EAT TO REPRODUCE: THE ROLE OF DIET QUANTITY ON HONEY BEE (*APIS MELLIFERA)* CASTE DETERMINATION

A Thesis
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

Garett Paul Slater

In Partial Fulfillment of the Requirements
for the Degree of
MASTER OF SCIENCE

Major Department:
Biological Sciences

March 2017

Fargo, North Dakota

North Dakota State University
Graduate School

Title

EAT TO REPRODUCE: THE ROLE OF DIET QUANTITY ON HONEY BEE (*APIS MELLIFERA)* CASTE DETERMINATION

By

Garett Paul Slater

The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

SUPERVISORY COMMITTEE:

Julia Bowsher
Chair

George Yocum

Kendall Swanson

Approved:

04/11/2017
Date

Wendy Reed
Department Chair

ABSTRACT

The received wisdom is that diet quality drives this queen-worker dichotomy, even though diet quantity differs significantly between queen and worker diet. Diet quantity likely determines honey bee caste; yet it has never been explicated tested. In this paper, we tested the hypothesis of diet quantity induced caste determination by; first, determine the ubiquity of quantity induced caste systems among highly-related taxa; second, determining the relative contributions of diet quantity vs. diet quality on adult caste in honey bees; and third, determine the influence of diet quantity on important cellular and physiological pathways known during caste determination. I found that: 1) diet quantity determines caste in honey bees and other eusocial hymenoptera, and 2) honey bees do not have a critical weight. This research likely will move forward the field of honey bee caste determination, which is a model for high-order sociality and phenotypic plasticity.

ACKNOWLEDGEMENTS

I would first like to thank my thesis advisor Dr. Julia H. Bowsher of the Department of Biology at North Dakota State University. The door to Prof. Bowsher office was always open whenever I ran into a trouble spot or had a question about my research or writing. She consistently allowed this paper to be my own work, but steered me in the right the direction whenever she thought I needed it.

I would also like to thank the experts who were involved in this research project: Dr. George Yocum, Dr. Joe Rinehart, Dr. Arun Rajmahoen and Dr. Kendra Greenlee. Without their passionate participation and input, this project could not have been successfully conducted.

I would also like to acknowledge my lab members, undergraduates, and other graduate students who were involved in this research project: Bryan Helm, Alex Torson, Dacotah Melicher, Marnie Larson, Raphael Royaute, Courtney Grulu, Kevin Cortez, Heather North, Micki Palmerson, Elizabeth Jensen, Lincoln Christenson, and Sean Nash. I am gratefully indebted to them for their valuable comments and input on this thesis.

Finally, I must express my very profound gratitude to my parents and to my Stephanie St. Aubin for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them. Thank you.

DEDICATION

To Stephanie for her unending support, level-headedness, and love. I wouldn't have gotten through my thesis without her.

To Wes for teaching me everything I know about honey bees. My thesis is impossible without him.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

QDL ..... Queen-Destined Larvae

WDL ..... Worker-Destined Larvae

PCA ..... Principal Component Analysis

PC1-2 ..... Principal Component 1-2

GLMM ..... Generalized Linear Mixed Model

MJRP1-3 ..... Major Royal Jelly Protein 1-3

MVM ..... Mean Viable Weight

MVC ..... Mean Viable Consumption

FOXO ..... Forkhead Box

TOR ..... Target of Rapmyacin

INR-2 ..... Insulin-Like Receptor 2

S6K2 ..... S6 Kinase 2

JHE ..... Juvenile Hormone Esterase

CNRQ ..... Calibrated Normalized Relative Quantities

LIST OF APPENDIX TABLES

CHAPTER 1: AN OVERVIEW OF CASTE DETERMINATION

1.1. Significance

Honey bee losses are a national concern, with an especially strong impact in the northern plains region of the USA. In 2015, 1.2 million honey bee colonies died in the United States, approximately 46% of all colonies (USDA, 2016). The decline in honeybee populations has affected production of 90 pollinator-dependent crops and over $215 billion dollars' worth of ecosystem services (K. M. Smith et al., 2013) Beekeepers in North Dakota and surrounding states lost an estimated $40 million dollar's worth of colonies in 2015, excluding lost revenue for pollination services and honey production (USDA, 2016) North Dakota suffers a disproportionate burden for hive losses because it remains first in number of hives and annual honey production per capita in the nation (USDA, 2016)Despite the economic and environmental importance of colony losses the causes remain enigmatic.

An emerging and understudied source of hive failure is poor queen performance. Beekeepers report high queen failure rates (50%) within 6 months (Pettis, Rice, & Joselow, 2016). Moreover, 15% of all colonies (2.68 million) are reported to contain low quality queens (USDA, 2016). These high failure rates and perceived risk of poor queens has led beekeepers to preemptively replace all queens annually. Historically, queens performed effectively for over three years (Mark L., 1991). Queens account for the entire reproductive output of each hive (Mark L., 1991) and are expensive ($19/queen (USDA, 2016)) to replace.

1.2. What Determines Caste in Other Eusocial Hymenoptera?

A polyphenism is when a single genotype produces multiple phenotypes (Simpson, Sword, & Lo, 2011). Many forms of polyphenisms have evolved, but the most profound form is arguably the reproductive caste system of eusocial insects (Simpson et al., 2011). In eusociality,

the reproductive and non-reproductive individuals display large differences in body size, fecundity and longevity (Oster & Wilson, 1978) and oftentimes, these traits are determined during juvenile development (Diana E. Wheeler, 1986). These life history traits are highly associated with growth during larval development. For example, body size reached during nutrient cessation determines the extent of adult reproductive capacity and longevity. Body size is highly correlated with nearly all aspects of fitness. But, caste in honeybees has never been assessed as a function of body size.

Eusociality is one of the most profound and diverse examples of complex behavior in the animal kingdom, and represents an extreme form of social organization. Three traits characterize eusociality: (1) cooperative brood care, (2) overlapping generations, and (3) reproductive division of labor, and this social structure epitomizes the transition from solitary to complex social behavior (Oster & Wilson, 1978). Because of this, eusociality is a model for sociobiology, evolutionary psychology, and selection at multiple levels of organization; and thus, eusociality elucidates the manifestation of social behavior within the animal kingdom. But despite the importance for understanding eusociality, the mechanisms that drove insects from solitary to complex sociality is poorly understand (Diana E. Wheeler, 1986), even though the mechanisms are likely conserved across taxa.

Genetically similar, but phenotypically diverse females structure insect societies. This is seen between reproductive (queens) and non-reproductive (workers) colony members, where individuals may display physiological, body size and/or morphology difference between reproductive castes (E. Wilson, 1998). Extreme morphology and body size differences can only occur during development; thus, there seems to be a developmental basis for complex societies. In Cyclical eusocial species, internal physiological systems and size differences distinguish

queens and workers, but in advanced eusocial species, they are differentiated by morphology, physiology, and/or size (Diana E. Wheeler, 1986). The increased gap between queens and workers correlates with the transition from simple social systems (cyclical eusociality) to complex social systems (permanent eusociality). The factor that determine caste and therefore facilitate eusociality, have only been broadly defined, and thus, the evolution of these mechanisms is poorly understood. Studies theorize that nutrition promotes discrete caste systems in eusocial hymenopteran, but a comparative analysis has never been performed before. ***Such an analysis would likely elucidate the evolution of social complexity in eusocial insects, and the environmental factors involved.*** In Paper 2, I performed a meta-analysis to determine which environmental and genetic factors contribute to caste determination across Hymenopterans.

1.3. Diet Quality Vs. Quantity

The life history traits between queen and worker honeybees differ tremendously, even though they are genetically analogous. Queen honeybees have 180 ovarioles, live over 2 years and are 300mg, whereas workers have 5 ovarioles, live 3 weeks and are 150mg (Seeley & Url, 1978; Snodgrass, 1910). Differences between Queen and worker nuances have been attributed to a labile substance found in the royal jelly provisioned to nascent queens, but not found in the worker jelly provisioned to larval workers (Rembold, 1965). Thus, although a labile substance has been sought after for over 100 years, quantity may be the important dietary attribute during juvenile caste determination.

Many substances have been attributed to caste determination in honeybees, which has increased the mystery of honeybee caste differentiation. Royalactin is the most abundant protein in both worker and royal jelly. Royalactin accounts for 44-48% of the water soluble proteins in royal jelly (Schmitzová et al., 1998)and recent evidence by (Kamakura, 2011)indicates this

protein acts as an epidermal growth factor for honeybees. When added to the diet of nascent larvae, royalactin decreased developmental time and increased the body mass and ovariole number of worker-destined larvae. But while MJRP-1 seems like a key nutrient in honeybee caste determination, worker jelly also contains royalactin. Moreover, these nascent larvae were provisioned ad-lib quantities of diet, increasing the likelihood of queen-like adults. Huang et al.(2012) provided evidence that MJRP-3 induces caste determination in honeybees. Nurse bees were induced to produce more nutrition diet by consuming diets with an inhibitor for histone deaceylase and the nurse produce diets higher in MJRP-3. Although these nurses induced higher larval growth rate, they did not indicate whether these adults differed in adult body size or caste. Because worker jelly also contains MJRP-3, the induction of caste by this protein seems unlikely. 10-HDA, an epigenetic regulator, is the major lipid component of royal jelly and may contain up to 80-90% of lipid portion (Antinelli et al.,2003). The portion is found in the mandibular gland, and may play a vital role in regulation of important caste differentiation. Although 10-HDA has been shown to act as an epigenetic regulator of larval weight and adult morphometrics (W. X. Wang, Tian, Huang, Wu, & Zeng, 2014) worker jelly also contains 10-HAD (Rembold, 1965). Sugar and moisture content have also been implicated as caste determining substances in honeybees (Asencot & Lensky, 1988; Alfred Dietz & Haydak., 1971). Because every major macronutrient in royal jelly has been suggested to be a determination substance, more research is needed to evaluate which nutrients are important for caste. Moreover, because worker jelly contains all the same nutrients as royal jelly (M. H. Haydak, 1970) quantity needs more attention in studies of caste determination.

Research comparing royal and worker jelly is limited, but evidence indicates miniscule differences between the two (Rembold, 1965). A biologically active substance necessary for

caste determination has not been found and evidence has shown the differences between worker and royal jelly is small (M. H. Haydak, 1970; von Planta, 1888; Y. Wang, Kaftanoglu, Fondrk, & Page, 2014). The long sought after queen determination substance has not been found in royal jelly, and it remains to be seen if there is a specific nutrient in royal jelly determining caste. Although many aspects of royal jelly have been illustrated as caste determination substances, these nutrients are seen in worker jelly. For example, royalactin is thought to determine caste in honeybees, yet, the major protein source in both worker and royal jelly is royalactin (Kamakura, 2011; Schmitzová et al., 1998). The nutrients necessary for caste determination are likely to be provisioned to both queen and worker larvae. The proportions between these major macronutrients are also unlikely to determine caste in honeybees.

Body size and caste is determined by dietary consumption in other insects (Jonathan Cnaani, Borst, Huang, Robinson, & Hefetz, 1997; Plowright & Jay, 1977). For example, developing queen *Bombus terrestris* received larger quantities of diet during the final instar than developing workers (Ribeiro, 1999). Although queens displayed a smaller growth rate than workers, queens eventually became larger due to the increased food availability (Ribeiro, 1994). Additionally, caste is determined in *Evylaeus calceatus* by dietary intake during juvenile development (Platt, Queller, & Strassmann, 2004).Although *Ceratina calcarata* or small carpenter bee does not display a complex caste system, this bee is also directly influenced by dietary consumption during juvenile development as adult body size and fecundity is directly correlated to dietary intake (Johnson,1990). Furthermore, dietary intake seems to not only influence adult body size, but also caste determination in many other insects in the family apidae. Therefore, honeybee castes may be determined by dietary intake, although dietary quality has long been hypothesized to determine caste in honeybees. The determination of honeybee caste by

dietary consumption is not ludicrous considering queens have 250 mg of excess food compared to workers (Rembold, 1965). Furthermore, my first aim indicates caste is determined by dietary consumption because as larval consume higher quantities of diet, they increase in body size, and become more queen like. Therefore, I aim to study caste as a function of dietary intake, although caste has often been seen as a function of a labile substance in royal jelly (Huang et al., 2012; Kamakura, 2011; Rembold, 1965). ***While growing evidence links caste determination in honeybees to diet quantity***, ***more research is needed to support this hypothesis.*** In Paper 3, I test the relative contribution of diet quantity and quality in honey bee caste determination using *in vitro* rearing.

1.4. The Physiological Basis of Caste Determination in Honey Bee

The environment is linked to development status by the endocrine system (Bloch, Shpigler, Wheeler, & Robinson, 2009). Environmental conditions induce changes in internal endocrinology, which mediate polyphenisms in many social insects. For examples, changes in nutrition mediate caste nuances through internal juvenile hormone titers (Diana E. Wheeler, 1986). Caste determination in most insects is regulated by internal juvenile hormone, as juvenile hormone is the common integrative hormone in insects. Exogenous application of JH can recover either a queen or worker phenotype when environmental conditions are not suitable (H. Frederik Nijhout & Wheeler, 1982) and thus, JH can directly mediate caste. For example, worker destined individuals can become queens when JH is applied, even if the environmental conditions necessary for queen development are not enforced (A. Dietz, Hermann, & Blum, 1979). Oftentimes, JH titer during development differ tremendously between castes during development (Rachinsky & Hartfelder, 1990). Therefore, JH is a good indicator of caste trajectory.

The corpora allata is located next to the corpora cadiaca and below the neurosecretory cells in the brain of insects. The corpora allata is known as a neurohemal organ because the corpora allata releases neurosecretory substances into the insect haemolymph (Nation, 2008). Because insects have an open circulatory system, any hormone released into the haemolymph directly interacts with any organ the hormone comes in contact with (Nation, 2008). JH is important during larval development because this hormone maintains the juvenile status of the nascent individual. Because holometabolous insects not only molt, but undergo complete metamorphosis, JH is the main mediator of the transition from growth to maturity (Nation, 2008). For example, ecdysone mediates both larval molting and larval to pupal metamorphosis and JH determines which one occurs. Larval to larval molting is initiated by ecdysone in the presence of JH, but larval to pupal molt is initiated by ecdysone when JH is not present. Juvenile hormone also influences gene expression, but the mode of action by JH is enigmatic. Many researchers believe JH acts similarity to ecdysteroids, i.e. bind to nuclear receptor and influence gene expression, but this pathway as not been identified (Nation, 2008).

Queen larvae have a significantly higher juvenile hormone titers than worker larvae (Rachinsky & Hartfelder, 1990) and an endogenous application of JH to worker-destined honeybees not only resulted in queens (Dietz, A., & Lambremont, 1970), but differential expression of 52 genes (Barchuk et al., 2007), which are mostly responsible for metabolic function. JH application has also been shown to mediate caste-specific motifs that have a strong influence on cis-regulatory networks (Cristino et al., 2006). Because JH integrates external cues into internal pathways, JH is a direct indicator of caste. Thus, by measuring JH titers of individuals consuming different quantities of diet, ***we can assess whether dietary consumption is integrated into internal JH titers***. We predict juveniles consuming lower quantities of diet

will have lower JH titers than individuals consuming more because we expect the assessment of food availability to be integrated into JH titers. Moreover, we also predict starvation is a cue for metamorphosis by increasing JH titers. Because aim 1 indicates size at pupation is directly associated with adult caste, starvation at a specific pupation size results in JH cessation and the entrance into metamorphosis. Regardless of JH titer, size reached during larval to pupal molts dictates the prospective adult caste. In Paper 4, I test whether the regulation of metamorphosis occurs through a critical weigh in honey bees.

1.5. Cellular Mechanisms

Information about the environment is conveyed to the corpora allata for juvenile hormone to be released. Because nutrition determines caste in honeybees, the individual must sense and convey nutritional status to internal pathways. The two main cellular pathways known for sensing nutritional status are the Target of Rapmyacin (TOR) and Insulin like-peptides (IlP) (Mutti et al., 2011). TOR and IlP sense nutritional status by sensing circulating amino acids and sugar respectively and these pathways not only convey this information to the corpora allata for JH release, but this information is used to mediate cell growth rate (cell growth and division). Therefore, TOR and IlP control both growth duration via juvenile hormone and cellular growth rate. Moreover, the mediation of insect body size is a combination between how long and fast an insect grows before entering metamorphosis, and IlP and TOR seem to have pivotal roles towards directly mediating caste determination.

Honeybees have two different IlP's; IlP-1 and IlP-2 (Corona, Libbrecht, & Wheeler, 2016). These Insulin-like peptides circulate and bind to cell-surface membranes and active gene transcription via a secondary messenger. IlP-1 is located in the pars intercerebralis, which are neurosecretory cells anterior of the corpora allata (Nation, 2008). When ILP-1 is released, this

peptide acts on the corpora allata through neural pathways, thus, mediating juvenile hormone release (Wolschin, Mutti, & Amdam, 2011). IlP-2 is in the fat body and new evidences points to a different role for IlP-2. When knock downed separately from IlP-1, IlP-2 lowered ovariole number and body size, whereas juvenile hormone titers were not lowered significantly. Juvenile hormone did decrease slightly, so IlP-2 may have a downfield role on JH mediation (Corona et al., 2016). TOR occurs downfield of the IlP pathway and may either be activated via IlP signal transduction, or TOR can activate the pathway by sensing circulating amino acids. When TOR is knockdown, queen differentiation does not occur and juvenile hormone titers decreased to worker-like levels (Mutti et al., 2011; Patel et al., 2007). This indicates TOR plays a major role during not only for larval growth, but also caste determination. Dietary consumption has been shown to influence insulin-like peptides and target of rapmyacin pathways. When larvae were switched from diet restricted worker cells to queen cells containing high quantities or vice versa, significant changes in insulin-like peptides and Target of rapmyacin occurred (D E Wheeler, Buck, & Evans, 2006). Even though differences in gene expression have been attributed to discrepancies between worker and royal jelly (Kamakura, 2011), this research indicates dietary consumption may have a primary role during caste determination. ***However, more research on how diet consumption regulates these cellular mechanisms is needed.*** In Paper 4, I determine the role of insulin and TOR signaling in mediating dietary signals during caste determination in honey bees.

1.6. Conclusion

Honey bees live in colonies that house thousands of workers and one queen. Because of this, it is difficult to study caste determination in an *in vivo* setting, which likely set back studies on honey bee caste determination. With the recent advent of *in vitro* rearing, honey bee caste

determination can be better understood. In this paper, we tested the hypothesis of diet quantity induced caste determination by; first, determine the ubiquity of quantity induced caste systems among highly-related taxa; second, determining the relative contributions of diet quantity vs. diet quality on final adult caste; and third, determine the influence of diet quantity on important cellular and physiological pathways known during caste determination. Diet quantity seems to be the cue that determines caste, not only in honey bees, but also other eusocial insects. Diet quantity has never been considered before, but may elucidate conserved mechanisms of caste systems in eusocial hymenoptera. JH and the cellular mechanisms did not differ across quantities; thus, more research is needed to understand how diet quantity is integrated into growth and cate determination in honey bees.

CHAPTER 2: DIET QUANTITY AND CASTE DETERMINATION IN EUSOCIAL HYMENOPTERA: META-ANALYSIS

2.1. Abstract

Eusociality explains the evolution of high-level social behavior in the animal kingdom. In social hymenoptera, eusociality evolved separately in multiple taxa; thus, hymenoptera species have abundant examples to examine the proximate mechanisms of eusociality. Reproductive caste systems characterize eusocial species and represents the differences between simple social behavior and higher-level social behavior seen in hymenopteran species. Moreover, queens and worker characteristics differ more so in eusocial species than within cyclical species, and oftentimes, environmental cues drive these caste differences; however, these cues are only characterized in a few well-studied model organisms. The studies aim is to characterize the cues that determine reproductive caste in all studied eusocial hymenoptera, which will not only generate a comparative framework, but also elucidate the cues important for both high level (permanent) and simple (cyclical) social behavior. We performed a meta-analysis on reproductive caste determination within eusocial hymenoptera, and found that diet quantity determined caste in most taxa within the *Apidae* and *Vespidae* families, whereas reproductive castes had a genetic predisposition in most examples within the *Formicidae* family. In these results, taxa seem to diverge into two paths: nutrition and genetic determination, and unsurprisingly, similar cues determine reproductive caste within entire taxonomic families, i.e. diet quantity in both *Apidae* and *Vespidae* and genetic predisposition in *Formicidae*. These proximate mechanisms elucidate the cues important for caste determination in eusocial hymenoptera, and help produce a comparative framework for understanding the ultimate reason for eusociality.

2.2. Introduction

Eusociality is one of the most profound and diverse examples of complex behavior in the animal kingdom, and represents an extreme form of social organization. Three traits characterize eusociality: (1) cooperative brood care, (2) overlapping generations, and (3) reproductive division of labor, and this social structure likely explains the evolution of social behavior in many animal species (Oster & Wilson, 1978). Eusociality is a model for sociobiology (E. Wilson, 1975), evolutionary psychology (E. Wilson, 1998), and selection at multiple levels of organization(Korb, 2010); and thus, eusociality elucidates the manifestation of social behavior within the animal kingdom (Bloch et al., 2009). Furthermore, eusocial animal species are dominant and successful because of both their biomass and impact on the environment, and this ultimate reasoning likely explains the abundance of eusocial animal species. However, the proximate mechanisms that drove insects from solitary to complex sociality is poorly understand, even though these mechanisms are likely conserved across taxa.

Genetically similar, but phenotypically diverse females structure hymenopteran societies. Within these societies are reproductive (queens) and non-reproductive (worker) colony members, which may display physiological, body size and/or morphology difference between castes (Brian, 1955). Extreme morphology and body size differences can only occur during development (Bloch et al., 2009); thus, there seems to be a developmental basis for highly social societies. In cyclical eusocial species (historically termed as primitive eusociality), internal physiological systems and size differences distinguish queens and workers, but in eusocial species (historically termed permanent eusociality) (KUKUK, 1994), they are differentiated by morphology, physiology, and/or size (Diana E. Wheeler, 1986). Cyclical eusocial species are defined as species with indistinguishable queens and workers, small populations, and an annual

life cycle; whereas, permanent eusocial species are defined as species that morphometrically distinguishable queens and workers, a perennial life cycle, and larger populations. Bumblebees are a common example of a cyclical eusocial species, and honey bees are an example of a permanent (Bloch et al., 2009) eusocial species. The gap between queens and workers is larger within permanent eusocial colonies than cyclical eusocial colonies, which correlates with the degree of social behavior (Michener, 1974), and thus, more profound developmental programs explain higher level social behavior.

Colony members control whether a juvenile will become the primary reproductive by exposing them to environmental cues. These cues promote or inhibit queen development at varying time-points of development. In cyclical eusocial species or during "critical periods" or specific time-points during development in permanent eusocial species (Diana E. Wheeler, 1986). Because of these "critical periods", permanent eusocial species display more control over queen development, which has lessened the struggle for reproductive control(Michener, 1974). In cyclical eusocial species, reproductive control is not resolved until adulthood because each individual(s) has reproductive potential, even though the largest individual often becomes the colony's queen. These differences between cyclical and permanent eusocial insects likely results from environmental factors, which not only increase the gap between queens and workers, but also give colony members complete control over reproduction. The proximate mechanisms (environmental factors), however, have only been broadly defined (Diana E. Wheeler, 1986), and thus, the evolution of these mechanisms are poorly understood. Studies theorize that nutrition promotes discrete caste systems in most eusocial hymenopteran, but a comparative analysis has never been performed. Such an analysis would likely elucidate the evolution of high level social in eusocial insects, and the proximate mechanisms (environmental factors) involved.

This study aims to determine whether nutrition is an evolutionary conserved mechanism between simple and complex social systems. We review and synthesize published literature on caste determination in eusocial hymenoptera to answer the following questions: (1) Does nutrition determine caste in the majority of social hymenoptera? (2) Or are other environmental and/or genetic factors more prominent? (3) Do the environmental and genetic factors differ between cyclical and permanent eusocial species? (4) Is there variation between family, genus, and species in their response to common environmental cues? (5) Is there a comparative viewpoint for caste determination? Our results indicate diet quantity is a major contributor towards eusocial caste determination, and likely explains physiological (reproduction, longevity), morphology, and/or size differences between reproductive and non-reproductive.

2.3. Materials and Methods

2.3.1. Literature Search

To determine which factor(s) determine caste, we performed a comprehensive review on studies pertaining to reproductive caste determination in eusocial hymenoptera. We obtained papers by using the search terms "caste determination", "queen determination", "gyne determination", and "insect genetic determination" on the ISI web of science database. In total, we collected 247 papers, some of which were collected from bibliographies of 9 caste determination review papers (Brian, 1957; Donnell, 1998; Fletcher & Ross, 1985; Kerr, Zucchi, Nakadaira, & Butolo, 2013; Lin, Norman, 1972; Markiewicz & O'Donnell, 2001; Schwander, Lo, Beekman, Oldroyd, & Keller, 2010; Weaver, 1966).

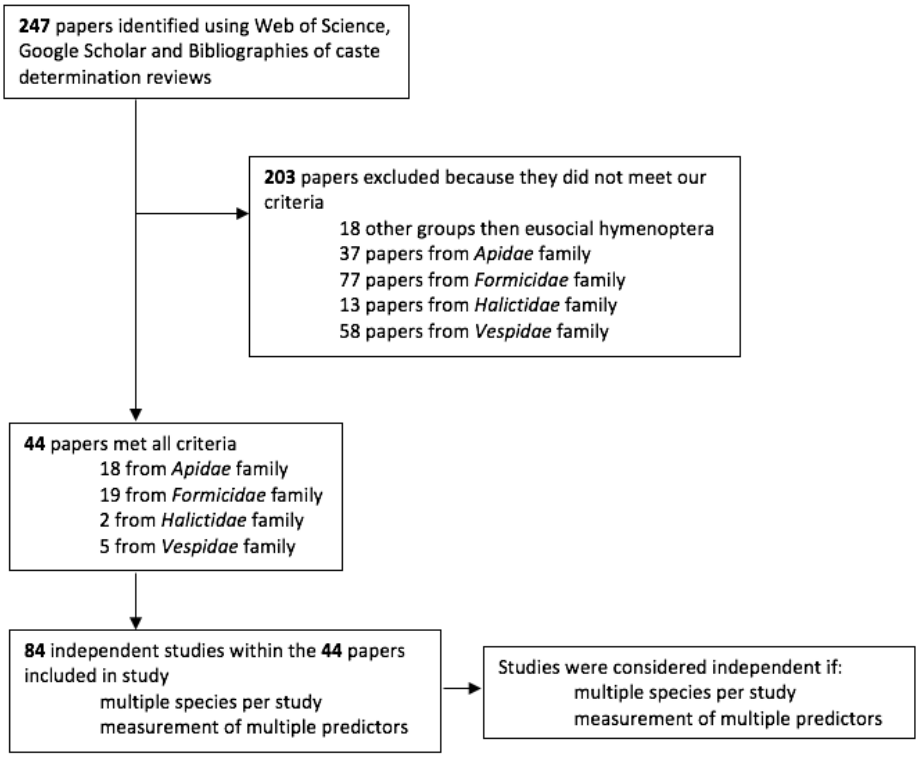

Figure 1: The Effect of Diet Quantity on Eusocial Hymenoptera

We selected papers based on specific criteria (Figure 1): the study (1) measures the effect of either an environmental factor or genetic influence on caste determination, (2) reports sufficient statistics to calculate cohen's d effect size in an online meta-analysis calculator (http://www.campbellcollaboration.org) , which can calculate cohen's d with different types of statistics, (3) presents data on hymenoptera, and (4) measures reproductive caste determination not determination of other castes, such as soldiers and workers. In criteria 2, the usable statistics include means and standard deviations, T-test, Chi-square analysis, F-test, two-way ANOVA, ANCOVA, Point-Biserial Correlation, PHI-Coefficient, Mean gain scores, and standardized regression coefficient. For criteria 4, we were interested in papers that measured the effect of a cue on the degree of queenliness. If papers explicitly measured the effect before and after the

application of the cue, they were included, even though most papers did measure queenliness differently, i.e. weight, queen-worker proportion, morphology, physiological processes (Appendix Table 1-4). Studies were also included if they measured quantitative differences between worker and queen diets; however, studies evaluating qualitative differences were excluded unless they tested the effects of diet quality on caste determination. Many studies tested qualitative differences between worker and queen diet, but not caste differences due to diet quality. In total based upon our criteria, our database contained 44 papers that met criteria, with 18 in the family Apidae, 5 from Vespidae, 2 from Halictidae, and 19 from Formicidae.

2.3.2. Data Extraction and Calculation of Effect Sizes

To calculate effect size, we extracted statistical data from these studies. Most studies included in text statistics, but for some studies, statistical information was not available in the text, so the data was extracted from the graphical representations via image J. All studies had a control worker treatment and an experimental queen treatment, most of which included data such as means, sample size, and standard deviations/errors. Furthermore, if used instead, confidence intervals were converted into standard error. In some cases, the studies only included sample size, median, range, and/or interquartile ranges, and if so, these values were converted into useable forms (means, standard deviations, etc.) according to (Wan, Wang, Liu, & Tong, 2014). Total number of queens and worker before and after a treatment was reported in most other studies, and in these cases, a 2X2 contingency table was used to calculate the chi-square value with a yate's correction. The rest of the studies used a ANOVA F-test, and to calculate effect size, the F-statistics and samples sizes were extracted.

Cohen's d effect size (unbiased standardized mean difference) was calculated for all studies using an online meta-analysis calculator (D. B. Wilson, 2011). The effect size is the

difference between the control (worker) and treatment (queen) in terms of standard deviation units, and the biggest effect size indicate the largest differences and lowest variation between control and treatment groups. The effect size, however, does not represent the absolute difference between the control or queen group; thus, we took the absolute value of the effect size to ensure a positive value. Appendix Table 1-4 show the effect sizes for each species.

2.3.3. Data Grouping and Analysis

Data was grouped into diet quantity (first analysis) induced determination, diet quantity differences between caste (second analysis), or genetic predisposed (third analysis) caste determination (Appendix Table 1-4). A fourth analysis included only factors that determined caste in honeybees, and we did this for three reasons: (1) honey bee caste determination is highly controversial, (2) it is not known which factors truly determine caste, and (3) many publications were found within this specific species. Other factors were shown to determine caste, i.e. pheromones, behavior, but a separate analysis was not performed because very few examples existed. Cyclical and permanent eusociality was considered in the model because I grouped/compared individuals based on type of eusociality, i.e. cyclical or permanent. I could compare effect sizes between cyclical and permanent groups with phylogenetic history considered. For the initial grouping, however, the model did not consider type of eusociality. In this way, eusociality was talked about as "one thing".

A multivariate/multi-level random effects model accounted for between-study variation. This model splits the studies variation into two parts, within- and between- study variation, and produces a correlation matrix using just the between-study variation. In this case, both phylogenetic history and method for measuring queenliness are between-study (random) variation; thus, these two between-study factors were included as a random effect, which allows

a more precise within-study outcome effect. Also, phylogenetic history deemed all studies not-independent because certain species may be more or less closely related, so this random effect model was necessary. Specifically, our model included genus as a random effect, which accounted for phylogenetic history. We produced this model in R (Team, 2016) by using the function rma.mv in the package metafor (Viechtbauer, 2010), and we fitted the model with a restricted maximum-likelihood (REML) (Viechtbauer, 2010).

We determined if the effect size variation was greater than expected by random chance (Cooper, 1998) by calculating heterogeneity, which was calculated via Q statistics (Hedges & Olkin, 1985). P-values associated with Q categories were also reported because they describe between effect size variation among taxa. The effect sizes were considered insignificant if the lower confidence intervals crossed 0, which was done because an effect size of 0.2 is considered slightly significant (Nakagawa & Cuthill, 2009). Along with each study, an overall effect size was calculated for each of the 4 analyses, and if applicable, the overall effect was calculated in a separate analysis for both cyclical and permanent eusocial species separately.

2.3.4. Phylogeny

To better visualize the study's results, we produced a phylogeny of all taxa used in the analysis. We used the rotl package to serve as an interface to the "Open Tree of Life"(Michonneau, Brown, & Winter, 2016), which allowed us to retrieve phylogenetic trees, and match taxonomic names to 'Open Tree identifiers'. The tree only includes taxa with cohen's d effect sizes over 0.2.

2.4. Results

2.4.1. Diet Quantity

In Figure 2, diet quantity did not have an overall significant effect size on reproductive caste determination (2.34, 95% CI=-0.94 to 5.18) because the confidence intervals crossed zero. However, diet quantity did have a significant effect on both cyclical (22.27, CI=18.29 to 27.12) and permanent (2.02, CI=1.71 to 2.40) species when they were separated. As for specific species, 15 of the 25 species had a significant effect size, and similarly, these species were shown to have a significant heterogeneity or between species variation ($Q=552.08$, $P<0.0001$). Moreover, species within cyclical ($Q=171.94$, $P<0.0001$) and permanent ($Q=53.63$, $P<0.0001$) eusocial groups also had significant heterogeneity.

Overall, queens were not shown to be fed significantly more than workers (2.85, 95% CI=-0.03 to 5.72) because the lower confidence intervals cross zero (Figure 3). But when analyzed by individual study, queens were fed more than workers in all 9 studies, and in both cyclical (13.35, 95% CI=10.56 to 16.88) and permanent (13.17, 95% CI=6.42).

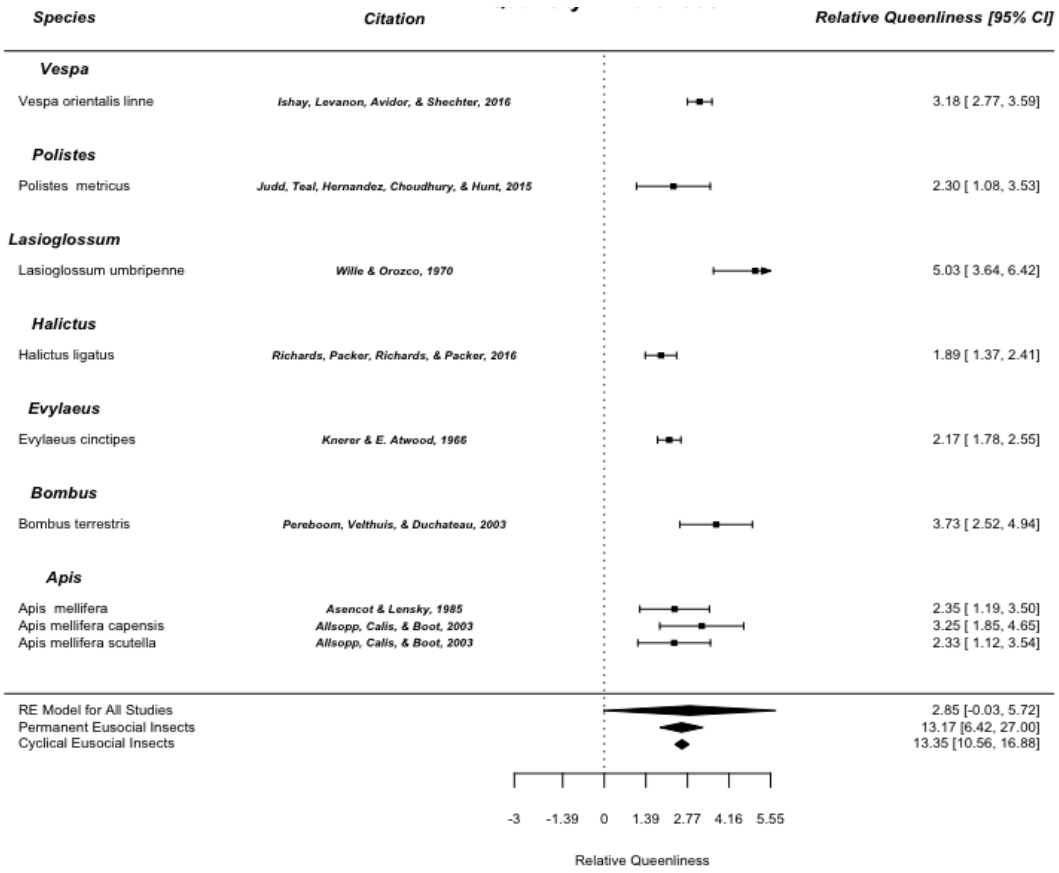

Figure 2: Quantity Differences Between Queen-Worker Diets in Eusocial Hymenoptera 27.00) Eusocial Groups. Between These 9 Studies, There was a Significant Heterogeneity (Q=36.19, P<0.0001) Overall, and Between Species Within Cyclical Eusocial Colonies (Q=34.98, P<0.0001). Interestingly Enough, Species Did Not Display Heterogeneity Within Permanent Eusocial Groups (Q=1.20, P<0.548), Meaning There Was Not Huge Differences Found Between Species

2.4.2. Genetic

As shown in Figure 4, queen development was genetically predisposed for 18 of 23 species of hymenoptera, despite an overall insignificant effect size (1.31, 95% CI -0.73 to 336). Species did exhibit significant heterogeneity between the 23 study species (Q=615.4138, P<0.0001), which may explain the highly variable effect size.

