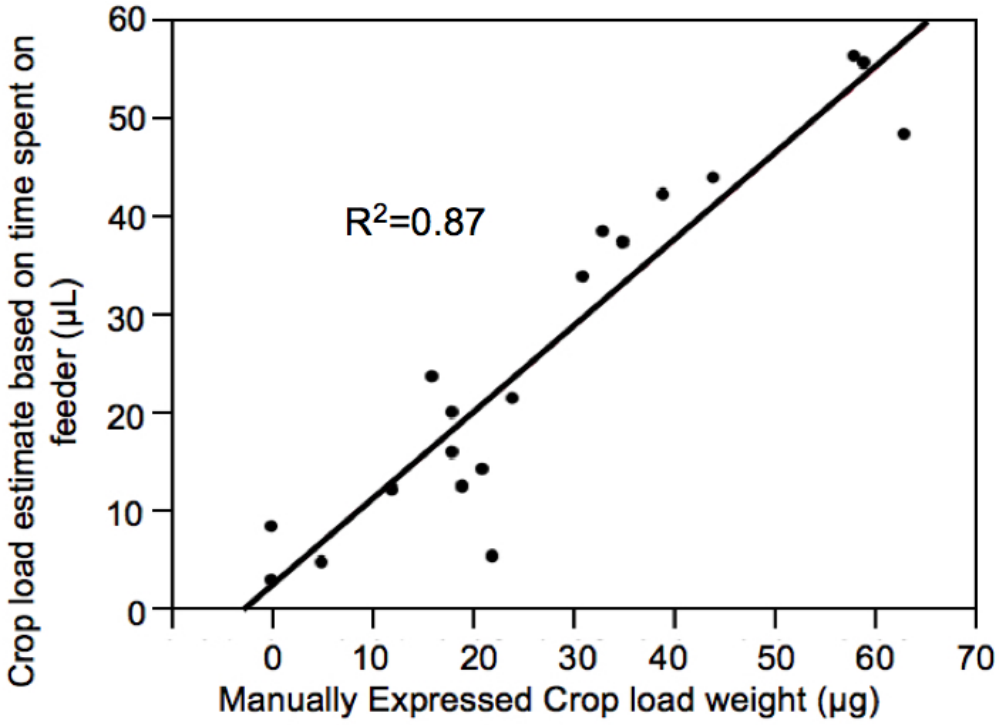

Figure 4.3. Linear relationship of crop load estimate (based on time spent on feeder) compared to manually expressed crop load weight.

