EFFECT OF POLLEN DIET AND HONEY BEE (*Apis mellifera* L.) PRIMER PHEROMONES ON WORKER BEE FOOD PRODUCING GLANDS

A Thesis

by

LIZETTE ALICE PETERS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2008

Major Subject: Entomology

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Approved by:

Chair of Committee,	Tanya Pankiw
Committee members,	Gil Rosenthal
	Keyan Zhu-Salzman
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ABSTRACT

Effect of Pollen Diet and Honey Bee (*Apis mellifera* L.) Primer Pheromones on Worker Bee Food Producing Glands. (December 2008)

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Chair of Advisory Committee: Dr. Tanya Pankiw

This thesis examines three factors that may influence the change in protein content and size of the brood food glands in honey bees. Effects on the mandibular gland, involved in the production of brood food and in royal jelly, have not been examined in relation to primer pheromones while effects on the hypopharyngeal glands, also involved in the production of brood food, have not been examined in relation to queen mandibular pheromone. This thesis provides preliminary insight into how these pheromones affect the extractable protein content of brood food glands.

The first study in this thesis assessed the effects of brood pheromone (BP), queen mandibular pheromone (QMP), and pollen presence on the protein content of hypopharyngeal and mandibular glands of the honey bee. In this study, newly emerged bees were caged for 12 days in one of eight treatments: Queenless state: 1) control (no pollen + no pheromone), 2) pollen, 3) BP, 4) BP + pollen; Queenright state: 1) QMP, 2) QMP + pollen, 3) BP + QMP, 4) BP + QMP + pollen. This study indicated that regardless of pheromone treatment, the most influential factor on gland protein content and size was pollen.

The second experiment examined effects of varying pollen dilution on hypopharyngeal and mandibular gland protein content, bee mass, and lipid content of the honey bee. In this experiment, newly emerged bees were caged for 7 days and fed one of five treatments: pollen, 1:1 pollen: cellulose (vol:vol), 1:2 pollen: cellulose (vol:vol); 1:3 pollen: cellulose (vol:vol), and cellulose. This study indicated that bees on the pollen diet were significantly greater than all other diluted diets in measurements of hypopharyngeal gland protein content, lipid content, and mass with significantly less consumption. However, mandibular gland protein content of bees on the pollen diet was significantly greater only from pure cellulose.

DEDICATION

I dedicate this thesis to my father who steadfastly supported and encouraged my education. It is by his constant recounting of his enjoyable experiences that I strived for a degree at Texas A&M University, by chance or fate, within the same building he achieved his doctorate.

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Over the course of this degree, I have faced obstacles that I would not have overcome without the aid and encouragement of many people. First and foremost, I would like to express my deepest gratitude to my advisor and committee chair Dr. Tanya Pankiw whose support and innovation made this thesis possible. I was very fortunate to have worked under the supervision of such an excellent and aspiring scientist. I would also like to acknowledge my committee members Dr. Keyan Zhu-Salzman who so graciously allowed the use of her lab and equipment and Dr. Gil Rosenthal whose enthusiasm for science and positive attitude aided in my own outlook.

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CHAPTER I

INTRODUCTION

This thesis is divided into four chapters. Chapter I is an introduction to honey bee colony division of labor, brood rearing, pheromones, and lists the objectives. Chapter II reports a study on effects of QMP, BP and pollen on hypopharyngeal and mandibular gland protein content, mandibular gland size, and pollen consumption. Chapter III describes a study on pollen diet dilution effects on mandibular and hypopharyngeal gland protein content. Chapter IV is an overall summary and conclusions pertaining to each chapter.

The honey bee is a eusocial insect, a term used to designate the highest level of social organization where there is reproductive division of labor, overlapping generations, cooperative care of young, and irreversible castes (Crespi and Yanega, 1995; Wilson, 1976). Reproductive division of labor in the honey bee is such that there is only one queen heading the colony as the only mated female and principal egg layer. Honey bee queens mate once in a lifetime usually in their second week of adult life with 5 to 20 or more males (Tarpy and Neilsen, 2003; Tarpy and Page, 2000). As a consequence of polyandry worker relatedness within colonies ranges from 0.25 to 0.75 (Page and Erber, 2002). There may be from 0 to 1000's of males living in the colony depending on time of year. Males called drones are haploid developing from

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This thesis follows the style of the Journal of Insect Physiology.

unfertilized eggs. The reproductive castes do no work and are solely engaged in individual reproduction. All the work associated with colony survival, growth, brood care, and colony-level reproduction is performed by the worker caste comprised of overlapping generations of 1000's of semi-sterile diploid females capable of laying unfertilized eggs but not of mating.

