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THE IMPACTS OF HONEY BEE QUEEN STRESS ON WORKER BEHAVIOR AND HEALTH

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THE IMPACTS OF HONEY BEE QUEEN STRESS ON WORKER BEHAVIOR AND HEALTH

Thesis

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
College of Agriculture, Food and Environment
at the University of Kentucky

By

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Lexington, Kentucky

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ABSTRACT OF THESIS

THE IMPACTS OF HONEY BEE QUEEN STRESS ON WORKER BEHAVIOR AND HEALTH

Pesticides, poor nutrition, parasites and diseases work synergistically to contribute to the decline of the honey bee. Heritable sub-lethal behavior/immune effects may also contribute to the decline. Maternal stress is a common source of heritable immune/behavior deficits in many species. A stressed honey bee queen has the potential to pass such deficits on to worker bees. Using a repeated measures design, this study will determine whether the health of worker bee is reduced by a cold stress on the queen by analyzing egg hatch rate and protein content, emergence rate, and adult aggression and immune function for offspring laid before and after the stressor. Results show that queen stress influences egg hatching rate and emergence rate but does not impact egg protein content, adult offspring immune function or aggressive behavior.

KEYWORDS: honey bee, worker bee health, worker bee behavior, maternal stress, queen stress

Sarah R. Preston

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I would like to dedicate this thesis to my mother, Sherry Mae Legier, and mother-in-law, Marilyn Diane Preston who passed away before I could celebrate this achievement. I will always hold dear the love and support you gave me to realize my dreams.

“A mother is someone who dreams great dreams for you, but then she lets you chase the dreams you have for yourself and loves you just the same”

Agatha Christie

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Chapter 1. Introduction

The decline of the honey bees populations around the world is mostly attributed to synergistic effects of pesticides, parasites, pathogens and poor nutrition (Smith et al. 2013, Goulson et al. 2015, McMenamin and Genersch 2015). In addition to these factors, colony survivorship is strongly influenced by the health and productivity of one critical member, the queen (Amiri et al. 2017). The main role of the queen is egg laying, up to 1500 eggs daily (Winston 1987), at a rate that replaces the entire worker population of the hive every 25-35 days (Amdam and Omholt 2002). A queen typically lives up to 3-4 years (Amiri et al. 2017); however a colony can detect the failure of a queen and will replace her when her pheromone production diminishes, she is injured or diseased, or when she is laying an insufficient number of fertilized eggs or a large amount of unfertilized eggs (Winston 1987). An apiculturist may also detect this failure and choose to remove the queen and replace her with a queen of a specific age and characteristic to maintain honey production. When a queen fails, colonies or the apiculturist must quickly replace her to maintain the necessary workforce and performance that contributes to colony survival (Tarpy et al. 2012, Pettis et al. 2016). Queenlessness for an extended period of time may result in one or more laying worker honey bees which can only lay unfertilized eggs.

Currently, honey bee queens are failing at record levels, with apiculturist replacing them at rates as high as every six months (Pettis et al. 2016, Amiri et al. 2017). Research shows that many of the factors that contribute to colony decline, such as pesticides (Williams et al. 2015), parasites and pathogens (Amiri et al. 2017), also affect

queen longevity. In addition to decreased fecundity, queen stress and poor health could also impact the quality of worker offspring. This type of indirect environmental effect on worker phenotypes is critical to consider, because it could have cumulative impacts on subsequent worker that are cared for by unhealthy sisters. Such a pattern would increase the probability of colony death over multiple worker cohorts.

An effect of queen health or stress on worker phenotype is a type of maternal effect. Maternal effects are a when the phenotype (and sometimes genotype) of a female affects the phenotype of her offspring (Räsänen and Kruuk 2007, Wolf and Wade 2009), and they can contribute greatly to offspring fitness. In some cases, maternal effects are adaptive and allow offspring to adjust to current environmental conditions; for example, in highly variable environments, the capacity for phenotypic plasticity of offspring in response to maternal experience may be a strong target of selection (Kuijper and Hoyle 2015). However, maternal effects can also reflect offspring response to maternal stressors or maternal genetic variation without clear adaptive value (Räsänen and Kruuk 2007). Evidence for maternal effects have been found across the animal kingdom including fish, reptiles, birds, mammals and insects (Räsänen and Kruuk 2007, Rowiński and Rogell 2017).

Adaptive maternal effects can involve a variety of environmental factors including temperature, photoperiod, predation risk, nutritional resource availability, and other influences (Mousseau and Fox 1998, Räsänen and Kruuk 2007, Sgrò et al. 2016) that impact offspring phenotype. For example, temperature and photoperiod, which signal the onset of winter, may induce a female to lay diapausing offspring, or switch to

winged offspring if conditions require dispersal in species such as aphids or grasshoppers (Mousseau and Fox 1998, Marshall and Uller 2007). Researchers have also documented that females can manipulate the sex of their offspring in relation to food availability or changes in temperature and photoperiod. Notably, in social Hymenoptera species, females may control the sex ratio of a colony by choosing whether or not to fertilize eggs, which is possible because of haplodiploid sex determination (Mousseau and Fox 1998). Additionally, predation and nutritional resources can influence where females oviposit and what resources her offspring will use early in life (Boggs 2009). Moreover, nutritional resources available to the female throughout her life can influence breeding time and quantity and size of eggs (Boggs 2009).

In honey bees, the relevance of maternal effects is unclear because of the nature of the social insect nest. First, the honey bee queen generates the entire colony and spends much of her mated life contained within the colony with optimal food, temperature, humidity and in constant darkness, and therefore gains little direct information from the external environment that she could transmit to worker offspring (Remolina and Hughes 2008). It is important to note, however, that few studies (if any) have investigated whether and how the queen could receive indirect information about the environment in the form of social interactions with workers. Second, queens play a relatively minimal role in rearing offspring after eggs are laid, as a specialized caste of worker bees (the nurse bee) is responsible for offspring rearing (Remolina and Hughes 2008), the mature forager worker bees interact with the environment and communicate to the colony perceived environmental changes. Several studies have shown that the

developmental environment controlled by adult worker bee influences the phenotype of larval and pupal bees. Thus, in the case of the honey bee, information transfer between workers and offspring may be more relevant than transfer between queens and offspring.

Several studies have shown how the immature environment can alter the adult honey bee. For example, Rittschof et al. (2015) found that the colony aggression level experienced during larval development has lasting effects on adult behavior and health. Colony aggression is a property of worker bees inside the colony who appear to transfer these characteristics to subsequent offspring (Rittschof et al. 2015). Additionally, the decrease of larval food provisioning by worker bees can have lasting effects on the subsequent cohort through a reduction in adult longevity, foraging activity, the communication of food location (Scofield and Mattila 2015), metabolic rate, respiration rate, and an increase in blood sugar preservation during adult starvation (Wang et al. 2016). Immature care is important to consider in the overall health of the colony; however, the queen may be contributing more than genetics to her offspring.