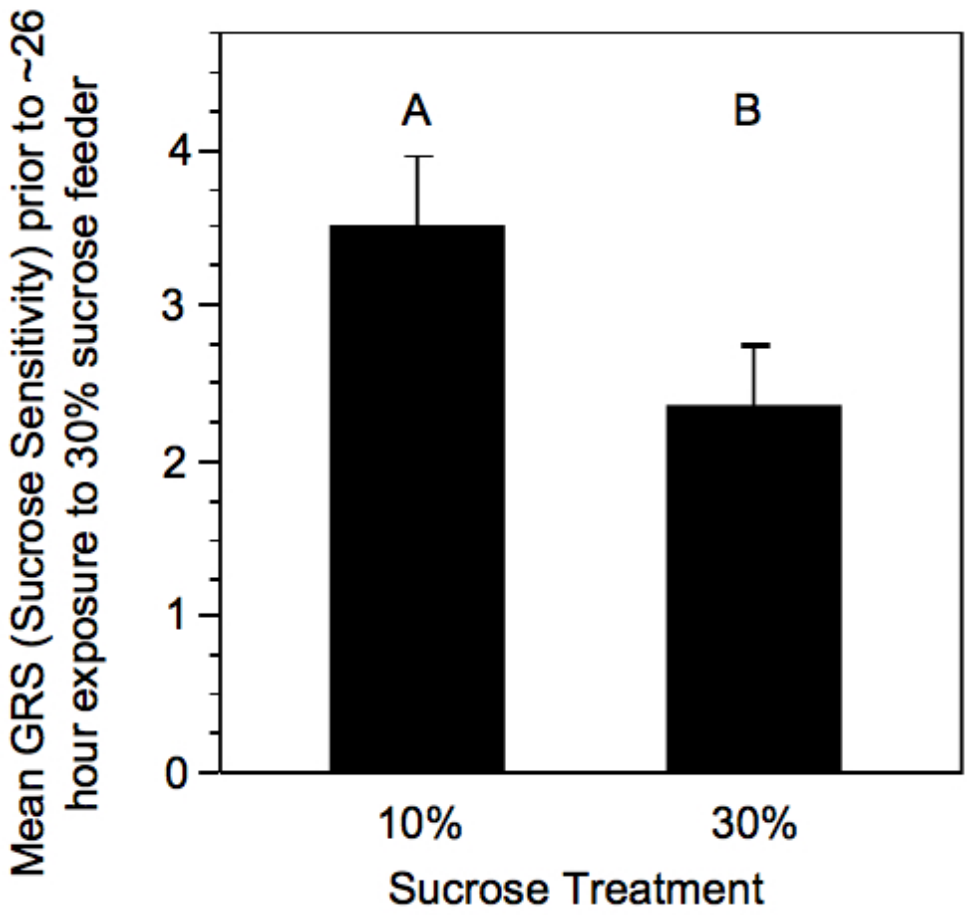

Figure 4.4. Mean (+SE) GRS (sucrose sensitivity) of bees after three days exposure to either 10% or 30% concentration sucrose solution. Letters signify significant difference ($p<0.05$).

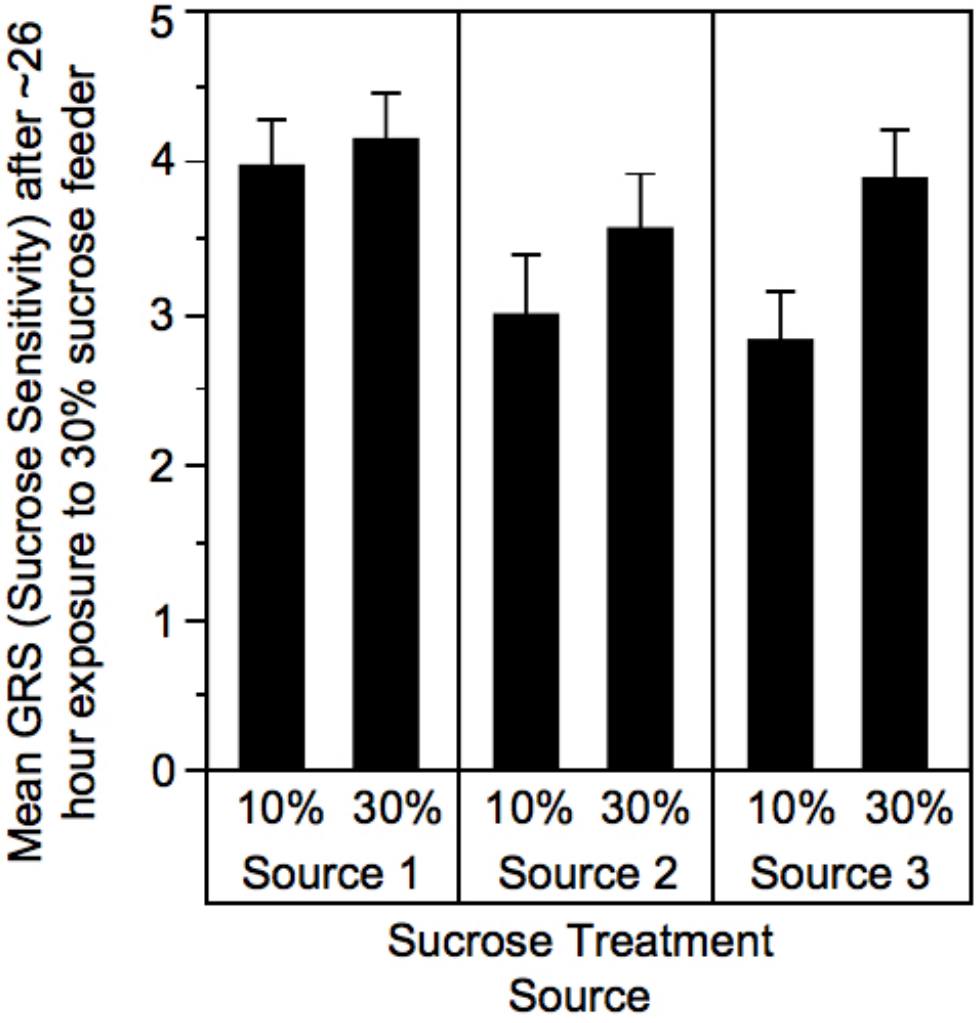

Figure 4.5. Mean (+SE) GRS (sucrose sensitivity) of bees after three days exposure to either 10% or 30% concentration sucrose solution followed by one day of additional exposure to 30% concentration sucrose solution. No significant differences in sensitivity were found regardless of original conditioning.

