Individuality in the hive: Behavioral variation and its proximate causes in insect societies

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Individuality in the hive: 
Behavioral variation and its proximate causes in insect societies

by

Alexander Walton

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Ecology and Evolutionary Biology

Program of Study Committee:
Amy Toth, Major Professor
Fredric Janzen
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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University

Ames, Iowa

2018

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DEDICATION

To my mother and father, Audrey and Dennis, who have nurtured in me an unquenchable curiosity. Thank you for always supporting me as I pursued all my various interests and passions; and for your love, which has always been my manna.
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ABSTRACT

A fundamental question in biology is “why does one individual have a particular phenotype while another individual of the same species has a different phenotype?” Research on behavioral phenotypes has demonstrated that consistent behavioral variation, termed personality, represents alternative solutions to the various challenges animals face throughout their lives. Although most animal personality research has been done with vertebrates, biologists have begun to accept that many lineages of invertebrates also exhibit personalities. Social insects in particular offer unique insights into how and why personalities exist in invertebrates and more broadly, in social animals. I conducted the first comprehensive study of personality differences across individuals in a eusocial insect. I defined and investigated three dimensions of personality within the worker caste of a model social insect, the honey bee *Apis mellifera*, as follows: 1) consistent individual behavioral differences over time, 2) consistent individual behavioral differences across contexts, and 3) the presence of correlated suites of behaviors. These findings suggest some individuals may be more likely to be highly interactive with other workers (e.g. engaging in food sharing), while other individuals are consistently less interactive. These results expand upon and contribute to previous models for the organization of worker division of labor in honey bees, suggesting that consistent behavioral differences (personalities) of workers within a behavioral caste have the potential to contribute to subcaste division of labor. I examined a potential proximate cause of this inter-individual behavioral variation in honey bee workers: differential nourishment. I tested the hypothesis that nutritionally stressed bees will be more likely to perform cooperative behavior than satiated worker, and found that diet restriction affected queen mandibular pheromone response (a cooperative behavior), but the direction of the effect was different.
depending on what life stage workers experience restriction. I suggest that these differences depend upon the extent of reproductive plasticity at these life stages, and that individual worker honey bees may adjust their behavioral and physiological traits in response to nutritional stress to invest nutritional resources in either their own or their colony’s reproduction. To further probe the generality of the phenomenon that nutritional stress promotes cooperation observed in honey bees, I expanded this work to investigate the link between nourishment and social cohesion in an independently evolved lineage of social insects, primitively eusocial paper wasps *Polistes fuscatus*. I found that diet restriction reduced aggressive interactions between nestmates, which we interpret as an indicator of high social cohesion. The research presented in this dissertation contributes to a growing body of work on how the nutritional environment, early-life effects, and tradeoffs between personal and group level reproduction affect cooperative behavior in social animals. This research will inform future investigation of how behavioral variation evolves and why it persists.
CHAPTER 1. INTRODUCTION

Inter-Individual Behavioral Variation

Phenotypic variation within populations is necessary for natural selection to operate, and yet the reasons as to why so much variation exists is still a fundamental question in biology. In the classical understanding, natural selection should remove variation within a population, and phenotypes should converge on a local fitness peak (Simpson, 1944). However, phenotypic variation in populations appears to be ubiquitous (Halama and Reznick, 2001; Bolnick et al., 2002), and is due to both genetic and environmental factors, as well as their interaction (Goldschmidt, 1940; Falconer and Mackay, 1996; Pigliucci, 2001; West-Eberhard, 2003). Lynch and Hill (1986) proposed that phenotypic variation is a byproduct of selectively neutral processes (Kimura, 1983), and suggested that much (though, not all) of this variation is “of little consequence.” More recently, with renewed interest in inter-individual variation, attention has focused on the potential ecological benefits of phenotypic variation, including alternative adaptive strategies to a dynamic environment (Mousseau et al., 2000; Halama and Reznick, 2001; Bolnick et al., 2002). After a long history of contentious debate (Grant and Price, 1981), phenotypic variation within populations is considered in modern evolutionary thought, not of little consequence, but as ecologically and evolutionarily important.

Behavior, like other phenotypic traits, can vary within a population. However, historically, behavioral variation was viewed in a manner similar to other phenotypic variation. Thus, many behavioral and ecological studies during the 20th century treated within-population variation as noise or as simply non-existent (Bolnick et al., 2002), despite both theoretical (Van Valen, 1965; Roughgarden, 1972) and empirical evidence (Grant et al.,
1976; Bernstein, 1979) to the contrary. Additionally, behavior has often been treated as not heritable. This is likely due to a deeply ingrained intellectual heritage in Western philosophy and psychology that has promoted the doctrine of the *tabula rasa*: that all human nature (and by extension animal behavior) can be explained by experience (Locke, 1690; Pinker, 2002). Modern studies have demonstrated, however, that animal behavior (human and non-human alike) is often highly heritable (Boake, 1994; Stirling et al., 2002; van Oers et al., 2005; Bell et al., 2005). With the emergence of animal personality research, the ecological and evolutionary importance of inter-individual behavioral differences has been reevaluated (Gosling, 2001; Sih et al., 2004; Réale et al., 2007; Dall et al., 2004).

Behavioral variation within populations has been studied extensively in vertebrates, and often with terms and definitions that are inconsistent from study to study. At various times, this variation has been termed “personality” (Gosling, 2001; Dall et al, 2004; Dingemanse and Réale, 2005; Bell and Sih, 2007; Biro and Stamps, 2008), “temperament” (Jones and Gosling, 2005; Réale et al., 2007; Freeman and Gosling, 2010), “coping styles” (Koolhaas et al., 1999; Koolhaas et al., 2010), and “behavioral syndromes” (Sih et al., 2004; Sih and Bell; 2008). Although these terms have varied, recent literature reviews (Sih et al 2004; Jandt et al, 2014) have called for more precise definitions. In this dissertation, I define consistent behavioral variation as belonging to three “dimensions of personality”: (1) behavioral consistency/repeatability over time, (2) behavioral consistency/repeatability across contexts, and (3) the presence of correlated suites of different behaviors (termed “behavioral syndromes”) (Walton and Toth, 2016).

Research on animal personalities has demonstrated that consistent, highly repeatable, behavioral variation is not “of little consequence”, but represents alternative solutions to the
various challenges animals face throughout their lives (Boake, 1989; Koolhaas et al., 1999; Dugatkin, 2013). For example, a bold individual may have increased opportunities to forage and mate than a shy individual, but be much more likely to fall victim to predators (Wilson, 1993; Wilson et al., 1994). Game theory predicts that different personalities can co-exist in a population, if they represent alternative strategies with equivalent fitness. In the case of different foraging strategies adopted by individuals in bird flocks, theory predicts that two personalities (either “producing” by finding new food sources or “scrounging” by following producers) can co-exist in equal proportion at equilibrium (Barnard and Sibly, 1981).

Empirical evidence on pigeons has confirmed these findings (Giraldeau and Lefebvre, 1986; 1987). Clearly, inter-individual behavioral variation is important to understanding how animals interact with their environment and each other.

Only recently have biologists begun to accept that invertebrates also exhibit personalities, and the dimensions of personality have been shown to exist in several lineages (Mather and Logue, 2013; Kralj-Fišer and Schuett, 2014). Much of this work has been done on cephalopods, which display strikingly robust personalities, comparable to those recorded in vertebrates (Red octopus, Octopus rubescens: Mather and Anderson, 1993; Dumpling squid, Euprymna tasmanica: Sinn et al., 2008). Additionally, a small but growing body of research has been done on insect personalities. The short length of their lifespan limits the length of time over which behavioral consistency can occur, but has the benefit of making it easy to observe behavior from birth to death (Kralj-Fišer and Schuett, 2014). Thus, insect systems (i.e., the water strider Aquarius remigis: Sih and Watters, 2005; Sih et al., 2014; Wey et al., 2015) have begun to emerge as models for understanding why animal personalities evolve and how they develop.
Social Insect Personality

Social insects offer unique insights into how and why personalities exist in invertebrates and, more broadly, in social animals. The most exaggerated form of sociality, eusociality, is defined by societies of animals with a reproductive division of labor, cooperative brood care, and overlapping generations living together (Wilson, 1971). Despite these shared defining traits, eusocial species possess a wide range of different forms of eusociality. The focal animals of this dissertation, the honey bee *Apis mellifera* and the paper wasp *Polistes fuscatus*, are eusocial, though they are generally categorized as advanced eusocial (large colonies with a fixed, often morphologically distinct, reproductive caste) and primitively eusocial (small annual colonies with overt reproductive conflict between nestmates), respectively (Wilson, 1971; Reeve, 1991). Due to their varying degree of cooperative behavior and colony-level adaptations, honey bees and paper wasps are model candidates for exploring social personality.

Variation in the behavior of individual members of a eusocial colony can be evident. In some cases, specialized groups of individuals in social insect colonies are so distinct and stereotyped, they are easily recognized as part of different “castes”, including reproductive castes, morphological castes, and behavioral castes (Oster and Wilson, 1978; Wilson, 1971). Examples of caste that are illustrative of just how specialized individuals within eusocial colonies can be include the “repletes” in colonies of honeypot ants in the genus *Myrmecocystus* that store large amounts of liquid food in their crops until times of shortage, whose gasters can swell to the size of a cherry (Hölldobler and Wilson, 2009), the “phragmatic” guard ants (of various genera) with shield-like heads that block nest entrances (Wheeler, 1927; Brandão et al., 2001), and the diminutive “minims” (some smaller than their larger sisters’ heads) in leaf-cutter ant societies that ride atop vegetation as it is transported to
the nest, chasing away phorid flies that attempt to parasitize the carrier ant (Hölldobler and Wilson, 2010). Because of the well-studied occurrence of distinct castes, it has been assumed that personalities are not a useful concept for understanding division of labor in these systems. Partly for this reason, individual differences in social insect behavior using a personality framework remain relatively unexplored.

Individual variation in some dimensions of personality has been recorded in several species of social invertebrates, though they may not have been explicitly studied within a personality framework (reviewed in Jandt et al. 2014). For example, aggression and boldness, one of the most stable and well-studied personality types in humans and non-human animals (Kagan, 1994; Dugatkin, 2013), were consistently correlated with each other in Myrmica rubra and M. ruginodis worker ants, and the boldness-aggressiveness syndrome was related to task choice (Chapman et al., 2011). Additionally, in colonies of ants in the genus Temnothorax, there is consistency in how active (Pinter-Wollman et al., 2012) or inactive (Charbonneau and Dornhaus, 2015) individuals are.

Researchers have begun to study personality in honey bees, though much of this work has been done on colony-level emergent personality (how entire colonies differ in behavior from each other) (Wray et al., 2011; Wray and Seeley, 2011). More recently, a burgeoning field of research has explored personality differences between honey bee workers. A great deal of the explanation for behavioral differences between honey bee workers can be attributed to their age, due to an age polyethism structure to their division of labor, in which workers switch tasks in a stereotyped manner as they age (Seeley, 1982; 1985). However, a few studies have demonstrated that honey bee workers of the same age exhibit behavioral differences that are broadly repeatable over time and across contexts. For instance, Robert E.
Page and colleagues have documented the social phenotypes of honey bee lines selected for either high or low pollen-hoarding (Page and Fondrk, 1995; Page, 2013). They found that not only is pollen-storage behavior (a colony-level trait) highly heritable (Hellmich et al., 1985), but it is coupled with suites of other heritable correlated behavioral traits at the individual level, including age of foraging onset (Page and Fondrk, 1995), biases toward either nectar or pollen foraging (Pankiw and Page, 2001), and learning (Scheiner et al., 2001a;b). The highly informative research on the pollen-hoarding syndrome illustrates that honey bee behavior can exist in heritable and correlated suites, but it has not focused on how behavior might differ between individuals within a colony. In a landmark study, Liang et al. (2012) found that individual bees in a swarm that scouted for new nest sites were more likely to scout for new food resources than individuals that did not scout for new nest sites, thus these individuals displayed a consistency in novelty-seeking across contexts. Walton and Toth (2016, Chapter 2 of this dissertation) conducted the first comprehensive investigation of all three dimensions of personality in worker honey bees. With a clearer understanding of what personality is and how it can exist, there has been an accumulation of evidence that individual variation in the behavior of social insect workers within castes (including worker honey bees from the same age cohort) is both real and important to the collective phenotype of the colony (Pinter-Wollman, 2012; Hui and Pinter-Wollman, 2014; Klein et al., 2017; Mosqueiro et al., 2017).

**Proximate Causes of Social Insect Inter-Individual Behavioral Variation**

A personality-based framework for understanding inter-individual behavioral sub-castes is novel, but is consistent with, and expands upon and extends, previous models for the organization of worker division of labor in social insects. An important idea related to the
organization of work, and individual variation in behavior, in social insect colonies is the response-threshold model (Page and Erber, 2002; Page and Mitchell, 1998). This model proposes that each worker has a specific threshold for responding to a stimulus (e.g. nest temperature), and that this threshold varies between individuals. In honey bees, genetic variation among workers in a colony is an important contributor to variation in behavioral thresholds (Calderone et al., 1989; Robinson and Page, 1989; Page, 2013). Honey bee queens mate with multiple males and store their sperm in a specialized organ, the spermatheca, for the duration of their life (Winston, 1987). Thus, a queen’s daughters, the colony’s work force, may derive from various patrilines. A worker’s patriline influences which tasks she is most likely to perform, including corpse-removal (Robinson and Page, 1988), nest-site scouting (Robinson and Page, 1989), and nectar and pollen foraging (Robinson and Page, 1989; Dreller et al., 1995). Although these studies did not explicitly investigate if these behavioral differences were consistent across time or context, patrilineal differences likely contribute to personality differences across workers.

Developmental and environmental factors can also contribute to inter-individual behavioral variation. For example, recent studies suggest life-long effects of rearing environment on levels of honey bee aggression (Rittschof et al., 2015; Rittschof, 2017). A difference in nourishment, in particular, is a likely proximate cause of intra-caste behavioral variation. In many social insects, nutritional differences organize social life as the major determinant of the reproductive division of labor (Wilson, 1971). The honey bee *Apis mellifera* serves as an illustrative model of how these early life differences in nutrition have permanent effects on an adult’s behavior, morphology, and physiology. Whether a developing larva will become a queen or worker depends on the diet she receives (Winston,
Additionally, adult nutritional state can affect behavior. A worker’s nutritional state acts in part to regulate behavioral caste, in that nurses tend to have higher lipid stores than foragers (Toth and Robinson, 2005), and reduced nutritional state causes early, and more frequent foraging (Mattila and Otis, 2006; Schulz et al., 1998; Toth et al., 2005). How variation in nourishment may affect long-term behavioral differences among workers is still an open area of research.

