INFLUENCE OF THE TYPE OF POLLEN DIET ON THE SURVIVAL, BODY WEIGHT, AND EXPRESSION OF IMMUNE-RESPONSE GENES OF THE HONEYBEE SUBSPECIES (*Apis mellifera scutellata)*

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Influence of the Type of Pollen Diet on the Survival, Body Weight, and Expression of Immune-Response Genes of the Honeybee Subspecies (*Apis mellifera scutellata)*

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

Signature…… Date…….……

Muturi Michael Njihia

This research proposal has been submitted for examination with our approval as supervisors.

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DEDICATION

I dedicate this research thesis to my beloved grandfather, Pharis Njihia Kwigita, and beloved parents, Paul and Jane Muturi.

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ABBREVIATIONS AND ACRONYMS

AMPs	Antimicrobial peptides
BSA	Bovine Serum Albumin
CCD	Colony Collapse Disorder
cDNA	Complementary Deoxyribonucleic Acid
Dcr2	Dicer-2
GLMM	Generalized Linear Mixed Model
GLD	Glucose Dehydrogenase
GOX	Glucose Oxidase
HD	Highly Diverse
LD	Lowly Diverse
LYS	Lysozyme
JAK	Janus kinase
JNK	c-Jun N-terminal Kinase
PAMPs	Pathogen Associated Molecular Patterns
PCR	Polymerase Chain Reaction
PRR	Pathogen Recognition Receptors
PO	Phenol Oxidase
NF-κβ	Nuclear Factor kβ
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid

RNase III Ribonuclease III

RISC RNA Induced Silencing Complex

siRNA small interfering RNA

STAT Signal Transducer and Activator of Transcription

TBP Tata Binding Protein

TE Tris-EDTA Buffer

ABSTRACT

The honeybee species, *Apis mellifera*, is recognized globally for its essential pollination services to plants. Plants, in turn, reward bees nutritionally through nectar and pollen. Pollen is vital for development of larvae and young worker bees. Honeybees forage on polyfloral pollen rather than monofloral pollen to satisfy their dietary needs. Increased land use and land cover changes, in sub-Saharan Africa, is reducing polyfloral pollen habitats towards monocultures or few floral habitats. The effect of these two pollen types on *Apis mellifera scutellata,* the predominant honeybee subspecies, in Kenya is poorly understood. Yet this knowledge is critical because poor diet for bees implies a decreased quantity of hive products and reduced income for beekeepers. To fill this knowledge gap, caged bees were fed with two pollen diets (i) lowly diverse (LD), monofloral pollen (ii) highly diverse (HD), polyfloral diet and their effects on four parameters, namely survival, pollen consumption, body weight, and immune response was tested. HD-fed bees had significantly higher survival ($p=0.001$) and greater pollen consumption (14 mg) than LD-fed bees (11.5 mg). However, LD-fed bees (101.7 mg) were heavier than HD-fed bees (109.5 mg). The correlation between pollen consumption and body weight gain was expressed strongly ($r=0.9$) in HD-fed bees than in LD-fed bees ($r=0.7$). Overall, this study reveals the benefits that the highly diverse diets provide to honeybee workers and how pollen diversity influences honeybee life-history traits. This informs the need for conserving the biodiversity of environments for safeguarding the health of the honeybees and other pollinators.

CHAPTER ONE

INTRODUCTION

1.1 Background Information.

Pollinators offer ecological services to both wild and food crops and are therefore an essential entity in maintaining the world's ecosystem (Potts *et al*., 2010). The estimated total economic value that pollination contributes to global food production is €153 billion and €11.9 billion for Africa (Gallai *et al.,* 2009). At least 20, 000 bee species have been described globally (Michener, 2000). However, out of this vast pool of bee species, the honeybee - *Apis mellifera*- remains the most significant pollinator globally (Morse & Calderone, 2000; Williams, 1994). They contribute to the production of 68.4% (39/57) of leading food crops used for human consumption (Klein *et al*., 2007). Many of these crops provide the bulk of micronutrients to the human diet (Chaplin-Kramer *et al*., 2014; Ellis *et al*., 2015; Smith *et al.,* 2015).

The recent decline in the *A. mellifera* population in the Northern Hemisphere occurring at an alarming rate has captured the world's attention (Neumann & Carreck, 2010). This decline described as Colony Collapse Disorder (CCD) has not been documented in Kenya because little surveillance has been so far conducted on honeybee populations and their pests and diseases (Muli *et al.,* 2014). But in North America and Europe, where it has been reported, it has been observed that worker honeybees mysteriously disappear leaving no traces of dead bees or any outward symptoms of the disease (Oldroyd, 2007; VanEngelsdorp *et al.*, 2009). The interaction of different factors and not just a single one accounts for the decline in honeybee populations (Strauss *et al.,* 2013; VanEngelsdorp *et al.*, 2009). The most significant stressors reported include the following; pathogens, parasite-mediated infections, exposure to pesticides, and poor nutrition which is the focus of my study (Collison *et al*., 2016; Meixner, 2010).

Honeybee nutrition is composed of plant-derived nectar and pollen (Roulston & Cane, 2000; Nicolson & Thornburg, 2007). Whereas nectar acts as the main carbohydrate source, pollen serves as the chief protein source for developing larvae and adult honeybees (Brodschneider & Crailsheim, 2010). Pollen varies with the host-plant species and thus some pollen species are considered nutritionally richer than others (Maurizio & Louveaux, 1965; Odoux *et al*., 2012), with noticeable differences in their protein, amino acid, lipid, micronutrient, and sugar content (Keller *et al*., 2005; Roulston & Cane, 2000). Honeybees being generalists collect a diversity of polyfloral pollen to constitute a balanced and optimal diet (Schmidt, 1984; Schmidt *et al*., 1987).

Pollen is vital for the development of the honeybee (Brodschneider & Crailsheim, 2010). For the larvae it is crucial for the development of the various larval instars. For the young adults, it is involved in the formation of the hypopharyngeal gland and the ovaries (Pernal and Currie, 2000; Pirk *et al*., 2010). In addition, pollen is involved in the formation of antimicrobial peptides which are controlled by immune-related genes such as: *defensin*, *hymenoptaecin* and *apidaecin*. In this study, two of these immune genes were used- *defensin-2* and *hymenoptaecin*.

The alteration of landscapes is contributing to the loss of pollen sources for honeybees. Consequently, rich habitats endowed with polyfloral pollen sources are slowly being altered towards habitats with monofloral (for monocultures) or few floral sources (Decourtye *et al*., 2010; Naug, 2009). This limits the quality and feeding choice of the honeybee, a trend also being witnessed in Africa's sub-Saharan region (Nkonya *et al*., 2016).

Multiple studies have already been conducted to investigate the influence of pollen and a no-pollen diet on the honeybee (Schmidt, 1984; Schmidt *et al*., 1987; Pernal & Currie 2000). Furthermore, the effects of polyfloral versus monofloral pollen diet on the European honeybee subspecies in the Northern Hemisphere have been studied extensively (Danihlík *et al*., 2018; Dolezal *et al*., 2019; Schmidt, 1984; Schmidt *et al*., 1987). However, this is little understood in African honeybees, amongst them the widely

distributed sub-species, *Apis mellifera scutellata* (Hepburn & Radloff 1998). This study utilized two pollen diets mimicking varying natural environments: (i) Highly diverse pollen diet reflecting a rich biodiversity (here abbreviated as HD), and (ii) Lowly diverse pollen diet corresponding to a degraded environment with fewer floral sources (abbreviated as LD).

Newly emerged honeybees were fed with the two pollen regimens- HD and LD pollen, and measured parameters related to physiology as weight, immune response, and survival were assessed. As hypothesized the honeybees fed on HD pollen had an increased survival and pollen consumption. However, their body weight was lower than the LD-fed bees. The expression of *defensin-2* and *hymenoptaecin* did not differ in the two categories of fed bees. This study is important in understanding the dietary requirements of the African honeybee and its physiological responses to varying diversities of pollen diets.

1.2 Problem statement

Kenya is endowed with rich biodiverse regions which form the bedrock of its agricultural heritage. However, this is currently under threat as more green spaces are shrinking to pave way for construction projects and urban expansion. Karura forest, for example, which hosts 2 experimental apiaries for *icipe* and several beekeeping groups over the recent past has been marked by decreased colony population. It has been increasingly difficult for beekeepers to trap swarming bees into empty hives. This has been associated with the decreased floral abundance and diversity. This observation coincides also with reports from interviews with beekeepers from other parts of the country. Honeybees feeding on such feeding sources with few flowers or monocultures are therefore limited in the feeding options and quality of diet that they can access.

1.3 Justification

Honeybees feed on polyfloral pollen for their optimal functioning. Due to the current changes in landscapes, bees are forced to tolerate with poor diet sourced from few flowers. Yet poor diets malnourish the bees thus resulting to a reduced amount and quality of their hive products. Consequently, this affects the income for beekeepers and other

stakeholders in the beekeeping industry. No study has investigated the effect of monofloral vs polyfloral pollen on the health of the honeybee subspecies in Kenya, *Apis mellifera scutellata*. This study exploits this knowledge gap in order to deliver evidence of the detrimental effects that monofloral diet could have on the honeybee that forms the livelihood of many rural households.

1.4 Research questions

i. What is the survival rate of *Apis mellifera scutellata* individuals fed on lowly and highly pollen diverse diets?
ii. What is the influence of a low versus highly diverse diet on the pollen consumption and bee body weight?
iii. What is the influence of a low versus highly diverse diet on the expression of the immune genes *defensin-2(def-2)* and *hymenoptaecin (Hym)* in *A. m. scutellata*?

1.5 Null hypothesis

There is no difference in the survival, pollen consumption, bee body weight, and the expression of *def-2* and *hym* genes between *A.m. scutellata* individuals fed on either a low or high diverse pollen diet.

1.6 Objectives

1.6.1 General objective

To determine the influence of the type of pollen diet on the survival, body weight and immune response of *Apis mellifera scutellata* individuals fed on different pollen diets.

1.6.2 Specific objectives

i. To compare the survival rate between *A.m. scutellata* individuals fed on lowly and highly pollen diverse diets.
ii. To compare the daily pollen consumption and honey bee body weight between *A. m. scutellata* individuals fed on lowly and highly pollen diverse diets

iii. To compare the relative gene expression of *def-2* and *hym* genes between *A. m. scutellata* individuals fed on lowly and highly pollen diverse diets.

CHAPTER TWO

LITERATURE REVIEW

2.1 The Honeybee, *Apis mellifera*

Many insects live in communities and among the best-known and studied social insects are the honeybees (Michener, 2000; Winston, 1991). They live in large colonies composed of females - a single female queen together with 20,000- 50,000 adult workers and the male individuals primarily composed of male drones (Sammataro *et al*., 2000). The young workers act as nurse bees that feed the queen and brood, inspect and clean the hive and maintain the nest homeostasis while the older nestmates forage for food and water, build and defend the colony (Schmickl & Crailsheim, 2004). The drones mate with the queen to raise a new generation (Weinstock *et al*., 2006).

The modern honeybee belongs to the genus *Apis* which consists of ten species. Two of the species - *A. mellifera* and *A. cerana* are the only currently 'domesticated' species. "The *A. mellifera* has 28 recognized subspecies which are categorized into six major lineages- A lineage (all African subspecies except Ethiopia), C lineage (Southern and Eastern Europe), M lineage (Western and northern Europe), O lineage (Caucasus, Middle East), S lineage (Syria and Lebanon) and Y lineage (Ethiopia and Indian Ocean) (Tihelka *et al*., 2020)". Africa boasts of an abundant and diverse population of honeybees as well as the richest records of the association of bees and human beings (Dietemann *et al*., 2009). In Kenya, five distinct subspecies of the *A. mellifera* African lineages have been identified but the predominant sub-species is *A. mellifera scutellata* (Hepburn & Radloff, 1998).

