Effects of Nutritional Stress on Aspects of Worker Performance in the Honey Bee (Apis mellifera)

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This material is submitted as partial fulfillment of a B.A. with honors in Biological Sciences.

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Abstract

Juvenile malnourishment affects learning and task performance in many species, and is well documented in mammals, but poorly studied in invertebrates. We examined the effect of nutritional stress during larval development on the longevity and task performance of honey bee (*Apis mellifera*) adults. Nutritional stress occurs naturally in honey bee colonies when pollen, which provides honey bees with essential nutrients, is in short supply. Workers can compensate for food shortages by distributing nutrients among fewer larvae, cannibalizing brood, or ceasing brood rearing altogether. Despite measures to ensure larvae are adequately nourished, observations of undersized adults suggest that worker quality suffers during pollen shortages. It is not known how pollen deprivation during larval development affects the performance of honey bee workers as adults. We compared foraging behavior between cohorts of workers that were reared under conditions of either pollen deprivation or abundance. A natural spring pollen dearth was simulated by placing frames of honey comb with adult workers, young larvae, and very little stored pollen in a cool incubator (5°C). After eclosion, cohorts of nutritionally stressed day-old adult workers were individually weighed, tagged, and introduced into an observation hive. Matching cohorts of day-old adult workers from the same colonies, but reared under free-flying conditions with access to pollen, were introduced into observation hives as controls. Nutritionally stressed workers were on average 33% lighter than control workers. Longevity, onset and duration of foraging, and waggle-dance activity of tagged workers were monitored to determine the effect of nutritional stress during larval development on the performance of workers as adults. Nutritional stress had a clear impact on worker weight and function, providing insight into the relationship between nutritional stress and performance in a model invertebrate and an economically important pollinator.
Introduction

European honey bees (*Apis mellifera*) have been critically important economic and ecological pollinators since they were first imported from the European continent to the Americas in 1622. Honey bees, along with other pollinators, are responsible for pollinating approximately 35% of the world’s food crops (Klein et al., 2007), with $15 billion (USD) of value added annually to North American crops from pollination services by honey bees alone (Calderone, 2012). Apart from their widespread use as pollinators, honey bees have long held the curiosity of researchers for their complex societies and social behavior. Honey bees also serve as a model organism for exploring genetics, the evolution of sociality, and the development of learning and memory in invertebrates. Their importance as a study subject is evident in the fact that they were the fifth insect to have their full genome sequenced (Honeybee Genome Sequencing Consortium, 2006).

As honey bee populations decline around the world (Ratnieks and Carreck, 2010; vanEnglesdorp et al., 2011), a priority has been placed on furthering our understanding of these small but important creatures.

Honey bee societies, called colonies, are divided into three castes, and each caste has its own function within the hive. Queens and drones comprise the two reproductive castes, and workers are the single nonreproductive caste. Queens, of which there is only one per colony, mate with multiple males during mating flights soon after they emerge as adults from the special cells in which they develop. These matings supply a queen with enough sperm to fertilize all of the eggs that she will lay in the colony throughout her lifetime, up to 1,500-2,000 eggs a day for 2-4 years (Winston, 1987). The eggs generate
all of the offspring in her colony at a given point in time, which includes a few hundred drones and up to 60,000 worker bees at the height of summer.

Honey bee drones are the only males produced by queens and they serve an exclusively reproductive purpose. Colonies rear drones so that their maturation corresponds to peaks in the mating flights of virgin queens, around late May and again in early August (Winston, 1987). Outside of these times, colonies support only limited numbers of drones because of the energy that is needed to rear and maintain them. Drones, unlike worker bees, do not work and need to be fed by worker bees for several days after they emerge as adults (Winston, 1987). Although they learn to feed themselves after three days, they consume considerable colony resources and are thus a liability for colony growth (Winston, 1987). Consequently, they are kicked out of colonies by workers when resources are scarce, such as during seasonal food dearths or during the winter months (Free and Williams, 1975).

Matings between queens and neighboring drones result in the production of the numerically vast worker caste, which consists of the sterile daughters of a colony’s queen. After their adult development is complete, worker bees immediately begin various tasks, such as cleaning cells, caring for developing brood (“nursing”), and building comb in the nest (Winston, 1987). As workers age, they transition through tasks in a phenomenon known as temporal polyethism, where each individual performs different types of jobs over her lifetime. Younger bees generally perform safer jobs inside the hive—from cleaning, to making comb and brood care, on to receiving nectar at the hive entrance from returning foragers—while the more dangerous outside jobs of foraging and guarding the nest are performed by older bees. Genetic components
contribute greatly in determining what jobs a worker is most likely to undertake and when she will start (Robinson, 1992). However, the specific needs of a colony at any given moment also have an enormous influence on task performance by its worker residents (Robinson, 1992).

Before performing these tasks as adults, each individual transitions through four life stages as it develops: from an egg, to a larva, and then to a pupa before finally emerging from a brood cell as an adult bee (Fig. 1). A worker’s life begins when the queen lays an egg in the center of a wax cell in the comb, where the egg will develop for the next three days before hatching into a small, white, and worm-like larva. After an average of 5.5 days in an uncapped honeycomb cell as a larva, the cell is covered with a wax cap by adult bees. Larvae in capped cells spin a silk cocoon around their bodies before undergoing a complete metamorphosis from legless larvae to winged and legged pupae. Over the next 12 days, they darken from white pupae to sclerotized adults. On the last day of their development, each individual scratches her way out of the wax cell cap to emerge into the colony as a fully developed adult worker. The total time from egg laying to emergence of worker adults for European honey bees is approximately 21 days. Honey bee queens take the shortest amount of time from egg to adult (only 16 days), while drone development is the longest (24 days; Fig. 1).

Honey bee larvae are meticulously attended to throughout their development by their older, adult worker sisters before transitioning to the quiescent pupal stage (Schmickl and Crailsheim, 2004). Larvae are fed almost continuously and are kept at a constant temperature of 35°C by active heating or cooling of the brood area by workers.
Figure 1. Development of honey bee castes from egg to adult. Drone development takes the longest of the three castes, requiring 24 days to go from egg to adult emergence from a cell. Queen bees only require 16 days, while workers generally require 21 days to complete their larval and pupal development. Image obtained from Winston (1987).
Developing worker larvae are fed for an average of five days by young workers (“nurses”) that synthesize nutritious secretions in the mandibular and hypopharageal glands in their heads (Jay, 1964). Younger worker larvae are fed worker jelly, which is comprised of honey mixed with gland secretions that contain proteins, lipids and vitamins, while older larvae are fed the same mixture with the addition of pollen (Haydak, 1970). The secretions of the mandibular and hypopharageal glands of the nurse bees are derived by ingesting pollen and honey and reconstituting it into the larval jelly (Winston, 1987). It has been reported that 125-187.5 mg of pollen (containing 25-37.5 mg of protein) is needed to rear one worker larva to the pupal stage (Brodschneider et al., 2009). At each feeding, a nurse bee inspects a larva before she deposits food near its head (Haydak, 1970). Larvae of different castes are fed in different amounts and on different diets (Haydak, 1970). For example, queen larvae receive a more lipid and protein-rich diet of “royal jelly”. Workers can identify different castes of larvae to make sure they are appropriately nourished. Nurse workers are also able to detect underfed larvae or larvae that are experimentally deprived of food for several hours because those individuals are fed significantly more food than their peers in the hours thereafter (Heimken et al., 2009). As a result of the ability of workers to monitor and respond to the hunger level of larvae, individuals of the same caste and age in a brood nest generally have the same amount food available to them at all times (Haydak, 1970). The activities of the nurse bees help to ensure that all offspring are of high and equal quality, which is an important part of maintaining nest homeostasis by honey bees (Schmickl and Crailsheim, 2004).
In part because of the regulation of food distribution by nurse bees, malnourished larvae can be difficult to find in healthy honey bee hives (Schmickl and Crailsheim, 2004). Under normal colony conditions, colonies tend to adjust the number of individuals that they rear in response to the availability of food (Mattila and Otis 2006a), rather than sacrificing the quality of those individuals (Mattila and Otis 2006b). Larvae reared under conditions of enforced nutritional stress (i.e., experimentally imposed malnutrition) have been shown to be of a poorer physiological quality; these larvae often emerge as adults with shortened lifespans, lower dry weight, and smaller body size (Brodschneider et al., 2009; Brodschneider and Crailsheim 2010). Decreased thorax weight and muscle mass, as well as smaller wing size and lower protein body content, are also seen in malnourished bees that are reared in incubators on artificial diets (Brodschneider et al., 2009). Severely malnourished bees that are artificially deprived of food during later stages of development suffer from debilitating physical malformations, such as deformed wings and an inability to complete pupal ecdysis (the final molt before emerging from the cell), and have high rates of mortality (Jay 1964).