In addition to genetics, honey bee queens may also be providing non-adaptive effects to their offspring. While the queen does not directly communicate environmental conditions, queen stress from disease, aging, and apicultural practices (pathogen and parasite treatment or shipment) can still have an effect on her offspring and may change the dynamic of the workers and lead to cascading changes within the colony (Barron 2015). Some properties of the queen are known to impact worker viability and health, regardless of the adaptive value of these effects. For example,

maternal age is negatively correlated with embryo size, embryo viability, and early larval development (Al-Lawati and Bienefeld 2009), and there is evidence of similar effects in other insects such as the house fly (McIntyre and Gooding 2000). While egg size is generally used to document maternal effects and likelihood of embryo survival in most species, egg provisioning (the quantity and ratio of three key macronutrients, protein, lipids, and carbohydrates) can vary within eggs of the same size (McIntyre and Gooding 2000, Al-Lawati and Bienefeld 2009), and in some cases may be a better predictor of offspring survival in the context of maternal effects. Other egg components may also be under maternal influence. For example, transgenerational immune priming (TGIP) refers to a phenomenon where offspring from a mother exposed to a pathogen are prepared via maternal effects to mount an immune response (Salmela et al. 2015). There is evidence of TGIP in honey bees: the yolk protein vitellogenin, binds to a pathogen within the queen, and as vitellogenin accumulates in the eggs during oogenesis, the pathogen is taken into the egg. As a result, offspring show decreased susceptibility to the pathogen (Salmela et al. 2015). Thus it is possible that a queen's status, e.g., disease state or age, directly impacts certain aspects of offspring phenotypes. However, no study has evaluated whether queen stress generally impacts health and behavior of adult offspring.

This study utilizes a repeated-measures design to compare the health and behavior of a queen's offspring before and after she experiences a two hour, 4°C cold stress treatment. I selected the cold stress from a study where the quality of sperm in the spermatheca of a honey bee queen was assessed after exposure to temperatures

that mimic the effects of shipping queens overnight in the mail (Pettis et al. 2016), a common apiculture practice. This test determined that the cold treatment decreased stored sperm viability by ~40%. This cold exposure likely exceeds stress experienced by a queen in a natural context, but it is a paradigm with proven biological impacts on queens, and thus provides an assessment of the potential impact of other more realistic queen stressors on offspring phenotypes.

To assess the offspring from before and after queen cold exposure, I selected tests to look for treatment effects at different life stages of the offspring. Developmental stages including egg hatching rate, egg protein content, and emergence rate were selected for their documented effects in maternal effects literature (Al-Lawati and Bienefeld 2009). To address permanent effects from cold stressed queens, adult offspring were assessed for immune function and behavior. It is often unclear how variation in behavior at the colony level predicts colony survivorship or health (Cremer 2018); however, worker aggression is a general indicator of health resilience in the honey bee. At the colony level, aggression is a strong positive predictor of foraging activity, honey and brood production, and overwintering success, as well as a negative predictor of *Varroa* mite loads (Wray *et al.* 2011; Rittschof *et al.* 2015). On the individual level, aggression predicts increased starvation and pesticide tolerance (Rittschof *et al.* 2015). Moreover, aggression appears to be a socially inherited behavior across worker generations (Rittschof *et al.* 2015), suggesting this behavior could be used to study how queen stress propagates throughout worker cohorts over time. To measure impacts of queen cold stress, I assessed offspring development rate (egg

hatching probability and emergence time), egg composition, adult immune gene expression, and adult aggressive behavior.

Chapter 2. Materials and Methods

Overview

The unit of replication within this study is the honey bee queen as only one queen is present in a honey bee colony. To limit the effects of this study on colonies within the apiary, I set up experimental colonies that I continuously reused. With the monthly replacement of the queen and removal of offspring, I anticipated that the experimental colonies would be greatly weakened and may not contain adequate nurse bees to successfully rear offspring if the ratio of brood to nurse bees declined below 2:1 (Amdam and Omholt 2002) as the study progressed. To mitigate the effects of the experimental colony, I randomized placement of offspring from each queen into strong foster colonies within 24 hours of ovipositing where they were allowed to mature.

Honey bee sources

Honey bee queen breeders can be a source of variation in queen quality. Although a large amount of variation can exist among queens within one breeder (Tarpy et al. 2012), I purchased same age mated queens from a single supplier (Guthries Naturals, Frankfort, KY, USA) at the start of each replicate to minimize the effect of queen breeder on the study. The Rittschof lab formed experimental colonies from splits of research colonies of mixed genotypic origin of *A. m. carnica* and *A.m. ligustica*, supplemented with a package of bees (Guthries Naturals, Frankfort, KY, USA). I allowed colonies to increase in population size to >10 frames of brood in 2 10-frame boxes for 4

weeks prior to the start of the experiment. Eight foster colonies were >1 year old, 2 10-frame box colonies containing a large number of bees, with >8 frames of brood (overwintered at the University of Kentucky or purchased from Hosey Honey, Midway, KY, USA).

Experimental Set-up

Between April and August 2017, I completed this study in 4 blocks of 8 queens. Start dates for each block were separated by 4-week time intervals. To test stress impacts on a queen of known age and origin (see below), I installed her into a pre-existing experimental colony, for practical reasons. Similarly, because behavioral assays had a strict timeline (see below), I started queens in pairs at 2-day intervals within each block (Fig 1). Since honey bee workers develop at a fairly uniform rate, staggering the start date across the different queens ensured that at least some offspring in all treatments would be assessed for behavior on the same phenological day (in case day of assay impacts behavioral expression). Having different treatments represented on overlapping days also allowed me to perform behavioral analyses blind to the treatment identity of the offspring (see below).

Within and among blocks, queens were identical in age on the block start date. To begin the experiment for a given queen, I located and removed the queen heading the experimental colony, and allowed the colony to remain queenless for two days. This allowed the colony to recognize the absence of the queen, which increases the likelihood the new queen will be accepted (Perez-Sato et al. 2015). After two queenless

days, I placed the queen within a wooden cage inside the colony, wedged between two frames to hold it in place, for four days until the workers of the colony were no longer aggressive (Graham 2015). During this period, workers can sense and feed the caged queen, but are unable to sting her. I then manually released the queen and left the colony undisturbed for the next 14 days in order to allow the queen to begin laying eggs. Honey bee worker eggs hatch within 72hrs (Winston 1987). Thus, due to the time that elapsed since removal of the original queen from the experimental colony, I could be sure that any eggs in the colony following this 14-day period were laid by the newly introduced queen.

Establishing treatment groups

To determine impacts of queen stress, I assessed three different sets of offspring. First, 14 days following queen installation, and prior to any additional disturbance to the queen or colony, I located a frame containing eggs that were approximately 24-hour old (I estimated age based on the vertical orientation of the egg within the honeycomb cell (Winston 1987)). I designated these eggs as the 'handling control', to control for the impacts of queen handling just prior to laying eggs (compared to my 'control' group described below), I then located the queen and caged her against an empty frame with drawn honeycomb using a 'push-in cage'. This cage (40.5cm long by 19cm wide by 3cm tall) consisted of hardware cloth (#8, Amazon.com, Seattle, WA, USA) around the perimeter of the cage and a plastic queen excluder (35cm long by 15cm wide, 0.5cm opening) glued into the center of the cage capable of cover 80% of one side

of frame (Fig. A1). This design trapped the queen so that I could collect eggs of a known age (compared to the handling control above), but also allowed workers access to the queen and the eggs. I caged the queen for 24 h and designated these eggs as 'Control'.

