The effects of pollen stress during larval development on the nursing behavior of adult honey bees (*Apis mellifera*)

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Abstract

The adverse effects of developmental nutritional stress on adult function are well-documented in vertebrates, but poorly studied in invertebrates. Our study examined the effects of larval pollen-stress in one of the world’s most important pollinators, *Apis mellifera*, the European honey bee. As honey bee workers age, they pass through important jobs in their colony in a process called temporal polyethism. Fundamental to colony health and population numbers is one of the first jobs a worker will do, nursing, or the feeding and general caring for of young larvae. To understand how larvae reared in a pollen-deprived environment affected nursing behavior, we divided natural colonies into three subunits to rear larvae under conditions of either abundant or limited pollen supply. We then observed focal workers for nursing behavior and hypopharyngeal gland growth (an important gland that nurses use to secrete “brood food”) during approximately the first three weeks of their adult life. Pollen-stressed workers had hypopharyngeal glands that were smaller than their unstressed cohorts’ in the middle of the typical period of nursing and followed a significantly different timeline of growth. Pollen-stressed workers also spent less mean time inspecting larvae in brood cells, but this difference was not statistically significant and interpretation would likely benefit from a larger sample size. As widespread pollinators in both commercial agriculture and natural settings, honey bees provide an invaluable ecological and economical service to our global society. In recent years, honey bee populations have been declining in part due to environmental stressors that are a result of food stress in colonies. This study provides insight into how a crucial honey bee behavior may be affected by such stress using honey bees reared under and living in a natural social setting.
Introduction

Pollinator populations are in decline

Since their introduction to North America in 1622, the western or European honey bee (Apis mellifera) has become an invaluable asset to modern agricultural practices in the U.S. Honey bees occur naturally only in Europe, Asia, and Africa (Wallberg et al., 2014), but they have been transported by humans to most corners of the globe. They remain ecologically successful outside of their native range because they are super-generalist pollinators, meaning they visit most plant species (Waser et al. 1996). This ability means that honey bees can produce honey and increase plant reproduction in a wide variety of locations. Furthermore, their eusocial nature and perennial life cycle makes them easy to tend and transport. As a result, honey bees are majority shareholders in animal pollination worldwide, contributing to over 87 leading global food crops and comprising approximately 50% of our global cultivated food supply (Klein et al., 2007). Out of the $15.12 billion (USD) that constitutes the annual economic value of direct insect pollination services in the United States, honey bees were estimated to contribute $11.68 billion (USD) of that value (Calderone 2012).

As key contributors to animal pollination, honey bees provide an irreplaceable and invaluable service to the global economy (Chopra et al., 2015; Potts et al., 2010; Genersch 2010). Unfortunately, while the agricultural demand for pollination is growing at a rate greater than 300%, the global population of managed honey bees has only increased by 45% (Aizen and Harder 2009). Factors such as stressful management practices, loss of foraging habitat, introduced pests and pathogens, and use of harmful pesticides are responsible in part for this growing disparity (Ricketts et al., 2008; Winfree et al., 2009; Potts et al., 2010; Karahan et al.,...
2015; Pacifico da Silva et al., 2015). With some of these stressors increasing in severity each growing season, there is a clear need for research and intervention that is aimed at maintaining a sustainable and diverse environment that can promote pollinator health. The issue of pollinator decline has received national attention in recent years and in 2014 President Barack Obama released a memorandum that recognized the severity of pollinator loss in the United States and established a Pollinator Health Task Force. As a call to develop a strategy to combat pollinator decline, the task force is responsible for mobilizing research, increasing and improving pollinator habitat, and educating the public about the steps that can be taken to address pollinator loss.

**Sources of pollen stress in honey bee colonies**

One outcome for honey bees in intensive management and with exposure to environmental pressures is a high level of nutritional stress in colonies. Honey bees consume two main food items: honey, a product derived from nectar, which provides carbohydrates for high-energy activities such as flying and thermoregulation, and pollen, which provides proteins, lipids, vitamins, and minerals that are essential for growth and normal function (Haydak 1935; De Groot 1953; Haydak 1970). The principal source of nutritional stress within a colony stems from inadequate access to pollen. While fully developed adult bees can subsist for some time on rich carbohydrate sources like honey (Haydak 1935), pollen stress is most damaging to larvae, who require its essential nutrients for proper growth during this early stage of development (De Groot 1953). In a colony, workers obtain the majority of pollen-derived nutrients from a bee-processed food store called “bee bread”. More nutritive than fresh pollen or any known pollen substitute, bee bread is made from a mix of freshly collected pollen, regurgitated nectar, honey,
and glandular secretions (Herbert and Shimanuki 1978; Hagedorn and Moeller, 1968; Dietz and Stevenson, 1980; Cremonez et al., 1998; Pernal and Currie, 2000). Pollen gets converted into bee bread after it is brought back to the hive and, in this form, it can be stored for long periods of time in a colony without losing much of its nutritive value.

A low supply or complete lack of either pollen or bee bread can occur in a colony for a number of reasons. One way is through seasonal variability, such as a long winter that prevents collection of more pollen in the spring, a period of intense brood rearing (i.e., care taking of larvae by honey bees) for colonies in temperate areas (Winston 1987). Limited foraging opportunities for pollen can also occur because of a loss of suitable foraging habitat (Brown and Paxton 2009; Potts et al., 2010; Vanbergen et al., 2013), as well as stressful management practices that place bees in high competition with one another among crop monocultures that have limited pollen diversity or poor nutritional value (O’Toole 1993; Williams et al., 1986). Unfortunately, these drivers of nutritional stress can occur simultaneously and can also be exacerbated by other stressors, including exposure to pesticides (Chauzat et al., 2009; Frazier et al., 2008) or pathogens (Cox-Foster and vanEngelsdorp, 2009). In response to pollen shortages, colonies may resort to one or more of the following emergency measures: they may decrease production of brood or cannibalize younger larvae so that resources can be reallocated to older larvae, in which heavy investment has already been made (Schmickl et al., 2001, 2002). Despite these protective measures, undersized and nutrient-depleted adults are sometimes reared (Jay 1964).

To properly address and reverse declines in honey bee health, it is crucial to understand exactly what role nutritional stress plays in the pollinator losses that are being observed around the world (Potts et al., 2010). A full-sized honey bee colony typically consists of 20,000-40,000
individuals (Page and Peng 2001) and, with eusocial insects like the honey bee, food stress is complicated by the many participants that are involved in day-to-day hive maintenance. In an environment where group behaviors have an unprecedented impact on colony success, understanding the effects of nutritional stress requires first understanding the structure of eusocial insect societies, including their basic colony structure, the role of nutrition in colony function, and how these elements interact when honey bees are faced with an environmental stressor like pollen deprivation.

**Basic colony structure, division of labor, and honey bee development**

Eusocial societies are identified by three main criteria: division of labor among colony members, cooperative care of brood (the eggs, larvae, and pupae that are cared for by adults), and overlapping generations of adults (Wilson 1971). To understand what is involved in cooperative brood care, which is the main category of behavior examined by this study, it is important to first understand the different castes that comprise a colony and the way labor is divided among them.