2.2 Pollen nutrition

Honeybees share a mutual relationship with the plants in their habitats. Plants rely on the pollination services offered by the honeybees for their reproduction and survival. Honeybees, in turn, accrue nutritional benefits from plants (de Sá-Otero *et al*., 2009). This

consists of two main food products- nectar and pollen (Nicolson & Thornburg, 2007; Roulston & Cane, 2000).

Just like any other typical organism, honeybees require macronutrients (proteins, carbohydrates, and lipids) and micronutrients (vitamins and minerals) for their survival and well-being. Nectar is the chief carbohydrate source primarily supplying fuel for energy tasks like flight muscle movement (Hrassnigg & Crailshem, 2005). The remaining part of the review will focus on the other plant product, pollen.

Pollen forms an important part of the diet of honeybees being the only source of protein (Brodschneider & Crailsheim, 2010). It contains the 10 essential amino acids (de Groot, 1953). Besides acting as the chief protein source, it also supplies other important nutrients like lipids, carbohydrates, and micronutrients namely minerals, vitamins, and phytochemicals (terpenoids, alkaloids, flavonoids, and phenolics) shown to possess antioxidant and antimicrobial activity (Campos *et al*., 2010; Roulston & Cane, 2000). The nutritional differences in pollen quality are due to the differences in terms of their protein, amino acids, lipid, and sugar content (Di Pasquale *et al*., 2013; Keller *et al.*, 2005).

The nutritional composition of pollen influences the foraging preferences of bees (Di Pasquale *et al*., 2013). Honeybees being generalists collect pollen from many, different types of flowers (Dimou & Thrasyvoulou, 2009; Vaudo *et al*., 2020). This results in a natural blend of different pollen types serving as an optimum diet for honeybees (Decourtye *et al*., 2009). Pollen obtained from different floral species differs in nutritional content suggesting that some pollen have higher nutritional content than others (Odoux *et al*., 2012; Roulston & Cane, 2000). It is generally accepted that monofloral pollen are inferior to polyfloral pollen diets (Alaux *et al*., 2011; Brodscheider & Crailshem, 2010). In both urbanized and monoculture areas with monofloral or few floral sources there have been reported high colony losses (Naug, 2009). Interestingly, in lowly degraded regions, a positive association has been established between uncultivated land and honeybee health status (Smart *et al*., 2016).

2.3 Role of pollen

Pollen is an indispensable requirement in the development of the honeybee. At the individual level, it is instrumental in the development and activation of the ovaries (Hoover *et al*., 2006; Human *et al*.,2007; Pirk *et al*., 2010), and formation of hypopharyngeal gland whose function is to convert digested pollen to royal jelly (Alqarni, 2006; Pernal & Currie, 2000). At the colony-level, pollen contributes to brood development (Brodschneider & Crailsheim, 2010) and longevity (Haydak, 1970).

Pollen deficiency has been "associated with the decline in colony population" (Keller *et al*., 2005). Low amounts of pollen in food stores make brood-rearing difficult (Brodschneider & Crailsheim, 2010; Kleinschmidt & Kondos, 1976). The adult workers may resort to cannibalizing some of the brood to obtain proteins needed to feed the remaining brood (Brodschneider & Crailsheim, 2010). When this pollen shortage persists, brood production is terminated (Brodschneider & Crailsheim, 2010).

2.4 Honeybee Immunity

A. mellifera being a eusocial insect has two main levels of immunity- individual and social immunity (Cremer & Sixt, 2009; Evans & Spivak, 2010). Individual immunity protects against parasitic infection at the individual level and it involves mechanical and physiological defenses (Cremer & Sixt, 2009). On the other hand, social immunity or group response immunity protects against parasitic infection at the colony level. This involves cooperation by individual members of the colony to deal with the high risk of disease transmission arising from communal living (Cremer *et al*., 2007). Social immunity is composed of behavioral and organizational defenses like self-grooming, grooming other bees, nest hygienic behavior and removal of dead bees from the nest(undertaking) (Cremer *et al*., 2018; Cremer & Sixt, 2009). The focus of the remaining part of the review will be on the physiological aspect of individual immunity.

The honeybee possesses an innate immune system (Strand, 2008). Its innate immune system is composed of the humoral and cellular defense system (Krautz *et al*., 2014; DeGrandi-Hoffman & Chen, 2015). Furthermore, these defense systems are coordinated

by the following immune signaling pathways: Toll, Imd, Janus kinase/Signal Transducer and Activator of Transcription (JAK/STAT), c-Jun Kinase (JNK), and RNA interference (Brutscher *et al*., 2015; Theopold & Dushay, 2007) which consist of three types of proteins namely; recognizing proteins, modulating proteins, and effector proteins (Lemaitre & Hoffmann, 2007).

The humoral defense system is controlled by immune-related genes (Christophides *et al*., 2002). At least four immune genes found in honeybees code for antimicrobial peptides (AMPs) and these include: *abaecin*, *defensin*, *hymenoptaecin and apidaecin*, (Casteels *et al*., 1993; Casteels *et al*., 1989; Yang & Cox-Foster, 2005). The fat body of insects an organ analogous to the liver in mammals secretes the AMPs (Ferrandon *et al*., 2007). Most of these AMPs have been discovered to act at the cell membrane (Lockey & Ourth, 1996). These AMPs complement each other rather than act synergistically in their defense against a wide spectrum of bacterial infections (Casteels *et al*., 1993).

The cellular defense system observed in honeybees, similar to other insects involves hemocytes and phagocytes circulating in the hemolymph which employ mechanisms like phagocytosis, encapsulation, and melanization (Brennan & Anderson, 2004). Cellular immunity depends on certain enzymes encoded by immune-related genes. Two of these enzymes, phenoloxidase (PO) and glucose dehydrogenase (GLD) are most critical for a cellular response. The PO enzyme is involved in melanization and encapsulation reactions while the GLD is involved in the killing pathogens via encapsulation reactions (Zaobidna *et al*., 2015). Two other enzymes – lysozyme (LYS) and glucose oxidase (GOX) are crucial in mounting a cellular immune response (Yang & Cox-Foster, 2005).

The role of the immune signaling pathways has been documented. They are activated when the host's recognition proteins -Pattern Recognition Receptors (PRRs) – detect and bind to the motifs found on the outer surfaces of the pathogens known as Pathogen Associating Molecular Patterns (PAMPs). PAMPS include; viral ds RNA, bacterial peptidoglycans, and fungal β-glucans (Brutscher *et al*., 2015). The role of the pathways has been briefly discussed in the subsequent paragraphs below.

The Toll pathway contains transmembrane proteins involved in signal transduction. Their critical role in immunity is to facilitate the insect's response to fungal and Gram-positive bacterial infections (Kurata, 2014). Phenoloxidase, antimicrobial peptides, and three lysozymes have been suggested as candidate effectors of the Toll pathway (Evans *et al*., 2006). The Imd pathway plays a critical role in the defense against Gram-negative bacteria (Kurata, 2014). It regulates a majority of the antimicrobial peptides in *Drosophila* (Hoffmann, 2003). These two pathways, the Imd and Toll pathways do not operate in isolation from each other and they cooperatively regulate the antimicrobial peptide *defensin* (Gregorio *et al*., 2002).

The "Jak/ STAT pathway regulates the antimicrobial effector proteins" while the JAK signaling system plays a role in AMP expression and apoptosis (Yang & Cox-Foster, 2005). The honeybee just like all other insects depends on RNA interference as its main antiviral defense mechanism (Kemp & Imler, 2009). RNA interference involves the enzyme Dicer-2 (Dcr2), an RNase III enzyme that cleaves the long stretches of ds RNA to short interference RNA (siRNA) which are "loaded onto RNA-induced Silencing Complex (RISC)". RISC complex is critical in silencing viral replication (Brutscher *et al.,* 2015). The Toll, Imd, and Jak-STAT pathways have also been shown to regulate infections by RNA viruses (Merkling & van Rij, 2013).

Despite the many similarities that the honeybee shares with other insects particularly social insects, there exist some unique features of their immune system (Barribeau *et al.,* 2015). The honeybee has only one-third of the immune genes presently found in two other existing model insects, *Drosophila* and *Anopheles*, whose genomes have already been described (Barribeau *et al*., 2015; Evans *et al.*, 2006). These distinct differences have however not been detcetd among the different bee species, the honeybee, *A. mellifera,* and other closely-related species like the "*Bombus impatiens, Bombus terrestris,* and *Megachile rotundata"* (Barribeau *et al.*, 2015).

2.5 Pollen nutrition and Honeybee Immunity

Nutrition has so far been pointed out as an important factor in developing an optimal immune response (Field *et al.,* 2002). Generally in animals, protein deficiency impairs immune function thus increasing the susceptibility of animals and human beings to diseases (Li *et al.*, 2007). Immunity particularly in insects is influenced by both protein quantity and protein quality (Lee *et al*., 2008).

Furthermore, the poor health status of the honeybees associated with pollen deficiency reduces their resistance threshold to stresses imposed by disease pathogens and pesticides (Le Conte *et al*., 2010; Naug, 2009). Pollen influences the honeybee immunocompetence (Alaux *et al*.,2011), thus enhancing the bee's tolerance to bacteria (Rinderer *et al.*, 1974), microsporidia (Rinderer & Elliott, 1977), and viral infections (De Grandi-Hoffman *et al*.,2010). Transcriptome studies involving healthy and *Varroa*-infested honeybees fed on pollen diet revealed that pollen positively influences the metabolic and nutrient-sensing pathways. It also stimulates the genes encoding for longevity and antimicrobial peptide functions (Alaux *et al*., 2011). Noteworthy is that the intermingled effects of malnutrition and disease have so far a threat to the health of the honeybee(Azzouz-olden *et al*., 2018). Therefore, different plant sources synergistically interact together to influence honeybee nutrition.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Site

This study was conducted at the International Centre of Insect Physiology and Ecology (*icipe*), Nairobi, Kenya (1.22°S, 36. 89°E) from August 2018 to April 2020.

3.2 Bee samples

Honeybee workers used in the study were obtained from three colonies maintained at the *icipe* experimental apiary. The colonies were headed by naturally mated queens and kept in standard Langstroth hives with ten frames. The local honeybee colonies belonged to the African honeybee subspecies, *Apis mellifera scutellata* (Muli *et al.,* 2014).

3.3 Establishment of laboratory honeybee colonies

Age-matched honeybees were obtained using sealed brood combs containing late-stage pupae which were incubated overnight at 34 ± 2°C and 50 - 70% relative humidity (Williams *et al*., 2013) (**Plate 3.3.1)**. The newly emerged workers (less than 24 h old) were transferred into cages and kept in an incubator at 34 ± 2°C and 50 - 70% relative humidity (**Plate 3.3.2)**. Metal cages measuring 10 cm × 8.5 cm × 5 cm and circular plastic cages measuring 12 cm diameter × 8 cm height accompanied by their feeders were used to confine cohorts of 5 and 50 honeybees, respectively.

