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Quantifying the effects of pollen nutrition on honey bee queen egg laying with a new laboratory system

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Abstract

Honey bee populations have been declining precipitously over the past decade, and multiple causative factors have been identified. Recent research indicates that these frequently co-occurring stressors interact, often in unpredictable ways, therefore it has become important to develop robust methods to assess their effects both in isolation and in combination. Most such efforts focus on honey bee workers, but the state of a colony also depends on the health and productivity of its queen. However, it is much more difficult to quantify the performance of queens relative to workers in the field, and there are no laboratory assays for queen performance. Here, we present a new system to monitor honey bee queen egg laying under laboratory conditions and report the results of experiments showing the effects of pollen nutrition on egg laying. These findings suggest that queen egg laying and worker physiology can be manipulated in this system through pollen nutrition, which is consistent with findings from field colonies. The results generated using this controlled, laboratory-based system suggest that worker physiology controls queen egg laying behavior. Additionally, the quantitative data generated in these experiments highlight the utility of the system for further use as a risk assessment tool.

Introduction

Managed honey bee pollinators contribute an estimated 15 billion dollars yearly to the United States economy [1], and they have become crucial to ensuring food security for a growing population world wide [1–3]. However, declines in populations of pollinators, including honey

specific roles of this author are articulated in the "author contributions" section.

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bees, have caused concern, and researchers have now identified four key factors that negatively impact honey bee health: poor nutrition, exposure to pesticides, pathogens, and parasites [4–6]. Importantly, these stressors can interact in unpredictable ways [5]. The effects of these frequently co-occurring stressors highlight the need for robust methods to assess risks to honey bee health so that they can be mitigated.

To study the effects of individual and interacting stressors on the complex biological processes that occur within a honey bee colony, many researchers conduct field experiments with full size colonies or experimental colonies that have reduced populations or demographies [7–11]. These experiments produce environmentally relevant data pertaining to colony-level effects, but their designs are often time- and resource-intensive, with challenges in controlling for variables such as the effects of agrochemical residues persisting in wax comb [12] and in the surrounding foraging landscapes [13], the sources of nutrition available to the colony [14,15], bee genetic variation [16,17], exposure to new or worsening pathogen infections [11], and queen failure events [4]. Laboratory-based assays generally afford researchers more control over experimental parameters, but there is currently no laboratory-based method to screen for effects of stressors on queen egg laying.

Following successful insemination, the queen is the sole producer of the fertilized eggs necessary for maintaining the colony population [18]. Therefore, the queen's health and productivity are critical to colony longevity [19]. Recently, high rates of queen failure and supercedure have been documented throughout the United States, and beekeepers have reported queen failure as a major cause of colony loss [20,21]. Several studies have indicated that queen failure could be due to agrochemical exposure [22,23] or stressful conditions during queen shipment affecting sperm viability in mated queens [24]. These observations highlight the need for controlled methods to study the effects of stressors on queen egg laying. However, the queen's unique life history poses considerable challenges to researchers seeking to dissect the effects of stressors on queen fecundity from other colony level effects.

The honey bee queen relies on constant care and feeding by young worker bees [18]. This behavior, which is referred to as retinue behavior [25], is elicited as a response to a semiochemical blend produced by the queen known as queen pheromone [26,27]. Sustained queen egg laying is not known to occur in the absence of honey bee workers, therefore, egg-laying is the product of the coordinated efforts of both the queen and the workers in the colony. The relatively small number of quantitative studies of queen egg laying behaviors have been performed in full-sized or reduced population colonies, and researchers either cage the queen to restrict her egg laying [28,29] or use glass-walled observation hives to perform daily egg counts and assessments [30–32]. These mostly field-based studies have yielded valuable insights into the queen's biology, life history, and the effects of stressors, but new laboratory-based methods that facilitate a higher degree of experimental control would speed the progress of queen health research. Additionally, federal regulators rely largely on laboratory-based tests to inform policy related to agrochemicals and honey bees [33], and the development of a laboratory-based screening system for effects of stressors on queens would help inform guidelines to protect pollinators.

Here, we describe a new laboratory-based system to quantitatively assess egg laying and use the system to examine the effects of pollen nutrition on queen fecundity. Within a colony, young adult nurse worker bees consume hive stored pollen, aka "bee bread," to develop their hypopharyngeal glands (HPGs) [34]. These glands produce the proteinaceous secretions that nurse bees use to provision members of the hive including developing larvae and the queen [25,35,36]. Bee bread is made by mixing pollen, honey, and honey bee salivary secretions that contain bacteria commonly found in the honey bee digestive track [37–39]. Cage studies have shown that while honey bees can survive and develop their HPGs and other tissues when fed artificial sources of protein, consuming bee bread results in the most developed HPGs [40,41].

The relationship between HPG development and retinue behavior is not well established, but it is known that queens are typically provisioned by bees less than 12 days old [25]. Bees in this age range typically have highly developed HPGs [42]. Egg production in queens is correlated with vitellogenin production [43], and vitellogenin may also play a role in increasing queen longevity by reducing oxidative stress [44, 45]. Vitellogenin levels in newly eclosed, unmated queens increase after feeding [43], suggesting that nutrition can influence queen vitellogenin production. Similarly, while there is no established correlation between retinue behavior and egg laying, a correlation between queen feedings and egg laying has been documented [25], and diet quality has been shown to influence reproduction in honey bee and ant colonies [10,46]. We therefore hypothesized that HPG development and egg laying in this system can be manipulated through worker pollen feeding, and that feeding caged bees bee bread will result in higher egg laying and HPG development relative to commercially sourced pollen.

Methods

Bees

Wax comb frames containing capped worker brood (pupae and older larvae) were obtained from colonies maintained according to standard commercial methods at the Bee Research Facility at the University of Illinois Urbana-Champaign, Urbana, Illinois (UIUC) during May-September 2017. They were placed in a warm room (34.5˚C) until adult eclosion. Newly eclosed worker bees were brushed off the frames and added to specially designed queen monitoring cages (QMCs; described below) by weight (10 g = approximately 100 bees). For each experiment, bees from 2–3 colonies were brushed from frames as they emerged and mixed before being added to cages to ensure a random distribution of bees from different colonies throughout the cages. Naturally mated queens of primarily Carniolan (subspecies) stock were purchased from Olivarez Honey Bees (Orland, CA). All queens used in these experiments were of the same genetic stock. Queen mortality was monitored and recorded throughout all experiments, and worker mortality was not quantitatively assessed.

Cage design

QMCs were composed of plexiglass with small holes in the walls for ventilation (Fig 1). Each QMC contained 1–2 egg laying plates (ELP) positioned vertically and serving as the inner walls of the cages. These custom made injection-molded, polystyrene plates were patterned with 264 hexagonal wells measuring 5.1 mm across and 11 mm deep, mimicking the dimensions of the cells in natural honey bee brood comb [47]. We chose to develop a system that does not require beeswax substrates because most samples of beeswax are contaminated with various agrochemical residues [12]. New ELPs were used for each experiment. Each QMC has four ports through which feeders containing pollen, sucrose solution, water, and honey can be inserted.

Three cage designs were used throughout our experiments to facilitate different experimental designs involving larger numbers of bees, and to explore how subtly different designs can facilitate the monitoring process.

Style 1. Style 1 is 8.3 cm X 2.8 cm X 12.3 cm (interior). A removable ELP is inserted into the back with a plexiglass insert behind it to block the bees from exiting the cage when the ELP is emptied or replaced. This QMC has four holes, each large enough to accommodate 2ml feeders.