After the control caging period, the queen was also removed using a queen clip catcher (Dadant and Sons Inc., Hamilton, IL, USA) and placed into a refrigerator at 4°C for 2 hours (following previously published methods in (Pettis et al. 2016)). The queen entered into a light chill coma during treatment, and revived within minutes of removal from the refrigerator. After the cold treatment, I placed the queen, still inside the queen clip, back into the experimental colony. I allowed the queen to recover from the treatment for one hour. Due to the design of the queen clip, workers were able to access the queen during this time. After 1 hour, I re-caged the queen under the push-in cage on a new frame with drawn out honeycomb. As with the control, I left the queen for 24 hours to lay eggs. Pilot studies during Summer 2016 showed that eggs laid during the first 24-hr following treatment often fail to hatch (25%, N=4 queens). Despite the cold stress decreasing the sperm viability within the queen, dead sperm will not alter the embryogenesis but will yield haploid eggs that are capable of hatching (Mackensen 1951, Baer et al. 2016), therefore the eggs from first 24-hr following treatment may fail to hatch due to the fragile state of the immature egg entering late stage of meiosis I, prior to fertilization and chorion formation (Yu and Omholt 1999, Rinderer 2008) during the cold stress. Eggs laid within the next 24 h period; however, were more likely to hatch (100%, N=4 queens). Therefore, I re-caged the queen for an additional 24 hours,

designating these offspring as "Cold treatment". Following this final 24 h caging period, I removed the queen from the colony to initiate the next block of the experiment.

I removed each frame of eggs (the handling-control, the control, and cold treatment frames) from the colony as soon as the 24-hour queen-caging period had lapsed (or in the case of the handling control, upon finding the appropriate frame in the colony). Within 30 minutes of removing a queen, I collected 20 eggs using a grafting tool (Mann Lake LTD, Hackensack, MN, USA) and stored them in pairs in microcentrifuge tubes at -20°C for later assessment of egg protein content. In my pilot study, eggs were evaluated for size differences to align my data collection to traditionally measured maternal effects. Egg size is variable, within queen (Q1 N=15 eggs, mean length ± s. e. = 1.5±0.2 and mean width ± s. e. = 0.3±0.1; Q2 N=18 eggs, mean length ± s. e. = 1.4±0.1 and mean width ± s. e. = 0.3±0.05) and among queens (N=2 queens, mean length ± s. e. = 1.4±0.2 and mean width ± s. e. = 0.3±0.07); however, the variation is not a function of treatment (Table A1) and I did not evaluate egg size in the larger study. I randomized all study frames into strong foster colonies to alleviate any experimental colony effects. Within 30 minutes of removal of experimental colonies, I introduced each frame of remaining eggs into a foster colony for the duration of development. Using a random number generator (Random.org c1998-2018), I assigned frames evenly across the eight colonies (3 frames per colony for each block). Using a prior established procedure (Rittschof et al. 2015), I placed frames in the brood nest in the lower box, placed a queen excluder between the bottom and top box, and moved the foster colony queen to the top box to prevent egg laying on study frames. Four days after I added the last

study frame to the foster colony (at which point all eggs from study frames would have hatched), I removed the queen excluder from the hive to allow the queen to move freely.

Assessments

Six days after each queen caging period (handling-control, control, cold treatment), I observed offspring for the presence for eggs or larvae (honey bee eggs typically hatch in 3 days after laying). I assessed hatching success, which was all or none for a given frame of eggs, as a binary response (Yes/No, N=20 queens). Other than this check, I allowed brood to develop undisturbed until one day prior to adult emergence (17 days after the queen was removed from the frame). On this day, I removed the frame and placed it in a circulated air incubator kept at 33.5±0.5°C and constant darkness. Twice a day, the morning and afternoon, I checked frames for one-day-old bee emergence following removal from the foster colony. Once the bees started to emerge, I recorded the number of bees that emerged from each frame each day. Some frames took multiple days to emerge. I report emergence time as the number of days between laying (the day the queen was released after 24 h of caging was day 1) and emergence.

As bees emerged (0-24h old), I placed them into Petri dishes (100mm x 15mm, Thermo Scientific, Waltham, MA, USA) modified with an entrance hole (4 bees/dish, 25 dishes/frame) and provisioned with 50% sucrose solution in a microcentrifuge tube (VWR International, Radnor, PA, USA) modified with two feeding holes. I labeled each

dish with a random number (Random.org c1998-2018). Once I transferred bees into their dishes, I returned dishes to the incubator until bees were 7 days old, at which point I assessed aggressive behavior using the Intruder Assay (described below). I placed an additional 25 emerging bees into 8 cm x 9.5 cm X 6.5 cm plexiglass boxes with ventilation holes, provisioned with 50% sucrose in a microcentrifuge tube (see above), to be used for immune competence testing (see below).

Egg Composition

I thawed samples (N=10 samples, 2 eggs/sample) on ice, added 200µL of distilled water and homogenized with a micro-pestle (Wegener et al. 2010, Foray et al. 2012). Utilizing 50 µL of homogenate, I quantified the protein with a Micro BCA Protein Assay kit following the manufactures protocol (Thermo Scientific, Waltham, MA, USA).

Adult Immune Competence - Fat Body Gene Expression Analysis

This study involves the comparison of immune competence of offspring exposed to a maternal cold stress. I used the fat body to allow for a more generalized analysis of immune competence. The fat body of an insect produces are variety of proteins including vitellogenin (Amdam and Omholt 2002) and antimicrobial peptides (Richard et al. 2012), functions as a part of the humoral immune system (Wilson-Rich et al. 2008) and is analogous to the liver and white adipose tissue of vertebrates (Nunes et al. 2013). The goal of my gene expression analysis is to determine if cold stress on a queen effects the immune competence of her offspring. Using the unbiased meta-analysis of a 19-transcriptome dataset (Doublet et al. 2017), I selected genes with the criteria that 1.)

that they are associated with the canonical immune system of the bee and 2.) their regulation is generalized across a range of pathogens and stressors (including *Varroa* mite feeding, viruses, and bacteria) because the direct nature of queen stress response is not known. The 8 candidate genes are described below.

From the genes selected for this study, I selected two of these genes from the prophenoloxidase genes that catalyze melanization in nodulation and encapsulation immune responses (Steinmann et al. 2015) of the Imd/JNK pathway (Doublet et al. 2017) and have been found to be up-regulated in a natural infection (Evans et al. 2006). I selected vitellogenin for its role in immune response (by transporting zinc throughout the worker bee to minimize pycnosis in the haemocytes (Amdam et al. 2004)). The other five candidate genes (abaecin, defensin-1, hymenoptaecin, lysozyme-2, and apidaecin) are known as antimicrobial peptides and are directly associated with the Toll pathway of insect immunity (Evans et al. 2006, Doublet et al. 2017).