A primary characteristic of eusocial life is an age-related division of labor. The temporal patterning of behavior is known as temporal, or age, polyethism (Hölldobler and Wilson, 1990; Jeanne, 1991; Robinson et al., 1992) and is expressed as apparent changes in probabilities that workers perform different behavioral tasks. In general, as bees age they make transitions from performing tasks in the center of the nest to performing tasks at the periphery, and finally they leave the nest to forage. Centrally located tasks include cell cleaning and tending to the needs of the brood and the queen. Medial tasks include comb building and food processing and storage. Peripheral tasks include receiving nectar from foragers, removing dead bodies, constructing comb, and guarding the colony entrance (Seeley, 1995; Winston, 1987). Progression from working in the nest to foraging marks a major transition in a worker honey bee's life. When workers are in about their third week of life they cease performing tasks within the nest and begin foraging outside for pollen, nectar, water, and propolis (a resinous material collected from plants used in nest construction). Once workers begin foraging, they seldom revert to perform within-nest tasks.

The timing of these behavioral transitions is not fixed; workers do not perform all possible tasks or necessarily develop into foragers. Factors such as genotype, the

demographic structure of the immature and adult worker population, and pheromones that communicate demographic structure affect behavioral development trajectories. For example, in the absence of older bees, worker bees will initiate foraging behavior at younger ages. In the absence of young bees, old bees may revert to performing within-nest tasks like feeding larvae (Huang and Robinson, 1996). Pheromones extracted from the surface of young non-foraging and foraging workers exert similar effects on foraging ontogeny suggesting that bees use pheromones to estimate amount of young and old bees and adjust their development accordingly (Leoncini et al., 2004; Pankiw, 2004c).

Honey bee queen mandibular pheromone also exerts similar effects; colonies given supplemental doses of synthetic pheromone show delayed onset of foraging relative to non-supplemented control colonies (Pankiw et al., 1998a). This is in addition to other effects of queen mandibular pheromone as a sex attractant, a releaser of retinue behavior and an inhibitor of queen-rearing behavior (Winston and Slessor, 1992). Nurse bees come into contact with the queen most frequently leading Pankiw et al. (1998a) to hypothesize that exposure to queen mandibular pheromone can extend the duration of the nursing phase to ensure more efficient brood rearing. The addition of larvae or their pheromones, called brood pheromone, to colonies also changes rate of behavioral development that is dose-dependent (Le Conte et al., 2001; Pankiw, 2004b). Additions of relatively small amounts of brood pheromone accelerate foraging ontogeny (Le Conte et al., 2001; Pankiw, 2004b; Pankiw et al., 2004; Sagili, 2007). Conversely, additions of relatively large amounts of brood pheromone delays foraging ontogeny and thus extends the duration a worker performs nursing duties (Le Conte et al., 2001; Pankiw, 2004b).

The principal function of nurses is to progressively provision larvae food produced from two glands found in the head, namely the hypopharyngeal and mandibular glands. Nurse bees feed on stored pollen as their sole source of protein and on recently collected nectar or stored honey as their sources of carbohydrate. The glands produce proteinaceous secretions deposited in a pool surrounding each larva. The ratio of hypopharyngeal to mandibular gland secretion deposited is varied depending on larva age, sex, and caste. Female larvae chosen to be reared as queens are mass provisioned nearly 100% mandibular gland secretion during the first 3 days of larval life followed by a 1:1 ratio of mandibular to hypopharyngeal gland secretion over the final 2 days as a larva (Beetsma, 1979; Brouwers et al., 1987). The mixture fed to queen larvae is commonly called "royal jelly" (Winston, 1987). Proteins belonging to the major royal jelly protein family constitute 90% of total royal jelly proteins (Santos et al., 2005; Scarselli et al., 2005). Worker larvae are progressively provisioned "brood food" in a 2:9:3 ratio of mandibular to hypopharyngeal gland secretions to pure pollen (Beetsma, 1979). Fourth and fifth instar worker larvae are given some honey inducing a phagostimulatory response, as well as pollen to accommodate the rapid rate of growth in these latter instars (Brouwers et al., 1987). Male larvae are provisioned food of lower protein quality than that provisioned to workers but of greater quantity due to their larger size and longer time of larval development (Brouwers et al., 1987; Winston, 1987).