A steady supply of pollen, which is the source of most of a colony’s essential lipids, proteins, vitamins, and minerals, is necessary for continued rearing of brood, and a colony will terminate brood rearing rather than rear grossly underfed bees (Farrar, 1934; Brodschneider and Crailsheim, 2010). Pollen shortages occur frequently throughout the year for colonies in temperate climates and honey bees have developed several feedback mechanisms that are designed to try to ensure that young bees are of a high quality, similar to those that are produced during times of plenty (Seeley, 1985). Pollen shortages are most commonly encountered in early spring when colonies recommence brood
rearing after a winter hiatus and, in so doing, often quickly deplete what remains of their winter pollen stores before additional pollen is available from the environment (Farrar, 1934; Mattila and Otis, 2006b). The overlap in brood rearing and lack of availability of pollen from the environment is commonly termed a “pollen dearth”. Cold snaps in spring in temperate climates also contribute to pollen shortages because they restrict foragers’ flight activity, thus shutting down a colony’s pollen intake (Winston, 1987). A colony that cannot acquire adequate pollen resources from the environment can continue to rear bees for only a short time. Nurse workers will first consume all in-colony stores and then they will deplete the nutritional reserves in their own bodies to support brood rearing, but once these emergency rations are used, rearing may cease until pollen foraging can resume once again (Schmickl and Crailsheim, 2004). To avoid rearing low quality adults, honey bees have developed another regulatory mechanism: when pollen supply can no longer meet the nutrient demand of brood rearing, younger larvae and eggs, in which workers have invested minimally, are cannibalized and their nutrients recycled to feed older larvae (Schmickl and Crailsheim, 2001; Schmickl et al., 2003). Despite these feedback mechanisms, honey bees that are reared by colonies during pollen dearths have been reported to be physically smaller than bees that are produced at other times of the year (Seeley, 1985). In the absence of an additional supply of pollen-based nutrients, brood rearing is typically discontinued, and resumes only when additional pollen is available (Schmickl and Crailsheim, 2001; 2002).

Management practices by commercial beekeepers can affect colony health negatively, so their impact is of interest to researchers, beekeepers, and crop growers alike (Klein, 2007). One way that beekeepers can reduce pollen stores in colonies is by
placing traps on hives that remove pollen from foragers as they return from the field (Duff and Furgala, 1986). This pollen is then sold as a hive product that is used as a dietary supplement for humans or fed back to colonies at later dates to boost brood rearing. Pollen shortages may also be produced when colonies are employed for commercial pollination, an agricultural service that offers beekeepers an opportunity to make more money than can be generated by honey production alone, but one that may have detrimental effects on colony health. Commercial pollination practices often deploy large numbers of colonies in high density within fields of bee-pollinated crops. By timing the presence of large numbers of colonies with a crop’s bloom, growers can greatly increase the yield of that crop. However, crowding colonies for pollination often creates stiff competition among them for limited food supplies (Jay and Jay 1993; Schmidt et al., 1995). Additionally, many crops that are pollinated by honey bees, such as blueberries, cherries, or almonds, are maintained in vast monocultures. Managing colonies within crop monocultures can be especially challenging if the pollen that the crop produces is of low quality, as plants of different species produce pollen with different nutritional values for bees (Haydak, 1970). Schmidt et al. (1995) suggested that honey bees that pollinate monoculture plants such as sunflowers (*Helianthus annuus*) and sesame (*Sesamum indicum*) need to be provided with nutritional supplements because of the low nutritional content of the pollen that is produced by these crops. The nutritional stress experienced by colonies from foraging on a nutritionally incomplete pollen source may be exacerbated by competition among colonies, leading to conditions that promote the rearing of malnourished bees.
Understanding the environmental factors that affect honey bee health is particularly important given the losses in honey bee colonies that have been occurring around the world, with particularly rapid declines documented in North America in recent years (vanEngelsdorp et al. 2007; 2008; 2010; 2011). Since 2006, as many as 30% percent of the colonies in the U.S. have disappeared annually in a phenomenon called Colony Collapse Disorder (CCD), a syndrome where large proportions of adult bees suddenly abandon their hives, leaving behind food and developing brood (Ratnieks and Carreck, 2010). The alarming nature of CCD has inspired research on its possible causes, however it remains poorly understood (Williams et al. 2010). Currently, CCD is believed to be caused by a variety of pathological and environmental agents that act in combination, and researchers are investigating interactions among pests, pathogens, environmental factors and management practices (Klein et al. 2007), as well as the role of the genetic background and nutritional state of the colonies themselves. Understanding the influence of worker nutritional state for maintaining colony health has been identified as a high priority for addressing honey bee losses in North America. Although we have some understanding of the physiological toll that severe malnutrition imposes on workers in a lab setting, we currently have little idea about how more typical levels of nutritional stress might affect worker performance under natural field conditions.

So how do honey bees acquire these critical pollen resources? Pollen stores are created by foraging workers who find, collect, and return with the floral resources that colonies need to support brood rearing and population growth (Winston, 1987). Workers generally forage at the end of their lives and, while onset of foraging can be highly variable (usually from 20-30 days of age), foragers will perform this final task until their
death (Winston, 1987). Worker lifespans can vary greatly depending on the season, with spring, fall, and winter bees living much longer than summer bees, who work the hardest over their 4–6 week adulthood (Rueppell et al., 2007). In addition to having the greatest nursing load early in their lives, the short lifespan of summer workers is partly predetermined because foraging is the most dangerous job a worker can do, and foraging is mainly undertaken by workers when it is warm. In fact, colonies in temperate climates acquire most of their annual food supply during a short 6–8 week period of time in the summer (Seeley and Visscher, 1985).

Foraging bees provide many critical services for their colony, namely the acquisition of food resources in the form of nectar and pollen. However, the task of foraging is far from simple. Efficient foraging is at the heart of a successful honey bee colony, particularly because honey bees remain active during the winter in temperate climates, clustered together in a shivering ball within the hive to stay warm, and they need these food reserves as an energy source over this time (Seeley, 1985). These winter food stores that sustain colony members’ activities are collected during the previous summer and early fall.