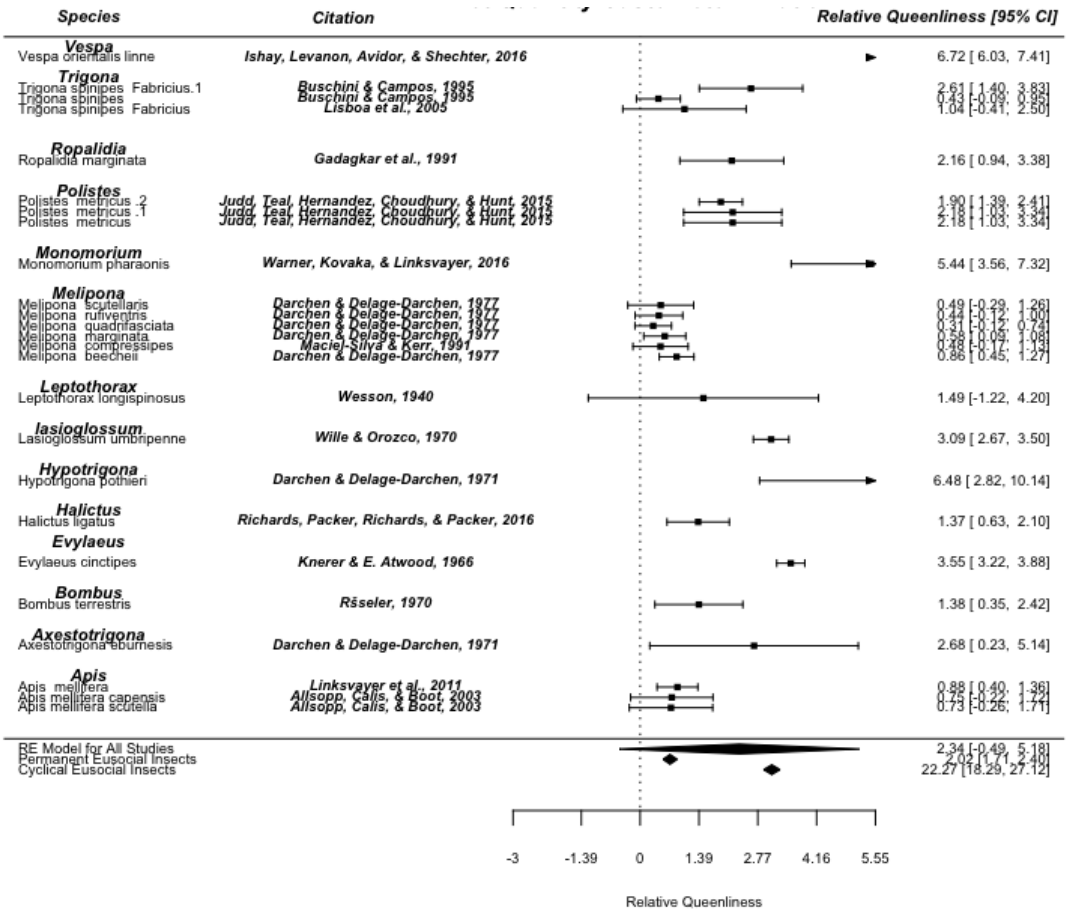

Figure 3: The Effect of Quantity on Eusocial Hymenoptera

All species used in this study that were genetically predisposed are permanent eusocial species, so the individuals could not be split into degree of sociality. Despite this, species did exhibit significant heterogeneity among effect sizes (Q=36.19, P<0.0001), which explains the high number of species that displayed significant effects sizes individually. Interestingly, many

species within the Genus *Melipona* were found to be genetically pre-disposed, which indicates that reproductive caste in some species may be mediated both genetically and nutritionally, or by some undetermined factor.

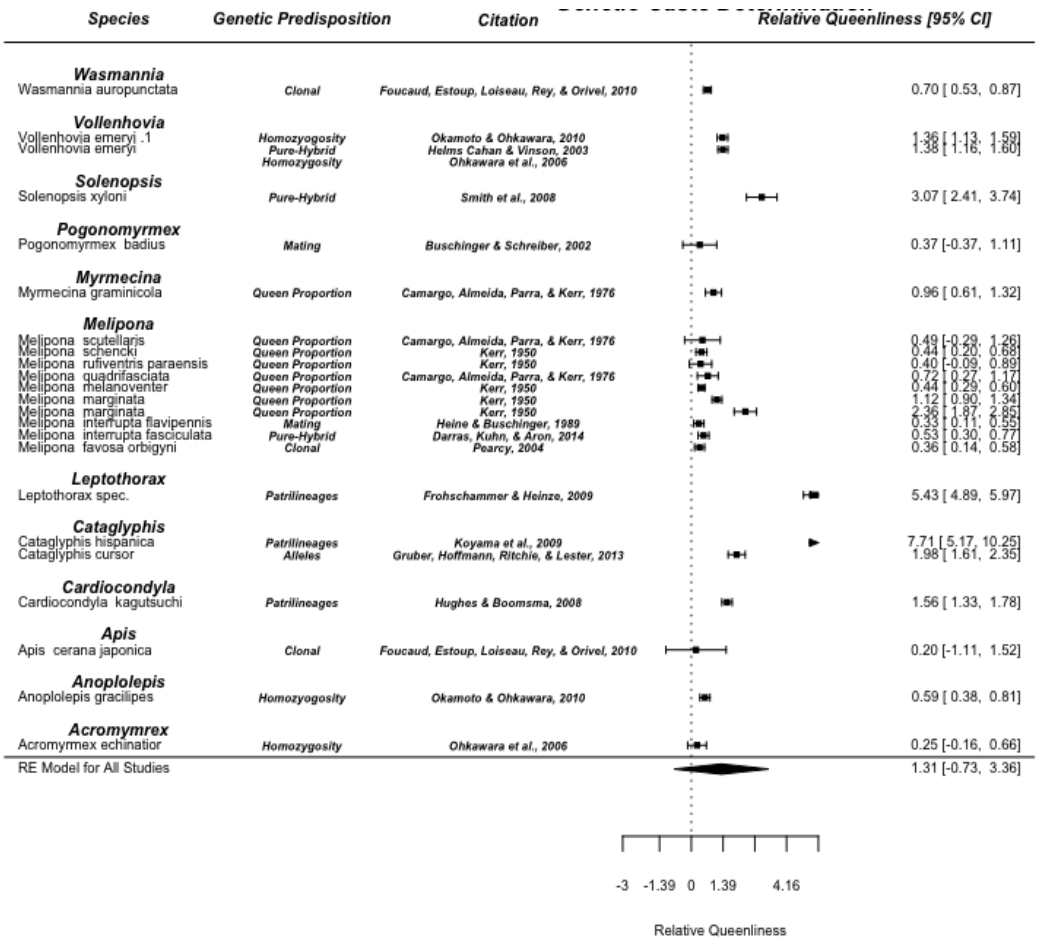

Figure 4: Genetic Predisposition in Eusocial Hymenoptera

2.4.3. Honey Bees

Five components were tested for their effect on honeybee caste determination: four qualitative and the other diet quantity (Figure 5). Overall, dietary factors did not have a significant effect on queen determination because the lower confidence interval of the effect size crossed zero (0.90, 95% CI=-0.98 to 2.78), and of the 5 qualitative components tested, p-

courmaric acid (0.13, 95% CI=-0.22 to 0.47) and protein/royalactin (0.16, 95% CI=-0.14 to 0.45) was insignificant, while protein/royalactin (1.54 ,95% CI=1.36 to 1.72), moisture (1.18 ,95% CI=0.36 to 2.00), and sugar (1.55, 95% CI=0.55 to 2.55) were all significant. Interestingly, royalactin (Major Royal Jelly Protein 1) was both found to be significant and non-significant in independent studies. Diet qualitative components had an overall significant effect size (1.54, 95% CI=1.37 to 1.71), as did diet quantity because the effect size was 0.88 (95% CI=0.40 to 1.36). Diet quantity impacted queen development in other sub-families of *Apis mellifera*; however, there was only one study for the European honey bee.

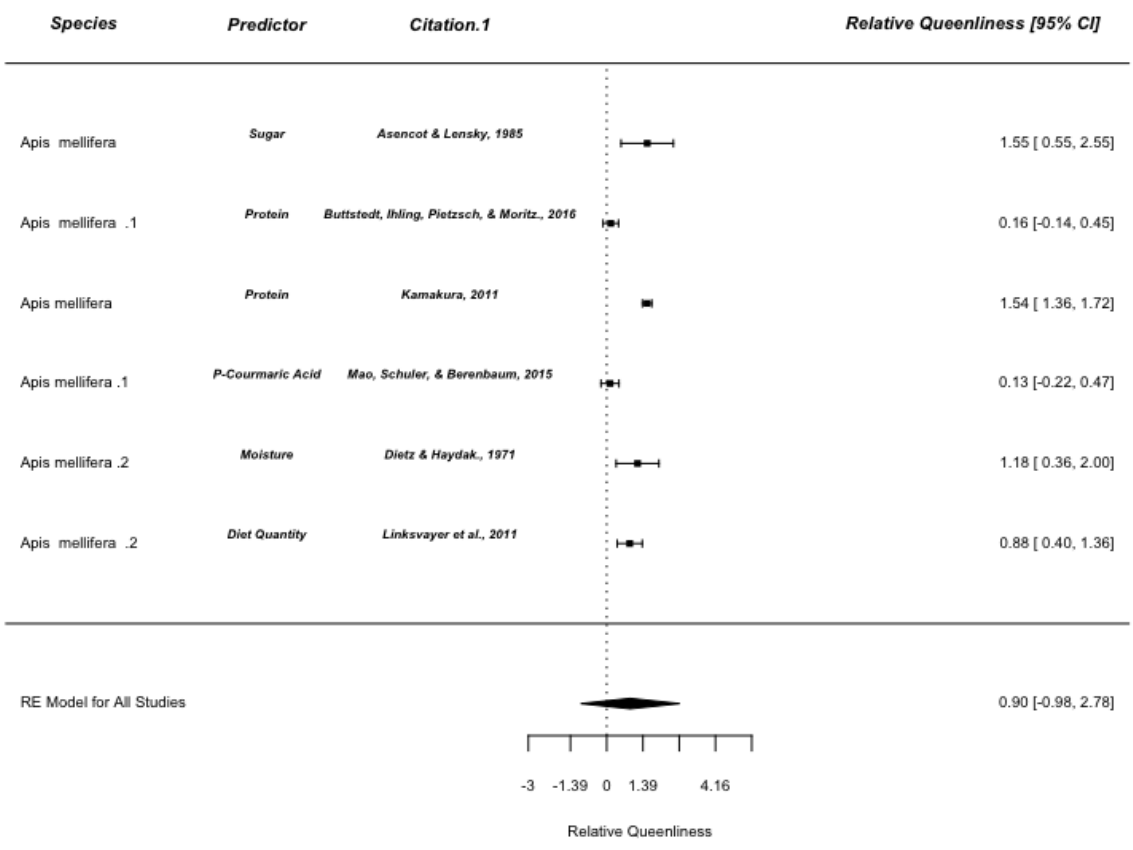

Figure 5: Honey Bee Caste Determination

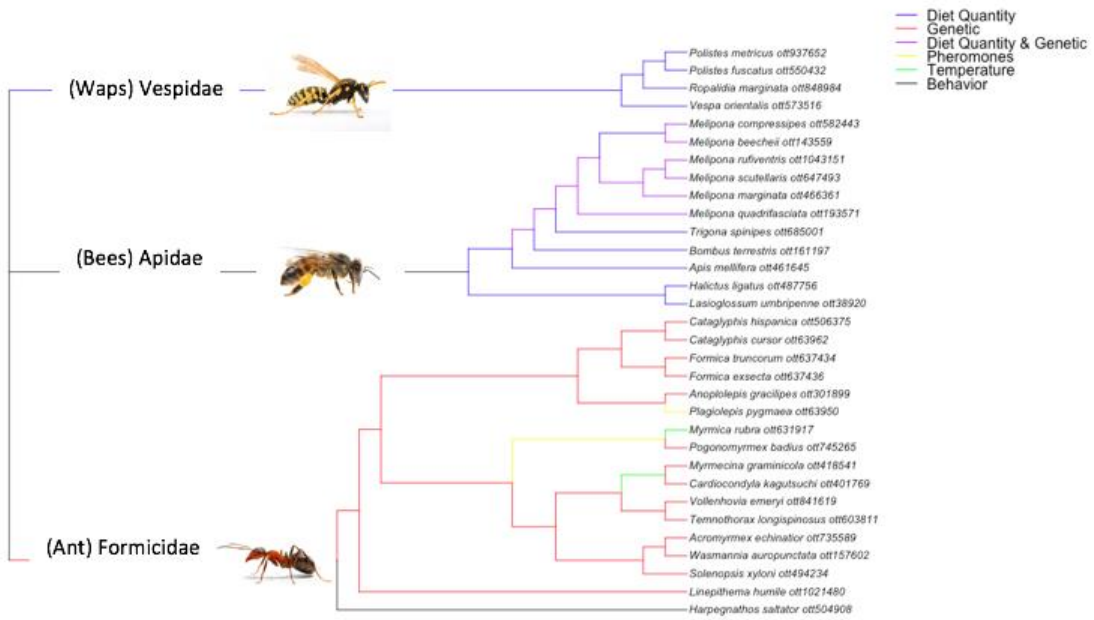

Figure 6: A Phylogeny of Caste Determination in Eusocial Hymenoptera

2.4.4. Phylogeny

A total of 31 different species were usable for the phylogeny because these studies had significant effect sizes (>0.2 Hedges' effect size) (Figure 6). Of the 31 species, 11 were from the *Apidae* family, 4 from the *Vespidae* family, and 17 from the *Formiculae* family. Within the *Apidae* family, reproductive caste was determined by diet quantity in every species, except for the queens in the genus *Melipona,* which were determined by both genetic predisposition and diet quantity. In the *Vespidae* family, all 4 species were determined by diet quantity. The *Formiculae* had 4 iterations of reproductive caste determination: genetic predisposition, temperature, pheromones, and behavioral dominance. For *Formiculae* genetic determination, these studies that found queens could be determined via genetic clones, pure bred, had specific patrilineages, or had certain alleles. Pheromone determination was found in the species

Plagiolepsis pygmea, temperature in the species *Mymrica rubra*, behavioral dominance in the species *Harpegnathos saltator*, and the rest of the species (14) were genetically determined. All species within the *Formiculae* family are permanent eusocial species, whereas all species within the *Vespidae* family are cyclical eusocial species. Within the *Apidae* family, all species were permanent except for *Bombus terrestris*, *Halictus ligatus*, and *Lassioglossum umbripenne.*

2.5. Discussion

Reproductive caste systems are a central characteristic of Eusociality, and likely explain the evolution of complex behavior within the animal kingdom. Environmental cues likely drove the evolution of reproductive castes due to the developmental basis of caste determination; thus, these cues likely elucidate the social evolution of Eusociality. These environmental cues, however, are only broadly defined because a comparative review of reproductive caste determination has not been done since (Wheeler, 1986). Moreover, this is the first study to develop a framework via a meta-analysis. Our data, in contrast to expectations (Brian, 1957; Weaver, 1966), suggests that diet quantity determines caste in many species within the families *Apidae* and *Vespidae* (Figure 2-3), but interestingly enough, the queens are genetically predisposed in many species of ants (Figure 4). Reproductive caste systems seem to have evolutionary conserved mechanisms; especially in taxonomic families as there is a clear divergence between ants, which have a genetic basis for caste determination, and both bees and wasps, which seem to be determined by diet quantity. Because these cues seem to be evolutionary conserved, this paper provides a useful framework for understanding the evolution of both cyclical and permanent eusocial groups.

2.5.1. How Can Quantitative Caste Determination Arise?

We suggest that *Vespidae* and *Apidae* colonies use diet quantity as a cue for queen-worker caste determination for 2 reasons: (1) Number of queens is easily manipulated; thus, lessening the struggle for reproductive control and (2) queens can receive higher maternal care to ensure optimal investment into colony reproduction, i.e. nutrient investment, development microclimate. *Apidae* and *Vespidae* colonies typically have 1 to 2 queens per colony (Norman & Michener, 1972) which differs from *Formicidae* colonies (Hölldobler & Wilson, 1977), and to ensure the optimal production of queens, worker cells are increased in order to promote queen development (Brian, 1955). While only 9 studies met our specific criteria for inclusion (Allsopp, Calis, & Boot, 2003; Asencot & Lensky, 1985; J. S. Ishay, Levanon, Avidor, & Shechter, 2016; Judd, Teal, Hernandez, Choudhury, & Hunt, 2015; Knerer & E. Atwood, 1966; Pereboom, Velthuis, & Duchateau, 2003; Richards, Packer, Richards, & Packer, 2016; Wille & Orozco, 1970) , many other studies suggest that queen cells are typically larger than worker cells (J. S. Ishay et al., 2016), and that queens are fed more.

In total, diet quantity determined queen development in 13 genera and 15 species of hymenoptera (Figure 2); thus, diet quantity regulates caste in more species than previously considered. For example, DE Wheeler stated "Caste differences reflect the discrete differences between the two [queen-worker] brood-rearing cycles in availability of food" based upon the cited examples of *Polistes fuscatus Lasioglossum cinctipes, Bombus hypnorum,*and *Apis mellifera* (diet quality not quantity)(Diana E. Wheeler, 1986). These organisms, while important models for eusocial caste determination, are a small percentage of eusocial insects in hymenoptera (Lin, Norman, 1972), and thus, this paper more specifically defines caste determination as "nutritionally" mediated. Our comparative analysis more specifically defines

eusocial caste determination, and reveals diet quantity as the predominant cue; both in cyclical and eusocial species.

Cyclical and permanent eusocial species are determined by diet quantity; thus, diet quantity likely increased the gap between queen-worker phenology commonly found between cyclical and permanent eusocial insects. For example, queens in eusocial colonies display extreme morphology, physiology, and size differences from workers (Darchen & Delage-Darchen, 1971, 1975; De Souza et al., 2015; Linksvayer, Wade, & Gordon, 2006) , whereas queens often only differ in size and certain physiology processes in more cyclical colonies (J. Ishay, 1975; Karsai & Hunt, 2002; Pereboom et al., 2003). Diet quantity may explain this evolutionary transition towards higher level eusociality, and may be the predictor that promotes or inhibits queen development "diffusely" in cyclical eusocial species or during "critical periods" in permanent eusocial species (Diana E. Wheeler, 1986). The advent of these critical periods, which are specific time-points of development after which the caste determination cannot be altered, likely increased colony member control over whether a juvenile will become the primary reproductive or not. These critical periods force colonies to rear reproductives earlier, to ensure these reproductives acquire the right development resources to ensure optimal reproduction. As for cyclical eusocial species, queen development occurs fluidly during the final instar, which lessens the investment into the reproductive caste. In more cyclical examples, queens and workers are both hardly distinguishable and have reproductive potential (Brian, 1957); queens stymie worker reproduction by inflicting behavioral dominance (Chandrashekara & Gadagkar, 1991; Creemers, Billen, & Gobin, 2003), and thus, workers are not inherently sterile. Diet quantity produces these larger and more dominant queens (Berens, Hunt, & Toth, 2015; Jeanne,

2009; Suryanarayanan et al., 2011), and it seems likely diet quantity drove the increased queen-worker divergence observed during the evolutionary history of eusocial hymenoptera.

2.5.2. How Can a Genetic Basis of Caste Determination Arise?

The genetic basis of *Formicidae* caste determination arose by multiple means, such as clonal (Foucaud et al., 2007; Pearcy, 2004) , degree of homozygosity (Ohkawara, Nakayama, Satoh, Trindl, & Rgen Heinze, 2006; Okamoto & Ohkawara, 2010), patriline lineages (Frohschammer & Heinze, 2009; Hughes & Boomsma, 2008; Koyama, Takagi, Martin, Yoshida, & Takahashi, 2009), hybridization (Darras, Kuhn, & Aron, 2014; Helms Cahan & Vinson, 2003; C. R. Smith, Anderson, Tillberg, Gadau, & Suarez, 2008), and advent of specific alleles (Gruber, Hoffmann, Ritchie, & Lester, 2013) (Appendix Tables 1-4). However, all these factors are similar in that genetic mechanisms underlying caste. Mate choice (Bushinger, n.d.; Heine & Buschinger, 1989) affects the genetic "predisposition" for laying queen destined eggs by regulating the degree of homozygosity. However, genetic mechanisms do not fully explain caste in ants. Our meta-analysis reveals, it seems, that other factors may be involved other than this ostensible genetic determination, i.e. environmental factors. For example, genetic determination had an insignificant effect size, which includes 10 of 13 genera and 19 of 23 species. It is possible that both genetic and environmental factors contribute to final adult caste, but very few studies have measured the relative contribution of both (Schwander et al., 2010; C. R. Smith et al., 2008). Moreover, a recent meta-analysis revealed that patrilineal lineages had a weaker effect on final adult caste than previously thought, stating that "their analysis of previous work failed to support the interpretation that caste is genetically biased in these species" (Wiernasz & Cole, 2010). Concurrently, overall insignificant results indicate that we failed to support this broader interpretation of genetic caste determination, even though most species and genera studied did

have a genetic bias towards reproductive caste. Despite this insignificant, the variation was extremely high (CI: -0.73 to 3.36), so more studies with higher sample sizes are needed to confirm these findings.

2.5.3. Are Honey Bees Truly an Anomaly?

Honey bees are a model for both eusocial species and nutritional-induced caste determination (Bloch et al., 2009), but despite their importance, it is not known whether diet quality, quantity, or some other underrepresented factor determines queen development (Leimar, Hartfelder, Laubichler, & Page, 2012; Rembold, 1965). Since the early 1800's (von Planta, 1888), a biological active substance is thought to drive queen development; thus, most royal jelly components were evaluated and tested for caste determination capabilities (Buttstedt, Ihling, Pietzsch, & Moritz., 2016; A. Dietz & Lambremont, 1970; Kamakura, 2011; Mao, Schuler, & Berenbaum, 2015a). We reveal that protein, carbohydrates, and moisture directly determined queen phenotype characteristics, whereas protein and p-courmaric acid did not. But despite qualitative components having positive effects, we found that diet quantity also influences final adult caste. Interestingly, two separate studies found the same protein, Royalactin or Major Royal Jelly Protein-1, had both a significant and negligible effect on final adult caste (Buttstedt et al., 2016; Kamakura, 2011). Royalactin did not significantly determine caste when diet quantity was accounted for (Buttstedt et al., 2016), so diet quantity may be more important than previously considered. Other qualitative components, such as sugar, were found to determine caste (Asencot & Lensky, 1985), but only when quantity was not controlled or limited. When controlled (Kaftanoglu, Linksvayer, & Page, n.d.), queen development was not possible despite higher amounts and concentrations of these qualitative components, so quantity may be the limiting factor. These studies need to be re-evaluated, diet quantity accounted for. In two

subspecies of the European Honey bee, African honey bee (*Apis mellifera scutella)* and the Cape honey bee (*Apis mellifera)* (Allsopp et al., 2003), diet quantity determined caste. And as see in other eusocial species within the *Apidae,* it is not far-fetched that quantity may determine caste in the European Honey bee.

2.5.4. Evolution of Multiple Cues

Multiple developmental programs are seen in eusocial hymenoptera, such as six in this study: temperature, pheromones, behavior, genetic, diet quantity and diet quality (Figure 6). Temperature, pheromones and behavior were only found in a few species (Brian & Hibble, 1963; Passera, 1980; Penick, Prager, & Liebig, 2012; Vargo & Passera, 1990), but they may be more common in more less studied species; however, it is likely these factors (pheromones, behavior and temperature) are aberrations, and merely divergent traits from the more commonly seen genetic and quantitative diet induced queen determination. Despite our specific criteria, most studies found that caste determination never occurred outside the larval stage, if nutritional mediated (Not shown).

This meta-analysis is limited for a few reasons: (1) the analysis is a product of subjective criteria and pre-set parameters, and (2) the included studies are a result of manuscript searches and limited studies on all hymenopteran species. For the first limitation, subjective criteria and pre-set parameters, I chose these criteria to make comparisons between studies. Because of this, many studies were excluded, which most likely included data on other species of hymenoptera. Even though meta-analysis produces a lot of information about a specific area of research, they are limited because of exclusion. Despite our findings, these two limitations are evident in this study. For example, other cues may be more prominent in more obscure species, but these species have not been well-studied, or in a manner available according to our criteria. As more

studies and species become available, researchers can build from this framework and produce a more supported framework.

2.5.5. Framework for Eusociality

This comparative study elucidates the cues driving caste determination in eusocial hymenoptera, offering a framework needed to understand the proximate mechanisms driving the evolution of Eusociality. The physiological and cellular mechanisms that drive caste divergence has widely eluded researchers, and thus, the evolution of complex caste systems. The two tiers of Eusociality, permanent and cyclical eusociality, were seen to have similar cues for caste determination (Figure 2-3). Even though eusocial organisms have extreme morphology differences from cyclical organisms (Diana E. Wheeler, 1986), the proximate cues are likely the same due to similar cues. Moreover, the increased gap between cyclical and permanent eusocial insects likely arose from selection of more pre-disposed queens. More profound development programs, increased diet quantity and queen-determined cells ensued; thus, increasing the gap between these phenotypically, yet genetically similar females.

CHAPTER 3: DIET QUANTITY AND CASTE DETERMINATION IN HONEY BEES (*APIS MELLIFERA)*

3.1. Abstract

In social hymenoptera, female offspring can become either reproductive queens or sterile workers. Environmental cues determine caste, and in the case of honey bees, nutrition drives queen and worker development. A qualitative substance in royal jelly is thought to drive this divergence; yet, this substance has not been found. Diet quantity regulates caste determination in many bee species; yet, studies on honey bees have focused on qualitative differences in diet. Our goal was to determine the relative contributions of diet quantity and quality to caste determination in honey bees. Larvae were reared *in vitro* on nine diets varying in the ratio of proteins to carbohydrates. These diets were fed to larvae in eight different quantities. The queenliness of eclosing adults was determined using Principal Component Analysis (PCA) on 7 morphological measurements. Larvae fed the largest quantities of diet were indistinguishable from commercially reared queens, independent of the protein carbohydrate ratio of the diet. Neither protein nor carbohydrate content had a significant influence on PC1, a component that explained 70.66% of the difference between queens and workers. Instead, the total quantity of diet explained a significant amount of the variation in PC1. Diet differences in the final instar were capable of inducing queen-like traits, contrary to received wisdom that caste determination occurs in the third instar. These results indicate that total diet quantity fed to larvae, instead of particular diet components, regulates the difference between castes in honey bees.

Keywords: Honey bees; *Apis mellifera;* Caste determination; Nutrition; Diet quantity

3.2. Introduction

Eusocial organisms have a division of labour of non-reproductive (worker) and reproductive (queen) individuals (Diana E. Wheeler, 1986). Oftentimes, queens and workers have analogous genotypes; yet, these similar genomes produce distinct queen-worker phenotypes via environmental cues (Mark L., 1991). These cues vary by species, but nutrition drives queen development in many social hymenoptera. In honey bees, diet quality is thought to drive queen development because nurse bees provide worker jelly to worker-destined larvae (WDL), and royal jelly to queen-destined larvae (QDL) (M. H. Haydak, 1970); however, qualitative differences between royal and worker jelly do not fully explain caste determination. Resent findings suggest that diet quantity may have a significant, but unrecognized, role in honey bee caste determination (Leimar et al., 2012; Linksvayer et al., 2011). However, the relative contribution of diet quality and quantity have not been tested in an experimental framework which would compare the influence of each.

Since the 1890's, diet quality has been thought to determine caste in honey bees. (M. Haydak, 1968; M. H. Haydak, 1943; Johansson, 1955; Rembold, 1965; Weever, 1965). These studies cite the existence of a "biological active substance" found only in royal jelly that activates queen determination (Rembold, 1965; Rembold, Lackner, & Geistbeck, 1974). The quantity hypothesis stems from early observations where researchers noticed queen and worker larvae receive different proportions of water-clear and milky-white secretions from nurse bee glands (M. H. Haydak, 1970; Johansson, 1955). Because of these observations, the major component driving queen development has been thoroughly searched. Nearly every major component in royal jelly has been tested for its effects on caste determination, with most of these studies finding positive results. For example, lipids (W. X. Wang et al., 2014), proteins (Huang

et al., 2012; Kamakura, 2011), carbohydrates (Asencot & Lensky, 1984, 1985, 1988), water (Alfred Dietz & Haydak., 1971), and pantothenic acid (Rembold, 1965) all contributed to queen development in honey bees under some experimental conditions. However, most of these studies did not control for quantity of diet, even though it may have a significant role in caste determination. For example, high sugar diets produce more queens when diet quantity is uncontrolled (Kaftanoglu et al., n.d.), but when accounted for, these studies could not distinguish queen development between diets (Aupinel et al., 2005). Even royalactin fails to produce queens when quantities are altered (Buttstedt et al., 2016) questioning its role as the queen determining substance. Diet quantity appears to have a significant, but unaccounted role in honey bee caste determination, and may explain why so many qualitative aspects of diet have been linked with caste.

Diet quantity controls queen-worker phenotypes in many other social hymenoptera (Allsopp et al., 2003; Camargo, Almeida, Parra, & Kerr, 1976; Judd et al., 2015; Karsai & Hunt, 2002; Pereboom, 2000). For example, higher provisioning drives queen differentiation in cape honey bees (Allsopp et al., 2003), bumble bees (Pereboom, 2000), and the oriental hornet (J. Ishay, 1975). Queen destined bumble bees receive large quantities of diet during the final instar, resulting in both increased and elongated growth period (Jonathan Cnaani et al., 1997; Plowright & Jay, 1977). In these examples, diet quantity elevates juvenile hormone, triggering development of reproductive potential (K Hartfelder, Cnaani, & Hefetz, 2000). While this pathway is known in these social hymenopterans, the dietary cue that triggers queen development in honey bees remains unknown. There is evidence, however, that diet quantity drives queen development in honey bees. Nurse bees not only provide queen-destined larvae with 5mg more diet than worker-destined larvae (WDL) (Elser, 1929; Rembold, 1965), but QDL are fed 1400 more times (M. H.

Haydak, 1970). But while queen and worker-destined larvae are fed significantly different amounts of diet during development, the role of quantity on honey bee caste differentiation is unrecognized.

Here we test a new hypothesis: diet quantity plays a significant role in determines caste in honey bees. To test the relative contributions of diet quantity and quality during caste determination, larvae reared in vitro on diets varying in quality (protein and carbohydrate proportion) and quantity in a factorial design. Using this experimental design, we test the effect of quantity and quality at the same time. Our results indicate that diet quantity influences queen differentiation in honey bees.

3.3. Materials and Methods

3.3.1. Artificial Rearing

Apis mellifera larvae were collected from nine hives near Fargo, Cass County, North Dakota during a three-week period in the summer of 2015. Hives were supplemented with pollen patties (Mann Lake,MN,USA) and a 1:1 fructose- water solution (Brushy Mountain Bee Farm, NC, USA) during poor foraging conditions. In order to collect 1st instar larvae, the hive queen was captured using a queen catcher (Mann Lake, MN, USA) and placed into a jenter box (Blue Sky Bee Supply, Ravenna, OH) for 24 hours. The jenter box was left in the hive for an additional 69 hours until the box contained 0-21 hour old larvae.

Larvae were transplanted into the wells of 24 well-plates using a size 0 paintbrush. Lifting carefully on the dorsal end of each individual, larvae were placed onto 10µl of artificial diet. The 24 well-plates were stored inside a modulator incubator chamber (Billups-Rothenberg, del Mar, CA, USA) containing a small volume of K_2SO_4 saturated solution for 96% RH. Chambers were maintained at a constant 34°C remainder of larval development. Also, larvae

were reared in constant darkness, except for daily feeding. Larvae were fed according to treatment, as described below. Once larvae finished their diet in the final instar, larvae were moved into pupation plates. Pupation plates are 24 well plates containing wipes (Kimtech Science, USA). that had been sterilized by soaking in EtOH and then dried. larvae were moved into these pupation plates using a detached blunger from a Chinese grafting tool (Mann Lake, MN, USA). Pupation plates were maintained at a constant 34°C and 75% RH (Saturated NaCl) until adult eclosion. Eclosion was determined by the development adult characteristics and full movement, at which time these individuals were immediately stored at -20°C.

3.3.2. Diet Treatments

Diets varying in both quantity and quality were provisioned to developing larvae to determine whether quantity regulates adult caste. The study consisted of 72 treatment groups: eight different diet qualities and nine different diet quantities (Table 1). Additionally, an *ad libitum* quantity treatment was added using the medium protein-medium carbohydrate diet (Table 1). Larvae from nine hives were reared into 24-well plates during a three week period. Each plate was randomly assigned a diet quantity treatment, and within the plates, each row was randomly assigned a diet quality treatment. Fresh diets were produced daily by homogenizing all dietary ingredients for 10 minutes, and then, placing the diets in a 34°C water bath for an additional 10 minutes before feeding. For all the treatments, larvae were fed the same amount until the 6^{th} day of development (see below), while diet quality remained the same throughout development.

Table 1: Dietary Treatments, Artificial Diet Components, and Macronutrient Content of Artificial Diets.