All honey bees start more or less on the same foot – as an egg laid by the female reproductive in a colony, the single queen. Whether the egg gives rise to a male or a female depends on whether it is fertilized before the queen deposits it in a cell: fertilized eggs become either female queens or workers, unfertilized eggs become males (called drones). Next comes the specialized developmental requirements that are reserved for rearing queens. While eggs destined to become drones or workers are deposited into normal cells in honeycomb, queens require larger cells, the royally titled “queen cup”, which projects outward from the smaller-
celled honeycomb. A larva, whether male or female, hatches from the egg several days after it is laid by the queen. At this stage, the cell is unsealed and “nurse” bees (young, adult workers) can actively clean and feed the growing larva. Queens are fed “royal jelly” exclusively, which is a protein-rich secretion that is made in the hypopharyngeal glands of nurses’ heads, while worker and drone larvae are fed a less nutritious diet of “brood food”, which include a combination of secretions from the nurse bees’ hypopharyngeal glands and mandibular glands (also found in the head), as well as straight honey and bee bread when they are older larvae. When larval feeding is complete for any of the castes (after approx. 5 days for workers, longer for drones and shorter for queens), the cell is sealed with a wax cap, the larva undergoes a final molt into the pupal stage (a quiescent stage during which wings and legs develop) before a fully formed adult chews through the wax capping and “ecloses” to join the adult colony population. The average metamorphosis time, from egg to emergence, takes approximately 16 days for queens and, the slightly longer, 21 days for workers and 24 for drones (Free 1987).

Division of reproductive labor separates queens and drones from the workers, who reproduce under only exceptional circumstances. Queens mate with drones from unrelated colonies early in their adult life – once mated, queens return to their colony and use the sperm they store from mating to fertilize eggs over the remainder of their lives. Drones spend their lives searching for queens with whom to mate and once mated, they die. Workers never mate and spend their entire lives performing the non-reproductive tasks that keep colonies functioning. The three castes are designated morphologically as well as behaviorally. The queen, the mother of all drones and workers in her colony, has an elongated abdomen which contains highly developed ovaries that are capable of laying over 1000 eggs per day. Drones can be identified by their large heads (with huge eyes and antennae for finding airborne queens), their boxy
abdomens (which contain complex genitalia for transferring sperm to queens), and they are equipped with powerful wings for catching queens on the wing. In contrast, workers are the sterile female work force. They are much smaller than queens and they have shriveled ovaries that are unable to produce eggs because of chemical signals that are released by queens to prevent their development. This chemical suppression ensures that workers do not overrule the queen’s reproductive dominance.

Among workers, there is further division of labor that is based on worker age, task location, and risk. While there is some flexibility in the tasks a worker takes up as she ages, there is a well-recognized pattern of task shifting based on age that is called temporal polyethism (Seeley 1982). Young workers tend to perform tasks within the hive (e.g., nursing, cell cleaning, comb building, and food processing), while older workers perform more dangerous outside jobs (e.g., foraging and colony defense). The mechanisms that regulate this task progression are still being actively investigated. However, physiological differences are known to characterize workers doing indoor versus outdoor tasks. Robinson et al. (1992) noted that changes in levels of juvenile hormone (JH) with the transition between indoor tasks and foraging. Younger workers performing brood care and other indoor activities have lower amounts of JH than the comparatively higher amounts that are found in older foragers (Rutz et al. 1976; Fluri et al. 1982; Robinson 1987, 1989; Huang et al. 1994; Huang and Robinson 1995). Furthermore, treating young bees with JH leads to precocious foraging (Jaycox 1976; Jaycox et al. 1974; Robinson 1985, 1987; Robinson and Ratnieks 1987; Robinson et al. 1989; Sasagawa et al. 1989), which shows that hormone levels play an important role in regulating the tasks that workers do.

There are many morphological differences between these various stages of temporal polyethism, including changes in gland structure, secretory products, musculature, and the
neuroanatomy that are required to perform very different tasks such as nursing brood versus foraging for food (Robinson 2009). For instance, worker brains change to increase the size of mushroom bodies associated with learning and memory when they first start foraging in order to better aid navigation and flower recognition – tools necessary for outdoor tasks (Fahrbach and Robinson 1996). Conversely, workers who are nursing brood have anatomy that supports the job of providing food to larvae. Thus, the development of adult worker hypopharyngeal glands, which have a large role in nursing behavior, can offer insight into a worker’s timeline of temporal polyethism, from which one can make inferences about how nutritional stress affects the development of the larvae they are feeding.

Figure 1. Anatomy of hypopharyngeal (or brood food) glands in *Apis mellifera* (Dade 1977; Tofilski 2012). Side view of worker showing location of hypopharyngeal glands in the head (A), paired “brood food” glands in dorsal view (B), dissected glands showing the “grapes on a vine” structure of the glands, with a close up of the acini that make up the glands (C).

(Refer to fig.1) Hypopharyngeal glands are paired glands that are found in the heads of worker bees and they are largest in nurse-aged workers that are 6-12 days old (Painter and Biesele 1966), the period of time when workers tend to do the most nursing (Seeley 1982). Each
gland consists of a lobe with a coiled-up “grapes on a vine” structure, the “grapes” being secretory units of the gland called acini (Painter and Biese 1966). Their secretion contributes greatly to the production of royal jelly (for queens) and to a lesser extent to brood food (for larval drones and workers). Once workers become foragers, the hypopharyngeal gland lobes and acini shrink, and the secretory protein components are broken down and re-used as digestive enzymes such as glucosidase, amylase, and glucose oxidase, which are used for honey making rather than brood food (Kubo et al., 1996; Ohashi et al., 1997, 1999). Degeneration of these glands is in part caused by increasing levels of JH (Kubo et al., 1996; Ohashi et al., 1997).

Interestingly, individuals are capable of accelerating, delaying, or even reversing behavioral development, which allows them to deviate from typical patterns of temporal polyethism in response to changing conditions within the hive (Huang and Robinson 1996). Moreover, results of additional experiments monitoring JH levels during changing colony conditions supported the idea that environmental stimuli specifically act on the endocrine system to cause changes in the rate (or reversal) of temporal polyethism (Robinson 1987). In fact, when nurse bees were removed from a colony, foragers demonstrated a decrease in Juvenile hormone levels as well as a reversal of hypopharyngeal gland degeneration, which allowed older workers to revert to a nursing state and serve the needs of the colony (Huang and Robinson 1996). It is this kind of behavioral and physiological flexibility that allows workers (and their colonies) to respond as required to changes in their environment and in the needs of their colony.
**Nutritional stress**

Poor nutrition during development can have a lasting impact on an animal’s life. The effects of early food stress have been documented across a wide array of species and showcase a myriad of physiological consequences for adults, including among zebra finches showing impaired immune response (Kriengwatana et al., 2013), fish with reduced muscle development and lower lipid content (Piccinetti et al., 2015; Rosenlund et al., 2004), and primates with diminished persistence and attention (Keenan et al., 2013). Invertebrate model organisms offer insight into possible wide-spread effects of nutritional stress during development. Insufficient access to food during development may result in delayed “rate of living” (Finkel et al., 2000). Thought of as an adaptive strategy for overcoming food scarcity, fruit flies (Carey et al., 1998, 2008), ladybeetles (Xie et al., 2015), and mosquitoes (Telang and Wells 2004; Takken et al., 2013) decrease their rate of growth and onset of reproduction when faced with limited resources. Although this strategy has the potential to offset stress by delaying development, individuals can experience subsequent fitness costs, such as lower adult body weights and associated reproductive costs (Bauerfiend et al., 2005; Barrett et al., 2009; Kolss et al., 2009; Dmitriew et al., 2011).

Even fewer studies have examined the effect of nutritional stress on non-reproductive behaviors in invertebrates. Nutritionally stressed fruit flies demonstrate significantly reduced learning acquisition and short-term memory compared to unstressed fruit flies, and these traits persisted in progeny (Xia et al., 1997), as well as significantly higher fecal output than their unstressed cohorts (Urquhart-Cronish and Sokolowski 2014). Food limitation in butterflies affects adult resource allocation (Boggs and Freeman 2005) and territory defense (Vande Velde
et al., 2013). Response to nutritional stress during development remains varied and, at times, its
effects can be far-reaching over an organism’s lifetime. Furthermore, with social insects like
honey bees, the effects of such stress can become increasingly complicated because of the
number of social interactions that occur within a colony. Therefore, understanding the role of
developmental food stress in an insect colony requires understanding how the specific behaviors
of individuals are affected by a dearth of resources, as well as the impact on the entire colony.