As workers age and transition from performing nursing tasks to tasks found in more peripheral regions of the nest, hypopharyngeal and mandibular gland activity and function may also change. For example, the hypopharyngeal glands in young nurse bees

are large and well developed producing primarily proteins. The function then switches in middle-aged bees engaged in food processing to produce α-glucosidase used to hydrolyze the sucrose of nectar into glucose and fructose (Deseyn and Billen, 2005; Kubo et al., 1996; Ohashi et al., 1999). Finally, the gland atrophies in foragers (Robinson, 1987; Sasagawa et al., 1989). The mandibular gland also changes with worker age related behaviors; however, the range of change is greater and more complex than what is currently known of the hypopharyngeal gland.

Like the hypopharyngeal gland, the mandibular gland has food producing activity among nurse bees in a colony with a queen (queenright) laying fertilized eggs that develop into worker bees. However as the worker ages in a queenright colony, the gland permanently switches to alarm pheromone production, principally 2-heptanone (Kerr et al., 1974). In a queenless colony or one in which the queen is no longer laying fertilized eggs, reproductive division of labor lines begin to blur and a form of social anarchy ensues where some workers grow well-developed ovaries and become egg layers (Oldroyd and Ratnieks, 2000). The mandibular glands of egg laying workers become queen-like, even producing queen-like mandibular gland pheromone, a blend of fatty acids and some aromatic compounds (Plettner et al., 1993). Two factors are associated with the loss of social cohesion in the honey bee, 1) the loss of the queen and, 2) the loss of diploid larvae which are communicated to the colony through queen mandibular gland (QMP) and brood (BP) primer pheromones, respectively. Primer pheromones produced by the queen and larvae affect worker bee endocrine, physiological and neurobiological systems (Pankiw, 2004b). Primers exert changes gradually and changes are permanent

even after the pheromone is no longer detectable and absent (Hölldobler and Wilson, 1990; Pankiw, 2004b).

The regulation of reproduction and cooperative brood care are critically important to eusocial species survival. As a consequence, pheromone mediated reproduction and cooperation systems regulating the worker caste have evolved. The first chemically characterized social insect primer pheromone is queen mandibular pheromone (Slessor et al., 1988). QMP induces workers to feed and groom the queen called retinue behavior, a releaser response (Slessor et al., 1988). QMP has a wide range of primer effects including the inhibition of queen rearing, regulation and timing of colony-level reproduction (swarming), partial inhibition of worker ovariole development, regulation of comb-building, regulation of foraging ontogeny, and modulation of worker brain dopamine function (Beggs et al., 2007; Hoover et al., 2003; Ledoux et al., 2004; Melathopoulos et al., 1996; Pankiw and Garza, 2007; Pankiw et al., 1998a; Pettis et al., 1995; Winston et al., 1991; Winston et al., 1990). The focal primer effect of QMP in this study is the regulation of worker mandibular gland size and amount of extractable protein because it is a key gland used for the production of royal jelly in queen rearing.

For social insect colonies, colony-level reproduction is the principal sources of fitness. As such, much of individual worker and colony behaviors are ultimately related to colony reproduction. Honey bee colonies reproduce through a process of colony budding, commonly referred to as swarming. In general, swarming is such that about half of the adult workers leave the parental nest with the old mother queen to initiate a

new nest elsewhere. Inheriting the parental nest is a new young daughter queen and the remaining workers to begin the colony life cycle anew. Colonies begin to rear queens approximately 10 days prior to swarming. That is, natural swarming does not occur without first initiating the process of queen rearing. Intra-colony factors that inhibited queen rearing are released and new queens are reared in the presence of the old queen (Winston and Slessor, 1992). One important queen rearing inhibitor is queen mandibular pheromone (Melathopoulos et al., 1996; Pettis et al., 1997; Winston et al., 1991; Winston et al., 1990).