Diet Treatments			Ingredients(g)					Macronutrient(%)			
Quantity	Protein	Carbs	Royal Jelly	Glucose	Fructose	Yeast	Water	Protein	Carbs	Water	P:C Ratio
370ul	High	High	65g	6g	12g	1g	35ml	6.80%	30.13%	60.51%	1/4.4
340ul		Medium	65g	4g	8g	1g	35ml	7.17%	26.38%	63.75%	1/3.7
310ul		Low	65g	2g	4g	1g	35ml	7.57%	22.22%	67.40%	1/3.0
280ul	Medium	High	50g	6g	12g	1g	35ml	5.23%	26.30%	66.10%	1/5.0
250ul		Medium	50g	4g	8g	1g	35ml	5.51%	22.77%	60.64%	1/4.1
220ul		Low	50g	2g	4g	1g	35ml	5.83%	18.34%	73.58%	1/3.1
190ul	Low	High	35g	6g	12g	1g	35ml	3.66%	23.30%	71.70%	1/6.4
160ul		Medium	35g	4g	8g	1g	35ml	3.86%	19.15%	75.54%	1/5.0
Ad libitum		Low	35g	2g	4g	1g	35ml	4.08%	14.58%	79.81%	1/3.6

The other eight diets were produced by altering carbohydrates and royal jelly (Pure Royal Jelly eBeeHoney.com, Ashland, OH,USA) in a full factorial design. The nine diets varied in protein to carbohydrate ratios by altering proportions of Royal jelly (protein) and carbohydrates (glucose and fructose). An artificial diet does not exist for honey bees and addition of non-royal jelly proteins such as casein significantly decreases survival (Singh, 1977). Altering amounts of royal jelly also affects sugar content because commercial royal jelly contains some sugars. The Dietary protein content and carbohydrate content were assessed when different concentrations of Royal jelly was ad-mixed (see below).

3.3.3. Determination of Protein, Carbohydrate, Lipid and Water Contents in Artificial Diets

Protein, carbohydrate, lipid and water content was assessed for each of the 9 diet quality treatments because Royal jelly, the only protein source in the artificial diets, contains varying amounts of protein, carbohydrates, lipids and water.

Protein content of Royal jelly was measured using a standard colorimetric Bradford assay (Sigma-Aldrich, MO, USA). Following protocols, a 200mg/ml protein albumin solution was

prepared and then diluted standards by dissolving 1µl, 2.5µl, and 5µl of the albumin solution in 1 ml of distilled H2O and 1mL of Royal jelly was diluted in 10 mL of water. 5ul aliquots were plated in a 96 well plate for each of the three standards and diluted Royal jelly. 250µl of the Bradford reagent was then added to each well and the plates were left to develop at room temperature for 20 minutes. The samples absorbance at 595nm was measured using a spectrophotometer (Thermo Scientific, MA, USA), and the Royal jelly protein contents were calculated from the spectrophotometers results based on a determined standard absorbance curve.

Carbohydrate, lipid and water contents of the jelly were calculated using a differential scanning calorimeters (DSC) (Perkin Elmer DSC Pyris 1, Waltham, MA, USA) by placing one to five mg of royal jelly into a sealed Perkin-Elmer aluminum DSC pan. The aluminum pan containing Royal jelly was then placed into the DSC in addition to the empty aluminum pan serving as the control. The sample chamber was perfused with helium gas at 10 ml/min during the scan, which occurred between 25°C and -100°C at a rate of 1°C /min using a liquid cooling accessory (Perkin Elmer Cryofill, Waltham, MA, USA) to determine both freezing and melting characteristics. Water melts near 0°C, generating an endotherm peak in the calorimetric scan, and the sugars caused distinct glass transitions between -20°C and -40°C. The area of the peaks generated during the events were calculated and used to determine the water content. The freezing point depression was measured in Royal jelly using a calibration based on glucose and fructose solutions in deionized water (5:7%, 7:10%, and 10:10% of glucose: fructose)(Simo & Christensen, 1962). In an Eppendorf tube, 100 µl of Royal jelly was diluted in 900 µl of 100% ethanol, vortexed for 1 minute, and chilled for 2 hours at -80°C. Then the tubes were centrifuged at 14000 rpm for 30 minutes. The supernatant was removed, dried in flowing nitrogen gas, and reconstituted in 1ml of deionized water. Five micro liters of this solution were analyzed in the

DSC in pentuplicates, and the freezing and melting points were noted and compared against glucose: fructose standards to obtain an estimate of the sugar content in Royal jelly.

3.3.4. Diet Quantities

The lowest dietary quantity (160µl) was adopted from previous *in vitro* methods because this quantity produces workers (De Souza et al., 2015). The 8 quantity treatments were produced by increasing quantities by 30µl increments from 160µl to 370µl (Table 1). There was an additional *ad libitum* treatment in which larvae were fed in excess of what they were able to consume. For all the treatments including the *ad libitum* treatment, individuals were given the same feeding regimen and a total of 110ul during the first 5 days of development (Day1:10µl, Day2:10µl, Day3:20µl, Day4:30µl, Day5:40µl). During the 6^{th} day of development, larvae were fed different amounts depending upon the diet quantity treatment (160µl treatment- fed 50µl, 190µl treatment- fed 80µl, 220µl treatment- fed 120µl, 250µl treatment- fed 150µl, 280µl treatment- fed 180µl, 310µl treatment – fed 220µl, 340µl treatment – fed 250µl, 370µl treatment – fed 280µl). As for the *ad libitum* treatment, larvae were fed 200µl per day until these larvae entered gut purging. Gut purging is a clear indicator of insect metamorphosis, and is seen when either frass or uric acid crystals are deposited (Nation, 2008). When either frass or uric acid crystals are seen, these individuals are moved into pupations plates.

3.3.5. Morphometrics

To determine whether quantity influences final adult caste, a measurement of queenliness was assessed by performing a Principal Component Analysis (PCA). This PCA determines whether an individual is a queen, worker or intercastes by comparing morphometric measurements between *in vitro* reared individuals and reference workers and queens. Adult morphometrics can separate and classify castes, even when traditional metrics such as ovariole

number and spermathecal size are excluded (De Souza et al., 2015). Ovariole number and spermathecal size are time-consuming measurements, and with this studies large sample size, these measurements were not feasible.

Adult morphometrics include total weight, and the width and length of the basitarsus, mandible, and head. The mandibles, basitarsus and head were dissected, and placed onto a slide with a calibration circle as seen in Figure 1, and image J software was used to both scale and take each width and length measurement. Adult morphometrics were taken from both *in vitro* reared adults and naturally reared queens and workers. The queens were purchased (Wildflower Meadows, Southern CA, USA) and the workers collected from the hive during early spring. After acquisition, the queens and workers were frozen at -20. Furthermore, the queens and workers served as the references for adult queenliness.

Figure 7: Honey Bee Head, Basitarsus and Mandible Measurements

3.3.6. Data Analysis and Presentation of Data

Statistical analyses were performed using R version 3.1.3 (R Core Team). Use of additional R packages are reported below where appropriate.

3.3.6.1. Principal Component Analysis

A Principal Component analysis (PCA) was performed to classify adult caste in honey bees. Hive-reared workers and commercially reared queens (Wildflower Meadows, Southern CA, USA) were used as reference samples for the analysis. The Principal Components were calculated from hive-reared workers and commercial queens using the *prcomp* function in the *stats* package (Team, 2016) . Once the principal components were calculated, the *predict* function in the stats R package was used to produce principal components for the *in vitro* reared individuals. PC1 and PC2 were plotted, and each treatment group was surrounded by an ellipse representing the 95% confidence interval. The assumptions for sphericity, sample adequacy, and determinant of the matrix were tested and met. Principle Component 1 (PC1) was used for downstream analysis because PC1 explained 70.66% of the variation between reference workers and queens. Thus, PC1 can be used as an indicator of queenliness in this study.

3.3.6.2. Clustering Analysis

A cluster analysis was used to classify and separate individuals into caste-related groups. Clustering is a typical statistical analysis for group classification; thus, this analysis was used to determine if *in vitro* reared individuals were grouped with reference queens and workers. In order to group individuals, linkage distances were calculating using the complete method for hierarchal clustering. The complete method, which is a type of agglomerate clustering, finds similar clusters by using a set of dissimilarities for *n* objects (Everitt, 1974). This method was used because it successfully grouped reference queens and workers into separate groups. The cluster analysis was performed using the 7 morphometric measurements with the *hclust* function in the *stats* package (Team, 2016). Once calculated, the linkage distance results were graphed using the *ColorDendrogram* function within the *sparcl* package in R (Daniela M. Witten and

Robert Tibshirani, 2013). Finally, the optimal number of clusters was calculated using the K-means clustering method. Each of the 8 treatments, *ad libitum* treatment, and reference workers and queens were color coded. After the cluster analysis, the dendrogram was cut into three groups to determine group classification.

3.3.6.3. Measurement of Contribution of Diet Quantity and Quality on PC1

The influence of diet quality and quantity on PC1 was compared for the 72 treatments. A generalized linear mixed model (GLMM) was performed to see which variable(s) had a significant influence on PC1. PC1 was used because this component explained 70.66% of the variation between reference workers and queens; thus, this component is a good indicator of queenliness. The linear mixed effects analysis used the *lme4* function, a package in R (Walker, 2015). The independent variables were: total diet quantity, the proportion of protein, the proportion of carbohydrates, and the proportion of water. Interaction term was excluded because there was not a significant interaction among the independent variables. Hive was included as a random effect to account for variation caused by parental genetics. The assumptions of collinearity, independence of data, and normality were calculated and met for all the models.

3.4. Results

3.4.1. Principal Component Analysis

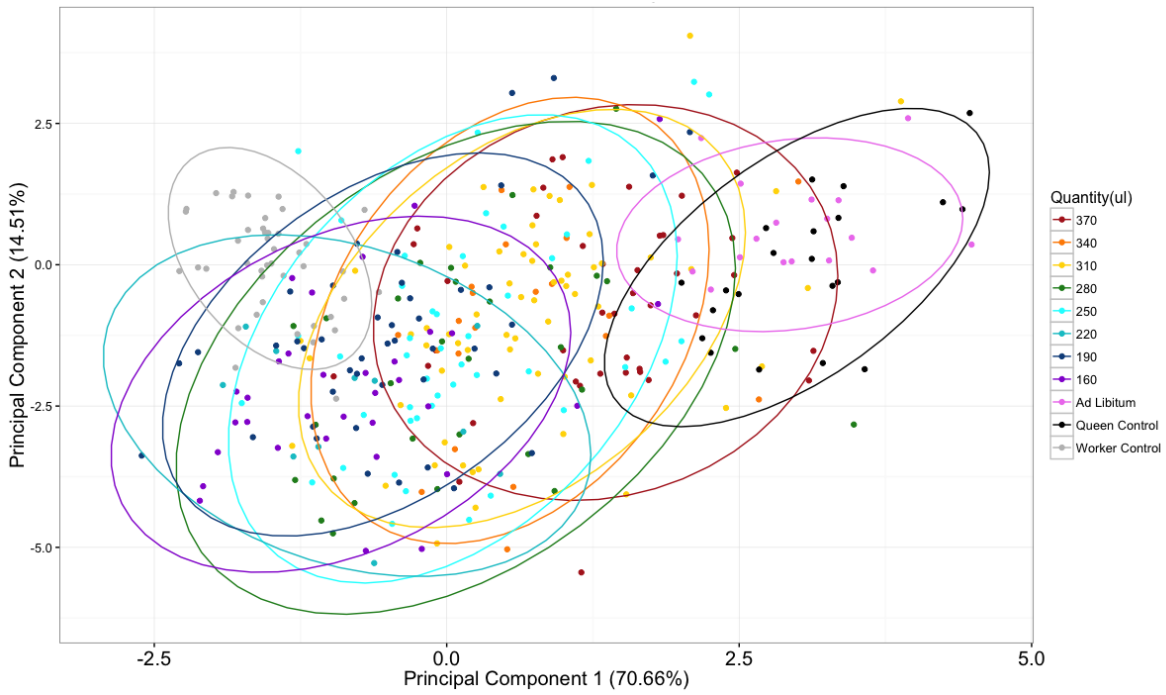

Figure 8: Principal Component Analysis(PCA) Using Principal Component 1(70.66%) and Principal Component 2 (14.51%). Each Color Represents Either a Quantitative Treatment or a Queen/Worker Control.

The Principal Component Analysis (PCA) reveals a clear separation between reference workers and queens (Figure 8), indicating morphometrics are a useful metric for caste classification. The results of the PCA also indicate that PC1 explains 70.66% of the variation between reference workers and queens, whereas PC2 explained only 14.51% of the variation. Furthermore, over 95% of the variation is explained by the first four components (PC1= 70.66, PC2=14.51, PC3=8.01, PC4=2.70). Five of the seven morphometric measurements had standardized loadings over 90%, indicating each morphometric had a high contribution to PC1 (Table 2). These five components had high commonality estimates, which is a measurement for

explained variation. The results of the PCA indicate PC1 clearly distinguishes caste; thus, we chose to use PC1 for downstream analysis.

Figure 9: Dendrogram Using Ward Linkage Method for Hierarchal Clustering. Each Color Represents Either a Quantitative Treatment or a Queen/Worker Control. The Line Crossing the Graphic Represents the Number of Significant Clusters in the Analysis,Which is Three. The Three Clusters are Labeled Among Worker, Queen or Intercaste.

Table 2: Principal Component Loadings

Morphometrics	PC1	PC2	h2	u2
Basitarsus Width	0.93	0.08	0.88	0.122
Basitarsus Length	0.06	0.99	0.99	0.014
Head Width	0.97	0.02	0.94	0.062
Head Length	0.92	0.06	0.85	0.14
Mandible Width	0.95	0.03	0.9	0.097
Mandible Length	0.94	0.02	0.88	0.116
Adult Weight	0.71	0.15	0.52	0.479

3.4.2. Clustering Analysis

The cluster analysis was used to classify and distinguish groups, and the analysis revealed 3 clear groups (Figure 9): queens, workers, and intercastes. Also, the K-means clustering analysis indicated that these three groups were optimal. The cluster analysis distinguished the reference workers and queen into separate groups, while the *in vitro* individuals occupied all three clusters. From the study, a total of 95 queens, 69 workers, and 143 intercastes were reared from the *in vitro* treatments. The high quantity treatments produced a high proportion of queens: first, the *ad libitum treatment* produced 100% (18/18) queens, and second, the 370µl treatment produced 77.5% queens (31/40) (Table 3). Also, 0% of queens were produced in the lower quantity treatments (220µl,190µl,160µl).

The results show a clear classification between queen and worker controls. Also, the *Ad libitum* treatment produced 100% queens.

The dendrogram shows three clear groups. The more-violet colors (high quantity treatments) occupied the queen cluster, while the more red-colors (low quantity treatments) occupied the worker cluster. Moreover, a general trend can be seen uses color representations of each quantity treatment.

Table 3: Number of Individuals Found in Each Cluster

Ad libitum	370ul	340ul	310ul	280ul	250ul	220ul	190ul	160ul	Queen Control	Worker Control
18	31	5	26	6	9	0	0	0	21	0
0	4	1	3	6	8	3	19	25	0	0
0	5	15	35	18	29	8	25	8	0	41

3.4.3. Diet Quantity and Quality on Final Adult Caste

A Generalized Linear Mixed Model (GLMM) was performed to determine whether PC1 was significantly influenced by diet quantity. PC1 was the dependent variable, whereas diet quantity and diet quality (protein, carbohydrate, and water proportion) were the independent variables. Protein, carbohydrate, and water proportion were calculated for each diet, which is shown in Table 1. Diet quantity is the total amount each individual larvae consumed throughout development. Hive was treated as a random effect because bees, which were reared from 9 different hives, are expected to differ in size and shape. The GLMM results in Table 4 indicate quantity has a significant influence on PC1, whereas quality (protein, carbohydrate and water proportion) does not. When quantity was plotted in Figure 10, a significant trend towards queenliness was seen; however, quality did not have a significant influence on queenliness, even when different metrics of quality were graphed in Figure 11 (P:C ratio, protein content, carbohydrate content, water content). Additionally, quantity influenced queenliness when each diet was calculated separately (Figure 12).

Table 4: Results of Generalized Linear Mixed Model. The Effects of Diet Quantity and Quality (Protein and Carbohydrate Proportion) Were Tested on Principal Component 1, Which Explains 70.66% of Variation Between the Reference Workers and Queens. There Was Not a Significant Influence of Among Carbohydrate Proportion (P=0.732), Protein Proportion (P=0.942), or Water Proportion (P=0.737); However, Quantity Significantly Influenced PC1. **(<0.001).**

		Principal Component 1(70.66%)		
		B	std. Error	p-value
Fixed Parts				
(Intercept)		-7.959	22.601	0.742
Total Quantity		0.289	0.025	**<0.0001**
Dietary Protein Proportion		-2.742	29.015	0.942
Dietary Carbohydrate Proportion		7.898	23.085	0.732
Dietary Water Proportion		7.619	22.742	0.737
Random Effects				
σ2			0.927	
T00, Hive			0.009	
N-Hive			8	
ICC-Hive			0.042	
Observations			289	
R2			0.322	
AIC			800.21	
Deviance			786.2	

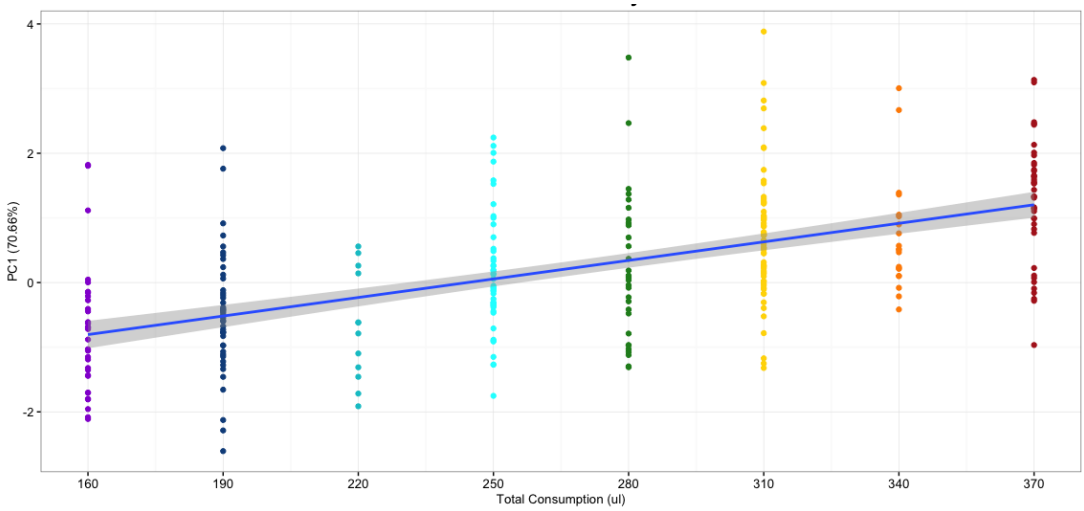

Figure 10: Influence of Diet Quantity on Principal Component 1 (70.66%). There was a Significant Influence of Quantity on PC1.

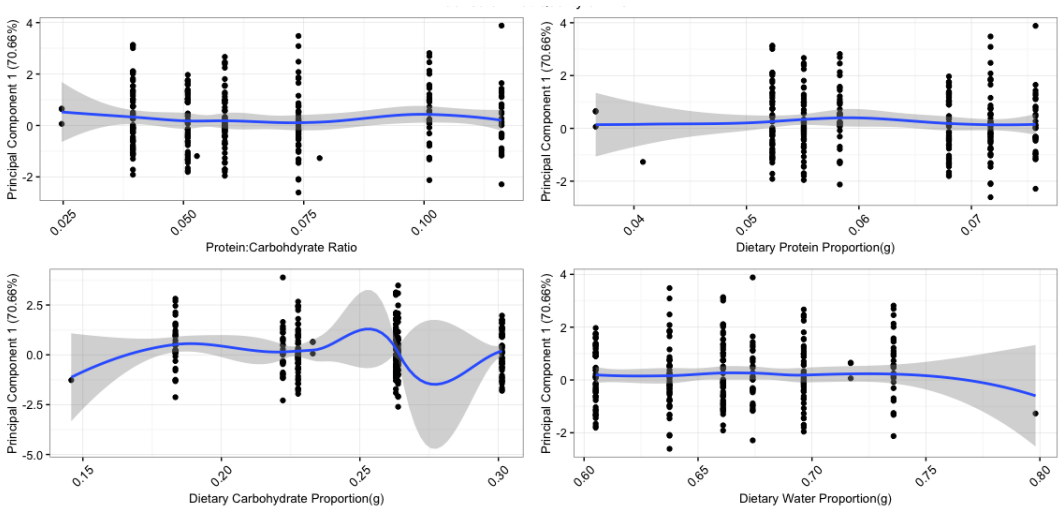

Figure 11: Influence of Diet Quality (P:C Ratio, Protein Proportion, Carbohydrate Proportion and Water Proportion) on Principal Component 1 (70.66%). There were No Significant Influences of Quantity on PC1.

3.5. Discussion

Eusocial insects are an excellent example of phenotypic plasticity because they have a division of labor of reproductive (queen) and non-reproductive (worker) individuals (Diana E. Wheeler, 1986). Along with reproductive viability, these individuals differ in physiological, morphometric, and behavioral characteristics. Oftentimes, environmental cues drive female larvae into irreversible queen or worker developmental pathways (Evans & Wheeler, 2001). These cues vary by species among eusocial hymenoptera; however, only a few studies have fully characterized the cues that drive this divergence. In honey bees, the cues driving caste bifurcation remains enigmatic, which is significant because honey bees are model for high-level Eusociality (Bloch et al., 2009). Thus, the studies goal was to determine the nutritional factors determining caste. In this study, we determined whether diet quantity has a significant role in queen differentiation. Because honey bees are model for eusociality, we

expect the results to provide material for understanding the mechanisms of cast determination and more importantly, the evolution of eusociality.

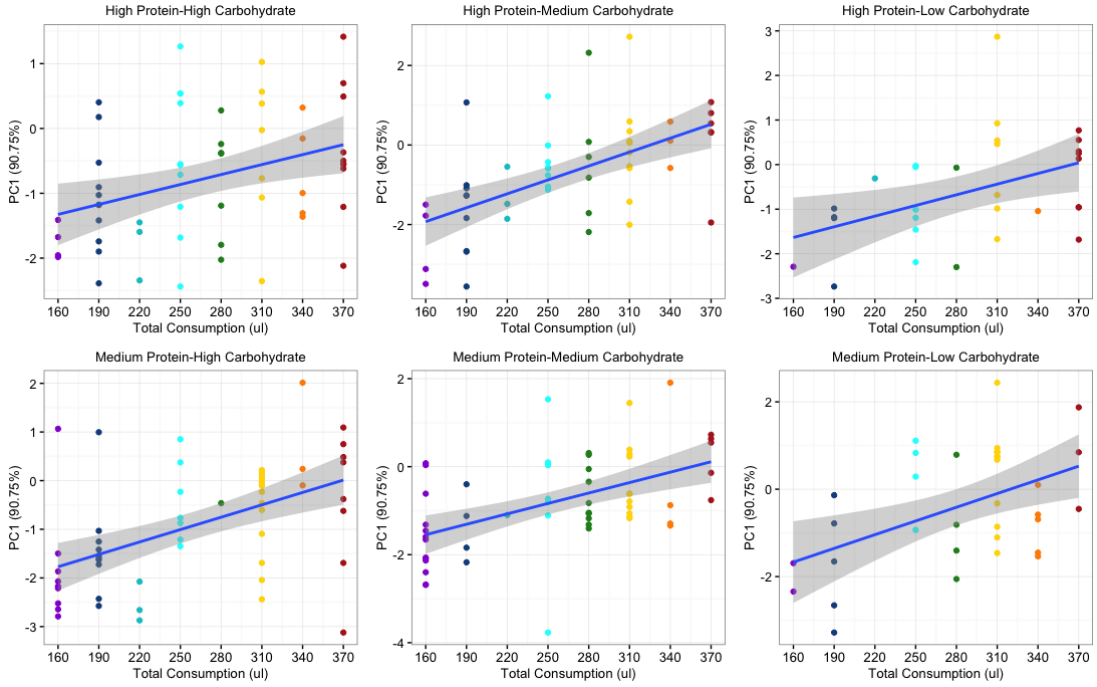

Figure 12: Effect of Diet Quantity on PC1 for the Diets (A-F). (A) High Protein-High Carbohydrates (R^2=0.126, P=**0.007**), (B) High Protein-Medium Carbohydrate (R^2=0.321, P<**0.001**), (C) High Protein-Low Carbohydrate (R^2=0.199, P=**0.013**), (D) Medium Protein-High Carbohydrate (R^2=0.257, P<**0.001**), (E) Medium Protein-Medium Carbohydrate (R^2=0.255, P<**0.001**), (F) Medium Protein-Low Carbohydrate (R^2=0.233, P=**0.004**).

3.5.1. PC1 and Queenliness

We produced queens, worker, and intercastes in the study, which was represented by the Principal Component 1 (PC1) graphic. The PCA graphical representation shows *in vitro* reared individuals are seen both between and within the worker and queen 95% confidence ellipse. This indicates that queens, workers, and intercastes were reared during this study. Also, the graphic clearly distinguishes the 9 quantity treatments by using different color ellipses. The results show that the more-red colored ellipses (low quantity treatment) were closer to the reference worker

cluster, whereas the more violet-colored ellipses (high quantity treatments) were closer to the reference queen cluster. Additionally, the graphic shows that nearly all the individuals reared *ad lib* were within the queen ellipse.

PC1 explained 70.66% of the variation between reference queens and workers; thus, caste can successfully be classified and separated using traditional morphometric. Furthermore, ovariole number and spermethecal size was excluded, and while these measurements are better predictors of queen reproduction viability, traditional morphometrics could still be used to calculate caste. De Souza found more separation between reference queens and workers when including ovariole number and spermathecal size, but separation could still be seen when excluding these morphometrics. Incidentally, PC1 in the study explained more of the variation between reference workers and queens than previous studies. PC1 was likely higher than the other studies because we performed the PCA on just the reference queens and workers, and then used a prediction model to determine the principal components for the *in vitro* reared individuals (De Souza et al., 2015). De Souza et al. (2015) ran a PCA on all the individuals in their study, excluding morphometrics contributing the least to the variation between the reference workers and queens. This difference in analysis is likely why the PC1 in this study explained a high amount of the variation.

3.5.2. Influence of Diet Quality

In the study, we found that neither protein, carbohydrates, or water had a significant influence on final adult caste. Counter to previous studies (Asencot & Lensky, 1988; Alfred Dietz & Haydak., 1971; Kamakura, 2011), these three qualitative components do not seem to determine caste when food quantity was controlled. While dietary quality is important for juvenile growth, development, and survival; diet quality does not appear to determine caste in

honey bees, despite the ubiquitous paradigm. While many studies believe royal jelly contains a "biological active substances" driving queen differentiation (Rembold, 1965), this was not evident in this study

Carbohydrates appear to act as phagostimulants (Asencot & Lensky, 1985). For example, high carbohydrate diets produced more queens when diet quantity was not controlled (Kaftanoglu et al., n.d.), but when accounted for, studies could not distinguish queen development between high and low carbohydrate diets (Aupinel et al., 2005). Concurrent with our study, we found queen development did not differ between high and low sugar diets. While we did not feed larvae *ad libitum* on different diets, we expect more queen development on higher sugar diets. There is evidence that carbohydrates act as a phagostimulant. Royal jelly stimulated larval chemoreceptors more so than worker jelly. Presumably, the higher stimulation is due to sugar content because the spike distribution of chemoreceptors was comparable between sugars alone and royal jelly (Goewie, 1978) . Royal jelly does contain significantly more carbohydrates than worker jelly (M. Haydak, 1968; Rembold, 1965; Y. Wang et al., 2016); thus, carbohydrates may be the phagostimulant that increases food consumption. In other insects, mass consumption requires phagostimulants (Cohen, 2015; Mitchell & Schoonhoven, 1974; Singer, Bernays, & Carrière, 2002), so it is not surprising that honey bees require one also.

We did not find evidence that proteins determine caste in honey bees, which contradicts recent studies. These studies found two proteins, Royalactin (MJRP-1) (Kamakura, 2011) and Major Royal Jelly Protein-3 (MJRP-3) (Huang et al., 2012), influenced queen development. But Despite these results, it is not known whether royal and worker jelly differ in proteins. For example, some studies found Royal jelly had higher protein content than worker jelly for 0-72 aged larvae (Y. Wang et al., 2016), whereas others reported Royal jelly having a lower protein

content (M. H. Haydak, 1943; Shuel & Dixon, 1959; von Planta, 1888). Moreover, no studies have even tested differences in MJRP's between Royal and worker jelly. Further supporting our findings, two new studies refuted Royalactin as a master "on-off" switch for queen development (Buttstedt et al., 2016; Kucharski, Foret, & Maleszka, 2015); thus, casting doubt on the authors original claim. When quantity was controlled at 300ul, queen development was not possible on diets containing high amounts of Royalactin. Therefore, quantity appears to be an unaccounted-for factor in previous caste determination studies. We conclude that protein, while necessary for larval growth and development, does control queen differentiation.

In this study, the diets were not wholly artificial because we used royal jelly as our protein source. We could not supplement non-royal jelly proteins for royal jelly proteins because they decreased larval survival (Singh, 1977), so to ensure maximum growth, development and survival; royal jelly proteins are necessary. Among protein, royal jelly also contains other nutrients, such as lipids, carbohydrates, micronutrients, etc. (Bărnuţiu, Mărghitaş, Dezmirean, Mihai, & Bobiş, 2011; Rembold, 1965); however, we designed this study to only analyze proteins, carbohydrates, and water. In previous studies, these three macronutrients influenced queen development the most (Asencot & Lensky, 1988; Alfred Dietz & Haydak., 1971; Kamakura, 2011); thus, others were not pursued. Our study found diet quality did not affect final adult caste, so these other nutrients were not further studied.

3.5.3. Influence of Diet Quantity

Diet quantity had a significant influence on adult caste. A high proportion of the *ad libitum* (18/18) and 370µl (31/40) treatment were grouped with queens, while zero queens were produced in the three lowest quantity treatments (160µl,190µl,220µl). In the GLMM, diet quantity significantly influences PC1 (70.66%), our final measurement of queenliness. This data

indicates that quantity may regulate caste, which is similar too other social hymenoptera (Allsopp et al., 2003; J Cnaani, Robinson, Bloch, Borst, & Hefetz, 2000; J. Ishay, 1975; Judd et al., 2015; Pereboom, 2000). In these taxa, diet quantity elevates juvenile hormone (Agrahari & Gadagkar, 2003; Penick et al., 2012), which then, triggers the development of reproductive potential. While previous studies show that honey bee queen larvae have significantly higher juvenile hormone titers than workers (Rachinsky, Strambi, Strambi, & Hartfelder, 1990), it has been generally accepted to be regulated by diet quality. These results indicate diet quantity may be the environmental cue that mediates internal physiological processes.

3.5.4. Implications for Honey Bee Caste Determination

A biological active substance in royal jelly has been thought to determine caste in honey bees since the late 1890's (M. H. Haydak, 1970; Rembold, 1965; von Planta, 1888). Since then, the search for a qualitative substance has eluded many researchers. Ours results, however, indicate diet quantity is a significant, but unacknowledged factor in caste determination. Previous studies already indicate quantity control caste determination in honey bees (Leimar et al., 2012; Linksvayer et al., 2011), but quantity has never been formally tested until now. Because of this novelty, future caste determination studies must account for diet quantity, including studies evaluating the physiological and cellular mechanisms of queen differentiation. In future studies, these techniques may elucidate the role of diet quantity on the physiological and cellular mechanisms of caste determination. Many studies have evaluated caste determination both *in vivo* and *in vitro* (de Azevedo & Hartfelder, 2008; Mutti et al., 2011; Patel et al., 2007; D. E. Wheeler, Buck, & Evans, 2014), and while these studies offer useful insights, diet quantity has not previously been considered.

Nearly major component in royal jelly has been shown to have positive results on caste determination (Asencot & Lensky, 1988; Alfred Dietz & Haydak., 1971; M. Haydak, 1968; Huang et al., 2012; Kamakura, 2011; Rembold, 1965; W. X. Wang et al., 2014); however, two of these studies have been refuted when diet quantity was accounted for (Aupinel et al., 2005; Buttstedt et al., 2016). So while other qualitative studies did find positive results, it must be considered that diet quantity was never considered nor controlled for. Diet quality had minimal influence in our study; thus, we consider diet quantity the significant and major factor in honey bee caste determination.

There is observational evidence that quantity determines caste in honey bees. Nurse bees provide queen larvae with more food than workers throughout development (Elser, 1929; M. H. Haydak, 1970), and when emergency queen rearing is necessary, nurses increase worker-destined larvae cell size in order to rear queens (Mark L., 1991). While not explicitly tested, increased cell size likely allows nurse bees to increase provisioning for queen rearing. It appears quantity is necessary for queen rearing, and in order for queen development to occur, nurse's must increase cell size to allow for massive provisioning.

Diet quantity may determine caste because lack of it may be a worker-induced stressor. In our study, low food availability inhibited queen development, which may explain why queen rearing is not possible after the third day. Nurse's may induce worker development by inhibiting queen development via stressors. In a recent study, p-courmaric acid was found to inhibit ovary development(Mao, Schuler, & Berenbaum, 2015b). While p-courmaric acid does not determine queens, the advent of this chemical is important to inhibit ovary development in adult workers. Thus, it seems queen development is impeded by worker-induced-stressors. Mae et al.(2015) and our study indicates that larvae are pre-determined queens, and larvae only become workers due

to dietary impediments, such as starvation and p-courmaric acid. These stressors are both implemented during the third day of larval development, which is the critical day for queen development. In this study, quantity was only altered during the 6th day of development; thus, queen development was still possible after this critical day. While this time-point has long been thought to decide queen-worker phenotypes (Evans & Wheeler, 2001), its seems queen development is still possible past this point. More research is needed, but larvae seem to be pre-determined queens inhibited by workers.

3.5.5. Relevance to Other Social Insects and Insect Polyphenisms Generally

Diet quality does not determine caste in other social hymenoptera; therefore, honey bees were one of the few exceptions. In other social hymenoptera, non-reproductive workers control whether the colony produces reproductive queens (Brian, 1955). Typically, workers increase cell size, and provision more diet to raise queens. While increased cell size is likely to allow development of larger, more reproductive individuals; larger cell sizes also allow more investment into colony reproduction. Because only a few individuals are reproductively viable (Diana E. Wheeler, 1986), increased cell size may be an evolutionarily conserved way for eusocial organisms to ensure the reproductive can maintain long-term colony viability.

CHAPTER 4: THE INFLUENCE OF DIET QUANTITY ON THE UNDERLYING PHYSIOLOGICAL MECHANISMS OF CASTE DETERMINATION IN HONEY BEES (*APIS MELLIFERA)*

4.1. Abstract

Eusocial insects have a reproductive division of labor, made of distinct queen-worker phenotypes with extreme morphometric, physiological and size differences. In honeybees, developmental programs drive these caste differences, and recent evidence indicates quantity determines queenliness. Thus, it seems the lack of food or starvation determines final adult caste. In the classical critical weight hypothesis, juveniles, if critical weight is not attained, postpone metamorphosis with the possibility of attaining more food. In honey bees, body size and caste are interconnected because queens are larger than workers, however, a classical critical weight study has never been done before in honey bees. To test this, we performed a classical critical weight study in honey bees, by starving individuals at different sizes. Then, to determined how diet quantity controlled underlying cellular mechanisms, we attained these different sizes by feeding individuals different quantities of food. We then looked at conserved pathways for caste determination to understand the regulation of body size, i.e. insulin and TOR pathways. Our results show that honey bees do not have a classical critical weight, which is counter to common belief. Intake, or total consumption seems to regulate body size so intake may extend develop for higher fed queens versus workers, and moreover, intake likely regulates nutritional mediated pathways, such as insulin and TOR, which were upregulated in larger individuals whom also consumed more food.