Style 2. Style 2 has the same dimensions as QMC Style 1, however, in place of a plexiglass insert, a plastic adapter was placed between the interior of the cage and the ELP. The adapter is a 3-D printed (Viper SI, 3D Systems Inc., material: WaterClear Ultra 10122) outline of the 264

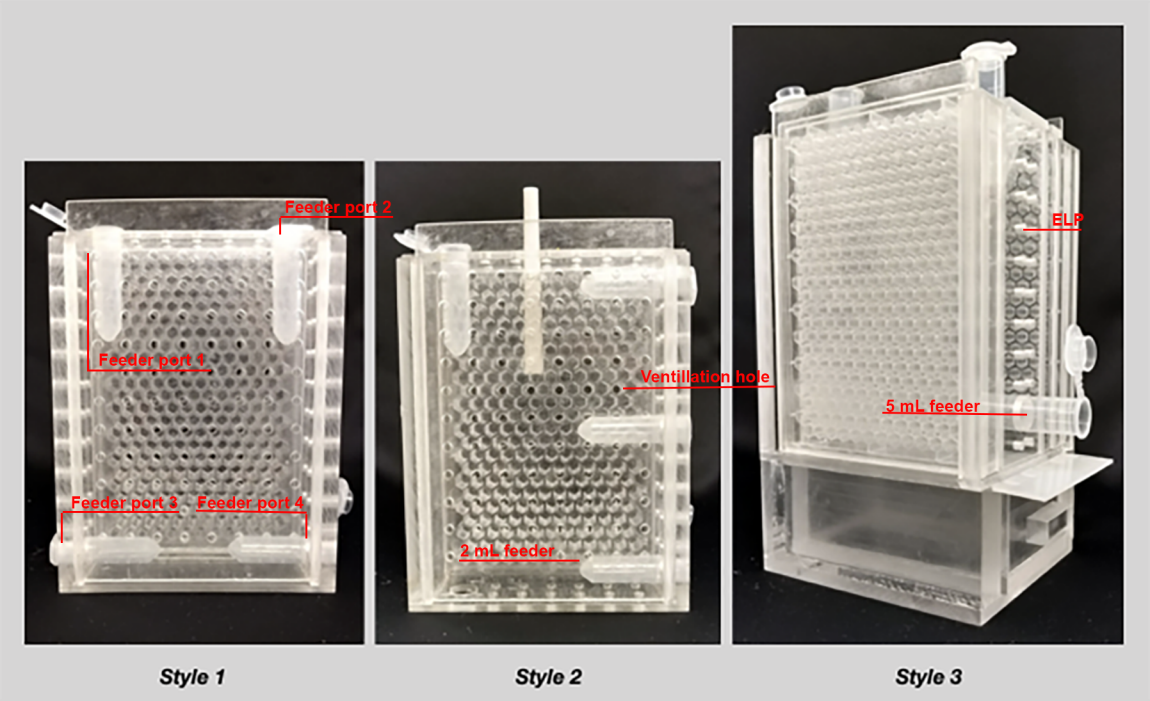

Fig 1. Queen monitoring cages (QMCs) styles 1, 2, and 3.

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cells and provides an interface between the bees and the ELP. This allows for the ELP to be easily removed without disturbing the bees. A flexible plastic film inserted between the adaptor and the ELP is used to keep the bees from exiting the cage while the ELP is emptied or replaced. These adapters tend to warp during cleaning, therefore care must be taken to ensure their continued utility.

Style 3. Style 3 measures 8.3 cm X 4.5 cm X 12.3 cm (interior) with a removable drawer for the introduction and removal of workers. This QMC has four holes large enough to accommodate 5 mL feeders and incorporates two parallel ELPs that face each other. Plexiglass inserts are used to prevent bees from exiting the cage when ELPs are emptied or replaced.

Styles 1 and 2 were used in Experiment 1, with an equal number of both styles used in each treatment, and Style 3 was used in Experiments 2 and 3. All 3 cage styles performed well in these experiments, though Style 3 allowed for more bees to be used, facilitating the sampling of adult bees throughout experiments.

Diets

Bee bread was collected from colonies by placing frames of empty wax comb in the center of the brood nest for three days. They were then removed and the bee bread was harvested from the wax comb cells. Fresh bee bread (BB) was fed to the caged bees after it was harvested without having been subjected to temperatures below 20˚C. Bee bread from the same frames was also harvested and stored in a freezer at -80˚C for at least 1 h before being thawed and fed to the caged bees (FBB) in order to test whether freezing could be implemented as a viable storage option for freshly collected bee bread. All of the bee bread in these experiments was stored in the colony for approximately 72 h based on research showing that nurse bees prefer freshly stored bee bread [40]. See S1 Supporting Text for further details on bee bread collections.

Commercial pollen was purchased from Betterbee Bee Supply (Greenwich, NY). Pollen paste diets were made using ground, commercial pollen stored at -20˚C. The diets were made less than one hour prior to use according to the following recipe:

45% pollen paste (PP-45)– 45% commercial pollen, 35% local honey, 20% sucrose solution (30% w/v).

70% pollen paste (PP-70)– 70% commercial pollen, 30% local honey.

Percentages were based on weight.

In addition to pollen diet, QMCs were supplied with feeders containing honey, water, and 30% sucrose solution, each administered in 2 mL or 5 mL feeders.

Incubator

QMCs were maintained in a Percival incubator with stable environmental conditions of 34˚±0.5˚C and 60%±10% relative humidity (RH), similar to the conditions inside a normal colony [18].

Experiment 1: Effects of fresh bee bread and 45% pollen paste on egg laying

Twenty cages were assembled on July 19–20, 2017, each containing 100 newly eclosed worker bees and a queen. Ten of these cages were provisioned with bee bread (BB), and 10 were provisioned with 45% commercial pollen paste (PP-45). Eggs were counted twice daily between 9–11:00 and again between 18–20:00. After counting, the eggs were removed from the ELPs, which were then reinserted to the QMCs. Pollen diet consumption was measured every 2 days by removing feeders and recording the change in weight. Pollen feeders were replaced every 2 days with feeders containing freshly collected or prepared diet according to treatment. Egg laying was tracked in each cage for 13 days.

Experiment 2: Effects of fresh bee bread vs. frozen bee bread vs. 45% pollen paste on egg laying

Forty-five QMCs were assembled on August 10–12, 2017, each containing 300 newly eclosed worker bees and a queen. Groups of 15 QMCs were each provisioned with either fresh bee bread (BB), frozen bee bread (FBB), or 45% commercial pollen paste (PP-45). BB and FBB were harvested from the same frames as described above. Egg laying and pollen consumption was monitored as in Experiment 1. Pollen feeders were replaced every 2 days with feeders containing freshly collected or prepared diet according to treatment, and 10 bees were removed through an empty feeder port using soft tweezers. The subsampled bees were flash-frozen in liquid nitrogen and stored at -80˚ C until they were dissected for HPG acinus measurement [48]. Egg laying was tracked in each cage for 14 days.

Bees subsampled on August 18th from 39 of the QMCs (14 BB, 13 FBB, and 12 PP-45) were selected for HPG dissection and measurement of acinus size. HPG dissections were performed by first removing the bee heads over dry ice, and the exoskeleton was removed in ethanol chilled with dry ice. The heads were then transferred to room temperature ethanol, and the glands were removed using a pair of forceps under an Olympus Szx12 stereomicroscope. Morphological measurements of the acini were performed on stored images taken with the stereo microscope as described by Hrassnigg et al. [48]. The average width (μm) of 10 acini from each bee was measured using the straight-line tool in ImageJ [49].

Experiment 3: Effects of frozen bee bread vs. 70% pollen paste on egg laying

Thirty cages were assembled on October 14, 2017, each containing 200 newly eclosed worker bees and a queen. Fifteen of the cages were provisioned with bee bread stored at -80˚C (FBB)

and the other 15 were provisioned with 70% commercial pollen paste (PP-70). Egg laying and pollen consumption was monitored as in Experiments 1 and 2. Pollen feeders were replaced every 2 days with feeders containing fresh diet. Egg laying was monitored and recorded daily for 10 days. The cages were disassembled on the 11th day due to observations of heavy mortality of worker bees. A smaller number of worker bees were added to each cage in this experiment because we did not sample worker bees.