Adult honeybees were fed *ad libitum* with one of the following diets; (i) Lowly diverse (LD) pollen and sugar solution (1 g icing sugar in 2 ml water, 50 % w/v), (ii) Highly diverse (HD) pollen and sugar solution. Each cage was also supplied with 2 ml of water and all cages were inspected daily to replace the food and water as well as remove any dead bees. At least 8 cages (replicate by cages) were used for each feeding assay. Pollen feeding in all setups was continued for six days (Muturi *et al*., 2022).

Plate 3.1: Selection of honeybee combs in the *icipe* experimental apiary for use in the lab experimental setups (Muturi *et al*., 2022).

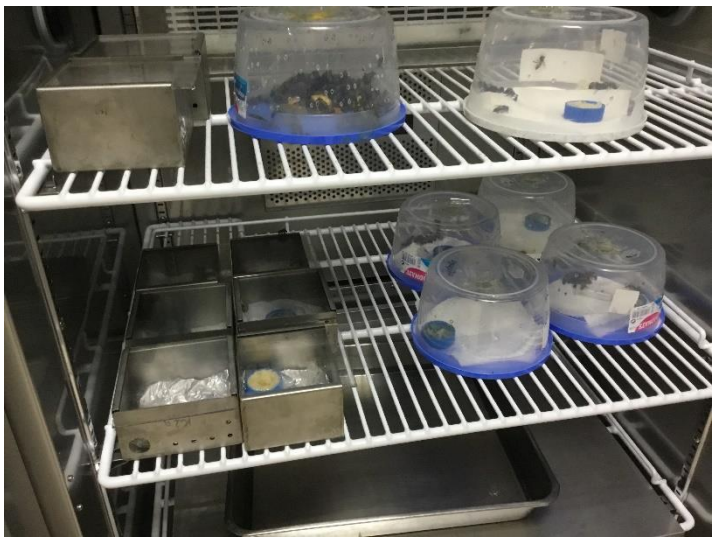

Plate 3.2: Setup of laboratory colonies in metallic cages (n=5 bees) and plastic cages (n=50 bees) (Muturi *et al*., 2022).

3.4 Pollen preparation and identification

Commercially available corbicular pollen (Aurica Naturheilmittel und Naturwaren GmbH, Schwalbach-Elm, Germany, originally collected from Spain) were used because locally available pollen was not sufficient for the experimental setups. The pollen loads were hand-sorted based on color and appearance (**Plate 3.4**). Further on, using microscopy, the hand-sorted pollen loads were examined to verify whether they were also morphologically distinct. A total of ten pollen morphotypes were obtained (**Table**. **4.1**). The pollen types were later constituted into two pollen diets: LD pollen consisting of a single pollen type and HD pollen comprising of ten pollen types at equal frequency. The pollen type which was most abundant was selected for the LD pollen. The pollen diets were immediately frozen at -20°C until use (Muturi *et al*., 2022).

Plate 3.3: Hand sorting pollen in the lab based on color (Muturi *et al*., 2022).

3.5 Bradford assay

The protein amount of the pollen diets were analyzed using the Bradford method with minor modifications (Bradford, 1976). Pollen was sampled from the daily food supplied to the bees kept in the metallic cages (n = 5 bees). Each pollen sample was crushed into a

fine powder using a pestle and mortar, transferred to a micro-centrifuge tube, and weighed on (Analytical Plus, Ohaus, Switzerland) weighing balance (accuracy ± 0.0001 g). Applying the method used by de Sá-Otero *et al*., (2009) with slight modifications (Ochungo *et al*., 2021), protein was extracted from the samples. Briefly, 5 ml of 30 mM TE-buffer was added to each sample, vortexed, and centrifuged for 10 min at 3,000 × g. The supernatant was collected in aliquots of 0.3 mL, added 4 mL of Bradford reagent and incubated the reaction mixture for 2 min at room temperature. This procedure was done in triplicates.

About 0.3 mL of TE buffer was used as blank and Bovine Serum Albumin (BSA) (Sigma-Aldrich- Kobian, Kenya) as the standard (concentration 2 mg/mL). Onto the various BSA dilutions prepared: 50 µg/mL, 150 µg/mL, 200 µg/mL, 250 µg/mL, and 400 µg/mL, 4 mL of Bradford reagent was added. Protein absorbances were measured at 595 nm using a Bio Spec-mini spectrophotometer (Shimadzu Corporation, Japan). Absorbance values of the standards were used to generate a calibration curve and its corresponding linear formula. From this formula, crude protein values (µg protein/mL of pollen) was afterwards converted to g protein/100 g pollen. A total of 19 and 16 replicates for HD and LD diets were used respectively (Muturi *et al*., 2022).

3.6 Assay for determining honeybee body weight, pollen consumption, and bee survival

Honeybees kept in small metallic cages in groups of five bees per cage were marked with different paint marks on their thoraces and were weighed to record the initial weight of individual bees using a weighing balance (Analytical Plus, Ohaus, Switzerland) (accuracy ± 0.0001 g). The honeybees were subjected to different diet regimens (LD and HD). The weight of the individual honeybees were recorded daily before supplying a fresh diet into the cages. This was done by individually placing a bee in a 1.5 mL Eppendorf tube and subtracting the weight of the empty tube from the total weight of the tube + weighed bee. Previous studies have measured the weight of whole cages (Harbo & Harris, 1999; Harbo & Hoopingarner, 1997), but the interest of the study was in the individual body weight

of honeybees. Since the bees were color-marked, the weight of individual bees in all cages were recorded on a daily basis. The weight of the daily pollen consumption per cage were further estimated after collecting the remaining diet the next day. This experimental setup was replicated four times.

To ascertain the pollen diets' effect on survival, groups of 50 honeybees were kept in plastic cages. Daily inspection of the bees was conducted to remove and score the number of dead honeybees as well as renew the food and water supply. The experimental setups were terminated when all honeybees in the cages died. The experiment was replicated four times (Muturi *et al*., 2022).

3.7 Setup for immune gene expression studies

For the immune gene expression assay, cages each consisting of 50 freshly emerged honeybee workers were set up. Subsequently, the bees received either of the two pollen treatments for six days. The food was changed and removed dead bees daily. On day six, ten bees per cage were harvested by freeze-killing at -80°C; the samples remained frozen until further processing (Muturi *et al*., 2022).

3.8 RNA extraction, cDNA synthesis, and qRT-PCR

Total RNA was extracted from individual bees (10 bees/cage x 3 replicates x 2 treatments) to quantify the expression of the target immune gene. This involved grounding the honeybee abdominal tissues in liquid nitrogen for RNA isolation using TRIzol (Invitrogen, USA) Evans (*et al.,* 2013). Onto the frozen bee abdomens 500 ul of TRIzol® was added and mashed with a pestle. Before adding an additional amount of 500 ul TRIzol and 200 ul of chloroform. The samples were then incubated at room temperature for 2-3 min before being spun at 4°C for 15 min at ~14,000 rpm. The upper supernatant was transferred into a fresh tube and 100% isopropanol was added, inverted to mix and centrifuged at 4°C for 10 min at full speed. The liquid was siphoned off and then 1 ml of cold 75-80% nuclease-free Ethanol added. The samples were spun at 4°C for 5 min and ethanol decanted carefully to avoid pouring the RNA pellet. The pellets were air dried and resuspended in 100 µl of RNase-free water. RNA samples were quantified at an

absorbance of 260 nm using a Nanodrop™ 2000 spectrophotometer (Thermofisher Scientific, USA) and quality was inferred from 260 / 280 nm ratio as well as from 230 / 260 nm ratio (**Table 3.1**).

10 µL total RNA was used to synthesize cDNA using High Capacity cDNA Reverse Transcription Kit that contained oligo-dT primers (Applied Biosystems, Lithuania). It was added to an RT master mix composed of: 10 X RT Buffer (2 µl), dNTP Mix (0.8 µl), oligo dT primers (2 µl), MultiScribe Transcriptase (1 µl), RNase Inhibitor (1 µl) and Nuclease-free H_2O (3.2 µl) making up a total reaction volume of 20 µL). The thermal conditions for the reaction were: 25°C for 10 min, 37°C for 120 min, 85°C for 5 min on T100™ Thermal Cycler (Bio-Rad, Singapore). Equal volumes of cDNA (10 µL) from two bees per treatment group were pooled as a template for qRT-PCR reactions.

Quantitative real-time PCR (qRT-PCR) were conducted on 96-well microtitre plates using HOT FIREPol EvaGreen® qPCR Mix Plus with ROX (Solis Biodyne, Estonia) and gene-specific primers **(Tab. 3.1)** for *def-2* and *hym* as the immune genes and Tata binding protein (TBP) as a housekeeping gene. A total reaction volume of 10 µl consisted of 2 µl Eva Green Ready Mix (5x), 0.5 µl each primer pair (10 µM), 6 µl RNase-free water, and 1 µl cDNA sample (~100 ng / µL). For each reaction, a no-template negative control was included and used two technical replicates. qRT-PCR was performed using the QuantStudio™ 3 System (Life Technologies Holding Pte Ltd, Singapore) using the following thermocycling conditions: initial activation (95°C for 30 s), denaturation (95°C for 30 s), annealing (gene-specific temperature in **Table 3.1**; for 30 s), extension (72°C for 30 s), and final extension (72°C for 10 min) running for 40 cycles. The fluorescence was measured during the annealing step (Muturi *et al*., 2022).

Table 3.1: List of primers for *TBP*, *defensin-2*, and *Hymenoptaecin* and their annealing temperatures.

Gene ID	Primer	Annealing temperature (°C)	Accession Number	Reference
Tata Binding Protein (TBP)	F: TTGGTTTCATTAGCTGCACAA R: ACTGCGGGAGTCAAATCTTC	53.5°C	XM_393492	(Tesovnik *et al*., 2017)
Defensin-2	F: GCAACTACCGCCTTTACGTC R: GGGTAACGTGCGACGTTTTA	55°C	**GB10036**	(Tesovnik *et al*., 2017)
Hymenoptaecin	F: ATGGATCCTCTTTCTTGTCG R: TTCATCGTGTTGGTTCTCTT	52.5°C	GB17538	(Zaobidna *et al*., 2015)

3.9 Data analysis

All data for normality was tested using the Shapiro-Wilkerson test. Since all my data were non-normally distributed, non-parametric tests was used for analysis. In order to compare differences in protein content between the LD and HD pollen diets, the Mann-Whitney-U-Test was used. The daily pollen consumption per cage for bees was compared using a generalized linear mixed model (GLMM) with the type of diet, feeding day, and their interaction as fixed factors and cage nested in replicate as random factors implemented in the *lme4* package within the R environment. (Bates *et al*., 2015). Similarly, the daily bee body weight was compared using a GLMM including bee ID as an additional random factor nested in cage. The relationship was analyzed between daily pollen consumption per bee and daily bee body weight change (loss/gain) using a Spearman rank correlation for each of the treatments separately. Day 6 was excluded from my analysis because several cages lacked surviving bees. Cox proportional-hazard regression analysis was used as implemented in the *survival* (Therneau, 2020) and *survminer* (Kassambara *et al.*, 2020) packages to compare the survival of the caged bees exposed to different diets.

Tata Binding Protein (TBP) served as a housekeeping gene (HKG), and *def-2 and hym* as the target genes (TG). Fluorescence measurements obtained during amplification were imported to LinReg 2020.0 (Ruijter *et al*., 2009). LinReg generated the quantitation cycle (Cq), equivalent to cycle threshold (C_T) and the PCR efficiency for each sample. The stability of TBP was determined using Reffinder (Xie *et al*., 2012).