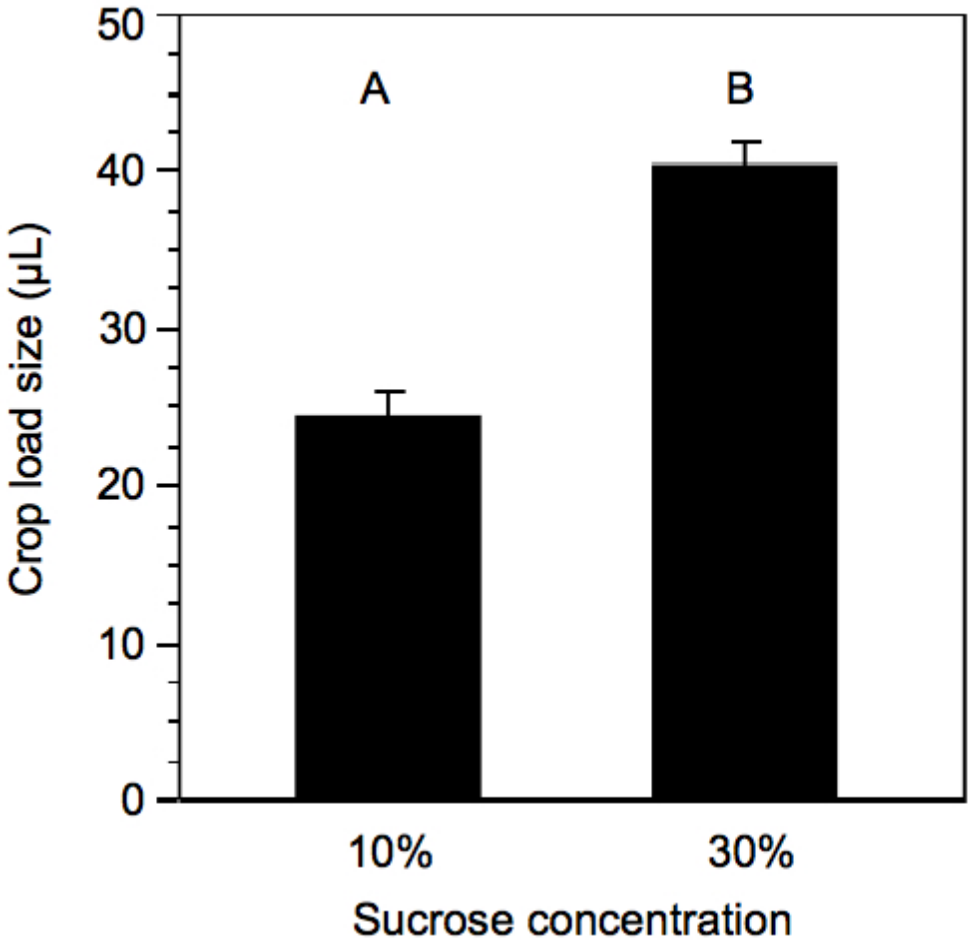

Figure 4.6. Mean (+SE) volume of sucrose collected by bees collected on 10% or 30% sucrose feeder. Letters signify significant difference ($p<0.05$).