Fat body dissection, RNA extraction, and gene expression quantification was completed in collaboration with an undergraduate student and Joseph Palmer, the Rittschof laboratory technician. The student dissected the abdomen to remove the fat body from frozen adult offspring in RNAlater (Thermo Scientific, Waltham, MA, USA) chilled on ice in order preserve the RNA. After dissecting the fat body, attached to the sclerite, from abdomen, the student extracted the RNA using E.Z.N.A. HP Total RNA kit (Omega Bio-Tek, Norcross, Georgia, USA), following the manufactures protocol, after homogenization in lysis buffer (from RNA kit) with four 0.7mm zirconia/silica beads (Bio-spec, Bartlettesville, OK, USA) in a bench top homogenizer (MP Biomedicals, Santa Anna,

CA, USA). The student quantified the RNA using a CLARIOstar microplate reader with LVis plate (BMG Labtech, Cary, NC, USA), synthesized cDNA using 200ng RNA and SensiFAST cDNA Synthesis Kit (Bioline, Taunton, MA, USA) and performed qPCR on a Quanta Studio 6 (Thermo Scientific, Waltham, MA, USA) with 10µL reactions (in triplicate) in 384-well plates using PerfeCTa SYBR green supermix (Quanta Bio, Beverly, MA, USA). The Rittschof laboratory technician and student assessed gene titers using previously published primers (Evans et al. 2006) and quantified against a DNA curve generated from whole DNA from a honey bee, with the exception of the target gene apidaecin (see below). To obtain a relative quantity, I normalized sample titers to the geometric mean of 2 continuously expressed control genes *gapdh* (GB50902) and *rp49* (AF41189). I selected these two controls based on preliminary data showing low expression variation in the fat body and I verified that these two endogenous controls had a coefficient of variation across all samples that was less than or equal to 20%, and that the controls were not differentially expressed across treatments. Due to the short exons, it was not possible to design primers to amplify the standard curve for the target gene apidaecin; therefore, I used the delta delta CT method to assess relative quantity.

Intruder Assay

To form a social group, I allowed bees to age together in dishes for 7 days. When bees were the appropriate age, I removed dishes to a temperature controlled ventilated laboratory space (25-30°C) with no light. I divided dishes from each treatment group on a given day evenly across observers (typically 2 observers per day), placed on a table top

in random order and I allowed bee to acclimate for 1 hour undisturbed prior to testing. Using the previously established Intruder Assay (Li-Byarlay et al. 2014, Rittschof et al. 2015, Rittschof 2017), I assessed aggression. At the start of the assay, one intruder bee (forager from a different colony) is marked on the thorax with a paint pen (Elmer's, High Point, NC, USA) for identification and introduced into the dish of 4 bees. Over 1 minute, I scored the following behaviors: antennation (scored as 1 point), movement of the antenna of the treatment group bee toward or on the intruder bee; antennation with mandibles open (scored as 2 points), similar to antennation but mandibles of the treatment group bee are open, possibly to release a pheromone to threaten intruder; biting (scored as 3 points), the mandibles of a group member clamp down or pull on the intruder bee; abdominal flexion (scored as 4 points), a group bee mounts or clings to intruder flexing its abdomen but not extruding stinger; sting (scored as 5 points per attempt or per 10 second duration), a group bee mounts or clings to intruder flexing its abdomen, extruding its stinger, and actively trying to sting the intruder. After behavioral assays, I examined dishes for *Varroa* mites. I calculated scores for each individual behavior as well as a total score (using individual behaviors with either sting attempt or duration) for each dish and then I divided each calculation by the total number of bees per dish for a final score. With stinging behavior, some bees may attempt to sting the intruder for a brief period of time and then return to the stinging behavior a few seconds later, while others may spend the entire 1 min test stinging the intruder. It is difficult to determine which stinging calculation (attempt or duration) best describes

stinging behavior; therefore, I will utilize individual behaviors for multivariate behavior analysis and both total scores (separately) will be used for any post-hoc anaylsis.

Statistical Analysis

All statistics were analyzed using JMP Pro 13.2 software package (JMP 2018).

The handling-control was difficult to obtain for each queen due to the variation of ages of offspring available and the detrimental effect of removing too much offspring; therefore, queen sample size of the handling-control group is much lower for egg hatching, larval development time, and behavior. To increase the power of my statistical tests, I analyzed the handling-control against the control and then I analyzed the control against the cold treatment for hatching success, emergence time, and behavior. I did not utilize the handling-control for egg protein content or immune competence testing due to low sample sizes and unreliable age when offspring were removed from the experimental colony (see results).

I treated hatching success (yes/no) as a nominal response variable, which I analyzed using a McNemar's Test, paired for queen. Handling-control vs control has a sample size of N=9 queens per treatment. Control vs. cold treatment has a sample size N=20 queens per treatment.

On a per-offspring basis, I calculated emergence time and treated it as a continuous response variable. For each data set, I used a linear mixed model to analyze emergence time with queen (random, categorical), treatment (categorical) and their

interaction as factors. Handling-control vs control has a sample size of N=4 queens. Control vs. cold treatment has a sample size N=11 queens.

I assessed egg protein content as a continuous response variable and constructed a mixed effect model using queen (random, categorical), treatment (categorical), and their interaction as factors.

Using the offspring from 3 queens (chosen at random), I assessed the immune competence of the offspring using the relative quantity of each target gene as a continuous variable. I analyzed each queen individually because cDNA was not synthesized at one time and used a non-parametric Wilcoxon/Kruskal-Wallis test with treatment as a categorical variable.

The observer of the intruder assay has an effect on the duration total score for behavior (N=3 observers; 18.4 ±0.2, 9.9 ±1.5, 11.8 ±1.0 scores; ANVOA, $F_{(2, 230)} = 5.4$, $P = 0.005$). Since this total score also accounts for individual scores, I transformed all individual and total scores into z-scores to account for the variability of the observer and to preserve power of my testing. I constructed a MANOVA model with individual behavior scores as continuous response variables with queen (categorical), treatment (categorical), and the interaction of queen and treatment as the predictor variables. Additionally, I also developed a linear mixed model with total scores (attempt or duration as z-scores) continuous response variable with queen (categorical), treatment (categorical), and the interaction of queen and treatment as the predictor variables. Handling-control vs control has a sample size of N=3 queens. Control vs. cold treatment has a sample size N=11 queens.

Figures for Methods

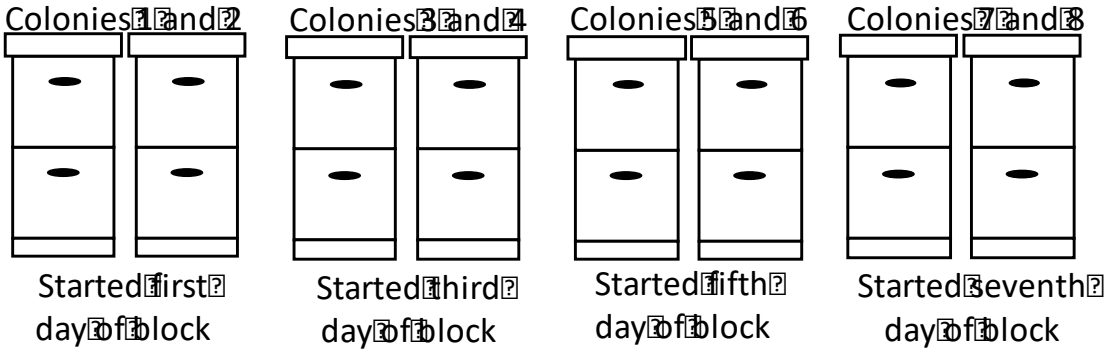

Figure 1. Experimental arrangement of treatments within a block.
Each experimental block is subdivided into four sections. Each section of colonies was then started every two days to allow for overlap of control/treatment brood development and eclosion.