Statistical analyses

A Student's *t*-test or one-way ANOVA with a post hoc Tukey's Honest Significant Difference test (Tukey HSD) was used to evaluate the effects of diet treatments on the total number of eggs laid in each experiment (JMP Pro 12). In Experiment 1, the cage styles were distributed equally across treatment, therefore we did not need to account for cage style in the analysis of treatment effects.

Poisson loglinear generalized estimating equations (GEE) with autoregressive (AR-1) correlation matrices (IBM SPSS Statistics 24) were used to assess the effects of pollen diet on egg laying across time, with each day and treatment group treated as factors and each queen treated as a subject effect with day as a within subject effect. We cannot confidently treat queen egg laying on a given day as independent from egg laying on an adjacent day, therefore the AR-1 correlation matrix structure was conservatively selected instead of an independent correlation matrix structure based on its appropriateness for data sets with a high number of factors that are not independent. GEE analysis accounts for within-subject variation and does not exclude subjects with incomplete datasets (as in the case of a queen death), and the GEE β-coefficients can be used to estimate the magnitude and direction of significant effects relative to a reference [50]. In our experiments, we used the treatment groups with the lowest average egg laying as the reference treatments (PP-45, PP-45, and PP-70 in Experiments 1, 2, and 3, respectively), and the reference time points were the final day of each experiment. A Wald Chi-Square post hoc test was used to determine the significance of treatments (when more than 2 were compared), each day, and each interaction term. A Bonferroni correction for multiple comparisons was applied to p-values when evaluating the effects of time (day) and interaction effects. In Experiment 2, no egg laying was observed in one or more treatments on the first 2 days of the experiment. To conform to the assumptions of the GEE analysis, these days were excluded from the GEE. In Experiment 3, no egg laying was observed in either treatment for the first 2 days of the experiment. These days were excluded from the GEE and additionally were not used to calculate average daily egg production.

To identify differences in pollen consumption between treatment groups, Wilcoxon Rank Sum tests and Kruskal Wallis tests were performed in JMP Pro 12. Correlations between time and pollen consumption were assessed using Spearman's ρ, estimated using JMP Pro 12. Significance was evaluated at a 0.05 level, and 0.1 was considered evidence of a trend. All experimental data are available in S1 File.

Results

Throughout our experiments, most queens in all treatment groups laid eggs in QMCs. See S1 Table for a summary of egg laying observed in all experiments. Variation in the onset of egg laying was observed during these experiments, with some queens beginning to lay after less than 24 hours and most laying within 72 hours. Because of this variation, an adaptation period during which the cages are not disturbed in the incubator may be considered for future experiments using this system.

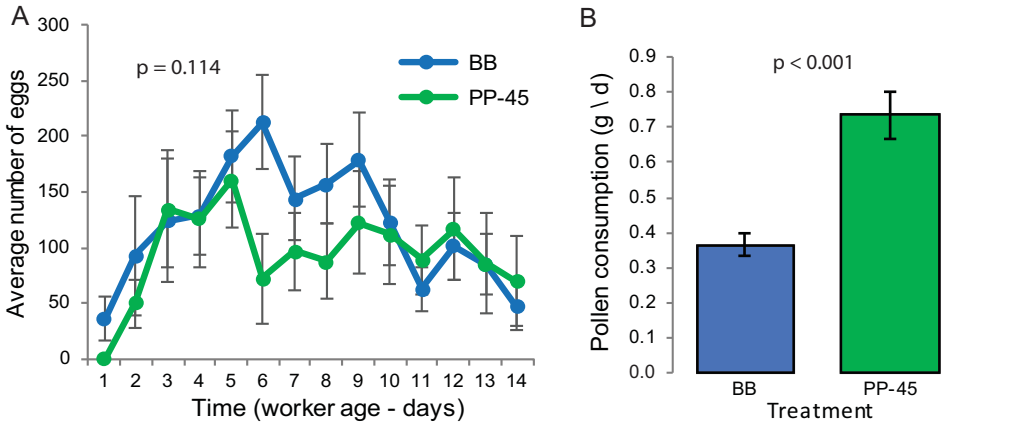

Fig 2. Effects of pollen diets on egg laying and pollen diet consumption (Experiment 1). A. Average ± SE number of eggs laid per day in QMCs provisioned with bee bread (BB, blue) or 45% pollen paste (PP-45, green). The p-value is the result of GEE analysis (p = 0.114, Wald Chi-Square = 2.3, df = 1). **B.** Average ± SE pollen diet consumed per day in BB (blue) and PP-45 (green) treatment groups. The p-value is the result of a Wilcoxon Rank Sum test (p<0.001, Chi-Square = 18.7, df = 1).

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During Experiment 1, one queen in the pollen paste treatment group died between the monitoring periods on day 6 and 7, and no data from this QMC after day 6 were used in any statistical analysis. No queen mortality was observed in Experiments 2 and 3.

Experiment 1: Effects of bee bread vs. 45% pollen paste on egg laying

There was no significant difference in total eggs laid by queens in QMCs provisioned with BB vs. PP-45 (p = 0.45, Student's t-test, t = -0.8, df = 18), and there was no significant difference in daily egg laying between QMCs provisioned with BB vs. PP-45 (p = 0.114, GEE, Wald Chi-Square = 2.3, df = 1, Fig 2A). Egg laying changed over time, with a statistically significant effect of time on egg laying (p<0.001, GEE, Wald Chi-Square = 1818.6, df = 13). Egg laying on day 1 was significantly lower than egg laying on the final day (Table 1). A significant interaction was detected between time and treatment (p<0.001, GEE, Wald Chi-Square = 280.5, df = 13). This interaction was evident on day 1 (Table 2). The positive β coefficient indicates that the increase in egg laying due to BB diet was greater on day 1 than on day 14.

Significantly greater amounts of pollen diet were consumed by bees in QMCs fed PP-45 relative to BB (p<0.001, Wilcoxon Rank Sum, Chi-Square = 18.7, df = 1, Fig 2B). A significant negative correlation was detected between time and pollen consumption (p≤0.0001, Spearman's ρ = -0.8).

Table 1. Effects of worker age on queen egg laying (GEE, Wald Chi-Square post hoc test). Directionality and magnitude can be interpreted from the β-coefficient with reference to the final time point (Day).

Experiment	Day	β-coefficient	Wald Chi-Square	p-value	Bonferroni adj. p-value
1	**1**	5.8±1.2	24.5	<0.001	<0.001
2	**10**	-1.7±0.4	14.5	<0.001	0.002
3	**3**	-4.0±0.7	34	<0.001	<0.001
	4	-1.6±0.4	16.5	<0.001	<0.001
	5	-0.6±0.2	6.3	0.012	0.084
	7	0.6±0.2	14.3	<0.001	0.001
	8	0.4±0.2	4.7	0.03	0.21
	9	0.3±0.1	5	0.03	0.182

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Table 2. Significant interaction effects of pollen diet and time on egg laying (GEE, Wald Chi-Square post hoc test). Directionality and magnitude can be interpreted from the β-coefficient with reference to PP-45 (Exp. 1 and 2) or PP-70 (Exp. 2) and the final time point (Day).