**Dissertation Organization**

In this dissertation I examine intra-caste, inter-individual behavioral variation among social insect workers. In chapter 2, I show that honey bee workers exhibit inter-individual behavioral differences in the form of personality. I use long-term behavioral observation techniques to track individual workers from the same age cohort throughout their lifetime to detect consistent behavioral differences, even as they switch behavioral regimes predicted by an age-related division of labor. I present evidence that honey bee workers exhibit all three dimensions of personality: behavioral variation that is consistent over time, across contexts, as well as the presence of suites of correlated behaviors.

In Chapter 3, I examine a potential proximate cause of inter-individual behavioral variation in honey bee workers: differential nourishment. In this experiment, I test the hypothesis that nutritionally stressed bees will be more likely to perform cooperative behavior than satiated workers. I restricted worker nutrition at both larval and adult life stages, and measured the effect on the cooperative behavior of response to queen mandibular pheromone. I found that nutritional restriction affected queen mandibular pheromone response, but the direction of the effect was different depending on life stage. I propose that these differences in how nourishment affects cooperative behavior are due to honey bee
worker reproductive physiology, which is still plastic as larvae, but more canalized by adulthood.

I suggest in Chapter 3 that examining the effects of nutritional stress on social behavior would be especially informative across species with gradients in reproductive plasticity, especially on other eusocial insects with higher levels of reproductive plasticity that persist through adulthood. In Chapter 4, I report the findings of such an experiment on the primitively eusocial insect, the paper wasp *Polistes fuscatus*, in which restricted nourishment led to higher aggression among nestmates.

In summary, this dissertation offers a novel exploration of the phenotypic variation of individuals in social insect societies. This research integrates ideas and techniques from nutritional ecology, evolutionary developmental biology, and ethology to address outstanding questions about how behavioral variation arises. In the future, these findings will help inform research on the origins of phenotypic variation and its role in evolution.

References


CHAPTER 2. VARIATION IN INDIVIDUAL WORKER HONEY BEE BEHAVIOR SHOWS HALLMARKS OF PERSONALITY

Modified from a paper published in *Behavioral Ecology and Sociobiology*

Alexander Walton and Amy L. Toth

Abstract

The existence of personalities has been explored in various invertebrates, but a comprehensive investigation of personality differences across individuals in a eusocial insect has not yet been conducted. The study of personality differences across individuals within the same behavioral caste may contribute to an understanding of how social insects divide labor within the nest. Here we define and investigate three dimensions of personality within the worker caste of a model social insect, the honey bee *Apis mellifera*, as follows: 1) consistent individual behavioral differences over time, 2) consistent individual behavioral differences across contexts, and 3) the presence of correlated suites of behaviors. To test whether honey bee workers exhibit dimensions 1 and 2, we repeatedly assessed responses of groups of same-age bees in cages to stimuli that are relevant to bee life history. To test for dimension 3, we examined behavior within a colony context by using observation hives to record the behaviors of individual bees across their lifetimes. Our results provide some evidence for all three dimensions of personality in honey bee workers. In particular, our data suggest some individuals may be more likely to be highly interactive with other workers (e.g. engaging in food sharing), while other individuals are consistently less interactive. These findings expand upon and contribute to previous models for the organization of worker division of labor in honey bees, suggesting that consistent behavioral differences (personalities) of
workers within a behavioral caste have the potential to contribute to subcaste division of labor.

**Introduction**

Recently, the study of personality has garnered increased interest in the field of animal behavior (Gosling 2001; Sih et al 2004; Réale et al 2007; Dall et al 2012; Dall et al 2004). Personalities are consistent behavioral differences between individuals within a population. Several different aspects of personality have been defined and well-established in the animal behavior literature based on studies in a wide variety of different organisms (reviewed in Mather and Logue 2013; Kralj-Fišer and Schuett 2014). Although definitions vary, the major aspects of personality can be encapsulated in three “dimensions” as follows: (1) consistency in individual behavior over time, (2) consistency in individual behavior across contexts, and (3) the presence of correlated suites of different behaviors (sometimes called behavioral syndromes). Individual variation in the behavior of eusocial insects is particularly striking, and easily recognized among different members of the society. In some cases, individual behavioral differences in social insect colonies are so distinct and stereotyped that completely separate groups of individuals are easily recognized as part of different “castes”, including reproductive castes, morphological castes, and behavioral castes (Oster and Wilson 1978; Hölldobler and Wilson 1990). Because of the presence of distinct castes, it has been assumed that personalities are not a useful concept for understanding division of labor; partly for this reason, an exploration of individual differences in social insect behavior using a personality framework is relatively unexplored (Jandt et al 2014). However, it is well recognized that there can be substantial variation in the behavior of individuals within a caste, and also differences between caste types and species as to how distinct or flexible castes are, leaving open the possibility of individual personality differences within castes. Although it is clear that individuals within a caste
vary, it is not entirely clear how much of this variation is attributable to individual behavioral consistencies over time and across contexts. If such “personality-based sub-castes” exist, then a consideration of personality differences can contribute to our understanding of division of labor within social insect colonies. For example, particular personalities might be more prone to perform particular tasks, even within the context of an established morphological or temporal caste system. This sub-caste variation in personality could be adaptive by contributing to a more finely-tuned division of labor, with tasks being performed more readily and or efficiently by individuals of different personality types.

Social insects live in highly integrated societies, where many individuals share the duties of maintaining the colony (Wilson 1971). The honey bee (Apis mellifera L.) is a highly eusocial insect, is an important behavioral model organism, and its division of labor has been extremely well-studied and characterized (Robinson 1992). Honey bees have colonies containing only one reproductive (the queen) and several thousand workers (Winston 1987). These individuals, the queen’s many daughters, make up a workforce that performs all non-reproductive tasks essential for the maintenance of the colony. It is well-established that a great deal of this division of labor is determined by age polyethism. As workers age, they generally transition from performing inside tasks (brood care, cleaning, building) to performing outside tasks (guarding, foraging for food) (Seeley 1985). However, variation does exist in which workers perform which tasks and when they perform them, though this variation remains to be broadly studied (Beshers and Fewell 2001). A personality framework has not yet been directly applied to the study of honey bee worker division of labor, but this perspective could be helpful for understanding how behavioral variation of individuals within a honey bee colony arises, and how this contributes to behavioral sub-caste division of labor.

The potential for personalities in an insect such as the honey bee has precedent in the
literature, as dimensions of personality have been shown to exist in several other groups of invertebrates (Mather and Logue 2013; Kralj-Fišer and Schuett 2014). The short length of an invertebrate lifespan creates a limit on the length of time over which behavioral consistency can occur, but has the benefit of making it easy to observe behavior from birth to death. Due to their highly cooperative behavior and colony-level adaptations, honey bees and other social insects may offer unique insights into how and why personalities exist in invertebrates and more broadly, in social animals. Individual variation in some of the dimensions of personality has been recorded in several species of social invertebrates (reviewed in Jandt et al 2014). For example, aggression and boldness were found to be consistently correlated with each other in *Myrmica rubra* and *Myrmica ruginodis* worker ants, and this boldness-aggressiveness syndrome was related to which tasks individuals performed (Chapman et al 2011). Additionally, in colonies of ants in the genus *Temnothorax*, there is consistency in how active (Pinter-Wollman et al 2012) or inactive (Charbonneau and Dornhaus 2015) individuals are. Because of highly integrated social group structures, social invertebrates may vary in behaviors that are unique to sociality (such as aggression toward nestmates or affiliation with nestmates). The variation in such interactive behaviors may contribute to a colony-level behavioral phenotype (LeBoeuf and Grozinger 2014). For example, in the social spider *Stegodyphus sarasinorum*, group-level personality in relation to boldness was reflective of the boldness of individuals that comprised the group (Pruitt et al 2013).

Researchers have begun to explore the potential for personalities in honey bees, at both the individual and entire colony levels. Honey bee colonies have displayed consistent differences in their propensity for group level activity in foraging, defense, undertaking (Wray et al 2011), and house-hunting behavior while swarming (Wray and Seeley 2011), and such differences have been suggested to represent colony-level personalities (Jandt et al 2014). Although accounts of
individuals that exhibit high specialization for allogrooming (Moore et al 1995), water collection (Robinson et al 1984), and dancing and vibration signals (Duong and Schneider 2008) have been recorded, there are still very few investigations into whether individual workers show the type of behavioral consistency that would qualify as personality. Liang et al (2012) found that individual bees in a swarm that scouted for new nest sites were more likely to scout for new food resources than individuals that did not scout for new nest sites, thus these individuals displayed a consistency in novelty-seeking across contexts. Correlations of behaviors have been documented in honey bees in relation to whether they are nectar or pollen foragers (Page et al 1998; Page 2013). Foraging type correlates with light sensitivity (Erber et al 2006), learning (Scheiner 2012), and collection of water and plant resin (Simone-Finstrom et al 2010). These studies show hallmarks of two of the dimensions of personality: behavioral consistencies across contexts and the existence of correlated suites of behaviors. Notably, the first personality dimension, consistency over time, has not yet been investigated in honey bees, likely because of the well-known and pronounced age polyethism of honey bees. Despite the fact that behaviors change predictably as worker bees age, there is substantial variation in task performance frequency and which tasks individual bees perform at any given age (Calderone and Page, 1988; Tenczar et al. 2014). Therefore, it is possible that personality types exist whereby individuals gravitate towards specific types of stimuli or tasks throughout their lifetimes (e.g. interacting with nestmates vs. solitary tasks), even within the context of an age polyethism.

In the current study we provide the first comprehensive investigation of three dimensions of personality in worker honey bees: (1) behavioral consistency over time, (2) behavioral consistency across contexts, and (3) the presence of correlated suites of different behaviors (Table 1). To do this, we used a two-pronged experimental approach. First, we used an artificial laboratory cage paradigm
to test whether honey bee workers exhibit personality dimensions 1 and 2. The cage setting, while undoubtedly artificial, is extremely useful because it allows for repeated observations of a large number of individuals over time and across contexts, which is logistically difficult in a natural colony context due to the low probability of repeatedly observing individual bees among thousands of nestmates. Using the cage paradigm, we assessed responses to well-established stimuli that are relevant to bee life history. These stimuli were queen mandibular pheromone (a pheromone produced by the queen that elicits a response from workers in which they antennate and feed her), alarm pheromone (which is produced by guard bees when the colony is under attack and induces a defensive response from other bees in the nest), and an intruder bee from another colony (often inducing an aggressive response) (Winston 1987). We repeatedly presented caged bees with these three different stimuli and recorded individual bee behavioral responses (such as aggression or food-sharing, i.e. trophallaxis). We then looked for consistency over time and across the contexts of the three different stimuli. Second, we utilized observation hives to provide complementary data on spontaneously occurring worker behavior (e.g. queen tending, brood care, trophallaxis, foraging, etc.) under more natural conditions. This allowed us to investigate dimension 3 by recording the behaviors of individual bees experiencing a full suite of colony stimuli across their lifetimes. We first used an exploratory approach to examine whether there were sets of correlated behaviors performed by individual colony-reared bees. In addition, we followed this up with a hypothesis-based approach that stemmed from our cage studies, where we observed that certain individuals appeared to be more likely to engage in trophallaxis. Because trophallaxis is a common interactive behavior exhibited throughout adult bee life, and is an important channel of communication in several contexts including food and pheromone exchange (Leoncini et al 2004), we hypothesized that naturally occurring colony-based behaviors would correspond to a distinct syndrome (correlated
suite of behaviors) with respect to an individual’s tendency to interact with other bees. These are defined as: 1) interactive: performing tasks that require direct physical interaction with other individuals, and 2) non-interactive: performing tasks that do not require direct interactions with others. We predicted that behaviors that require direct contact with other bees would be more likely to co-occur in some individuals, even in the context of age polyethism in which workers are switching task regimes as they age.

Methods

Experiment 1: Cage-based Stimuli Response Assays

Bees

The bees in this experiment were collected from hives at the Iowa State University Horticulture Research Station in Ames, Iowa during the winter of 2014 and summer of 2015. The bees from winter 2014 were reared during winter months when normal hives are not producing brood; this was done in order to continue experimentation through the winter months. To stimulate brood production and summer-like colony activity, we placed colonies in a heated room, approximately 25°C with timed lights to reflect summer day length. Colonies were fed field-collected pollen patties, artificial pollen supplements, and 1:1 sucrose syrup. Frames containing pupae about to emerge were removed and placed in a 33°C incubator overnight. Newly emerged day-old adults were collected from brood frames from 2 different colonies for winter replicates. Although brood was collected over the winter months, these colonies were still active and producing brood, so we did not expect them to behave differently from summer bees. In summer 2015, we similarly collected frames of capped brood from a mix of 5 different field colonies, placed bees in an incubator overnight,
and collected one day-old newly emerged bees for cage experiments. Bees from summer 2015 came from colonies that experienced natural outside summer conditions.

Cage assays

Within 24 hours of eclosion, groups of 9 adult day-old bees were marked, each individual with a unique color of paint, and placed in Plexiglas cages (dimensions: 10.16 cm x 10.16 cm x 7.62 cm) and kept in an incubator at 33°C and fed 50% sucrose solution ad libitum. We performed a series of three behavioral assays and recorded how every single individual bee responded to particular stimuli. Every other day for up to 17 days (the maximum age that experimental bees survived) we introduced one of three of the following stimuli to the entire cage of bees: 1) a microscope slide with synthetic QMP (which can elicit retinue response, to assess queen responsiveness). QMP (Pherotech International, Delta, British Colombia) was diluted in 1% water/isopropanol to 0.01 queen equivalents, which has been shown to elicit a normal queen response (Pankiw et al 1994), 2) a microscope slide with crushed bee stingers and venom sacs (which emit alarm pheromone, to assess aggressiveness), or 3) a live “intruder” bee taken directly from a different colony (which is perceived as an intruder, to assess sociability and aggressiveness). Each stimulus was presented sequentially to cages once every other day throughout the course of the experiment with five minutes between stimuli. The order of stimulus presentation was randomly assigned each day.