4.2. Introduction

Reproductive caste systems are a key characteristic of insect Eusociality, and queen-worker castes display large differences in body size (Oster & Wilson, 1978). Many of the adult characteristics, such as body size and caste morphometrics, are determined solely during juvenile development; thus, caste is a result of a certain developmental program (Diana E. Wheeler, 1986). Within this development program, two major components are important for body size development, and likely caste development: (1) Growth during juvenile development and (2) onset of metamorphosis (H. F. Nijhout, 2003). These two components are important because in holometabolous insects, growth only occurs during juvenile development, so once growth ceases, final adult caste is set.

In honeybees, this developmental program (larval growth and onset of metamorphosis) have rarely been studied because of the challenges of rear larvae outside of the hive. In a previous study (Paper 2), provision quantity seems to regulate caste and body size in many eusocial hymenoptera within the *Apidae* family; however, honeybees have been a rare exception, because a "biological active" substance in royal jelly is widely thought to determine queens (Rembold, 1965). In our previous study (Paper 3), diet quantity not only determines final caste phenotype, but also, diet quantity determined extent of "queenliness". For example, different quantities produced honey bees with seemingly fluid castes because individuals fed on high quantities displayed all queen traits; individuals on low quantities displayed worker traits; and individuals fed in between displayed a mixture of both, called intercastes. If a "biological active substance" existed, then caste would be a binary switch between queens and worker, but because caste is fluid and ostensibly mediated by quantity, a different framework for understanding the regulation (developmental program) of honey bee castes are needed.

In all holometabolous insects, including honey bees, the timing of molting and metamorphosis is controlled by juvenile hormone. When juvenile hormone is absent, the ecdysone hormone initiates metamorphosis via a metamorphic molt, whereas the presence of juvenile hormone (JH) ensures non-metamorphic molts (Nation, 2008). The cessation of juvenile hormone and thus, the onset of metamorphosis is controlled by the critical weight in some holometabolous species, including *Manduca sexta* (Davidowitz, D'Amico, & Nijhout, 2003). The classical critical weight is defined as "the minimal mass at which further growth is not necessary for a normal time course to pupation", and has been identified in various species (Stieper, Kupershtok, Driscoll, & Shingleton, 2008). However, it is not known whether honey bees have a traditional critical weight. A critical weigh does not exist in *Osmia lignaria*, the blue orchard bee, which is the only bee in which the presence of a crticial weight has been tested (Helm et al *in review*). Because of this, the onset of metamorphosis seems to be mediated differently in honey bees, but a classical critical weight study has never been done before on honey bees.

Honey bee caste determination and body size is a product of growth rate and extent of body size growth during juvenile development, which is regulated on two different levels: physiological and cellular level (Corona et al., 2016). The physiological level controls rate of juvenile hormone (JH) production and degradation(Asencot & Lensky, 1984; Rachinsky et al., 1990), and thus, amount of circulating JH. While JH is necessary for non-metamorphic molts (Nation, 2008), JH is invariably necessary for increase growth because this hormone regulates downstream growth mechanisms (Mutti et al., 2011).. These growth mechanisms, which are on the cellular level and include Insulin (de Azevedo & Hartfelder, 2008; Mutti et al., 2011; Y. Wang, Azevedo, Hartfelder, & Amdam, 2013; D. E. Wheeler et al., 2014; D E Wheeler et al.,

2006; Wolschin et al., 2011), and Target of Rapmyacin (TOR) (Mutti et al., 2011; Patel et al., 2007; D. E. Wheeler et al., 2014), controls body size via both cell proliferation and cell growth (Wu & Brown, 2006), and while these pathways are upregulated by JH, they can also control growth independently (Mutti et al., 2011). Ostensibly, insulin and TOR pathways can either trigger JH release from the corpora allata (Rachinsky & Hartfelder, 1990) or be triggered by JH (Mutti et al., 2011), but the contribution of both is unknown. By controlling nutritional factors *in vitro*, we can better understand the interaction between JH, TOR pathway and Insulin pathway during honey bee caste determination.

Diet quantity controls body size and caste (Chapter 3), but the link between quantity and caste mechanisms, growth rate and onset of metamorphosis, has never been done before. Previous experiments have assessed caste mechanisms; however, their experimental designs included only binary caste outcomes *in vivo* (de Azevedo & Hartfelder, 2008; D. E. Wheeler et al., 2014; D E Wheeler et al., 2006; Kamakura, 2011; Mutti et al., 2011; Patel et al., 2007). Our previous results indicate caste is a reaction norm, so caste mechanisms may be more complicated than previously considered. In the past, these mechanisms have only been compared between queens and workers, and at the point of analysis, these individuals are already set on two different pathways and are consuming different diets, i.e. larger cell size. Because of this, diet quantity is a useful conceptual framework for honeybee, and allows a more precise evaluation of the physiological mechanisms controlling body size and caste. Moreover, body size and metamorphosis likely respond to different diet quantities, which results in this reaction norm. Our study has two components: (1) the onset of metamorphosis and (2) the physiological mechanisms of growth rate. For the first component, we will perform a classical critical weight study in honey bees. For the second component, we will look at the relative expression of

nutrient signaling pathways (Insulin and TOR) and JH degradation pathways in response to differing dietary consumptions, i.e. quantities for rearing workers, intercastes and queens. This study hopefully elucidates the key mechanisms controlling caste in honey bees.

4.3. Materials and Methods

4.3.1. Artificial Rearing

Our *in vitro* rearing protocol is based upon previous studies (Aupinel et al., 2005; Kaftanoglu, Osman, Linksvayer, & Page, 2011). *In vitro* experiments were conducted during July, 2016 using *Apis mellifera* larvae from honey bee colonies near Fargo, Cass County, North Dakota. The study used only 1st instar larvae to limit exposure to extraneous environmental factors. To get 1st instar larvae, queen egg laying was restricted for 24 hours within a jenter box (Blue Sky Bee Supply, Ravenna, OH). Once eggs were oviposited, they were left to incubate within the hive for an additional 72 hours to ensure eggs were fully hatched into 0-24 hour old larvae.

Larvae were transferred into the wells of 24 well-plates using a size 0 paintbrush. Lifting carefully on the dorsal end of each individual, larvae were placed onto 10µl of artificial diet. The 24 well-plates were stored inside a modulator incubator chamber (Billups-Rothenberg, del Mar, CA, USA) containing a small volume of K_2SO_4 saturated solution for 96% RH. Chambers were maintained at 34°C and constant darkness for the remainder of larval development, except for feeding and survival assessments. Larvae were fed daily according to the dietary regimen described below until larva were removed from their food as part of an experimental treatment, or defecated or completely finished the diet. At this point, larvae were moved into pupation plates, which are 24-well plates containing kim wipes soaked and then dried in EtOH. We moved larvae into these plates using a detached blunger from a Chinese grafting tool (Mann Lake, MN,

USA). Pupation plates were maintained at a constant 34°C and 75% RH (Saturated NaCl) until adult eclosion. Eclosion was determined by the development adult characteristics and full movement, at which time these individuals were immediately stored at -20°C.

4.3.2. Diet Treatments and Starvation

All larvae were fed the same artificial diet (also termed "MPMS" in Paper 3), which was similar to past *in vitro* methods (Aupinel et al., 2005; Kaftanoglu et al., 2011). The diet contains all necessary nutrients for growth and development: protein (Royal Jelly (Pure Royal Jelly eBeeHoney.com, Ashland, OH, USA)), carbohydrates (glucose and fructose), vitamins (yeast extract) and water. We supplemented diets with royal jelly instead of purer protein sources for two reasons: (1) other proteins affect survival significantly(Singh, 1977), and (2) other protocols observed successful adult growth and development(De Souza et al., 2015; Kaftanoglu et al., 2011). Diets were produced daily in order to ensure larvae were fed fresh diets. We performed a chemical analysis on this diet so protein and carbohydrate content are known, which is shown in Table 1 and section 3.3.3.

As for feeding regimen, larvae were place into two treatments: worker and queen (Figure 13). Within the worker treatment, individuals were fed a total of 160µl (Day1:10µl, Day2:10µl, Day3:20µl, Day4:30µl, Day5:40µl, Day6:50µl), which followed past protocols (Paper 3-Section 3.3.5). For the queen regimen, larval provisioning followed previous protocols (Paper 3-Section 3.3.5), except larvae were fed 200µl each day starting on Day 6, which continued until the larvae defecated (Day1:10µl, Day2:10µl, Day3:20µl, Day4:30µl, Day5:40µl, Day6 to Defication:200µl).

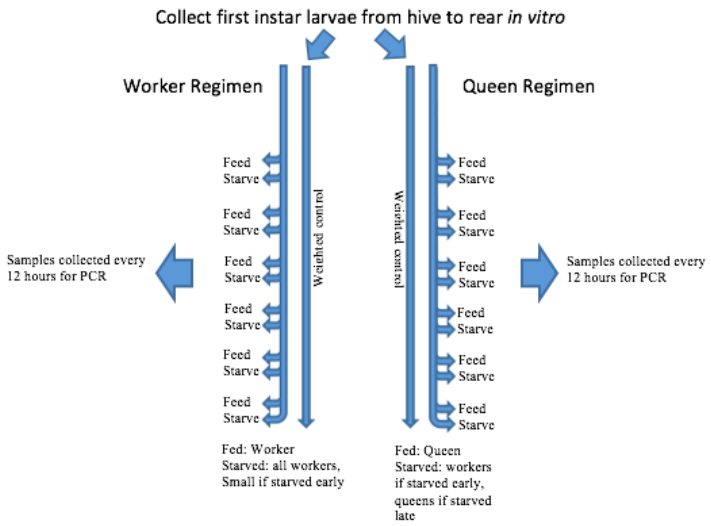

Figure 13: Paper 3 Experimental Design

We originally grafted 960 larvae, and removed 60 larvae every 12 hours. Of the 60 larvae, 10 were flash frozen in liquid nitrogen and stored at -80°C for qPCR, while the other 50 larvae were starved by placing them into pupation plates. After larvae were starved, we checked larvae every 6 hours for deposition of frass, which is an indicator of gut purging and the entrance of individuals into metamorphosis. These starvation treatments continued every 12 hours until all larvae defecated within their diet before we were able to starve them.

4.3.3. Measurement of Critical Size and Other Development Parameters

If honey bees have a critical weight, then larvae would not delay metamorphosis (gut purge) after they attain a certain weight (Davidowitz et al., 2003); thus, we starved individuals at different weights and measured their Delay to Metamorphosis (gut purging) post-starvation. To calculate time to metamorphosis, certain time-points were recorded: Age at Starvation and Age

at Gut Purge, and the differences between these two results in Delay to Metamorphosis (Equation 1).

Equation 1: Delay to Metamorphosis = Age at Gut Purge – Age at Starvation

Once Delay to Metamorphosis was calculated, we performed a Davies test to determines if the regression has a significant change in slope or a "breaking point"(Stieper et al., 2008), but only for individuals that survived to eclosion. If the Davies test is significant and reveals a significant change in slope; then, a critical weight is likely and we would plot a bi-segmental linear regression to determine weight. We used the *davies.test* function in the package *segmented* with R (Muggeo, 2003) to perform the Davies test.

Minimal Viable Weight (MVM) is that weight at which 50% of larvae survive to pupation post-starvation (Stieper et al., 2008). The MVM, which has never been calculated before in honey bees, determines an important developmental time-point in which a 50% of larvae survive into pupation. To calculate, we used a logistic regression model to predict the 50% survival infliction point within a binomial linear regression, which was calculated in R (R Development Core Team, 2011). We also calculated the Minimal Viable Consumption (MVC), which is the diet consumption necessary for a 50% of larvae-to-pupation survival.

Larvae did not consume a set amount of diet because they were starved at varying time-points throughout develop, so in order to determine consumption, we measured amount of food remaining post-starvation. To do this, 1 ml of water was admixed with the remaining larval diet to lower viscosity, and this water-diet mixture was mixed via pipette. Then, the solution was removed and weighed for a final measurement, and intake was calculated by subtracting 1 ml from the weight-diet weight mixture.

4.3.4. Caste Measurement and Principal Component Analysis

Final adult caste was measured using a Principal Component Analysis (PCA), which is an established method for distinguishing queens, workers and intercastes (De Souza et al., 2015; Chapter 3-Section 3.3.5 & 3.3.6.1). A total of 7 morphometric measurements were used: basitarsus length and width, head length and width, mandible length and width, and final adult weight. The analysis was first done between control workers and queens in order to produce components that explain the variation between known queens and workers. The reference queens were purchased (Wildflower Meadows, Southern CA, USA), whereas the control workers were taken from a hive during late spring. The *prcomp* function in the stats package calculated Principal Component 1 (PC1) and Principal Component 2(PC2) (Team, 2016), which explained the majority of the variation between control workers and queens (PC1=70.66%, PC2=14.51%) . To produce Principal Components for the *in vitro* reared individuals, the *predict* function within the *stats* package in R (Team, 2016) was used to produce predicted principal components based upon the control queens and workers. PC1 and PC2 were plotted, with the control workers and queens separated into groups surround by 95% confidence interval ellipses and the *in vitro* reared individuals plotted separately without an ellipse. The assumptions for sphericity, sample adequacy, and determinant of the matrix were tested and met.

4.3.5. Quantitative Real-Time PCR

We quantified expression of four transcripts: Forkhead Box (FOXO), Target of Rapmyacin (TOR), Insulin-Like Receptor 2 (INR-2), S6 Kinase 2 (S6K2), and Juvenile Hormone Esterase (JHE), because three are involved in the Insulin/TOR pathway (FOXO, TOR, INR-2, S6K2) (Wu & Brown, 2006) and the fourth, JHE, is important during entrance into metamorphosis (Nation, 2008). Insulin-like receptor 2 is regulated by circulating sugars (Y.

Wang et al., 2013), whereas TOR is regulated by circulating amino acids; however, FOXO and S6K2 are key kinases that connect Insulin and TOR pathways (Soulard, Cohen, & Hall, 2009), and is shown to regulate important life history traits in honey bees (Kamakura, 2011). Because of this, TOR, INR-2 and FOXO are key genes for growth and caste determination in honey bees. Moreover, these genes were significant in previous studies, thus, these genes were logical candidates (D. E. Wheeler et al., 2014).

We collected RNA from three *Apis mellifera* larvae per weight by using the Invitrogen Trizol protocol (Carlsbad, CA, USA). Larvae were collected at different sizes; 40mg, 70mg, 100mg, 130mg, 190mg, and 240mg (18 bees total), all of which were reared *in vitro* under the queen protocol detailed above. These flash frozen larvae were immediately stored at -80°C until RNA extraction, and once RNA was extracted from the frozen samples, RNA was stored under ethanol at -80°C until needed.

The IDR program from Integrated DNA Technologies (Coralville, IA, USA) designed all primers for all targets and reference genes, which were chosen based on previous publications (Lourenço, Mackert, Cristino, & Simões, 2008; D. E. Wheeler et al., 2014; D E Wheeler et al., 2006). Two primer sets were designed for all target transcripts, and specific primers for quantitative PCR are given in Table 5. Four reference genes were used in the study because these genes showed consistent expression across our different time-points (Torson, Yocum, Rinehart, Kemp, & Bowsher, 2015), and to run the qBasePLUS (Biogazelle, Ghent, Belgium) statistical program, a minimum of 3 were needed. These reference genes, which were used in previous studies, include Calmodulin, Actin, Elongation Factor, Ribosomal Protein L32 (Lourenço et al., 2008).

Table 5: Primers used for Quantitative real-time PCR in This Study

AmInR2	F-CCAGGAGATGAACGACACTTAC	R-ACAGCAGCCAGAACACTATC
	F-GGAGTGTGCCAAAGCATAGA	R-GCTGGTTCCAACAGAGGTATT
AmS6K2	F-CAGTGTGGAACAGCAATAAA	R-AGCACCTAAACTCCACCAATC
	F-GCACTTGATTGGTGGAGTTAG	R-CCTTGGTTGAGCACACATTTC
AmFOXO	F-CCTCATCCTGTGGTCGATTAAG	R-CGATCCTGACGACAAGCTATT
	F-CTCGCGGTCAAAGTGGATTA	R-TCTACGTGATTTCCCTGGTTC
AmTOR	F-GAATGGCTTGGTGGTGATAGA	R-CTGTTCCCTCTGTGCAGTAA
	F-GGTCAATGGCCACAGGTATATG	R-CGACGTACAGTCAAAGGATAA
AmJHE	F-GAAGATGCGCCCAGAGTAAA	R-CCACCGGCACTTAAACCTATTA
	F-GGTGGTGCTAGCGTTCATTA	R-TTTCGATCATTTCGCCCATTTC

As modified from (Torson et al., 2015), the qPCR RNA samples were diluted to a 0.33 μg μl −1 concentration before the samples were treated with DNAase I (Invitrogen, Carlsbad, CA, USA). Then, I used Super Script III first strand synthesis system for RT-PCR (Invitrogen) for first strand cDNA synthesis. For all the samples and primer sets, we ran a dissociation plate using a 1:10 dilution in order to test both primer efficiency and usable reference genes. SYBR Green I Master Mix with ROX (FastStart Universal, Indianapolis, IN, USA) protocol and reagents initiated qPCR reactions, before the LightCycler 480 (Roche, Indianapolis, IN, USA) conducted the qPCR. A total of 3 controls were used during qPCR: (1) no-template control (all enzymatic components except RNA template), (2) negative RT control (no reverse transcriptase) and (3) positive RT control (control RNA template). qBasePLUS (Biogazelle, Ghent, Belgium) was used for analysis of the qPCR data.

4.4. Results

4.4.1. Minimal Viable Weight (MVW) and Critical Size

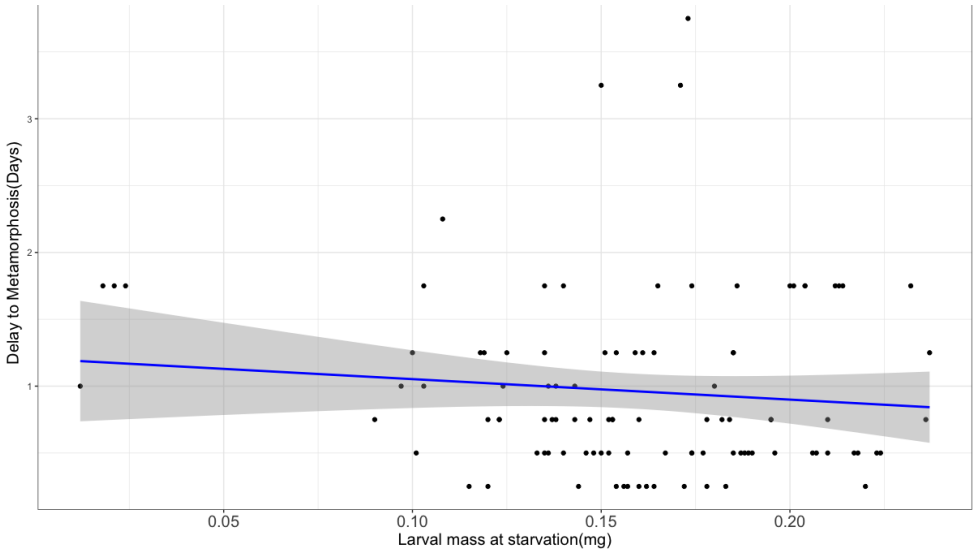

Figure 14: A Critical weight was not Revealed Because The Davies Analysis Determined There was Not a Breaking Point (P<0.0001). Overall, There was An Insignificant Correlation Between Delay To Metamorphosis and Larval Mass At Starvation (Cor=-0.103, P=0.3046).

If honey bee larvae delay before a certain weight, then they likely have a critical weight (H. F. Nijhout, 2003). To calculate this, we used a Davies test, which determines if the regression has a significant change in slope or a "breaking point"(Stieper et al., 2008). The Davies test did not reveal a change in the slope (P<0.0001), and thus, we did not use a bi-segmental linear regression (Cor=-0.103, P=0.3046) to plot the delay from starvation to metamorphosis (Figure 14).

The Minimum Viable Weight (MVM) was calculated using a logistic regression model, which predicted the infliction point at which weight 50% of larvae survive to pupation post-

starvation (Figure 15). The MVW for pupation is 123 mg (95% CI) For all starved larvae that weighed 120-129 mg, the average consumption or Mean Viable Consumption (MVC) was 299.38 µl, and the minimal viable weight, which is the minimum weight needed to possibly survive to adult, was 85 mg and the minimum weight need to pupate is 42 mg. Mean viable weight is the weight at which 50% of larvae survived to adult, which is 123 mg, whereas minimal viable weight is the minimum weight necessary to pupate or for adults to eclose.

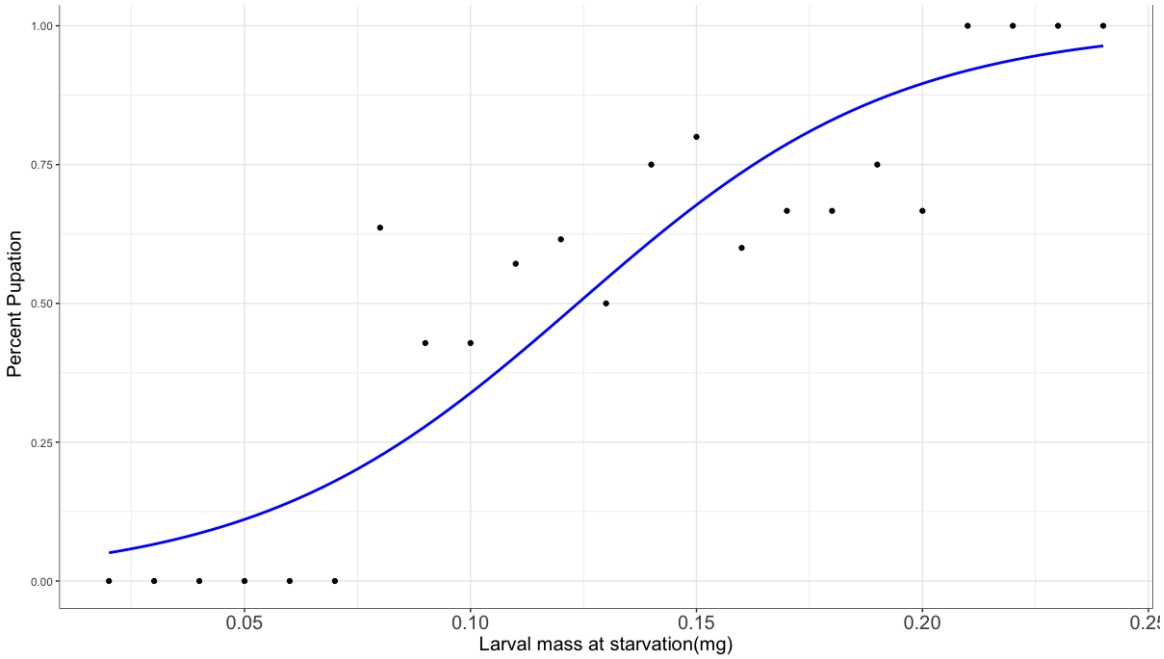

Figure 15: The Mean Viable Weight (MVW(P)) for 50% Survival to Pupation is 123 mg with 95% CI.

4.4.2. Principal Component Analysis

The Principal Component Analysis (PCA) separate references queens and workers (Figure 16), which is similar to Figure 8 because the same reference queens and workers were used (Paper 3-Section 3.4.1). Similar to previous studies(De Souza et al., 2015), traditional morphometrics that exclude reproductive characteristics (ovariole number and spermathecal size) can successfully classify caste. In total, PC1 explains 70.66% of reference queen-worker

variation, while PC2 explains 14.51%, PC3 explains 8.01%, and PC4 explains 2.70. Table 2 (Paper 2- section 3.4.1) shows standardized loadings (contribution to Principal Components) and commonality estimates (explained variation). PC1 clearly distinguished and classified caste, so PC1 was used for future analysis as a caste indicator.

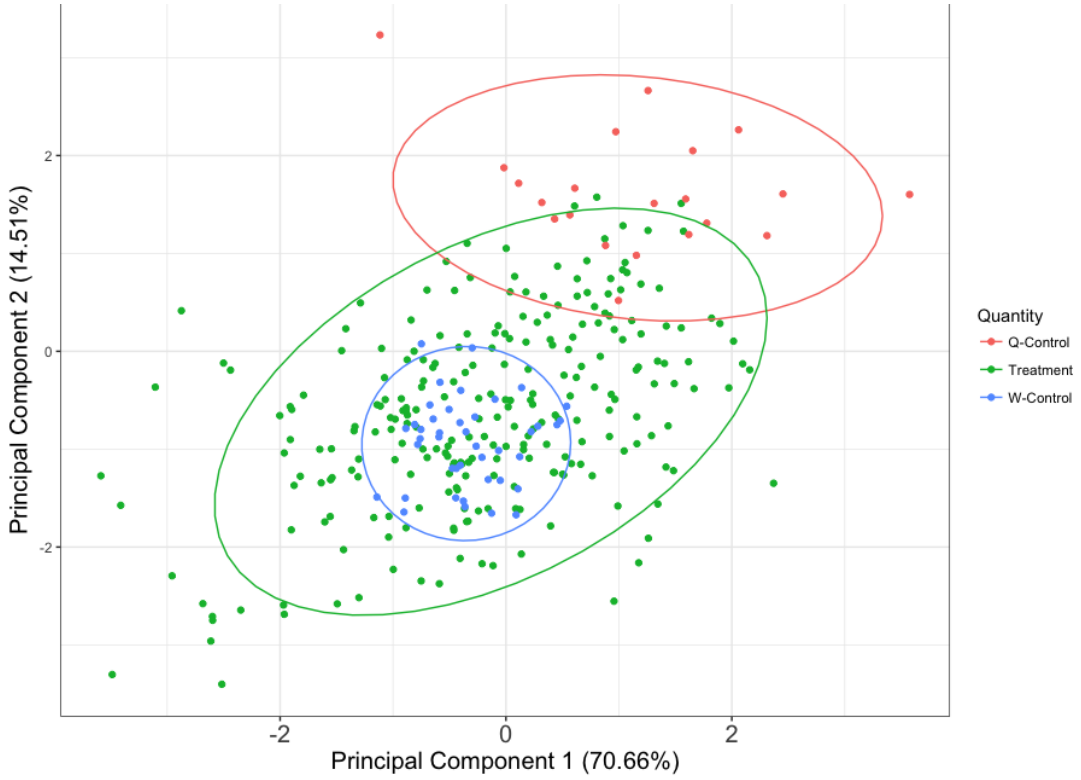

Figure 16: Principal Component Analysis (PCA) using Principal Component 1 (70.66%) and Principal Component 2 (14.51%). Treatment Represents Starved Groups.

4.4.3. Effect of Consumption on Size

Development time did not differ between treatments (F=21.2, P=0.54), but there was huge variation in larval sizes. In order to explain this variation, we measured the amount of food individuals consumed before starvation. There was not only a strong correlation between starvation size and total consumption (0.674), but intake explained 45.36% of variation in adult

weight (F= 324.6, df=1,391, P<0.0001). Figure 17 graphically represents the influence of dietary larval intake on both final adult caste (PC1) and final larval weight. Intake had a significant influence on final adult caste or PC1 (F=27.25, P=<0.001), and explained a high proportion of caste variation (R^2=0.2248)

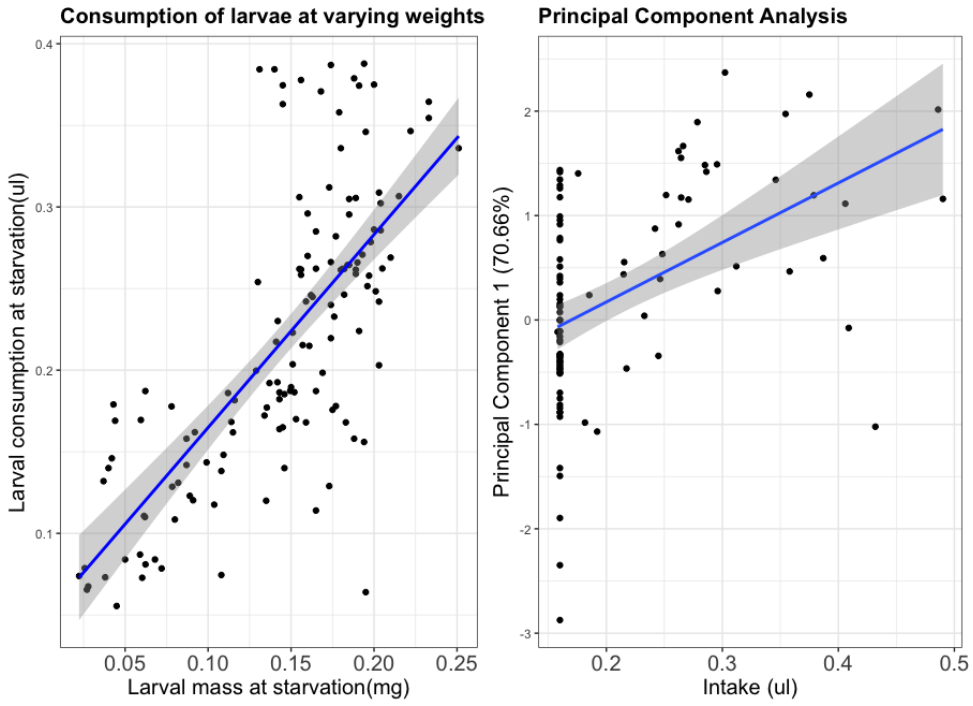

Figure 17: Correlation between Larval Consumption and Both Starvation Weight and Final Adult Caste. Consumption had a Significant Effect On Both Final Adult Caste PC1 (F=27.25, P=<0.001, R^2=0.2248) and Larval Starvation Weight (F= 142 ,P=<0.001,R^2= 0.5164)

4.4.4. qPCR

We were interested in five genes known to be important during caste determination: TOR, INR-2, FOXO, S6K2, and JHE. These five genes were chosen because they were significantly different between queens and workers *in vivo;* thus, we expect larger individuals to have a higher expression of these genes. We measured relative gene expression across different weights, which include 40mg, 70mg, 100mg, 130mg, 190mg, 240mg and explores a wide range

of weights seen during honey bee development. The results are shown in Figure 18, with the relative expression in terms of Calibrated Normalized Relative Quantities (CNRQ). A one-way Anova was performed in qBase on each gene to determine whether gene expression varied across weight. The first gene, FOXO, was not significantly different across treatments (F=4.13, P=0.112), but comparison between 40mg and 190mg revealed a significant difference (ratio= 0.127, CI: 0.026 to 0.624). The second gene, TOR, was also not significantly different across treatments (F=2.048, P=0.226); however, the 40 mg treatment different significantly from both the 100mg (ratio= 0.42, CI: 0.177 to 0.994). and 190mg (ratio= 0.422, CI: 0.178 to 0.998) treatment. Furthermore, INR-2 (F=3.523, P=0.134), JHE (F=0.279, P=0.625) and S6K2 (F=0.564, P=0.494) did not differ significantly across treatments, nor did comparisons reveal significant differences between treatments.

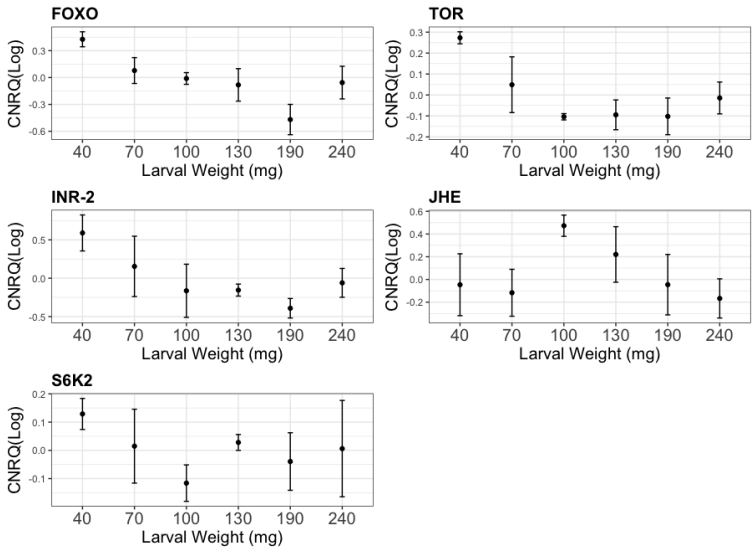

Figure 18: Relative Gene Expression using CNRQ or Calibrated Normalized Relative Quantities. The Graphic Represents the Mean CNRQ for 3 Samples and Their Standard Error.None of the Genes Experience Significant Differences Across Weights (FOXO- F=4.13,P=0.112; TOR- F=2.048, P=0.226; INR-2- F=3.523, P=0.134; JHE- F=0.279,P=0.625; S6K2- F=0.564, P=0.494) However, 40mg Differed From 190mg Treatment In Both FOXO and TOR Genes, and Also From 100mg Within the TOR Gene Treatment.

4.5. Discussion

Developmental programs control final caste phenotype (Diana E. Wheeler, 1986); however, these programs are poorly understood in honey bees. With the recent evidence that diet quantity, not quality determines caste, the two components of developmental programs can be better understood. Since the 1800's (von Planta, 1888), researchers believed diet quality determined queen phenotype, and research has detailed honey bee development based upon diet quality or a "biological active substance" (Rembold, 1965). This has been a problem for one reason: past experiments are based upon binary caste outcome (Kamakura, 2011; D. E. Wheeler et al., 2014; D E Wheeler et al., 2006). According to Figure 19, caste is seemingly fluid as intercastes can be produced. Moreover, not only are intercastes produced from intermediate quantities of diet, but queens are produced from high quantities and workers are produced from lower quantities (Paper 3), so a reaction norm can be produced from seemingly different diets

and likely development programs. Here, we carefully measured diet consumption and its effect on larval growth mechanisms and onset of metamorphosis in the form of a critical weight.

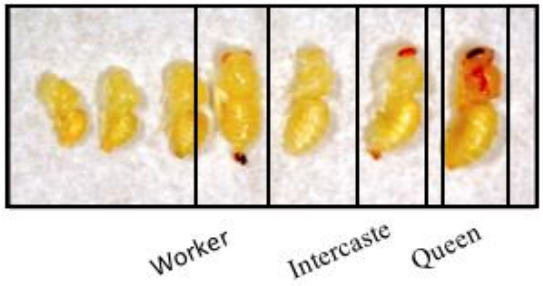

Figure 19: Honey Bee Pupae on Different Quantities

4.5.1. The Onset of Metamorphosis

We tested whether honey bees have a critical weight, and found that they do not have a critical weight (Davies test-P<0.0001). These results indicate honey bees likely enter metamorphosis via a different cue; however, this cue is not known nor well-studied. In some insects, a critical weight determines an individual's decisions to enter metamorphosis. For a well-studied example, *Manduca sexta*, once an individual's reaches a certain weight, Juvenile Hormone titers decrease, and once these titers reach a certain limit, non-metamorphic molts are no longer possible (Davidowitz et al., 2003; Nation, 2008; H. F. Nijhout, 2003). But according to these results, honey bees onset into metamorphosis is determined by another cue or stressor.