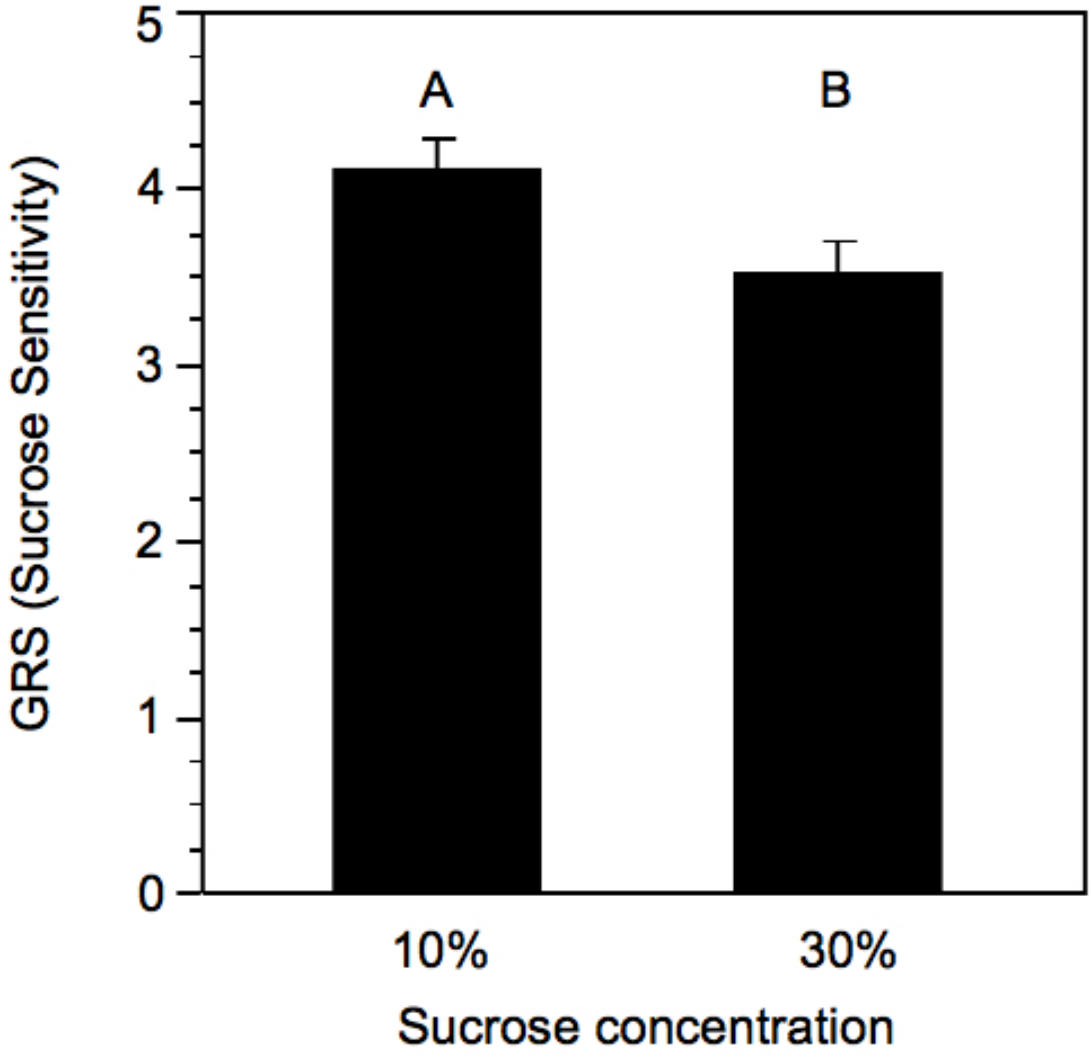

Figure 4.7. Mean (+SE) GRS (sucrose sensitivity) of bees collected on 10% or 30% sucrose feeder. Letters signify significant difference ($p<0.05$).

Table 4.1. Factors impacting volume of 10% sucrose solution collected treating GRS as an Ordinal Variable. Note that there is a significant interaction effect of ovariole number and GRS (sucrose sensitivity). Hive ID includes error caused by Colony source of bees and temporal pattern of data collection (Generalized Linear Mixed Model, N = 131, * = p <0.05, **** = p < .0001)

Table 4.1

GLMM PARAMETER ESTIMATES (EST.), STANDARD ERRORS (SE), AND P VALUES OF POTENTIAL FACTORS IMPACTING 10% SUCROSE SOLUTION LOAD SIZE

PARAMETER	EST.	SE	STATISTICAL SIGNIFICANCE
Intercept	25.05	5.54	****
Total Ovariole Number	-0.11	0.49	-
GRS	0.12	0.85	-
Total Ovariole Number × GRS	-0.49	0.23	*
Hive ID	2.69	2.33	-

Table 4.2. Factors impacting volume of 30% sucrose solution collected. * Hive ID includes error caused by colony source of bees and temporal pattern of data collection. Hive ID includes error caused by Colony source of bees and temporal pattern of data collection (Generalized Linear Mixed Model, N = 138, **** = $p < .0001$)

Table 4.2

GLMM PARAMETER ESTIMATES (EST.), STANDARD ERRORS (SE), AND P VALUES OF POTENTIAL FACTORS IMPACTING 30% SUCROSE SOLUTION LOAD SIZE

PARAMETER	EST.	SE	P VALUE
Intercept	45.32	4.49	<.0001
Total Ovariole Number	-0.08	0.47	0.85
GRS	-0.71	0.70	0.31
Total Ovariole Number × GRS	-0.02	0.19	0.94
Hive ID	-0.48	2.29	0.84

BIOGRAPHICAL SKETCH

Adam Joshua Siegel was born to Jack Stephen Siegel, M.D. F.A.C.C. and Carole Jean Siegel (née Milstein), L.C.S.W. on June 30th, 1981 in Schenectady, New York. He grew up in Los Gatos, California. Adam earned a B.S. in biology from Tufts University in 2004, and a Ph.D. in biology from Arizona State University in 2011.