The purpose of this study is to investigate the consequences of larval food stress on the
adult nursing behavior of honey bee workers. A previous study conducted by Scofield and
Mattila (2015) examined the effects of larval nutritional stress on foraging, an essential behavior
of workers that ensures colonies have enough food to support their members. When focal
workers experienced conditions of pollen stress during larval development, they were lighter,
lived shorter lives, and were much less likely to participate in foraging than workers who were
reared without pollen stress (Scofield and Mattila 2015). When workers who were pollen
stressed as larvae did forage as adults, they did so precociously and for fewer days in total that
adult workers who were not stressed as larvae. One important question raised by this study is:
Why do nutritionally stressed workers forage earlier than unstressed workers? One possibility
driving this observation is that early foraging results from a compressed period of nursing, which
precedes it and may be similarly compressed by poor nutrition during development. It is already
known that poor access to pollen during the nursing period can shorten the period that a worker
can nurse (Maurizio and Hodges 1950), but it is possible that poor nutrition prior to nursing may
affect nursing ability as well.

Following the methods developed previously in our lab (Scofield and Mattila 2015), our
study aims to create as natural a setting as possible in order to realistically assess the effects of
larval nutritional stress on adult nursing behavior. First, we split colonies into three different treatments: pollen-stressed and confined to prevent further pollen collection, unstressed and confined (a control for the effect of confinement), and unstressed and unconfined. All focal workers were reared by nestmates in a natural social setting with access to either limited (pollen-stressed) or adequate (unstressed) pollen stores. Once workers emerged as adults, they were weighed, tagged, and introduced into observation hives that provided focal workers with a normal social environment. Over the course of 20 days, focal workers from all treatments were monitored for three different measures of nursing behavior to determine whether larval pollen stress affected adult nursing capacity. Additional bees were dissected over the same period to assess changes in hypopharyngeal gland development across the three treatments. Our findings will help elucidate how developmental nutritional stress affects the behavioral and physiological trajectory of adult workers, which will provide insight into the potential effects of such stress on the function of the entire honey bee colony.
**Materials and Methods**

To examine the effects of larval pollen stress on adult nursing behavior and physiology, we manipulated pollen availability during worker bee development by providing colony subunits with either an abundant or limited pollen supply to rear larval brood and then monitoring the activity of reared workers as adults. Honey bee (*Apis mellifera*) colonies used in the study had been established at the Wellesley College research apiary (Wellesley, MA, USA) within the year of study and were either of Italian descent (purchased in 2014 from Wood’s Beekeeping Supply, Lincoln, RI, USA) or of Carniolan decent (purchased in 2015 from Autumn Morning Farms Beekeeping Supply, Barre, MA, USA). All queens were open-mated varroa-suppression hybrids of Italian descent and were no more than one year old. All field work was conducted at Wellesley College during June and July 2015.

**Manipulating pollen availability during larval development**

Focal honey bee workers were reared under conditions of either limited or abundant pollen supply and then monitored as adults for nursing behavior and hypopharyngeal gland development during a period of 20 days when bees were between the ages of one and twenty-two days. To manipulate pollen stress during larval development, seven source colonies were split into three subunits so that the larvae they contained could be reared under different conditions of pollen availability: “pollen-stressed, confined,” “unstressed, confined,” and “unstressed, unconfined”. Source colonies had many frames of open brood (i.e., larvae and/or eggs) occurring in a consistent laying pattern and appeared otherwise healthy upon visual inspection.
All subunits were either transferred to screened up, 5-frame hives (confined treatments) or left in their original hive (unconfined treatment). Pollen-stressed workers were created by limiting the area of honey comb that contained pollen (with the intent of forcing workers to exhaust their pollen supply while rearing focal workers) and confining all workers to their hive so that they would be unable to forage for more pollen while focal larvae were undergoing development (pollen-stressed, confined treatment). In contrast, the other two subunits were given abundant supplies of pollen during this developmental period so that workers had sufficient pollen resources for brood rearing. Of these two subunits, one was allowed to continue pollen foraging (unstressed, unconfined treatment) and the other was confined to control for the effect of confinement on the pollen-limiting treatment (unstressed, confined treatment). The unstressed, unconfined colony subunits were kept outside so that workers could forage; they also had the original source queen. Confined subunits were kept indoors at a cooler temperature (20 °C) to limit colony overheating; they were given queen lures that were impregnated with queen pheromone to prevent queen supersedure and were replaced every two days. Subunits remained in this state for at least eight days after they were assembled, which ensured that focal workers remained under these conditions during their entire larval development (including if they were in the egg stage when subunits were assembled). After the majority of the brood were sealed into their cells for pupation, the subunits were disassembled, the frames were returned to the original source hive, and brood frames from all three treatments were placed in an incubator (35 °C) until pupating worker emerged as adults.

Each subunit contained 1-2 frames of open brood, plus additional honey frames to ensure that honey-derived nutrients were not limited in any treatment. The area of stored pollen (assessed using a 2.54 x 2.54 cm grid) was measured on each frame before they were distributed.
among the subunits. Subunits undergoing the pollen-stress treatment had an average of 52.1 (± 4.2) cm² of pollen at the start of brood rearing, whereas control treatments had 619.8 (± 125.1) and 568.2 (+/- 192.9) cm² of pollen for confined and unconfined subunits, respectively (one-way ANOVA, treatment effect: df = 2, 18, F = 5.8, p = 0.01). Pollen-stressed subunits had significantly less pollen at the end of the rearing period compared to the beginning (t-test: t = 8.4, df = 12, p < 0.0001), while those in the unstressed, confined treatment (the only treatment for which post-rearing pollen reserves were assessed) showed no change in pollen availability before and after the rearing of focal larvae was completed (t = 1.1, df = 12, p = 0.15). Pollen-stressed bees had significantly less pollen remaining in comb (2.8 ± 4.2 cm²) than those reared under unstressed, confined conditions (429.5 ± 125.1 cm²) at the end of the rearing period (t-test: t = 3.2, df = 6, p = 0.02). Unstressed, unconfined bees, as free foragers, had unlimited access to pollen and visual inspection after brood rearing was complete confirmed that they still had adequate pollen stores at the end of the rearing period.

Once focal workers began to emerge from sealed cells, they were weighed individually (to the nearest 0.001 g, using a Mettler Toledo AB-104S scale, Columbus, OH, USA) and marked on the thorax with uniquely identifiable colored and numbered plastic tags (from Beeworks, Oro-Medonte, Ontario, Canada and BioQuip, Rancho Dominguez, CA), which provided us with information corresponding to a worker’s treatment, age, and source colony. Young bees were then introduced as day-old adults into one of two observation hives with all subunits from each source colony introduced into the same hive. Hive one received bees from five source colonies and hive two hosted tagged workers from two source colonies. Each observation hive had two frames filled with brood and food, as well as a queen that was reared in the year of study, providing a typical and shared social environment for focal adults where their
nursing behavior could be easily monitored. Observation hives were maintained indoors in a bee house that is part of the Wellesley College research apiary. All workers in the observation hives could access the outdoors via a tube that connected the observation hive to the outside wall of the bee house.