Experiment	Day	Treatments compared	β-coefficient	Wald Chi- Square	p-value	Bonerroni adj. p-value
1	1	BB vs. PP-45	-5.6±1.3	17.7	<0.001	<0.001
2	10	BB vs. PP-45	1.8±0.5	15.2	<0.001	0.002
	3	FBB vs. PP-45	-1.7±0.5	12.1	0.001	0.024
	4	FBB vs. PP-45	-1.0±0.4	6.7	0.009	0.198
	5	FBB vs. PP-45	-1.0±0.4	5.7	0.017	0.374
	6	FBB vs. PP-45	-0.8±0.3	9.6	0.002	0.044
	7	FBB vs. PP-45	-0.8±0.2	13.5	<0.001	0.005
	10	FBB vs. PP-45	1.1±0.5	5.7	0.017	0.374
3	3	FBB vs. PP-70	2.4±0.8	9.8	0.002	0.014
	4	FBB vs. PP-70	1.2±0.5	6.5	0.011	0.077
	5	FBB vs. PP-70	0.7±0.3	5.8	0.016	0.112

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Experiment 2: Effects of fresh bee bread vs. frozen bee bread vs. 45% pollen paste on egg laying

A significant effect of diet on total eggs laid by queens was observed (p = 0.01, ANOVA, F = 5.1, df = 2), and queens in PP-45 provisioned QMCs laid fewer eggs than queens in BB or FBB provisioned QMCs (Tukey HSD). A significant effect of pollen diet type on daily egg laying was observed (p = 0.001, GEE, Wald Chi-Square = 13.8, df = 2), with queens in FBB QMCs laying significantly more eggs per day than queens in PP-45 QMCs (p = 0.001, Wald Chi-Square = 11.4, df = 1, Fig 3A). A trend was observed for queens in BB QMCs to lay more eggs on a daily basis than PP-45 QMCs (p = 0.06, Wald Chi-Square = 3.7, df = 1, Fig 3A). Time significantly affected egg laying (p<0.001, GEE, Wald Chi-Square = 63.5, df = 11). Significantly lower egg laying was observed on day 10 relative to the final time point (Table 1).

A significant interaction was detected between time and treatment (p<0.001, Wald Chi-Square = 174.6, GEE, df = 22). This interaction was observed in QMCs provisioned with BB relative to PP-45 on day 10. The positive β coefficient of the interaction term indicates that the

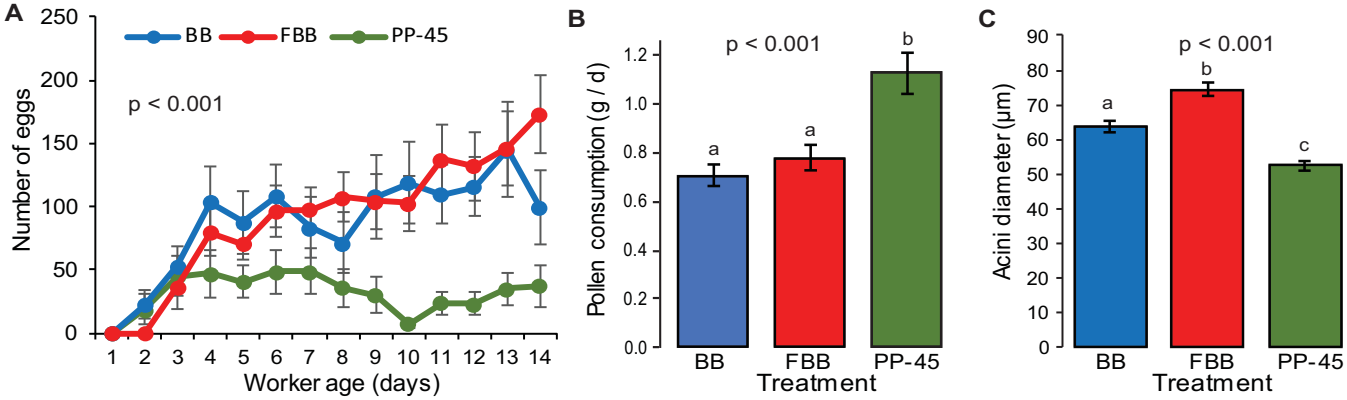

Fig 3. Effects of pollen diets on egg laying, pollen diet consumption and hypopharyngeal gland acinus diameter (Experiment 2). A. Average ± SE number of eggs laid per day in QMCs provisioned with bee bread (BB, blue); frozen bee bread (FBB, red) or with 45% pollen paste (PP-45, green). The p-value is the result of GEE analysis (p = 0.001, Wald Chi-Square = 13.8, df = 2). **B.** Average ± SE pollen diet consumed per day in BB (blue); FBB (red) and PP-45 (green) treatment groups. The p-value is the result of a Kruskal Wallis test (p = 0.0006, Chi-Square = 14.9). **C.** Average ± SE acini diameter of worker bees sampled on day 7 and 8 from QMCs provisioned with BB (blue), FBB (red), and PP-45 (green). The p-value is the result of a Kruskal Wallis test (p<0.0001, Chi-Square = 50.0).

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increase in egg laying related to BB treatment was greater on day 10 relative to the increase at the final time point. This is explained by a decrease in the number of eggs laid in PP-45 QMCs but not in BB QMCs on day 10. In QMCs provisioned with FBB, 6 time points are affected by an interaction between time and treatment, but only 3, days 3, 6, and 7 were significant after a Bonferroni p-value correction for multiple tests. On these days, the negative β coefficients of the interaction terms indicate that the increases in egg laying related to FBB treatment was less than the increase at the final time point. This is explained by continuing increase in average eggs laid per day in FBB provisioned cages until the conclusion of the experiment. See Table 2 for a summary of the affected time points by treatment comparisons.

Diet type significantly affected pollen consumption in QMCs ($p = 0.0006$, Kruskal Wallis test, Chi-Square = 14.9). Significantly greater amounts of pollen diet were consumed by bees in QMCs provisioned with PP-45 relative to BB or FBB (BB: $p = 0.0002$, Wilcoxon Rank Sum, Chi-Square = 13.7, df = 1; FBB: $p = 0.005$, Chi-Square = 8.0, df = 1, Fig 3B). No differences in pollen consumption were detected between QMCs provisioned with BB or FBB ($p = 0.4$, Wilcoxon Rank Sum, Chi-Square = 0.7, df = 1). A significant negative correlation was detected between time and pollen consumption ($p \leq 0.0001$, Spearman's $\rho = -0.7$).

Diet type had a significant effect on the average acini width in worker bees sampled on day 7 or 8 of this experiment ($p < 0.0001$, Kruskal Wallis test, Chi-Square = 50.0). The average acini diameters were significantly different among the 3 treatments (BB vs. FBB: $p = 0.006$, Wilcoxon Rank Sum, Chi-Square = 7.5, df = 1; BB vs. PP-45: $p \leq 0.0001$, Chi-Square = 19.7, df = 1; FBB vs. PP-45: $p \leq 0.0001$, Chi-Square = 48.5, df = 1, Fig 3C). The bees in QMCs provisioned with FBB had the largest HPG acini, followed by BB and PP-45, in that order.

Experiment 3: Effects of frozen bee bread vs. 70% pollen paste on egg laying

No significant effect of pollen diet on total eggs laid was observed ($p = 0.3$, Student's *t*-test, $t = -1.1$, df = 28). However, as in Experiment 2, there was a significant effect of diet type on daily egg laying ($p = 0.019$, GEE, Wald Chi-Square = 5.5, df = 1, Fig 4A). Time significantly affected egg laying ($p < 0.001$, GEE, Wald Chi-Square = 204.8, df = 7). Significantly lower egg laying was observed on the days 3 and 4 relative to the final time point after Bonferroni correction, but significantly higher egg laying was observed on day 7, indicating that peak performance was achieved at this time (Table 1).