A critical weight is not the only cue for metamorphosis known; stretch receptors stimulate molting in *Rhodnius* (Nation, 2008; Osborne & Finlayson, 1962)*;* cold exposure and other environmental cues can also determine molting (Nation, 2008). These three, while known to determine the onset of metamorphosis in well-studied examples, have not been tested in many other organisms, including honey bees. This study elucidates that a critical weight does not drive

metamorphosis, but can these others cues be viable in honey bees? The first, stretch receptors, were hypothesized to determine intake (Asencot & Lensky, 1985), and thus, caste in honey bees; but, honey bees likely don't have stretch receptors because these receptors are not viable in closely related species (Osborne & Finlayson, 1962) and moreover, zero studies indicate bee guts contain such receptors. The second and third known cue, which are cold exposure and temperature, likely do not influence the onset of metamorphosis for two reasons: (1) larval development occurs only in the late spring to summer (Mark L., 1991) and (2) colony nurses maintain precise brood rearing conditions, i.e. constant darkness, temperature, humidity. These factors are especially true for queen rearing, which is under more vigorous rearing conditions(Seeley, 2009). Honey bees metamorphosis is unclear, and other well-studied cues offer zero clues into what determines the onset of metamorphosis.

Our lab found that a closely related species, *Osmia lignaria*, also does not have a critical weight (Unpublished), which indicates these closely related hymenoptera (and likely other related species) are determined by other cues or stressors. Interestingly enough, starvation seemed to initiate metamorphosis in *Osmia lignaria*, which challenges the traditional critical weight model. In these species, larvae initiated metamorphosis immediately post-starvation, and at a wide range of body sizes. We repeated this study in honey bees and found that metamorphosis was initiated within 24-48 hours; however, the delay to metamorphosis post-starvation was consistent across body sizes. So, it is possible starvation plays a key role in metamorphosis.

Unlike the solitary bee *Osmia lignaria*, honey bees have a reproductive caste system, with reproductive queens and sterile workers from genetically similar females. Because of this caste system, we may expect nuances between honey bee and *Osmia lignaria* metamorphosis,

such as immediate entrance into metamorphosis for *Osmia lignaria* and a slight delay across body sizes for honey bees; however, diet quantity (Paper 3) highly influence final adult caste, which indicates that starvation may be a conserved cue. This seems true, not only for maternal control over offspring (as is seen in the solitary *Osmia lignaria),* but an evolutionary conserved cue to produced castrated sterile workers and highly reproductive queens. In both examples, food quantity determines body size as does starvation, because both *Osmia lignaria* and Honey bee growth only occurs during development due to most holometabolous insect display of determinant growth; thus, size is permanent once larvae enter metamorphosis. Diet quantity and starvation go hand and hand and likely determine both body size and caste in honey bees, but even though starvation is a logical cue for metamorphosis, more studies are needed.

Starvation offers a plausible hypothesis for honey bee metamorphosis, which has remained enigmatic. Honey bee caste determination has been constrained to the "third day of development", (M. H. Haydak, 1943) which is the worker larval age at which queens cannot develop from. This third day is important because worker and queen larvae begin to diverge in weight (Rembold, 1965), which slowly restricts the possibility of worker development into queens. We did not change diet quantity until the final instar, and both queens and workers still developed. Intake directly regulated both larval size and final adult caste, well-past the third day of development. Because of this, increasing diet may drive queen development, regardless of developmental day. In other eusocial hymenoptera, diet quantity during the final instar increases both development time and growth, so food quantity may act similarity in honey bees. Quantity determined final adult caste as did starvation weight, so these two results support our hypothesis: a starvation stressor controls body size in honey bees.

4.5.2. Larval Growth

Larval growth determined both final adult caste and body size, because honey bees display determinant growth. Two pathways drive growth: Insulin and TOR, which are well-known to not only determine caste, but insect/honey bee body size (Ament, Corona, Pollock, & Robinson, 2008; de Azevedo & Hartfelder, 2008; Klaus Hartfelder & Azevedo, 2007; Mutti et al., 2011; Patel et al., 2007; Y. Wang et al., 2013; D. E. Wheeler et al., 2014; D E Wheeler et al., 2006; Wolschin et al., 2011). Our results indicated that Insulin and TOR pathways are consistent across development (weights) (Figure 18); thus, our results differ from previous studies. These individuals were all fed *ad libitum*, but individuals were starved at different time-points (every 12 hours). For the qPCR, we only looked at gene expression for individuals of different weights consuming diet, not starved. Because of this, it seems, some other cue may regulated these pathways.

According to this study, pathway thresholds are likely maintained consistently across development (40mg larvae to 240mg larvae), driving increased growth at an ostensibly consistent relative rate. Which is unsurprising considering our framework for body size in honey bees: quantity increases body size, and once individuals are starved, metamorphosis shortly ensues. If starvation drives metamorphosis, starvation would have a precipitous impact on Insulin and TOR pathways; priming larvae for pupation. For example, we did not measure gene expression in individual's post-starvation, which may be useful in understanding body size and regulation of metamorphosis. Also, we only measured expression in five genes, which have important roles, but likely do not elucidate entire caste mechanisms. Even though these genes were chosen based upon expression in previous studies (D. E. Wheeler et al., 2014), other genes need to be considered.

Insulin and TOR pathways drive honey bee growth and caste determination, and the pathways are well-understood due to the abundance of studies (Ament et al., 2008; de Azevedo & Hartfelder, 2008; Klaus Hartfelder & Azevedo, 2007; Mutti et al., 2011; Patel et al., 2007; Y. Wang et al., 2013; D. E. Wheeler et al., 2014; D E Wheeler et al., 2006; Wolschin et al., 2011); however, this study is unique because manipulated larvae produce a reaction norm, not just a binary caste outcome. Previous studies, both *in vivo* and *in vitro* tested Insulin and TOR pathways in two ways: (1) look at gene expression differences between queens and workers *in vivo* (D. E. Wheeler et al., 2014; D E Wheeler et al., 2006), or (2) use RNAi to knockdown genes (Mutti et al., 2011), and elucidate these pathways. These studies have limitations because Insulin and TOR are up-regulated by circulating nutrients that cannot be controlled in an *in vivo* setting (Nation, 2008; Wu & Brown, 2006); thus, it is difficult to understand these mechanisms in an hive setting. Despite this, larval nutrition is not well-known in honey bees because (1) a biological active substance is thought to determine caste (Rembold, 1965) and (2) nutrition is difficult to manipulated *in vivo.* With the advent of *in vitro* techniques (Aupinel et al., 2005; Kaftanoglu et al., n.d.), and the ability to rear queens (and both workers and intercastes) based upon diet quantity, the impact of nutrition can be tested on these pathways. Controlling for nutritional factors, and other extraneous factors, may explain why our results differ from previous studies, and while these differences between studies are interesting, they indicate more studies are needed to truly understand these pathways.

4.5.3. Diet Quantity and Honey Bee Caste Determination

Colony behavior make studying caste determination difficult; thus, many aspects of honey bee development are not well-developed. With our advent of *in vitro* queen rearing techniques, and our ability to rear queens, workers and intercaste based upon diet quantity, we

can hopefully better understand these development programs. The two main components of developmental programs, the onset of metamorphosis and larval growth, were tested in this paper. First, starvation seems to control metamorphosis and second, diet quantity consistently regulates growth throughout development. More studies are needed, but these results likely contribute and perpetuate the field of honey bee caste determination.

CHAPTER 5: CONCLUSIONS

Honey bee reproductive castes, queens and workers, are genetically analogous, yet phenotypically distinct. Diet quality is thought to determine caste in honey bees; however, diet quantity may play a larger role that previously thought, and for 3 reasons: 1) a biological active substance that determines caste has not been found, 2) queens are fed more than workers, and 3) other closely related hymenopteran are determined by diet quantity. Diet quantity has never been assessed for its role in honey bee caste determination; thus, we 1) determine whether diet quantity determines caste in other eusocial hymenoptera, 2) test the relative contribution of diet quantity and quality on final adult caste, and 3) study the role of diet quantity on caste mechanisms and developmental programs of honey bee caste determination.

5.1. Chapter 2: Does Diet Quantity Determine Caste in Eusocial Hymenoptera?

Reproductive caste systems are model organisms for the evolution of complex social behavior in the animal kingdom. Especially in eusocial hymenoptera, this complex behavior likely arose synchronously with reproductive caste systems; and in the case of many examples of eusociality, environmental cues determined whether an individual becomes a highly reproductive queen or non-reproducing worker. These cues drive the developmental divergence of genetically analogous individuals into reproductive castes, i.e. queens and workers. Most importantly, these environmental cues are a possible selectable source of variation that may explain the evolution of these complex caste systems. In this paper, we reveal many species within the families *Vespidae* and *Apidae* were determined by diet quantity. Interestingly enough, honey bees are closely related to these species determined by diet quantity. Thus, it seems according to the historical paradigm, diet quality determines caste only in honey bees, not other closely related

hymenopterans. Because of this possible paradox, more studies are needed, such as Paper 2, to reveal the true cue that determines caste in honey bees.

This paper also shows 2 trends: 1) caste determining cues are family specific and may be derived, and 2) environmental cues are not only the mechanisms mediating the gap between reproductive queens and non-reproductive workers (proximate cues), but these phenotypically plastic mechanism provide a sources of variation that evolution has selected upon to produce these dimorphic castes (ultimate cues). Diet quantity is easily regulated by worker behavior, such as number of times the cell is visited, providing a simple mechanism through which caste can evolve from extant body size variation. This model could elucidate the evolution of reproductive caste systems, including complex social behavior, i.e. sociobiology, evolutionary psychology.

5.2. Chapter 3: Does Diet Quantity Determine Caste in Honey Bees?

Honey bees contain both reproductive females (queens) and non-reproductive females (workers), which derive from nutrition during larval development. Queens consume royal jelly whereas worker consume worker jelly; but these glandular secretions are difficult to study, let alone manipulate for caste determination studies. In this paper, we reveal diet quantity determines caste in honey bees. Honey bee larvae were fed diets of both quantitative and qualitative differences; however, quality had a lesser role than predicted, whereas quantity directly influences final adult queenliness. Because of this, diet quantity may be an underrepresented nutrient past researchers have not considered, yet, seems to have huge implications on caste during development. While this study reveals diet quantity determines caste in honey bees, more studies are needed.

This paper offers three major developments: 1) diet quantity determines caste via underlying physiological and cellular mechanisms, developments impossible to understand *in*

vivo, 2) caste is a reaction norm under which a continuum of interacastes can be produced rather than a pholyphenism in which only two distinct castes are possible. Intercastes are developmentally possible, but nurse bee behavior ensure that they are not produced in the hive. And finally 3) The critical period for determining queens extends into the final instar and is not restricted to the third instar. This paper, along with the development of *in vitro* rearing, not only advances our understanding growth and development of honey bees, but allows more controlled research on honey bee caste determination.

5.3. Chapter 4: Does Diet Quantity Regulate Development Caste Mechanisms in Honey Bees?

Honey bee caste determination occurs during development, because much of adult size is determined by larval development. Specifically, caste is determined by multiple mechanisms: growth rate and developmental length. In this paper, the cue for metamorphosis remains unknown; but, we reveal honey bees do not have a critical weight, which is the cue for metamorphosis in some holometabolous insects. By possibly excluding a critical weight as the cue for honey bee metamorphosis, other research can focus on other cues for metamorphosis. As for growth mechanisms, Insulin and TOR pathway gene expression differed between our study and from past research. More research is needed to understand why and how these mechanisms differed between studies.

The development programs for honey bees, metamorphosis and growth rate, are enigmatic because it is difficult to study honey bee development *in vivo.* With *in vitro* rearing, it is easier to control for all environmental factors. Because of this, honey bee development can be better understood. This is one of the first studies *in vitro* that specifically tested honey bee development, but more can be understood with this method of rearing.

5.4. General Conclusion

The research has implications for not only honey bee research, but also the field of insect eusociality. Researchers use honey bees as model for eusociality and the evolution of social behavior, and because of this, our results have far reaching implications. Diet quantity seems to be the cue for caste for both honey bees and other closely related hymenopterans. With this research, our understanding of insect caste systems can be greatly elucidated and understood.

This research has significant implications for the challenges facing beekeeping in the US. Many factors contribute to annual hive losses, and the interactions between these factors remains unknown. Beekeepers have observed a decline in queen quality over the last decade, with increase failure rates and decreased longevity. However, measuring queen quality is difficult because many of the metrics require killing the queen and doing time consuming dissection. The results of this thesis have the following implications for solving the problem of queen quality, 1) morphological measurement could be used to assess queenliness during queen breeding, 2) nutrition might be a causal factor in queen failure through the production of queens that are slightly intercaste and therefore of poor quality.

REFERENCES

Agrahari, M., & Gadagkar, R. (2003). Juvenile hormone accelerates ovarian development and does not affect age polyethism in the primitively eusocial wasp, Ropalidia marginata. *Journal of Insect Physiology*, *49*(3), 217–222. https://doi.org/10.1016/S0022-1910(02)00268-8

Allsopp, M. H., Calis, J. N. M., & Boot, W. J. (2003). Differential feeding of worker larvae affects caste characters in the Cape honeybee, Apis mellifera capensis. *Behavioral Ecology and Sociobiology*, *54*(6), 555–561. https://doi.org/10.1007/s00265-003-0666-4

Ament, S. A., Corona, M., Pollock, H. S., & Robinson, G. E. (2008). Insulin signaling is involved in the regulation of worker division of labor in honey bee colonies. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(11), 4226–31. https://doi.org/10.1073/pnas.0800630105

Asencot, M., & Lensky, Y. (1984). Juvenile hormone induction of "queenliness" on female honey bee (Apis mellifera L.) larvae reared on worker jelly and on stored royal jelly. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, *78*(1), 109–117. https://doi.org/10.1016/0305-0491(84)90153-6

Asencot, M., & Lensky, Y. (1985). The phagostimulatory effect of sugars on the induction of "queenliness" in female honeybee (Apis mellifera L.) larvae. *Comparative Biochemistry and Physiology Part A: Physiology*, *81*(1), 203–208. https://doi.org/10.1016/0300-9629(85)90289-0

Asencot, M., & Lensky, Y. (1988). The effect of soluble sugars in stored royal jelly on the differentiation of female honeybee (Apis mellifera L.) larvae to queens. *Insect Biochemistry*, *18*(2), 127–133. https://doi.org/10.1016/0020-1790(88)90016-9

Aupinel, P., Fortini, D., Dufour, H., Tasei, J., Michaud, B., Odoux, J., & Pham-Delegue, M. (2005). Improvement of artificial feeding in a standard in vitro method for rearing Apis mellifera larvae. *Bulletin of Insectology*, *58*(2), 107–111.

Barchuk, A. R., Cristino, A. S., Kucharski, R., Costa, L. F., Simões, Z. L., & Maleszka, R. (2007). Molecular determinants of caste differentiation in the highly eusocial honeybee Apis mellifera. *BMC Developmental Biology*, *7*(1), 1. https://doi.org/10.1186/1471-213X-7-70

Bărnuţiu, L. I., Mărghitaş, L. Al, Dezmirean, D. S., Mihai, C. M., & Bobiş, O. (2011). Chemical Composition and Antimicrobial Activity of Royal Jelly - Review. *Scientific Papers Animal Science and Biotechnologies*, *44*(2), 67–72.

Berens, A. J., Hunt, J. H., & Toth, A. L. (2015). Nourishment level affects caste-related gene expression in Polistes wasps. *BMC Genomics*, *16*(1), 235. https://doi.org/10.1186/s12864-015-1410-y

Bloch, G., Shpigler, H., Wheeler, D. E., & Robinson, G. E. (2009). 30 endocrine influences on the organization of insect societies. *Hormones*, *2*, 1027–1068.

Brian, M. . (1955). CASTE DETERMINATION IN SOCIAL INSECTS.

Brian, M. V. (1957). CASTE DETERMINATION IN SOCIAL INSECTS. *Annual Review of Entomology*, *2*(1), 107–120.

Brian, M. V., & Hibble, J. (1963). Larval size and the influence of the queen on growth in Myrmica. *Insectes Sociaux*, *10*(1), 71–82.

Bushinger, von A. (n.d.). Polymorphismus und Kastendetermination im Ameisentribus Leptothoracini.

Buttstedt, A., Ihling, C. H., Pietzsch, M., & Moritz., R. F. (2016). Royalactin is not a royal making of a queen. *Nature*, *537*(7621), E10–E12. https://doi.org/10.1038/nature19349

Camargo, C. A. de, Almeida, M. G. de, Parra, M. G. N., & Kerr, W. E. (1976). Genetics of Sex Determination in Bees . IX . Frequencies of Queens and Workers from Larvae under Controlled Conditions (Hymenoptera : Apoidea). *Journal of Kansas Entomology*, *49*(1), 120–125.

Chandrashekara, K., & Gadagkar, R. (1991). Behavioural Castes, Dominance and Division of Labour in a Primitively Eusocial Wasp. *Ethology*, *87*, 269–283. https://doi.org/10.1111/j.1439-0310.1991.tb00252.x

Cnaani, J., Borst, D. W., Huang, Z.-Y., Robinson, G. E., & Hefetz, A. (1997). Caste determination in Bombus terrestris: differences in development and rates of JH biosynthesis between queen and worker larvae. *Journal of Insect Physiology*, *43*(4), 373–381.

Cnaani, J., Robinson, G. E., Bloch, G., Borst, D., & Hefetz, A. (2000). The effect of queen-worker conflict on caste detennination in the bumblebee Bombus terrestris. *Behaviour Ecology and Sociobiology*, *47*(5), 346–352.

Cohen, A. C. (2015). *Insect diets: science and technology*. CRC press.

Cooper, H. M. (1998). *Synthesizing research: A guide for literature reviews*. (Sage, Ed.).

Corona, M., Libbrecht, R., & Wheeler, D. E. (2016). Molecular mechanisms of phenotypic plasticity in social insects. *Current Opinion in Insect Science*, *13*, 55–60. https://doi.org/10.1016/j.cois.2015.12.003

Creemers, B., Billen, J., & Gobin, B. (2003). Larval begging behaviour in the ant Myrmica rubra. *Ethology Ecology & Evolution*, *15*(February 2015), 261–272. https://doi.org/10.1080/08927014.2003.9522671

Cristino, A. S., Nunes, F. M. F., Lobo, C. H., Bitondi, M. M. G., Simões, Z. L. P., Da Fontoura Costa, L., … Hartfelder, K. (2006). Caste development and reproduction: A genome-wide analysis of hallmarks of insect eusociality. *Insect Molecular Biology*, *15*(5), 703–714. https://doi.org/10.1111/j.1365-2583.2006.00696.x

Daniela M. Witten and Robert Tibshirani. (2013). sparcl: Perform sparse hierarchical clustering and sparse k-means clustering.

Darchen, R., & Delage-Darchen, B. (1971). Le determinisme des castes chez les trigones (Hymenop-tere Apides). *Insectes Sociaux*, 121–134.

Darchen, R., & Delage-Darchen, B. (1975). CONTRIBUTION A L'ETUDE D'UNE ABEILLE DU MEXIQUE MELIPONA BEECHEII B.(HYMENOPT ERE : APIDE). LE D ETERMINISME DES CASTES CHEZ LES M ELIPONES. *Apidologie*, *6*(4), 295–339.

Darras, H., Kuhn, A., & Aron, S. (2014). Genetic determination of female castes in a hybridogenetic desert ant. *Journal of Evolutionary Biology*, *27*(10), 2265–2271. https://doi.org/10.1111/jeb.12470

Davidowitz, G., D'Amico, L. J., & Nijhout, H. F. (2003). Critical weight in the development of insect body size. *Evolution and Development*, *5*(2), 188–197. https://doi.org/10.1046/j.1525-142X.2003.03026.x

de Azevedo, S. V., & Hartfelder, K. (2008). The insulin signaling pathway in honey bee (Apis mellifera) caste development—differential expression of insulin-like peptides and insulin receptors in queen and worker larvae. *Journal of Insect Physiology*, *54*(6), 1064–1071. https://doi.org/10.1016/j.jinsphys.2008.04.009

De Souza, D. A., Wang, Y., Kaftanoglu, O., De Jong, D., Amdam, G. V., Gonçalves, L. S., & Francoy, T. M. (2015). Morphometric identification of queens, workers, intermediates in in

vitro reared honey bees (Apis mellifera). *PLoS ONE*, *10*(4), 1–14. https://doi.org/10.1371/journal.pone.0123663
Dietz, A., & Lambremont, E. N. (1970). A method of studying food consumption of live honey bee larvae by liquid scintillation counting. *Annals of the Entomological Society of America*, *63*(5), 1340–1345.
Dietz, A., & Haydak., M. H. (1971). Caste determination in honey bees. I. The significance of moisture in larval food. *Journal of Experimental Zoology*, *177*(3), 353–357.
Dietz, A., Hermann, H. R., & Blum, M. S. (1979). The role of exogenous JH I, JH III and Anti-JH (precocene II) on queen induction of 4.5-day-old worker honey bee larvae. *Journal of Insect Physiology*, *25*(6), 503–512. https://doi.org/10.1016/S0022-1910(79)80009-8
Dietz, A., & Lambremont, E. N. (1970). Caste determination in honey bees. II. Food consumption of individual honey bee larvae, determined with 32P-labeled royal jelly. *Annals of the Entomological Society of America*, *63*(5), 1342–1345.
Donnell, S. O. (1998). Reproductive Caste Determination in Eusocial Wasps (Hymenoptera : Vespidae), (7).
Elser, E. (1929). Die chemische Zusammensetzung der Nahrungstoffe der Biene. *Markische Bienenzeitung*, *19*, 211-215-235-252.
Evans, J. D., & Wheeler, D. E. (2001). Expression profiles during honeybee caste determination. *Genome Biology*, *2*(1), 1. https://doi.org/10.1186/gb-2000-2-1-research0001
Everitt, B. (1974). *Cluster Analysis: an SSRC review of recent research*.
Fletcher, D. J. C., & Ross, K. G. (1985). Regulation of reproduction in eusocial Hymenoptera. *Analysis*, *30*(1), 319–343.
Foucaud, J., Fournier, D., Orivel, J., Delabie, J. H. C., Loiseau, A., Le Breton, J., … Estoup, A. (2007). Sex and clonality in the little fire ant. *Molecular Biology and Evolution*, *24*(11), 2465–2473. https://doi.org/10.1093/molbev/msm180
Frohschammer, S., & Heinze, J. (2009). A heritable component in sex ratio and caste determination in a Cardiocondyla ant. *Frontiers in Zoology*, *6*, 27. https://doi.org/10.1186/1742-9994-6-27
Goewie, E. A. (1978). Regulation of caste differentiation in the honey bee (Apis mellifera L.). *Meded. Landbouwhogesch. Wageninge*, *78*, 1–76.
Gruber, M. A. M., Hoffmann, B. D., Ritchie, P. A., & Lester, P. J. (2013). The conundrum of the yellow crazy ant (Anoplolepis gracilipes) reproductive mode: No evidence for dependent lineage genetic caste determination. *Insectes Sociaux*, *60*(2), 135–145. https://doi.org/10.1007/s00040-012-0277-z
Hartfelder, K., & Azevedo, S. V. (2007). Expression of insulin receptor isoforms in honey bee (Apis mellifera) caste development. *Developmental Biology*, *306*(1), 339. https://doi.org/10.1016/j.ydbio.2007.03.181
Hartfelder, K., Cnaani, J., & Hefetz, a. (2000). Caste-specific differences in ecdysteroid titers in early larval stages of the bumble bee Bombus terrestris. *J Insect Physiol*, *46*(11), 1433–1439. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10891571
Haydak, M. (1968). III. Nutrition Des Larves D'abeilles. *Traite de Biologie de L'abeille*, *1*, 302–333.
Haydak, M. H. (1943). Larval food and development of castes in the honeybee. *Journal of Economic Entomology*, *36*(5), 778–792. https://doi.org/10.1093/jee/36.5.778
Haydak, M. H. (1970). Honey bee nutrition. *Annual Review of Entomology*, *15*(1), 143–156.
Hedges, L. V., & Olkin, I. (1985). *Statistical Methods for Meta-analysis*. (A. Press, Ed.). New

York, New York. USA.
Heine, J., & Buschinger, A. (1989). Queen polymorphism in Leptothorax spec. A: Its genetic and ecological background (Hymenoptera: Formicidae). *Insectes Sociaux*, *36*(2), 139–155. https://doi.org/10.1007/BF02225909
Helms Cahan, S., & Vinson, S. B. (2003). Reproductive division of labor between hybrid and nonhybrid offspring in a fire ant hybrid zone. *Evolution; International Journal of Organic Evolution*, *57*(7), 1562–1570. https://doi.org/http://dx.doi.org/10.1554/02-518
Hölldobler, B., & Wilson, E. O. (1977). The number of queens: an important trait in ant evolution. *Naturwissenschaften*, *64*(1), 8–15.
Huang, C. Y., Chi, L. L., Huang, W. J., Chen, Y. W., Chen, W. J., Kuo, Y. C., … Chen, C. N. (2012). Growth stimulating effect on queen bee larvae of histone deacetylase inhibitors. *Journal of Agricultural and Food Chemistry*, *60*(24), 6139–6149. https://doi.org/10.1021/jf300815b
Hughes, W. O. H., & Boomsma, J. J. (2008). Genetic royal cheats in leaf-cutting ant societies. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(13), 5150–5153. https://doi.org/10.1073/pnas.0710262105
Ishay, J. (1975). Caste determination by social wasps: Cell size and building behaviour. *Animal Behaviour*, *23*(PART 2), 425–431. https://doi.org/10.1016/0003-3472(75)90090-1
Ishay, J. S., Levanon, D., Avidor, N., & Shechter, O. S. (2016). PHOTOPERIODICITY STIMULATES CELL SIZE AND CASTE DETERMINATION IN THE ORIENTAL HORNET VESPA orientalis Linne, 1775. Retrieved from http://www.tandfonline.com/action/journalInformation?journalCode=wsub20
Jeanne, R. L. (2009). Vibrational signals in Social Wasps: a role in caste determination? *Organization of Insect Societies*.
Johansson, T. S. (1955). Royal Jelly. *Bee World*, *36*(2), 21–32.
Judd, T. M., Teal, P. E. A., Hernandez, E. J., Choudhury, T., & Hunt, J. H. (2015). Quantitative differences in nourishment affect caste-related physiology and development in the paper wasp Polistes metricus. *PLoS ONE*, *10*(2), 1–18. https://doi.org/10.1371/journal.pone.0116199
Kaftanoglu, O., Linksvayer, T. A., & Page, R. E. (n.d.). Rearing honey bees , Apis mellifera , in vitro 1 : Effects of sugar concentrations on survival and development, *11*(96), 1–10.
Kaftanoglu, Osman, T. A., Linksvayer, & Page, R. E. (2011). Rearing honey bees, Apis mellifera, in vitro I: Effects of sugar concentrations on survival and development. *Journal of Insect Science*, *11*(1), 96. https://doi.org/10.1673/031.011.9601
Kamakura, M. (2011). Royalactin induces queen differentiation in honeybees. *Nature*, *473*(7348), 478–483. https://doi.org/10.1038/nature10093
Karsai, I., & Hunt, J. H. (2002). Food quantity affects traits of offspring in the paper wasp *Polistes metricus* (Hymenoptera : Vespidae). *Population Ecology*, *31*(1), 99–106. https://doi.org/http://dx.doi.org/10.1603/0046-225X-31.1.99
Kerr, W., Zucchi, R., Nakadaira, J., & Butolo, J. (2013). Reproduction in the Social Bees (Hymenoptera : Apidae). *Social Bee Reproduction*, *70*(4), 265–276.
Knerer, G., & E. Atwood, C. (1966). Polymorphism in Some Nearctic Halictine Bees. *Science*, *152*(3726), 1262–1263.
Korb, J. (2010). Social insects, major evolutionary transitions and multilevel selection. *Animal Behaviour: Evolution and Mechanisms*, 179–211.
Koyama, S., Takagi, T., Martin, S. J., Yoshida, T., & Takahashi, J. (2009). Absence of

reproductive conflict during queen rearing in Apis cerana. *Insectes Sociaux*, *56*(2), 171–175. https://doi.org/10.1007/s00040-009-0008-2

Kucharski, R., Foret, S., & Maleszka, R. (2015). EGFR gene methylation is not involved in Royalactin controlled phenotypic polymorphism in honey bees. *Scientific Reports*, *5*(August), 14070. https://doi.org/10.1038/srep14070

KUKUK, P. F. (1994). Replacing the Terms Primitive and Advanced - New Modifiers for the Term Eusocial. *Animal Behaviour*. https://doi.org/http://dx.doi.org/10.1006/anbe.1994.1198

Leimar, O., Hartfelder, K., Laubichler, M. D., & Page, R. E. (2012). Development and evolution of caste dimorphism in honeybees–a modeling approach. *Ecology and Evolution*, *2*(12), 3098–3109. https://doi.org/10.1002/ece3.414

Lin, Norman, and C. D. M. (1972). Evolution of Sociality in Insects. *Quarterly Review of Biology*, 131–159.

Linksvayer, T. A., Kaftanoglu, O., Akyol, E., Blatch, S., Amdam, G. V., & Page, R. E. (2011). Larval and nurse worker control of developmental plasticity and the evolution of honey bee queen–worker dimorphism. *Journal of Evolutionary Biology*, *24*(9), 1939–1948. https://doi.org/10.3851/IMP2701.Changes

Linksvayer, T. A., Wade, M. J., & Gordon, D. M. (2006). Genetic caste determination in harvester ants: Possible origin and maintenance by cyto-nuclear epistasis. *Ecology*, *87*(9), 2185–2193. https://doi.org/10.1890/0012-9658(2006)87[2185:GCDIHA]2.0.CO;2

Lourenço, A. P., Mackert, A., Cristino, A. S., & Simões, Z. L. P. (2008). Validation of reference genes for gene expression studies in the honey bee , Apis mellifera , by quantitative real-time RT-PCR *. *Apidologie*, *39*, 372–385. https://doi.org/10.1051/apido

Mao, W., Schuler, M. A., & Berenbaum, M. R. (2015a). A dietary phytochemical alters caste-associated gene expression in honey bees. *Science Advances*, *1*(7), e1500795. https://doi.org/10.1126/sciadv.1500795

Mao, W., Schuler, M. A., & Berenbaum, M. R. (2015b). A dietary phytochemical alters caste-associated gene expression in honey bees. *Science Advances*, *1*(7), e1500795. https://doi.org/10.1126/sciadv.1500795

Mark L., W. (1991). *The biology of the honey bee*. *harvard university press*.

Markiewicz, D. A., & O'Donnell, S. (2001). Social dominance, task performance and nutrition: Implications for reproduction in eusocial wasps. *Journal of Comparative Physiology - A Sensory, Neural, and Behavioral Physiology*, *187*(5), 327–333. https://doi.org/10.1007/s003590100204

Michener, C. D. (1974). *The social behavior of the bees: a comparative study*.

Michonneau, F., Brown, J., & Winter, D. (2016). rotl an R package to interact with the Open Tree of Life Data. *Methods in Ecology and Evolution*, *7*(12), 1476–1481.

Mitchell, B. K., & Schoonhoven, L. M. (1974). Taste Receptors in Colorado Larvae Beetle. *Journal of Insect Physiology*, *20*(9), 1787–1793.

Muggeo, V. M. R. (2003). Estimating regression models with unknown break-points. *Statistics in Medicine*, *22*, 3055–3071.

Mutti, N. S., Dolezal, A. G., Wolschin, F., Mutti, J. S., Gill, K. S., & Amdam, G. V. (2011). IRS and TOR nutrient-signaling pathways act via juvenile hormone to influence honey bee caste fate. *Journal of Experimental Biology*, *214*(23), 3977–3984. https://doi.org/10.1242/jeb.061499

Nakagawa, S., & Cuthill, I. C. (2009). Effect size, confidence interval and statistical significance: A practical guide for biologists: (Biological Reviews (2007) 82 (591-605)).

Biological Reviews, *84*(3), 515. https://doi.org/10.1111/j.1469-185X.2009.00083.x
Nation, J. L. (2008). *Insect physiology and biochemistry*.
Nijhout, H. F. (2003). The control of body size in insects. *Developmental Biology*, *261*(1), 1–9. https://doi.org/10.1016/S0012-1606(03)00276-8
Nijhout, H. F., & Wheeler, D. E. (1982). Juvenile hormone and the physiological basis of insect polymorphisms. *The Quarterly Review of Biology*, *57*(2), 109–133.
Norman, L., & Michener, C. D. (1972). Evolution of sociality in insects. *The Quarterly Review of Biology*, *47*(2), 131–159.
Ohkawara, K., Nakayama, M., Satoh, A., Trindl, A., & Rgen Heinze, J. (2006). Clonal reproduction and genetic caste differences in a queen-polymorphic ant, Vollenhovia emeryi. *Biol. Lett*, *2*(May), 359–363. https://doi.org/10.1098/rsbl.2006.0491
Okamoto, M., & Ohkawara, K. (2010). Egg production and caste allocation in the clonally reproductive ant Vollenhovia emeryi. *Behavioral Ecology*, *21*(5), 1005–1010. https://doi.org/10.1093/beheco/arq093
Osborne, B. M. P., & Finlayson, L. H. (1962). The structure and topography of stretch receptors in representatives of seven orders of insects, *103*(June), 227–242.
Oster, G. F., & Wilson, E. O. (1978). *Caste and ecology in the social insects*.
Passera, L. (1980). La fonction inhibitrice des reines de la fourmi Plagiolepis pygmaea latr.: Role des pheromones. *Insectes Sociaux*, *27*(3), 212–225.
Patel, A., Fondrk, M. K., Kaftanoglu, O., Emore, C., Hunt, G., Frederick, K., & Amdam, G. V. (2007). The Making of a Queen: TOR Pathway Is a Key Player in Diphenic Caste Development. *PLoS ONE*, *2*(6), 1–7. https://doi.org/10.1371/journal.pone.0000509
Pearcy, M. (2004). Conditional Use of Sex and Parthenogenesis for Worker and Queen Production in Ants. *Science*, *306*(5702), 1780–1783. https://doi.org/10.1126/science.1105453
Penick, C. A., Prager, S. S., & Liebig, J. (2012). Juvenile hormone induces queen development in late-stage larvae of the ant Harpegnathos saltator. *Journal of Insect Physiology*, *58*(12), 1643–1649. https://doi.org/10.1016/j.jinsphys.2012.10.004
Pereboom, J. J. M. (2000). The composition of larvae fool and the significance of exocrine secretiosn in the bumblebee Bombus terrestris. *Insectes Sociaux*, *47*(1), 11–20. https://doi.org/10.1007/s000400050003
Pereboom, J. J. M., Velthuis, H. H. W., & Duchateau, M. J. (2003). The organisation of larval feeding in bumblebees (Hymenoptera, Apidae) and its significance to caste differentiation. *Insectes Sociaux*, *50*(2), 127–133. https://doi.org/10.1007/s00040-003-0639-7
Pettis, J. S., Rice, N., & Joselow, K. (2016). Colony Failure Linked to Low Sperm Viability in Honey Bee (Apis mellifera) Queens and an Exploration of Potential Causative Factors, 1–10. https://doi.org/10.1371/journal.pone.0147220
Platt, T. G., Queller, D. C., & Strassmann, J. E. (2004). Aggression and worker control of caste fate in a multiple-queen wasp, Parachartergus colobopterus. *Animal Behaviour*, *67*(1), 1–10. https://doi.org/10.1016/j.anbehav.2003.01.005
Plowright, R. C., & Jay, S. C. (1977). On the size determination of bumble bee castes (Hymenoptera: Apidae). *Canadian Journal of Zoology*, *55*(7), 1133–1138. https://doi.org/10.1139/z77-146
Rachinsky, A., & Hartfelder, K. (1990). Corpora allata activity, a prime regulating element for caste-specific juvenile hormone titre in honey bee larvae (Apis mellifera carnica). *Journal of Insect Physiology*, *36*(3), 189–194. https://doi.org/10.1016/0022-1910(90)90121-U

Rachinsky, A., Strambi, C., Strambi, A., & Hartfelder, K. (1990). Caste and metamorphosis: Hemolymph titers of juvenile hormone and ecdysteroids in last instar honeybee larvae. *General and Comparative Endocrinology*, *79*(1), 31–38. https://doi.org/10.1016/0016-6480(90)90085-Z

R Development Core Team, R. (2011). *R: A Language and Environment for Statistical Computing*. *R Foundation for Statistical Computing* (Vol. 1). https://doi.org/10.1007/978-3-540-74686-7

Rembold, H. (1965). Biologically active substances in royal jelly. *Vitamins and Hormones*. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/5326344

Rembold, H., Lackner, B., & Geistbeck, I. (1974). The chemical basis of honeybee, Apis mellifera, caste formation. Partial purification of queen bee determinator from royal jelly. *Journal of Insect Physiology*, *20*(2), 307–314. https://doi.org/10.1016/0022-1910(74)90063-8

Ribeiro, M. F. (1994). Growth in bumble bee larvae: relation between development time, mass, and amount of pollen ingested. *Canadian Journal of Zoology*, *72*(11), 1978–1985. https://doi.org/10.1139/z94-270

Ribeiro, M. F. (1999). Long-duration feedings and caste differentiation in Bombus terrestris larvae. *Insectes Sociaux*, *46*(4), 315–322. https://doi.org/10.1007/s000400050151

Richards, M. H., Packer, L., Richards, M. H., & Packer, L. (2016). Trophic Aspects of Caste Determination in Halictus ligatus , a Primitively Eusocial Sweat bee, *34*(6), 385–391.