**Worker survivorship**

Each day, twice a day, a honey bee “estimate of worker survival” was taken by visually scanning the observation hive and noting which bees were present by tag color and number to determine who was accepted by the observation hive populations after introduction, as well as the identity of the tagged individuals that were alive in the observation hives when estimates of participation in nursing were made thereafter. Most estimates of worker survival were taken once in the early morning and once in the afternoon when foraging activity was lowest and there was a greater chance of seeing as many tagged bees as possible. These data were used to determine the survival of cohorts of introduced workers over time and across treatments. The maximum number of days that the survival of the last-introduced cohort of workers was monitored was until 22 days of age. Thus, this age was used as the cut-off for survival for all workers, even if they were introduced to observation hives earlier and had their survivorship monitored longer. Because we used a 22-days-of-age as the maximum cutoff for observations of survival for all workers, the data were right-censored. Including right-censoring data means that the calculation of the survival function for each treatment took into account that some of the workers had a time of death at some time beyond the period of observation (see statistical methods below for more information).
Estimating participation in nursing activities

After manipulating developmental conditions when focal workers were larvae, their nursing behavior as adults was assessed across treatments through three estimates of nursing behavior over the first 20 days of the adult workers’ lives: hive-wide scan sampling for nursing behavior, recording the occurrence of nursing behaviors by individuals within smaller brood areas, and tracking of focal bees over time. Each assessment consisted of the measurement of two known indicators of nursing behavior, “inspecting” (workers ducking their heads and sometimes whole bodies into brood cells in order to check on and/or feed brood open brood) and “mouthing” (workers running their mandibles over the wax seals of cells containing pupating workers, with their antennae pointing downward to the capped cells, which is thought to be the shaping of wax around brood (Kolmes 1985).

Hive-wide scan sampling was conducted two times per day for each observation hive, once in the morning and in the afternoon. Each hive had a grid consisting of 5 x 5 cm squares. During a scan, an observer would scan the grid on an observation hive, from top to bottom on each side, to record the identity of any tagged worker who was encountered that was observed either inspecting open brood cells or mouthing sealed brood (or both). Such scans were employed to compare across treatments the proportion of focal bees from each treatment that participated in nursing and per capita rates of nursing per day (both for all workers and only those who were observed nursing at some point over the observation period). Nursing activity was always corrected for and then compared among same-aged workers (rather than comparing across actual date) because workers were introduced over a four-day period.
Nursing behaviors performed within smaller areas of the hive that consisted primarily of brood were also conducted by recording the identity of tagged workers who were seen performing any behaviors within a 10 x 10 cm brood area (open and/or sealed brood) during a 5-minute period. An assessment of nursing within these smaller brood areas was made up to ten times per day in each observation hive (range 2–10 times per day). A different brood area in each hive was used on the same day. Again, comparisons were made between similarly aged workers of different treatments, rather than actual date, to adjust for differences among workers in their adult emergence and introduction into the observation hive. The period of observation that all workers had in common was 7 to 22 days of age for this estimate of nursing activity.

For focal bee tracking, we randomly selected by identifying a color of interest according to treatment, then going to the brood area and select a focal worker based on tag color and said treatment. and then followed individual workers during a 2-minute period and counted the number of times that individual inspected a cell and the amount of time she spent mouthing sealed brood. Twenty focal bees from each treatment (10 per observation hive) were randomly selected each day for a total of 60 bees tracked daily to measure which tagged bees were engaging in nursing behavior and, if so, through nursing or mouthing – the goal was 20 days but actual numbers were, tailing off at the end when workers were dying and moving away from the brood area. For this estimate, the workers were sampled for their behavior over a 19-day period, when workers were 4-25 days of age (depending on when they were introduced to the observation hive over the four days that workers were tagged). Because workers were selected randomly, it was difficult to get reliable sample sizes of each worker age and treatment combination, so data were pooled across ages and compared between treatments instead.
Both observation hives were examined daily for signs of disease (none were seen) and the presence of pollen and honey stores to ensure that focal workers were not subjected to conditions of food stress as adults in the observation hives. One of the observation hives was supplied with sugar water (a syrup containing a 1:1 ratio of water and sugar) during a period when few floral resources were available and honey stores in the colony were low.

Assessment of hypopharyngeal gland development

Additional focal workers from each treatment were painted according to treatment and age so that they could be sampled later to assess hypopharyngeal gland development. Painted bees from all seven of the source colonies were placed in an outdoor colony, which was a separate colony from the observation hives that held the tagged bees. Because the painted workers needed to be sampled every three days, putting them in a separate colony avoided the disturbance of opening and closing observation hives when retrieving bees that were to be dissected. The colony that hosted the painted bees was kept in the same apiary as the observation hives and was from the same stock as the source colonies for the tagged bees (see above). During the same observation period as the tagged bees, 8-15 painted workers from each treatment were collected every three days (when bees were aged 5, 8, 11, 14, 17, 20 days old). These workers were frozen until they were dead and then dissected the same day to determine the development of their hypopharyngeal glands. This was assessed by measuring the size of acini over time (Wegener et al. 2009). Painted worker bees were selected according to color, which represented one age group (but range of source colonies), in order to compare changes in the size of acini over time and between treatment groups. Once painted bees were removed from
the common hive in which they were kept and were ready for dissection, their heads were mounted in wax and the head’s mask removed. Glands were then removed and the diameter of 3 acini per gland were measured for a total of 6 acini per bee. Acini were measured at 100x magnification on a compound scope using the micrometer in the ocular lens, which was rotated until the ruler went across the widest part of the acini that was parallel to the main duct. Head dissections were performed dry and then hypopharyngeal glands were floated in Ringer solution (to produce a standard isotonic solution 6.5g NaCl, 0.42g KCl, 0.25g CaCl2 and 0.2g of sodium bicarbonate is dissolved in one liter of distilled water), according to Wegener et al. (2009).

**Statistical approach**

To determine if weights of newly emerged worker bees were affected by conditions of pollen stress during the rearing period, a two-way ANOVA was performed to examine the effects on adult emergence weight of treatment (pollen-stressed or unstressed controls) and source colony. The survival of workers reared in pollen-limited and pollen-abundant treatment conditions was compared with Kaplan-Meier estimates of survival; post-hoc comparisons were made with Šidák adjustments to log-rank tests. Differences in nursing activity (mean number of nursing acts per day per capita for nursing scans and mean number of nursing acts per minute per capita for smaller brood scans) between pollen-stressed and unstressed rearing environments were estimated using repeated measures ANOVAs. A Greenhouse-Geisser epsilon correction factor was applied to p values when the assumption of sphericity that repeated measures assume was violated. A 2x3 contingency table was used to assess differences in nursing participation between treatments (the number of workers that were seen nursing at least once between the ages
of 6 and 22 compared to the number of workers that were never seen nursing during this period), pooling across source colony replicates. When we tracked individual bees to look for differences in nursing – referred to as focal following – a two-way ANOVA was used to examine the effects of source colony and treatment on mean time spent inspecting or mouthing. To determine the effects of age and treatment on acini diameter in hypopharyngeal glands, a two-way ANOVA was used. Where ANOVA tests showed significant differences among treatments, source colonies, or their interaction, a Tukey-Kramer multiple comparisons test was used to compare means and determine where these differences were found. All tests were performed using SAS version 9.3 (SAS Institute Inc., Cary, North Carolina, U.S.A.), except for 2x3 contingency tables which were conducted with an online calculator (physics.csbsju.edu/stats/contingency. Html)
Results

Introducing focal workers

A total of 1,668 workers were successfully introduced into the observation hives of the 1,973 workers who were originally placed in the hives (n = 611 out of 702 or 87% of workers in unstressed and unconfined colonies; n = 528 out of 616 or 86% of workers in unstressed and confined colonies; n = 529 out of 655 or 80% of workers in stressed and confined colonies). A range of 175–275 workers from each source colony were introduced into their respective observation hive (mean 238 workers per source colony).