For assessing relative gene expression, the target gene expression values was normalized to the HKG expression levels. To compare the ratio between the expression of *def-2* and TBP, I used the formula

$$r = (E_{TG}\,\hat{}\,C_{T\,TG})/(E_{HKG}\,\hat{}\,C_{T\,HKG}) \qquad (1)$$

where; E_{TG} - PCR efficiency of *def-2* and *hym*, E_{HKG} - PCR efficiency of TBP, $C_{T\,TG}$ - cycle threshold of *def-2,* and $C_{T\,HKG}$ - cycle threshold of TBP (Pfaffl, 2001). The PCR efficiency obtained from LinReg replaced the optimal PCR efficiency of 2. The relative expression

values were log_2-transformed. To analyze the differences in gene expression, the Mann-Whitney U-test were used. The effect size using G-power was calculated (Faul *et al*., 2007).

All the statistical analyses were performed using R software 3.6.2 version (RCore Team, 2020) with appropriate packages: *survival*, *survminer*, and *lme4*. For all tests, a level of significance at I was assumed at $p<0.05$.

CHAPTER FOUR

RESULTS

4.1 Pollen identification

Fig 4.1 shows 10 pollen morphotypes sorted based on color and appearance. LD pollen diet comprised of pollen type 1 while HD pollen diet comprised of pollen types 1-10. The color codes correspond each individual pollen morphotype.

1-Dark yellow*, 2- Light Yellow, 3- Orange, 4-Light Purple, 5-Dark Purple, 6-Red, 7-Dark Grey, 8-White Brown, 9-Light Brown & 10- Grey

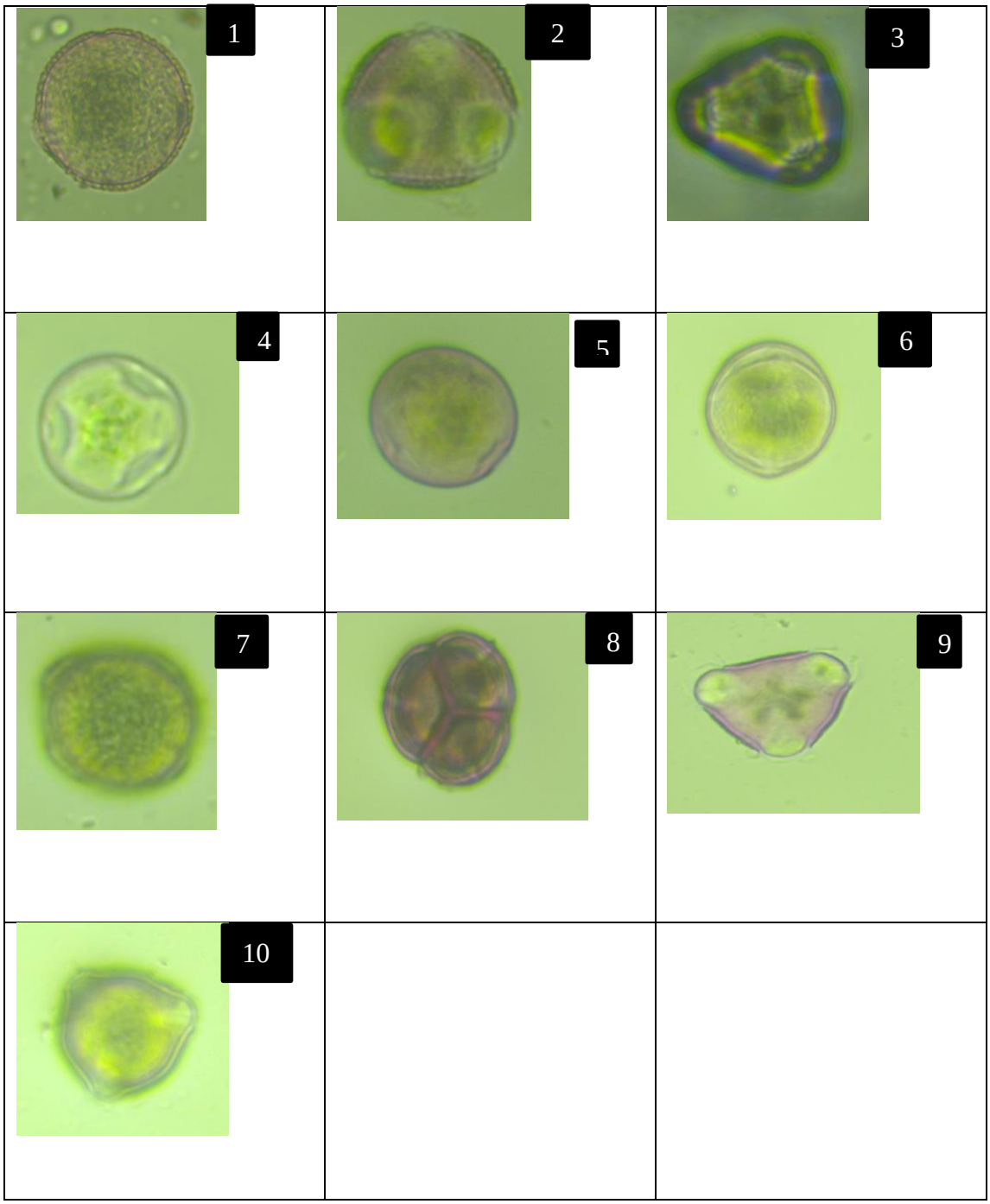

Figure 4.1: The 10 pollen morphotypes used to constitute HD and LD diet

1-Dark yellow*, 2- Light Yellow, 3- Orange, 4-Light Purple, 5-Dark Purple, 6-Red, 7-Dark Grey, 8-White Brown, 9-Light Brown & 10- Grey (Muturi *et al*., 2022)

4.2 Protein content of LD and HD pollen

The protein content of the different pollen diets was similar (LD pollen: 5.3 ± 4.4 mg / 100 mg, HD pollen: 5.6 ± 7.3 mg / 100 mg; Mann-Whitney U-test: w = 210, p = 0.52; **Figure 4.2**).

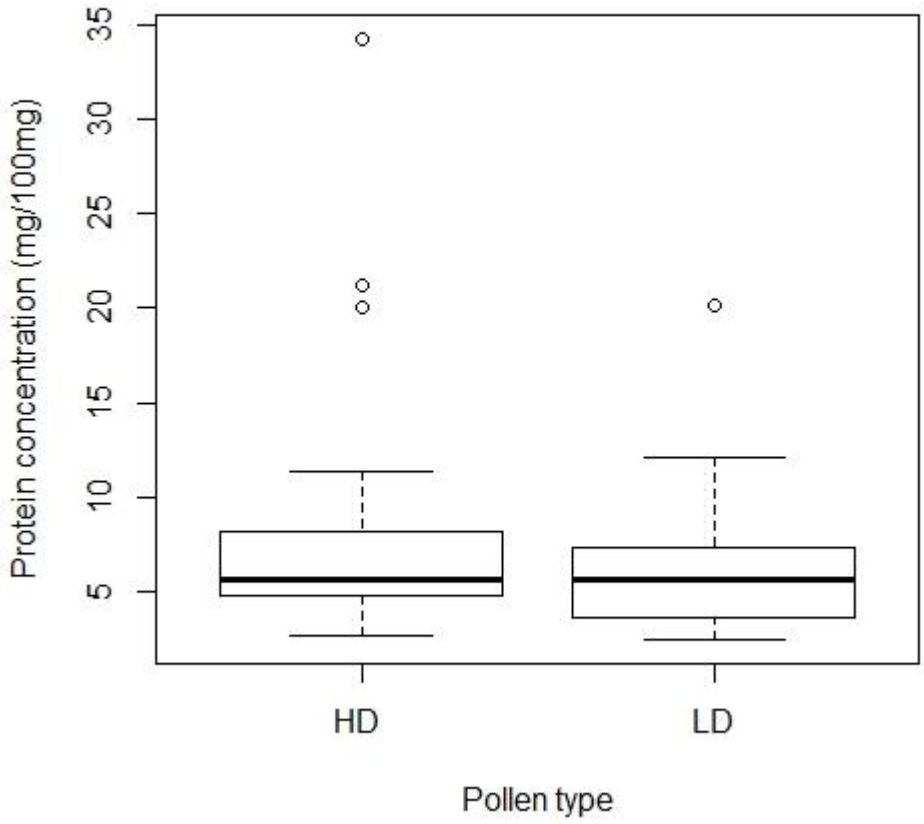

Figure 4.2: Protein content of different pollen diets. HD – highly diverse pollen diet, LD – lowly diverse pollen diet (Muturi *et al*., 2022).

4.3 Survival of LD and HD-fed bees

HD pollen diet significantly increased the lifespan of the bees relative to those supplied with LD pollen diet (Cox hazard proportion test; Cox log-rank score = 10.56; df = 1; p = 0.001; **Figure 4.3**).

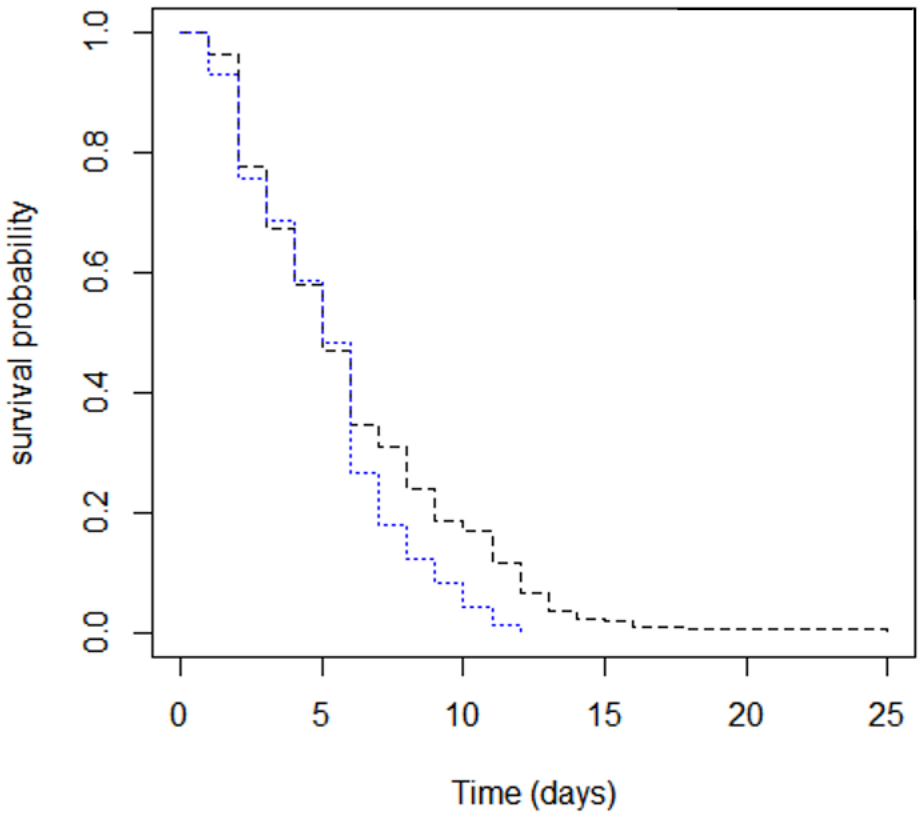

Figure 4.3: Survival analysis for different feeding regimens. Black dashed line - HD (highly diverse pollen diet), blue dotted line - LD (lowly diverse pollen diet).