In general, QMP communicates queen presence to the colony. One queen equivalent (QEq) of QMP extractable from the paired mandibular glands of a mated, egg laying queen is approximately, 200 µg 9-keto2-(E)-decenoic acid (9-ODA), 100 µg 9-hydroxy-2(E)-decenoic acid [88%R-(-) and 12% S-(+)] (9-HDA), 20 µg methyl p-hydroxybenzoate (HOB), and 2 µg 4-hydroxy-3-methoxyphenylethanol (HVA) (Pankiw et al., 1996; Slessor et al., 1988). While some honey bee pheromones are transmitted by diffusion through the air, many are non-volatile, and are transmitted by contacts between bees. Queen mandibular pheromone is considered non-volatile having a very short volatile space of approximately 12 mm, thus transmission throughout the nest is principally through a series of contacts between the queen and retinue bees, and retinue bees with other bees (Naumann, 1991; Naumann et al., 1993; Naumann et al., 1992; Naumann et al., 1991). Retinue response is characterized as a dynamic group of workers surrounding the queen or source of QMP, frequently antennating, licking, grooming and sometimes feeding (Kaminski et al., 1990; Naumann, 1991; Pankiw et al., 1994). QMP

is dispersed throughout the nest in part by the movement of the queen, who remains in the brood nest area, and through serial worker-to-worker transmissions (Naumann, 1991; Naumann et al., 1993; Naumann et al., 1992). As colonies grow in worker number, the amount of QMP reaching individuals decreases due to a simple dilution effect and due to colony crowding that obstructs transmission (Watmough et al., 1998). Once QMP reaching individuals drops below detectable levels, estimated to be from 10^{-5} to 10^{-7} QEq (Slessor et al., 1988), queen rearing may be initiated.

Female caste development in the honey bee is triggered solely by the diet of the larvae. Larvae fed royal jelly throughout their larval life become queens; those that are not become workers. Proteinaceous secretions of the nurse bee mandibular gland are a major component of royal jelly. While the role of QMP is well understood for the initiation of queen rearing and the regulation and timing of colony-level reproduction, effects of QMP on the mandibular gland of workers are not understood, even at the most rudimentary level. One objective of this thesis is to compare the effect of QMP and non-pheromone rearing environments on worker mandibular gland size and extractable protein.

Colony-level reproduction is not the only context in which queens are reared. Queens are reared to replace dead, injured, old, or sick queens (Winston, 1987). Emergency queen rearing takes place when a queen is killed or removed consequently placing the colony in a sudden ‘queenless’ state (Winston, 1987). Within about 20 min after a queen is removed from a colony or killed, workers become agitated, and about 2 hrs later queen rearing is initiated (Seeley, 1985). The loss of a queen triggers

emergency queen rearing because colonies have approximately 6 days to choose larvae from among the remaining larvae that are 3 days old or younger to rear queens (Pettis et al., 1997; Winston, 1987; Winston and Slessor, 1992). If colonies do not successfully rear a queen to replace the dead queen, it is placed in a "hopelessly queenless" state and the colony eventually dies. In addition to QMP, the communication of larva sex, age, and caste of larvae clearly play a critical role in the regulation of nurse bee food producing glands.

Pettis et al. (1997) demonstrated that the presence of young larvae (1-3 d) may play a role in the regulation of queen rearing. The first of three experiments was designed to examine the effects of synthetic queen mandibular gland pheromone and the addition of young larvae plus QMP on the number of queens reared and time to queen rearing in queenless colonies (emergency queen rearing). The number of queens reared and timing were significantly negatively correlated with amount of young larvae added to colonies (Pettis et al., 1997). In this experiment addition of QMP confounded the effects of larvae on queen rearing, however results suggested larvae play a role in regulating queen rearing. In a second experiment queenless colonies received either young larvae (1-3 d) or old larvae (3-5 d) over a 3 day period (Pettis et al., 1997). In colonies treated with young larvae, significantly fewer queens were initiated, and significantly fewer queen cells were prepared. Only in colonies containing young larvae was no queen rearing observed in the first 24 hr period of the experiment (Pettis et al., 1997). In a third experiment colonies with queens were manipulated such that 1) young larvae were removed and thus contained mostly old larvae, 2) colonies contained mostly

young larvae, and 3) controls with no larva manipulation. Adult population growth was controlled by allowing equal amounts of pupae to emerge in colonies. Colonies were measured for amount of queen rearing over a 14 day period. Colonies containing mostly old larvae reared significantly more queen cells in a significantly shorter period of time compared to colonies with mostly young larvae and controls (Pettis et al., 1997). Combined, these experiments strongly suggest that larvae play a key role in regulating the timing and amount of queen rearing. Pettis et al. (1997) concluded that colonies perceive the presence of young larvae as a queen fecundity cue that feeds back on worker queen rearing behaviors, even in queenless colonies.