Many social insects rely on a complex cooperative system of recruitment signaling to collect food, and honey bees are no exception (Seeley and Visscher, 1985). To increase a colony’s ability to gather as many reserves as possible before winter, honey bees utilize several types of signals to organize their foraging efforts, from the chemical signals that are typically used by many types of social insects, to a unique dance language that only honey bees employ. Waggle dances communicate the quality, location, and distance of food resources, which allows a colony’s forager workforce to act as vast
information sharing center where individual foragers report particularly good food sources to their peers (Seeley, 1985). This process enables foragers to extract resources from the environment in a highly efficient manner by focusing available bee-power (foragers) on only particularly fruitful patches of flowers (Seeley and Vischer, 1985). Foragers who find superior food sources can recruit new foragers to that source by waggle dancing, which consists of a series of movements that convey the journey of the dancing forager (von Frisch, 1967). When a forager returns from a successful foraging trip, she moves to the comb just inside the entrance of the nest (the “dance floor”). Here, she begins to turn a tight figure-eight pattern, placing particular emphasis on the straight section between the loops by shaking her abdomen back and forth laterally, or “wagging,” before running into the next loop (Fig. 2; von Frisch, 1967). She will augment her waggle run by emitting a buzzing sound that is created by vibrating her flight muscles (von Frisch, 1967). A dancer usually has several other bees that attempt to follow her quick movements, extending their antennae to gather tactile information about the dancer’s waggle run in the dark interior of the hive (Tanner and Visscher, 2009). In this way, followers pick up several types of information from dancers: the direction of the food relative to the sun is encoded by the angle of the dancer’s run and the distance to the food is given by the length of her run, with longer distances corresponding to longer waggles (von Frisch, 1967). Also provided by the dance is information about the quality of food because, when prompted by followers, dancers give out regurgitated samples of nectar (Seeley, 1985). Odors from flowers that are carried back on dancers’ bodies also seems to be important; intensifying odors aids in recruitment, as foragers appear to use odor to pinpoint the location of the food source.
Figure 2. Honey bee foragers use the “waggle dance” to communicate the location of floral resources to other foragers within the colony, moving in a figure-eight pattern as they dance. Figure obtained from Winston (1987).
after using the waggle dance to find its approximate location (von Frish, 1967; Seeley, 1985).

Learning and memory have been shown to be critically important elements of the foraging performance of workers (Dukas and Visscher, 1993). The task of foraging requires sophisticated learning and cognitive abilities as workers learn to navigate to and from their home, to manipulate flower types, and to make decisions about the profitability of particular floral sources compared to other resources, either novel ones or those advertised by their peers (Dukas and Visscher, 1993). The performance of foragers increases as they gain experience with the task over a week or more, which suggests that foragers spend a good deal of time learning and improving their collection abilities (Dukas and Visscher, 1993). While honey bees can learn to handle flowers or navigate to and from their hive over only a few trials (von Frisch, 1967), workers probably need to employ longer-term learning on subtler skills (Dukas and Visscher, 1993). These skills might include learning to associate changes in forage quality over the course of a day or making assessments about whether to follow dances to new foraging locations or to search for new sources on their own (Dukas and Visscher, 1993).

The memory and learning abilities that foragers employ in their acquisition of food resources can be affected by environmental factors, such as contact with pesticides (Decourtye et al., 2005), temperature differences during pupation (Tautz et al., 2003), and levels of mite infestation (Krali et al., 2007). These environmental factors are even known in some cases to affect aspects of recruitment, such as the consistency of foragers’ dance communication (Tautz et al., 2003). “Bad” dancers may ultimately reduce a colony’s acquisition of resources (Seeley, 1985) because short dances or fewer dances on
the dance floor attract fewer potential recruits (Von Frisch, 1967). Mattila and Smith (2008) found no effect of colony-level pollen deprivation on associative learning abilities of individuals because colonies responded to nutrient stress by adjusting brood numbers, but they suggested that prolonged seasonal dearths or management stresses could impose greater nutritional stress at the individual level, which could ultimately lead to impaired learning and memory.

Although not well understood in invertebrates, nutritional stress has been well documented to have adverse effects on task performance and learning in vertebrates, including humans (Pravosudov et al 2005; Santos de Souza et al, 2011; Alamy and Bengelloun 2012). Cognitive impairment is particularly associated with nutritional stress during early development, and a growing body of evidence indicates that these impairments can lead to long-term deficits in learning and behavior (Alamy and Bengelloun, 2012). For example, scrub jay hatchlings that experience nutritional stress perform poorly in spatial memory tasks later in life compared to control birds, and subsequent nutritional rehabilitation does not improve later performance (Pravosudov et al. 2005). Decreased cognitive abilities due to malnutrition, particularly protein deprivation in early stages of life, are also well documented in mammals. In one study, rat pups that experienced postnatal protein malnutrition had impaired learning and retention in navigational tasks compared to controls (Almay and Bengelloun, 2012). Very few studies have examined the effects of nutritional stress on invertebrates (Mattila and Smith, 2008). Only one invertebrate model organism, *Drosophila melanogaster*, has demonstrated a reduction in performance as a consequence of malnutrition. *Drosophila*
fed a low-protein diet as larvae exhibited decreased learning acquisition and memory retention compared to larvae fed on a diet with adequate protein (Xia et al. 1997).

Despite honey bees’ importance as a model organism for invertebrate learning and memory, their economic importance, and persistent suspicions that poor nutrition plays a role in their recent decline, no study has examined the behavioral effects of malnutrition on the performance of free-flying honey bee workers. This oversight is especially surprising considering the important role that learning and memory play in honey bee foraging, and the fact that colonies are often put in nutritionally stressful conditions as the result of seasonal changes and commercial practices. If malnutrition early in development affects the ability of foragers to perform their job later in life, then it could have serious implications for the overall health and survival of colonies. In this study, we seek to address this knowledge gap by assessing the foraging performance of adult workers that were reared under nutritionally stressful conditions during larval development. To do this, we simulated a natural spring pollen dearth by placing frames of honey comb with adult workers, young larvae, and very little stored pollen in a cool incubator. Once workers had reared new individuals under these nutritionally restricted conditions, we individually tagged emerging adults, matched them with normally nourished workers from the parental colonies, and then compared the longevity, foraging effort, and dancing ability of these two groups over their lifetimes. The outcome of this study will identify the effects of nutritional stress on worker performance, providing insight into the relationship between malnutrition and colony function for an economically important pollinator and a model invertebrate.
Materials and Methods

Source colonies

To investigate the effect of early nutritional stress on the foraging and dancing performance of honey bee (*Apis mellifera*) workers as adults, we manipulated levels of nutritional stress for workers as they underwent larval development and then introduced them as new adults into a two-frame observation hive to monitor their behavior. Bees used in the study were a mix of Carniolan and Italian European descent and were obtained from source colonies in Wellesley College’s research apiary that were purchased that year as five-frame nucleus colonies (nucs) from Beehavin’ Apiaries (Smithfield, Rhode Island, U.S.A.). All queens were naturally mated and reared in the year of study. All colonies were managed similarly for pests and pathogens (e.g., varroa mites, Nosema).

Manipulating nutritional stress during larval development

Levels of nutritional stress to which workers were exposed during larval development were altered by creating rearing environments that either had restricted (nutritionally stressed) or normal (control) quantities of pollen available to nursing workers that were rearing larvae. To create conditions of nutritional stress for larvae, one or two brood frames (depending on the amount of larvae on frames) with minimal pollen (insufficient to rear the larvae on the frame), young larvae, and eggs were obtained from three source colonies. Each colony’s brood frames were placed in a cardboard nuc boxes with two half-sized frames of honey; adult workers from 3-4 frames were shaken from each source colony into its nuc box. The nuc boxes were adapted for extra ventilation by
creating an 18x5 cm gap along the base and a 4 cm diameter circle on each side box, which were covered with wire mesh (see Figure 3). All nuc boxes were created over a three-day period (June 18-21, 2012). Once filled with frames and bees, nuc boxes were placed in a low-temperature incubator (VWR, Radnor, Pennsylvania, U.S.A.; manufactured by Sheldon Manufacturing Inc., Corenlius, Oregon, U.S.A.) at 5°C to simulate spring conditions, when colonies in temperate zones often find pollen in short supply. Approximately four days into the incubator treatment, the nucs were briefly removed and frames were visually examined to monitor levels of brood cannibalism, refresh honey supplies if necessary (a source of energy for adults to keep frames warm), and to check the progression of brood development.