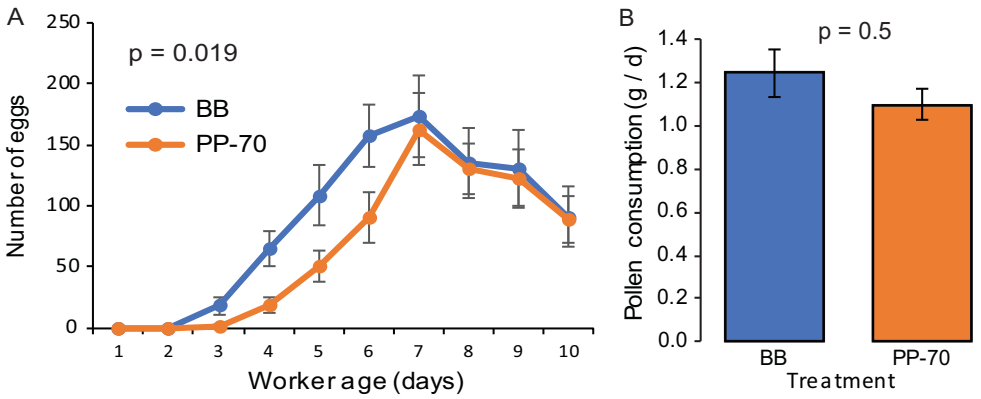

Fig 4. Effects of pollen diets on egg laying and pollen diet consumption (Experiment 3). A. Average ± SE number of eggs laid per day in QMCs provisioned with bee bread (BB, blue) or 70% pollen paste (PP-70, orange). The p-value is the result of GEE analysis ($p = 0.019$, Wald Chi-Square = 5.5, df = 1). **B.** Average ± SE pollen diet consumed per day in BB (blue) and PP-70 (orange) treatment groups. The p-value is the result of a Wilcoxon Rank Sum test ($p = 0.5$, Chi-Square = 0.4, df = 1).

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A significant interaction was detected between time and treatment ($p \leq 0.005$, GEE, Wald Chi-Square = 20.1, df = 7). The interaction was evident on days 3, 4, and 5, but only day 3 was significant after Bonferroni adjustment. The positive β coefficient of this interaction indicates that the increase in egg laying related to BB treatment was greater on day 3 relative to the final time point (Table 2).

No significant difference in pollen consumption was detected between treatments ($p = 0.5$, Wilcoxon Rank Sum, Chi-Square = 0.4, df = 1, Fig 4B). A significant negative correlation was detected between time and pollen consumption ($p \leq 0.0001$, Spearman's $\rho = -0.7$).

Discussion

We have presented a new method to track and quantify queen egg laying under controlled laboratory conditions and used it to test the effect of pollen diet on egg laying. The use of Queen Monitoring Cages (QMCs) allowed us to examine the relationship between worker diet and physiology and queen fecundity, yielding robust data from a large sample size. This versatile system could be adapted for many purposes including honey bee risk assessment and egg collection to produce transgenic bees [51].

During these experiments, the number of eggs laid did not reach the high end for queens in full sized colonies reported in the literature [18], suggesting that further manipulations can increase egg production in this system. However, the majority of queens readily laid eggs in QMCs and responded to diet treatments, suggesting that the system is also suitable for risk assessment experiments.

The results of Experiments 2 and 3 indicate that provisioning QMCs with bee bread can positively influence queen egg laying. This difference was particularly striking in Experiment 2, when provisioning QMCs with frozen bee bread resulted in nearly 3 times more eggs than 45% pollen paste. This may be due in part to the differences in pollen composition of the diets. Percentages of pollen in bee bread as high as 88% have been reported [52], and, as the primary source of protein, lipids, and many vitamins and minerals for honey bees, pollen is essential to the health of a colony [36,53]. The relatively higher consumption of pollen paste in Experiment 2 may have been a compensatory response to the lower percentage of pollen relative to the other diets. This is consistent with the finding that when the percentage of pollen in pollen paste was increased to 70%, no difference could be detected in total eggs laid, and a much smaller disparity in the daily number eggs laid between bee bread and pollen paste-provisioned QMCs that diminished over time was observed.

Bee bread was also shown to positively affect the size of worker bee hypopharyngeal glands (HPGs), suggesting a mechanism for the effect of worker nutrition on queen egg laying. Within a colony, the queen receives her nutrition through trophallaxis from young workers who form a retinue around her [18]. Although a direct relationship between HPG development and queen retinue behaviors has not been established, the results of Experiment 2 strongly suggest that worker HPG development influences queen egg laying productivity. This may be directly related to the ability of worker bees to provision the queen with proteinaceous secretions produced by the HPGs [25,36]. In other insect species it is well known that reproduction is heavily dependent on individual nutrition [54], but in these experiments, the pollen diet was not directly consumed by the queen. These results suggest that the egg laying of the honey bee queen is dependent on worker nutrition, providing another example by which the colony functions as a superorganism[55].

Average HPG acinus diameter in bees from QMCs provisioned with bee bread was still smaller than what has been reported in the literature for similarly aged bees [48]. Perhaps this is because at the time the bees were sampled, egg laying had not yet peaked, and worker HPG

development also had not yet peaked. An alternative explanation is that because QMCs were populated only with younger bees, some bees may have experienced accelerated development resulting in more forager-like physiology, with smaller HPGs. This phenomenon is based on social inhibition of adult maturation and has been previously reported in single-cohort colonies initially composed of all young worker bees [56,57].

Although there were no differences in egg laying between queens in QMCs provisioned with bee bread or frozen bee bread, workers from QMCs provisioned with frozen bee bread had higher average HPG gland sizes. This may be because freezing plant material degrades the cell wall components [58], potentially making pollen easier to digest. Our results demonstrate that freezing bee bread at -80˚C is an acceptable form of short term storage and may even contribute to successful egg laying in QMCs. The duration and conditions of pollen storage are known to affect its quality and suitability for brood rearing [59], therefore more research is needed to determine if bee bread can be stored in this manner for longer periods of time. Additionally, more research is needed to determine what components of bee bread contribute positively to egg laying.

In all experiments, egg laying rates were low initially and increased over the first few days. This may be because the bees require time to adjust to the cage after their introduction, but another compelling hypothesis is that worker age has an effect on queen egg laying in QMCs. This would not be surprising, as worker honey bees exhibit striking patterns of physiological and behavioral maturation, a function of the colony's age-related division of labor [60]. This also has been observed in laboratory cages [61], and was likely the cause of the patterns observed here. This may explain the time by treatment interaction effects on queen egg laying seen in Experiments 2 and 3. This interaction was particularly strong in Experiment 2, where we observed egg laying continuing to increase until the final time point in bee bread provisioned QMCs relative to pollen paste.

Variation in egg laying between experiments suggests that factors external to this study can influence egg laying in QMCs. Overall, the highest egg laying was observed in Experiment 1, which was conducted in July. Experiment 2 was conducted less than 1 month later, but much lower rates of egg laying were observed in cages fed PP-45 relative to BB and FBB. In Experiment 3, higher rates of egg laying were evident in BB fed cages relative to PP-70, but only during the first half of the experiment. Worker physiology varies with season [62–66] and with conditions during pre-adult development [67,68]. The workers used in Experiment 1 may have been better suited for the tasks associated with supporting queen egg laying due to physiological differences caused by either of these factors. Additionally, pathogen abundance within colonies can change over time, often increasing throughout the active season [69]. It is not clear how pathogen infections would affect egg laying in QMCs, but behavioral effects associated with viral infections, including learning deficits and increased aggression, have been documented [70,71], suggesting that pathogen infections would have a negative effect on egg production in QMCs. One interpretation of the results of all 3 experiments taken together is that diet can buffer the effects of developmental background on worker physiology, better equipping them to care for and provision the queen.

Other sources of variation that may have contributed to the differences in results between experiments and that would be worth considering in the future are seasonal variation in bee bread composition and the effects of worker honey bee genetic variation. A recent study by Degrandi-Hoffman et al. [72] found that gene expression profiles in honey bee fat bodies vary with the time of year and the seasonality of their pollen diet, suggesting that bees in QMCs can also be manipulated this way in the future. Additionally, genetic variation among workers has been shown to affect virtually every trait studied, at the molecular, physiological, and behavioral levels [73,74], so it is possible that there also is variation for physiological and behavioral traits that affect queen egg laying.