The fatty acid esters extractable from the surface of larvae induce the greatest number of known primer pheromone responses in honey bees (Pankiw, 2004b). The ten fatty acid esters of honey bee larvae that have been reported as pheromonal are methyl and ethyl esters of linoleate, linolenate, oleate, palmitate, and stearate of male or drone larvae (LeConte et al., 1990), as well as queens and workers (Trouiller, 1993; Trouiller et al., 1994; Trouiller et al., 1991). Total amount of esters are reported to change with larval instar for all castes. In general, total amount of detectable esters increase with age (Le Conte et al., 1994; Trouiller et al., 1994; Trouiller et al., 1991). Although weight and surface area measures have not been considered, increased size with age is likely in part due to increased total amount of esters. Proportion of ethyl to methyl esters also changes with age. "Young" larvae (estimated as 3rd to 4th instars) secrete about 64% ethyl esters (ratio of 1.7 ethyl to methyl esters) and "old" larvae (estimated as 5th instar to prepupa), about 69% methyl esters (ethyl: methyl ratio of 0.4; Le Conte et al. (1994)).

Mature drone larvae have greater proportions of methyl and ethyl palmitate than queens or workers. Workers have greater proportions of methyl stearate and linolenate and, queens have greater proportions of methyl and ethyl oleate (Trouiller et al., 1994). For all sexes and castes, total extractable esters peak for several hours prior to and after pupation, triggering cell capping by adults (Le Conte et al., 1994; Trouiller et al., 1994; Trouiller et al., 1991). Larval esters regulate worker hypopharyngeal gland development and protein biosynthesis of nurse bees in cages (Mohammedi et al., 1996) as well as in colonies (Pankiw et al., 2004). The 10-component blend is as follows; 1% ethyl linoleate, 13% ethyl linolenate, 8% ethyl oleate, 3% ethyl palmitate, 7% ethyl sterarate, 2% methyl linoleate, 21% methyl linolenate, 25% methyl oleate, 3% methyl palmitate and, 17% methyl stearate. Brood pheromone acts as a releaser of multiple individual forager behaviors and primer on foraging behavior development (Le Conte et al., 2001; Pankiw, 2004a, b; Pankiw and Page, 2001a; Pankiw et al., 1998b; Pankiw et al., 2004; Pankiw and Rubink, 2002). Nurse bees, aged from around 7 to14 days consume pollen and convert it into proteinaceous secretions provisioned to larvae. In this way larvae consume pollen via nurse bees. Larval cues and pollen are necessary for hypopharyngeal gland development, activity and, protein production (Brouwers, 1982, 1983; Hrassnigg and Crailsheim, 1998; Huang and Otis, 1989; Huang et al., 1989; Mohammedi et al., 1996). Larvae or their esters stimulate hypopharyngeal gland development even in the absence of a pollen diet (Mohammedi et al., 1996). However a protein source is necessary for glandular protein biosynthesis resulting in greater amounts of extractable protein (Brouwers, 1983; Huang and Otis, 1989; Huang et al.,

1989; Mohammedi et al., 1996). Larvae and larval esters have clearly been demonstrated to prime hypopharyngeal gland development and, pollen provides the protein source fueling brood-food production. Addition of larval esters to average colonies also increases amount of protein extractable from hypopharyngeal glands even in the winter when few to no larvae are being reared in colonies (Pankiw et al., 2004; Pankiw et al., 2008).

This review reveals that changes in amounts and proportions of larval esters (ester profile) can result in changes in adult behavioral responses. Despite what seems like a wealth of information on honey bee larval esters, important primer effects on nurse bee mandibular glands are unknown. Additionally, a more integrative approach has not been previously examined measuring the effects of QMP + BP on worker food producing glands. The objectives of this thesis were to 1) analyze effects of QMP, BP, and pollen on hypopharyngeal and mandibular gland protein content, mandibular gland size, and pollen consumption; and 2) evaluate the effects of pollen dilutions on honey bee hypopharyngeal and mandibular gland extractable protein.