Emergence weights of focal workers

The weights of accepted workers at adult emergence were marginally higher for individuals reared in unstressed and unconfined colonies, but this difference was not significant compared to the other two treatments (Figure 2; two-way ANOVA, treatment effect: F = 1.4, df = 2, 1643, p = 0.25). While there were differences among source colonies in the weights of workers at emergence (data not shown; source effect: F = 2.5, df = 6, 1643, p = 0.02), the nature of these differences was not influenced by the stress conditions under which workers were reared (treatment x source interaction: F = 1.5, df = 12, 1643, p = 0.10).
Survivorship of focal workers

Mean survivorship of workers accepted into the observation hives differed significantly among treatments (Figure 3; based on a Kaplan-Meier log-rank test of survival function among treatments, p < 0.0001). Survival over time was highest for workers reared under the condition of abundant pollen supply and confinement, intermediate for workers reared with little available pollen, and lowest for workers reared in a pollen unlimited environment and access to environment pollen (Figure 3; Šidák adjustment; all comparisons: p < 0.01).

Assessing the nursing behavior of focal workers

Nursing acts per day per capita (whole colony scans). When the number of nursing acts performed per day per capita was considered for all tagged workers (combining inspecting and mouthing acts), no difference in nursing was found based on access to pollen during brood rearing or confinement to the colony to prevent further pollen collection (Figure 4A; treatment effect: F = 0.32, df = 2, 18, p = 0.73). A lack of difference between treatments persisted over time (age effect: F = 1.44, df = 16, 288, p = 0.20; treatment x age interaction: F = 1.08, df = 16, 288, p = 0.39). Even when the number of nursing acts performed per day per capita was considered for only workers that had been seen nursing at least once between the ages of 6 and 22 days (as opposed to all workers; above), there was still no difference in nursing behavior among the treatments (Figure 4B; treatment effect: F = 0.04, df = 2, 18, p = 0.97). The frequency of nursing changed as nursing workers aged. (age effect: F = 2.15, df = 16, 288, p = 0.045), but this was not affected by the conditions under which nurses were reared (treatment x age interaction: F = 1.12, df = 16, 266, p = 0.32). Nursing workers maintained consistently higher
levels of nursing early on in their lives, with peaks across treatments around ages 7 and 15, but then nursing acts declined from that point onward (Figure 4B).

There was a significant difference between treatments in the number of workers that did (and, conversely, did not) participate in nursing at some point between 6 to 22 days of age (2x3 contingency table: $X^2 = 17.2$, df = 2, $p < 0.0001$). The proportion of the accepted workers that were observed nursing at least once during their lifetime was 55% and 43% of workers reared with lots of pollen while their colony was confined or unconfined (the control treatments), respectively, and 50% for workers reared under pollen stress and confined conditions.

*Number of nursing acts per minute per capita (brood area scans).* There was no difference between treatments or over time in the number of nursing acts (inspecting larvae and/or mouthing sealed brood) that were observed per worker during a one-minute period when a 10x10 cm$^2$ area of brood comb was monitored for nursing workers (Figure 5; treatment effect: $F = 0.26$, df = 2, 18, $p = 0.77$; age effect: $F = 0.74$, df = 15, 270, $p = 0.60$; treatment x age effect: $F = 0.74$, df = 30, 270, $p = 0.69$). Records of nursing from this method of assessing behavior were relatively consistent over time.

*Following focal workers.* Although workers reared under conditions of pollen stress tended to have the lowest mean rate of inspection activity when they were followed throughout the colony for a 5-minute period, the difference between treatments was not significant (Figure 6A; treatment effect: $F = 1.1$, df = 2, 517, $p = 0.34$). Rates if inspection activity differed for workers depending on the source colony from which they were derived (source effect: $F = 3.9$, df = 2, 517, $p = 0.0007$), but this difference was not affected by pollen treatment (data not shown; treatment x source interaction: $F = 1.7$, df = 12, 517, $p = 0.07$).
During the 5-minute period when focal individuals were followed, the time spent “mouthing” sealed brood, a behavior performed by nurse-aged workers, was affected by the conditions of pollen availability experienced by workers when they were reared as larvae (Figure 6B; treatment effect: $F = 7.5$, df = 2, 517, $p = 0.0006$), but not by the source of the workers (source effect: $F = 1.4$, df = 6, 517, $p = 0.22$; treatment x source interaction: $F = 1.2$, df = 12, 517, $p = 0.25$). Workers reared in unstressed and confined colonies spent the most time as adults tending to brood in this way. Mouthing time was lowest for workers reared in pollen stressed colonies and slightly higher (but not significantly so) for workers reared without pollen stress and in colonies that were not confined during the rearing period.

**Hypopharyngeal gland development**

The interaction between treatment and age on acini diameter differed significantly (Fig 7; ANOVA; interaction effect: $F = 1.953$, df = 10, $p = 0.0385$; treatment effect: $F = 33.204$, df = 2, $p < 0.0001$; age effect: $F = 18.275$, df = 5, $p < 0.0001$). Acini size in both unstressed control treatments tended to decrease significantly toward the end of the sample period, whereas acini size in the pollen stressed treatment stayed uniformly low when acini size was examined for each treatment over time (Figure 7). For bees reared in unstressed and unconfined control colonies, their hypopharyngeal glands were larger early on in the workers’ lives, when they were 5 to 14 days of age, compared to the last day of sampling, when the workers were 20 days of age. Change in worker acini size in the other control group, where bees were reared in unstressed and confined colonies, mirrored the unconfined control group. Acini size was large early in the workers’ lives (5 to 14 days of age), then dropped significantly in size between 14 and 17 days of
age, and then stayed low after that point (20 days of age). In contrast, workers reared in pollen stressed and confined colonies were generally low and uniform, except for a slight but insignificant increase in acini size at 8 days of age. When individual sample days were compared across the three treatments, acini size differed between treatments on three of six sample days (Figure 7). When workers were 5, 11, and 14 days of age, acini size was significantly larger for workers reared in unstressed and confined colonies compared to workers reared in pollen-stressed and confined colonies. Acini size was intermediate for workers reared in unstressed and unconfined colonies on these same days.

Because of the number of means comparisons that were made for this analysis, the threshold for significance was considered at both $p = 0.05$ and $p = 0.01$ for comparisons of means where effects were significant. The functional significance of the results did not change under either scenario, so statistical differences were reported for the more conventional $p = 0.05$. 
Figure 2. Mean weight (±SE) of newly emerged worker bees reared under pollen stress (stressed, confined) or control conditions (unstressed and unconfined, unstressed and confined). After emerging from sealed brood cells, workers were weighed before they were tagged and introduced into host observation hives. Mean weights across treatment and source colony were compared in a two-way ANOVA (see table 1).

Table 1. Comparison of mean weights of newly emerged workers across treatment and source colony in a two-way ANOVA (see figure 2).