One potential application of the QMC system is the collection of honey bee embryos for genome editing via CRISPR/Cas9 [75]. Because of the fragility of honey bee eggs, special care must be taken when manipulating them, and eggs are typically taken directly from full sized colonies for these purposes using a specialized queen cage (Jenter Queen Rearing Kit) [76]. Although we did not collect eggs during these experiments, this system could be easily adapted for this purpose. CRISPR/Cas9 has been used successfully in four hymenopteran species including honey bees [51,77–80], and its utility in studying the roles of specific genes in social evolution has already been demonstrated [77,78]. In the future, gene editing techniques could be applied to honey bee embryos collected using QMCs to further our understanding of honey bee biology, health, and responses to stress.

There is currently a pressing need to develop systems to examine and quantify the effects of single and multiple stressors on honey bee queen health. Queen egg laying is affected by a variety of seasonal, nutritional and social factors [10,18,25,26], and research suggests it may be vulnerable to disruption via these stressors [21,23,24,30,81]. In the future, the system presented here should be used for experiments to assess the single and combined effects of pesticides, pathogens, parasites and nutrition on egg laying. Paired with extant and additional field study data, the findings of experiments performed with QMCs could greatly aid in predicting, assessing, and mitigating health risks to the honey bee population and pollination services.

Supporting information

S1 Supporting Text. Bee bread collection.
(DOCX)

S1 File. Egg laying data.
(XLSX)

S1 Table. Average eggs laid per day, maximum eggs laid per day, and laying vs. non-laying queens by experiment and treatment.
(DOCX)

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References

1. Calderone NW. Insect Pollinated Crops, Insect Pollinators and US agriculture: Trend analysis of aggregate data for the period 1992–2009. Smagghe G, editor. PLOS One. 2012; 7: e37235. https://doi.org/10.1371/journal.pone.0037235 PMID: 22629374
2. Southwick EE, Southwick L. Estimating the economic value of honey bees (Hymenoptera: Apidae) as agricultural pollinators in the United States. J Econ Entomol. 1992; 85:621–33. https://doi.org/10.1093/jee/85.3.621
3. Aizen MA, Garibaldi LA, Cunningham SA, Klein AM. Long-term global trends in crop yield and production reveal no current pollination shortage but increasing pollinator dependency. Curr Biol. 18:1572–1575. https://doi.org/10.1016/j.cub.2008.08.066 PMID: 18926704
4. vanEngelsdorp D, Evans JD, Saegerman C, Mullin C, Haubruge E, Nguyen BK, et al. Colony collapse disorder: A descriptive study. PLOS ONE. 2009; 4:e6481. https://doi.org/10.1371/journal.pone.0006481 PMID: 19649264
5. Goulson D, Nicholls E, Botías C, Rotheray EL. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. Science. 2015;347. Available: http://www.sciencemag.org/content/347/6229/1255957.abstract https://doi.org/10.1126/science.aaa2958 PMID: 25765066
6. Spivak M, Mader E, Vaughan M, Euliss NH. The plight of the bees. Environ Sci Technol. 2011; 45: 34–38. https://doi.org/10.1021/es101468w PMID: 20839858
7. Tsvetkov N, Samson-Robert O, Sood K, Patel HS, Malena DA, Gajiwala PH, et al. Chronic exposure to neonicotinoids reduces honey bee health near corn crops. Science. 2017; 356: 1395–1397. https://doi.org/10.1126/science.aam7470 PMID: 28663503
8. Woodcock BA, Bullock JM, Shore RF, Heard MS, Pereira MG, Redhead J, et al. Country-specific effects of neonicotinoid pesticides on honey bees and wild bees. Science. 2017; 356: 1393–1395. https://doi.org/10.1126/science.aaa1190 PMID: 28663502
9. Huang ZY, Hanley AV, Pett WL, Langenberger M, Duan JJ. Field and semifield evaluation of impacts of transgenic canola pollen on survival and development of worker honey bees. J Econ Entomol. 2004; 97: 1517–1523. PMID: 15568338
10. DeGrandi-Hoffman G, Wardell G, Ahumada-Segura F, Rinderer T, Danka R, Pettis J, et al. Comparisons of pollen substitute diets for honey bees: consumption rates by colonies and effects on brood and adult populations. J Apic Res. 2008; 47:265–270. https://doi.org/10.1080/00218839.2008.11101473
11. Cavigli I, Daughenbaugh KF, Martin M, Lerch M, Banner K, Garcia E, et al. Pathogen prevalence and abundance in honey bee colonies involved in almond pollination. Apidologie. 2016; 47: 251–266. https://doi.org/10.1007/s13592-015-0395-5 PMID: 27053820
12. Mullin CA, Frazier M, Frazier JL, Ashcraft S, Simonds R, vanEngelsdorp D, et al. Hive levels of miticides and agrochemicals in North American apiaries: Implications for honey bee health. PLOS One. 2010; 5. https://doi.org/10.1371/journal.pone.0009754 PMID: 20333298
13. Chauzat M-P, Faucon J-P, Martel A-C, Lachaize J, Cougoule N, Aubert M, et al. A survey of pesticide residues in pollen loads collected by honey bees in France. J Econ Entomol. 2006; 99:253–262. https://doi.org/10.1093/jee/99.2.253 PMID: 16686121
14. Di Pasquale G, Salignon M, Le Conte Y, Belzunces LP, Decourtye A, Kretzschmar A, et al. Influence of pollen nutrition on honey bee health: Do pollen quality and diversity matter? Zeil J, editor. PLOS One. 2013; 8: e72016. https://doi.org/10.1371/journal.pone.0072016 PMID: 23940803
15. Donkersley P, Rhodes G, Pickup RW, Jones KC, Wilson K. Honeybee nutrition is linked to landscape composition. Ecol Evol. 2014; 4: 4195–4206. https://doi.org/10.1002/ece3.1293 PMID: 25505544
16. Navajas M, Migeon A, Alaux C, Martin-Magniette M, Robinson G, Evans J, et al. Differential gene expression of the honey bee *Apis mellifera* associated with Varroa destructor infection. BMC Genomics. 2008; 9: 301–301. https://doi.org/10.1186/1471-2164-9-301 PMID: 18578863
17. Page RE Jr, Erber J, Fondrk MK. The effect of genotype on response thresholds to sucrose and foraging behavior of honey bees (*Apis mellifera* L.). J Comp Physiol A. 1998; 182: 489–500. https://doi.org/10.1007/s003590050196 PMID: 9565478