CHAPTER II

EFFECTS OF PRIMER PHEROMONES AND POLLEN ON BROOD FOOD GLANDS

Introduction

Primer pheromones induce long term effects on endocrine, reproductive, and neurobiological systems of worker bees ultimately affecting individual bee behavior (Pankiw, 2004c; Pankiw and Page, 2003). Two primers: queen mandibular pheromone (QMP), produced by the mandibular glands of mated, egg laying queens (Pankiw et al., 1996; Slessor et al., 1988); and brood pheromone (BP), extractable from the cuticle of larva (Le Conte et al., 1989; Mohammedi et al., 1996; Trouiller et al., 1991), were used with a pollen diet to measure effects on amount of extractable protein on brood food producing glands and size of the mandibular glands.

QMP aids in colony organization through both its releaser and primer effects. Primer effects of QMP include partial inhibition of worker ovary development (Hoover et al., 2003; Lin and Winston, 1998; Willis et al., 1990), delayed foraging onset (Pankiw et al., 1998a), and inhibition of queen rearing (Melathopoulos et al., 1996; Pettis et al., 1995; Winston et al., 1991; Winston et al., 1990). Colonies with a queen are termed queenright, those without are termed queenless. Workers in a queenless colony (Melathopoulos et al., 1996) or in a highly congested queenright colony (Watmough et al., 1998) no longer inhibited by QMP may initiate queen rearing. Both hypopharyngeal and mandibular

glands produce necessary components of royal jelly fed to queen larvae (Lensky and Rakover, 1983).

Royal jelly is comprised of carbohydrates (11.9 ± 0.7), lipids (6.1 ± 0.4), proteins (12.7 ± 0.8), and moisture content (68.3 ± 1.4) (Takenaka and Takenaka, 1996). The proteins comprising royal jelly are not entirely known (Schonleben et al., 2007). What is known is that a series of major royal jelly proteins or MRJPs of which there are five main members comprise 82-90% of royal jelly proteins . These main proteins are identical to those found in worker jelly fed to worker larvae (Schmitzova et al., 1998). Most of the proteins are synthesized by the hypopharyngeal glands and secreted in royal jelly (Hanes and Simuth, 1992; Kubo et al., 1996; Santos et al., 2005). Mandibular glands also produce some proteins present within royal jelly (Lensky and Rakover, 1983) as well as royal jelly acid or 10-hydroxy-dec-2-enoic acid (Barbier, 1981).

Because nurse bees without mandibular glands are unable to rear queens (Peng and Jay, 1977, 1979), it is likely that treatments without QMP will have greater amounts of extractable protein from mandibular glands as well as a potentially larger gland. If the reverse is found, then QMP will have failed to suppress a key gland used to produce royal jelly. Because hypopharyngeal glands produce the majority of worker brood food protein content (Brouwers, 1982; Lensky and Rakover, 1983), it was expected that QMP would not regulate the extractable protein from this gland. However, because royal jelly is a 1:1 mandibular to hypopharyngeal gland secretion, then it is possible that treatments without QMP would have greater amounts of extractable hypopharyngeal glands. Regardless of

the outcome, the findings of this study will be novel: there is currently no data on primer pheromone effects on worker mandibular gland food producing ability.

Brood pheromone (BP) induces effects that stimulate brood care. BP aids QMP by inducing partial inhibition of worker ovariole development (Arnold et al., 1994; Mohammedi et al., 1998; Pankiw and Garza, 2007). There is a direct correlation in the amount of stored pollen and the amount of brood successfully reared (Allen and Jeffree, 1956). BP has been shown to increase colony number of pollen foragers (Pankiw, 2004a, c; Pankiw and Garza, 2007; Pankiw and Page, 2001b; Pankiw et al., 1998b; Pankiw et al., 2004; Schulz et al., 2002), increase the forager returning load weights of pollen (Pankiw, 2004a), and increase the number of trips an individual pollen forager takes per unit time (Pankiw, 2007). All of these effects help ensure that the brood are tended to properly. It is therefore not surprising that brood stimulate hypopharyngeal gland development even in the absence of a pollen diet (Mohammedi et al., 1996) resulting in an increased amount of extractable hypopharyngeal glands (Pankiw et al., 2004). The mandibular glands aid in brood food production (Barker et al., 1959; Lensky and Rakover, 1983); therefore, it is likely that brood pheromone will increase mandibular gland size and protein content.