During each presentation of a given stimulus, each cage was observed every 30 seconds for 10 minutes and the following behaviors were recorded, along with the identity of the individual bee performing the behavior:
- Trophallaxing with a cage-mate (all contexts)
- Attacking a cage-mate (all contexts)
- Responding to QMP (contacting the slide with antennae, QMP context only)
- Attacking the intruder bee (intruder context only)
- Responding to stinger slide (contacting the slide with antennae, stinger context only)

The behaviors of trophallaxis and attacking a cage-mate were used to assess cross-context consistency because they were the only two behaviors consistently observable across all contexts. A total of 13 cages of bees were observed during the winter of 2014 and 22 cages of bees were observed during the summer of 2015. Each cage started with 9 bees, but bees that died within the first two days of observations were removed from the analyses. The average lifespan of bees in these cages was 9.54 days. This is lower than the average lifespan of free-living summer bees, which has been reported from 15 – 38 days (Winston, 1987). Although the lifespan of caged bees was short, it served the experimental purpose to repeatedly assess behavior of individual bees. The total number of individual bees with behavioral data in our study was 171. Although these cages represent unnatural conditions for bees, the cage paradigm is a well-established method for honey bee experimentation (Williams et al 2013). Additionally, testing personality in unnatural conditions such as cages is common practice for many animal behavior studies (e.g. spider aggression and boldness tested in plastic enclosures in Pruitt et al 2013, and the personality traits of blue tits tested in cages which reflected personality traits when tested in the wild in Herborn et al 2010). Cages ensured that individuals would be observed many times during their lifetime and across contexts, which is nearly impossible to achieve when observing an actual hive due to the
large number of bees (many thousands) and the small chances of being able to observe the same bee responding to many stimuli (e.g. queens, intruders, disturbances, etc.). Each bee was tested with each stimulus every other day until she died. Because the average lifespan of bees in this experiment was 9.54 days, this means that the average number of times an individual was tested with each stimulus was 4.77.

Statistics

Statistical analyses were performed using R version 3.1.1. For each behavior, a “response rate” was calculated by counting the number of times a bee performed any behavior and dividing by the number of days she remained alive during the course of the experiment in order to account for differences in total behavioral performance due to mortality. To measure behavioral consistency over time (personality dimension 1), we measured Kendall’s coefficient of concordance (KCC), a non-parametric estimator of repeatability commonly used in personality studies (e.g. cats: Durr and Smith 1997; hermit crabs: Briffa et al 2008; honey bee colonies: Wray et al 2011). A KCC was ascertained based on individual bees’ behavioral response rates in each context (during the presence of each stimulus) using the “kendall.global” function in the R package “vegan” (Oksanen et al 2013).

To assess the consistency of individuals across the three contexts (personality dimension 2) we assigned individuals to a category based on how they behaved in one context and then examined the response rate of the same behavior in the two other contexts. Individuals were assigned to the categories of “aggressors” or “non-aggressors” based on whether they were ever aggressive toward cage-mates during the stingers assay. If a bee lunged at a cage-mate or mauled a cage-mate, i.e., clutched and pulled at another bee with
her mandibles (Butler and Free 1952; Sakagami 1954), it was recorded as an aggression incident. If they exhibited aggression toward a cage-mate at least one time during a stinger assay they were categorized as aggressors (n = 43), and if they never exhibited aggression toward cage-mates in the stinger assay they were categorized as non-aggressors (n = 128). The stinger context was used as the basis for assigning bees to the aggressor categories because this context had the highest number of aggression incidents, allowing for a more even number of aggressors and non-aggressors than if another context was used to assign individuals to these categories. We compared the aggressive response rate of these aggressors and non-aggressors in the two other contexts (intruder and QMP contexts) and performed a Mann-Whitney U test to compare the response rate in each context.

Additionally, individuals were assigned to the categories of “trophallaxers” or “non-trophallaxers” based on whether they engaged in trophallaxis during the QMP-slide assay. If they participated in trophallaxis at least one time during a QMP-slide context assay, they were categorized as trophallaxers (n = 103). If not, they were categorized as non-trophallaxers (n = 68). The QMP-slide context was selected because it had the highest number of trophallaxis incidents and thus provided us with a roughly equal sample size of bees in the trophallaxer and non-trophallaxer categories. A Mann-Whitney U test was used to compare the difference in trophallaxis response rate between these two groups in both the stinger slide context and the intruder bee context. A preliminary ANOVA was run for each behavior in each context to test whether there was a significant difference between bees’ behavior during summer and winter. We found that season was not significant for 7 of the 9 behaviors (data not shown). Because there was not a systematic bias of season, we pooled data from both seasons.
All pairwise Pearson correlations of behaviors measured were calculated in R using the “psych” package (Revelle 2014) and adjusted to control the false discovery rate using the Benjamini-Hochberg procedure (Benjamini and Hochberg 1995).

**Experiment 2: Observation Hives**

To track and record behavioral data of individuals throughout their lifetimes, we utilized observation hives constructed from wood and clear Plexiglas. These transparent-walled bee hives allowed us to monitor activity within the colony without disturbing the bees. Although the chances of observing an individual many times throughout her life is much less than the cage assays, the observation hives simulate a much more natural environment in which to observe these behaviors and allow us to observe a much wider array of behaviors that cannot be performed in cages (e.g. foraging, brood care, etc.). Each individual in an experimental age cohort was marked with a unique number tag, which was glued to the top of each bee’s thorax. We recorded 17 different behaviors and categorized these behaviors into the categories of "interactive" (require direct contact or interaction with another adult or larval individual) and "non-interactive" (do not require interaction with another individual), see Table 2. At the start of the experiment 485 individually marked bees of the same age were added to each colony (n = 4 observation hives). Twice daily for 4 weeks these hives were observed for an hour each. The observer recorded the behaviors of every marked bee visible during the observation period. When a bee was observed, her behavior was recorded and she was not observed again for that observation period. Thus, an individual bee could have a maximum of two behaviors recorded every day. Observation hives were set up and observed during August and September of 2013. A total of 1107 bees
were observed at least twice during the course of the entire four colony trials of this experiment. The average number of observations per bee used for analysis was 4.77.

**Statistics**

The number of times a bee performed one of the 17 behaviors (Table 1) was calculated. Bees observed less than two times were removed from all subsequent analyses. A correspondence analysis was performed on the lifetime counts for all behaviors observed to visualize whether groups of behaviors are performed by the same individuals. The correspondence analysis was performed in R version 3.1.1 using the “cca” function in the “vegan” package (Oksanen et al 2013). A Mann-Whitney U Test was used to compare the CA scores of behaviors assigned to be “interactive” vs “non-interactive” (as listed in Table 1) using values derived from the first axis of the correspondence analysis.

For pairwise correlations of behaviors, the number of times a bee performed one of the 17 behaviors was divided by the number of times she was observed throughout the entirety of the experiment, thus each bee had a “behavior frequency score” for each behavior. Pairwise correlations between “behavior frequency scores” for all behaviors were examined using Spearman correlations in R with the “psyche” package (Revelle 2014). All pairwise correlations of behaviors observed in the observation hives were calculated and adjusted to control the false discovery rate using the Benjamini-Hochberg procedure (Benjamini and Hochberg 1995).
Results

Experiment 1: Cage-Based Stimulus Response Assays

**Dimension 1: Are individuals consistent in how they behave over time?**

All behaviors (response to stimulus, trophallaxis with cage-mate, and aggression towards cage-mate) in all contexts (QMP assay, stinger assay, and intruder assay) were significantly repeatable (*Figure 1*). Kendall’s coefficient of concordance (KCC) for behaviors observed in this study ranged from 0.08 to 0.14.

**Dimension 2: Are individuals consistent in how they behave across contexts?**

Individuals observed performing aggression toward cage-mates (“aggressors”) in the stinger assay were consistently more aggressive than individuals not observed performing aggression toward cage-mates (“non-aggressors”) in the context of the QMP slide assay (Mann-Whitney U test: \( W = 2433; p\text{-value} = 0.02851 \)). However, there was no difference between aggressors and non-aggressors in how aggressive they were to cage mates in the context of the intruder assay (Mann-Whitney U test: \( W = 2737.5; p\text{-value} = 0.6867 \)) (*Figure 2a*).

Individuals observed performing trophallaxis in the QMP slide assay (“trophallaxers”) showed consistent behavior in other contexts—they participated in more trophallaxis events than “non-trophallaxers” when present (Mann-Whitney U test: \( W = 2584; p\text{-value} = 0.00087 \)), and when stingers were present (Mann-Whitney U test: \( W = 2822.5; p\text{-value} = 0.01899 \)) (Figure 2b).

Additionally, we tested all pairwise correlations between response rates of all behaviors (both within and across contexts) and found several pairs of behaviors were correlated. Notably, we found instances of the same behavior being significantly correlated across
contexts. Trophallaxis response rates in all three contexts were positively correlated with each other (Table 3). Our data demonstrate that individuals with a high trophallaxis rate in one context were more likely to have a high trophallaxis rate in both other contexts (Figure 3). In addition, response to QMP was positively correlated with trophallaxis rate in both the QMP context and the stingers context (Spearman rank correlation: $r = 0.0721$; p-value = 0.0414), suggesting a connection between queen responsiveness and tendency to engage in trophallaxis. In addition to the aforementioned examples, we also found some additional correlations between different behaviors within the same context, and different behaviors across different contexts (Supplemental Table 1).

**Experiment 2: Observation Hives**

Before examining the third dimension of personality, we first used our observation hive data to explore the frequency of performance of all 17 behaviors in relation to bee age. The purpose was two-fold. First, we wanted to confirm that we conducted sufficient observations to replicate well-known patterns of age polyethism in honey bees (e.g. nursing before foraging). Second, we wanted to see if any behaviors (such as trophallaxis, as examined in Experiment 1) were age-independent. As expected, we found that bees in our observation hives exhibited a clear age polyethism, e.g. on average nursing behavior occurred much earlier than foraging behavior (as in Seeley 1982; Winston 1987). We also found that a few behaviors, most prominently trophallaxis, were performed broadly by bees of different ages (Supplemental Figure 1). This result lends support to the idea that trophallaxis is used in many social contexts throughout a bee’s behavioral development, and thus trophallaxis is a good candidate behavior for exploring personality differences and is a potential biomarker for an individual bee’s interactive tendency.
Are there pairwise correlations between tasks?

To investigate whether some tasks are associated with each other, we investigated whether pairs of tasks showed significant correlations in their incidence of performance by individual bees. Essentially, we were asking: if a bee performs a particular task, is she more likely to also perform a different task? All pairwise correlations were calculated and adjusted to control the false discovery rate using the Benjamini-Hochberg procedure (Benjamini and Hochberg 1995). All significant pairwise correlations are reported in Table 4. Out of a total of 136 tested correlations, we found only 6 pairs of behaviors were significantly correlated with each other. Four of the 6 significantly correlated pairs were positive correlations between two “non-interactive” tasks, and two were negative correlations between two “non-interactive” tasks.

Do interactive tasks group together?

To examine whether different groups of individuals perform interactive and non-interactive tasks, we performed a correspondence analysis (CA) using the full suite of behavior performance scores for all individual bees (Figure 4). We found a trend across CA axis 1 (CA1), in which interactive tasks had lower CA1 scores and non-interactive tasks had higher CA1 scores. We performed a Mann-Whitney U test on CA1 scores and found a trend, but no significant difference, between the CA1 scores of interactive and non-interactive tasks (Mann-Whitney U test: W = 47; p-value = 0.11).

As is apparent in Figure 4, queen retinue behavior appears to be an outlier task that is not grouping together with all of the other tasks. Retinue behavior was characterized as an interactive task, but all other interactive tasks involve interacting with another worker bee, whereas retinue behavior is the only one of these behaviors that involves interactions with the queen. Thus, we
repeated the CA1 score analysis after removing retinue behavior. In this case, we found a significant difference between CA1 scores between interactive and non-interactive tasks (Mann-Whitney U test: \( W = 47; p\)-value 0.018).

**Discussion**

This study provides the first comprehensive investigation of whether individual worker honey bees show hallmarks of personality. We investigated three dimensions of personality (Table 1)—consistency over time, consistency across contexts, and correlated suites of behaviors—and found some evidence for all three dimensions of personality in worker bees. Although age polyethism may be a stronger determinant of individual behavioral choices during an individual worker bee’s lifetime, our data suggest individual personalities may still have the potential to contribute to variation between individuals in their tendency to perform different tasks at a given age.

A first step in establishing personalities is to demonstrate that behaviors are repeatable, addressing the first dimension of personality. All behaviors measured in these assays were demonstrated to be significantly repeatable. However, the repeatability values calculated for all honey bee behaviors (range 0.08-0.2) were on the low side, below average for repeatability values of behavioral traits reported in other organisms (Figure 1, reviewed in Bell *et al* 2009). Nonetheless, repeatability values ranging between 0.1 and 0.2 are not uncommon for behavioral studies. Of the 759 repeatability measurements of behavioral traits compiled by Bell *et al* (2009) from 114 studies, 100 fell within this range. A problem with measuring repeatability of behavior for honey bees is the strong age-dependency of behavior (Seeley 1982). Thus, we do not fully expect all behaviors to be highly repeatable. Even though bees clearly changed behaviors as they aged (Supplemental Figure 1), there were still
significant consistencies over time in all behaviors measured, and these consistencies varied between individuals, a hallmark of personality.

In addition to behavioral consistencies over time, we found evidence for the second dimension of personality, individual behavioral consistency across contexts. Individual behavior across contexts was only partially consistent for aggression, but was highly consistent for trophallaxis. For aggression, individuals that were aggressive toward a cage-mate in the presence of stingers were more likely to be aggressive toward cage-mates in the QMP-context, but this was not seen in the presence of an intruder (Figure 2a). On the other hand, for trophallaxis, individuals that performed trophallaxis in the context of queen mandibular pheromone were more likely to perform trophallaxis in both of the other two contexts (presence of an intruder and presence of stingers) (Figures 2b and 3). These data suggest consistency in trophallaxis across contexts is relatively robust. Trophallaxis is an important component of colony communication; it is related to food exchange and provides the conduit for the “social stomach” of the colony, aids in exchange of queen and forager pheromones (Crailsheim 1998), spreads information regarding the nutritional needs of the colony (Camazine et al 1997) and the quality of forage resources (Farina and Núñez 1991; Farina 1996, Grüter et al 2006), and is also used by guard bees at the entrance to distinguish between nestmates and intruders (Kirchner and Gadagkar 1994). Our data suggest some bees may be more likely to engage in trophallaxis throughout their lifetimes and across contexts, and this result led us to hypothesize correlated suites of interactive behaviors would co-occur in individual bees.