4.4 Pollen consumption and body weight

HD pollen consumption per day ranged between 0.3 mg - 14 mg, while daily LD pollen consumption ranged between 0.4 mg - 11.5 mg. HD-fed bees showed a higher pollen

consumption relative to LD-fed bees across the days (**Figure 4.4**). The null model only including random effects (cage and replicate) was better than the full model (GLMM: LRT: 0.4532 p = 0.32; AIC: null model: -338.74; full model: -335.57) implying that the fixed effects; feeding day and diet, and their interaction had no significant effect on daily pollen consumption.

The range in individual body weight of HD-fed bees was 59.6 mg - 101.7 mg and LD-fed bees 48.9 mg - 109.5 mg. LD and HD-fed bees shared a common trend from day 1-3. Past day 3, the HD-reared bees experienced a decrease in weight persisting to day 4 before slightly increasing towards day 5 (**Figure 4.4**). The LD-reared bees recorded an onward sharp increase in weight past day 3 up to day 5 (**Figure 4.5)**. Bee weight was affected by the feeding day (GLMM: df = 7, t = -4.948, $p < 0.0001$) and not by the type of diet (GLMM: df = 7, t = 0.972, p = 0.33) or interaction between the two factors (GLMM; df = 7, t = -1.720, p = 0.09). HD pollen consumption was correlated to the bee weight gain and marginally significant (r = 0.9, p = 0.08) while this effect was less strong in LD (r = 0.7, p = 0.23) (**Figure 4.5**)

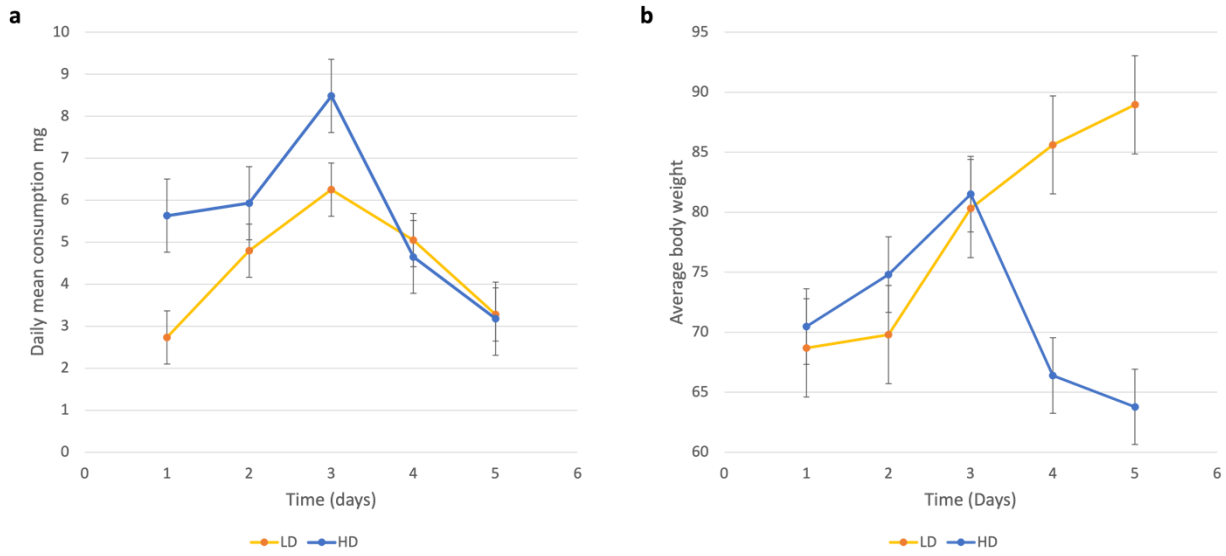

Figure 4.4: Interaction plot for pollen consumption (a) and daily bee body weight (b) from Day 1-5. blue solid line - HD (highly diverse pollen diet), yellow solid line - LD (lowly diverse pollen diet) (Muturi *et al*., 2022)

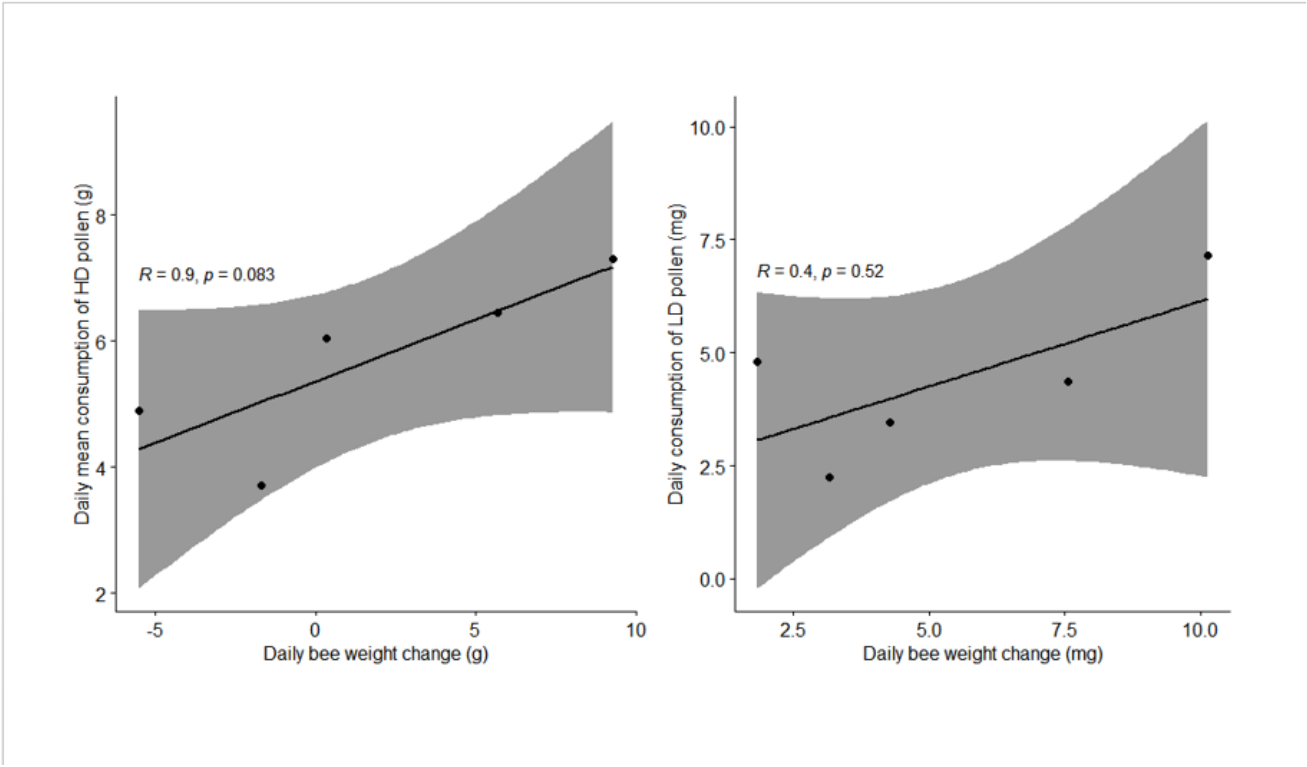

Figure 4.5: Correlation of daily pollen consumption and daily weight change for highly diverse(HD pollen diet and lowly diverse (LD) pollen diet (Muturi *et al*., 2022).

4.5 Immune gene expression

LD pollen diet increased the expression levels of *def-2* more than the HD pollen but this wasn't significant (p=0.32) (**Figure 4.6**). The expression levels of *Hym* were similar in HD-fed bees and LD-fed bees (p=0.6) **(Fig 4.6)**. The power of the analysis was, however, low (1- β = 0.16 (*Def*), 0.118(*Hym*)), potentially due to the low sample size (n= 8 HD-fed bees, 7 LD-fed bees).

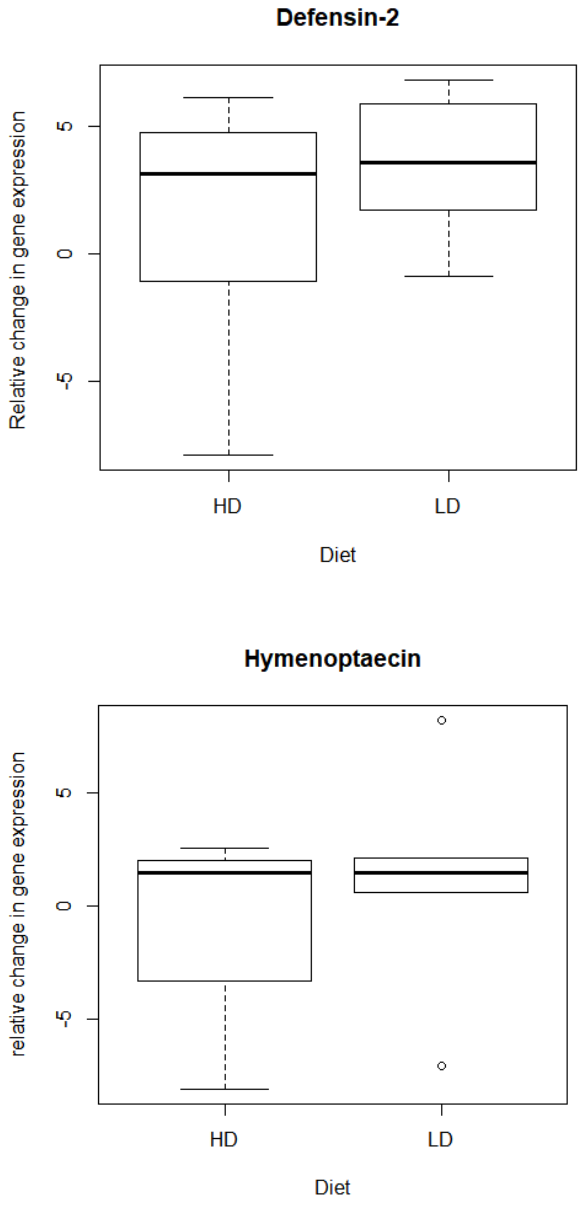

Figure 4.6: Gene expression levels of *defensin-2* and *hymenoptaecin* for HD and LD-reared bees (Muturi *et al*., 2022).

CHAPTER FIVE

DISCUSSION

5.1 Pollen identification

Diet comparisons can be investigated at two levels (i) pollen vs. no-pollen diet, and (ii) HD vs. LD pollen diet. Previous studies have already investigated the effect of the 1st level of comparison on honeybee health (Schmidt, 1984; Schmidt *et al*., 1987; Pernal & Currie 2000). The focus of the study was on the influence of the 2nd level of comparison, the quality of the pollen diet, by comparing the HD vs. LD pollen diet on bee health. The study highlights the effects of the pollen diets on four parameters relating to the honeybee development and wellbeing; survival, pollen consumption, bee body weight change, and immune response. The LD pollen had one predominant pollen based on color. HD pollen was a blend of ten equally frequent pollen morphotypes. From the microscopic examination, the different pollen load colours were verified to be morphologically distinct. Pollen types can share the same color shade yet belong to different plant species owing to other morphological differences in shape, size, and weight (Almeida-Muradian *et al.*, 2005; Komosinska-Vassev, 2015).