Protein is necessary for development of larvae. Adult worker bee hypopharyngeal glands produce the protein component of brood food (Patel et al., 1960). The rate of protein synthesis peaks at 8-16 days of age (Knecht and Kaatz, 1990). A protein source is necessary for glandular protein biosynthesis resulting in greater amounts of extractable protein (Brouwers, 1983; Huang and Otis, 1989; Huang et al.,

1989; Mohammedi et al., 1996). However, adult honey bees will temporarily utilize proteins from their own bodies to feed brood if pollen is not available (Haydak, 1970). Poor gland development and a shorter length of life can result from insufficient pollen consumption early in adult life (Haydak, 1970; Maurizio, 1950); therefore, I hypothesized that pollen would increase the amount of extractable protein content of both the mandibular and hypopharyngeal glands.

In this study, I measured effects of QMP, BP, and pollen on amount of extractable protein from hypopharyngeal and mandibular glands as well as mandibular gland size. Pollen diet consumption was recorded. This study is the first to measure the effects of a synthetic pheromone environment on mandibular gland protein content and to measure the effects of synthetic QMP on hypopharyngeal glands of honey bees.

Methods

Combs containing pupae about to emerge as adult bees were placed inside an incubator (32°C, 50% RH) for 24 h. Three hundred (300) newly emerged bees from multiple colony sources were placed in plexiglass/wiremesh cages (15 cm x 11 cm x 8 cm). Bees were reared for 12 days in an incubator maintained at hive conditions (32°C, 50% RH) with one of the following pheromone treatments: control, QMP, BP, or QMP + BP. Each pheromone treatment was replicated with and without pollen diet resulting in a total of 8 treatments (see Table 1). Due to logistical constraints and availability of newly emerged bees, only 4 cages were reared in the incubator at a time. Treatments were replicated 3 times. The third replicate contained 50 additional older bees collected

directly from the brood nest area to compensate for mortality in this replicate. These 50 bees were paint-marked for distinction. A fourth replicate was conducted with 6 cages in the incubator: control, pollen, QMP, QMP + pollen, QMP + BP, and QMP + BP + pollen.

Every 24 h, each cage was provisioned 30 ml of 30% sucrose solution and 30 ml of distilled H_2O. Pollen (#78-23063) was obtained from Glory Bee Foods (Eugene, OR. USA) and sucrose from the Imperial Sugar Company (Sugar Land, TX. USA). To make a homogenized pollen diet, 200 g of powdered pollen was blended with 40 ml of 30% sucrose (13 g sucrose, 32 ml distilled H_2O) using an Artisan Series Tilt-Head Stand Mixer (KitchenAid, St. Joseph, MI. USA). Pollen diet was provisioned in a 15 mm x 10 mm (depth x radius) plastic test tube cap placed in the bottom of the rearing cage. Treatments without pollen received an empty plastic cap. After each 24 h period, remaining sucrose and water volumes were recorded. Pollen diet consumption was measured by subtracting post-feeding weight from pre-feeding weight.

The blend of brood pheromone used here was as follows; 1% ethyl linoleate, 13% ethyl linolenate, 8% ethyl oleate, 3% ethyl palmitate, 7% ethyl stearate, 2% methyl linoleate, 21% methyl linolenate, 25% methyl oleate, 3% methyl palmitate, and 17% methyl stearate (Sigma-Adrich, St. Louis, MO, USA). Isopropanol was used as the solvent to formulate daily doses of 560 ng/µl per bee. Brood pheromone was delivered on a glass plate (7.5 cm x 8 cm) and suspended from the center of each plexiglass/ wiremesh cage. Cages with no BP received a glass plate rinsed in isopropyl alcohol.

Table 1. Cages received one of the following treatments: control, QMP, BP, or QMP + BP. Each pheromone treatment was conducted both with and without pollen resulting in a total of 8 treatments. Treatments were replicated at least 3 times at the cage level. A fourth replicate included: control, pollen, QMP, QMP + pollen, QMP + BP, and QMP + BP + pollen.