18. Winston M. The Biology of the Honey Bee. Cambridge, MA: Harvard University Press; 1987.
19. Moore PA, Wilson ME, Skinner JA. Honey bee queens: Evaluating the most important colony member. 2015 Oct [cited 2 Jan 2018]. In: eXtension [Internet]. Available from: http://articles.extension.org/pages/73133/honey-bee-queens:-evaluating-the-most-important-colony-member
20. vanEngelsdorp D, Jr JH, Underwood RM, Pettis J. A survey of honey bee colony losses in the U.S., Fall 2007 to Spring 2008. PLOS One. 2008; 3: e4071. https://doi.org/10.1371/journal.pone.0004071 PMID: 19115015
21. vanEngelsdorp D, Tarpy DR, Lengerich EJ, Pettis JS. Idiopathic brood disease syndrome and queen events as precursors of colony mortality in migratory beekeeping operations in the eastern United States. Prev Vet Med. 2013; 108: 225–233. https://doi.org/10.1016/j.prevetmed.2012.08.004 PMID: 22939774
22. Dively GP, Embrey MS, Kamel A, Hawthorne DJ, Pettis JS. Assessment of chronic sublethal effects of imidacloprid on honey bee colony health. PLOS One. 2015; 10: e0118748. https://doi.org/10.1371/journal.pone.0118748 PMID: 25786127
23. Sandrock C, Tanadini M, Tanadini LG, Fauser-Misslin A, Potts SG, Neumann P, et al. Impact of chronic neonicotinoid exposure on honeybee colony performance and queen supersedure. PLOS One. 2014; 9: e103592. https://doi.org/10.1371/journal.pone.0103592 PMID: 25084279
24. Pettis JS, Rice N, Joselow K, vanEngelsdorp D, Chaimanee V. Colony failure linked to low sperm viability in honey bee (*Apis mellifera*) queens and an exploration of potential causative Factors. PLOS ONE. 2016; 11: e0147220. https://doi.org/10.1371/journal.pone.0147220 PMID: 26863438
25. Allen MD. The honeybee queen and her attendants. Anim Behav. 1960; 8: 201–208. https://doi.org/10.1016/0003-3472(60)90028-2
26. Keeling CI, Slessor KN, Higo HA, Winston ML. New components of the honey bee (*Apis mellifera* L.) queen retinue pheromone. Proc Natl Acad Sci. 2003; 100: 4486–4491. https://doi.org/10.1073/pnas.0836984100 PMID: 12676987
27. Butler CG, Callow RK, Koster CG, Simpson J. Perception of the queen by workers in the honeybee colony. J Apic Res. 1973; 12: 159–166. https://doi.org/10.1080/00218839.1973.11099744
28. Miranda CRE, Bitondi MMG, Simões ZLP. Effect of proctolin on the egg-laying activity of *Apis mellifera* queens. J Apic Res. 2003; 42: 35–38. https://doi.org/10.1080/00218839.2003.11101086
29. Fine JD, Mullin CA, Frazier MT, Reynolds RD. Field residues and effects of the insect growth regulator novaluron and its major co-formulant *N*-Methyl-2-Pyrrolidone on honey bee reproduction and development. J Econ Entomol. 2017; 110: 1993–2001. https://doi.org/10.1093/jee/tox220 PMID: 28961741
30. Wu-Smart J, Spivak M. Sub-lethal effects of dietary neonicotinoid insecticide exposure on honey bee queen fecundity and colony development. Sci Rep. 2016; 6: 32108. https://doi.org/10.1038/srep32108 PMID: 27562025
31. DeGrandi-Hoffman G, Martin JH. Behaviour of egg-laying virgin and mated queen honey bees (*Apis mellifera* L.) and the composition of brood in their colonies. J Apic Res. 1993; 32: 19–26. https://doi.org/10.1080/00218839.1993.11101283
32. Dunham WE. Temperature gradient in the egg-laying activities of the queen bee. Ohio J Sci. 1930; 30. Available: http://hdl.handle.net/1811/2481
33. US EPA O. How we assess risks to pollinators. In: US EPA [Internet]. 16 Sep 2013 [cited 13 Mar 2018]. Available: https://www.epa.gov/pollinator-protection/how-we-assess-risks-pollinators
34. Crailsheim K, Schneider LHW, Hrassnigg N, Bühlmann G, Brosch U, Gmeinbauer R, et al. Pollen consumption and utilization in worker honeybees (*Apis mellifera* carnica): Dependence on individual age and function. J Insect Physiol. 1992; 38: 409–419. https://doi.org/10.1016/0022-1910(92)90117-V
35. Crailsheim K. The flow of jelly within a honeybee colony. J Comp Physiol B. 1992; 162: 681–689. https://doi.org/10.1007/BF00301617
36. Haydak MH. Honey bee nutrition. Annu Rev Entomol. 1970; 15: 143–156. https://doi.org/10.1146/annurev.en.15.010170.001043
37. Vásquez A, Olofsson TC. The lactic acid bacteria involved in the production of bee pollen and bee bread. J Apic Res. 2009; 48: 189–195. https://doi.org/10.3896/IBRA.1.48.3.07
38. Herbert EWJ, Shimanuki H. Chemical composition and nutritive value of bee-collected and bee-stored pollen. Apidologie. 1978; 9: 33–40. https://doi.org/10.1051/apido:19780103
39. Carroll MJ, Brown N, Goodall C, Downs AM, Sheenan TH, Anderson KE, et al. Honey bees preferentially consume freshly-stored pollen. PLOS ONE. 2017; 12: e0175933. https://doi.org/10.1371/journal.pone.0175933 PMID: 28430801
40. Alquarni A. Influence of some protein diets on the longevity and some physiological conditions of honeybee *Apis mellifera* L. Workers. J Biol Sci. 2006; 6. https://doi.org/10.3923/jbs.2006.734.737

41. Al-Ghamdi AA, Al-Khaibari AM, Omar MO. Consumption rate of some proteinic diets affecting hypopharyngeal glands development in honeybee workers. Saudi J Biol Sci. 2011; 18: 73–77. https://doi.org/10.1016/j.sjbs.2010.10.001 PMID: 23961106

42. Free JB. Hypopharyngeal gland development and division of labour in honey-bee (*Apis mellifera* L.) colonies. Proc R Entomol Soc Lond Ser Gen Entomol. 1961; 36: 5–8. https://doi.org/10.1111/j.1365-3032.1961.tb00253.x

43. Engels W. Occurrence and significance of vitellogenins in female castes of social hymenoptera. American Zoologist. 1974; 14: 1229–1237.

44. Corona M, Velarde RA, Remolina S, Moran-Lauter A, Wang Y, Hughes KA, et al. Vitellogenin, juvenile hormone, insulin signaling, and queen honey bee longevity. Proc Natl Acad Sci. 2007; 104: 7128–7133. https://doi.org/10.1073/pnas.0701909104 PMID: 17438290

45. Seehuus S-C, Norberg K, Gimsa U, Krekling T, Amdam GV. Reproductive protein protects functionally sterile honey bee workers from oxidative stress. Proc natl acad sci. 2006; 103: 962–967. https://doi.org/10.1073/pnas.0502681103 PMID: 16418279

46. Dussutour A, Simpson SJ. Description of a simple synthetic diet for studying nutritional responses in ants. Insectes Sociaux. 2008; 55: 329–333. https://doi.org/10.1007/s00040-008-1008-3

47. Piccirillo GA, De Jong D. The influence of brood comb cell size on the reproductive behavior of the ectoparasitic mite Varroa destructor in Africanized honey bee colonies. Genet Mol Res. 2003; 2: 471–473.

48. Hrassnigg N, Crailsheim K. Adaptation of hypopharyngeal gland development to the brood status of honeybee (*Apis mellifera* L.) colonies. J Insect Physiol. 1998; 44: 929–939. https://doi.org/10.1016/S0022-1910(98)00058-4 PMID: 12770429

49. Rasband W.S., ImageJ [software]. U. S. National Institutes of Health, Bethesda, MD. 1997–2016 [cited 26 Mar 2018]., https://imagej.nih.gov/ij/, 1997–2016. Available from: https://imagej.nih.gov/ij/

50. Hanley JA, Negassa A, Edwardes MD deB, Forrester JE. Statistical Analysis of Correlated Data Using Generalized Estimating Equations: An Orientation. Am J Epidemiol. 2003; 157: 364–375. https://doi.org/10.1093/aje/kwf215 PMID: 12578807

51. Schulte C, Theilenberg E, Müller-Borg M, Gempe T, Beye M. Highly efficient integration and expression of piggyBac-derived cassettes in the honeybee (*Apis mellifera*). Proc Natl Acad Sci. 2014; 111: 9003–9008. https://doi.org/10.1073/pnas.1402341111 PMID: 24821811

52. Kaplan M, Karaoglu Ö, Eroglu N, Silici S. Fatty acid and proximate composition of bee bread. Food Technol Biotechnol. 2016; 54: 497–504. https://doi.org/10.17113/ftb.54.04.16.4635 PMID: 28115909

53. DeGroot AP. Protein and amino acid requirements of the honeybee (Apis mellifica L.). Physiol Comp Oecologia. 1953; 3: 197–285.