After all viable larvae (i.e., those that were not cannibalized because of nutritional stress) were sealed into cells for pupal development by adult workers, the nucs were removed from the incubator and all the adult bees that had reared the brood were removed from the frames and returned to their source colonies. Sealed brood and honey frames were returned to the nucs and placed into a heated incubator at 35°C. This allowed developing workers in the frames (now without adult bees to care for them) to be exposed to stable developmental temperatures, similar to what adult workers would maintain for pupating brood, a developmental stage that is particularly sensitive to fluctuations in nest temperature. Nucs were checked daily for the emergence of new adults from sealed cells. Adults that emerged from day 10-17 of the study had been sealed before or shortly after being placed in the cold incubator (and thus had not been subjected to nutritional stress during their larval development), so they were not used for the study.
To get matching workers from the source colonies that were not exposed to conditions of nutritional stress during larval development (control workers), brood frames were removed from the source colonies (where they had had normal pollen supplies during development) and placed into empty nucs, two days before nutritionally stressed workers were expected to emerge from sealed cells. This second set of nucs were also given frames of honey and housed in the heated incubator.

Once new adult workers emerged from sealed cells in both sets of nucs, they were weighed, tagged, and introduced into a single observation hive so that their adult behavior could be monitored. Focal workers emerged as new adults over a period of 7 days; they were tagged and weighed daily as they emerged. Fresh weights for focal workers from both treatments were obtained by transferring newly emerged workers into containers that were chilled for several minutes on ice until adult movement slowed enough that each worker could be weighed to the nearest 0.001 g on a analytical balance (Mettler Toledo AB104-S; Mettler Toledo, Columbus, Ohio, U.S.A.). Nutritionally stressed workers that were visually small, and control workers that appeared typically sized were targeted for tagging. Immediately after being weighed, each worker was tagged on her thorax with a colored and numbered tag (The Bee Works, Orillia, Ontario, Canada), giving each worker a unique mark for identification throughout the study. Colors did not code for treatment so that observers were blind to nutritional conditions that workers experienced when behavioral data were collected. All bees were tagged and introduced into the observation hive within 24 hours of emergence from sealed cells.

Making the observation hive
Focal workers were introduced into a two-frame observation hive that was housed in a building on the Wellesley campus (the research apiary’s bee house). Workers in the observation hive were free flying, meaning they had normal access to food resources in the environment. The observation hive had approximately 7,000 adult workers (and their queen) from an unrelated colony that had been established that spring in the Wellesley College Apiary; their queen was naturally mated and raised in the year of study, and was also supplied by Beehavin’ Apiaries.

The observation hive was installed three weeks before the first group of focal workers were introduced into it. It had one empty frame and one frame that was a mix of brood and food. Tagged focal bees were introduced in the evening by putting them in a screened cage, attaching the cage to a hole above the top frame of the hives, and then lightly spraying with cage with dilute sugar water to make the tagged bees more acceptable to the workers in the hive as they moved from the cage and onto the top frame.

Focal bees were introduced daily as adults emerged over the next seven days, pairing workers from nutritionally stressed and control groups as much as possible, although pairing by treatment as well as source colony was constrained by the number of workers that emerged from sealed cells on a daily basis. In total, 464 nutritionally stressed workers and 457 control workers were introduced into the observation hive between July 10-17.

To determine how many nutritionally stressed and control workers were successfully introduced and accepted by workers in the host observation hive, marked workers who were introduced but never observed thereafter were considered to be rejected by the observation hive. Only workers who were successfully introduced and
accepted (e.g., seen during attendance following introduction; see below) were considered as part of the starting population for the study. Numbers of rejected workers were compared between treatments to determine whether nutritional state during larval development affected worker acceptance.

**Longevity**

To assess differences in the longevity of workers that were reared under nutritionally stressed versus control condition, the presence of all focal bees in the observation hive was recorded twice a day between the hours of 8 A.M. and 9 P.M. for 57 days following the last introduction of new adults (observations were made only once a day on 7 out 57 days). Attendance records were taken from the first day tagged cohorts were introduced on July 10 until September 12, when tagged bees were no longer seen in the observation hive. Workers were presumed to have died the day that they were no longer recorded as present in the hive. Observations were recorded a minimum of two hours apart, preferably before or after workers foraged to maximize the probability of observing as many living bees in the hive as possible within a single day. Mean longevity per worker was compared between treatments to determine whether nutritional stress affected worker lifespan.

**Foraging activity**

To determine whether nutritional stress during larval development alters the foraging behavior of workers, foraging activity was monitored by observing marked workers as they entered and exited the hive over a two-hour period every day (between 9
A.M. and 4 P.M.). A wooden runway (approximately 26 cm long, 9.5 cm wide, and 3 cm tall) that covered with plexiglass was attached to the front of the hive prior to introduction of focal bees to facilitate observation of foragers (Figs. 4, 5). Two wooden baffles the height of the runway and stretching 6 cm of the way across were inserted approximately 5 cm apart on opposite sides to slow exiting and entering bees and allow observers greater probability of seeing marked foragers as they moved in and out of the hive. Observations of foraging activity began seven days after the introduction of the first marked workers into the observation hive (on July 18), and concluded on day 50 of the study (August 30), when the number of foraging workers had declined to the point that they were rarely observed at the entrance.

A worker was deemed as performing an “orientation flight” (where a worker explores the area outside her hive to learn how to locate it once she commences foraging) and not counted as foraging if a single observation of her entering or exiting the hive had occurred more than 10 days before a subsequent record of foraging activity was made for that worker. Bees who were introduced but not accepted by the colony in the observation hive, but were recorded as foraging, were also excluded as mistake in tag identification by observers at the hive entrance (i.e., “attendance” data recorded from inside the hive was considered more accurate than foraging data taken from fast moving bees at the entrance). If a worker was not observed foraging every day after she was first observed foraging, it was assumed that she foraged every day between the first and last time that she was observed at hive entrance.

These data were used to compare differences between treatment groups in the onset of foraging (the first day a bee foraged) and total days each worker foraged over
her lifetime (foraging lifespan). These estimates of foraging activity were also correlated against the fresh weight of workers to explore the relationships between these variables.

**Dance activity**

To determine whether level of nutritional stress during larval development affected dancing activity, the observation hive was videotaped for two hours every day between July 27-August 30 to record the behavior of marked workers if they waggle danced. From these recordings, 10 consecutive days, totaling 20 hours of observation between August 2-12 were selected for use in this study because they coincided with the peak in dancing behavior of focal workers, with a total of 50 individual bees observed waggle dancing at least once during that 10-day period. Video recordings of the dance floor were made in one-hour increments between 9 A.M. and 4 P.M. (depending on weather and hive activity levels) using video cameras (Sony HandyCam, model DCR-HC62 digital video camera, Tokyo, Japan). Placement of a shunt at the hive entrance forced all bees to enter or exit on one side of the hive frame, allowing for videotaping of dancing behavior in one location on the bottom frame near the hive entrance (the “dance floor”). Each dancing forager was pointed out and named by an observer so that she could be easily identified during video analysis. All videos were analyzed using video editing software (Final Cut Express 4.0.1, Apple Inc., Cupertino, California, U.S.A.), which allowed for frame-by-frame analysis (one frame = 1/30 s).