Using our observation hives, we addressed this hypothesis, finding some evidence for the third dimension of personality, correlated suites of behaviors performed by different
individuals in the colony. In examining pairwise correlations of behaviors, we found very few behaviors that were significantly correlated, i.e., likely to be co-performed by the same sets of bees. The small number of significant correlations may be due to the fact that we had low statistical power due to having relatively few observations on any given bee. Based on this limited dataset, we did find that most correlations were between pairs of non-interactive tasks (Table 4), and the strongest correlation was a pair of non-interactive tasks. Taking a multivariate view of the data with a correspondence analysis, we found a tendency for tasks that do not involve direct worker-worker interactions to be performed by different individuals than tasks that are interactive in nature (Figure 4); however, this separation was only significant when queen retinue behavior was removed from the analysis. Although the separation of interactive and non-interactive behaviors was not perfect, these data provide a starting place for future investigations into whether certain individuals in a colony are more interactive members of a colony-wide social network.

Of all the behaviors observed in the observation hives, the most highly correlated pair of behaviors (i.e. performed by the same individuals) was washboarding (when a worker repeatedly scrapes her tarsi and mandibles across a wooden surface inside or on the hive itself) and waxwork, i.e., the application and manipulation of wax within the hive (Table 4). Although beekeepers and bee researchers have long been aware of washboarding behavior, its purpose remains unknown and under-studied (Seeley and Morse 1976). Washboarding has been described as a task that may smooth the entrance of the hive (i.e. Seeley and Morse 1976; Johnson 2008), but it has never been confirmed that this behavior has an effect on the coarseness of the wood. Washboarding was a fairly uncommon behavior (performed by 66 individuals, in contrast to the more general performance of brood care, performed by 431
individuals) suggesting this behavior may be restricted to a relatively specialized group of workers. Perhaps washboarding is a nest maintenance task as previously hypothesized (Seeley and Morse 1976) and its association with wax work is evidence that it is part of a series of maintenance-related subtasks, or more broadly, that it is part of a hive maintenance personality type. The purpose of washboarding continues to be a mystery to bee researchers, so exploring its significance within the framework of personality could offer fresh and meaningful insights into the function and significance of this behavior.

Our results raise the question—could the hallmarks of personality found in honey bee workers be an adaptive part of honey bee division of labor, or are they an artifact of constraints on behavioral flexibility? Although we cannot answer this question directly from the data provided in the current study, we suggest that personality-level variation within the worker caste has the potential to provide a benefit to colony division of labor. Conventional representations of honey bee temporal castes describe individuals as switching from one task to another as they age (Seeley 1982). Our results suggest that some behavioral elements remain consistent as individuals age (e.g. tendency to engage in trophallaxis or interactions with nestmates) even as they switch temporal behavioral castes. If certain individuals gravitate more toward certain tasks, they may become better at performing those and related tasks (due to learning), potentially providing a personality-based benefit to individual consistency. Such explanations have previously been suggested to explain the adaptive value of individual bees that are ultra-specialists on specific tasks such as water collecting (Robinson et al. 1984). Sub-caste behavioral variation, in the form of individual differences in personality, could fine tune age-related division of labor and thus contribute to division of labor efficiency and colony fitness. Further studies that examine how individual worker
personality variation is manifest at the whole colony level are needed to better understand the implications of such variation for colony division of labor.

The idea of personality-based behavioral sub-castes is consistent with, but expands upon and extends, previous models for the organization of worker division of labor in social insects. One dominant explanation for honey bee (and several other species of social insect) worker division of labor is age polyethism (Seeley, 1982), which our data also clearly support as a strong determinant of individual behavioral task performance (Supplemental Figure 1). In addition, another important idea related to the organization of work in social insect colonies is the response-threshold model (Page and Erber 2002; Page and Mitchell 1991). This model proposes that each worker has a specific threshold for responding to a stimulus (e.g. nest temperature), and that this threshold varies between individuals. An important element of the response threshold model of division of labor is that response thresholds are expected to change with experience and age (Pankiw et al 2004; Pankiw and Page 1999). The idea of personality-based sub-castes has the potential to expand this model of division of labor by emphasizing that there are elements of individual behavior that may remain consistent over time and across contexts. This idea is thus consistent with response threshold models, but brings up the possibility that response thresholds to some stimuli could be related to personality type, and thus somewhat consistent over the lifetime of an individual. The rigidity in behavioral responses prescribed by personality types could contribute to more robust differences in behavior between individuals, thus reinforcing a division of labor even when individuals age together or share similar experiences.

The mechanisms that lead to consistent personality differences in individual honey bees will require further study. One likely explanation is that consistent individual behavioral
differences may arise due to genetic differences across workers. A worker’s patriline can influence which tasks she performs in the hive (Robinson and Page 1988; Robinson and Page 1989), so it is likely that genetics plays a role in the development of consistent behavior over time and across contexts. Mechanistic studies on personality variation in humans and other vertebrates also strongly implicate environmental effects, particularly early life exposure to environmental stressors or maternal factors mediated by epigenetic mechanisms such as DNA methylation (reviewed in Powledge 2011). Recent studies suggest life-long effects of rearing environment on levels of honey bee aggression (Rittschof et al 2015), and DNA methylation has also been linked to age polyethism and queen-worker caste differences in honey bees (reviewed in Rasmussen and Amdam 2015). Further research is needed to better understand the genetic, environmental, and epigenetic factors that may contribute to individual differences in honey bee worker behavior, including personalities.

Although our data on individual behavioral differences in worker honey bees show hallmarks of personality, we suggest that personality differences could be more pronounced and possibly more important to division of labor in other types of insect societies. Honey bees exhibit a highly derived form of sociality with many group-level adaptations and a robust worker division of labor. Thus, the adaptive benefits of individual personality may be muted by factors such as a well-developed age polyethism, the large number of individuals that make up a colony, a highly derived social integration that favors a lack of behavioral individuality (like the cells of a particular tissue in a multicellular organism). Variation in personality could potentially be more important in a social insect colony with fewer individuals and with a weaker age polyethism, such as more intermediately eusocial species such as bumble bees or paper wasps, or more incipiently social species such as some sweat or
carpenter bees. Future comparative work on groups of organisms of varying levels of sociality will be highly informative for understanding the evolution and possible adaptive role of personality in social insects.

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We would like to thank members of the Toth lab for helpful suggestions on these experiments and feedback on the manuscript. We would like to thank Kate Hunter for assistance with behavioral observations and Eric Gangloff for guidance on statistical analyses.

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Figures

**Figure 1.** Repeatabilities of honey bee behaviors over time, placed in context with studies from other organisms. All honey bee behaviors in all stimulus contexts were significantly repeatable (from 171 bees), but fell within the lower end of the range of significant repeatabilities obtained for behaviors in other animals (reviewed in Bell et al 2009). Note, the repeatability values reported for honey bee behaviors here are Kendall’s coefficients of concordance (KCC), whereas the repeatabilities from other animal behavior studies are intraclass correlation coefficients (ICC). Although they are different methods, they are both used to calculate repeatability and are on the same scale (0 to 1), so they are roughly comparable.

<table>
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<th>Context</th>
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</tr>
<tr>
<td>2 Stinger</td>
<td>Response</td>
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<tr>
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<tr>
<td>4 Intruder</td>
<td>Response</td>
<td>0.1</td>
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<tr>
<td>5 Intruder</td>
<td>Aggression</td>
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<td>6 Stinger</td>
<td>Aggression</td>
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<td>9 Stinger</td>
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Figure 2. Consistency of behaviors across contexts. a) The aggressiveness of individuals that were aggressive toward cage-mates in the stinger assay (aggressors, n = 43) or not aggressive (non-aggressors, n = 128), in two other contexts. Aggressors were more aggressive than non-aggressors in the QMP context (Mann-Whitney U test: W = 2433; p-value = 0.02851), but there was no difference in the intruder context (Mann-Whitney U test: W = 2737.5 p-value = 0.6867).  
b) The number of trophallaxis incidences involving individuals that trophallaxed with a cage-mate during the QMP assay (n = 103) or not (n = 68), in the two other contexts. Trophallaxers trophallaxed more than non-trophallaxers in the intruder (Mann-Whitney U test: W = 2584; p-value = 0.00087) context and the stingers context (Mann-Whitney U test: W = 2822.5; p-value = 0.01899).
Figure 3. Three-dimensional scatterplot of trophallaxis in different contexts. Axes are response rates for trophallaxis in each context. Dots represent individual bees. The plane illustrates the positive correlations in individuals’ performance of trophallaxis in all three contexts (Spearman correlation: QMP and Intruder: $r = 0.167$, p-value = 6.99E-08; QMP and Stingers: $r = 0.128$, p-value = 5.75E-05; Intruder and Stingers: $r = 0.129$, p-value = 5.09E-05)
Figure 4. Correspondence Analysis on tasks observed in observation hives, denoting relationship of behaviors observed in observation hives. Behaviors close to each other were performed by more of the same bees than behaviors further from each other. Circles indicate “interactive” behaviors and triangles indicate “non-interactive” behaviors.
CHAPTER 3. HUNGRY FOR THE QUEEN: HONEY BEE NUTRITIONAL ENVIRONMENT AFFECTS WORKER PHEROMONE RESPONSE IN A LIFE-STAGE DEPENDENT MANNER

Modified from a paper in revision for *Functional Ecology*

Alexander Walton, Adam G. Dolezal, Marit A. Bakken and Amy L. Toth

Abstract

Animal nutritional state can profoundly affect behavior, including various forms of cooperative behavior. In highly cooperative societies of the eusocial honey bee, nutritional differences during development are important regulators of stark differences in reproductive caste and worker behavioral development. However, it is not known whether nutritional variation affects differences between individual workers in their extent of cooperative behavior. In this study we investigate how nutritional state affects a honey bee worker’s likeliness to respond to queen pheromone, a measurement of cooperativeness. We found that nutritional restriction affects a worker’s queen pheromone response, but the direction of this effect depends on the life-stage when restriction occurs. Nutritional restriction at the larval stage leads to increased queen pheromone response, but nutritional restriction at the adult stage leads to reduced queen pheromone response. We suggest that these differences depend upon the extent of reproductive plasticity at these life stages, and that individual worker honey bees may adjust their behavioral and physiological traits in response to nutritional stress to invest nutritional resources in either their own or their colony’s reproduction.
Introduction

Nutritional regulation of behavior via deeply conserved pathways may reflect the conditions that led to the origin and evolutionary maintenance of cooperation. When nutritional resources are scarce, studies from several systems suggest cooperative behaviors may be pronounced. This trend has been observed across many animal lineages, from blood-meal sharing in vampire bats (Wilkinson, 1984) to social foraging in tadpoles (Sontag, Wilson, and Wilcox, 2006), to the multicellular aggregations of otherwise solitary Dictyostelium amoebae (Kessin, 2001). However, resource limitation in some species, e.g. baboons and other primates, may also lead to increased competition and aggression (Vitousek, Manke, Gray, and Vitousek, 2004). The decision to invest in cooperative behavior vs. self under nutritional duress may depend on reproductive options available to an individual, but we lack a solid understanding of how these tradeoffs are mediated within a species. The social insects, a pinnacle of cooperative evolution, are an ideal system to study how nutrition can regulate social behavior. Not only is there variation in cooperative behavior between species, but also between different castes (e.g. queens vs workers) as well as between individuals of the same caste.

In social insects, nutritional differences organize social life as the major determinant of the reproductive division of labor. In many social Hymenoptera (ants, social bees, and social wasps), early life nutrition of a female has a drastic effect on adult phenotype. The honey bee Apis mellifera serves as an illustrative model of how these early life differences in nutrition have permanent effects on an adult’s behavior, morphology, and physiology. Honey bees live in a colony of several thousand sterile workers, and one reproductive: the queen. Whether a developing larva will become a queen or worker depends on the diet she
receives (Winston, 1987). Additionally, adult nutritional state can affect behavior. A worker’s nutritional state acts in part to regulate behavioral caste, in that nurses tend to have higher lipid stores than foragers (Toth and Robinson, 2005) and reduced nutritional state causes early, and more frequent foraging (Mattila and Otis, 2006; Schulz, Huang, and Robinson, 1998; Toth, Kantarovich, Meisel, and Robinson, 2005). In other social insects, differential nutrition during larval development can also lead to differences in size and behavior, contributing to a division of labor among the work force, such as in the bumble bee *Bombus impatiens* (Couvillon and Dornhaus, 2009). As in other social insects, consistent behavioral differences between same-aged honey bee workers within a colony do exist (Walton and Toth, 2016), but the mechanisms that mediate these differences are not yet known. In this study, we explore whether differential nutrition may be a factor in the regulation of inter-individual differences in cooperative behavior between individuals.

Nutritional regulation of cooperative behavior may be especially important in social insects and the balance between “me” and “we” modes of reproduction. If nutrient availability is high, investment in “me” (one’s own) reproduction is favorable, even in a highly social species with limited (but non-zero) personal reproductive opportunities. But, if nutritional resources are scarce, investment in “we” (a group of relatives) reproduction may be the best option, especially when personal reproductive probabilities approach zero (Wheeler, 1986; Hunt, 1991; Rossi and Hunt, 1988). Thus, in environments where nutrition is limited, cooperation may offer a selective advantage. It has been suggested that historical nutritional scarcity could have contributed to the evolution of extreme forms of cooperation, such as insect eusociality (Hunt and Nalepa, 1994). If the molecular and physiological pathways that contributed to these behavioral options continue to modulate behavioral
differences in honey bees, we expect workers that receive a high nutrition diet should shunt investment to their own ovaries and behave less cooperatively. Conversely, a nutritionally restricted worker should be unable to invest in her own ovaries and behave more cooperatively.

One potential regulatory link between nutritional state and behavior in worker honey bees is the ovaries. Although under normal colony conditions a honey bee worker’s ovaries are inactive, natural variation in the size of worker ovaries (the number of ovarioles that make up each ovary) does exist. The ovary is uncoupled from direct reproduction in workers in queenright colonies, yet the ovary and conserved reproductive pathways may regulate aspects of worker behavior such as nursing and pollen foraging, as proposed by the ground plan hypotheses of West-Eberhard, Amdam, and colleagues (West-Eberhard, 1987; Amdam, Norberg, Fondrk, and Page, 2004; Amdam, Csondes, Fondrk, and Page, 2006, Amdam & Page, 2010). These hypotheses are supported by evidence that variation in ovariole number contributes to honey bee behavioral maturation and the division of labor (Wang, Kaftanoglu, Siegel, Page and Amdam, 2010; Wang et al., 2012). Although worker ovariole number is affected by genotype (Makert, Paxton, and Hartfelder, 2006; Robinson, Page, and Fondrk,1990), ovariole number is also highly affected by environmental factors (Backx, Guzman-Novoa, and Thompson, 2012). For example, seasonal variation in nutritional availability influences ovariole number; workers that develop during periods of high pollen availability have higher ovariole number than those during pollen dearth (Hoover, Higo, and Winston, 2005). Thus, ovaries are likely targets for reduced allocation during nutritional stress, which in turn may affect behavior in the long term. This is especially true in honey bee workers because, although they do not normally reproduce, variation in worker ovary
size determines which workers will lay unfertilized eggs if a colony becomes queenless (Ratnieks, 1993). Because of the potentially important role of the ovaries as a site of nutritional and reproductive tradeoffs, in this study we integrated information about ovariole number and lipid stores with an indicator cooperative behavior, response to queen pheromone.