Schmitzová, J., Klaudiny, J., Albert, S., Schröder, W., Schreckengost, W., Hanes, J., … Simúth, J. (1998). A family of major royal jelly proteins of the honeybee *Apis mellifera L*. *Cellular and Molecular Life Sciences : CMLS*, *54*(9), 1020–30. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9791542

Schwander, T., Lo, N., Beekman, M., Oldroyd, B. P., & Keller, L. (2010). Nature versus nurture in social insect caste differentiation. *Trends in Ecology and Evolution*, *25*(5), 275–282. https://doi.org/10.1016/j.tree.2009.12.001

Seeley, T. D. (2009). *The wisdom of the hive: the social physiology of honey bee colonies*. Harvard University Press.

Seeley, T. D., & Url, S. (1978). International Association for Ecology Life History Strategy of the Honey Bee , Apis mellifera, *32*(1), 109–118.

Shuel, R. W., & Dixon, S. E. (1959). Studies in the mode of action of royal jelly in honeybee development: ii. respiration of newly emerged larvae on various substrates. *Canadian Journal of Zoology*, *37*(5), 803–813.

Simo, K., & Christensen, G. M. (1962). Quantitative analysis of sugars in royal jelly. *Nature*, 1208–1209.

Simpson, S. J., Sword, G. A., & Lo, N. (2011). Polyphenism in insects. *Current Biology*, *21*(18), R738–R749. https://doi.org/10.1016/j.cub.2011.06.006

Singer, M. S., Bernays, E. a, & Carrière, Y. (2002). The interplay between nutrient balancing and toxin dilution in foraging by a generalist insect herbivore. *Animal Behaviour*, *64*(4), 629–643. https://doi.org/10.1006/anbe.2002.3082

Singh, P. (1977). *Artificial Diets for insects, mites and spiders*.

Smith, C. R., Anderson, K. E., Tillberg, C. V, Gadau, J., & Suarez, A. V. (2008). Caste determination in a polymorphic social insect: nutritional, social, and genetic factors. *The American Naturalist*, *172*(4), 497–507. https://doi.org/10.1086/590961

Smith, K. M., Loh, E. H., Rostal, M. K., Zambrana-Torrelio, C. M., Mendiola, L., & Daszak, P.

(2013). Pathogens, pests, and economics: Drivers of honey bee colony declines and losses. *EcoHealth*, *10*(4), 434–445. https://doi.org/10.1007/s10393-013-0870-2
Snodgrass, R. E. (1910). The Anatomy of the Honey Bee. *Nature*, *85*(2145), 169–169. https://doi.org/10.1038/085169b0
Soulard, A., Cohen, A., & Hall, M. N. (2009). TOR signaling in invertebrates. *Current Opinion in Cell Biology*, *21*(6), 825–836. https://doi.org/10.1016/j.ceb.2009.08.007
Stieper, B. C., Kupershtok, M., Driscoll, M. V., & Shingleton, A. W. (2008). Imaginal discs regulate developmental timing in Drosophila melanogaster. *Developmental Biology*, *321*(1), 18–26. https://doi.org/10.1016/j.ydbio.2008.05.556
Suryanarayanan, S., Hermanson, J. C., Jeanne, R. L., Wi, M., Gifford, O., & Drive, P. (2011). A Mechanical Signal Biases Caste Development in a Social Wasp. *Current Biology*, *21*(3), 231–235. https://doi.org/10.1016/j.cub.2011.01.003
Team, R. C. (2016). R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing. Retrieved from https://www.r-project.org/
Torson, A. S., Yocum, G. D., Rinehart, J. P., Kemp, W. P., & Bowsher, J. H. (2015). Transcriptional responses to fluctuating thermal regimes underpinning differences in survival in the solitary bee Megachile rotundata. *Journal of Experimental Biology*, *218*(7), 1060–1068. https://doi.org/10.1242/jeb.113829
USDA. (2016). Honey Bee Colonies, (March), 1–17.
Vargo, E. L., & Passera, L. (1990). Pheromonal and Behavioral Queen Control over the Production of Gynes in the Argentine Ant Iridomyrmex humilis (Mayr). *Behavioral Ecology and Sociobiology*, *28*(3), 161–169.
Viechtbauer, W. (2010). Conducting meta-analyses in R with the metafor package. *J Stat Softw*, *36*(3), 1–48.
von Planta, A. (1888). Ueber den Futtersaft der Bienen. *Zeitschrift Für Physiologische Chemie*, *12.4*, 327–354.
Walker, D. B. and M. M. and B. B. and S. (2015). Fitting Linear Mixed-Effects Models Using {lme4}. *Journal of Statistical Software*, *67*, 1–48. https://doi.org/10.18637/jss.v067.i01
Wan, X., Wang, W., Liu, J., & Tong, T. (2014). Estimating the sample mean and standard deviation from the sample size, median, range and/or interquartile range. *BMC Medical Research Methodology*, *14*(1), 135. https://doi.org/10.1186/1471-2288-14-135
Wang, W. X., Tian, L. Q., Huang, Q., Wu, X. B., & Zeng, Z. J. (2014). Effects of 10-Hydroxy-2-decenoic acid on the development of honey bee (Apis mellifera) larvae. *Journal of Apicultural Research*, *53*(1), 171–176. https://doi.org/10.3896/IBRA.1.53.1.19
Wang, Y., Azevedo, S. V, Hartfelder, K., & Amdam, G. V. (2013). Insulin-like peptides (AmILP1 and AmILP2) differentially affect female caste development in the honey bee (Apis mellifera L.). *The Journal of Experimental Biology*, *216*(Pt 23), 4347–57. https://doi.org/10.1242/jeb.085779
Wang, Y., Kaftanoglu, O., Fondrk, M. K., & Page, R. E. (2014). Nurse bee behaviour manipulates worker honeybee (Apis mellifera L.) reproductive development. *Animal Behaviour*, *92*, 253–261. https://doi.org/10.1016/j.anbehav.2014.02.012
Wang, Y., Ma, L., Zhang, W., Cui, X., Wang, H., & Xu, B. (2016). Comparison of the nutrient composition of royal jelly and worker jelly of honey bees (Apis mellifera). *Apidologie*, *47*(1), 48–56. https://doi.org/10.1007/s13592-015-0374-x
Weaver, N. (1966). Physiology of Caste Determination. *Annual Review of Entomology*, *11*(1), 79–102d.

Weever, N. (1965). Physiology of caste determination. *Annual Review of Entomology*, *11*(1), 79–102.
Wheeler, D. E. (1986). Developmental and physiological determinants of caste in social Hymenoptera: evolutionary implications. *The American Naturalist*, *128*(1), 13–34.
Wheeler, D. E., Buck, N. A., & Evans, J. D. (2014). Expression of insulin/insulin-like signalling and TOR pathway genes in honey bee caste determination. *Insect Molecular Biology*, *23*(1), 113–121. https://doi.org/10.1111/imb.12065
Wheeler, D. E., Buck, N. a, & Evans, J. D. (2006). Expression of insulin pathwaygenes during the period of caste determination in the honey bee, Apis mellifera. *Insect Molecular Biology*, *15*, 597–602. https://doi.org/10.1111/j.1365-2583.2006.00681.x
Wiernasz, D. C., & Cole, B. J. (2010). Patriline shifting leads to apparent genetic caste determination in harvester ants. *Proceedings of the National Academy of Sciences of the United States of America*, *107*, 12958–12962. https://doi.org/10.1073/pnas.1003299107
Wille, A., & Orozco, E. (1970). The life cycle and behaviour of the social bee Lasioglossum (Dialictus) umbripenne (Hymenoptera: Halictidae). *Revista de Biologia Tropical*, *17*(2), 199–245.
Wilson, D. B. (2011). Practical meta-analysis effect size calculator.
Wilson, E. (1975). *Sociobiology*. Cambridge, MA: Belk-nap/Harvard University Press.
Wilson, E. (1998). *Consilience*. (T. unity Ofknowledge, Ed.). New York.
Wolschin, F., Mutti, N. S., & Amdam, G. V. (2011). Insulin receptor substrate influences female caste development in honeybees. *Biology Letters*, *7*(1), 112–5. https://doi.org/10.1098/rsbl.2010.0463
Wu, Q., & Brown, M. R. (2006). Signaling and Function of Insulin-Like Peptides in Insects. *Annual Review of Entomology*, *51*(1), 1–24. https://doi.org/10.1146/annurev.ento.51.110104.151011

APPENDIX

Table A1: This Table Shows All Information, Including Effect Size, Species Information, and Caste Determination Factor, For All Species Determined by Factors Other Than Diet Quantity or Genetic Predisposition.

Citation	Order	Family	Genus	Species	Subspecies	Eusocial	Broad Determination	Queen Measurment	d	Lower CI	Upper CI	Var
(Dietz & Haydak., 1971)	Hymenoptera	*Apidae*	*Apis*	*mellifera*		Permanent	Moisture	Weight	1.180	0.358	1.993	0.174
(Mao, Schuler, & Berenbaum, 2016)	Hymenoptera	*Apidae*	*Apis*	*mellifera*		Permanent	p-courmaric acid	ovary score	0.125	-0.219	0.470	0.031
(Kamakura, 2011)	Hymenoptera	*Apidae*	*Apis*	*mellifera*		Permanent	Protein	weight	1.544	1.365	1.724	0.008
(Dietz & Haydak., 1971)	Hymenoptera	*Apidae*	*Apis*	*mellifera*		Permanent	Diet Quality	quantity	3.340	1.830	4.440	0.450
(Buttstedt et al., 2016)	Hymenoptera	*Apidae*	*Apis*	*mellifera*		Permanent	Protein	Weight	0.156	-0.139	0.452	0.023
(Asencot & Lensky, 1985)	Hymenoptera	*Apidae*	*Apis*	*mellifera*		Permanent	Sugar	Weight	2.190	1.090	3.300	0.321
(Asencot & Lensky, 1985)	Hymenoptera	*Apidae*	*Apis*	*mellifera*		Permanent	Sugar	Weight	1.550	0.552	2.550	0.260
(Asencot & Lensky, 1985)	Hymenoptera	*Apidae*	*Apis*	*mellifera*		Permanent	Sugar	Consumption	7.710	5.160	10.240	1.680
(Penick & Liebig, 2012)	Hymenoptera	*Formicidae*	*Harpegnathos*	*saltator*		Permanent	Behavior	Dominance	8.390	4.850	11.930	3.260
(Vargo & Passera, 1990)	Hymenoptera	*Formicidae*	*Iridomyrmex*	*humilis*		Advanced	Environmental	pheromone	10.260	5.590	14.930	5.660
(Vargo & Passera, 1992)	Hymenoptera	*Formicidae*	*Iridomyrmex*	*humilis*		Permanent	Temperature	overwintering	1.190	0.360	2.010	0.176
(Jarau et al., 2010)	Hymenoptera	*Apidae*	*Melipona*	*beecheii*		Permanent	Diet Quantity	Geraniol	0.410	0.197	0.610	0.011
(Warner, Kovaka, & Linksvayer, 2016)	Hymenoptera	*Formicidae*	*Mycocepurus*	*smithii*		Permanent	Fungi	Queen Proportion	0.073	-0.263	0.409	0.031
(Brian & Hibble, 1963)	Hymenoptera	*Formicidae*	*Myrmica*	*rubra*	*L*	Permanent	Diet Quality	Protein shortage	0.339	0.037	0.643	0.024
(Brian & Hibble, 1963)	Hymenoptera	*Formicidae*	*Myrmica*	*rubra*	*L*	Permanent	Diet Quality	Carb shortage	0.409	0.073	0.746	0.030
(Brian & Hibble, 1963)	Hymenoptera	*Formicidae*	*Myrmica*	*rubra*	*L*	Permanent	Environmental	Temperature	1.980	1.498	2.454	0.059
(Brian & Hibble, 1963)	Hymenoptera	*Formicidae*	*Myrmica*	*rubra*	*L*	Permanent	Environmental	Queen influence	0.821	0.282	1.359	0.075
(Passera, 1980)	Hymenoptera	*Formicidae*	*Plagiolepis*	*pygmaea*		Permanent	Environmental	Pheromone	0.397	0.328	0.466	0.001
(Smith, Anderson, Tillberg, Gadau, & Suarez, 2008)	Hymenoptera	*Formicidae*	*Pogonomyrmex*	*badius*		Permanent	Diet Quality	Weight	1.158	0.956	1.361	0.011
(Smith et al., 2008)	Hymenoptera	*Formicidae*	*Pogonomyrmex*	*badius*		Permanent	Diet Quality	C:N	0.520	-0.372	1.414	0.207
(Passera, 1980)	Hymenoptera	*Formicidae*	*Pogonomyrmex*	*badius*		Permanent	Environmental	Colony size	0.037	-0.976	1.049	0.267

A.1. References

Agrahari, M., & Gadagkar, R. (2003). Juvenile hormone accelerates ovarian development and does not affect age polyethism in the primitively eusocial wasp, Ropalidia marginata. *Journal of Insect Physiology*, *49*(3), 217–222. https://doi.org/10.1016/S0022-1910(02)00268-8

Allsopp, M. H., Calis, J. N. M., & Boot, W. J. (2003). Differential feeding of worker larvae affects caste characters in the Cape honeybee, Apis mellifera capensis. *Behavioral Ecology and Sociobiology*, *54*(6), 555–561. https://doi.org/10.1007/s00265-003-0666-4

Ament, S. A., Corona, M., Pollock, H. S., & Robinson, G. E. (2008). Insulin signaling is involved in the regulation of worker division of labor in honey bee colonies. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(11), 4226–31. https://doi.org/10.1073/pnas.0800630105

Asencot, M., & Lensky, Y. (1984). Juvenile hormone induction of "queenliness" on female honey bee (Apis mellifera L.) larvae reared on worker jelly and on stored royal jelly. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, *78*(1), 109–

117. https://doi.org/10.1016/0305-0491(84)90153-6
Asencot, M., & Lensky, Y. (1985). The phagostimulatory effect of sugars on the induction of "queenliness" in female honeybee (Apis mellifera L.) larvae. *Comparative Biochemistry and Physiology Part A: Physiology*, *81*(1), 203–208. https://doi.org/10.1016/0300-9629(85)90289-0
Asencot, M., & Lensky, Y. (1988). The effect of soluble sugars in stored royal jelly on the differentiation of female honeybee (Apis mellifera L.) larvae to queens. *Insect Biochemistry*, *18*(2), 127–133. https://doi.org/10.1016/0020-1790(88)90016-9
Aupinel, P., Fortini, D., Dufour, H., Tasei, J., Michaud, B., Odoux, J., & Pham-Delegue, M. (2005). Improvement of artificial feeding in a standard in vitro method for rearing Apis mellifera larvae. *Bulletin of Insectology*, *58*(2), 107–111.
Barchuk, A. R., Cristino, A. S., Kucharski, R., Costa, L. F., Simões, Z. L., & Maleszka, R. (2007). Molecular determinants of caste differentiation in the highly eusocial honeybee Apis mellifera. *BMC Developmental Biology*, *7*(1), 1. https://doi.org/10.1186/1471-213X-7-70
Bărnuţiu, L. I., Mărghitaş, L. Al, Dezmirean, D. S., Mihai, C. M., & Bobiş, O. (2011). Chemical Composition and Antimicrobial Activity of Royal Jelly - Review. *Scientific Papers Animal Science and Biotechnologies*, *44*(2), 67–72.
Berens, A. J., Hunt, J. H., & Toth, A. L. (2015). Nourishment level affects caste-related gene expression in Polistes wasps. *BMC Genomics*, *16*(1), 235. https://doi.org/10.1186/s12864-015-1410-y
Bloch, G., Shpigler, H., Wheeler, D. E., & Robinson, G. E. (2009). 30 endocrine influences on the organization of insect societies. *Hormones*, *2*, 1027–1068.
Brian, M. . (1955). CASTE DETERMINATION IN SOCIAL INSECTS.
Brian, M. V. (1957). CASTE DETERMINATION IN SOCIAL INSECTS. *Annual Review of Entomology*, *2*(1), 107–120.
Brian, M. V., & Hibble, J. (1963). Larval size and the influence of the queen on growth in Myrmica. *Insectes Sociaux*, *10*(1), 71–82.
Bushinger, von A. (n.d.). Polymorphismus und Kastendetermination im Ameisentribus Leptothoracini.
Buttstedt, A., Ihling, C. H., Pietzsch, M., & Moritz., R. F. (2016). Royalactin is not a royal making of a queen. *Nature*, *537*(7621), E10–E12. https://doi.org/10.1038/nature19349
Camargo, C. A. de, Almeida, M. G. de, Parra, M. G. N., & Kerr, W. E. (1976). Genetics of Sex Determination in Bees . IX . Frequencies of Queens and Workers from Larvae under Controlled Conditions (Hymenoptera : Apoidea). *Journal of Kansas Entomology*, *49*(1), 120–125.
Chandrashekara, K., & Gadagkar, R. (1991). Behavioural Castes, Dominance and Division of Labour in a Primitively Eusocial Wasp. *Ethology*, *87*, 269–283. https://doi.org/10.1111/j.1439-0310.1991.tb00252.x
Cnaani, J., Borst, D. W., Huang, Z.-Y., Robinson, G. E., & Hefetz, A. (1997). Caste determination in Bombus terrestris: differences in development and rates of JH biosynthesis between queen and worker larvae. *Journal of Insect Physiology*, *43*(4), 373–381.
Cnaani, J., Robinson, G. E., Bloch, G., Borst, D., & Hefetz, A. (2000). The effect of queen-worker conflict on caste detennination in the bumblebee Bombus terrestris. *Behaviour Ecology and Sociobiology*, *47*(5), 346–352.
Cohen, A. C. (2015). *Insect diets: science and technology*. CRC press.

Cooper, H. M. (1998). *Synthesizing research: A guide for literature reviews*. (Sage, Ed.).
Corona, M., Libbrecht, R., & Wheeler, D. E. (2016). Molecular mechanisms of phenotypic plasticity in social insects. *Current Opinion in Insect Science*, *13*, 55–60. https://doi.org/10.1016/j.cois.2015.12.003
Creemers, B., Billen, J., & Gobin, B. (2003). Larval begging behaviour in the ant Myrmica rubra. *Ethology Ecology & Evolution*, *15*(February 2015), 261–272. https://doi.org/10.1080/08927014.2003.9522671
Cristino, A. S., Nunes, F. M. F., Lobo, C. H., Bitondi, M. M. G., Simões, Z. L. P., Da Fontoura Costa, L., … Hartfelder, K. (2006). Caste development and reproduction: A genome-wide analysis of hallmarks of insect eusociality. *Insect Molecular Biology*, *15*(5), 703–714. https://doi.org/10.1111/j.1365-2583.2006.00696.x
Daniela M. Witten and Robert Tibshirani. (2013). sparcl: Perform sparse hierarchical clustering and sparse k-means clustering.
Darchen, R., & Delage-Darchen, B. (1971). Le determinisme des castes chez les trigones (Hymenop-tere Apides). *Insectes Sociaux*, 121–134.
Darchen, R., & Delage-Darchen, B. (1975). CONTRIBUTION A L'ETUDE D'UNE ABEILLE DU MEXIQUE MELIPONA BEECHEII B.(HYMENOPT ERE : APIDE). LE D ETERMINISME DES CASTES CHEZ LES M ELIPONES. *Apidologie*, *6*(4), 295–339.
Darras, H., Kuhn, A., & Aron, S. (2014). Genetic determination of female castes in a hybridogenetic desert ant. *Journal of Evolutionary Biology*, *27*(10), 2265–2271. https://doi.org/10.1111/jeb.12470
Davidowitz, G., D'Amico, L. J., & Nijhout, H. F. (2003). Critical weight in the development of insect body size. *Evolution and Development*, *5*(2), 188–197. https://doi.org/10.1046/j.1525-142X.2003.03026.x
de Azevedo, S. V., & Hartfelder, K. (2008). The insulin signaling pathway in honey bee (Apis mellifera) caste development—differential expression of insulin-like peptides and insulin receptors in queen and worker larvae. *Journal of Insect Physiology*, *54*(6), 1064–1071. https://doi.org/10.1016/j.jinsphys.2008.04.009
De Souza, D. A., Wang, Y., Kaftanoglu, O., De Jong, D., Amdam, G. V., Gonçalves, L. S., & Francoy, T. M. (2015). Morphometric identification of queens, workers, intermediates in in vitro reared honey bees (Apis mellifera). *PLoS ONE*, *10*(4), 1–14. https://doi.org/10.1371/journal.pone.0123663
Dietz, A., & Lambremont, E. N. (1970). A method of studying food consumption of live honey bee larvae by liquid scintillation counting. *Annals of the Entomological Society of America*, *63*(5), 1340–1345.
Dietz, A., & Haydak., M. H. (1971). Caste determination in honey bees. I. The significance of moisture in larval food. *Journal of Experimental Zoology*, *177*(3), 353–357.
Dietz, A., Hermann, H. R., & Blum, M. S. (1979). The role of exogenous JH I, JH III and Anti-JH (precocene II) on queen induction of 4.5-day-old worker honey bee larvae. *Journal of Insect Physiology*, *25*(6), 503–512. https://doi.org/10.1016/S0022-1910(79)80009-8
Dietz, A., & Lambremont, E. N. (1970). Caste determination in honey bees. II. Food consumption of individual honey bee larvae, determined with 32P-labeled royal jelly. *Annals of the Entomological Society of America*, *63*(5), 1342–1345.
Donnell, S. O. (1998). Reproductive Caste Determination in Eusocial Wasps (Hymenoptera : Vespidae), (7).
Elser, E. (1929). Die chemische Zusammensetzung der Nahrungstoffe der Biene. *Markische*

Bienenzeitung, *19*, 211-215-235-252.
Evans, J. D., & Wheeler, D. E. (2001). Expression profiles during honeybee caste determination. *Genome Biology*, *2*(1), 1. https://doi.org/10.1186/gb-2000-2-1-research0001
Everitt, B. (1974). *Cluster Analysis: an SSRC review of recent research*.
Fletcher, D. J. C., & Ross, K. G. (1985). Regulation of reproduction in eusocial Hymenoptera. *Analysis*, *30*(1), 319–343.
Foucaud, J., Fournier, D., Orivel, J., Delabie, J. H. C., Loiseau, A., Le Breton, J., … Estoup, A. (2007). Sex and clonality in the little fire ant. *Molecular Biology and Evolution*, *24*(11), 2465–2473. https://doi.org/10.1093/molbev/msm180
Frohschammer, S., & Heinze, J. (2009). A heritable component in sex ratio and caste determination in a Cardiocondyla ant. *Frontiers in Zoology*, *6*, 27. https://doi.org/10.1186/1742-9994-6-27
Goewie, E. A. (1978). Regulation of caste differentiation in the honey bee (Apis mellifera L.). *Meded. Landbouwhogesch. Wageninge*, *78*, 1–76.
Gruber, M. A. M., Hoffmann, B. D., Ritchie, P. A., & Lester, P. J. (2013). The conundrum of the yellow crazy ant (Anoplolepis gracilipes) reproductive mode: No evidence for dependent lineage genetic caste determination. *Insectes Sociaux*, *60*(2), 135–145. https://doi.org/10.1007/s00040-012-0277-z
Hartfelder, K., & Azevedo, S. V. (2007). Expression of insulin receptor isoforms in honey bee (Apis mellifera) caste development. *Developmental Biology*, *306*(1), 339. https://doi.org/10.1016/j.ydbio.2007.03.181
Hartfelder, K., Cnaani, J., & Hefetz, a. (2000). Caste-specific differences in ecdysteroid titers in early larval stages of the bumble bee Bombus terrestris. *J Insect Physiol*, *46*(11), 1433–1439. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10891571
Haydak, M. (1968). III. Nutrition Des Larves D'abeilles. *Traite de Biologie de L'abeille*, *1*, 302–333.
Haydak, M. H. (1943). Larval food and development of castes in the honeybee. *Journal of Economic Entomology*, *36*(5), 778–792. https://doi.org/10.1093/jee/36.5.778
Haydak, M. H. (1970). Honey bee nutrition. *Annual Review of Entomology*, *15*(1), 143–156.
Hedges, L. V., & Olkin, I. (1985). *Statistical Methods for Meta-analysis*. (A. Press, Ed.). New York, New York. USA.
Heine, J., & Buschinger, A. (1989). Queen polymorphism in Leptothorax spec. A: Its genetic and ecological background (Hymenoptera: Formicidae). *Insectes Sociaux*, *36*(2), 139–155. https://doi.org/10.1007/BF02225909
Helms Cahan, S., & Vinson, S. B. (2003). Reproductive division of labor between hybrid and nonhybrid offspring in a fire ant hybrid zone. *Evolution; International Journal of Organic Evolution*, *57*(7), 1562–1570. https://doi.org/http://dx.doi.org/10.1554/02-518
Hölldobler, B., & Wilson, E. O. (1977). The number of queens: an important trait in ant evolution. *Naturwissenschaften*, *64*(1), 8–15.
Huang, C. Y., Chi, L. L., Huang, W. J., Chen, Y. W., Chen, W. J., Kuo, Y. C., … Chen, C. N. (2012). Growth stimulating effect on queen bee larvae of histone deacetylase inhibitors. *Journal of Agricultural and Food Chemistry*, *60*(24), 6139–6149. https://doi.org/10.1021/jf300815b
Hughes, W. O. H., & Boomsma, J. J. (2008). Genetic royal cheats in leaf-cutting ant societies. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(13), 5150–5153. https://doi.org/10.1073/pnas.0710262105

Ishay, J. (1975). Caste determination by social wasps: Cell size and building behaviour. *Animal Behaviour*, *23*(PART 2), 425–431. https://doi.org/10.1016/0003-3472(75)90090-1

Ishay, J. S., Levanon, D., Avidor, N., & Shechter, O. S. (2016). PHOTOPERIODICITY STIMULATES CELL SIZE AND CASTE DETERMINATION IN THE ORIENTAL HORNET VESPA orientalis Linne, 1775. Retrieved from http://www.tandfonline.com/action/journalInformation?journalCode=wsub20

Jeanne, R. L. (2009). Vibrational signals in Social Wasps: a role in caste determination? *Organization of Insect Societies*.

Johansson, T. S. (1955). Royal Jelly. *Bee World*, *36*(2), 21–32.

Judd, T. M., Teal, P. E. A., Hernandez, E. J., Choudhury, T., & Hunt, J. H. (2015). Quantitative differences in nourishment affect caste-related physiology and development in the paper wasp Polistes metricus. *PLoS ONE*, *10*(2), 1–18. https://doi.org/10.1371/journal.pone.0116199

Kaftanoglu, O., Linksvayer, T. A., & Page, R. E. (n.d.). Rearing honey bees , Apis mellifera , in vitro 1 : Effects of sugar concentrations on survival and development, *11*(96), 1–10.

Kaftanoglu, Osman, T. A., Linksvayer, & Page, R. E. (2011). Rearing honey bees, Apis mellifera, in vitro I: Effects of sugar concentrations on survival and development. *Journal of Insect Science*, *11*(1), 96. https://doi.org/10.1673/031.011.9601

Kamakura, M. (2011). Royalactin induces queen differentiation in honeybees. *Nature*, *473*(7348), 478–483. https://doi.org/10.1038/nature10093

Karsai, I., & Hunt, J. H. (2002). Food quantity affects traits of offspring in the paper wasp *Polistes metricus* (Hymenoptera : Vespidae). *Population Ecology*, *31*(1), 99–106. https://doi.org/http://dx.doi.org/10.1603/0046-225X-31.1.99

Kerr, W., Zucchi, R., Nakadaira, J., & Butolo, J. (2013). Reproduction in the Social Bees (Hymenoptera : Apidae). *Social Bee Reproduction*, *70*(4), 265–276.

Knerer, G., & E. Atwood, C. (1966). Polymorphism in Some Nearctic Halictine Bees. *Science*, *152*(3726), 1262–1263.

Korb, J. (2010). Social insects, major evolutionary transitions and multilevel selection. *Animal Behaviour: Evolution and Mechanisms*, 179–211.

Koyama, S., Takagi, T., Martin, S. J., Yoshida, T., & Takahashi, J. (2009). Absence of reproductive conflict during queen rearing in Apis cerana. *Insectes Sociaux*, *56*(2), 171–175. https://doi.org/10.1007/s00040-009-0008-2

Kucharski, R., Foret, S., & Maleszka, R. (2015). EGFR gene methylation is not involved in Royalactin controlled phenotypic polymorphism in honey bees. *Scientific Reports*, *5*(August), 14070. https://doi.org/10.1038/srep14070

KUKUK, P. F. (1994). Replacing the Terms Primitive and Advanced - New Modifiers for the Term Eusocial. *Animal Behaviour*. https://doi.org/http://dx.doi.org/10.1006/anbe.1994.1198

Leimar, O., Hartfelder, K., Laubichler, M. D., & Page, R. E. (2012). Development and evolution of caste dimorphism in honeybees–a modeling approach. *Ecology and Evolution*, *2*(12), 3098–3109. https://doi.org/10.1002/ece3.414

Lin, Norman, and C. D. M. (1972). Evolution of Sociality in Insects. *Quarterly Review of Biology*, 131–159.

Linksvayer, T. A., Kaftanoglu, O., Akyol, E., Blatch, S., Amdam, G. V., & Page, R. E. (2011). Larval and nurse worker control of developmental plasticity and the evolution of honey bee queen–worker dimorphism. *Journal of Evolutionary Biology*, *24*(9), 1939–1948. https://doi.org/10.3851/IMP2701.Changes

Linksvayer, T. A., Wade, M. J., & Gordon, D. M. (2006). Genetic caste determination in harvester ants: Possible origin and maintenance by cyto-nuclear epistasis. *Ecology*, *87*(9), 2185–2193. https://doi.org/10.1890/0012-9658(2006)87[2185:GCDIHA]2.0.CO;2

Lourenço, A. P., Mackert, A., Cristino, A. S., & Simões, Z. L. P. (2008). Validation of reference genes for gene expression studies in the honey bee , Apis mellifera , by quantitative real-time RT-PCR *. *Apidologie*, *39*, 372–385. https://doi.org/10.1051/apido

Mao, W., Schuler, M. A., & Berenbaum, M. R. (2015a). A dietary phytochemical alters caste-associated gene expression in honey bees. *Science Advances*, *1*(7), e1500795. https://doi.org/10.1126/sciadv.1500795

Mao, W., Schuler, M. A., & Berenbaum, M. R. (2015b). A dietary phytochemical alters caste-associated gene expression in honey bees. *Science Advances*, *1*(7), e1500795. https://doi.org/10.1126/sciadv.1500795

Mark L., W. (1991). *The biology of the honey bee*. *harvard university press*.

Markiewicz, D. A., & O'Donnell, S. (2001). Social dominance, task performance and nutrition: Implications for reproduction in eusocial wasps. *Journal of Comparative Physiology - A Sensory, Neural, and Behavioral Physiology*, *187*(5), 327–333. https://doi.org/10.1007/s003590100204

Michener, C. D. (1974). *The social behavior of the bees: a comparative study*.

Michonneau, F., Brown, J., & Winter, D. (2016). rotl an R package to interact with the Open Tree of Life Data. *Methods in Ecology and Evolution*, *7*(12), 1476–1481.

Mitchell, B. K., & Schoonhoven, L. M. (1974). Taste Receptors in Colorado Larvae Beetle. *Journal of Insect Physiology*, *20*(9), 1787–1793.

Muggeo, V. M. R. (2003). Estimating regression models with unknown break-points. *Statistics in Medicine*, *22*, 3055–3071.

Mutti, N. S., Dolezal, A. G., Wolschin, F., Mutti, J. S., Gill, K. S., & Amdam, G. V. (2011). IRS and TOR nutrient-signaling pathways act via juvenile hormone to influence honey bee caste fate. *Journal of Experimental Biology*, *214*(23), 3977–3984. https://doi.org/10.1242/jeb.061499

Nakagawa, S., & Cuthill, I. C. (2009). Effect size, confidence interval and statistical significance: A practical guide for biologists: (Biological Reviews (2007) 82 (591-605)). *Biological Reviews*, *84*(3), 515. https://doi.org/10.1111/j.1469-185X.2009.00083.x

Nation, J. L. (2008). *Insect physiology and biochemistry*.

Nijhout, H. F. (2003). The control of body size in insects. *Developmental Biology*, *261*(1), 1–9. https://doi.org/10.1016/S0012-1606(03)00276-8

Nijhout, H. F., & Wheeler, D. E. (1982). Juvenile hormone and the physiological basis of insect polymorphisms. *The Quarterly Review of Biology*, *57*(2), 109–133.

Norman, L., & Michener, C. D. (1972). Evolution of sociality in insects. *The Quarterly Review of Biology*, *47*(2), 131–159.

Ohkawara, K., Nakayama, M., Satoh, A., Trindl, A., & Rgen Heinze, J. (2006). Clonal reproduction and genetic caste differences in a queen-polymorphic ant, Vollenhovia emeryi. *Biol. Lett*, *2*(May), 359–363. https://doi.org/10.1098/rsbl.2006.0491

Okamoto, M., & Ohkawara, K. (2010). Egg production and caste allocation in the clonally reproductive ant Vollenhovia emeryi. *Behavioral Ecology*, *21*(5), 1005–1010. https://doi.org/10.1093/beheco/arq093

Osborne, B. M. P., & Finlayson, L. H. (1962). The structure and topography of stretch receptors in representatives of seven orders of insects, *103*(June), 227–242.