Two types of dancing behavior were examined: tremble dances and waggle dances. Because of the extent of tremble dancing that was performed by workers (over one quarter of all focal bees were seen tremble dancing at least once in the 10 day
sample), the duration of many of tremble dances could not be determined because their start and/or finish was not captured within the one-hour time frame of the videos. Thus, only data on the frequency of tremble dancing was extracted from the videos. These data were used to determine whether nutritionally stressed bees were less inclined to tremble dance than their normally nourished counterparts.

A single waggle dance is often paused for several seconds because a dancer frequently stops to exchange nectar with surrounding workers or to wander around the comb before continuing the waggle dance. Thus, a single “dance” was defined as all the sections of waggle dancing between when a marked worker entered the dance floor to begin dancing and when she either ceased dancing altogether or left the hive to recommence foraging. If a bee was not seen on the video for more than 15 minutes between sections of dancing, the sections were recorded as separate dances. A “pause” in a dance was defined as starting when a marked worker ceased dancing for more than one second to either walk around the comb or to begin a nectar exchange and then ending when she resumed waggle dancing. Thus, a single dance by a focal dancer often consisted of multiple dance sections broken up by brief pauses. Three aspects of waggle dance activity were compared between marked workers in each treatment group: the length of focal dances (the sum of all dance sections in a single dance), the number of pauses in a single dance, and the number of times a worker was observed waggle dancing in the 10-day sample period.
Statistical Analysis

Fisher’s exact tests (2x2 contingency tables) were used to determine whether workers from either treatment were more likely to be accepted into the observation hive, to forage during their lifespan, and to be seen tremble or waggle dancing. Two-way ANOVAs were performed to determine the effects of nutritional state and source colony on measures of fresh weight, longevity, foraging, and dancing behavior. Data were log transformed prior to analysis to improve their normality. Where ANOVA tests showed differences among source colonies or treatments or their interaction, Tukey-Kramer multiple comparison tests were used to compare means and determine where these differences were found. An ANCOVA was done to explore the effects of treatment, with worker lifespan as a covariate, on the number of days workers foraged to determine whether treatment affected foraging lifespan when overall worker lifespan was taken into account. Relationships between fresh weights, longevity, foraging behavior, and dancing behavior were analyzed using Spearman correlations on untransformed data. All tests except for Fischer’s exact tests were performed using SAS version 9.1 (SAS Institute Inc., Cary, North Carolina, U.S.A.). All other tests were performed using GraphPad (2013 GraphPad Software, Inc., San Diego California, U.S.A.; http://www.graphpad.com/).
Fig. 1. To create nutritionally stressed workers, brood frames with little or no pollen (but lots of access to honey) were placed in nuc boxes with adult bees. Nucs were placed in an incubator at 5°C until brood had entered the pupation stage. Nuc boxes were altered to provide ventilation through two ventilation panels (18 x 5 cm gap along the base and a 4 cm diameter circle on each side). Frames containing control workers were placed in the same kinds of boxes after pupation until adult emergence (nutritionally stressed and controlled workers were maintained in a 34°C incubator over that time).
Fig 2. A plexiglass-covered runway (26 x 9.5 cm) was attached to the entrance of the observation hive to facilitate observation of foraging focal workers (below: picture of the front entrance into the observation hive; above: top view of the runway). The runway had two baffles (6 cm long and 5 cm apart) to slow incoming and outgoing bees.
Fig. 3. The runway was attached to a two-frame observation hive where all focal bees were housed with a host colony of unrelated workers and their queen. All observations took place within the observation hive frames, dance floor, or the foraging runway.
Results

Fresh weights

Mean fresh weight of nutritionally stressed workers after emergence from sealed brood cells was significantly lower than the mean fresh weights of emerging controls workers (Fig. 6; two-way ANOVA; treatment effect: $F_{(1,1024)} = 3591.64, p < 0.0001$). The extent of weight difference between treatments was affected by the source colony from which workers were derived (Fig. 6; interaction of effects: $F_{(2,1024)} = 11.67, p < 0.0001$; source colony effect: $F_{(2,1024)} = 74.34 p < 0.0001$). Pooled across source colonies, there was a 33% decrease in the fresh weights of workers if they experienced nutritional stress during larval development.

Nutritionally stressed workers were also more likely to be rejected after introduction to the host colony than were control bees, with only 83% of nutritionally stressed workers present in the colony 24 hours after their introduction as day-old adults to the observation hive, compared to 97% of control workers that were accepted after introduction (Table 1; Fisher’s exact test, 2x2 contingency table: $p< 0.0001$ $n = 1035$).

Longevity

Workers that were nutritionally stressed during larval development had significantly shorter adult lifespans than workers that were not stressed as larvae, living approximately one week less on average. (Fig. 7; two-way ANOVA; treatment effect: $F_{(1,914)} = 44.58, p < 0.0001$). Workers’ source colony influenced the degree to which longevity changed depending on treatment (Fig. 7: source colony effect: $F_{(2,914)} = 16.23$, 914).
p < 0.0001; interaction: F(2, 914) = 6.63, p = 0.0014). Consistent differences in survivorship over time were apparent for nutritionally stressed workers after tagged workers were five days of age, and these differences in survivorship persisted over the remainder of their adult lives (Fig 8). Workers’ fresh weights were positively correlated with lifespan (Fig. 9; Spearman correlation: df = 915, ρ = 0.27, p < 0.0001). Workers that were lighter upon adult emergence generally live shorter lives than heavier workers (Fig. 9).

**Foraging**

When only workers who were successfully introduced to the observation hive were considered for each treatment, those that experienced nutritional stress as larvae were significantly less likely to be seen foraging as adults compared to control workers, with only 62.5% of stressed workers compared to 81.4% of control workers observed foraging at least once during the study (Table 2; Fisher’s exact test; p < 0.0001, n = 921 workers in total).
Table 1: Numbers of nutritionally stressed and control workers accepted or rejected by workers in the host observation hive 24 hours after their introduction as newly emerged adults.

<table>
<thead>
<tr>
<th></th>
<th>Accepted</th>
<th>Rejected</th>
<th>Total Introduced</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stressed</td>
<td>464</td>
<td>98</td>
<td>562</td>
</tr>
<tr>
<td>Control</td>
<td>457</td>
<td>16</td>
<td>473</td>
</tr>
</tbody>
</table>

Table 2: Number of accepted workers that were observed exiting or entering the observation hive at least once to forage compared to workers that were not seen foraging at all. Workers either experienced nutritional stress or control conditions during larval development.

<table>
<thead>
<tr>
<th></th>
<th>Foraged</th>
<th>Did not forage</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stressed</td>
<td>290</td>
<td>174</td>
<td>464</td>
</tr>
<tr>
<td>Control</td>
<td>372</td>
<td>85</td>
<td>457</td>
</tr>
</tbody>
</table>
Of the workers who were successfully introduced to the observation hive, nutritionally stressed workers had foraging lifespans that were approximately one week less than control bees (Fig. 10; two-way ANOVA: treatment effect: $F_{(1,653)} = 28.61$, $p < 0.0001$; source colony effect: $F_{(2,653)} = 0.61$, $p = 0.55$; interaction: $F_{(2,653)} = 0.14$, $p = 0.87$), which is likely linked to their generally shorter lifespan. When overall longevity was examined as a covariate for foraging lifespan, the difference between treatments in foraging longevity became insignificant (ANCOVA; treatment effect: $F_{(1,658)} = 0.17$, $p = 0.68$; effect of covariate: $F_{(1,658)} = 197.87$, $p < 0.0001$).