Chapter 3. Results

I found no evidence that queen handling alone impacted egg hatching success of the offspring (N=9 queens, 9 sets of handling-control eggs hatched, 8 sets of control eggs hatched, McNemar test, $X^2_{(1).} = 0$, P=1, Table 1). Cold stress significantly decreased egg hatching success relative to the control (N=20 queens, 17 sets of control eggs hatched, 13 sets of cold treatment eggs hatched, McNemar test, $X^2_{(1).} = 4$, P<0.046, Table 2). Cold stress also significantly impacted offspring emergence time. Queen identity, cold treatment, and their interaction all significantly impacted offspring emergence time, a measure of developmental rate (LMM: queen: Wald P = 0.03, treatment: $F_{(1, 2183.1)} = 10.4$, P = 0.001, interaction of queen and treatment: $F_{(10, 2177.2)} = 123.0$, P <0.0001). Control bees took less time to develop into adults compared to cold treatment (N=11 queens, control: 20.8 ±0.7 days versus cold treatment: 21.3 ±0.5 days, Fig 2). Offspring from 8 of 11 queens showed evidence of extended emergence time following cold stress. I found some evidence of an effect of handling on emergence rate (N=4; handling-control: 19.7 ±0.4 days versus control: 20.3 ±0.7 days; LMM: queen: Wald P = 0.2, treatment: $F_{(1, 836.8)} = 3713.3$, P <0.0001, interaction of queen and treatment: $F_{(3, 836.4)} = 772.3$, P <0.0001; Fig. A2), but I observed a strong queen by treatment interaction effect that reflects variable patterns for two of the four queens. Thus, while emergence time differs across my controls, unlike for the cold treatment and control comparison, there is no clear pattern in the direction of this effect.

Despite variation in egg hatching success and emergence time, offspring egg protein content showed no effect of queen cold treatment (N=8, control: 6.1 ±2.7µg

versus treatment: 6.0 ±2.1µg, LMM: queen: Wald P = 0.09, treatment: $F_{(1, 141.1)} = 0.4$, P = 0.5, interaction of the queen and treatment: $F_{(7, 141.0)} = 1.8$, P = 0.09, Fig. 3). There were also no consistent significant impacts of queen cold stress on offspring immune gene expression for any of the 8 target genes tested. Results for the statistical analyses are listed in Table 3.

The queen variation found in the emergence time of the offspring is continued with the comparison of six individual behaviors between control and cold treatment eggs. The MANOVA model is significant for queen (N=11 queens; MANOVA overall model: $F_{(21, 333)} = 2.6$, P = 0.002; sphericity: $X^2_{(14)} = 391.1$, P < 0.001; queen: Pallai's Trace $F_{(50, 1665)} = 2.0$, P < 0.001). With further analysis of univariate models for queen, using ANOVA, the following behaviors are significantly influenced by queen identity: antennation ($F_{(10, 344)} = 3.31$, P = 0.004), bite ($F_{(10, 344)} = 2.10$, P < 0.02), flexion ($F_{(10, 344)} = 3.00$, P = 0.001), sting attempt ($F_{(10,344)} = 4.86$, P < 0.001), and sting duration ($F_{(10,344)} = 4.85$, P < 0.001). The control and cold treatment samples sizes (listed by queen) for are list in Table A2. Multivariate and univariate statistics for control and cold treatment behaviors are listed in Table A3. I found some evidence that handling impacts adult behavior with the comparison of the handling-control and control using MANOVA model with a significant treatment (N=3 queens; MANOVA overall model: $F_{(5, 118)} = 3.0$, P = 0.01; treatment: $F_{(1, 118)} = 5.5$, P = 0.02). For the 6 individual behaviors analyzed for handling-control and control, univariate models using an ANOVA for treatment, mandibles open is the only significant response ($F_{(1, 122)} = 5.4$, P = 0.02; treatment: $t_{(1, 122)} = 2.3$, P = 0.02). The control for the individual mandibles open behavior shows a

consistent increased score compared to the handling-control for all three queens (mean z-score ±s.d., control: 0.3±1.1 versus handling-control: -0.1 ±0.9). Multivariate and univariate statistics for handling-control and control behaviors are listed in Table A4. For the aggression attempt total score, the handling-control versus the control shows of individual behaviors (N=3 queens, score, handling-control: 0.2 ±1.0 versus control: -0.1 ±0.6, LLM: queen: Wald P = 0.9, treatment: $F_{(1, 118.7)} = 5.6$, P = 0.02, interaction of the queen and treatment: $F_{(2, 118.5)} = 2.5$, P = 0.09). The aggression duration total score shows very similar results to the aggression attempt total score.

Additionally, I observed a significant negative correlation between both aggression attempt total score (z-score averaged by queen, $R^2=0.49$, LMM: $F_{(1,8)} = 9.75$, P<0.014; Fig. 4) and a similar correlation for duration total score (z-score averaged by queen) for behavior and egg protein content, averaged by queen.

Tables and Figures for Results

Table 1. Egg hatching rate of offspring before and after caging queen.

	N = 9 queens	
	No Hatch	Hatch
Handling-control	0	9
Control	1	8
McNemar's test p-value	1.0	

Table 2. Egg hatching rate of offspring from queen before and 48-hr after temperature stress.

	N = 20 queens	
	No Hatch	Hatch
Control	3	17
Cold Treatment	6	14
McNemar's test p-value 0.046		

Table 3. Mean immune competence target gene expression among offspring produced by different honey bee queens before and 48-hr after cold treatment.