Oster, G. F., & Wilson, E. O. (1978). *Caste and ecology in the social insects*.
Passera, L. (1980). La fonction inhibitrice des reines de la fourmi Plagiolepis pygmaea latr.: Role des pheromones. *Insectes Sociaux*, *27*(3), 212–225.
Patel, A., Fondrk, M. K., Kaftanoglu, O., Emore, C., Hunt, G., Frederick, K., & Amdam, G. V. (2007). The Making of a Queen: TOR Pathway Is a Key Player in Diphenic Caste Development. *PLoS ONE*, *2*(6), 1–7. https://doi.org/10.1371/journal.pone.0000509
Pearcy, M. (2004). Conditional Use of Sex and Parthenogenesis for Worker and Queen Production in Ants. *Science*, *306*(5702), 1780–1783. https://doi.org/10.1126/science.1105453
Penick, C. A., Prager, S. S., & Liebig, J. (2012). Juvenile hormone induces queen development in late-stage larvae of the ant Harpegnathos saltator. *Journal of Insect Physiology*, *58*(12), 1643–1649. https://doi.org/10.1016/j.jinsphys.2012.10.004
Pereboom, J. J. M. (2000). The composition of larvae fool and the significance of exocrine secretiosn in the bumblebee Bombus terrestris. *Insectes Sociaux*, *47*(1), 11–20. https://doi.org/10.1007/s000400050003
Pereboom, J. J. M., Velthuis, H. H. W., & Duchateau, M. J. (2003). The organisation of larval feeding in bumblebees (Hymenoptera, Apidae) and its significance to caste differentiation. *Insectes Sociaux*, *50*(2), 127–133. https://doi.org/10.1007/s00040-003-0639-7
Pettis, J. S., Rice, N., & Joselow, K. (2016). Colony Failure Linked to Low Sperm Viability in Honey Bee (Apis mellifera) Queens and an Exploration of Potential Causative Factors, 1–10. https://doi.org/10.1371/journal.pone.0147220
Platt, T. G., Queller, D. C., & Strassmann, J. E. (2004). Aggression and worker control of caste fate in a multiple-queen wasp, Parachartergus colobopterus. *Animal Behaviour*, *67*(1), 1–10. https://doi.org/10.1016/j.anbehav.2003.01.005
Plowright, R. C., & Jay, S. C. (1977). On the size determination of bumble bee castes (Hymenoptera: Apidae). *Canadian Journal of Zoology*, *55*(7), 1133–1138. https://doi.org/10.1139/z77-146
Rachinsky, A., & Hartfelder, K. (1990). Corpora allata activity, a prime regulating element for caste-specific juvenile hormone titre in honey bee larvae (Apis mellifera carnica). *Journal of Insect Physiology*, *36*(3), 189–194. https://doi.org/10.1016/0022-1910(90)90121-U
Rachinsky, A., Strambi, C., Strambi, A., & Hartfelder, K. (1990). Caste and metamorphosis: Hemolymph titers of juvenile hormone and ecdysteroids in last instar honeybee larvae. *General and Comparative Endocrinology*, *79*(1), 31–38. https://doi.org/10.1016/0016-6480(90)90085-Z
R Development Core Team, R. (2011). *R: A Language and Environment for Statistical Computing*. *R Foundation for Statistical Computing* (Vol. 1). https://doi.org/10.1007/978-3-540-74686-7
Rembold, H. (1965). Biologically active substances in royal jelly. *Vitamins and Hormones*. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/5326344
Rembold, H., Lackner, B., & Geistbeck, I. (1974). The chemical basis of honeybee, Apis mellifera, caste formation. Partial purification of queen bee determinator from royal jelly. *Journal of Insect Physiology*, *20*(2), 307–314. https://doi.org/10.1016/0022-1910(74)90063-8
Ribeiro, M. F. (1994). Growth in bumble bee larvae: relation between development time, mass, and amount of pollen ingested. *Canadian Journal of Zoology*, *72*(11), 1978–1985. https://doi.org/10.1139/z94-270

Ribeiro, M. F. (1999). Long-duration feedings and caste differentiation in Bombus terrestris larvae. *Insectes Sociaux*, *46*(4), 315–322. https://doi.org/10.1007/s000400050151
Richards, M. H., Packer, L., Richards, M. H., & Packer, L. (2016). Trophic Aspects of Caste Determination in Halictus ligatus , a Primitively Eusocial Sweat bee, *34*(6), 385–391.
Schmitzová, J., Klaudiny, J., Albert, S., Schröder, W., Schreckengost, W., Hanes, J., … Simúth, J. (1998). A family of major royal jelly proteins of the honeybee *Apis mellifera L*. *Cellular and Molecular Life Sciences : CMLS*, *54*(9), 1020–30. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9791542
Schwander, T., Lo, N., Beekman, M., Oldroyd, B. P., & Keller, L. (2010). Nature versus nurture in social insect caste differentiation. *Trends in Ecology and Evolution*, *25*(5), 275–282. https://doi.org/10.1016/j.tree.2009.12.001
Seeley, T. D. (2009). *The wisdom of the hive: the social physiology of honey bee colonies*. Harvard University Press.
Seeley, T. D., & Url, S. (1978). International Association for Ecology Life History Strategy of the Honey Bee , Apis mellifera, *32*(1), 109–118.
Shuel, R. W., & Dixon, S. E. (1959). Studies in the mode of action of royal jelly in honeybee development: ii. respiration of newly emerged larvae on various substrates. *Canadian Journal of Zoology*, *37*(5), 803–813.
Simo, K., & Christensen, G. M. (1962). Quantitative analysis of sugars in royal jelly. *Nature*, 1208–1209.
Simpson, S. J., Sword, G. A., & Lo, N. (2011). Polyphenism in insects. *Current Biology*, *21*(18), R738–R749. https://doi.org/10.1016/j.cub.2011.06.006
Singer, M. S., Bernays, E. a, & Carrière, Y. (2002). The interplay between nutrient balancing and toxin dilution in foraging by a generalist insect herbivore. *Animal Behaviour*, *64*(4), 629–643. https://doi.org/10.1006/anbe.2002.3082
Singh, P. (1977). *Artificial Diets for insects, mites and spiders*.
Smith, C. R., Anderson, K. E., Tillberg, C. V, Gadau, J., & Suarez, A. V. (2008). Caste determination in a polymorphic social insect: nutritional, social, and genetic factors. *The American Naturalist*, *172*(4), 497–507. https://doi.org/10.1086/590961
Smith, K. M., Loh, E. H., Rostal, M. K., Zambrana-Torrelio, C. M., Mendiola, L., & Daszak, P. (2013). Pathogens, pests, and economics: Drivers of honey bee colony declines and losses. *EcoHealth*, *10*(4), 434–445. https://doi.org/10.1007/s10393-013-0870-2
Snodgrass, R. E. (1910). The Anatomy of the Honey Bee. *Nature*, *85*(2145), 169–169. https://doi.org/10.1038/085169b0
Soulard, A., Cohen, A., & Hall, M. N. (2009). TOR signaling in invertebrates. *Current Opinion in Cell Biology*, *21*(6), 825–836. https://doi.org/10.1016/j.ceb.2009.08.007
Stieper, B. C., Kupershtok, M., Driscoll, M. V., & Shingleton, A. W. (2008). Imaginal discs regulate developmental timing in Drosophila melanogaster. *Developmental Biology*, *321*(1), 18–26. https://doi.org/10.1016/j.ydbio.2008.05.556
Suryanarayanan, S., Hermanson, J. C., Jeanne, R. L., Wi, M., Gifford, O., & Drive, P. (2011). A Mechanical Signal Biases Caste Development in a Social Wasp. *Current Biology*, *21*(3), 231–235. https://doi.org/10.1016/j.cub.2011.01.003
Team, R. C. (2016). R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing. Retrieved from https://www.r-project.org/
Torson, A. S., Yocum, G. D., Rinehart, J. P., Kemp, W. P., & Bowsher, J. H. (2015). Transcriptional responses to fluctuating thermal regimes underpinning differences in

survival in the solitary bee Megachile rotundata. *Journal of Experimental Biology*, *218*(7), 1060–1068. https://doi.org/10.1242/jeb.113829
USDA. (2016). Honey Bee Colonies, (March), 1–17.
Vargo, E. L., & Passera, L. (1990). Pheromonal and Behavioral Queen Control over the Production of Gynes in the Argentine Ant Iridomyrmex humilis (Mayr). *Behavioral Ecology and Sociobiology*, *28*(3), 161–169.
Viechtbauer, W. (2010). Conducting meta-analyses in R with the metafor package. *J Stat Softw*, *36*(3), 1–48.
von Planta, A. (1888). Ueber den Futtersaft der Bienen. *Zeitschrift Für Physiologische Chemie*, *12.4*, 327–354.
Walker, D. B. and M. M. and B. B. and S. (2015). Fitting Linear Mixed-Effects Models Using {lme4}. *Journal of Statistical Software*, *67*, 1–48. https://doi.org/10.18637/jss.v067.i01
Wan, X., Wang, W., Liu, J., & Tong, T. (2014). Estimating the sample mean and standard deviation from the sample size, median, range and/or interquartile range. *BMC Medical Research Methodology*, *14*(1), 135. https://doi.org/10.1186/1471-2288-14-135
Wang, W. X., Tian, L. Q., Huang, Q., Wu, X. B., & Zeng, Z. J. (2014). Effects of 10-Hydroxy-2-decenoic acid on the development of honey bee (Apis mellifera) larvae. *Journal of Apicultural Research*, *53*(1), 171–176. https://doi.org/10.3896/IBRA.1.53.1.19
Wang, Y., Azevedo, S. V, Hartfelder, K., & Amdam, G. V. (2013). Insulin-like peptides (AmILP1 and AmILP2) differentially affect female caste development in the honey bee (Apis mellifera L.). *The Journal of Experimental Biology*, *216*(Pt 23), 4347–57. https://doi.org/10.1242/jeb.085779
Wang, Y., Kaftanoglu, O., Fondrk, M. K., & Page, R. E. (2014). Nurse bee behaviour manipulates worker honeybee (Apis mellifera L.) reproductive development. *Animal Behaviour*, *92*, 253–261. https://doi.org/10.1016/j.anbehav.2014.02.012
Wang, Y., Ma, L., Zhang, W., Cui, X., Wang, H., & Xu, B. (2016). Comparison of the nutrient composition of royal jelly and worker jelly of honey bees (Apis mellifera). *Apidologie*, *47*(1), 48–56. https://doi.org/10.1007/s13592-015-0374-x
Weaver, N. (1966). Physiology of Caste Determination. *Annual Review of Entomology*, *11*(1), 79–102d.
Weever, N. (1965). Physiology of caste determination. *Annual Review of Entomology*, *11*(1), 79–102.
Wheeler, D. E. (1986). Developmental and physiological determinants of caste in social Hymenoptera: evolutionary implications. *The American Naturalist*, *128*(1), 13–34.
Wheeler, D. E., Buck, N. A., & Evans, J. D. (2014). Expression of insulin/insulin-like signalling and TOR pathway genes in honey bee caste determination. *Insect Molecular Biology*, *23*(1), 113–121. https://doi.org/10.1111/imb.12065
Wheeler, D. E., Buck, N. a, & Evans, J. D. (2006). Expression of insulin pathwaygenes during the period of caste determination in the honey bee, Apis mellifera. *Insect Molecular Biology*, *15*, 597–602. https://doi.org/10.1111/j.1365-2583.2006.00681.x
Wiernasz, D. C., & Cole, B. J. (2010). Patriline shifting leads to apparent genetic caste determination in harvester ants. *Proceedings of the National Academy of Sciences of the United States of America*, *107*, 12958–12962. https://doi.org/10.1073/pnas.1003299107
Wille, A., & Orozco, E. (1970). The life cycle and behaviour of the social bee Lasioglossum (Dialictus) umbripenne (Hymenoptera: Halictidae). *Revista de Biologia Tropical*, *17*(2), 199–245.

Wilson, D. B. (2011). Practical meta-analysis effect size calculator.
Wilson, E. (1975). *Sociobiology*. Cambridge, MA: Belk-nap/Harvard University Press.
Wilson, E. (1998). *Consilience*. (T. unity Ofknowledge, Ed.). New York.
Wolschin, F., Mutti, N. S., & Amdam, G. V. (2011). Insulin receptor substrate influences female caste development in honeybees. *Biology Letters*, *7*(1), 112–5. https://doi.org/10.1098/rsbl.2010.0463
Wu, Q., & Brown, M. R. (2006). Signaling and Function of Insulin-Like Peptides in Insects. *Annual Review of Entomology*, *51*(1), 1–24.
https://doi.org/10.1146/annurev.ento.51.110104.151011

Table A2: This Table Shows All Information, Including Effect Size, Species Information, and Caste Determination Factor, For All Species Determined by Diet Quantity.

Citation	Order	Family	Genus	Species	Subspecies	Eusocial	Broad Determination	Queen Measurment	d	Lower CI	Upper CI	Var
(Allsopp et al., 2003)	Hymenoptera	*Apidae*	*Apis*	*mellifera*	*scutella*	Permanent	Diet Quantity	Hairs	0.430	-0.511	1.370	0.230
(Allsopp et al., 2003)	Hymenoptera	*Apidae*	*Apis*	*mellifera*	*capensis*	Permanent	Diet Quantity	Hairs	0.499	-0.440	1.440	0.230
(Allsopp et al., 2003)	Hymenoptera	*Apidae*	*Apis*	*mellifera*	*scutella*	Permanent	Diet Quantity	morphometrics	0.727	-0.250	1.700	0.251
(Allsopp et al., 2003)	Hymenoptera	*Apidae*	*Apis*	*mellifera*	*capensis*	Permanent	Diet Quantity	morphometrics	0.746	-0.223	1.718	0.245
(Allsopp et al., 2003)	Hymenoptera	*Apidae*	*Apis*	*mellifera*	*scutella*	Permanent	Diet Quantity	Ovariole	0.123	-0.810	1.050	0.225
(Allsopp et al., 2003)	Hymenoptera	*Apidae*	*Apis*	*mellifera*	*capensis*	Permanent	Diet Quantity	Ovariole	0.095	-0.830	1.020	0.220
(Allsopp et al., 2003)	Hymenoptera	*Apidae*	*Apis*	*mellifera*	*scutella*	Permanent	Diet Quantity	Spermetheca	1.710	0.630	2.790	0.310
(Allsopp et al., 2003)	Hymenoptera	*Apidae*	*Apis*	*mellifera*	*capensis*	Permanent	Diet Quantity	Spermetheca	1.480	0.440	2.530	0.280
(Allsopp et al., 2003)	Hymenoptera	*Apidae*	*Apis*	*mellifera*	*scutella*	Permanent	Diet Quantity	Weight	0.645	-0.310	1.590	0.240
(Allsopp et al., 2003)	Hymenoptera	*Apidae*	*Apis*	*mellifera*	*capensis*	Permanent	Diet Quantity	Weight	0.910	-0.060	1.880	0.250
(Linksvayer et al., 2011)	Hymenoptera	*Apidae*	*Apis*	*mellifera*		Permanent	Diet Quantity	morphometrics	0.880	0.401	1.360	0.060
(Buttstedt et al., 2016)	Hymenoptera	*Apidae*	*Apis*	*mellifera*		Permanent	Diet Quantity	Ovariole number	0.369	-0.094	0.830	0.056
(Buttstedt et al., 2016)	Hymenoptera	*Apidae*	*Apis*	*mellifera*		Permanent	Diet Quantity	Weight	1.390	0.897	1.890	0.064
(Linksvayer et al., 2011)	Hymenoptera	*Apidae*	*Axestotrigona*	*eburnesis*		Permanent	Diet Quantity	morphometrics	0.027	-1.544	1.599	0.643
(Linksvayer et al., 2011)	Hymenoptera	*Apidae*	*Axestotrigona*	*eburnesis*		Permanent	Diet Quantity	morphometrics	0.421	-1.163	2.004	0.653
(Linksvayer et al., 2011)	Hymenoptera	*Apidae*	*Axestotrigona*	*eburnesis*		Permanent	Diet Quantity	morphometrics	6.452	3.083	9.820	2.955
(Linksvayer et al., 2011)	Hymenoptera	*Apidae*	*Axestotrigona*	*eburnesis*		Permanent	Diet Quantity	morphometrics	6.467	3.092	9.843	2.966
(Linksvayer et al., 2011)	Hymenoptera	*Apidae*	*Axestotrigona*	*eburnesis*		Permanent	Diet Quantity	morphometrics	0.056	-1.515	1.628	0.643
(Darchen & Delage-Darchen, 1971)	Hymenoptera	*Apidae*	*Axestotrigona*	*eburnesis*		Permanent	Diet Quantity	morphometrics	2.685	0.390	4.979	1.572
(Röseler, 1970)	Hymenoptera	*Apidae*	*Bombus*	*terrestris*		Cyclical	Diet Quantity	morphometrics	1.384	-0.136	2.405	0.280
(Pereboom, Velthuis, & Duchateau, 2003)	Hymenoptera	*Apidae*	*Bombus*	*terrestris*		Cyclical	Diet Quantity	Weight	1.960	0.108	2.810	0.189
(Pereboom et al., 2003)	Hymenoptera	*Apidae*	*Bombus*	*terrestris*		Cyclical	Diet Quantity	Wing Span	0.808	-0.380	2.000	0.370
(Knerer & E. Atwood, 1966)	Hymenoptera	*Halictidae*	*Evylaeus*	*cinctipes*		Cyclical	Diet Quantity	wing length	3.550	3.221	3.882	0.029
(Richards, Packer, Richards, & Packer, 2016)	Hymenoptera	*Halictidae*	*Halictus*	*ligatus*		Cyclical	Diet Quantity	Fat Content	1.420	0.678	2.156	0.142
(Richards et al., 2016)	Hymenoptera	*Halictidae*	*Halictus*	*ligatus*		Cyclical	Diet Quantity	Head Width	1.323	0.593	2.052	0.139
(Richards et al., 2016)	Hymenoptera	*Halictidae*	*Halictus*	*ligatus*		Cyclical	Diet Quantity	morphometrics	1.368	0.630	2.099	0.140
(Richards et al., 2016)	Hymenoptera	*Halictidae*	*Halictus*	*ligatus*		Cyclical	Diet Quantity	Weight	1.360	0.620	2.088	0.140
(Röseler, 1970)	Hymenoptera	*Apidae*	*Hypotrigona*	*pothieri*		Permanent	Diet Quantity	morphometrics	2.989	1.057	4.922	0.972
(Röseler, 1970)	Hymenoptera	*Apidae*	*Hypotrigona*	*pothieri*		Permanent	Diet Quantity	morphometrics	6.069	3.206	8.934	2.135
(Röseler, 1970)	Hymenoptera	*Apidae*	*Hypotrigona*	*pothieri*		Permanent	Diet Quantity	morphometrics	16.690	9.800	23.500	12.210
(Röseler, 1970)	Hymenoptera	*Apidae*	*Hypotrigona*	*pothieri*		Permanent	Diet Quantity	morphometrics	2.670	0.816	4.529	0.898
(Röseler, 1970)	Hymenoptera	*Apidae*	*Hypotrigona*	*pothieri*		Permanent	Diet Quantity	morphometrics	3.980	1.780	6.180	1.260
(Darchen & Delage-Darchen, 1971)1	Hymenoptera	*Apidae*	*Hypotrigona*	*pothieri*		Permanent	Diet Quantity	morphometrics	6.480	3.332	9.613	3.495
(Wille & Orozco, 1970)	Hymenoptera	*Halictidae*	*Lasioglossum*	*umbripenne*		Cyclical	Diet Quantity	head width	4.087	3.599	4.576	0.062
(Wille & Orozco, 1970)	Hymenoptera	*Halictidae*	*Lasioglossum*	*umbripenne*		Cyclical	Diet Quantity	morphometrics	3.086	2.668	3.499	0.045
(Wille & Orozco, 1970)	Hymenoptera	*Halictidae*	*Lasioglossum*	*umbripenne*		Cyclical	Diet Quantity	total length	2.350	1.986	2.710	0.034
(Wille & Orozco, 1970)	Hymenoptera	*Halictidae*	*Lasioglossum*	*umbripenne*		Cyclical	Diet Quantity	wing length	2.822	2.420	3.210	0.040
(Darchen & Delage-Darchen, 1977)	Hymenoptera	*Apidae*	*Melipona*	*beecheii*		Permanent	Diet Quantity	Queen Proportion	0.859	0.451	1.268	0.044

A.2. References

Agrahari, M., & Gadagkar, R. (2003). Juvenile hormone accelerates ovarian development and does not affect age polyethism in the primitively eusocial wasp, Ropalidia marginata. *Journal of Insect Physiology*, *49*(3), 217–222. https://doi.org/10.1016/S0022-1910(02)00268-8

Allsopp, M. H., Calis, J. N. M., & Boot, W. J. (2003). Differential feeding of worker larvae affects caste characters in the Cape honeybee, Apis mellifera capensis. *Behavioral Ecology and Sociobiology*, *54*(6), 555–561. https://doi.org/10.1007/s00265-003-0666-4

Ament, S. A., Corona, M., Pollock, H. S., & Robinson, G. E. (2008). Insulin signaling is involved in the regulation of worker division of labor in honey bee colonies. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(11), 4226–31. https://doi.org/10.1073/pnas.0800630105

Asencot, M., & Lensky, Y. (1984). Juvenile hormone induction of "queenliness" on female honey bee (Apis mellifera L.) larvae reared on worker jelly and on stored royal jelly. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, *78*(1), 109–117. https://doi.org/10.1016/0305-0491(84)90153-6

Asencot, M., & Lensky, Y. (1985). The phagostimulatory effect of sugars on the induction of "queenliness" in female honeybee (Apis mellifera L.) larvae. *Comparative Biochemistry and Physiology Part A: Physiology*, *81*(1), 203–208. https://doi.org/10.1016/0300-9629(85)90289-0

Asencot, M., & Lensky, Y. (1988). The effect of soluble sugars in stored royal jelly on the differentiation of female honeybee (Apis mellifera L.) larvae to queens. *Insect Biochemistry*, *18*(2), 127–133. https://doi.org/10.1016/0020-1790(88)90016-9

Aupinel, P., Fortini, D., Dufour, H., Tasei, J., Michaud, B., Odoux, J., & Pham-Delegue, M. (2005). Improvement of artificial feeding in a standard in vitro method for rearing Apis mellifera larvae. *Bulletin of Insectology*, *58*(2), 107–111.

Barchuk, A. R., Cristino, A. S., Kucharski, R., Costa, L. F., Simões, Z. L., & Maleszka, R. (2007). Molecular determinants of caste differentiation in the highly eusocial honeybee Apis mellifera. *BMC Developmental Biology*, *7*(1), 1. https://doi.org/10.1186/1471-213X-7-70

Bărnuţiu, L. I., Mărghitaş, L. Al, Dezmirean, D. S., Mihai, C. M., & Bobiş, O. (2011). Chemical Composition and Antimicrobial Activity of Royal Jelly - Review. *Scientific Papers Animal Science and Biotechnologies*, *44*(2), 67–72.

Berens, A. J., Hunt, J. H., & Toth, A. L. (2015). Nourishment level affects caste-related gene expression in Polistes wasps. *BMC Genomics*, *16*(1), 235. https://doi.org/10.1186/s12864-015-1410-y

Bloch, G., Shpigler, H., Wheeler, D. E., & Robinson, G. E. (2009). 30 endocrine influences on the organization of insect societies. *Hormones*, *2*, 1027–1068.

Brian, M. . (1955). CASTE DETERMINATION IN SOCIAL INSECTS.

Brian, M. V. (1957). CASTE DETERMINATION IN SOCIAL INSECTS. *Annual Review of Entomology*, *2*(1), 107–120.

Brian, M. V., & Hibble, J. (1963). Larval size and the influence of the queen on growth in Myrmica. *Insectes Sociaux*, *10*(1), 71–82.

Bushinger, von A. (n.d.). Polymorphismus und Kastendetermination im Ameisentribus Leptothoracini.

Buttstedt, A., Ihling, C. H., Pietzsch, M., & Moritz., R. F. (2016). Royalactin is not a royal making of a queen. *Nature*, *537*(7621), E10–E12. https://doi.org/10.1038/nature19349

Camargo, C. A. de, Almeida, M. G. de, Parra, M. G. N., & Kerr, W. E. (1976). Genetics of Sex Determination in Bees . IX . Frequencies of Queens and Workers from Larvae under Controlled Conditions (Hymenoptera : Apoidea). *Journal of Kansas Entomology*, *49*(1), 120–125.

Chandrashekara, K., & Gadagkar, R. (1991). Behavioural Castes, Dominance and Division of Labour in a Primitively Eusocial Wasp. *Ethology*, *87*, 269–283. https://doi.org/10.1111/j.1439-0310.1991.tb00252.x

Cnaani, J., Borst, D. W., Huang, Z.-Y., Robinson, G. E., & Hefetz, A. (1997). Caste determination in Bombus terrestris: differences in development and rates of JH biosynthesis between queen and worker larvae. *Journal of Insect Physiology*, *43*(4), 373–381.

Cnaani, J., Robinson, G. E., Bloch, G., Borst, D., & Hefetz, A. (2000). The effect of queen-worker conflict on caste detennination in the bumblebee Bombus terrestris. *Behaviour Ecology and Sociobiology*, *47*(5), 346–352.

Cohen, A. C. (2015). *Insect diets: science and technology*. CRC press.

Cooper, H. M. (1998). *Synthesizing research: A guide for literature reviews*. (Sage, Ed.).

Corona, M., Libbrecht, R., & Wheeler, D. E. (2016). Molecular mechanisms of phenotypic plasticity in social insects. *Current Opinion in Insect Science*, *13*, 55–60. https://doi.org/10.1016/j.cois.2015.12.003

Creemers, B., Billen, J., & Gobin, B. (2003). Larval begging behaviour in the ant Myrmica rubra. *Ethology Ecology & Evolution*, *15*(February 2015), 261–272. https://doi.org/10.1080/08927014.2003.9522671

Cristino, A. S., Nunes, F. M. F., Lobo, C. H., Bitondi, M. M. G., Simões, Z. L. P., Da Fontoura Costa, L., … Hartfelder, K. (2006). Caste development and reproduction: A genome-wide analysis of hallmarks of insect eusociality. *Insect Molecular Biology*, *15*(5), 703–714. https://doi.org/10.1111/j.1365-2583.2006.00696.x

Daniela M. Witten and Robert Tibshirani. (2013). sparcl: Perform sparse hierarchical clustering and sparse k-means clustering.

Darchen, R., & Delage-Darchen, B. (1971). Le determinisme des castes chez les trigones (Hymenop-tere Apides). *Insectes Sociaux*, 121–134.

Darchen, R., & Delage-Darchen, B. (1975). CONTRIBUTION A L'ETUDE D'UNE ABEILLE DU MEXIQUE MELIPONA BEECHEII B.(HYMENOPT ERE : APIDE). LE D ETERMINISME DES CASTES CHEZ LES M ELIPONES. *Apidologie*, *6*(4), 295–339.

Darras, H., Kuhn, A., & Aron, S. (2014). Genetic determination of female castes in a hybridogenetic desert ant. *Journal of Evolutionary Biology*, *27*(10), 2265–2271. https://doi.org/10.1111/jeb.12470

Davidowitz, G., D'Amico, L. J., & Nijhout, H. F. (2003). Critical weight in the development of insect body size. *Evolution and Development*, *5*(2), 188–197. https://doi.org/10.1046/j.1525-142X.2003.03026.x

de Azevedo, S. V., & Hartfelder, K. (2008). The insulin signaling pathway in honey bee (Apis mellifera) caste development—differential expression of insulin-like peptides and insulin receptors in queen and worker larvae. *Journal of Insect Physiology*, *54*(6), 1064–1071. https://doi.org/10.1016/j.jinsphys.2008.04.009

De Souza, D. A., Wang, Y., Kaftanoglu, O., De Jong, D., Amdam, G. V., Gonçalves, L. S., & Francoy, T. M. (2015). Morphometric identification of queens, workers, intermediates in in vitro reared honey bees (Apis mellifera). *PLoS ONE*, *10*(4), 1–14. https://doi.org/10.1371/journal.pone.0123663

Dietz, A., & Lambremont, E. N. (1970). A method of studying food consumption of live honey bee larvae by liquid scintillation counting. *Annals of the Entomological Society of America*, *63*(5), 1340–1345.

Dietz, A., & Haydak., M. H. (1971). Caste determination in honey bees. I. The significance of

moisture in larval food. *Journal of Experimental Zoology*, *177*(3), 353–357.
Dietz, A., Hermann, H. R., & Blum, M. S. (1979). The role of exogenous JH I, JH III and Anti-JH (precocene II) on queen induction of 4.5-day-old worker honey bee larvae. *Journal of Insect Physiology*, *25*(6), 503–512. https://doi.org/10.1016/S0022-1910(79)80009-8
Dietz, A., & Lambremont, E. N. (1970). Caste determination in honey bees. II. Food consumption of individual honey bee larvae, determined with 32P-labeled royal jelly. *Annals of the Entomological Society of America*, *63*(5), 1342–1345.
Donnell, S. O. (1998). Reproductive Caste Determination in Eusocial Wasps (Hymenoptera : Vespidae), (7).
Elser, E. (1929). Die chemische Zusammensetzung der Nahrungstoffe der Biene. *Markische Bienenzeitung*, *19*, 211-215-235-252.
Evans, J. D., & Wheeler, D. E. (2001). Expression profiles during honeybee caste determination. *Genome Biology*, *2*(1), 1. https://doi.org/10.1186/gb-2000-2-1-research0001
Everitt, B. (1974). *Cluster Analysis: an SSRC review of recent research*.
Fletcher, D. J. C., & Ross, K. G. (1985). Regulation of reproduction in eusocial Hymenoptera. *Analysis*, *30*(1), 319–343.
Foucaud, J., Fournier, D., Orivel, J., Delabie, J. H. C., Loiseau, A., Le Breton, J., … Estoup, A. (2007). Sex and clonality in the little fire ant. *Molecular Biology and Evolution*, *24*(11), 2465–2473. https://doi.org/10.1093/molbev/msm180
Frohschammer, S., & Heinze, J. (2009). A heritable component in sex ratio and caste determination in a Cardiocondyla ant. *Frontiers in Zoology*, *6*, 27. https://doi.org/10.1186/1742-9994-6-27
Goewie, E. A. (1978). Regulation of caste differentiation in the honey bee (Apis mellifera L.). *Meded. Landbouwhogesch. Wageninge*, *78*, 1–76.
Gruber, M. A. M., Hoffmann, B. D., Ritchie, P. A., & Lester, P. J. (2013). The conundrum of the yellow crazy ant (Anoplolepis gracilipes) reproductive mode: No evidence for dependent lineage genetic caste determination. *Insectes Sociaux*, *60*(2), 135–145. https://doi.org/10.1007/s00040-012-0277-z
Hartfelder, K., & Azevedo, S. V. (2007). Expression of insulin receptor isoforms in honey bee (Apis mellifera) caste development. *Developmental Biology*, *306*(1), 339. https://doi.org/10.1016/j.ydbio.2007.03.181
Hartfelder, K., Cnaani, J., & Hefetz, a. (2000). Caste-specific differences in ecdysteroid titers in early larval stages of the bumble bee Bombus terrestris. *J Insect Physiol*, *46*(11), 1433–1439. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10891571
Haydak, M. (1968). III. Nutrition Des Larves D'abeilles. *Traite de Biologie de L'abeille*, *1*, 302–333.
Haydak, M. H. (1943). Larval food and development of castes in the honeybee. *Journal of Economic Entomology*, *36*(5), 778–792. https://doi.org/10.1093/jee/36.5.778
Haydak, M. H. (1970). Honey bee nutrition. *Annual Review of Entomology*, *15*(1), 143–156.
Hedges, L. V., & Olkin, I. (1985). *Statistical Methods for Meta-analysis*. (A. Press, Ed.). New York, New York. USA.
Heine, J., & Buschinger, A. (1989). Queen polymorphism in Leptothorax spec. A: Its genetic and ecological background (Hymenoptera: Formicidae). *Insectes Sociaux*, *36*(2), 139–155. https://doi.org/10.1007/BF02225909
Helms Cahan, S., & Vinson, S. B. (2003). Reproductive division of labor between hybrid and nonhybrid offspring in a fire ant hybrid zone. *Evolution; International Journal of Organic*

Evolution, *57*(7), 1562–1570. https://doi.org/http://dx.doi.org/10.1554/02-518
Hölldobler, B., & Wilson, E. O. (1977). The number of queens: an important trait in ant evolution. *Naturwissenschaften*, *64*(1), 8–15.
Huang, C. Y., Chi, L. L., Huang, W. J., Chen, Y. W., Chen, W. J., Kuo, Y. C., … Chen, C. N. (2012). Growth stimulating effect on queen bee larvae of histone deacetylase inhibitors. *Journal of Agricultural and Food Chemistry*, *60*(24), 6139–6149. https://doi.org/10.1021/jf300815b
Hughes, W. O. H., & Boomsma, J. J. (2008). Genetic royal cheats in leaf-cutting ant societies. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(13), 5150–5153. https://doi.org/10.1073/pnas.0710262105
Ishay, J. (1975). Caste determination by social wasps: Cell size and building behaviour. *Animal Behaviour*, *23*(PART 2), 425–431. https://doi.org/10.1016/0003-3472(75)90090-1
Ishay, J. S., Levanon, D., Avidor, N., & Shechter, O. S. (2016). PHOTOPERIODICITY STIMULATES CELL SIZE AND CASTE DETERMINATION IN THE ORIENTAL HORNET VESPA orientalis Linne, 1775. Retrieved from http://www.tandfonline.com/action/journalInformation?journalCode=wsub20
Jeanne, R. L. (2009). Vibrational signals in Social Wasps: a role in caste determination? *Organization of Insect Societies*.
Johansson, T. S. (1955). Royal Jelly. *Bee World*, *36*(2), 21–32.
Judd, T. M., Teal, P. E. A., Hernandez, E. J., Choudhury, T., & Hunt, J. H. (2015). Quantitative differences in nourishment affect caste-related physiology and development in the paper wasp Polistes metricus. *PLoS ONE*, *10*(2), 1–18. https://doi.org/10.1371/journal.pone.0116199
Kaftanoglu, O., Linksvayer, T. A., & Page, R. E. (n.d.). Rearing honey bees , Apis mellifera , in vitro 1 : Effects of sugar concentrations on survival and development, *11*(96), 1–10.
Kaftanoglu, Osman, T. A., Linksvayer, & Page, R. E. (2011). Rearing honey bees, Apis mellifera, in vitro I: Effects of sugar concentrations on survival and development. *Journal of Insect Science*, *11*(1), 96. https://doi.org/10.1673/031.011.9601
Kamakura, M. (2011). Royalactin induces queen differentiation in honeybees. *Nature*, *473*(7348), 478–483. https://doi.org/10.1038/nature10093
Karsai, I., & Hunt, J. H. (2002). Food quantity affects traits of offspring in the paper wasp *Polistes metricus* (Hymenoptera : Vespidae). *Population Ecology*, *31*(1), 99–106. https://doi.org/http://dx.doi.org/10.1603/0046-225X-31.1.99
Kerr, W., Zucchi, R., Nakadaira, J., & Butolo, J. (2013). Reproduction in the Social Bees (Hymenoptera : Apidae). *Social Bee Reproduction*, *70*(4), 265–276.
Knerer, G., & E. Atwood, C. (1966). Polymorphism in Some Nearctic Halictine Bees. *Science*, *152*(3726), 1262–1263.
Korb, J. (2010). Social insects, major evolutionary transitions and multilevel selection. *Animal Behaviour: Evolution and Mechanisms*, 179–211.
Koyama, S., Takagi, T., Martin, S. J., Yoshida, T., & Takahashi, J. (2009). Absence of reproductive conflict during queen rearing in Apis cerana. *Insectes Sociaux*, *56*(2), 171–175. https://doi.org/10.1007/s00040-009-0008-2
Kucharski, R., Foret, S., & Maleszka, R. (2015). EGFR gene methylation is not involved in Royalactin controlled phenotypic polymorphism in honey bees. *Scientific Reports*, *5*(August), 14070. https://doi.org/10.1038/srep14070
KUKUK, P. F. (1994). Replacing the Terms Primitive and Advanced - New Modifiers for the

Term Eusocial. *Animal Behaviour*. https://doi.org/http://dx.doi.org/10.1006/anbe.1994.1198
Leimar, O., Hartfelder, K., Laubichler, M. D., & Page, R. E. (2012). Development and evolution of caste dimorphism in honeybees–a modeling approach. *Ecology and Evolution*, *2*(12), 3098–3109. https://doi.org/10.1002/ece3.414
Lin, Norman, and C. D. M. (1972). Evolution of Sociality in Insects. *Quarterly Review of Biology*, 131–159.
Linksvayer, T. A., Kaftanoglu, O., Akyol, E., Blatch, S., Amdam, G. V., & Page, R. E. (2011). Larval and nurse worker control of developmental plasticity and the evolution of honey bee queen–worker dimorphism. *Journal of Evolutionary Biology*, *24*(9), 1939–1948. https://doi.org/10.3851/IMP2701.Changes
Linksvayer, T. A., Wade, M. J., & Gordon, D. M. (2006). Genetic caste determination in harvester ants: Possible origin and maintenance by cyto-nuclear epistasis. *Ecology*, *87*(9), 2185–2193. https://doi.org/10.1890/0012-9658(2006)87[2185:GCDIHA]2.0.CO;2
Lourenço, A. P., Mackert, A., Cristino, A. S., & Simões, Z. L. P. (2008). Validation of reference genes for gene expression studies in the honey bee , Apis mellifera , by quantitative real-time RT-PCR *. *Apidologie*, *39*, 372–385. https://doi.org/10.1051/apido
Mao, W., Schuler, M. A., & Berenbaum, M. R. (2015a). A dietary phytochemical alters caste-associated gene expression in honey bees. *Science Advances*, *1*(7), e1500795. https://doi.org/10.1126/sciadv.1500795
Mao, W., Schuler, M. A., & Berenbaum, M. R. (2015b). A dietary phytochemical alters caste-associated gene expression in honey bees. *Science Advances*, *1*(7), e1500795. https://doi.org/10.1126/sciadv.1500795
Mark L., W. (1991). *The biology of the honey bee*. *harvard university press*.
Markiewicz, D. A., & O'Donnell, S. (2001). Social dominance, task performance and nutrition: Implications for reproduction in eusocial wasps. *Journal of Comparative Physiology - A Sensory, Neural, and Behavioral Physiology*, *187*(5), 327–333. https://doi.org/10.1007/s003590100204
Michener, C. D. (1974). *The social behavior of the bees: a comparative study*.
Michonneau, F., Brown, J., & Winter, D. (2016). rotl an R package to interact with the Open Tree of Life Data. *Methods in Ecology and Evolution*, *7*(12), 1476–1481.
Mitchell, B. K., & Schoonhoven, L. M. (1974). Taste Receptors in Colorado Larvae Beetle. *Journal of Insect Physiology*, *20*(9), 1787–1793.
Muggeo, V. M. R. (2003). Estimating regression models with unknown break-points. *Statistics in Medicine*, *22*, 3055–3071.
Mutti, N. S., Dolezal, A. G., Wolschin, F., Mutti, J. S., Gill, K. S., & Amdam, G. V. (2011). IRS and TOR nutrient-signaling pathways act via juvenile hormone to influence honey bee caste fate. *Journal of Experimental Biology*, *214*(23), 3977–3984. https://doi.org/10.1242/jeb.061499
Nakagawa, S., & Cuthill, I. C. (2009). Effect size, confidence interval and statistical significance: A practical guide for biologists: (Biological Reviews (2007) 82 (591-605)). *Biological Reviews*, *84*(3), 515. https://doi.org/10.1111/j.1469-185X.2009.00083.x
Nation, J. L. (2008). *Insect physiology and biochemistry*.
Nijhout, H. F. (2003). The control of body size in insects. *Developmental Biology*, *261*(1), 1–9. https://doi.org/10.1016/S0012-1606(03)00276-8
Nijhout, H. F., & Wheeler, D. E. (1982). Juvenile hormone and the physiological basis of insect polymorphisms. *The Quarterly Review of Biology*, *57*(2), 109–133.