Of the workers that were successfully introduced into the observation hive, nutritionally stressed workers experienced an earlier onset of foraging than control workers (Fig. 11). Control bees started foraging at an average of 23 days of age compared to 21 days of age for nutritionally stressed bees (Fig. 11; two-way ANOVA; treatment effect: $F_{(1,653)} = 9.45$, $p = 0.002$). Source colony and the interaction between source colony and treatment also influenced the age at which workers first foraged (source colony effect: $F_{(2,653)} = 59.79$, $p < 0.0001$; interaction: $F_{(2,653)} = 63.36$, $p = 0.035$). Overall, control workers in one colony (CH11) had workers who lived longest before they started foraging compared to other treatment combinations; amongst these latter groups, substantial differences were not found between workers who were nutritionally stressed versus those who were controls (Fig. 11).

Worker fresh weights were correlated against foraging lifespan, days foraged (i.e., the actual number of days that a worker was observed at the hive entrance), and the age of foraging onset to determine whether there were relationships between these variables. Weight was positively correlated with foraging lifespan for workers who were
successfully introduced into the hive and later observed foraging (Fig. 12; Spearman correlation: df = 657, ρ = 0.20, p < 0.0001). Across treatment groups, workers who were lighter upon emergence as adults generally had reduced foraging lifespans compared to heavier workers. Worker fresh weights were also positively correlated with the actual number of days that a worker was observed at the hive entrance, (Fig. 13; Spearman correlation: df = 657 ρ = 0.17, p < 0.0001). Workers that were lighter upon emergence as adults were generally observed foraging fewer days than heavier workers (Fig. 13).

For the same group of workers, fresh weights were also positively correlated with worker age at the onset of foraging (Fig. 14; Spearman correlation; df = 657, ρ = 0.16, p < 0.0001). Workers that were lighter upon adult emergence generally started to forage at a younger age than then heavier workers (Fig. 12).

**Differences in dancing behavior**

Of 51 accepted workers that waggle danced at least once over 10 days of observation, control bees were more likely to be observed waggle dancing than bees that had been nutritionally stressed (Table 3; Fisher’s exact test: p = 0.035). Similarly, of the 264 accepted bees that were observed tremble dancing over the same period, 61.3% were control workers compared to 38.6% nutritionally stressed workers (Table 4; Fisher’s exact test: p < 0.0001).
Table 3. Number of accepted workers reared under either nutritional stress or control conditions that were observed waggle dancing over 10 days.

<table>
<thead>
<tr>
<th></th>
<th>Did not Waggle Dance</th>
<th>Waggle Danced</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stressed</td>
<td>423</td>
<td>41</td>
<td>464</td>
</tr>
<tr>
<td>Control</td>
<td>396</td>
<td>61</td>
<td>457</td>
</tr>
</tbody>
</table>

Table 4.
Number of accepted workers reared under either nutritional stress or control conditions that were observed tremble dancing over 10 days.

<table>
<thead>
<tr>
<th></th>
<th>Did not Tremble Dance</th>
<th>Tremble Danced</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stressed</td>
<td>362</td>
<td>102</td>
<td>464</td>
</tr>
<tr>
<td>Control</td>
<td>295</td>
<td>162</td>
<td>457</td>
</tr>
</tbody>
</table>
Once accepted by the host colony, the number of days a worker was observed waggle dancing on average over a 10-day period was not affected by their nutritional state when they were larvae (Fig. 15; ANOVA, $F_{(1,45)} = 0.23, p = 0.64$) or the colony from which they were derived (source colony effect: $F_{(2,45)} = 0.48, p = 0.62$; interaction effect: $F_{(2,45)} = 0.37, p = 0.69$). The same was also true for the total amount of time that a worker spent waggle dancing over the observation period (Fig. 16; two-way ANOVA; treatment effect: $F_{(1,45)} = 0.22, p = 0.64$; source colony effect: $F_{(2,45)} = 0.13, p = 0.88$; interaction: $F_{(2,45)} = 1.24, p = 0.30$), and the average duration of individual dances (Fig. 17; two-way ANOVA: $F_{(1,45)} = 0.07, p = 0.79$; source colony effect: $F_{(2,45)} = 0.11, p = 0.89$; interaction: $F_{(2,45)} = 0.96, p = 0.39$).

There was no relationship between worker fresh weights and mean duration of workers’ dances for those workers who danced (Fig. 18; Spearman correlation; $df = 657, \rho = 0.05, p = 0.71$), the total time workers spent dancing over the ten-day observation period (Fig. 19; Spearman correlation: $df = 657, \rho = -0.02, p = 0.86$), or dancing frequency (Fig 20; Spearman correlation: $df = 657, \rho = -0.16, p = 0.27$).

Although no differences were found between nutritionally stressed and control workers in the duration of each dance or the number of dances workers performed over ten days, it was noted in the field that dances performed by nutritionally stressed workers seemed to be more irregular than those of control bees. In an attempt to assess this irregularity, the number times a dancer paused during a waggle dance was compared between treatment groups to determine whether this measure reflected perceptions of dance irregularity. However, number of pauses per dance did not differ between
nutritionally stressed and control workers (Fig. 21; two-way ANOVA; treatment effect: 
$F_{(1, 45)} = 0.01, p = 0.92$; source colony effect: $F_{(2, 45)} = 0.79, p = 0.46$; effects interaction: 
$F_{(2, 45)} = 2.11, p = 0.13$).
Figure 6. Worker bees reared as larvae under nutritional stress or control conditions in three source colonies were weighed live less than 24 hours after eclosion from sealed brood cells. Fresh weights (± SE) were compared in a two-way ANOVA. Data were log transformed for analysis; untransformed data are presented here.
Fig. 7. Mean lifespan (days ± SE) of all accepted, adult workers reared under nutritional stress or control conditions and compared by treatment and source colony in a two-way ANOVA. Data were log transformed for analysis; untransformed data are presented here.
Fig 8. Survivorship curves for cohorts of marked workers that were reared under nutritional stress or control conditions and then introduced into an observation have, where their survival over time was subsequently monitored.
Fig 9. Workers reared as larvae under either nutritional stress or control conditions were weighed after emergence from sealed brood cells. Workers were introduced into an observation hive where their longevity over time was subsequently monitored. Fresh weight was correlated against longevity using a Spearman correlation on untransformed data.
Fig. 10. Mean foraging lifespan (± SE) for workers that were accepted by the host observation hive for workers that were reared as larvae under nutritional stress or control conditions. To control for lifespan on the span of days a worker spent foraging, lifespan, foraging lifespan and treatment were compared using an ANCOVA test. Data were log transformed for analysis; untransformed data are shown here.
Fig. 11. Mean age at foraging onset (days ± SE) of all accepted, adult workers that were seen foraging. Worker bees were reared as larvae under nutritional stress or control conditions and compared by treatment and source colony in a two-way ANOVA. Data were log transformed for analysis; untransformed data are shown here.
Fig. 12. Workers that were reared as larvae under either nutritional stress or control conditions were weighed as day-old adults after emergence from sealed brood cells. Workers were introduced into an observation hive where their foraging behavior was subsequently monitored. Foraging lifespan was estimated as the number of days between the first and last time a worker was observed entering or exiting the observation hive. Fresh weight was correlated against foraging lifespan using a Spearman correlation on untransformed data.
Fig. 13. Workers that were reared as larvae under either nutritional stress or control conditions were weighed as day-old adults after emergence from sealed brood cells. Workers were introduced into an observation hive where their foraging behavior was subsequently monitored. The number of days workers were observed on the foraging runway was correlated against fresh weight using a Spearman correlation on untransformed data.
Fig. 14. Workers that were reared as larvae under either nutritional stress or control conditions were weighed as day-old adults after emergence from sealed brood cells. Workers were introduced into an observation hive where their foraging behavior was subsequently monitored. First day of foraging was estimated as the age of a worker when she was first observed leaving the foraging runway. Fresh weight was correlated against the age of workers at the onset of foraging behavior using a Spearman correlation on untransformed data.
Fig. 15. The waggle dance behavior of worker bees reared under nutritional stress and control conditions was monitored for two hours per day over a ten-day period. The number of days in the 10 day period that an individual bee was observed waggle dancing during the observation period (±SE) was compared by a two-way ANOVA. Data were log transformed for analysis; untransformed data are shown here.
Figure 16. The total time spent waggle dancing per worker (± SE) reared as larvae under nutritional stress and control conditions was monitored for two hours per day over a ten-day period. The total time each worker spent waggle dancing during the observation period was compared by a two-way ANOVA. Data were log transformed for analysis; untransformed data are shown here.
Fig. 17. The mean time spent waggle dancing per dance (± SE) by worker bees that were reared as larvae under nutritional stress and control conditions were monitored for two hours per day over a ten-day period. The average amount of time each worker bee waggle danced per dance was compared by a two-way ANOVA. Data were log transformed for analysis; untransformed data are shown here.
Figure 18. Workers that were reared as larvae under either nutritional stress or control conditions were weighed after emergence as adults from sealed brood cells. Workers were then introduced into an observation hive where their dance behavior was monitored later in their lives over 10 consecutive days (2 hours per day). The total time spent waggle dancing during that time was correlated against worker fresh weights.
Figure 19. Workers reared under either nutritional stress or control conditions were weighed after emergence as adults from sealed brood cells. Workers were then introduced into an observation hive where their dance behavior was monitored later in their lives over 10 consecutive days (2 hours per day). The total time spent waggle dancing during that time was correlated against worker fresh weights.
Figure 20. Workers reared under either nutritional stress or control conditions were weighed after emergence as adults from sealed brood cells. Workers were then introduced into an observation hive where their dance behavior was monitored later in their lives over 10 consecutive days (2 hours per day). The frequency of waggle dance behavior during that time was correlated against worker fresh weights using a Spearman correlation on untransformed data.
Fig. 21. Mean number of pauses per waggle dance (± SE) for dances performed by workers reared as larvae under nutritional stress and control conditions. Workers were then introduced into an observation hive where their dance behavior was monitored later in their lives over 10 consecutive days (2 hours per day). The mean number of pauses in all observed waggle dances was compared by a two-way ANOVA. Data were log transformed for analysis; untransformed data are shown here.
Discussion