5.2 Protein content of LD and HD pollen

The protein values, on average, for both pollen types were within the acceptable range of 2.5% - 61% (Roulston *et al*., 2000). However, the low amount of crude protein values is attributed to the stored, dried pollen used in this study. Drying pollen significantly reduces the crude protein content of pollen (Human & Nicolson, 2006). LD-fed bees received the same quantity of protein as HD-fed bees. Although the method used - Bradford assay - provides a general guideline on the protein quality, it is limited to determining the number of specific amino acids (Pernal & Currie, 2000). Therefore, any difference elicited in the phenotype of the bees could be due to differing amounts of amino acids and fatty acids in the pollen. Previously, different pollen types have been reported to share a similar concentration of proteins yet differ in their amino acids and fatty acids concentration

(DeGrandi-Hoffman *et al*., 2018). This may have drastic implications for either a sufficiency or scarcity of certain essential amino acids like tryptophan and phenylalanine (Roulston & Cane, 2000).

5.3 Survival of LD vs HD-fed bees

The longer lifespan registered by HD-fed bees could be attributed to the nutritional disparities in the two pollen regimens. Besides proteins, pollen is packed with fatty acids and micronutrients varying in amount and composition in different pollen types (Di Pasquale *et al*., 2013; Keller *et al*., 2005). The findings from this study coincide with previous reports which demonstrated that caged bees fed on polyfloral pollen survived longer than their counterparts fed on monofloral pollen (Di Pasquale *et al*., 2013; Dolezal *et al*., 2019; Schmidt *et al*., 1987). A polyfloral diet mimics the natural pollen diet used by honeybees (Schmidt *et al*., 1987). Honeybees are generalists that collect diverse multifloral pollen to constitute a balanced and diverse diet (Dimou & Thrasyvoulou, 2009). A high protein diet seems to boost the honeybee's survival even under stressful conditions (Archer *et al*., 2014).

In field studies, the quality and availability of pollen have been shown to influence colony survival and productivity; pollen supply remains a limiting factor in the longevity of the honeybee colony (Brodschneider & Crailsheim, 2010; DeGrandi-Hoffman *et al*., 2008). Both, low amounts of pollen food stores and pollen of low nutritional value makes brood-rearing difficult (Brodschneider & Crailsheim, 2010; Kleinschmidt & Kondos, 1976) and might even lead to absconding (Cheruiyot *et al.*, 2020).

5.4 Pollen consumption and body weight

There was a noticeable increase in pollen consumption in HD and LD fed bees in the first three days which was consistent with other studies (Alqarni, 2006). Possibly, this increased appetite was to meet the nutritional demands of the honeybees during this early, critical phase of growth and development. Pollen diet is crucial for the first seven days of the adult honeybee development (Brodschneider & Crailsheim, 2010). In addition, it promotes hypopharyngeal glands development in brood and young bees (Di Pasquale *et*

al., 2013) and determines the lifespan of the honeybee workers (Amdam & Omholt, 2002).

The honeybees fed under the LD pollen regimen consumed a lower amount of pollen. The pollen consumed is a reliable measure of the palatability of the diet (Pernal & Currie, 2000). This reduced palatability in the LD diet could be associated with its poor unbalanced nutritional state. It would be expected that LD-fed bees would ingest more pollen to compensate for any lacking nutrients. However, honeybees do not ingest more of a poor pollen diet to compensate for any nutritional deficiencies in their diet or counter stressful conditions *(*Knox *et al*., 1971; Archer *et al.*, 2014). If they are forcefully fed, this results in lowered survival and compromises health status (Paoli *et al*., 2014). Under field conditions, whether bees evaluate which pollen to consume is presently unclear. While foragers have been shown to select nutritionally dense pollen (Hendriksma & Shafir, 2016), nurse bees do not evaluate their choice of pollen diet based on nutrition (Corby-Harris *et al*., 2018). Noteworthy is that besides the nutritional quality of pollen, the amount of pollen consumed is determined by physical and chemical cues (Schmidt & Johnson, 1984). One example deserving special mention are phagostimulants which occur in different combinations for different pollen types (Schmidt, 1985).

LD-fed bees increased in weight over time. However, there is a relatively weaker correlation of change in body weight to the pollen they consumed relative to the HD-fed bees. The higher weight recorded could not be traced only to pollen consumed. The LD-fed bees reverted to ingesting more of the sugar solution additionally supplied possibly to compensate for the nutritional deficiency in the LD diet. Previously, it has been reported that honeybees under laboratory conditions regulate their nutrient intake by shifting to a carbohydrate-biased diet (Altaye *et al*., 2010). However, sugar solution consumption was not quantified in this study.

A low number of bees (n=5) was used because weighing bees individually generally induces stress (Sgolastra *et al*., 2017). A previous study used a similar number of bees (Arien *et al*., 2018). The pollen consumption and bee body weight was measured up to

day five as some cages had no surviving bees on day 6. Honeybees endure stress when kept in small numbers resulting in a shorter survival period (Rinderer & Baxter, 1978). Furthermore, this has been demonstrated in ants. Isolated ants (n = 1 or 2) were more hyperactive and increased their energy demand compared to grouped ants (n = 10), thus leading to higher mortality (Koto *et al*., 2015). In bumblebees, isolated bees showed a reduced immune response compared to bees kept in groups (Richter *et al*., 2012).

5.5 Immune gene expression

Def-2 and *hym* was not significantly expressed in the HD and LD diet comparison. These two genes were selected because they code for antimicrobial peptides and have been investigated in previous studies, which showed a strong up-regulation under pathogen exposure. (Ilyasov *et al*., 2012; Li *et al*., 2017; Erler *et al*., 2011). For newly-emerged bees fed with pollen for six days, their immune system at the baseline level seems to be unrelated to the quality of the diet. A closer look at the survival curve (**Fig 4.3**) reveals a significant difference in mortality after day 6. This phenotypic difference could imply a genotypic difference. Thus, a significant difference in the expression of the *def-2* and *hym* gene would be expected if bees analyzed were from the 7^{th} day onwards. Another possible explanation to the result obtained is this; the activation of the immune system in honeybees is a costly process in terms of energy required (Laughton *et al*., 2011; Moret & Schmid-Hempel, 2000). Honeybees, like other insects, will mount an immune system when it is only necessary, that is- upon pathogen exposure or an immunological challenge (Tyler *et al*., 2006). When the immune system is stimulated, other bee genera like *Bombus* have been shown to consume more food. This compensates for the significant amount of energy investment needed for mounting an immune response (Tyler *et al*., 2006).

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

In summary, although HD and LD pollen diets appear to be similar in their protein quantity, there were detectable differences in their influence on the survival and physiology of the caged honeybees. The findings from this study reveal that the

a) HD pollen diet was consumed more than the LD pollen diet
b) HD promoted higher survivorship as hypothesized.
c) The LD-fed bees didn't ingest more pollen than the HD-fed bees but recorded a higher weight.
d) Finally, the HD-fed bees had a stronger correlation of pollen consumption and bee weight than in the LD-fed bees.

Therefore, implying a stronger influence that HD pollen has on the honeybee physiology.

6.2 Recommendations

Since polyfloral pollen diet confers benefits to the wellbeing of the honeybee, there is need to conserve existing diverse environments. This is indeed a prerequisite for securing the health of the honeybee and other pollinators thus ensure optimum delivery of the ecosystem service of pollination.

The study has established that pollen with similar protein amounts elicit different changes in life-history traits of the honeybee implying that other components in pollen such as fatty acids and phytochemicals play a pivotal role in honeybee. Future studies should highlight the role of other pollen components like lipids, vitamins and phytochemicals on bee health.

REFERENCES

Alaux, C., Dantec, C., Parrinello, H., & Le Conte, Y. (2011). Nutrigenomics in honey bees : digital gene expression analysis of pollen's nutritive effects on healthy and varroa-parasitized bees. *BMC Genomics*, *12*, 496.

Alaux, C., Ducloz, F., Crauser, D., & Le Conte, Y. (2010). Diet effects on honeybee immunocompetence. *Biol. Lett*, *6*, 562–565.

Almeida-Muradian, Pamplona, L. C., Silvia, C., & Barth, O. M. (2005). Chemical composition and botanical evaluation of dried bee pollen pellets. *Journal of Food Composition and Analysis*, *18*, 105–111.

Alqarni, A. S. (2006). Influence of Some Protein Diets on the Longevity and Some Physiological Conditions of Honeybee *Apis mellifera L*. Workers. *Journal of Biological Sciences*, *6*, 734–737.

Altaye, S. Z., Pirk, C. W. W., Crewe, R. M., & Nicolson, S. W. (2010). Convergence of carbohydrate-biased intake targets in caged worker honeybees fed different protein sources. *The Journal of Experimental Biology*, *213*, 3311–3318.

Archer, C. . R., Pirk, C. W. W., Wright, G. A., & Sue, W. N. (2014). Nutrition affects survival in African honeybees exposed to interacting stressors. *Functional Ecology*, *28*, 913–923.

Amdam, G. V., & Omholt, S. W. (2002). The regulatory anatomy of honeybee lifespan. *Journal of theoretical biology*, *216*, 209-228.

Azzouz-olden, F., Hunt, A., & Degrandi-hoffman, G. (2018). Transcriptional response of honey bee (*Apis mellifera*) to differential nutritional status and Nosema infection. *BMC Genomics*, *19*, 1–20.

Barribeau, S. M., Sadd, B. M., Plessis, L., Brown, M. J. F., Buechel, S. D., Cappelle, K., … Karaus, E. (2015). A depauperate immune repertoire precedes evolution of sociality in bees A depauperate immune repertoire precedes evolution of sociality in bees. *Genome Biology*, *16*, 1–20.

Bates, D., Mächler, M., Bolker, B. M., & Walker, S. C. (2015). Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software*, *67*, 1–48.

Bradford, M. M. (1976). A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye binding. *Analytical Biochemistry*, *72*, 248–254.

Brennan, C. A., & Anderson, K. V. (2004). Drosophila : The Genetics of Innate Immune Recognition and Response. *Annual Review of Immunology*, *22*, 457–483.

Brodschneider, R., & Crailsheim, K. (2010). Nutrition and health in honey bees. *Apidologie*, *41*, 278–294.

Brutscher, L. M., Daughenbaugh, K. F., & Flenniken, M. L. (2015). Antiviral defense mechanisms in honey bees. *Current Opinion in Insect Science*, *10*, 71–82.

Campos, M. G. R., Frigerio, C., Lopes, J., & Bogdanov, S. (2010). *What is the future of Bee-Pollen ? 2*, 131–144.

Casteels, P., Ampe, C., Jacobs, F., & Tempst, P. (1993). Functional and chemical characterization of hymenoptaecin, an antibacterial polypeptide that is infection-inducible in the honeybee (Apis mellifera). *Journal of Biological Chemistry*, *268*, 7044–7054.

Casteels, Peter, Ampe, C., Jacobs, F., Vaeck, M., & Tempst, P. (1989). Apidaecins: antibacterial peptides from honeybees. *EMBO*, *8*, 2387–2391.

Chaplin-Kramer, R., Dombeck, E., Gerber, J., Knuth, K. A., Mueller, N. D., Mueller, M., … Klein, A. M. (2014). Global malnutrition overlaps with pollinator-dependent micronutrient production. *Proceedings of the Royal Society B: Biological Sciences*, *281*, 1–7.

Cheruiyot, S. K., Kahuthia-Gathu, R., Mbugi, J. P., Muli, E., & Lattorff, H. M. G. (2020). Population abundance of Varroa destructor and its effects on Apis mellifera scutellata colonies in Kenya. *Experimental and Applied Acarology*, *82*, 171-184.

Christophides, G. K., Zdobnov, E., Barillas-Mury, C., Birney, E., Blandin, S., Blass, C., … Kafatos, F. C. (2002). Immunity-related genes and gene families in Anopheles gambiae. *Science*, *298*, 159–165.