Social insect queens can enforce worker cooperation and sterility in several ways, including physical aggression (Reeve, 1991) and chemical communication (Slessor, Winston, and Le Conte, 2005; Kocher and Grozinger, 2011). In the honey bee, the queen utilizes queen mandibular pheromone (QMP), which prevents worker ovarian activation (Slessor, Winston, and Le Conte, 2005). QMP also elicits a “retinue response” from workers, in which they face the queen, and antennate and tend her (Slessor, Kaminski, King, Borden, and Winston, 1988). The task of queen tending (feeding, examining, and grooming the queen) is a form of worker-queen cooperation necessary to colony function. The queen is singly occupied by the task of laying eggs, so the workers must feed and maintain her. Thus, the workers’ response to the queen is of key importance to colony health. Natural variation in response to the queen exists among the workers of a honey bee colony (Kocher, Ayroles, Stone, and Grozinger, 2010; Walton and Toth, 2016). This variation in response may contribute to the colony’s division of labor (specific individuals are more likely to respond to, and thus care for, the queen).

In this study, we assayed individual variation in QMP response to test the hypothesis that nutritional restriction enhances cooperation. We manipulated the nutritional environment of honey bee workers in two separate ways, adult pollen deprivation (Experiment 1 and 2), and acute larval starvation (Experiment 2). In Experiment 1, we
manipulated adult diets by providing caged adult workers with either a pollen supplemented diet or a pollen deprived diet. In Experiment 2 we investigated the effects of diet manipulation at both larval and adult life stages. We predicted that nutritionally-stressed larvae would exhibit a higher response to QMP as adults. We predicted that the effect of adult diet would follow the same pattern: pollen-supplemented adults would be less responsive to QMP than adults deprived of pollen. If nutrition mediates cooperative behavior via reproductive physiology, we predict bees that experienced high nutrition to invest these resources in their own reproductive potential, and thus have larger ovaries and higher lipid stores. We found evidence that nutritional stress during larval development does lead to enhanced QMP response and smaller ovaries, suggesting nutritional stress leads bees to divest their own reproduction and invest in their colonies. Interestingly, we found the opposite pattern in adults, suggesting different strategies for dealing with nutritional stress depending on life stage and level of reproductive plasticity.

**Methods**

**Bees**

Honey bee (*Apis mellifera* L.) colonies were maintained at the Iowa State University Horticulture Research Station in Ames, Iowa, during the summers of 2015, 2016, and 2017. Adult bees were transferred to rearing facilities at Iowa State University, and all observational data was collected there.
**Experiment 1 adult restriction: pollen deprivation**

Brood frames containing pre-eclosion workers were removed from 6 un-manipulated hives at the Iowa State University Horticulture Research Station apiary and placed in a 33 °C incubator overnight to emerge. Upon emergence, adult bees were divided into cages (see *Cage Assays* below). These cages were subdivided into pollen-fed (49 cages) or pollen-deprived treatments (55 cages). In the pollen-fed treatment, cages were fed 1 gram of bee-collected chestnut (Pollenergie, France) pollen daily for the course of the experiment (seven days).

**Experiment 2 larval and adult restriction: acute larval starvation and adult pollen deprivation**

Four queens in four different colonies were caged over a frame of empty drawn comb with a push-in cage and allowed to lay eggs for 48-hours, after which the cage was removed and the comb placed in a separate colony. At 180 hours after eggs were laid, a starvation procedure was performed (Wang, Kaftanoglu, Fondrk and Page, 2014; Wang, Kaftanoglu, Brent, Page, and Amdam, 2016; Wang et al., 2016). Nurse bees were removed from the frame, then a wire push-in cage was placed over half of the larvae, preventing nurses from feeding or in any way caring for them. The other half of the larvae were left uncovered and nurses able to feed and care for them. The cages were removed after 10 hours and the larvae allowed to pupate normally. The day before adult bees emerged, these frames were removed and placed in a 33 °C incubator overnight.

When adults emerged, they were divided into cages. These cages were further
divided into pollen-fed or pollen-deprived treatments. In the pollen-fed treatment, cages were fed 1 gram of bee-collected pollen daily for the course of the experiment (seven days). Thus, in this experiment there were two possible larval treatments (starved vs. not starved) and two following adult treatments (pollen-fed vs. pollen-deprived) resulting in a total of four possible cage-level treatments (starved larvae + pollen-deprived, starved larvae + pollen-fed, not starved larvae + pollen-deprived, and not starved larvae + pollen-fed).

Cage Assays

When adult bees from each experiment emerged, groups of 30 day-old bees were placed in acrylic cages (dimensions: 10.6 × 10.16 × 7.62 cm) and kept in an incubator at 33°C and 50% relative humidity and fed 50% sucrose solution ad libitum. Each day, any dead bees were removed and a glass microscope slide containing synthetic QMP (Pherotech International, Delta, British Colombia) was inserted. QMP was diluted with 1% water/isopropanol to 0.01 queen equivalents, which has been shown to elicit a strong queen response (Pankiw, Winston, and Slessor, 1994). A queen equivalent is equal to the average amount of pheromone in the mandibular glands of a laying queen (Slessor, Kaminski, King, Borden, and Winston, 1988). When the bees were 7-days old, response to the QMP slide was recorded. The number of individuals contacting the slide was recorded every 5 minutes for 30 minutes. This assay has been shown to elicit natural queen response and has been well established in the literature (Kocher, Ayroles, Stone, and Grozinger, 2010; Slessor, Kaminski, King, Borden, and Winston, 1988; Pankiw, Winston, Fondrk, and Slessor, 2000; Hoover, Keeling, Winston, and Slessor, 2003). We confirmed the efficacy of this assay in our
experimental setup, and confirmed that 0.01 queen-equivalents of QMP elicits a strong retinue response from young worker bees (Fig. S1).

**Physiological Measurements**

Newly emerged bees were collected on dry ice. We removed the gut to prevent lipid contamination from any food stored in the gut, and we measured the mass of each. Bees were processed for lipid quantification using a phospho-vanillin spectrophotometric assay (Toth and Robinson, 2005). Lipid concentrations from 15 bees per treatment were compared. We also dissected out the ovaries of newly emerged bees from larval diet manipulation experiments. The total number of ovarioles in both ovaries was recorded.

**Statistics**

Statistical analyses were performed using R version 3.3.1 (R Core Team, 2016). The QMP response rate per cage was calculated as: the number of individuals responding to the QMP microscope slide divided by the number of bees in the cage (which was different in each cage, due to mortality). For each cage, the QMP response rate was averaged across the 6 observation periods.

To analyze the effect of diet treatment on queen response, we used a generalized linear mixed effects model with a binomial error structure using the function “`glmer`” in the R package “`lme4`” (Bates, Maechler, Bolker, and Walker, 2015), controlling for hive source and trial. For analyses of queen response in Experiment 3, post-hoc contrasts between treatment groups were performed using the function “`lsmeans`” in the R package “`lsmeans`” (Lenth, 2016).
Results

Experiment 1: Effects on Behavior

Bees fed pollen as adults showed a higher response to QMP than adults deprived of pollen (GLMM: z-ratio = 7.69, p-value < 0.0001, n = 49 pollen-fed cages and 55 pollen-restricted cages) (Fig. 1).

Experiment 2: Effects on Behavior

Adult bees that had been restricted from contact with nurses as larvae exhibited a higher response to QMP than those that were never restricted (generalized linear mixed model, z-ratio = -5.35, p-value < 0.0001, n = 25 cages per treatment, larval diet contrast results averaged over adult diet treatment) (Fig. 2, Table S1). Adult bees fed supplemental pollen showed a higher response to QMP than adult bees not supplemented with pollen (generalized linear mixed model: z-ratio = -8.28, p-value < 0.0001, n = 25 cages per treatment, adult diet contrast results averaged over larval diet treatment) (Fig. 2; Table S1). There was no interaction effect of larval and adult diet treatments on QMP response (generalized linear mixed model: z-value = 0.83, p-value = 0.40).

Experiment 2: Effects on Physiology

Bees fed pollen as adults had higher percent lipid content than bees deprived of pollen (linear model, t-ratio = -3.72, p-value = 0.0005, n = 29 pollen-fed bees and 30 pollen-restricted bees, adult diet contrast results averaged over larval diet treatment) (Fig. 3A), and pollen-fed adults had a higher average mass than bees deprived of pollen (linear model: t-
ratio = -4.35, p-value = 0.0001, n = 29 pollen-fed bees and 30 pollen-restricted bees, adult
diet contrast results averaged over larval diet treatment) (Fig. 3A). Percent lipid content was
not affected by acute larval starvation (linear model, t-ratio = -0.45, p-value = 0.66, n = 30
restricted diet bees and 29 unrestricted diet bees, larval diet contrast results averaged over
adult diet treatment) (Fig. 3A), nor did acute larval starvation affect mass (linear model: t-
ratio = -1.59, p-value = 0.16, n = 30 low larval diet bees and 29 high larval diet bees, larval
diet contrast results averaged over adult diet treatment) (Fig. 3A). Bees from the starved
larval treatment had fewer ovarioles than those from the unstarved larval treatment (t-test: p-
value = 0.0005, n = 55 unrestricted bees and 65 restricted bees) (Fig. 3B), replicating the
findings of Wang, Kaftanoglu, Fondrk, and Page, (2014), Wang, Kaftanoglu, Brent, Page,
and Amdam (2016) and Wang et al. (2016) and confirming the efficacy of our treatment
regime.

Discussion

Early life environments have the potential to affect an animal’s life-history strategy
through adjustments in plastic phenotypic traits (Monaghan, 2008). In this study, we provide
evidence that individual worker honey bees may adjust their behavioral and physiological
traits in response to nutritional stress. Specifically, we found a relationship between the
nutritional environment a honey bee worker experiences and her likeliness to respond to
queen pheromone, an indicator of investment in colony reproduction. When developing
larvae experience a period of acute starvation, they become more responsive to queen
pheromone later in life no matter their adult diet. Interestingly, adult nutritional stress had
the opposite effect on behavior. Adult bees deprived of pollen had a lower response to queen
pheromone than adult bees fed pollen. Together, these data suggest nutritional stress at
different life stages can have differential effects on bees’ investment in colony reproduction.

The fact that larval nutritional stress also influences ovary development suggests possible connections between individual and colony reproductive tradeoffs in worker bees. In concurrence with previous studies (Wang, Kaftanoglu, Fondrk and Page, 2014; Wang, Kaftanoglu, Brent, Page, and Amdam, 2016), we found that diet quantity deprivation (restricted access to nurse bees) during the 5th instar of larval development resulted in decreased ovariole number. This manipulation of larval diet supports the hypothesis that, in honey bee workers, nutritional stress leads to divestment in ovarian development and an increase in cooperative behavior.

Diet stress had strikingly opposite effects on behavior and physiology of larval and adult honey bees. We hypothesized that cooperative behavior would be promoted by nutritional stress, and therefore we predicted increased response to queen pheromone from bees that experienced diet restriction, both as larvae and as adults. However, this relationship was only evident in bees that experienced diet restriction as larvae, and was accompanied by decreased ovary development. The exact opposite effect occurred in honey bees that experienced diet restriction as adults. In addition, while larvae invested nutritional resources in their ovaries, adults invested nutritional resources in their abdominal fat stores. Adult fat stores are likely to be metabolized for fueling colony level activities such as wax production and brood food production (Hepburn et al., 1991; Toth and Robinson 2005). Thus, how nutrition mediates cooperative behavior differs greatly depending on the life stage at which individuals experience a nutritional environment. We suggest this life stage-dependent effect of nutrition may be, in part, due to the different degree of developmental plasticity honey bees have at these different life stages (Fig. 4).
Female honey bee larvae are reproductively totipotent (they can develop into either a queen or a worker) for their first 3-4 days of age (Weaver, 1957). After this point, worker-destined larvae can no longer develop into viable queens (Winston, 1987). However, their reproductive potential is not yet entirely fixed, as worker ovaries (the number of ovarioles) only begin to reduce via programmed cell death in the fifth larval instar (Hartfelder and Steinbrück, 1997). Diet restriction appears to mediate ovariole programmed cell death, as nurse bees can control the food quantity developing larvae receive at this sensitive stage (Wang, Kaftanoglu, Fondrk, and Page, 2014). Thus, workers retain developmental plasticity through the fifth larval instar, in the form of variable numbers of ovarioles. This correlates with adult reproductive potential, as workers with more ovarioles are more likely to lay eggs of their own (Makert, Paxton, and Hartfelder, 2006). As an adult, however, a worker’s ovariole number is fixed, and diet can no longer influence this aspect of her reproductive physiology (Hartfelder and Steinbrück, 1997). Thus, reproductive traits remain somewhat plastic as larvae, but are predominately fixed by adulthood.

Consequently, if as we hypothesize, nutritional resource availability mediates cooperative behavior via reproductive pathways, then nutrition’s effect on cooperative behavior may depend on the degree of reproductive plasticity present. Therefore, we hypothesize that nutritional stress promotes cooperation, but this effect is limited to situations in which individual have greater plasticity in reproductive potential. In other words, if an individual is unable to shunt adequate nutritional resources towards sustaining reproductive development, cooperation with others may be the best option to increase their fitness. We predict that when an individual’s reproductive potential is plastic (as in larval honey bees), nutritional resource availability will negatively correlate with cooperative behavior. In such
situations, resources may be shunted to an individual’s own reproductive development (favoring “me” instead of “we”), as in the case of increased ovariole number in larval honey bee workers. Higher ovariole number will correlate with a reduction in cooperative behaviors as an adult, such as reduced response to the queen (Kocher, Ayroles, Stone, and Grozinger, 2010). In addition, we surmise that when an individual’s reproductive potential is fixed (as in adult honey bees with generally low reproductive potential), nutritional resource availability will positively correlate with cooperative behavior. Because energy obtained from nutritional resources can no longer be used to bolster the individual’s own reproductive development, these resources should be invested in the group (favoring “we” instead of “me”) (Wheeler, 1986). We observed that adult worker honey bees invested nutritional resources in increased queen responsiveness and lipid stores, which are likely metabolized to fuel cooperative activities such as brood rearing, queen rearing, and wax production (Hepburn et al., 1991; Svoboda, Thompson, Herbert, Shortino, and Szczepanik-Vanleeuwen, 1982).