		Control		Cold Treatment		Wilcoxon/Kruskal-Wallis	
	Queen	bees/treatment	Mean expression ± s.d.	bees/treatment	Mean expression ± s.d.	X^2	*P* -value
Abaecin	1	9	0.7 (±0.9)	6	26.6 (±36.5)	2.35	0.13
	2	9	0.4 (±0.6)	9	0.07 (±0.2)	0.02	0.89
	3	8	1.9 (±5.2)	10	0.4 (±0.9)	0.03	0.86
Defensin-1	1	9	1.7 (±3.6)	6	6.4 (±8.9)	0.35	0.56
	2	9	0.2 (±0.2)	9	0.3 (±0.4)	0.02	0.89
	3	8	2.5 (±2.5)	10	1.5 (±2.7)	1.55	0.21
Hymenoptaecin	1	9	9.3 (±24.3)	6	70.9 (±99.3)	0.35	0.56
	2	9	0.2 (±0.2)	9	1.0 (±2.2)	0.44	0.51
	3	8	27.0 (±37.3)	10	20.1 (±29.0)	0.96	0.33
Prophenoloxidase	1	9	0.06 (±0.03)	6	0.06 (±0.03)	0.07	0.79
	2	9	0.1 (±0.04)	9	0.07 (±0.04)	1.87	0.17
	3	8	0.04 (±0.02)	10	0.03 (±0.01)	0.2	0.66
Prophenoloxidase activator	1	9	0.05 (±0.02)	6	0.06 (±0.01)	0.06	0.81
	2	9	0.04 (±0.01)	9	0.03 (±0.02)	1.03	0.31
	3	8	0.03 (±0.02)	10	0.03 (±0.01)	0.2	0.66
Vitellogenin	1	9	0.4 (±0.4)	6	0.1 (±0.2)	1.39	0.24
	2	9	0.4 (±0.3)	9	0.5 (±0.4)	0.1	0.76
	3	8	0.06 (±0.03)	10	0.2 (±0.2)	1.33	0.25
Apidaecin	1	9	4.1 (±0.9)	6	0.9 (±1.3)	2.34	0.12
	2	9	2.5 (±2.1)	9	7.1 (±15.2)	0.002	0.96
	3	8	273.4 (±770.7)	10	1.4 (±1.9)	0.23	0.63
Lysozyme-2	1	9	0.06 (±0.4)	6	0.5 (±0.4)	0.68	0.41
	2	9	0.06 (±0.04)	9	0.2(±0.2)	1.22	0.27
	3	8	0.5 (±0.3)	10	0.3 (±0.2)	2.02	0.15
Queen and cold treatment of queen have no effect on normalized gene expression of offspring.							

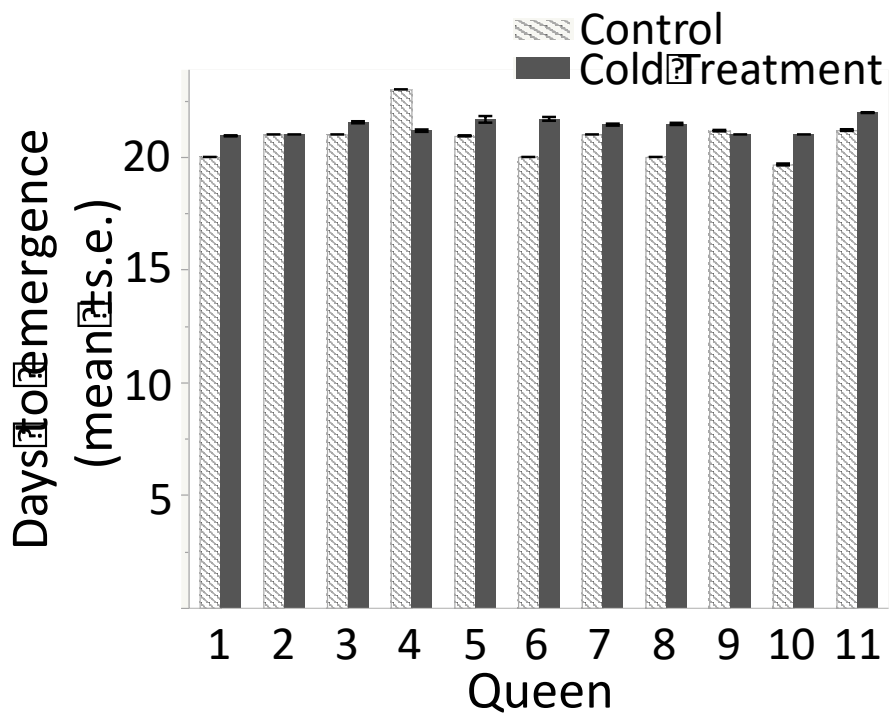

Figure 2. Emergence time of honey bee workers laid by queens increased following cold exposure.

For 8 of 11 queens, the control offspring took less time to emerge than the offspring following queen cold stress. Queen (Wald $p = 0.04$), cold treatment ($p = 0.01$), and their interaction ($p < 0.0001$) all impacted emergence time.

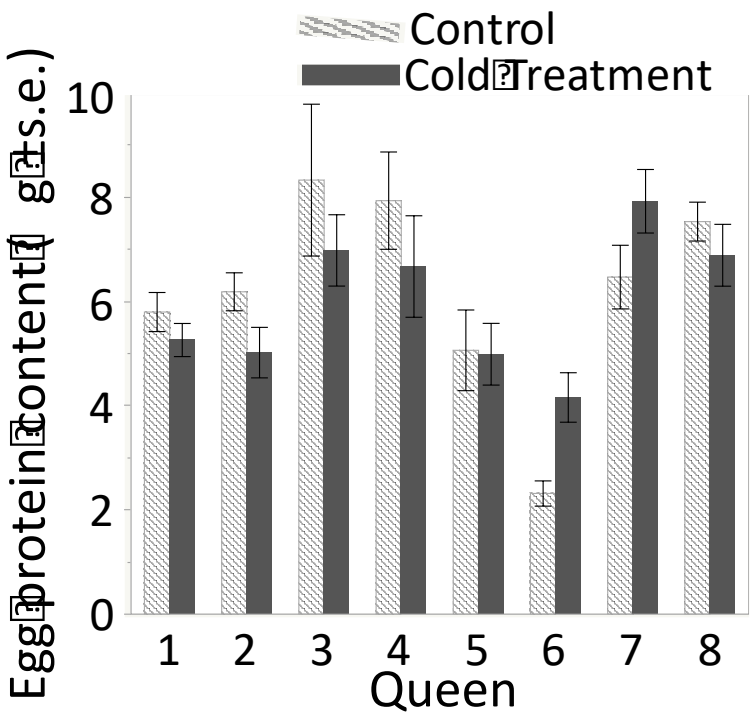

Figure 3. Offspring egg protein content did not differ as a function of queen cold stress. Protein content did not differ as a function of queen identity (Wald p = 0.08) or queen cold treatment (p-value 0.5).