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type III SS</th>
<th>Mean Square</th>
<th>F Value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>2</td>
<td>0.01201928</td>
<td>0.00600964</td>
<td>1.39</td>
<td>0.2488</td>
</tr>
<tr>
<td>Repetition</td>
<td>6</td>
<td>0.06492271</td>
<td>0.01082045</td>
<td>2.51</td>
<td>0.0203</td>
</tr>
<tr>
<td>Treatment*repetition</td>
<td>12</td>
<td>0.07973630</td>
<td>0.00664469</td>
<td>1.54</td>
<td>0.1034</td>
</tr>
<tr>
<td>Error</td>
<td>1643</td>
<td>7.09273082</td>
<td>0.00431694</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Workers were reared under treatments of pollen stress (stressed, confined) or control conditions (unstressed, unconfined and unstressed, confined). After emerging from sealed brood cells, workers were weighed before being tagged and introduced into host observation hives.
Figure 3. Mean survival (± SE) of cohorts of workers over time for workers reared in pollen stressed or control conditions. A survival curve was determined for cohorts of workers from each source colony per treatment (n=7 colonies per treatment). Means presented here are the average of those 7 survival curves per treatment. Actual data are shown for average survival of cohorts over time, not the survival function as calculated by the Kaplan-Meier estimator. Treatments not sharing the same letter differed significantly in survival function.
Figure 4. Mean number of nursing acts (± SE), inspecting cells and mouthing sealed brood combined, per day per capita across worker bees reared in pollen-stress or control conditions. The nursing behavior of all focal bees was considered in the means presented (A) or the nursing behavior of only those workers who were seen performing at least one nursing act (B). Nursing was examined when focal workers were 6-22 days of age (the age range for which all workers were observed, regardless of date of introduction into the observation hive). Focal bees were monitored twice daily through a visual scan of each observation hive and noted for performing known nursing acts (mouthing or inspecting). Colony averages (across workers) were determined for each source colony per treatment, and these averages were used to calculate treatment means. Nursing acts were compared across treatments and source colonies with repeated-measures ANOVAs (see tables 2 and 3).
Table 2. Comparison of mean nursing acts per day per capita of all observed focal workers across source, treatment and age in a repeated-measures ANOVA (see figure 4A).

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
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<th>Mean Square</th>
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<th>P value</th>
<th>Adjusted P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
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<td>0.01569600</td>
<td>0.00784800</td>
<td>0.32</td>
<td>0.7302</td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>18</td>
<td>0.44150821</td>
<td>0.02452823</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>16</td>
<td>0.06531119</td>
<td>0.00408195</td>
<td>1.44</td>
<td>0.1210</td>
<td>0.2024</td>
</tr>
<tr>
<td>Age*treatment</td>
<td>32</td>
<td>0.09742620</td>
<td>0.00304457</td>
<td>1.08</td>
<td>0.3631</td>
<td>0.3870</td>
</tr>
<tr>
<td>Error(trial)</td>
<td>288</td>
<td>0.81493849</td>
<td>0.00282965</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Workers were reared in treatments of pollen stress (pollen stressed, confined) or control conditions (unstressed confined, unstressed unconfined) and then monitored for nursing acts (mouthing and inspecting) twice-daily between six and twenty-two days of age.

Table 3. Comparison of mean number nursing acts per day per capita of focal workers seen nursing at least once across source, treatment and age in a repeated-measures ANOVA (see figure 4B).

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
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<th>Mean Square</th>
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<th>P value</th>
<th>Adjusted P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>2</td>
<td>0.00176578</td>
<td>0.00088289</td>
<td>0.04</td>
<td>0.9657</td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>18</td>
<td>0.45388920</td>
<td>0.02521607</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trial</td>
<td>16</td>
<td>0.25656560</td>
<td>0.01603535</td>
<td>2.15</td>
<td>0.0068</td>
<td>0.0456</td>
</tr>
<tr>
<td>Trial*treatment</td>
<td>32</td>
<td>0.26793801</td>
<td>0.00837306</td>
<td>1.12</td>
<td>0.3027</td>
<td>0.3457</td>
</tr>
<tr>
<td>Error(trial)</td>
<td>288</td>
<td>2.14715935</td>
<td>0.00745541</td>
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<td></td>
</tr>
</tbody>
</table>

Workers were reared in treatments of pollen stress (pollen stressed, confined) or control conditions (unstressed confined, unstressed unconfined) and then monitored for nursing acts (mouthing and inspecting) twice-daily between six and twenty-two days of age.
Figure 5. Mean number of nursing acts (inspecting and mouthing combined) (± SE) per bee per minute. These data are based on monitoring a 10x10 cm² area of brood for 5-minute periods. Mean activity per worker per minute was determined for each source colony; all bees were included in the analysis, not just those who were seen nursing at some point when brood areas were monitored. Source colony means were determined (across workers from each source colony) and these source colony means were used as the replicates for each treatment. Means were compared with a repeated-measures ANOVA.

Table 4. Comparison of mean number of nursing acts (inspecting and mouthing combined) per bee per minute across source and treatment in a repeated-measures ANOVA (see figure 5).

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type III SS</th>
<th>Mean Square</th>
<th>F Value</th>
<th>P value</th>
<th>Adjusted P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>2</td>
<td>0.00002347</td>
<td>0.00001174</td>
<td>0.26</td>
<td>0.7702</td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>18</td>
<td>0.00079729</td>
<td>0.00004429</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trial</td>
<td>15</td>
<td>0.00006602</td>
<td>0.00000440</td>
<td>0.74</td>
<td>0.7428</td>
<td>0.5987</td>
</tr>
<tr>
<td>Trial*treatment</td>
<td>30</td>
<td>0.00013182</td>
<td>0.00000439</td>
<td>0.74</td>
<td>0.8396</td>
<td>0.6896</td>
</tr>
<tr>
<td>Error(trial)</td>
<td>270</td>
<td>0.00160584</td>
<td>0.00000595</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Focal bees were monitored in a 10x10 cm² area of brood for 5-minute periods and mean activity was determined for each source colony. Source colony means were determined (across workers from each source colony) and these source colony means were used as the replicates for each treatment.
Figure 6. Mean amount of time (+/-SE) workers were observed inspecting brood cells (A) or mouthing sealed brood (B) for workers reared under different conditions of pollen availability. Nursing activity was determined by observing randomly selected workers for 2 minutes and recording observed nursing behaviors during that period. Workers were between 7 and 22 days of age at observation (data pooled). Both activities were analyzed with two-way ANOVAs (treatment and source colony effects) (see tables 5 and 6). If a worker was selected randomly for observation more than one time, her nursing activity was averaged over all observation periods and a single data value was included in the dataset prior to analysis (to avoid pseudo-replication).

Table 5. Comparison of mean inspecting acts across treatment and source colony in a two-way ANOVA (see figure 6A).

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
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<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>2</td>
<td>50.2725317</td>
<td>25.1362658</td>
<td>1.07</td>
<td>0.3426</td>
</tr>
<tr>
<td>Repetition</td>
<td>6</td>
<td>553.4905769</td>
<td>92.2484295</td>
<td>3.94</td>
<td>0.0007</td>
</tr>
<tr>
<td>Treatment*repetition</td>
<td>12</td>
<td>470.7822385</td>
<td>39.2318532</td>
<td>1.68</td>
<td>0.0689</td>
</tr>
<tr>
<td>Error</td>
<td>497</td>
<td>11638.54991</td>
<td>23.41761</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Workers were reared under conditions of limited (pollen stressed, confine) or abundant pollen supply (unstressed, confined and unstressed unconfined), randomly selected, and then monitored for inspecting acts during at 2-minute period between 7 and 22 days of age.

Table 6. Comparison of mean mouthing acts across treatment and source colony in a two-way ANOVA (see figure 6B).