In this study, workers reared under nutritionally stressful conditions weighed approximately one third less than workers reared under control conditions after all workers emerged from brood cells as day-old adults. Because of the substantial differences between treatment groups in the fresh weights of newly emerged adults, we are confident that we were able to develop for this study a novel method for rearing sizeable numbers of nutritionally stressed workers under natural conditions, something that has to date eluded researchers who are interested in exploring nutritional stress in honey bees. Overall, nutritional stress during larval development caused dramatic and long-lasting effects on most aspects of worker longevity and forager performance as adults. Importantly, we demonstrated that nutritionally stressed workers foraged fewer days on average and had shorter lifespans compared to workers reared under conditions of normal pollen availability. Furthermore, we found a positive relationship between fresh body weight at emergence and foraging lifespan. Nutritionally stressed workers were also less likely to perform waggle and tremble dances compared to control workers. However, if workers started dancing, there was no differences between treatment types in the amount of time a worker spent waggle dancing or average length of her dance per foraging trip. These results, in combination with our successful methods for imposing non-fatal nutritional stress on workers in the larval stage, open the door for further research on the effects of nutritional stress on honey bees, particularly aspects of learning and memory that are already known to be important in other model organisms (Halas et al., 1978; Xia et al., 1997; Alamy and Bengelloun, 2012). Such research has been highlighted by the U.S. Department of Agriculture as one of today’s most important
research priorities for investigating declines in honey bee health in North America (Honey Bees and Colony Collapse Disorder: Research Directions; http://www.ars.usda.gov/News/docs.htm?docid=15572).

**Broader Impacts**

Honey bees are ecologically and economically important pollinators that have experienced alarming declines in recent years (vanEngelsdorp et al. 2007; 2008; 2010; 2011). Despite widespread suspicion that poor nutrition plays an important role in honey bee decline (Broodschneider and Crailsheim, 2010; Alaux et al., 2010), few studies have addressed the effect of subleathal larval nutritional stress on honey bee adults. Understanding the impact of nutritional stress is especially crucial for colonies that are managed for commercial pollination. Under current pollination management practices, honey bees are widely subjected to multiple stressors from transport, dense packing into apiaries, and subsequent widespread competition for limited floral resources and variety in monocultures (Kevan et al., 2007; Girard et al., 2012). Colonies placed in crop monocultures with low protein-content pollen experience decreased brood rearing compared to colonies that have access to additional forage, demonstrating a colony-level response to nutritional stress (Girard et al. 2012). Our study is one of the first to give insight into how such stress affects individual workers.

In addition to exposure to low quality or nutritionally incomplete forage in a monoculture, renting hives for large blooming events forces colonies to endure stressful travel, only to encounter pollen dearths before and after the blooming event. For example, the world’s largest hive-migration event occurs each spring in California, where
1.1 million colonies are transported across North America to pollinate almonds crops. Once colonies arrive in California, they are often placed in holding lots for days, where thousands of hives are densely stacked in open, desert-like conditions. Immediately before and after almond pollination and while in almond crops, competition for any non-crop forage or supplemental food is inevitably intense, likely exacerbating the pre- and post-bloom pollen dearths. Our study shows how this kind of stress may impact the function of adult workers in these colonies over the remainder of their lifetime, even if foraging conditions improve after larval development is complete.

In addition to environmental stressors, honey bees have a variety of ecto- and endo-parasites, including *Varroa destructor* and *Nosema ceranae*, that may contribute to the energetic stress that honey bees regularly experience as adults (Naug, 2009). *Varroa spp.* mites suck hemolymph from developing worker larvae (Schneider, 1986) and *Nosema spp.* infections develop in adult guts, interrupting nutrient absorption and reducing worker longevity (Rinderer and Sylvester, 1978). As the prevalence of these parasites in hobbyist and commercial apiaries increases (Guzmán-Novoa et al., 2010) the case for building a better understanding of the effects of nutritional stress during development is strengthened, especially considering we know that supplementing colonies with additional protein helps to offset the effects of nutritional stress that these pests impose on workers (Janmaat and Winston, 2000; Mattila and Otis, 2006c).

It will be important going forward that these studies examine colonies and workers that are functioning naturally (i.e., outside of a lab setting) and as a social collective (i.e., allowing the dynamics of colony structure to affect the system). Early work on the effects of severe malnutrition during larvae development on worker viability
was completed over 40 years ago (Jay, 1964; Haydak, 1970), but these studies used lab techniques to alter nutrient input, such that stressed and control larvae were not reared by adult workers. Overcoming this obstacle by investigating nutritional stress under natural colony conditions is difficult because adults alter brood rearing to avoid larval stress, in part by cannibalizing young larvae to redirect nutrients to older larvae (Schmickl and Crailsheim, 2001) or ceasing brood rearing altogether. Although inroads have been made in rearing larvae on artificial diets in the lab (Aupinel et al. 2005), it is labor intensive to use artificial rearing to produce workers in large numbers and it is virtually impossible to manipulate nutrient input in a way that mimics the activity of nurse workers. In this way, artificial-rearing techniques provide a poor substitute for the natural process of brood rearing by the colony collective. To date, the lack of reliable methods to encourage stressed workers to complete larval development without high levels of cannibalism, thus producing functional adult workers in large numbers, has been a barrier for the study of larval nutritional stress and its impacts on honey bee behavior, learning, and memory. Our study provides a breakthrough in this regard.