Norman, L., & Michener, C. D. (1972). Evolution of sociality in insects. *The Quarterly Review of Biology*, *47*(2), 131–159.
Ohkawara, K., Nakayama, M., Satoh, A., Trindl, A., & Rgen Heinze, J. (2006). Clonal reproduction and genetic caste differences in a queen-polymorphic ant, Vollenhovia emeryi. *Biol. Lett*, *2*(May), 359–363. https://doi.org/10.1098/rsbl.2006.0491
Okamoto, M., & Ohkawara, K. (2010). Egg production and caste allocation in the clonally reproductive ant Vollenhovia emeryi. *Behavioral Ecology*, *21*(5), 1005–1010. https://doi.org/10.1093/beheco/arq093
Osborne, B. M. P., & Finlayson, L. H. (1962). The structure and topography of stretch receptors in representatives of seven orders of insects, *103*(June), 227–242.
Oster, G. F., & Wilson, E. O. (1978). *Caste and ecology in the social insects*.
Passera, L. (1980). La fonction inhibitrice des reines de la fourmi Plagiolepis pygmaea latr.: Role des pheromones. *Insectes Sociaux*, *27*(3), 212–225.
Patel, A., Fondrk, M. K., Kaftanoglu, O., Emore, C., Hunt, G., Frederick, K., & Amdam, G. V. (2007). The Making of a Queen: TOR Pathway Is a Key Player in Diphenic Caste Development. *PLoS ONE*, *2*(6), 1–7. https://doi.org/10.1371/journal.pone.0000509
Pearcy, M. (2004). Conditional Use of Sex and Parthenogenesis for Worker and Queen Production in Ants. *Science*, *306*(5702), 1780–1783. https://doi.org/10.1126/science.1105453
Penick, C. A., Prager, S. S., & Liebig, J. (2012). Juvenile hormone induces queen development in late-stage larvae of the ant Harpegnathos saltator. *Journal of Insect Physiology*, *58*(12), 1643–1649. https://doi.org/10.1016/j.jinsphys.2012.10.004
Pereboom, J. J. M. (2000). The composition of larvae fool and the significance of exocrine secretiosn in the bumblebee Bombus terrestris. *Insectes Sociaux*, *47*(1), 11–20. https://doi.org/10.1007/s000400050003
Pereboom, J. J. M., Velthuis, H. H. W., & Duchateau, M. J. (2003). The organisation of larval feeding in bumblebees (Hymenoptera, Apidae) and its significance to caste differentiation. *Insectes Sociaux*, *50*(2), 127–133. https://doi.org/10.1007/s00040-003-0639-7
Pettis, J. S., Rice, N., & Joselow, K. (2016). Colony Failure Linked to Low Sperm Viability in Honey Bee (Apis mellifera) Queens and an Exploration of Potential Causative Factors, 1–10. https://doi.org/10.1371/journal.pone.0147220
Platt, T. G., Queller, D. C., & Strassmann, J. E. (2004). Aggression and worker control of caste fate in a multiple-queen wasp, Parachartergus colobopterus. *Animal Behaviour*, *67*(1), 1–10. https://doi.org/10.1016/j.anbehav.2003.01.005
Plowright, R. C., & Jay, S. C. (1977). On the size determination of bumble bee castes (Hymenoptera: Apidae). *Canadian Journal of Zoology*, *55*(7), 1133–1138. https://doi.org/10.1139/z77-146
Rachinsky, A., & Hartfelder, K. (1990). Corpora allata activity, a prime regulating element for caste-specific juvenile hormone titre in honey bee larvae (Apis mellifera carnica). *Journal of Insect Physiology*, *36*(3), 189–194. https://doi.org/10.1016/0022-1910(90)90121-U
Rachinsky, A., Strambi, C., Strambi, A., & Hartfelder, K. (1990). Caste and metamorphosis: Hemolymph titers of juvenile hormone and ecdysteroids in last instar honeybee larvae. *General and Comparative Endocrinology*, *79*(1), 31–38. https://doi.org/10.1016/0016-6480(90)90085-Z
R Development Core Team, R. (2011). *R: A Language and Environment for Statistical Computing*. *R Foundation for Statistical Computing* (Vol. 1). https://doi.org/10.1007/978-3-

540-74686-7
Rembold, H. (1965). Biologically active substances in royal jelly. *Vitamins and Hormones*. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/5326344
Rembold, H., Lackner, B., & Geistbeck, I. (1974). The chemical basis of honeybee, Apis mellifera, caste formation. Partial purification of queen bee determinator from royal jelly. *Journal of Insect Physiology*, *20*(2), 307–314. https://doi.org/10.1016/0022-1910(74)90063-8
Ribeiro, M. F. (1994). Growth in bumble bee larvae: relation between development time, mass, and amount of pollen ingested. *Canadian Journal of Zoology*, *72*(11), 1978–1985. https://doi.org/10.1139/z94-270
Ribeiro, M. F. (1999). Long-duration feedings and caste differentiation in Bombus terrestris larvae. *Insectes Sociaux*, *46*(4), 315–322. https://doi.org/10.1007/s000400050151
Richards, M. H., Packer, L., Richards, M. H., & Packer, L. (2016). Trophic Aspects of Caste Determination in Halictus ligatus , a Primitively Eusocial Sweat bee, *34*(6), 385–391.
Schmitzová, J., Klaudiny, J., Albert, S., Schröder, W., Schreckengost, W., Hanes, J., … Simúth, J. (1998). A family of major royal jelly proteins of the honeybee *Apis mellifera L*. *Cellular and Molecular Life Sciences : CMLS*, *54*(9), 1020–30. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9791542
Schwander, T., Lo, N., Beekman, M., Oldroyd, B. P., & Keller, L. (2010). Nature versus nurture in social insect caste differentiation. *Trends in Ecology and Evolution*, *25*(5), 275–282. https://doi.org/10.1016/j.tree.2009.12.001
Seeley, T. D. (2009). *The wisdom of the hive: the social physiology of honey bee colonies*. Harvard University Press.
Seeley, T. D., & Url, S. (1978). International Association for Ecology Life History Strategy of the Honey Bee , Apis mellifera, *32*(1), 109–118.
Shuel, R. W., & Dixon, S. E. (1959). Studies in the mode of action of royal jelly in honeybee development: ii. respiration of newly emerged larvae on various substrates. *Canadian Journal of Zoology*, *37*(5), 803–813.
Simo, K., & Christensen, G. M. (1962). Quantitative analysis of sugars in royal jelly. *Nature*, 1208–1209.
Simpson, S. J., Sword, G. A., & Lo, N. (2011). Polyphenism in insects. *Current Biology*, *21*(18), R738–R749. https://doi.org/10.1016/j.cub.2011.06.006
Singer, M. S., Bernays, E. a, & Carrière, Y. (2002). The interplay between nutrient balancing and toxin dilution in foraging by a generalist insect herbivore. *Animal Behaviour*, *64*(4), 629–643. https://doi.org/10.1006/anbe.2002.3082
Singh, P. (1977). *Artificial Diets for insects, mites and spiders*.
Smith, C. R., Anderson, K. E., Tillberg, C. V, Gadau, J., & Suarez, A. V. (2008). Caste determination in a polymorphic social insect: nutritional, social, and genetic factors. *The American Naturalist*, *172*(4), 497–507. https://doi.org/10.1086/590961
Smith, K. M., Loh, E. H., Rostal, M. K., Zambrana-Torrelio, C. M., Mendiola, L., & Daszak, P. (2013). Pathogens, pests, and economics: Drivers of honey bee colony declines and losses. *EcoHealth*, *10*(4), 434–445. https://doi.org/10.1007/s10393-013-0870-2
Snodgrass, R. E. (1910). The Anatomy of the Honey Bee. *Nature*, *85*(2145), 169–169. https://doi.org/10.1038/085169b0
Soulard, A., Cohen, A., & Hall, M. N. (2009). TOR signaling in invertebrates. *Current Opinion in Cell Biology*, *21*(6), 825–836. https://doi.org/10.1016/j.ceb.2009.08.007

Stieper, B. C., Kupershtok, M., Driscoll, M. V., & Shingleton, A. W. (2008). Imaginal discs regulate developmental timing in Drosophila melanogaster. *Developmental Biology*, *321*(1), 18–26. https://doi.org/10.1016/j.ydbio.2008.05.556

Suryanarayanan, S., Hermanson, J. C., Jeanne, R. L., Wi, M., Gifford, O., & Drive, P. (2011). A Mechanical Signal Biases Caste Development in a Social Wasp. *Current Biology*, *21*(3), 231–235. https://doi.org/10.1016/j.cub.2011.01.003

Team, R. C. (2016). R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing. Retrieved from https://www.r-project.org/

Torson, A. S., Yocum, G. D., Rinehart, J. P., Kemp, W. P., & Bowsher, J. H. (2015). Transcriptional responses to fluctuating thermal regimes underpinning differences in survival in the solitary bee Megachile rotundata. *Journal of Experimental Biology*, *218*(7), 1060–1068. https://doi.org/10.1242/jeb.113829

USDA. (2016). Honey Bee Colonies, (March), 1–17.

Vargo, E. L., & Passera, L. (1990). Pheromonal and Behavioral Queen Control over the Production of Gynes in the Argentine Ant Iridomyrmex humilis (Mayr). *Behavioral Ecology and Sociobiology*, *28*(3), 161–169.

Viechtbauer, W. (2010). Conducting meta-analyses in R with the metafor package. *J Stat Softw*, *36*(3), 1–48.

von Planta, A. (1888). Ueber den Futtersaft der Bienen. *Zeitschrift Für Physiologische Chemie*, *12.4*, 327–354.

Walker, D. B. and M. M. and B. B. and S. (2015). Fitting Linear Mixed-Effects Models Using {lme4}. *Journal of Statistical Software*, *67*, 1–48. https://doi.org/10.18637/jss.v067.i01

Wan, X., Wang, W., Liu, J., & Tong, T. (2014). Estimating the sample mean and standard deviation from the sample size, median, range and/or interquartile range. *BMC Medical Research Methodology*, *14*(1), 135. https://doi.org/10.1186/1471-2288-14-135

Wang, W. X., Tian, L. Q., Huang, Q., Wu, X. B., & Zeng, Z. J. (2014). Effects of 10-Hydroxy-2-decenoic acid on the development of honey bee (Apis mellifera) larvae. *Journal of Apicultural Research*, *53*(1), 171–176. https://doi.org/10.3896/IBRA.1.53.1.19

Wang, Y., Azevedo, S. V, Hartfelder, K., & Amdam, G. V. (2013). Insulin-like peptides (AmILP1 and AmILP2) differentially affect female caste development in the honey bee (Apis mellifera L.). *The Journal of Experimental Biology*, *216*(Pt 23), 4347–57. https://doi.org/10.1242/jeb.085779

Wang, Y., Kaftanoglu, O., Fondrk, M. K., & Page, R. E. (2014). Nurse bee behaviour manipulates worker honeybee (Apis mellifera L.) reproductive development. *Animal Behaviour*, *92*, 253–261. https://doi.org/10.1016/j.anbehav.2014.02.012

Wang, Y., Ma, L., Zhang, W., Cui, X., Wang, H., & Xu, B. (2016). Comparison of the nutrient composition of royal jelly and worker jelly of honey bees (Apis mellifera). *Apidologie*, *47*(1), 48–56. https://doi.org/10.1007/s13592-015-0374-x

Weaver, N. (1966). Physiology of Caste Determination. *Annual Review of Entomology*, *11*(1), 79–102d.

Weever, N. (1965). Physiology of caste determination. *Annual Review of Entomology*, *11*(1), 79–102.

Wheeler, D. E. (1986). Developmental and physiological determinants of caste in social Hymenoptera: evolutionary implications. *The American Naturalist*, *128*(1), 13–34.

Wheeler, D. E., Buck, N. A., & Evans, J. D. (2014). Expression of insulin/insulin-like signalling and TOR pathway genes in honey bee caste determination. *Insect Molecular Biology*, *23*(1),

113–121. https://doi.org/10.1111/imb.12065
Wheeler, D. E., Buck, N. a, & Evans, J. D. (2006). Expression of insulin pathwaygenes during the period of caste determination in the honey bee, Apis mellifera. *Insect Molecular Biology*, *15*, 597–602. https://doi.org/10.1111/j.1365-2583.2006.00681.x
Wiernasz, D. C., & Cole, B. J. (2010). Patriline shifting leads to apparent genetic caste determination in harvester ants. *Proceedings of the National Academy of Sciences of the United States of America*, *107*, 12958–12962. https://doi.org/10.1073/pnas.1003299107
Wille, A., & Orozco, E. (1970). The life cycle and behaviour of the social bee Lasioglossum (Dialictus) umbripenne (Hymenoptera: Halictidae). *Revista de Biologia Tropical*, *17*(2), 199–245.
Wilson, D. B. (2011). Practical meta-analysis effect size calculator.
Wilson, E. (1975). *Sociobiology*. Cambridge, MA: Belk-nap/Harvard University Press.
Wilson, E. (1998). *Consilience*. (T. unity Ofknowledge, Ed.). New York.
Wolschin, F., Mutti, N. S., & Amdam, G. V. (2011). Insulin receptor substrate influences female caste development in honeybees. *Biology Letters*, *7*(1), 112–5. https://doi.org/10.1098/rsbl.2010.0463
Wu, Q., & Brown, M. R. (2006). Signaling and Function of Insulin-Like Peptides in Insects. *Annual Review of Entomology*, *51*(1), 1–24. https://doi.org/10.1146/annurev.ento.51.110104.151011

Table A3: This Table Shows All Information, Including Effect Size, Species Information, and Caste Determination Factors, For All Species Where Quantity Differences were Compared Between Queens-Workers.

Citation	Order	Family	Genus	Species	Subspecies	Eusocial	Broad Determination	Queen Measurement	d	Lower CI	Upper CI	Var
(Allsopp et al., 2003)	Hymenoptera	*Apidae*	*Apis*	*mellifera*	*scutella*	Permanent	Diet Quantity	quantity	2.33	1.13	3.54	0.38
(Allsopp et al., 2003)	Hymenoptera	*Apidae*	*Apis*	*mellifera*	*capensis*	Permanent	Diet Quantity	quantity	3.25	1.84	4.65	0.51
(Asencot & Lensky, 1985)	Hymenoptera	*Apidae*	*Apis*	*mellifera*		Permanent	Diet Quantity	quantity	#####	1.105	3.38	0.35
(Asencot & Lensky, 1985)	Hymenoptera	*Apidae*	*Apis*	*mellifera*		Permanent	Diet Quantity	Consumption	1.35	0.38	2.32	0.25
(Pereboom et al., 2003)	Hymenoptera	*Apidae*	*Bombus*	*terrestris*		Cyclical	Diet Quantity	quantity	3.73	2.53	4.93	0.38
(Knerer & E. Atwood, 1966)	Hymenoptera	*Halictidae*	*Evylaeus*	*cinctipes*		Cyclical	Diet Quantity	quantity	#####	1.778	2.555	0.039
(Richards et al., 2016)	Hymenoptera	*Halictidae*	*Halictus*	*ligatus*		Cyclical	Diet Quantity	quantity	1.89	1.37	2.41	0.071
(Richards et al., 2016)	Hymenoptera	*Halictidae*	*Lasioglossum*	*umbripenne*		Cyclical	Diet Quantity	quantity	#####	7.057	11.07	1.051
(Richards et al., 2016)	Hymenoptera	*Halictidae*	*Lasioglossum*	*umbripenne*		Cyclical	Diet Quantity	quantity	#####	1.766	3.457	0.186
(Richards et al., 2016)	Hymenoptera	*Halictidae*	*Lasioglossum*	*umbripenne*		Cyclical	Diet Quantity	quantity	3.42	2.39	4.45	0.274
(Wille & Orozco, 1970)	Hymenoptera	*Halictidae*	*Lasioglossum*	*umbripenne*		Cyclical	Diet Quantity	quantity	#####	3.738	6.326	0.504
(Wesson, 1940)	Hymenoptera	*Formicidae*	*Leptothorax*	*longispinosus*		Permanent	Diet Quantity	Colony supplemented	1.49	0.9	1.22	1.916
(Warner et al., 2016)	Hymenoptera	*Formicidae*	*Monomorium*	*pharaonis*		Permanent	Diet Quantity	Colony supplemented	5.44	1	2.33	0.921
(Judd et al., 2015)	Hymenoptera	*Vespidae*	*Polistes*	*metricus*		Cyclical	Diet Quantity	quantity	#####	0.034	1.94	0.23
(Judd et al., 2015)	Hymenoptera	*Vespidae*	*Polistes*	*metricus*		Cyclical	Diet Quantity	quantity	#####	1.097	3.51	0.393
(Judd et al., 2015)	Hymenoptera	*Vespidae*	*Polistes*	*metricus*		Cyclical	Diet Quantity	cell height	3.62	2.16	5.08	0.556
(J. S. Ishay et al., 2016)	Hymenoptera	*Vespidae*	*Vespa*	*orientalis*	*linne*	Cyclical	Diet Quantity	quantity	3.18	2.78	3.59	###

A.3. References

Allsopp, M. H., Calis, J. N. M., & Boot, W. J. (2003). Differential feeding of worker larvae affects caste characters in the Cape honeybee, Apis mellifera capensis. *Behavioral Ecology and Sociobiology*, *54*(6), 555–561. https://doi.org/10.1007/s00265-003-0666-4

Asencot, M., & Lensky, Y. (1985). Thephagostimulatory effect of sugars on the induction of "queenliness" in female honeybee (Apis mellifera L.) larvae. *Comparative Biochemistry and Physiology Part A: Physiology*, *81*(1), 203–208. https://doi.org/10.1016/0300-9629(85)90289-0

Ishay, J. S., Levanon, D., Avidor, N., & Shechter, O. S. (2016). PHOTOPERIODICITY STIMULATES CELL SIZE AND CASTE DETERMINATION IN THE ORIENTAL HORNET VESPA orientalis Linne, 1775. Retrieved from http://www.tandfonline.com/action/journalInformation?journalCode=wsub20

Judd, T. M., Teal, P. E. A., Hernandez, E. J., Choudhury, T., & Hunt, J. H. (2015).Quantitative differences in nourishment affect caste-related physiology and development in the paper wasp Polistes metricus. *PLoS ONE*, *10*(2), 1–18. https://doi.org/10.1371/journal.pone.0116199

Knerer, G., & E. Atwood, C. (1966). Polymorphism in Some Nearctic Halictine Bees. *Science*, *152*(3726), 1262–1263.

Pereboom, J. J. M., Velthuis, H. H. W., & Duchateau, M. J. (2003). The organisation of larval feeding in bumblebees (Hymenoptera, Apidae) and its significance to caste differentiation. *Insectes Sociaux*, *50*(2), 127–133. https://doi.org/10.1007/s00040-003-0639-7

Richards, M. H., Packer, L., Richards, M. H., & Packer, L. (2016). Trophic Aspects of Caste Determination in Halictus ligatus , a Primitively Eusocial Sweat bee, *34*(6), 385–391.

Warner, M. R., Kovaka, K., & Linksvayer, T. A. (2016). Late-instar ant worker larvae play a prominent role in colony-level caste regulation. *Insectes Sociaux*, 1–9. https://doi.org/10.1007/s00040-016-0501-3

Wesson, L. G. J. (1940). An experimental study on caste determination in ants.

Wille, A., & Orozco, E. (1970). The life cycle and behaviour of the social bee Lasioglossum (Dialictus) umbripenne (Hymenoptera: Halictidae). *Revista de Biologia Tropical*, *17*(2), 199 245.

Table A4: This Table Shows All Information, Including Effect Size, Species Information, and Caste Determination Factors, For All Species Determined By Genetic Predisposition.

Citation	Order	Family	Genus	Species	Subspecies	Eusocial	Broad Determination	Queen Measurment	d	Lower CI	Upper CI	Var
(Hughes & Boomsma, 2008)	Hymenoptera	*Formicidae*	*Acromyrmex*	*echinatior*		Permanent	Genetic	Patriline Prop	0.250	-0.139	0.639	0.043
(Gruber, Hoffmann, Ritchie, & Lester, 2013)	Hymenoptera	*Formicidae*	*Anoplolepis*	*gracilipes*		Permanent	Genetic	Allele	0.595	0.377	0.816	0.013
(Koyama, Takagi, Martin, Yoshida, & Takahashi, 2009)	Hymenoptera	*Apidae*	*Apis*	*cerana*	*japonica*	Permanent	Genetic	Patriline Prop	0.205	-1.108	1.520	0.450
(Frohschammer & Heinze, 2009)	Hymenoptera	*Formicidae*	*Cardiocondyla*	*kagutsuchi*		Permanent	Genetic	Patriline Prop	1.559	0.973	1.409	0.013
(Darras, Kuhn, & Aron, 2014)	Hymenoptera	*Formicidae*	*Cataglyphis*	*hispanica*		Permanent	Genetic	Pure-hybrid	7.710	5.160	10.240	1.680
(Pearcy, 2004)	Hymenoptera	*Formicidae*	*Cataglyphis*	*cursor*		Permanent	Genetic	Clonal	1.980	1.614	2.360	0.036
(Darras et al., 2014)	Hymenoptera	*Formicidae*	*Formica*	*truncorum*		Permanent	Genetic	monandrous	0.000	-0.877	0.877	0.200
(Darras et al., 2014)	Hymenoptera	*Formicidae*	*Formica*	*truncorum*		Permanent	Genetic	polyandrous	0.300	-0.582	1.181	0.202
(Darras et al., 2014)	Hymenoptera	*Formicidae*	*Formica*	*truncorum*		Permanent	Genetic	mating	0.150	-0.729	1.029	0.201
(Darras et al., 2014)	Hymenoptera	*Formicidae*	*Formica*	*exsecta*		Permanent	Genetic	Patriline Prop	0.106	-0.073	0.284	0.008
(Darras et al., 2014)	Hymenoptera	*Formicidae*	*Formica*	*truncorum*		Permanent	Genetic	Patriline Prop	0.111	-0.068	0.290	0.008
(Heine & Buschinger, 1989)	Hymenoptera	*Formicidae*	*Leptothorax*	*spec.*		Permanent	Genetic	Mating	5.430	0.988	1.450	0.075
(Kerr, 1950)	Hymenoptera	*Apidae*	*Melipona*	*favosa*	*orbigyni*	Permanent	Genetic	Queen Proportion	0.361	0.142	0.580	0.012
(Kerr, 1950)	Hymenoptera	*Apidae*	*Melipona*	*interrupta*	*fasciculata*	Permanent	Genetic	Queen Proportion	0.533	0.295	0.771	0.015
(Kerr, 1950)	Hymenoptera	*Apidae*	*Melipona*	*interrupta*	*flavipennis*	Permanent	Genetic	Queen Proportion	0.330	0.110	0.550	0.013
(Kerr, 1950)	Hymenoptera	*Apidae*	*Melipona*	*marginata*		Permanent	Genetic	Queen Proportion	0.630	-0.031	1.570	0.232
(Camargo et al., 1976)	Hymenoptera	*Apidae*	*Melipona*	*marginata*		Permanent	Genetic	Queen Proportion	2.359	3.599	4.576	0.062
(Kerr, 1950)	Hymenoptera	*Apidae*	*Melipona*	*marginata*		Permanent	Genetic	Queen Proportion	1.120	0.900	1.340	0.012
(Kerr, 1950)	Hymenoptera	*Apidae*	*Melipona*	*melanoventer*		Permanent	Genetic	Queen Proportion	0.442	0.280	0.589	0.006
(Kerr, 1950)	Hymenoptera	*Apidae*	*Melipona*	*quadrifasciata*		Permanent	Genetic	Queen Proportion	0.750	0.650	0.840	0.003
(Kerr, 1950)	Hymenoptera	*Apidae*	*Melipona*	*quadrifasciata*		Permanent	Genetic	Queen Proportion	0.360	-0.310	1.040	0.118
(Camargo et al., 1976)	Hymenoptera	*Apidae*	*Melipona*	*quadrifasciata*		Permanent	Genetic	Queen Proportion	0.720	0.268	1.172	0.054
(Darchen & Delage-Darchen, 1977)	Hymenoptera	*Apidae*	*Melipona*	*rufiventris*	*paraensis*	Permanent	Genetic	Queen Proportion	0.227	-0.153	0.610	0.038
(Darchen & Delage-Darchen, 1977)	Hymenoptera	*Apidae*	*Melipona*	*rufiventris*		Permanent	Genetic	Queen Proportion	0.550	-0.230	1.340	0.160
(Camargo et al., 1976)	Hymenoptera	*Apidae*	*Melipona*	*rufiventris*	*paraensis*	Permanent	Genetic	Queen Proportion	0.401	-0.015	0.815	0.062
(Kerr, 1950)	Hymenoptera	*Apidae*	*Melipona*	*schencki*		Permanent	Genetic	Queen Proportion	0.440	0.199	0.680	0.015
(Camargo et al., 1976)	Hymenoptera	*Apidae*	*Melipona*	*scutellaris*		Permanent	Genetic	Queen Proportion	0.486	-0.290	1.260	0.157
(Buschinger & Schreiber, 2002)	Hymenoptera	*Formicidae*	*Myrmecina*	*graminicola*		Permanent	Genetic	Monogynous	0.965	0.613	1.320	0.032
(Smith et al., 2008)	Hymenoptera	*Formicidae*	*Pogonomyrmex*	*badius*		Permanent	Genetic	Patriline Prop	0.368	-0.370	1.106	0.143
(Smith et al., 2008)	Hymenoptera	*Formicidae*	*Pogonomyrmex*	*occidentalis*		Permanent	Genetic	Patriline Prop	0.028	-0.848	0.905	0.200
(Smith et al., 2008)	Hymenoptera	*Formicidae*	*Solenopsis*	*geminata*		Permanent	Genetic	Pure-hybrid	0.021	-0.512	0.555	0.074
(Helms Cahan & Vinson, 2003)	Hymenoptera	*Formicidae*	*Solenopsis*	*xyloni*		Permanent	Genetic	Pure-hybrid	3.073	2.406	3.740	0.116
(Ohkawara, Nakayama, Satoh, Trindl, & Rgen Heinze, 2006)d	Hymenoptera	*Formicidae*	*Vollenhovia*	*emeryi*		Permanent	Genetic	homozygosity	1.378	1.159	1.591	0.013
(Okamoto & Ohkawara, 2010)	Hymenoptera	*Formicidae*	*Vollenhovia*	*emeryi*		Permanent	Genetic	homozygosity	1.360	1.125	1.590	0.014
(Foucaud, Estoup, Loiseau, Rey, & Orivel, 2010)	Hymenoptera	*Formicidae*	*Wasmannia*	*auropunctata*		Permanent	Genetic	Clonal	0.699	0.527	0.871	0.008

A.4. References

Buschinger, A., & Schreiber, M. (2002). Queen polymorphism and queen-morph related facultative polygyny in the ant, *Myrmecina graminicola* (Hymenoptera, Formicidae). *Insectes Sociaux*, *49*(4), 344–353. https://doi.org/10.1007/PL00012658

Camargo, C. A. de, Almeida, M. G. de, Parra, M. G. N., & Kerr, W. E. (1976). Genetics of Sex Determination in Bees . IX . Frequencies of Queens and Workers from Larvae under

Controlled Conditions (Hymenoptera : Apoidea). *Journal of Kansas Entomology*, *49*(1), 120–125.
Darchen, R., & Delage-Darchen, B. (1977). Sur Le Determinisme Des Castes Chez Les Melipones (Hymenopteres Apides). *Bulletin Biologique De La France Et De La Belgique*.
Darras, H., Kuhn, A., & Aron, S. (2014). Genetic determination of female castes in a hybridogenetic desert ant. *Journal of Evolutionary Biology*, *27*(10), 2265– 2271. https://doi.org/10.1111/jeb.12470
Foucaud, J., Estoup, a, Loiseau, a, Rey, O., & Orivel, J. (2010). Thelytokous parthenogenesis, male clonality and genetic caste determination in the little fire ant: new evidence and insights from the lab. *Heredity*, *105*(2), 205–212. https://doi.org/10.1038/hdy.2009.169
Frohschammer, S., & Heinze, J. (2009). A heritable component in sex ratio and caste determination in a Cardiocondyla ant. *Frontiers in Zoology*, *6*, 27. https://doi.org/10.1186/1742-9994-6-27
Gruber, M. A. M., Hoffmann, B. D., Ritchie, P. A., & Lester, P. J. (2013). The conundrum of the yellow crazy ant (Anoplolepis gracilipes) reproductive mode: No evidence for dependent lineage genetic caste determination. *Insectes Sociaux*, *60*(2), 135–145. https://doi.org/10.1007/s00040-012-0277-z
Heine, J., & Buschinger, A. (1989). Queen polymorphism in Leptothorax spec. A: Its genetic and ecological background (Hymenoptera: Formicidae). *Insectes Sociaux*, *36*(2), 139–155. https://doi.org/10.1007/BF02225909
Helms Cahan, S., & Vinson, S. B. (2003). Reproductive division of labor between hybrid and nonhybrid offspring in a fire ant hybrid zone. *Evolution; International Journal of Organic Evolution*, *57*(7), 1562–1570. https://doi.org/http://dx.doi.org/10.1554/02-518
Hughes, W. O. H., & Boomsma, J. J. (2008). Genetic royal cheats in leaf-cutting ant societies. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(13), 5150–5153. https://doi.org/10.1073/pnas.0710262105
Kerr, W. E. (1950). Genetic determination of castes in the genus Melipona. *Genetics*, *35*(2),143. Retrieved from papers2://publication/uuid/CA3CE576-398B-47BE-921E-AA586B02DE92
Koyama, S., Takagi, T., Martin, S. J., Yoshida, T., & Takahashi, J. (2009). Absence of reproductive conflict during queen rearing in Apis cerana. *Insectes Sociaux*, *56*(2), 171–175. https://doi.org/10.1007/s00040-009-0008-2
Ohkawara, K., Nakayama, M., Satoh, A., Trindl, A., & Rgen Heinze, J. (2006). Clonal reproduction and genetic caste differences in a queen-polymorphic ant, Vollenhovia emeryi. *Biol. Lett*, *2*(May), 359–363. https://doi.org/10.1098/rsbl.2006.0491
Okamoto, M., & Ohkawara, K. (2010). Egg production and caste allocation in the clonally reproductive ant Vollenhovia emeryi. *Behavioral Ecology*, *21*(5), 1005–1010. https://doi.org/10.1093/beheco/arq093
Pearcy, M. (2004). Conditional Use of Sex and Parthenogenesis for Worker and Queen Production in Ants. *Science*, *306*(5702), 1780–1783. https://doi.org/10.1126/science.1105453
Smith, C. R., Anderson, K. E., Tillberg, C. V, Gadau, J., & Suarez, A. V. (2008). Caste determination in a polymorphic social insect: nutritional, social, and genetic factors. *The American Naturalist*, *172*(4), 497–507. https://doi.org/10.1086/590961