<table>
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<tr>
<th>Source</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
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<td>11679.61350</td>
<td>5839.80675</td>
<td>7.48</td>
<td>0.0006</td>
</tr>
<tr>
<td>Repetition</td>
<td>6</td>
<td>6428.03120</td>
<td>1071.33853</td>
<td>1.37</td>
<td>0.2241</td>
</tr>
<tr>
<td>Treatment*repetition</td>
<td>12</td>
<td>11639.98757</td>
<td>969.99896</td>
<td>1.24</td>
<td>0.2508</td>
</tr>
<tr>
<td>Error</td>
<td>497</td>
<td>388153.8529</td>
<td>780.9937</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Workers were reared under conditions of limited (pollen stressed, confine) or abundant pollen supply (unstressed, confined and unstressed unconfined), randomly selected, and then monitored for mouthing acts during at 2-minute period between 7 and 22 days of age.
Figure 7. Acini size of hypopharyngeal glands (mean +/- SE) across different ages of adult honey bees reared under pollen stress or control conditions. Honey bees were raised under three treatments: unstressed/unconfined, unstressed/confined, or pollen-stressed/confined, painted, and introduced into an outdoor colony as one day-old adults. Every three days, over the course of 20 days, 8-15 bees from each treatment were collected, dissected, and measured for acini diameter of their hypopharyngeal glands (three acini per gland, with an average sized determined per sampled bee). Interactions between age and treatment were compared in a two-way ANOVA (see table 7); means not sharing the same letter are statistically different.

Table 7. Comparison of mean acini size of hypopharyngeal glands across age, treatment and source in a two-way ANOVA (see figure 7).

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
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</tr>
</thead>
<tbody>
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<td>Treatment</td>
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<td>0.03254959</td>
<td>0.01627479</td>
<td>19.72</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Age</td>
<td>5</td>
<td>0.08375704</td>
<td>0.01675141</td>
<td>20.30</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Treatment*age</td>
<td>10</td>
<td>0.01611491</td>
<td>0.00161149</td>
<td>1.95</td>
<td>0.0385</td>
</tr>
<tr>
<td>Error</td>
<td>280</td>
<td>0.23109400</td>
<td>0.00082534</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Honey bees were raised under three treatments: unstressed/unconfined, unstressed/confined, or pollen-stressed/confined, painted, and introduced into an outdoor colony as one day-old adults. Every three days, over the course of 20 days, 8-15 bees from each treatment were collected, dissected, and measured for acini diameter of their hypopharyngeal glands (three acini per gland, with an average sized determined per sampled bee).
**Discussion**

The effect of nutritional stress on honey bees is an increasingly relevant area of study with declines in honey bee health and productivity in recent years (van Engelsdorp 2010). Concerns about poor nutrition are owed in part to factors such as erratic weather (such as harsh winters or dry seasons), loss of quality foraging habitat, and stressful management practices, all of which are challenging on their own, but can also coalesce to degrade foraging opportunities and increase nutritional stress in colonies (Ricketts et al., 2008; Winfree et al., 2008; Potts et al., 2010; Karahan et al., 2015; Pacifico da Silva et al., 2015). Many assessments of the effects of poor nutrition involve measures of colony growth or worker longevity (Maurizio and Hodges 1950; Amdam and Ohmolt, 2002, 2003; Manning et al., 2007; Sagili and Pankiw 2007). Far less is known about the effects of nutritional stress on adult worker behavior, both for short-term stress (such as stress during larval development) and for cross-generational, chronic stress (such as stress over both developmental and adult periods that can last more than one generation).

Understanding the impact on colonies of nutritional stress is especially critical given their eusocial nature, given that colony growth and maintenance relies on group behaviors that may be compromised if workers underperform certain tasks (Smart et al. 2016). A past experiment in our lab demonstrated that workers reared under conditions of pollen stress grew up to be precocious foragers, foraging both earlier in their lives and for less time (Scofield and Mattila 2015). This outcome raised important questions about the performance of other tasks by these stressed workers. We hypothesized that precocious foraging could be caused by accelerated temporal polyethism, where the timeline of the stages and roles that adult honey bees pass through as they age may be compressed by poor access to food during development. To test the effect of larval
pollen stress on adult nursing behavior, we manipulated pollen availability among brood-rearing subunits of source colonies, providing them with either an abundant or limited pollen supply, and then monitored nursing performance, an important pre-foraging behavior, by adult workers that were raised under such conditions.

By manipulating pollen availability during the larval rearing period, we were able to compare the physiology and behavior of workers reared with access to adequate or limited pollen-derived nutrients. While physiological markers of an effect on nursing from food stress were evident, the effects of this stress on nursing behavior were not as clear. Hypopharyngeal glands, which secrete brood food for feeding the larvae that adult nurses tend, stayed more uniformly low over time for bees reared under pollen stress, whereas they were often larger for nurse-aged workers in both controls. Specifically, acini were larger early in workers’ lives if they were reared with abundant pollen (at 5 to 14 days of age), and did not become significantly reduced in size until after 14 days of age, staying low after that point. In contrast, workers that were pollen-stressed as larvae only showed acini growth at 8 days of age, which after they returned to their previously smaller acini size.

In contrast, the differences in the behavior of similarly aged workers in the observation hives were not as clear between treatments. Pollen-stressed workers showed intermediate survivorship compared to the survivorship of the cohorts of workers from the two control groups. When monitoring each treatment for number and type of nursing acts performed, participation in nursing was also intermediate for the pollen-stressed workers compared to the controls. Scanning the observation hives to record incidences of nursing among the focal bees, either the entire colony or with more focused scans of smaller brood areas, did not reveal any differences between treatments in nursing behaviors (inspecting larvae or mouthing sealed brood), regardless
of whether all bees or only those seen nursing at some point in their lives were considered.  
Because these colony and brood area scans yielded no significant effects of treatment, it is  
difficult to know whether there are no real differences between treatments or if scans were not an  
effective means of recording potential differences in nursing behavior by workers.  When focal  
individuals were followed for two minutes to record incidences of nursing, the results were more  
in line with the hypopharyngeal gland data that were obtained.  The mean inspection rate of focal  
workers was lowest for pollen-stressed workers (although this trend was not significant) and  
these workers also mouthed sealed brood at a significantly lower rate than workers that were  
reared with abundant pollen during confinement (although mouthing by stressed workers was  
statistically similar to the other control, where workers were reared with abundant pollen and no  
confinement).

**Impact of larval pollen stress on adult nursing behavior**

Honey bee workers reared under conditions of pollen-stress had hypopharyngeal glands  
that were smaller than their unstressed cohorts’ at certain ages, particularly those following the  
anticipated “mid-point” in a worker’s nursing career, and followed a significantly different  
timeline of growth. With the function of secreting nutritionally dense brood food,  
hypopharyngeal glands are largest in nurse-aged workers that are 6-12 days old during the period  
in which workers perform the most nursing (Painter and Biese 1966; Seeley 1982). Workers  
reared under conditions of abundant pollen supply conformed to this timeline, from which  
inferences can be made about the temporal polyethism timeline based on the physiological  
development the gland acini (Kubo et al., 1996; Ohashi et al., 1997, 1999). With the ability of
workers to nurse so incredibly valuable to brood production and subsequent colony growth, underperforming hypopharyngeal glands could result in a poor ability to rear brood, which would perpetuate the rearing of pollen-stressed honey bees and potentially lead to declining colony population over the long term.

Relating these physiological measures, which were made in a separate colony from the observation hives where behavioral measures were made, is not as easy to reconcile. Inspecting, one of the two nursing acts that we observed, most directly relies on hypopharyngeal gland use as the nurses duck their heads into uncapped cells to feed young larvae with the secretions that these glands produce. Workers reared under conditions of pollen stress, which showed the aforementioned smaller sizes of hypopharyngeal gland acini toward the end of the typical nursing period, also spent marginally less mean time inspecting when focal individuals were followed for two minutes (although this comparison was not statistically significant). It is possible that, although nurse bees across treatments and ages performed the same amount of nursing in our nursing scans, honey bees that were pollen stressed as larvae could be doing so less efficiently by engaging in fewer nurse-related tasks during their nursing timeline or transferring smaller amounts of food to larvae (something that we did not measure). It is possible that the marginal difference we observed between stressed and unstressed workers as focal individuals were followed (outside of the behavioral scans) could be strengthened with a larger sample size (with the goal of verifying this hypothesis).