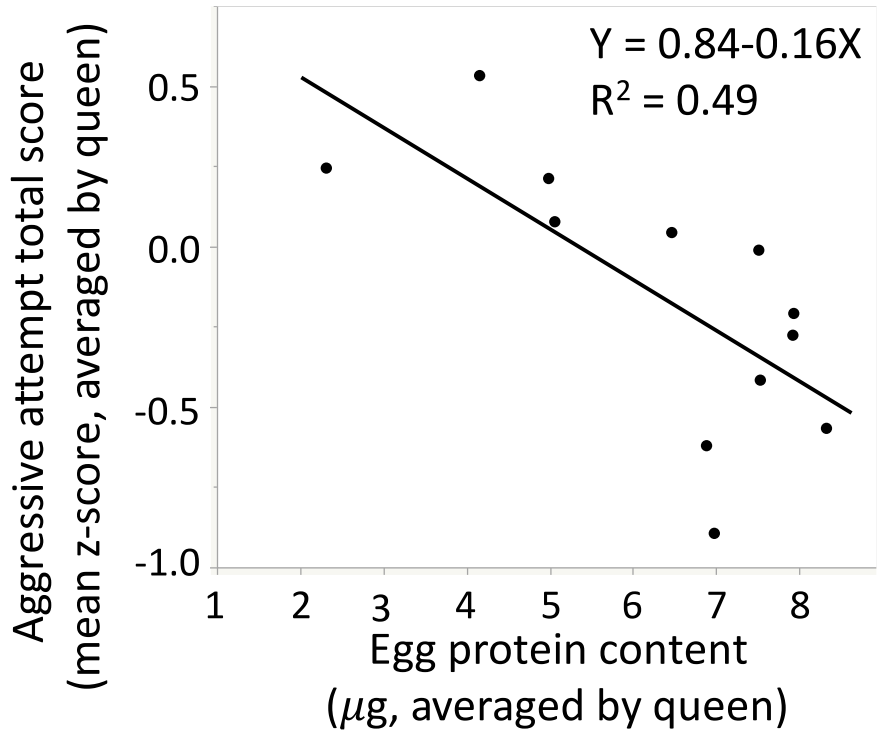

Figure 4. Worker aggression is negatively correlated with egg protein content. Mean aggressive behavior attempt (z-score, averaged by queen) is significantly negatively correlated with mean egg protein concentration (averaged by queen) and significant ($p = 0.01$).

Chapter 4. Discussion

Over all of the tests conducted to measure the impacts of queen stress on honey bee workers, egg hatching rate and emergence time were affected by the queen cold treatment. Offspring analyzed for gg protein content, adult immune competence, and adult aggressive behavior did not show an effect of the queen stress; however, the immune competence and behavior of the offspring did vary by queen.

Cold treatment impacted early developmental processes, including egg hatching success and offspring emergence time, but it did not impact behavior or immune function during the offspring adult stages. These results suggest that while queen stress impacts the early life stages, surviving adults show no lasting effects. One implication of this finding is that queen stress impacts adult worker bee quantity but not quality. Decreased egg hatching success and emergence delay could affect the overall health of the colony by reducing the population over time with a smaller number of adults per worker cohort, and a delay in worker turnover. Inviable female eggs may make it more difficult for the colony to replace the stressed queen, or expend more effort attempting to do so, because there are fewer viable choices among female offspring. Given a strong enough deficit in productivity, queen replacement is essential to colony survival without the intervention of the apiculturist.

The cold stress effects I observed could be a direct result of egg exposure to cold temperatures, as opposed to an indirect effect of queen stress on some feature of egg provisioning or development. Studies determining the cryopreservation temperatures of honey bee embryos found that embryos (less than 2hr after laying) have a low

survival rate following cold (0°C) temperature exposure, because they are in the pre-cellular, syncytial state (Collins and Mazur 2006). Though this study evaluated embryos (2 hours following oviposition) while I exposed offspring to cold prior to oviposition, the sensitivity to direct cold exposure may also extend to the late stage oocytes within the queen prior to fertilization. Additionally, I observed high egg hatching failure for eggs laid during the 24 h directly following queen cold stress suggesting eggs closer to oviposition are relatively more sensitive to cold than less developed eggs. I did not track offspring beyond 48 h following the queen cold temperature stress; it is possible that over a longer time frame and turnover of eggs directly exposed to temperature stress, offspring would return to normal. Nonetheless, even a temporary decrease in worker number could have lasting impacts on the colony.

In addition to environmental factors, genotype affects several characteristics I measured in this study, including developmental pacing (Amdam et al. 2010) and behaviors including aggression (Guzmán-Novoa and Page Jr. 1999). Thus unsurprisingly, I observed substantial variation in several measured variables as a function of queen identity. Queen identity affected offspring emergence time, and it had consistent effects on aggressive behavior regardless of treatment. The consistent queen effect suggests that my behavioral analysis was sensitive enough to identify genetic differences in behavior, despite no evidence of additional treatment effects. Though there was a non-significant trend for an effect of queen on egg protein content, and the strong correlation between aggression and egg protein content provides additional evidence of a genetic basis for two factors.

The strong, negative correlation between total aggressive behavioral scores and egg protein content was an unanticipated result of this study. In the gypsy moth, low egg protein content is correlated with small adult body size (Diss 1996); a similar relationship could exist for honey bees. Comparing highly aggressive Africanized sub-species of honey bees to the more docile European-derived sub-species, adult body size (forewing length) is negatively correlated with aggression (Guzmán-Novoa and Page Jr. 1999), further suggesting that egg protein content may be serve as a predictive measure for aggression within a colony. More research needs to be completed on the relationship between egg content, adult body size and behavior, across several genotypes, determine the definitive relationship.

While the relationship of treatment within emergence time and egg protein content is fairly consistent across most queens, some queens did not follow the same relationship leading to an interaction between queen and treatment. Although the control emerged 3% faster than the treatment day 2 for emergence time of the offspring (9 of 11 queens), this trend was not consistent across all queens (2 of 11 queens). The trend continues with egg protein content where the control has an average of higher mean protein (3%) than the treatment day 2 (6 of 8 queens). Factors outside the scope this study may be the source variation between the treatment and the queens.

Contrary to the results of environmental impact and genotype, there was no detected impact on worker innate immune competence from cold stress to the queen. The selected genes for this study did not measure the direct response to cold stress in

the worker, rather I selected them to measure baseline immune activity. It may be that the effect of queen cold stress is only evident on her offspring's immune function when the immune system is activated. A potential way to activate these pathways in future studies would be to use an immune challenge with yeast (Di Prisco et al. 2013) and compare the target gene activation for challenged and non-challenged bees.

In addition to the environment and genotypic effects on offspring, queen caging has an effect on emergence time and aggressive behavior. While the act of caging the queen may be producing a stress that is detectable in the offspring, an alternative explanation to the caging effect is that the sample size of this portion of my study is too low to adequately estimate the population mean and the selection of the handling-control was imprecise in the age estimation of the eggs. Further replication of this portion of the study would be needed to draw conclusive determination of the treatment effects between the handling-control and control but any effect of caging is standardized across my study due to the repeated measures experimental design and I feel that the results between the control and treatment groups is valid.

The role of maternal effects is debated in honey bees with the queen not conveying information about the environment to her offspring, however queen stress can be transmitted to her offspring. Cold stress on the queen affects the early life stages of her offspring, which will have a lasting effect in the colony. Once the offspring matures to an adult, however queen cold stress does not appear to have an effect on the health of the colony because the behavior and immune competence of her offspring does not change. Healthy sisters are still raising healthy sisters, although the reduced

colony population may still result in the collapse of the colony. To mitigate the effect of queen cold stress, the colony must quickly replace the effected queen to maintain a minimal population level within the colony.

Appendix

Table A1. Morphometric egg measurement.

			Length		Width		Ratio width:length	
	Number of eggs measured		mean µg ± sd		mean µg ± sd		mean µg ± sd	
Queen	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment
1	6	9	1.4±0.2	1.5±0.2	0.3±0.05	0.3±0.1	0.2±0.02	0.2±0.07
2	8	10	1.3±0.1	1.4±0.1	0.3±0.04	0.3±0.05	0.2±0.04	0.2±0.03
Overall	14	19	1.4±0.1	1.4±0.2	0.3±0.05	0.3±0.09	0.2±0.03	0.2±0.05

Table A2. Aggressive behavior sample sizes among offspring produced by different honey bee queens before and 48-hr after cold treatment.