Collison, E., Hird, H., Cresswell, J., & Tyler, C. (2016). Interactive effects of pesticide exposure and pathogen infection on bee health- a critical analysis. *Biological*

Reviews, *91*, 1006–1019.

Corby-Harris, V., Snyder, L., Meador, C., & Ayotte, T. (2018). Honey bee (Apis mellifera) nurses do not consume pollens based on their nutritional quality. *PLoS ONE*, *13*, 1–21.

Cremer, S., Armitage, S. A. O., & Schmid-Hempel, P. (2007). Social Immunity. *Current Biology*, *17*, 693–702.

Cremer, S., Pull, C. D., & Fürst, M. A. (2018). Social Immunity: Emergence and Evolution of Colony-Level Disease Protection. *Annual Review of Entomology*, *63*(1), annurev-ento-020117-043110.

Cremer, S., & Sixt, M. (2009). Analogies in the evolution of individual and social immunity. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *364*, 129–142.

Danihlík, J., Škrabišová, M., Lenobel, R., Šebela, M., Omar, E., Petřivalský, M., ... & Brodschneider, R. (2018). Does the pollen diet influence the production and expression of antimicrobial peptides in individual honey bees?. *Insects*, *9*, 79.

de Sá-Otero, M. D. P., Armesto-Baztan, S., & Díaz-Losada, E. (2009). Analysis of protein content in pollen loads produced in north-west Spain. *Grana*, *48*, 290–296.

Decourtye, A., Mader, E., & Desneux, N. (2010). Landscape enhancement of floral resources for honey bees in agro-ecosystems. *Apidologie*, *41*, 264–277.

Degrandi-hoffman, G., & Chen, Y. (2015). Nutrition , immunity and viral infections in honey bees. *Current Opinion in Insect Science*, *10*, 170–176.

DeGrandi-hoffman, G., Chen, Y., Huang, E., & Huang, M. H. (2010). The effect of diet on protein concentration , hypopharyngeal gland development and virus load in worker honey bees (Apis mellifera L .). *Journal of Insect Physiology*, *56*, 1184–1191.

DeGrandi-Hoffman, G., Gage, S. L., Corby-harris, V., Carroll, M., Chambers, M., Graham, H., … Ziolkowski, N. (2018). Connecting the nutrient composition of seasonal pollens with changing nutritional needs of honey bee (Apis mellifera L .) colonies. *Journal of Insect Physiology*, *109*, 114–124.

DeGrandi-Hoffman, G., Wardell, G., Ahumada-segura, F., Rinderer, T., Danka, R., & Pettis, J. (2008). Comparisons of pollen substitute diets for honey bees : consumption rates by colonies and effects on brood and adult populations . *Journal of Apicultural Research and Bee World*, *47*, 265–270.

Di Pasquale, G., Salignon, M., Le Conte, Y., Belzunces, L. P., Decourtye, A., Kretzschmar, A., … Alaux, C. (2013). Influence of Pollen Nutrition on Honey Bee Health : Do Pollen Quality and Diversity Matter ? *PLoS ONE*, *8*, 1–13.

Dietemann, V., Pirk, C. W. W., & Crewe, R. (2009). Is there a need for conservation of honeybees in Africa? *Apidologie*, *40*, 285–295.

Dimou, M., & Thrasyvoulou, A. (2009). Pollen analysis of honeybee rectum as a method to record the bee pollen flora of an area. *Apidologie*, *40*, 124–133.

Dolezal, A. G., Carrillo-Tripp, J., Judd, T. M., Allen Miller, W., Bonning, B. C., & Toth, A. L. (2019). Interacting stressors matter: Diet quality and virus infection in honeybee health. *Royal Society Open Science*, *6*, 1–12.

Ellis, A. M., Myers, S. S., & Ricketts, T. H. (2015). Do pollinators contribute to nutritional health? *PLoS ONE*, *10*, 1–17.

Evans, J. D., Aronstein, K., Chen, Y. P., Hetru, C., Imler, J. L., Jiang, H., … Hultmark, D. (2006). Immune pathways and defence mechanisms in honey bees Apis mellifera. *Insect Molecular Biology*, *15*, 645–656.

Evans, Jay D, & Spivak, M. (2010). Socialized medicine: Individual and communal disease barriers in honey bees. *Journal of Invertebrate Pathology*, *103*, S62–S72.

Evans, J. D., Schwarz, R. S., Chen, Y. P., Budge, G., Cornman, R. S., De la Rua, P., ... & Pinto, M. A. (2013). Standard methods for molecular research in Apis mellifera. *Journal of Apicultural Research*, *52*, 1-54.

Erler, S., Popp, M., & Lattorff, H. M. G. (2011). Dynamics of immune system gene expression upon bacterial challenge and wounding in a social insect (Bombus terrestris). *PLoS One*, *6*, e18126.

Faul, F., Erdfelder, E., Lang, A.-G., & Buchner, A. (2007). G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical

sciences. *Behavior Research Methods*, *39*, 175–191.

Ferrandon, D., Imler, J.L., Hetru, C., & Hoffmann, J. A. (2007). The Drosophila systemic immune response: Sensing and signalling during bacterial and fungal infections. *Nature Reviews Immunology*, *7*, 862–874.

Field, C. J., Johnson, I. R., & Schley, P. D. (2002). *Nutrients and their role in host resistance to infection*. *71*, 3–18.

Gallai, N., Salles, J. M., Settele, J., & Vaissière, B. E. (2009). Economic valuation of the vunerability of world agriculture confronted with pollinator decline. *Ecological Economics*, *68*, 810–821.

Groot, A. P. D. (1953). Protein and amino acid requirements of the honeybee (*Apis mellifica* L.).

Gregorio, E. De, Spellman, P. T., Tzou, P., Rubin, G. M., & Lemaitre, B. (2002). The Toll and Imd pathways are the major regulators of the immune response in Drosophila. *EMBO*, *21*, 2568–2579.

Harbo, J. R., & Harris, J. W. (1999). Heritability in honey bees (Hymenoptera: Apidae) of characteristics associated with resistance to Varroa jacobsoni (Mesostigmata: Varroidae). *Journal of economic Entomology*, *92*, 261-265.

Harbo, J. R., & Hoopingarner, R. A. (1997). Honey bees (Hyntenoptera: Apidae) in the United States that express resistance to Varroa jacobsoni (Mesostigmata: Varroidae). *Journal of Economic Entomology*, *90*, 893-898.

Haydak, M. H. (1970). Honey Bee Nutrition. *Annual Review of Entomology*, *15*, 143–156.

Hendriksma, H. P., & Shafir, S. (2016). Honey bee foragers balance colony nutritional deficiencies. *Behavioral Ecology and Sociobiology*, *70*, 509–517.

Hepburn R. H., & Radloff E. S. (1998). *Honeybees of Africa*.

Hoffmann, J. A. (2003). The immune response of Drosophila. *Nature Publishing Group*, *426*, 33–38.

Hoover, S. E. R., Higo, Æ. H. A., & Winston, M. L. (2006). Worker honey bee ovary development : seasonal variation and the influence of larval and adult nutrition. *J*

Comp Physiol B, *176*, 55–63.

Human, H., & Nicolson, S. W. (2006). Nutritional content of fresh, bee-collected and stored pollen of Aloe greatheadii var. davyana (Asphodelaceae). *Phytochemistry*, *67*, 1486-1492.

Human, H., Nicolson, S. W., Strauss, K., Pirk, C. W. W., & Dietemann, V. (2007). Influence of pollen quality on ovarian development in honeybee workers (Apis mellifera scutellata). *Journal of Insect Physiology*, *53*, 649-655.

Hrassnigg, N., & Crailshem, K. (2005). Differences in drone and worker physiology in honeybees (Apis mellifera). *Apidologie*, *36*, 255–277.

Ilyasov, R., Gaifullina, L. R., Saltykova, E. S., Poskryakov, A., & Nikolenko, A. G. (2012). Review of the expression of antimicrobial peptide defensin in honey bees Apis mellifera L. *Journal of Apicultural Science*, *56*, 115-124.

Kassambara, A., Kosinski, M., Biecek, P., & Fabian, S. (2020). *Drawing Survival Curves using "ggplot2" R package version 0.4-8*.

Keller, I., Fluri, P., & Imdorf, A. (2005). Pollen nutrition and colony development in honey bees: Part I. *Bee World*, *86*, 3–10.

Kemp, C., & Imler, J.-L. (2009). Antiviral immunity in drosophila. *Curr Opin Immunol*, *201*, 359–365.

Klein, A. M., Vaissière, B. E., Cane, J. H., Steffan-Dewenter, I., Cunningham, S. A., Kremen, C., & Tscharntke, T. (2007). Importance of pollinators in changing landscapes for world crops. *Proceedings. Biological Sciences / The Royal Society*, *274*, 303–310.

Kleinschmidt, G. J., & Kondos, A. C. (1976). Influence of crude protein levels on colony production. *Australasian Beekeeper*.

Knox, D. A., Shimanuki, H., & Herbert, E. W. (1971). Diet and the Longevity of Adult Honey Bees1. *Journal of Economic Entomology*, *64*, 1415–1416.

Komosinska-Vassev, K., Olczyk, P., Kazfmierczak, J., Mencner, L., & Olczyk, K. (2015). Bee Pollen: Chemical Composition and Therapeutic Application. *Evidence-Based Complementary and Alternative Medicine*, *2015*, 6.

Koto, A., Mersch, D., Hollis, B., & Keller, L. (2015). Social isolation causes mortality by disrupting energy homeostasis in ants. *Behavioral Ecology and Sociobiology*, *69*, 583-591.

Krautz, R., Arefin, B., & Theopold, U. (2014). Damage signals in the insect immune response. *Frontiers in Plant Science*, *5*, 1–11.

Kurata, S. (2014). Peptidoglycan recognition proteins in Drosophila immunity Shoichiro. *Developmental & Comparative Immunology*, *42*, 1–7.

Laughton, A. M., Boots, M., & Siva-Jothy, M. T. (2011). The ontogeny of immunity in the honey bee, Apis mellifera L. following an immune challenge. *Journal of insect physiology*, *57*(7), 1023-1032.

Le Conte, Y., Ellis, M., & Ritter, W. (2010). Varroa mites and honey bee health: Can Varroa explain part of the colony losses? *Apidologie*, *41*, 353–363.

Lee, K. P., Simpson, S. J., & Wilson, K. (2008). Dietary protein-quality influences melanization and immune function in an insect. *Functional Ecology*, *22*, 1052–1061.

Lemaitre, B., & Hoffmann, J. (2007). The Host Defense of Drosophila melanogaster. *Annual Review of Immunology*, *25*, 697–743.

Li, W., Evans, J. D., Li, J., Su, S., Hamilton, M., & Chen, Y. (2017). Spore load and immune response of honey bees naturally infected by Nosema ceranae. *Parasitology research*, *116*, 3265-3274.

Li, P., Yin, Y., Li, D., Kim, S. W., & Wu, G. (2007). Review Article Amino acids and immune function. *British Journal of Nutrition*, *98*, 237–252.

Lockey, T. D., & Ourth, D. D. (1996). Formation of pores in Escherichia coli cell membranes by a cecropin isolated from hemolymph of Heliothis virescens larvae. *Eur. J. Biochem.*, *236*, 263–271.

Maurizio, A., & Louveaux, J. (1965). Pollens de plantes mellifères d'Europe.

Meixner, M. D. (2010). A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. *Journal of invertebrate pathology*, *103*, S80-S95.