In contrast to inspecting, the function of mouthing sealed brood is less well understood, but has traditionally been associated with the suite of behaviors of nurse-aged bees. Time spent on this nursing-related behavior was lowest in honey bee workers reared under pollen stress than those workers raised by unstressed and confined workers, which was one of the control
treatments (but similar to workers reared under unconfined control conditions). Along with intermediate participation in nursing activities by pollen-stressed workers (i.e., lower participation in nursing by workers from confined controls, but higher than workers from the unconfined controls) and the lower (but statistically insignificant) mean time spent inspecting, these results suggest the possibility of a lower efficiency of nursing behaviors in pollen-stressed bees. Although little is known on the exact function of mouthing, it may play an important role in facilitating larvae growth and emergence, as workers use mouthing to make the wax surrounding capped brood conform to a specific shape and perhaps make it more readily penetrable when larvae are ready to emerge (Kolmes 1985). It may also be similar to the cleaning behavior ants perform to keep cocooned pupae clean, which are not held in cells like honey bee larvae, but rather in curated pupae piles on the floor of ant nursery chambers (Tragust et al., 2013). Since this behavior seems to be related to the cleanliness and maintenance of the brood area and its important young members, fewer mouthing acts as a result of being pollen-stressed could mean that underperforming nurses might adversely affect brood production and limit colony growth, but more research would have to be done to make this connection.

**Future experimentation**

Some of our results did not conform to our hypothesis that nursing measures would be suppressed or reduced by poor nutrition during larval development. For instance, data collected on survivorship and newly emerged bee weights do not align with trends observed in past experiments (Scofield and Mattila 2015). Survivorship in the present study was lowest for well-supplied colonies that were able to continue foraging for pollen focal workers were being reared,
instead having the lowest survivorship for workers reared in pollen-stressed colony subunits, as observed by Scofield and Mattila (2015). This difference suggests that our natural colony controls may have been experiencing a baseline level of pollen stress that we did not anticipate, perhaps making them an incomparable “well-supplied” control. In a previous lab study that used the same rearing conditions (Scofield and Mattila 2015), honey bees that were reared under conditions of pollen stress had low weights and decreased survivorship that were in the range of the current study. However, the fresh weights of emerging focal adults in control treatments in this study were much lower than the weights of the control workers from our previous work, in which mean survivorship was highest for the two similar control treatments and lowest for those workers who were reared in pollen-stressed colonies (Scofield and Mattila 2015). These results suggest that workers reared in our control colonies, and particularly those reared in unconfined controls with low survivorship, may have been experiencing external nutritional stressors that were not present in our previous work where the differences between stressed and unstressed treatments were clearer.

One of our working hypotheses to explain this difference between years (the summers of 2012 and 2013 reported by Scofield Mattila [2015] versus the present work, which was conducted during the summer of 2015) is related to the general perception that honey bee colonies across the region suffered from the long-term effects of a prolonged winter in Massachusetts in early 2015, when record snowfalls occurred. If colonies were still recovering from the general nutritional stress caused by the long, snowy spring before our summer fieldwork began, it could mean that all colonies in general were exhibiting the results of long-term pollen-stress, even under “control” conditions. This would explain why all treatments had substantially lower weights than workers reared by similar methods in previous years (Scofield
and Mattila 2015). Based on the differences we observed here between control treatments, it is possible that honey bees residing in a natural setting (e.g., unstressed and unconfined) were more adversely affected by long-term effects of pollen stress and therefore reared more nutritionally stressed brood than was expected. With the understanding that pollen stress results in precocious foraging (Scofield and Mattila 2015), it could be that bees in our natural colony were not foraging as effectively as in previous years compared to our confined controls, who were given ample supplies of pollen and did not need to expend energy to forage during the treatment rearing period. In the future, we plan to collect more data during the upcoming 2016 summer field season, which will allow us to increase our sample size, focus on the nursing estimates that these pilot studies showed to be most promising, and collect additional data that may be useful, such as the number of pollen stores in unstressed and unconfined colonies at the end of the rearing period. These data will allow us to better understand whether honey bees in “control” colonies were actually experiencing conditions of pollen stress, or whether another compounding factor, such as long-term environmental pollen stress, resulted in the development of underweight and poorly performing bees.

Finally, we also hope in our studies going forward to increase our sample size for promising measures of nursing behavior, such as focal following, which yielded cleaner data on the frequency or rate of performance of individual nursing behaviors than did whole-colony scans or scans of smaller brood areas. This path would allow us to spend less time on behavioral estimates that were harder to interpret, such as the nursing scans, which showed a lot of noise between treatments and over time. Increasing our sample size would also mean collecting more data on hypopharyngeal gland development over time, which would provide clearer insight into the physiological effects of developmental pollen stress on honey bees.
Broader impacts

Honey bees are invaluable pollinators and provide a critical ecological and economical service to our global society (Chopra et al., 2015; Potts et al., 2010; Genersch 2010). In their natural environment, honey bee larvae may be routinely exposed to the same level of pollen stress experienced by our focal workers. Aforementioned stressors, such as inclement weather that damage pollen-yielding plants or stressful management practices that place bees in very competitive monocultures with limited nutrients, are real world practices that may induce some of the same trends that were observed in this study. Other non-environmental factors like pesticides and pathogens may exacerbate the effects of these trends and have reiterated long-term negative effects on colonies (Alaux et al., 2010). Broadly, nutritionally stressed bees tend to have a weakened immune system and be more susceptible to pathogens, parasites and pesticides (Alaux et al., 2010, Wahl and Ulm, 1983). Additionally, these non-environmental stressors may affect developmental physiology (Robinson and Vargo 1997). One mechanism by which hypopharyngeal glands expand and retract is through hormone signaling. Juvenile hormone (JH) functions as a developmental signaling mechanism in honey bees, driving the sequences of behavior that are observed as part of temporal polyethism. They are at lower levels while bees are nursing and at higher levels when they start foraging (Rutz et al. 1976; Fluri et al. 1982; Robinson 1987, 1989; Huang et al. 1994; Huang and Robinson 1995). Pathogens and parasites can increase the expression of JH and could, along with pollen stress, diminish nursing efficiency through underdeveloped or prematurely shrinking hypopharyngeal glands (Robinson and Vargo 1997). The microsporidial parasite *Nosema ceranae* is a common and widespread pathogen that can trigger premature foraging and shorten the lifespan of infected workers (Goblirsch et al.
Based on our above hypothesis, which suggests a compressed temporal polyethism timeline for many of the physiological and behavioral measures that we monitored, exposure to additional stressors such as parasites could further decrease foraging activity under the same mechanisms that poor nutrition is expected to operate. Nursing efficiency may also be similarly affected by exposure to parasites such as *Nosema ceranae*, which infect the midgut of workers and make it difficult to digest the nutrients that they have consumed (Wojcik et al., 2014). Understanding how the effects of stressors that impair nutrient absorption synergistically exacerbate the effects of pollen stress is an important relationship to explore, and why natural colony settings, where workers forage for and distribute food socially, are so useful to study.

**Conclusion**

Honey bees play an imperative ecological and economical role in our world, pollinating an estimated 50% of our global cultivated food supply and providing a service valued at approximately 12 billion dollars (USD) while doing so (Klein et al., 2007, Calderone 2012). With populations of honey bees declining, it has become even more important that we understand how naturally occurring nutritional stress affects honey bee behavior and general hive health. Our study indicated that workers that were raised under conditions of pollen-stress grew up to be nurses with diminished hypopharyngeal glands and some compromised elements of their nursing performance, which may in turn adversely affect their efficacy as nurses and ability to support colony growth.


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