	N = 11 queens	
	assays/queen	
Queen	Control	Cold Treatment
1	20	12
2	20	20
3	20	12
4	20	15
5	20	6
6	20	21
7	13	11
8	17	10
9	18	19
10	7	18
11	16	20
Overall	191	164

Table A3. Aggressive behavior of adult offspring from queen before and 48-hr after cold stress, a. multivariate, b. univariate models.

a. Behavior MANOVA results for control and cold treatment.

	Exact F value	df	Error df	p-value
All Between	2.6123	21	333	0.0002
Queen	4.4015	10	333	<0.0001
Treatment	1.2087	1	333	0.2724
Queen*Treatment	0.7272	10	333	0.6988
Within Subjects	Chi-square	Df		P-value
Sphericity	391.09	14		<0.0001
	Pallai's Trace	df	Error df	p-value
All Within	1.3431	105	1665	0.0179
Behavior	1.02341	5	329	0.4033
Behavior*Queen	1.9557	50	1665	<0.0001
Behavior*Treatment	1.1652	5	329	0.326
Behavior*Queen*Treatme	0.7017	50	1665	0.9437

b. Behavior univariate results for control and cold treatment.

		Exact F value	p-value	df
Antennation	Overall model	3.31	0.0004	10
	Queen effect	3.31	0.0004	10
Mandibles Open	Overall model	1.01	0.43	10
	Queen effect	1.01	0.43	10
Bite	Overall model	2.1	0.02	10
	Queen effect	2.1	0.02	10
Flexion	Overall model	3	0.001	10
	Queen effect	3	0.001	10
Sting Attempt	Overall model	4.86	<0.0001	10
	Queen effect	4.86	<0.0001	10
Sting Duration	Overall model	4.85	<0.0001	10
	Queen effect	4.85	<0.0001	10

Table A4. Aggressive behavior of adult offspring from queen before and after caging, a. multivariate, b. univariate models.

a. Behavior MANOVA results for handling-control and control.				
	Exact F value	df	Error df	p-value
All Between	3.02	5	118	0.01
Queen	2.03	2	118	0.14
Treatment	5.54	1	118	0.02
Queen*Treatment	2.62	2	118	0.08
Within Subjects	Pallai's Trace	df	df of error	p-value
All Within	1.41	25	590	0.09
Behavior	1.06	5	114	0.38
Behavior*Queen	1.45	10	230	0.16
Behavior*Treatment	2.2	5	114	0.06
Behavior*Queen*Treatment	1.08	10	230	0.37
b. Behavior univariate results for handling-control and control.				
Behavior	Effect	F Ratio	P-value	Df
Antennation	Overall model	2.28	0.13	1
	Treatment effect	1.51	0.13	1
Mandibles Open	Overall model	5.41	0.02	1
	Treatment effect	2.33	0.02	1
Bite	Overall model	0.008	0.93	1
	Treatment effect	0.09	0.093	1
Flexion	Overall model	2.88	0.09	1
	Treatment effect	1.7	0.09	1
Sting Attempt	Overall model	2.12	0.15	1
	Treatment effect	1.46	0.15	1
Sting Duration	Overall model	0.35	0.55	1
	Treatment effect	-0.6	0.55	1

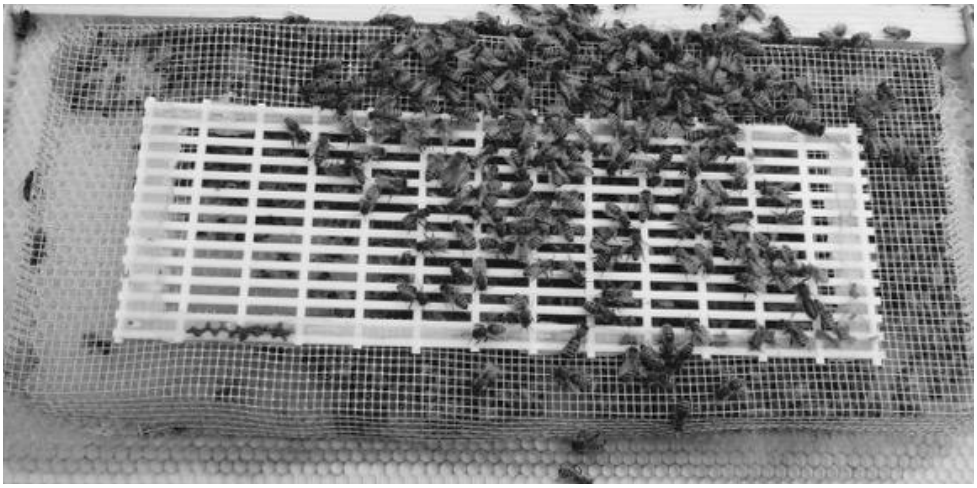

Figure A1. 'Push-in cage' made of hardware cloth and plastic queen-excluder.
This cage restrained queen to one frame to allow age of eggs to be known, but allowed the workers to enter the cage to care for queen and eggs.

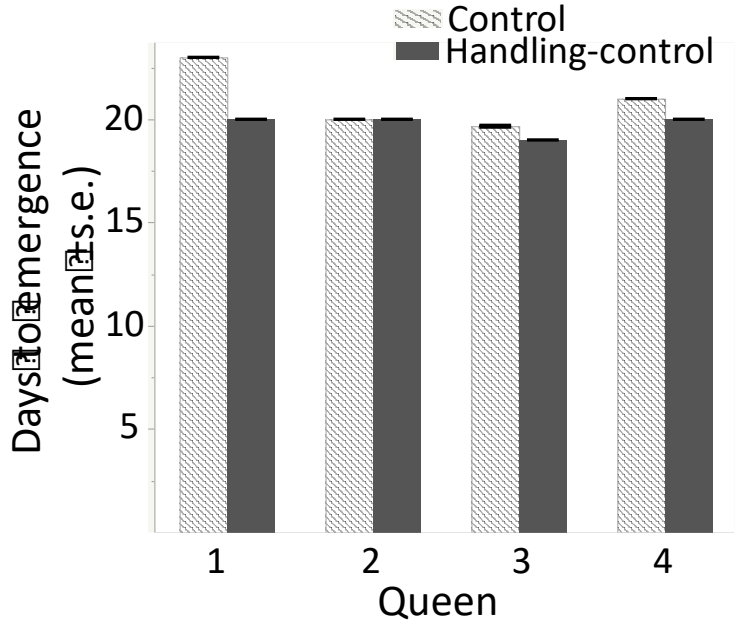

Figure A2. Mean emergence time of honey bee workers laid by queens before and after caging.
Emergence time of offspring for handling-control and control did not varied by queen (Wald P = 0.2), but the caging of the queen did have an effect on the emergence time of the offspring (P <0.0001) and an interaction of queen and treatment (P <0.0001).

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