Merkling, S. H., & van Rij, R. P. (2013). Beyond RNAi: antiviral defense strategies in

Drosophila and mosquito. *Journal of insect physiology*, *59*, 159-170.

Michener, C. D. (2000). The bees of the world. In *The Johns Hopkins University Press* (Vol. 85).

Moret, Y., & Schmid-Hempel, P. (2000). Survival for immunity: activation of the immune system has a price for bumblebee workers. *Science*, *290*, 1166-1168.

Morse, R. A., & Calderone, N. W. (2000). The value of honey bees as pollinators of US crops in 2000. *Bee culture*, *128*, 1-15.

Muli, E., Patch, H., Frazier, M., Frazier, J., Torto, B., Baumgarten, T., … Grozinger, C. (2014). Evaluation of the Distribution and Impacts of Parasites, Pathogens, and Pesticides on Honey Bee (Apis mellifera) populations in East Africa. *PLoS ONE*, *9*, 1–12.

Muturi, M. N., Bargul, J. L., & Lattorff, H. M. G. (2022). Influence of the Type of Pollen Diet on the Survival, Body Weight, and Immune Response in the African Honeybee. *Journal of Apicultural Science*, *66*, 29-43.

Naug, D. (2009). Nutritional stress due to habitat loss may explain recent honeybee colony collapses. *Biological Conservation*, *142*, 2369–2372.

Neumann, P., & Carreck, N. L. (2010). Honey bee colony losses. *Journal of Apicultural Research*, *49*, 1–6.

Nicolson, S. W., & Thornburg, R. W. (2007). Nectaries and Nectar. In *Nectaries and Nectar* (pp. 215–263).

Nkonya, E., Johnson, T., Kwon, H. Y., & Kato, E. (2015). Economics of Land Degradation and Improvement - A Global Assessment for Sustainable Development. In *Economics of Land Degradation in Sub-Saharan Africa*.

Ochungo, P., Veldtman, R., Kinyanjui, R., Abdel-Rahman, E. M., Muli, E., Muturi, M. N. K., … Landmann, T. (2021). Pollen diversity and protein content in differentially degraded semi-arid landscapes in Kenya. *Journal of Apicultural Research*, *60*, 828-841

Odoux, J. F., Feuillet, D., Aupinel, P., Loublier, Y., Tasei, J. N., & Mateescu, C. (2012). Territorial biodiversity and consequences on physico-chemical characteristics of

pollen collected by honey bee colonies. *Apidologie*, *43*, 561–575.

Oldroyd, B. P. (2007). What's Killing American Honey Bees ? *PLoS BIOLOGY*, *5*, e168.

Paoli, P. P., Donley, D., Stabler, D., Saseendranath, A., Nicolson, S. W., Simpson, S. J., & Wright, G. A. (2014). Nutritional balance of essential amino acids and carbohydrates of the adult worker honeybee depends on age. *Amino Acids*, *46*, 1449–1458.

Pernal, S. F., & Currie, R. W. (2000). Pollen quality of fresh and 1-year-old single pollen diets for worker honey bees (Apis mellifera L.). *Apidologie*, *31*, 387–409.

Pfaffl, W. M. (2001). A new mathematical model for relative quantifocation in real-time RT-PCR. *Nucleic Acids Research*, *29*, 2002–2007.

Pirk, C. W. W., Boodhoo, C., Human, H., & Nicolson, S. W. (2010). The importance of protein type and protein to carbohydrate ratio for survival and ovarian activation of caged honeybees (Apis mellifera scutellata). *Apidologie*, *41*, 62–72.

Potts, S. G., Biesmeijer, J. C., Kremen, C., Neumann, P., Schweiger, O., & Kunin, W. E. (2010). Global pollinator declines: Trends, impacts and drivers. *Trends in Ecology and Evolution*, *25*, 345–353.

Rinderer, T. E., & Elliott, K. D. (1977). Worker Honey Bee Response with *Nosema Apis*: Influence of Diet. *Economic Entomology*, *70*, 431–433.

Rinderer, T. E., Rothenbuhler, W. C., & Gochnauer, T. A. (1974). The influence of pollen on the susceptibility of honey-bee larvae to *Bacillus larvae*. *Journal of Invertebrate Pathology*, *23*, 347–350.

Rinderer, T. E., & Baxter, J. R. (1978). Effect of empty comb on hoarding behavior and honey production of the honey bee. *Journal of Economic Entomology*, *71*, 757-759.

Roulston, T. H., & Cane, J. H. (2000). Pollen nutritional content and digestibility for animals. *Plant Systematics and Evolution*, *222*, 187–209.

Ruijter, J. M., Ramakers, C., Hoogaars, W. M. H., Karlen, Y., Bakker, O., van den hoff, M. J. B., & Moorman, A. F. M. (2009). Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Research*, *37*, 1–12.

Ruttner, F. (1988). *Biogeography and Taxonomy of Honeybees*.

Sammataro, D., Gerson, U., & Needham, G. (2000). Parasitic Mites of the Honey Bees: Life History, Implications and Impact. *Annual Review of Entomology*, *45*, 519–548.

Schmickl, T., & Crailsheim, K. (2004). Inner nest homeostasis in a changing environment with special emphasis on honey bee brood nursing and pollen supply. *Apidologie*, *35*, 249–263.

Schmidt, J. O. (1984). Feeding preferences of Apis mellifera L. (Hymenoptera: Apidae): individual versus mixed pollen species. *Journal of the Kansas entomological society*, 323-327.

Schmidt, J. O., & Johnson, B. E. (1984). Pollen feeding preference of Apis-Mellifera (Hymenoptera, Apidae) a polylectic bee. *Southwestern Entomologist*, *9*, 41-47.

Schmidt, J. (1985). Phagostimulants in pollen. *Journal of Apicultural Research*, *24*, 107-114.

Schmidt, J. O., Thoenes, S. C., & Levin, M. D. (1987). Survival of Honey Bees, *Apis mellifera* (Hymenoptera: Apidae), Fed Various Pollen Sources. *Ann. Entomol. Soc. Am*, *80*, 176–183.

Sgolastra, F., Medrzycki, P., Bortolotti, L., Renzi, M. T., Tosi, S., Bogo, G., ... & Bosch, J. (2017). Synergistic mortality between a neonicotinoid insecticide and an ergosterol-biosynthesis-inhibiting fungicide in three bee species. *Pest Management Science*, *73*, 1236-1243.

Smart, M. D., Pettis, J. S., Euliss, N., & Spivak, M. S. (2016). Land use in the Northern Great Plains region of the U.S. influences the survival and productivity of honey bee colonies. *Agriculture, Ecosystems and Environment*, *230*, 139–149.

Smith, M. R., Singh, G. M., Mozaffarian, D., & Myers, S. S. (2015). Effects of decreases of animal pollinators on human nutrition and global health: A modelling analysis. *The Lancet*, *386*, 1964–1972.

Strand, M. R. (2008). The insect cellular immune response. *Insect Science*, *15*, 1–14.

Strauss, U., Human, H., Gauthier, L., Crewe, R. M., Dietemann, V., & Pirk, C. W. W. (2013). Seasonal prevalence of pathogens and parasites in the savannah honeybee (*Apis mellifera scutellata*). *Journal of Invertebrate Pathology*, *114*, 45–52.

Tesovnik, T., Cizelj, I., Zorc, M., Manuela, Č., Bozič, J., Glavan, G., & Narat, M. (2017). Immune related gene expression in worker honey bee (*Apis mellifera carnica*) pupae exposed to neonicotinoid thiamethoxam and Varroa mites (*Varroa destructor*). *PLoS ONE*, *12*, 1–15.

Theopold, U., & Dushay, M. S. (2007). Mechanisms of Drosophila immunity-an innate immune system at work. *Current Immunology Reviews*, *3*, 276-288.

Therneau, M. T. (2020). *Therneau T (2020). A Package for Survival Analysis in R. R package version 3*,2-3.

Tihelka, E., Cai, C., Pisani, D., & Donoghue, P. C. (2020). Mitochondrial genomes illuminate the evolutionary history of the Western honey bee (Apis mellifera). *Scientific reports*, *10*, 1-10.

Tyler, E. R., Adams, S., & Mallon, E. B. (2006). An immune response in the bumblebee, Bombus terrestris leads to increased food consumption. *BMC physiology*, *6*, 1-4.

VanEngelsdorp, D., Evans, J. D., Saegerman, C., Mullin, C., Haubruge, E., Nguyen, B. K., … Pettis, J. S. (2009). Colony collapse disorder: A descriptive study. *PLoS ONE*, *4*, e6481.

Vaudo, A. D., Tooker, J. F., Biddinger, D., & Crone, M. (2020). Pollen Protein : Lipid Macronutrient Ratios May Guide Broad Patterns of Bee Species Floral Preferences. *Insects*, *11*, 0–14.

Weinstock, G. M., Robinson, G. E., Gibbs, R. A., Weinstock, G. M., Weinstock, G. M., Robinson, G. E., … Wright, R. (2006). Insights into social insects from the genome of the honeybee *Apis mellifera*. *Nature*, *443*, 931–949.

Williams, G. R., Alaux, C., Costa, C., Csáki, T., Doublet, V., Eisenhardt, D., … Brodschneider, R. (2013). Standard methods for maintaining adult *Apis mellifera* in cages under in vitro laboratory conditions.In V Dietemann; J D Ellis; P Neumann (Eds) The COLOSS BEEBOOK, Volume I: standard methods for Apis mellifera research. *Journal of Apicultural Research*, *52*, 1–36.

Winston, M. L. (1991). *The biology of the honey bee*. Harvard university press.

Xie, F., Xiao, P., Chen, D., Xu, L., & Zhang, B. (2012). miRDeepFinder: a miRNA

analysis tool for deep sequencing of plant small RNAs. *Plant molecular biology*, *80*, 75-84.

Yang, X., & Cox-Foster, D. L. (2005). Impact of an ectoparasite on the immunity and pathology of an invertebrate: Evidence for host immunosuppression and viral amplification. *Proceedings of the National Academy of Sciences of the United States of America*, *102*, 7470–7475.

Zaobidna, E. A., Żółtowska, K., & Łopieńska-Biernat, E. (2015). Expression of the prophenoloxidase gene and phenoloxidase activity, during the development of *Apis mellifera* brood infected with varroa destructor. *Journal of Apicultural Science*, *59*, 85–93.

APPENDICES

Appendix I: RNA nanodrop results

Bee No	260/230 Ratio	260/280 Ratio
B1	0.5	1.94
B2	0.7	1.7
B3	1.06	1.99
B4	0.55	1.63
B5	0.21	1.54
B6	0.22	1.83
B7	0.11	1.73
B8	0.33	1.89
B9	0.18	1.82
B10	0.11	1.76
B1	0.33	1.64
B2	0.69	1.8
B3	0.3	1.48
B4	0.33	1.54
B5	0.36	1.6
B6	1.14	1.96
B7	1.19	1.88
B8	0.78	1.79
B9	1.05	1.89
B1	0.87	1.77
B2	0.29	1.14
B3	0.81	1.74
B4	10.19	1.63
B5	0.5	1.57
B6	0.72	1.85
B7	1.12	1.96

B8	1.11	1.96
B9	0.41	1.39
B10	0.41	1.39
B1	0.47	2.41
B2	0.48	2.02
B3	0.7	2
B4	0.34	2.27
B5	0.48	2
B6	0.64	2.09
B7	0.37	2.5
B8	0.62	2.06
B9	0.51	2
B10	0.43	2.53
B11	0.39	2.6