54. Rivero A, Giron D, Casas J. Lifetime allocation of juvenile and adult nutritional resources to egg production in a holometabolous insect. Proc Biol Sci. 2001; 268: 1231–1237. https://doi.org/10.1098/rspb.2001.1645 PMID: 11410148

55. Hölldobler B. The superorganism: the beauty, elegance, and strangeness of insect societies /. 1st ed. New York: W.W. Norton; c2009.

56. Crailsheim K, Stolberg E. Influence of diet, age and colony condition upon intestinal proteolytic activity and size of the hypopharyngeal glands in the honeybee (*Apis mellifera* L.). J Insect Physiol. 1989; 35: 595–602. https://doi.org/10.1016/0022-1910(89)90121-2

57. Giray T, Robinson GE. Effects of intracolony variability in behavioral development on plasticity of division of labor in honey bee colonies. Behav Ecol Sociobiol. 1994; 35: 13–20.

58. Zaritzky NE. Chemical and physical deterioration of frozen foods. In: Skibsted LH, Risbo J, Andersen ML, editors. Chemical deterioration and physical instability of food and beverages. Woodhead Publishing; 2010. p. 561–607. https://doi.org/10.1533/9781845699260.3.561

59. Dietz A, Stevenson HR. Influence of long term storage on the nutritional value of frozen pollen for brood rearing of honey bees. Apidologie. 1980; 11: 143–151. https://doi.org/10.1051/apido:19800204

60. Robinson GE. Regulation of division of labor in insect societies. Annu Rev Entomol. 1992; 37: 637–665. https://doi.org/10.1146/annurev.en.37.010192.003225 PMID: 1539941

61. Huang ZY, Robinson GE. Honeybee colony integration: worker-worker interactions mediate hormonally regulated plasticity in division of labor. Proc Natl Acad Sci U S A. 1992; 89: 11726–11729. PMID: 1465390

62. Kunert K, Crailsheim K. Seasonal changes in carbohydrate, lipid and protein content in emerging worker honeybees and their mortality. J Apic Res. 1988; 27: 13–21. https://doi.org/10.1080/00218839.1988.11100775

63. Harris JW, Woodring J. Effects of stress, age, season, and source colony on levels of octopamine, dopamine and serotonin in the honey bee (*Apis mellifera* L.) brain. J Insect Physiol. 1992; 38: 29–35. https://doi.org/10.1016/0022-1910(92)90019-A

64. Fluri P, Wille H, Gerig L, Lüscher M. Juvenile hormone, vitellogenin and haemocyte composition in winter worker honeybees (*Apis mellifera*). Experientia. 1977; 33: 1240–1241. https://doi.org/10.1007/BF01922354

65. Goblirsch M, Huang ZY, Spivak M. Physiological and behavioral changes in honey bees (*Apis mellifera*) induced by *Nosema ceranae* infection. PLoS ONE. 2013; 8. https://doi.org/10.1371/journal.pone.0058165 PMID: 23483987

66. Huang ZY, Robinson GE. Seasonal changes in juvenile hormone titers and rates of biosynthesis in honey bees. J Comp Physiol [B]. 1995; 165: 18–28.

67. Eishchen FA, Rothenbuhler WC, Kulinčević JM. Length of life and dry weight of worker honeybees reared in colonies with different worker-larva ratios. J Apic Res. 1982; 21: 19–25. https://doi.org/10.1080/00218839.1982.11100511

68. Mattila HR, Otis GW. The effects of pollen availability during larval development on the behaviour and physiology of spring-reared honey bee workers. Apidologie. 2006; 37: 533–546. https://doi.org/10.1051/apido:2006037

69. Tentcheva D, Gauthier L, Zappulla N, Dainat B, Cousserans F, Colin ME, et al. Prevalence and seasonal variations of six bee viruses in *Apis mellifera* L. and *Varroa destructor* mite populations in France. Appl Environ Microbiol. 2004; 70: 7185–7191. https://doi.org/10.1128/AEM.70.12.7185-7191.2004 PMID: 15574916

70. Fujiyuki T, Takeuchi H, Ono M, Ohka S, Sasaki T, Nomoto A, et al. Novel Insect Picorna-Like Virus Identified in the Brains of Aggressive Worker Honeybees. J Virol. 2004; 78: 1093–1100. https://doi.org/10.1128/JVI.78.3.1093-1100.2004 PMID: 14722264

71. Iqbal J, Mueller U. Virus infection causes specific learning deficits in honeybee foragers. Proc R Soc B Biol Sci. 2007; 274: 1517–1521. https://doi.org/10.1098/rspb.2007.0022 PMID: 17439851

72. DeGrandi-Hoffman G, Gage SL, Corby-Harris V, Carroll M, Chambers M, Graham H, et al. Connecting the nutrient composition of seasonal pollens with changing nutritional needs of honey bee (*Apis mellifera* L.) colonies. J Insect Physiol. 2018; 109: 114–124. https://doi.org/10.1016/j.jinsphys.2018.07.002 PMID: 29990468

73. Page RE, Robinson GE. The genetics of division of labour in honey bee colonies. In: Evans PD, editor. Advances in Insect Physiology. Academic Press; 1991. pp. 117–169. https://doi.org/10.1016/S0065-2806(08)60093-4

74. Zayed A, Robinson GE. Understanding the relationship between brain gene expression and social behavior: Lessons from the honey bee. Annu Rev Genet. 2012; 46: 591–615. https://doi.org/10.1146/annurev-genet-110711-155517 PMID: 22994354

75. Doudna JA, Charpentier E. The new frontier of genome engineering with CRISPR-Cas9. Science. 2014; 346: 1258096. https://doi.org/10.1126/science.1258096 PMID: 25430774

76. Lene T.Aase A O., Amdam G V., Hagen A, Omholt S W. A new method for rearing genetically manipulated honey bee workers. Apidologie. 2005; 36: 293–299.

77. Yan H, Opachaloemphan C, Mancini G, Yang H, Gallitto M, Mlejnek J, et al. An engineered orco mutation produces aberrant social behavior and defective neural development in ants. Cell. 2017; 170: 736–747.e9. https://doi.org/10.1016/j.cell.2017.06.051 PMID: 28802043

78. Trible W, Olivos-Cisneros L, McKenzie SK, Saragosti J, Chang N-C, Matthews BJ, et al. orco mutagenesis causes loss of antennal lobe glomeruli and impaired social behavior in ants. Cell. 2017; 170: 727–735.e10. https://doi.org/10.1016/j.cell.2017.07.001 PMID: 28802042

79. Li M, Au LYC, Douglah D, Chong A, White BJ, Ferree PM, et al. Generation of heritable germline mutations in the jewel wasp *Nasonia vitripennis* using CRISPR/Cas9. Sci Rep. 2017; 7: 901. https://doi.org/10.1038/s41598-017-00990-3 PMID: 28424460

80. Schulte C, Theilenberg E, Müller-Borg M, Gempe T, Beye M. Highly efficient integration and expression of piggyBac-derived cassettes in the honeybee (*Apis mellifera*). Proc Natl Acad Sci. 2014; 111: 9003–9008. https://doi.org/10.1073/pnas.1402341111 PMID: 24821811

81. Francis RM, Nielsen SL, Kryger P. Patterns of viral infection in honey bee queens. J Gen Virol. 2013; 94: 668–676. https://doi.org/10.1099/vir.0.047019-0 PMID: 23223622