Although our data are consistent with the argument that nutritional stress leads to changes in physiological and behavioral life history strategies in honey bees, there are other possible explanations. The observed connection between larval nutritional stress and increased queen pheromone response could instead be a form of worker emergency response. Perhaps experiencing nutritional stress as larvae cues workers to exhibit higher queen care, protecting the queen when the hive is in dire condition. Further experimentation with other potential colony “emergency” status cues (i.e. high pest pressure, heat stress, toxin exposure, disease) could help elucidate whether developing larvae can sense colony stressors and adjust their behavior upon eclosion.
The results of this study support the hypothesis that nutritional stress can affect cooperation, but further research on cooperative behaviors other than queen pheromone response could further cement this idea. Honey bees exhibit many cooperative and selfish behaviors (Walton and Toth, 2016), and testing whether these behaviors are also influenced by nutrition could further clarify the nutritional environment’s role in cooperative behavior. Additionally, comparative studies can illuminate how universal this connection may be, and enhance understanding of how plasticity of reproductive potential affects how nutrition mediates cooperation. Experiments examining the effects of nutritional stress on cooperation would be especially informative across species with gradients in reproductive plasticity, especially on other eusocial insects with higher levels of reproductive plasticity that persist through adulthood (e.g. Polistes wasps: Reeve, 1991). The general principle that nutritional stress fuels cooperation may extend much more broadly than social insects. In the future, broad scale comparative studies can address whether the patterns recorded in this study persist across different levels of reproductive plasticity, and across lineages through evolutionary time.

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References


**Figure 1.** Effect of adult pollen deprivation. Bees fed pollen as adults showed a higher response to QMP than bees deprived of pollen (GLMM: z-value = 7.69, p-value = <0.0001, n = 49 pollen-fed cages and 55 pollen-deprived cages). Boxplots display median, interquartile range, and full range of the data.
Figure 2. Effects of acute larval starvation and adult pollen deprivation on QMP response. Bees from low larval quantity diet treatments (Low L) exhibited a higher response to QMP than bees from high larval quantity diet treatment (High L). Letters denote significant differences (GLMM: \( z \)-ratio = -5.35, p-value < 0.0001, \( n = 25 \) cages per treatment, larval diet contrast results averaged over adult diet treatment). Adult bees fed supplemental pollen (High A) showed a higher response to QMP than adult bees not supplemented with pollen (Low A) (GLMM: \( z \)-ratio = -8.28, p-value < 0.0001, \( n = 25 \) cages per treatment, adult diet contrast results averaged over larval diet treatment). There was no interaction effect of larval and adult diet treatments on QMP response (\( z \)-value = 0.83, p-value = 0.40). Boxplots display median, interquartile range, and full range of the data.
Figure 3. Physiological effects of acute larval starvation and adult pollen deprivation. A) Bees fed pollen as adults (High A) had higher percent lipid content than bees not fed pollen (Low A) (lm: t-ratio = -3.72, p-value = 0.0005, n = 30 Low A and 29 High A bees, adult diet contrast results averaged over larval diet treatment) and greater mass (lm: t-ratio = -4.35, p-value = 0.0001, n = 30 Low A and 29 High A bees, adult diet contrast results averaged over larval diet treatment). Percent lipid content was not affected by larval quantity diet treatment (lm: t-ratio = -0.45, p-value = 0.66, n = 30 Low L and 29 High L bees, larval diet contrast results averaged over adult diet treatment) nor was mass (lm: t-ratio = -1.59, p-value = 0.16, n = 30 Low L and 29 High L bees, larval diet contrast results averaged over adult diet treatment). B) Bees from low larval quantity diets treatment had fewer ovarioles than those from the high larval quantity (t-test: p-value = 0.0005, n = 55 High L and 65 Low L).

Boxplots display median, interquartile range, and full range of the data.
Figure 4. Different strategies for investment of nutritional resources, depending reproductive plasticity. When reproductive potential is plastic, as in larvae, a worker invests nutritional resources in her ovaries and exhibit low cooperation. When reproductive potential is fixed, as in adults, a worker invests nutritional resources in lipid stores and exhibit high cooperation.
CHAPTER 4. DOES RESOURCE LIMITATION PROMOTE SOCIAL COHESION? DIET RESTRICTION AND AGGRESSION IN THE PAPER WASP *POLISTES FUSCATUS*

Alexander Walton and Amy L. Toth

**Abstract**

Nourishment can have profound effects on social behavior. Previous studies suggest that when nutritional resources are limited, social cohesion in animal groups may be enhanced. This may be especially true in social insect colonies, where nourishment is often important in determining differences between reproductive caste and worker behavioral development. We investigated how nourishment affects social cohesion in colonies of the paper wasp *Polistes fuscatus*. Field-collected colonies were maintained in the lab and assigned to either a high or low feeding treatment. Nests in the high treatment group were fed prey daily, and the low treatment nests were fed every fourth day, and behaviors were recorded throughout the experiment. We find that nutritional restriction reduced aggressive interactions, which we interpret as an indicator of high social cohesion. Additionally, we found that adult wasp diet restriction led to decreased abdominal mass in workers. However, diet restriction did not affect lipid content. Thus, although diet restriction led to decreased mass, workers from the restricted treatment were able to maintain normal fat body lipid stores, possibly by increased sugar intake. We also investigated whether expression in the brain of 7 genes related to reproduction and nutrient signaling differed between wasps under high and low nutritional availability treatments, but we did not detect any transcriptional differences. We suggest that individual worker paper wasps may adjust their behavior (i.e.,
reduced aggression) in response to nutritional stress to invest nutritional resources in either their own or their colony’s reproduction.

**Introduction**

Resource limitation may favor enhanced cooperation between members of the same species, even when individuals are unrelated to each other (Pfeiffer et al., 2001; Requejo et al., 2011). There are many examples across taxa demonstrating that individuals tend toward enhanced cooperation when resources are limited, from blood meal sharing in vampire bats (Wilkinson, 1984) to the formation of multi-cellular fruiting bodies in aggregations of free-living *Dictyostelium discoideum* amoebas (Kuzdzal-Fick et al., 2007; Kessin, 2001; Bonner, 1982). In some animal cases, caloric restriction can lead to aggression amongst non-relatives (Vitousek et al., 2004). In kin groups, however, where individuals can increase their inclusive fitness through cooperation, conditions where food resources are consistently scarce may select for increased cooperation and increasingly cohesive group behavior. If so, could nutritional resource limitation also be an important contributor to a major transition in evolution (Maynard Smith and Szathmary, 1995), such as from solitary living to highly integrated eusocial colonies? If nutritional limitation was a factor in the evolution of insect sociality, then nutritional restriction should increase social cohesion in extant insect societies.

Eusocial insects (ants, termites, and the social bees and wasps) have been called a “pinnacle” of cooperation (Wilson, 1971). These groups live in highly integrated societies, where many individuals share the duties of maintaining the colony (Wilson, 1971). These insect colonies are illustrative examples of cooperation, wherein group members work together to complete tasks or share information. Additionally, insect societies display remarkable social cohesion, defined here as the degree of how all individuals act toward
promoting the interests of the group over the interests of the individual. Different eusocial species show a continuum of degrees of cooperation and cohesion, with some more “primitively” eusocial species showing marked conflict within the society (Pardi, 1948; West-Eberhard, 1967; Strassmann, 1981; Ratnieks and Reeve, 1992). Examining how nutritional resource limitation affects social cohesion in different lineages across this continuum allows for testing of the generality of the resource limitation hypothesis across different levels of sociality and independently evolved eusocial lineages.

At the primitive end of the eusociality continuum are the paper wasps (e.g. *Polistes fuscatu*s). Paper wasps live on an open nest with only, at most, a few dozen individuals. There is just one dominant reproductive (the queen), who exerts her reproductive dominance over workers through physical aggression. In a typical worker phase, colony social cohesion is high, as colony members work together to build and defend the nest, collectively forage, allogroom each other, and share food. However, social cohesion can sometimes be low (e.g. when a dominant wasp dies, leading to elevated colony-wide aggression among nestmates), and cooperative individuals (workers or subordinate queens) may even abandon the nest to reproduce and found colonies of their own (Reeve, 1991; Hunt 2007).

Previous research supports the supposition that many pathways related to reproductive physiology and nutrient signaling are associated with social traits in wasps, as well as bees. Ovarian development and activation is linked to pollen foraging behavior in worker honey bees (Amdam et al., 2006; Wang et al., 2010; Kocher et al., 2010) and reproductive dominance in worker paper wasps (Fletcher and Ross, 1985). This link may be in part regulated by the yolk precursor protein vitellogenin and the gonadotropin juvenile hormone (Röseler, Röseler, and Strambi, 1985; Hartfelder, 2000; Amdam and Omholt,
2003). Additional work on both honey bees and paper wasps also suggests genes related to nutrient signaling, in the deeply conserved insulin pathway, are also related to the regulation of worker foraging behavior (Ament et al., 2008; Daugherty et al., 2011).

Based on prior work on honey bees, Walton et al. (in review) proposed that the way in which nutritional stress affects cooperative behavior in eusocial insects is related to the level of reproductive plasticity individuals possess. In honey bees, reproductively plastic larvae develop into more cooperative adults when they experience nutritional stress, whereas reproductively fixed adults exhibit lower cooperative behavior. These findings suggest that, when workers are reproductively plastic, they may selfishly invest excess nutritional resources in their own reproduction. But, if nutritional resources are low, or if they cannot invest resources in their own reproduction, workers invest energy and resources in the colony’s fitness. To test the generality of these results in social insects, we explored the role of nutritional restriction on cooperation and aggression in reproductively totipotent adult paper wasps.

In Polistes sp., which have evolved sociality independently from honey bees, adult paper wasp workers have plastic reproductive potential, and can mate, lay eggs, and take over as queen of the colony if the resident queen dies (Reeve, 1991). Thus, adult paper wasps more closely resemble larval honey bees in their flexibility in reproductive potential. In a more primitively social system such as Polistes wasps, environmental conditions may have an even stronger effect on the cooperativeness of individuals than in the more derived and likely more canalized society of the honey bee. For example, Jandt et al. (2015) found that when Polistes fuscatus nests are reared in the lab, which usually involves ad libitum food provisions, individuals tend to spend more time performing self-maintenance behaviors such
as sugar feeding, and nearly completely cease working tasks such as nest building. This study also suggested a nutritional connection to selfish behavior—wasps reared in the laboratory had higher fat stores and spent less time on the nest than wasps reared in the field. However, this study did not address whether the opposite effect – nutritional restriction – would lead to higher levels of cooperative behavior. Here, we describe findings that investigate connections between nutritional restriction, behavior, reproductive and nutritional physiology, and reproductive and nutrient signaling pathways in the brain in the paper wasp *Polistes fuscatus*. Thus, the goal of this study was to extend previous work to address whether the connection between nutrient limitation and cooperation is a more general, conserved phenomenon on social insects, and therefore contribute to our understanding of the forces that favor the evolution of extreme forms of cooperation such as eusociality.

**Methods**

**Field Collection**

*Polistes fuscatus* nests were collected from field sites in central Iowa. To attract wasps to construct nests, wooden boxes (14cm x 14cm x 14cm) were set out in April 2017 at two sites: The Iowa 4-H Center (Madrid, IA) and Chichaqua Bottoms Greenbelt (Maxwell, IA). Boxes were affixed to the top of metal posts, and wasps were able to enter and build nests by entering boxes through the bottoms, which were open but for a coarse wire screen. Boxes were regularly monitored, and foundresses were marked with paint pens to distinguish them from workers later during experimentation.

Wasp nests (3 from the 4-H Center and 14 from Chichaqua Bottoms Greenbelt) were collected from the field and moved into the lab in June 2017, just before adult workers began emerging. Wasps built their nests on the roof of each box, which were detachable. To
collect a nest, the wood lid containing nest and foundress was removed and placed onto the top of a laboratory nest box (see below). These boxes were closed with duct tape to prevent escape during transportation. Nest were collected at night, between 9PM and 5AM, to ensure that colony members were present.

Additionally, 9 already established nests were collected from parking canopies at Brighton Park Apartments in Ames, IA. These nests were also collected before workers emerged. To collect these nests, Ziploc bags were placed over the nest and foundress and the nest’s pedicel was severed from the parking enclosure ceiling with scissors. Foundresses were marked with paint pens and nests were affixed to cardboard squares with a hot glue gun, and then placed onto the tops of laboratory nest boxes.

**Lab Conditions**

Moving colonies to the lab in late June 2017 ensured that all workers in the experiment would be from the foundress’ first batch of brood. Thus, any pre-overwintering queens, or “gynes”, which appear later in the season, were excluded. Colonies were subsequently maintained in an indoor rearing room at Iowa State University in Ames, IA. Wasp nests were placed in 30cm x 30cm x 30cm clear Plexiglas laboratory nest boxes, with a 9cm x 9cm opening at the top, where nests (affixed to either wooden field box roofs or glued to cardboard) were placed and secured. Full-spectrum lights were set to a day-night cycle, with lights coming on at 6:00 and turning off at 20:00. Temperature was maintained at 27 °C. Colonies were provided with construction paper to build and maintain their nests, water and sugar rock candy *ad libitum*, and prey according to feeding treatment (see below). Every day, nest box positions were rotated, so that each nest experienced any potential rearing room
microclimate equally, and so that nests were not observed in the same order during behavioral observations.

**Feeding Treatments**

Colonies were provided with prey (*Galleria mellonella* waxworms purchased from local bait vendors, or *Trichoplusia ni* cabbage loopers from Frontier Scientific Services) according to their adult and larval population (0.5 larvae per adult and 0.083 larvae per adult) (as per Daugherty et al., 2011). Nest population was censused by counting adults and larvae every other day. Colonies were randomly assigned to either a high or low feeding treatment (n = 13 nests per treatment). Nests in the high treatment group were fed prey daily, and the low treatment nests were fed prey every fourth day. Prior to treatment assignment, all nests were fed a high diet treatment for the two days following their move to the lab. Upon treatment assignment, nests were fed their respective diets for four days before behavioral observations commenced, and continued throughout the experiment until all wasps were sampled at the end of the experiment.