Unlike adult honey bees, for which carbohydrates compose a substantial part of their diet, larvae depend largely on protein-based, pollen-derived, secretions that are supplied by nurse bees (Winston, 1967). In mammals (Halas et al., 1978; Alamy and Bengelloun, 2012) and the invertebrate Drosophila melanogaster (Xia et al., 1997), the only other insect for which such a similar study has been done, a lack of adequate protein during early development negatively impacts learning and memory in adults. A crucial time for learning in honey bees is when they transition from in-hive activities to foraging, when they must learn to navigate their environment and handle numerous types of
flowers. During this time, protein is crucial for growth of the mushroom bodies, a part of the honey bee brain that is responsible for memory, learning, and navigation. Mushroom bodies expand in size when workers initiate foraging (Farris et al. 1999), suggesting that, as in other creatures, nutritional stress during development may have adverse affects on lifelong learning and performance. One direction for future work will be to investigate the effect of nutritional stress on brain development in honey bee adults.

If stressed larvae exhibit decreased participation as adults in foraging for floral resources, a colony that is forced to produce nutritionally stressed workers could perpetuate a continual state of low pollen stores, subjecting future workers to conditions of nutritional stress as larvae, who would then mature to adulthood only to continue the cycle. It is possible that, over the long-term, early seasonal or commercial nutritional stress could lead to weakening of colonies if dearths persist. Clearly, a colony has the capacity to withstand initial pollen shortages in the spring, with most colonies recovering later in the season when food sources become more plentiful (Seeley, 1985; Mattila and Otis, 2006a). However, as nutritional stress in adult bees has also been shown to decrease immune defense to pathogens and parasites (Alaux et al., 2009) as well as resistance to pesticides (Wahl and Ulm, 1983), it is conceivable that poor nutrition could contribute to more rapid decline of weakened colonies, in concert with other stress factors.

**Impact of nutritional stress on colony function and productivity**

This work shows that nutritionally stressed workers, in part through decreases in longevity, contribute less frequently to important colony tasks, which likely has effects on overall colony productivity. It has been suggested previously that lighter,
malnourished workers have reduced longevity (Jay and Jay, 1993; Brodschneider and Crailsheim, 2010), which we have confirmed here through a demonstration that worker lifespan is directly related to nutritional stress during development. The reductions in adult longevity that we observed here are significant. Summer workers live 25-35 days on average (Maurizio 1950), so a decrease in longevity for nutritionally stressed workers of seven days constitutes a substantial part of their lives. Such a drastic reduction in longevity has the potential to adversely impact the productivity of the entire colony. Not all adult workers were observed foraging, but nutritionally stressed workers were less likely to be seen foraging during their lifetime than control bees. Nutritionally stressed workers did show a predisposition to precocial foraging, in accordance with Toth et al. (2005) who found that adults that experienced nutritional stress immediately after adult emergence experienced earlier onset of foraging behavior later in their lives. Precocial foraging by stressed workers in our study did not mean that workers were able to forage more over their lifetimes. The reduced longevity and foraging lifespans of nutritionally stressed workers, combined with a reduced likelihood to begin foraging at all, likely means that nutritionally stressed workers contributed less to the critical job of food acquisition compared to their counterparts in the well-nourished cohort. Critically, as a group, nutritionally stressed workers also did not contribute as frequently to the pool of information about available food resources that workers share through waggle and tremble dancing (although once workers took up the task of recruitment dancing, their per capita performance was equivalent to control workers). Without communicating successful foraging through dancing, there can be no recruitment of other foragers to capitalize on potential floral rewards, further lessening possibility that a nutritionally
stressed colony will bring adequate resources into the hive to support normal function and population growth.

**Future directions**

This project served largely as the first successful demonstration of the effects of larval nutritional stress on adult workers that were reared under “normal” (i.e., worker managed) brood rearing conditions by nurse workers. Although we were successful in developing a method to produce large numbers of malnourished, worker-reared individuals under fairly natural conditions, our methods are not yet perfect and there are a number of ways that our treatments could be better implemented. In general, workers confined in the incubator in treatment nucs were subjected to multiple stressors besides pollen limitation, including undergoing development in the absence of a queen. While control larvae were reared in queenright colonies, larvae subjected to nutritional stress were confined to frames with workers that lacked a queen. While we do not believe an absence of queen pheromones would significantly affect worker development in a way that could produce the dramatic differences in behavior and longevity that were observed here for adults in a queenright host colony, future work should seek to rule out possible behavioral effects on adults of being without a queen during larval development. Additionally, while stressed bees were confined to incubator boxes, control bees were reared in free-flying colonies. For increased comparability in future work, control workers should also be confined with workers on frames to nucs in an incubator, and all nucs should have a queen in them.
Although all source colonies exhibited a clear 30% reduction in fresh weight for nutritionally stressed workers, genetic and other environmental factors specific to each of the three source colonies may have played a role in the behavioral affects of malnutrition. Source colony had a significant effect in several measures: fresh weight at emergence, longevity and the age of foraging onset were all influenced by which colony larvae originated from. Larvae obtained from colony GH10 did not exhibit any differences between control and stressed bees in foraging lifespan and the age of foraging onset. This suggests that colony GH10 control larvae were subjected to some type of additional stress during development that affected the performance of all workers from that colony as adults, a stress that was perhaps not experienced (or experienced as keenly) by other control groups in colonies CH10 and CH11. Control bees from GH10 consistently performed no differently from nutritionally stressed bees from the other two source colonies. GH10 was housed in a different campus apiary than CH10 and CH11, so this stressor may have been unique to this location, or to the hive (although they are only about 1 km apart), indicating that careful screening for healthy colonies is essential before future work begins and that many factors likely play a role in the effects of larval stress on adult workers.

Although no differences were found in the amount of time individual dancers spent waggle dancing between the two groups, the measurements taken in this study such as total time danced, may be too generalized to detect behavioral differences between stressed and control workers. For example, nutritionally stressed bees appeared to dance with more inconsistent waggle circuit frequency, as individual foragers alternated between slow and fast circuits in the same waggle dance. Stressed bees may also have
greater variation in orientation angle of the dance, which could have serious implications for the accuracy of communication about the location of floral resources to nestmates. Training workers to visit a specific food resource and then evaluating their dance accuracy would help to reveal such effects. Additionally, nutritionally stressed workers who were observed waggle dancing may in some cases have fewer dance followers than workers reared under control conditions, which is another way that recruitment signaling can be hampered by stress, potentially reducing overall colony productivity and food-gathering ability (Girard et al., 2011). While no measurements were taken on these more specific dance parameters in this study, they remain important avenues for future research on the behavioral effects of nutritional stress on worker performance and colony function.

Conclusion

Honey bees serve as important ecological and economical pollinators and add an estimated $15 billion annually in added value to bee-pollinated crops in the U.S. (Southwick and Southwick, 1992; Calderone, 2012). As honey bee declines continue around the world, it is becoming increasingly important that we understand the effects of nutritional stress on honey bee behavior and foraging performance. The findings of this study indicate that workers that experience nutritional stress as larvae have reduced longevity, foraging performance, and probability of participating in recruitment signaling, which likely impedes the contributions that they can make to hive productivity in comparison to workers that are well nourished as larvae. The methods developed by this study will allow for future work that more closely examines the impact of these
nutritional and performance deficiencies on aspects of social behavior. We hope that this research creates an opportunity to investigate the synergistic effects of nutritional stress with other stressors experienced by declining honey bee populations in North America’s agricultural landscape.
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