**Behavioral Observations**

Twice daily (mornings and afternoons) for 8 days behavioral observations were recorded. Each colony was observed for two periods of 5 minutes (during two sequential observational rotations), and instances of trophallaxis (food-sharing), foraging (on caterpillars, sugar, and paper), and aggression (lunging, biting, and grappling) were tallied. Separate analyses were done for all behavioral observation periods (averaged), to investigate long-term effects of the treatments, as well as to focus on just the behavioral observation
periods that occurred after all nests had been fed (so that behaviors related to prey capture and processing were not biased to one treatment).

**Ovaries, Mass, and Lipids**

Abdomens of workers were dissected at the conclusion of experimentation to remove organs, leaving the fat body adhered to the cuticle. Ovaries were removed and ovarian development was scored in a manner similar to the protocol used for honey bees (Velthuis, 1970) and other polistine studies (Gelin et al., 2008; Daugherty et al., 2011) (see Supplemental Table 1).

Dissected abdomens were weighed, and lipids were extracted in 2:1 chloroform: methanol and quantified using a sulphophospho-vanillin spectrophotometry assay (Toth and Robinson, 2005). In this way, mass, total lipid content, and calculated percent lipid content (lipid content per mass) were measured.

**Gene Expression**

Wasp worker heads were freeze dried at 300 mTorr for 60 minutes, and brains were dissected over dry ice (n = 10 wasp brains per diet treatment). Cuticle, fat, and glands were carefully removed with a scalpel to isolate the brain from surrounding tissue.

To identify candidate genes related to social cohesion and nutrition in *Polistes fuscatus*, we selected genes that have showed associations with nutrient-sensing, reproduction, and social behavior in wasps and honey bees (Badisco et al., 2013; Wheeler et al., 2006; Azevedo and Hartfelder, 2008; Amdam et al, 2003; Nelson et al., 2007; Ament et al., 2008; Wang et al., 2009; Wang et al., 2010; Mutti et al., 2011; Manfredini et al., 2018). Seven genes were selected as candidates for differential gene expression across diet
treatments: the insulin-like peptide ilp1, the insulin-like receptor InR1, the insulin-like receptor InR2, the nutrient sensing kinase gene TOR (target-of-rapamycin), the ecdysone-inducible nuclear hormone receptor gene HR46, the egg-yolk precursor vitellogenin gene vg, and the vitellogenin receptor vgr. Gene sequences were identified by BLASTing previously published Apis mellifera sequences for each gene (Honeybee Genome Sequencing Consortium, 2006) against a Polistes fuscatus Transcriptome Shotgun Assembly (Berens et al., 2014). Primers were designed with the Primer Quest (Integrated DNA Technologies). Primer sequences of focal genes are found in the supplementary materials (Supplemental Table 2).

Brain RNA was extracted using a Qiagen RNeasy Mini Kit and protocol (Qiagen, Valencia, CA), and treated with DNaseI (Ambion, Austin, Texas). To control for technical errors that may occur during cDNA synthesis or pipetting error, an external reference gene, mCherry (RNA isolated from a cnidarian of the genus Discosoma, Carrillo-Tripp, 2014) was spiked in. 200 ng of isolated RNA was used as a template for cDNA synthesis with SuperScript III First-Strand Synthesis System (Invitrogen).

For RT-qPCR, 2 µL of cDNA was used in 10 µL volume reactions of the 2X SYBR® Green PCR Master Mix (Applied Biosystems) with the CFX384 Touch™ Real-Time PCR Detection System. Samples were run in triplicate as technical replicates. An internal reference gene, rp49 was used to normalize gene expression data. The internal reference gene and the external reference gene cycle thresholds did not differ across treatments (rp49: linear model: F = 0.20, df = 1, p-value = 0.66; mCherry: linear model: F = 0.35; df = 1; p-value = 0.56). The $2^{-\Delta\Delta C_T}$ method was used to calculate relative gene expression (Livak and Schmittgen, 2001).
Statistics

Statistical analyses were performed using R version 3.4.3 (R Core Team, 2017). Behavioral data were analyzed with Wilcoxon Rank-Sum tests using the “wilcox.test” function. Relative gene expression for each gene of interest was compared across treatments with one-way ANOVAs using the “aov” function. Linear mixed models for mass and lipids were made with nest as a random factor using the “lmer” function in the “lme4” package (Bates et al., 2015). Post-hoc comparisons were done using the “lsmeans” functions in the package “lsmeans” (Lenth, 2016). Ovary scores were compared using a t-test with the “t.test” function. Plots were generated using R package ggplot2 (Wickham, 2009).

Results

Behavioral Observations

All observations

When behaviors were averaged across all observations, wasp nests from the low diet treatment foraged on sugar more than nests from the high diet treatment (Wilcoxon rank sum test: $W = 31$, p-value: 0.007; Figure 1A). Conversely, nests from the high diet treatment foraged on caterpillars more than nests from the low diet treatment (Wilcoxon rank-sum test: $W = 142$, p-value = 0.002; Figure 1A). All other behaviors were not significantly different across treatments (Supplemental Table 3).

Observations at specific time points

Because behaviors may be affected by immediate access to prey, behavior was examined only in observation periods that occurred directly after all nests in both treatments...
had been fed caterpillars. Wasps nests in the high diet treatment exhibited higher aggression than those from the low diet treatment (Wilcoxon rank-sum test: $W = 120$, p-value = 0.022, n= 13 nests per treatment; Figure 1B). Wasp nests did not differ in trophallaxis rates across treatments (Wilcoxon rank-sum test: $W = 53.5$, p-value = 0.11, n = 13 nests per treatment; Figure 1B), nor in foraging behaviors (Supplemental Table 4).

Additionally, behavioral observations were compared from the morning before experiment-wide prey feeding occurred to confirm that the pattern observed above was robust when no nests had prey available as well. Here, too, nests in the high diet treatment exhibited higher aggression than those from the low diet treatment (Wilcoxon rank-sum test: $W = 124$, p-value = 0.042; Figure 1C), and this was the only behavior that differed significantly between treatments (Supplemental Table 5).

Mass and physiological measurements

Workers from the high diet treatment had a higher mass than workers from the low diet treatment (linear mixed model: t-ratio = 3.01, p-value = 0.004, n= 29 high and 24 low; Figure 2A). Total lipid content did not differ between treatment groups (linear mixed model: t-ratio = 1.13, p-value = 0.27, n= 29 high and 24 low; Figure 2B). Relative lipid (lipid content divided by mass) did not differ between treatment groups (Linear mixed model: t-ratio = -0.88, p-value = 0.38, n= 29 high and 24 low; Figure 2C).

Ovary scores did not differ between diet treatment groups (t-test: t = 0.23, df = 45.89, p-value = 0.82, 24 wasps per feeding treatment; Figure 2D).
Gene Expression

Workers from each treatment (n = 10 wasp brains per treatment) did not differ in brain gene expression for the insulin-like peptide *ilp1* (one-way ANOVA: F = 1.18, df = 1, p-value = 0.29), the insulin-like receptor *InR1* (one-way ANOVA: F = 3.53, df = 1, p-value = 0.08), the insulin-like receptor *InR2* (one-way ANOVA: F = 1.34, df = 1, p-value = 0.26), *TOR* (one-way ANOVA: F = 1.76, df = 1, p-value = 0.20), *HR46* (one-way ANOVA: F = 1.05, df = 1, p-value = 0.32), vitellogenin *vg* (one-way ANOVA: F = 0.59, df = 1, p-value = 0.45), or the vitellogenin receptor *vgr* (one-way ANOVA: F = 0.57, df = 1, p-value = 0.46) (Figure 3). However, all genes measured trended toward higher expression in the high diet treatment than in the low diet treatment. An exact binomial test of the null hypothesis that these genes would be equally expressed across treatments was performed, which showed a pattern of higher brain gene expression in wasps from the high diet treatment than the low diet treatment (Exact binomial test: p = 0.016).

Discussion

Here we present evidence that the nutritional environment has the potential to affect social cohesion in colonies of the paper wasp *Polistes fuscatus*. Specifically, we found that low food availability is associated with less aggression toward nestmates. Although not significantly different, the cooperative behavior of trophallaxis trended toward an increase in diet restricted nests. Previous research in this species demonstrated that high food availability is associated with decreased cooperative behavior (Jandt et al. 2015). Together, these studies suggest that paper wasps may adjust their cooperative strategies in relation to the nutritional environment they are currently experiencing. When nutritional resources are scarce, it may be most beneficial to behave cooperatively to promote group welfare and inclusive fitness.
Alternatively, when resources are abundant, individuals can more readily invest in their own fitness, and group cohesion may begin to degrade. We observed enhanced nutrition leading to increased aggressive interactions, a trait more typical of loosely social groups than highly cooperative eusocial societies where social cohesion is strong. Thus, we suggest continual nutritional deprivation of workers may be an important mechanism for the promotion of cooperation in social insect societies (Hunt and Nalepa, 1994). These results may be indicative of a general trend in social behavior (Wheeler, 1986; Hunt, 1991; Rossi and Hunt, 1988), especially in organisms with maternal care and in kin groups.

Importantly, we were able to verify that our nutritional restriction treatment was successful—diet restriction led to decreased abdominal mass in workers. However, diet restriction did not affect lipid content or relative lipid content. Thus, although diet restriction led to decreased mass, workers from this treatment were able to maintain normal fat body lipid stores. This was likely accomplished by increased sugar consumption, which is corroborated by the increased sugar foraging observed in prey diet restricted nests. Together, the decrease in mass and the increase in sugar foraging confirm the efficacy of the diet restriction method used in the study.

Our results show that nourishment affects social behavior in paper wasp workers. To investigate how nutritional state influences cooperative behaviors, we focused on aggression and trophallaxis during observation periods following prey feeding for all experimental nests (every 4 days). We focused on these times because these were the only observation periods in which the high and low diet treatments were on a “level playing field” with respect to prey availability. During these observation periods, aggression was higher in the high diet treatment. However, it could be possible that the low diet treatment spent more effort
foraging for prey during these observation periods because prey is rarer and thus of higher priority to wasps in this treatment. If that were so, we would predict higher prey foraging post-feeding in low diet treatments than high diet treatments, as observed when behavior rates were averaged across the course of the experiment (Figure 1A). Yet, there was no difference in prey foraging post-feeding between treatments following prey feeding (Figure 1B, Supplemental Table 4). Further, we recorded behaviors on the mornings prior to experiment-wide prey feeding, when no nests had prey to forage. Here, we observed the same pattern as post-feeding: higher aggression in the high diet treatment (Figure 1C, Supplemental Table 5). Thus, lower aggression in the low diet treatment was not a result of wasps in this treatment focusing on prey foraging during observation. When nutritionally restricted, wasps are less aggressive toward nestmates.

We predicted that nutritional deprivation would result in decreased ovary size in wasp workers, as is true of honey bees that experience nutritional stress (albeit, as larvae, not as adults) (Hoover et al., 2006; Wang et al., 2014; 2016a; 2016b; Walton et al., in review). However, we did not observe a difference in ovarian size between diet treatments. Although wasps examined in this study had very low variation in ovary size, the ovary sizes observed are typical of Polistes workers (Toth et al., 2009). Thus, differences may have been non-existent or too small to detect. Alternatively, it is possible that the level of nutritional stress imposed by our treatment was not severe enough to result in a change in ovary size. Also, the timing of this experiment may not have been long enough for changes in gene expression to occur. Perhaps, if diet treatments had continued for longer, candidate genes may have begun to exhibit differences in expression profiles.
We measured expression levels in the brain of seven genes associated with nutrient signaling and reproduction in insects that are putatively important in caste determination and social behavior in eusocial insects (Badisco et al., 2013; Wheeler et al., 2006; Azevedo and Hartfelder, 2008; Amdam et al., 2003; Nelson et al., 2007; Ament et al., 2008; Wang et al., 2009; Wang et al., 2010; Mutti et al., 2011). Manfredini et al. (2018) recently showed levels of vg to be good predictors of social dominance in *Polistes dominula* wasps. In the current study, none of the genes we investigated were significantly differentially expressed in the brains of workers across diet treatments. This outcome suggests these pathways related to egg yolk protein production and insulin signaling are not involved in the regulation of nutritionally-mediated changes in social behavior in wasps. However, although no individual gene differed in expression level between diet treatment groups, brain gene expression patterns for all gene combined showed a significant difference, suggesting that there may be diet-induced shifts in brain gene expression. It is possible that other conserved pathways not examined here, or novel, species-specific pathways may regulate these nutritionally mediated changes in behavior. Although we did not find statistical differences in brain gene expression in the genes we measured, some of these genes may be differentially expressed in other body parts, such as the fat body, which has been documented in relation to social behavior in honey bees (i.e., insulin-like peptides: Nilsen et al., 2011).

Although this study examined an extant species and the immediate effect of nutritional restriction on social cohesion, the results have the potential to be reflective of the conditions that promoted social insect evolution historically (Hunt and Nalepa, 1994). Lack of nutrition can make personal reproduction difficult or impossible, and so investing in the fitness of the group (via cooperative behavior) may be the best or only option under these
circumstances (Hunt, 1991). This approach would be especially true of kin groups with maternal care, where individuals are closely related and the inclusive fitness payoff of cooperation is highest. The physiological and molecular pathways that promoted cooperation at the origins of insect sociality may still reinforce cooperative behavior in modern social insect systems (Toth and Robinson, 2007). Thus, in social Hymenoptera colonies, decreased individual conflict and increased group cohesion may be achieved by maintaining a workforce of nutritionally restricted daughter helpers (Rossi and Hunt, 1991; Wheeler, 1986). We suggest nutritional restriction could be an important internal regulator of cooperation amongst the individuals that make up social insect colonies, in turn promoting the emergent group trait of cohesion and maintenance of the superorganism.

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**References**


Figure 1. Rates of behaviors in high and low diet-treated nests. A) Behavior rates averaged across the course of the experiment. Wasp nests from the low diet treatment foraged on sugar more than nests from the high diet treatment (Wilcoxon rank sum test: $W = 31$, $p$-value: 0.007, $n = 13$ nests per treatment) and nests from the high diet treatment foraged on caterpillars more than nests from the low diet treatment (Wilcoxon rank-sum test: $W = 142$, $p$-value = 0.002, $n = 13$ nests per treatment). B) Average rates of all behaviors recorded immediately following experiment-wide prey feeding. Only aggression was significantly different between treatments (Wilcoxon Rank-Sum Test: $W = 120$, $p$-value = 0.022, $n = 13$ nests per treatment). C) Average rates of all behaviors recorded prior to experiment-wide prey feeding. Only aggression was significantly different between treatments (Wilcoxon Rank-Sum Test: $W = 124$, $p$-value = 0.042, $n = 13$ nests per treatment).
Figure 2. Mass and physiological measurements. A) Workers from the high diet treatment had a higher mass than workers from the low diet treatment (linear mixed model: t-ratio = 3.01, p-value = 0.004, n= 29 high and 24 low). B) Total lipid content did not differ between treatment groups ((linear mixed model: t-ratio = 1.13, p-value = 0.27, n= 29 high and 24 low). C) Relative lipid did not differ between treatment groups (Linear mixed model: t-ratio = -0.88, p-value = 0.38, n= 29 high and 24 low). D) Ovary scores. There was no difference in average ovary score between diet treatments (T-test: t = 0.23, df = 45.89, p-value = 0.82, n = 24 wasps per treatment).
Figure 3. Brain gene expression for 7 candidate genes, as determined by real-time quantitative RT-PCR. There were no significant differences in normalized expression fold change across high and low diet treatments (one-way ANOVAs, all p-values > 0.05, n = 10 wasp brains per treatment). We confirmed that the internal reference gene (rp49) and the external reference gene (mCherry) cycle thresholds did not differ across treatments. Overall, brain gene expression was higher in wasps from the high diet treatment than the low diet treatment (Exact binomial test: p = 0.016).
CHAPTER 5. GENERAL CONCLUSIONS

A fundamental question in biology is “why does one individual have a particular phenotype while another individual of the same species has a different phenotype?” This is especially interesting to behavioral biologists who observe animals in the same population adopt consistently different behavioral strategies. My dissertation work explores this question in the context of social insect societies, where behavioral phenotypes of individuals can affect the emergent behavior and fitness of the entire colony. The main aims of this dissertation were to establish whether honey bee workers exhibit the hallmarks of personality, to investigate how nourishment contributes to behavioral variation among honey bee workers, and to address the generality of this pattern by comparing the effects of nourishment on social behavior in honey bees to its effects in paper wasps.

This dissertation provides the first comprehensive investigation of whether workers of the highly eusocial honey bee show hallmarks of personality (Chapter 2, Walton and Toth 2016). Although these workers demonstrate well documented behavioral changes as they age, I uncovered some aspects of their behavior, in particular their tendency to physically interact with other bees, remain somewhat consistent even as they age and change roles within the colony. I suggest that individual-level personality differences have the potential to contribute to colony division of labor by creating variation in individual tendencies to perform different tasks. However, honey bees showed fairly “weak” personalities (i.e. low but significant behavioral repeatability) compared to other non-eusocial insects and vertebrates. Honey bees exhibit “advanced eusociality”, a highly derived form of sociality with many group-level adaptations and a robust worker division of labor. Thus, the adaptive
benefits of individual personality may be muted by factors such as a well-developed age polyethism, the large number of individuals that make up a colony, and a highly derived social cohesion that favors a lack of behavioral individuality (like the cells of a multicellular organism). Variation in personality could potentially be more important in animal social groups with fewer individuals and lacking other forms of division of labor (e.g. age polyethism), such as more intermediately eusocial species like bumble bees or paper wasps or vertebrate societies without highly structured caste systems. Future comparative work on groups of organisms of varying levels of sociality will be highly informative for understanding the evolution and possible adaptive role of personality in animal societies.

Given the large amount of behavioral variation present within a species, it is of great interest to understand the genetic and environmental factors that give rise to such individual differences. It is well known from studies across a wide range of animal taxa that nutritional state can greatly influence behavioral choices and nutrition during development can lead to long-term, stable differences in individuals’ behavioral tendencies (Birkhead et al., 1999; Lummaa and Clutton-Brock, 2002; Barrett et al., 2009). In the honey bee, nutritional differences during development are important regulators of large differences in reproductive caste and worker behavioral development. In Chapter 3 (Walton et al. in revision), I investigated how nutritional variation affects one aspect of worker cooperative behavior, specifically their response to queen mandibular pheromone. I found that nutritional restriction affects the direction of this effect depending on the life-stage when restriction occurs. Nutritional restriction at the larval stage increases queen pheromone response, but nutritional restriction at the adult stage reduces queen pheromone response. This pattern was coupled with differences in ovarian development and lipid stores, which suggests a link
between nutrition and tradeoffs between personal and group-level reproduction. Honey bees exhibit many other cooperative behaviors (including brood care, trophallaxis, etc.), and testing whether these behaviors are also influenced by nutrition could further clarify the nutritional environment’s role in cooperative behavior. The general principle that nutritional stress fuels cooperation (Hunt and Nalepa, 1994; Pfeiffer et al., 2001; Requejo et al., 2011) may extend much more broadly than social insects. In the future, broad scale comparative analyses can address whether the patterns recorded in this study persist across different levels of reproductive plasticity, and across lineages through evolutionary time.

To further probe the generality of the phenomenon observed in honey bees—namely that nutritional stress promotes cooperation—I expanded this work to an independently evolved lineage of social insects, primitively eusocial paper wasps Polistes fuscatus (Chapter 4). In paper wasp societies, reproductive roles are flexible (as compared to the honey bee’s rigid reproductive division of labor between queen and worker). Thus, adult workers could potentially use nutritional resources to invest in their own reproductive potential (as I demonstrated with larval honey bees). I found that nourishment affected paper wasp worker social behavior, with a pattern similar to larval honey bees. When provided with higher prey availability, wasp nests exhibited higher rates of aggression. However, the differences in nourishment used in this experiment did not affect ovarian development, nor expression of several canonical nutrient-signaling or reproduction-linked genes. Future research should focus on other potential mechanisms for the observed behavioral effect of differential nourishment, including gene expression profiles in other tissues.

This research provides important evidence that behavioral differences between individuals in insect societies can be subtler than previously thought by manifesting at an
organization level below morphological and temporal caste. Moreover, these behavioral differences can be more persistent than previously believed by enduring throughout individuals’ lifetimes, even as they age and change roles within the colony. Additionally, the findings reported in this dissertation contribute to a growing body of work on how the nutritional environment, early-life effects, and tradeoffs between personal and group level reproduction affect cooperative behavior in social animals. This research will inform future investigation on how behavioral variation evolves and why it persists.

References


Supplemental Figure 1. Box-and-whisker plots of age polyethism of bees in observation hives. The pattern of behavioral transition observed in this study reflected patterns observed in previous studies (Seeley 1982; Wintson 1987). Red represents interactive tasks and blue represents non-interactive tasks.
**Supplemental Table 1.** Spearman rank correlations of same behaviors performed within a context and different behaviors performed in different contexts. P-values are shown after a Benjamini-Hochberg correction.

<table>
<thead>
<tr>
<th>Behavior 1</th>
<th>Behavior 2</th>
<th>R</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Different behaviors performed in different contexts</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QMP response</td>
<td>Intruder response</td>
<td>0.1370</td>
<td>0.00002</td>
</tr>
<tr>
<td>QMP response</td>
<td>Trophallaxis during stingers context</td>
<td>0.0721</td>
<td>0.0414</td>
</tr>
<tr>
<td>Stinger response</td>
<td>Intruder response</td>
<td>0.1165</td>
<td>0.00028</td>
</tr>
<tr>
<td>Stinger response</td>
<td>Trophallaxis during intruder response</td>
<td>0.0983</td>
<td>0.00297</td>
</tr>
<tr>
<td>Stinger response</td>
<td>QMP response</td>
<td>0.0760</td>
<td>0.0308</td>
</tr>
<tr>
<td>Trophallaxis in</td>
<td>Aggression toward cage-mate in Stingers context</td>
<td>0.0824</td>
<td>0.0174</td>
</tr>
<tr>
<td>Intruder context</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Different behaviors performed in the same context</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QMP response</td>
<td>Trophallaxis in QMP context</td>
<td>0.2411</td>
<td>1.45E-12</td>
</tr>
<tr>
<td>Stinger response</td>
<td>Aggression toward cage-mate in Stingers context</td>
<td>0.2063</td>
<td>1.03E-11</td>
</tr>
</tbody>
</table>
Supplemental figure 1. Confirmation of QMP assay. 40 7-day-old bees were placed in Plexiglas cages with a microscope slide treated with either QMP or control solvent (isopropanol). After 5 minutes, we counted the number of bees contacting the slide. Bees contacted QMP-laden slides more than control slides ($t = 12.6$, $df = 14.5$, p-value = 3.109e-09, n = 10 cages per treatment). Bar charts display means and standard errors.
Supplemental Table 1. Post-hoc comparisons of QMP response from dietary treatments in the acute larval starvation and adult pollen deprivation experiment. The four treatment groups are bees starved as larvae and deprived of pollen as adults (Low L/Low A), starved as larvae and fed pollen as adults (Low L/High A), not starved as larvae and deprived of pollen as adults (High L/Low A), and not starved as larvae and fed pollen as adults (High L/High A). Bolded names and p-values indicate significant differences. All comparisons were significantly different, except Low L/Low A vs. High L/High A. The non-significance of this comparison is likely because diet restriction had opposite effects in larvae and adults.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Estimate</th>
<th>z-ratio</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low L/Low A – Low L/High A</td>
<td>-0.77</td>
<td>-7.01</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Low L/Low A – High L/Low A</td>
<td>0.39</td>
<td>2.82</td>
<td>0.02</td>
</tr>
<tr>
<td>Low L/Low A – High L/High A</td>
<td>-0.24</td>
<td>-1.87</td>
<td>0.24</td>
</tr>
<tr>
<td>Low L/High A – High L/Low A</td>
<td>1.16</td>
<td>9.07</td>
<td>&lt;0.0001</td>
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<tr>
<td>Low L/High A – High L/High A</td>
<td>0.54</td>
<td>4.71</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>High L / Low A – High L / High A</td>
<td>-0.63</td>
<td>-4.53</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
APPENDIX C. ADDITIONAL TABLES FOR CHAPTER 4

Supplemental Table 1. Criteria for scoring the level of ovarian development.

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>Ovaries are formless strings</td>
</tr>
<tr>
<td>1.5</td>
<td>Ovaries are puffy, slightly swollen</td>
</tr>
<tr>
<td>2.0</td>
<td>Presence of 1-2 under-developed oocytes on ovary</td>
</tr>
<tr>
<td>2.5</td>
<td>&gt;2 under-developed oocytes on ovary</td>
</tr>
<tr>
<td>3.0</td>
<td>Presence of a mature oocyte</td>
</tr>
<tr>
<td>3.5</td>
<td>&gt;1 mature oocyte</td>
</tr>
<tr>
<td>4.0</td>
<td>Queen, many mature oocytes (excluded from analysis)</td>
</tr>
</tbody>
</table>

Supplemental Table 2. Primers used for real-time RT-qPCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5’ to 3’)</th>
<th>Reverse (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vg</td>
<td>GTCGGTCGATTATACGAGTCTTT</td>
<td>GAACTACTTTGCAGCGACAATTC</td>
</tr>
<tr>
<td>Vgr</td>
<td>TCACGAGGGTTCTTGCATATC</td>
<td>ACCATCCTTGCGAGTTACCTAAA</td>
</tr>
<tr>
<td>Ilp1</td>
<td>GCGACAACATGTGAGTGAATAAAA</td>
<td>GTCTTCGTCCGACAAATCCTTT</td>
</tr>
<tr>
<td>InR1</td>
<td>CTCAGTGTCCTGTTGGTTATTTG</td>
<td>CCCCTCGTGCTATAGTGGTTAC</td>
</tr>
<tr>
<td>InR2</td>
<td>GTCAACGAGTTGGAGATAGTT</td>
<td>CCAGAAGCGGCGAGGTATATTT</td>
</tr>
<tr>
<td>HR46</td>
<td>CCAGGACGACCAGATTGTGGTT</td>
<td>CCGTTTCGGTTAGCTTTCAATTC</td>
</tr>
<tr>
<td>TOR</td>
<td>GCTTGGAGGTGATAGGAATGAG</td>
<td>CCGTACTGACGGTAAACACTA</td>
</tr>
</tbody>
</table>
Supplemental Table 3. Wilcoxon rank-sum comparisons of all behaviors in high vs. low diet treatments (8 days, totaling 16 observation periods). Bolded behaviors were significantly different between treatments (n = 13 nests per treatment). The higher rate of caterpillar foraging in the high diet treatment can most likely be explained by the increased caterpillar availability in this treatment.

<table>
<thead>
<tr>
<th>Behavior</th>
<th>W</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trophallaxis</td>
<td>53.5</td>
<td>0.11</td>
</tr>
<tr>
<td>Caterpillar foraging</td>
<td>142</td>
<td>0.002</td>
</tr>
<tr>
<td>Sugar foraging</td>
<td>31</td>
<td>0.007</td>
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<tr>
<td>Aggression</td>
<td>102</td>
<td>0.38</td>
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</tbody>
</table>

Supplemental Table 4. Wilcoxon rank-sum comparisons of all behaviors in High vs. Low diet treatments, during observation periods immediately following experiment-wide prey feeding. The bolded behavior was significantly different between treatments.

<table>
<thead>
<tr>
<th>Behavior</th>
<th>W</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trophallaxis</td>
<td>63.5</td>
<td>0.13</td>
</tr>
<tr>
<td>Caterpillar foraging</td>
<td>56.5</td>
<td>0.16</td>
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<tr>
<td>Sugar foraging</td>
<td>99</td>
<td>0.33</td>
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<tr>
<td>Aggression</td>
<td>120</td>
<td>0.022</td>
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</table>
Supplemental Table 5. Wilcoxon rank-sum comparisons of all behaviors in high vs. low diet treatments, during observation periods immediately prior to experiment-wide prey feeding. The bolded behavior was significantly different between treatments. Caterpillar foraging was not observed in either group (since neither had yet been fed).

<table>
<thead>
<tr>
<th>Behavior</th>
<th>W</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trophallaxis</td>
<td>63</td>
<td>0.12</td>
</tr>
<tr>
<td>Caterpillar foraging</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Sugar foraging</td>
<td>90</td>
<td>0.79</td>
</tr>
<tr>
<td>Aggression</td>
<td>124</td>
<td>0.042</td>
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</table>