Treatment	QMP[1]	BP[2]	Pollen
Control	-	-	-
Pollen	-	-	+
BP	-	+	-
BP + pollen	-	+	+
QMP	+	-	-
QMP + pollen	+	-	+
QMP + BP	+	+	-
QMP + BP + pollen	+	+	+

[1] +QMP is queenright, –QMP is queenless

[2] +BP is broodright, -BP is broodless

Bee Boost® (Pherotech International Inc., Delta, B.C. Canada) was the source of QMP. Bee Boost® delivers approximately 1 queen equivalent (QEq) of QMP per day, (200 µg 9-keto-2 (*E*)-decenoic acid (ODA), 80 µg for 9-hydroxy-2-(E)-decenoic acid (HDA), 20 µg methyl *p*-hydroxybenzoate and 2 µg homovanillyl alcohol (HVA) (Pankiw et al., 1996)). Bee Boost® is loaded with 30 QEq of QMP. Treatments receiving QMP simulated colonies with a queen and were therefore termed "queenright," while those not receiving QMP simulated colonies without a queen present were therefore termed "queenless." One Bee Boost® stick was placed in each QMP + treatment cage for the duration of the experiment (Table 1).

Sub-samples of 20 bees per treatment were collected every 3 days from each cage, cold anesthetized, and stored at -20 °C until dissected for protein quantification of hypopharyngeal and mandibular glands and mandibular gland measurement. For each dissection the bee was first decapitated. Next, the head was pinned on a wax mount and dissected under a Stereo Zoom Binocular microscope body (CO-SZ-600 on Boom Stand, Sciencescope, Chino, CA. USA), objective: 10x. The head was dissected from right to left. An incision was made to separate the right compound eye and ommatidia from the rest of the head. Then, the right hypopharyngeal gland was removed, rinsed in distilled H_2O, and placed into a vial containing 20 µl Tris buffer, (0.1 M Tris-HCl, pH 7.5). Following the removal of the hypopharyngeal gland, the mandible and all attached glandular tissue were removed to measure width and length of the gland using a microscope reticle. The mandible with gland attached was rinsed with distilled H_2O and placed into a second vial of 20 µl Tris buffer, (0.1 M Tris-HCl, pH 7.5). This procedure

was repeated to remove glands on the left. The vials, each containing 2 hypopharyngeal or 2 mandibular glands were stored at -20 °C prior to protein quantification using the Bradford assay (Bradford, 1976).

The Bradford assay methods were after Sagili et al. (2005). Glands were homogenized using a homogenizer that tightly fitted in 1.5 µl microcentrifuge tubes used to store the glands. Next, tubes were centrifuged at 10,000 rpm for 5 min. Supernatant from each tube was used for analysis. The 500-0202 Quick Start Bradford Protein Assay Kit 2 was used (Bio-Rad Laboratories, CA, and U.S.A.) containing all necessary reagents and dyes. The dye reagent was prepared by adding 1 part Coomassie Brilliant Blue G-250 dye reagent to 4 parts distilled water. Subsequently, 2 µl or 5 µl aliquots were added from each sample to be analyzed to a microcentrifuge tube containing 1 ml Bradford reagent. Tubes were vortexed to homogenize the contents, and then incubated for 10 min at room temperature (approximately 24° C). Standard-curves were prepared using bovine serum albumin (BSA). Protein absorbance was measured at 595 nm against blank reagent using a Thermo Genesys 10 UV Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA). A standard curve was generated by plotting the known weight of BSA against the corresponding absorbance values measured (SPSS, 2007). Protein extracted from the glands was estimated using the linear regression equation generated from the BSA standard curve assay.

Protein quantity was statistically analyzed using analysis of variance (ANOVA) (Sokal and Rohlf, 1995; SPSS, 2007). The hypopharyngeal gland data were natural log transformed prior to analysis to normalize the distribution (Sokal and Rohlf, 1995). To

reduce the probability of a type 1 error (a false positive result), the Bonferroni post-hoc test was used to analyze differences between treatments (Sokal and Rohlf, 1995).

Results

In the queenless environment, there were no significant differences between replicates for sucrose consumption per bee (ANOVA $F_{2, 141} = 0.593$, $P = 0.554$). Therefore, replicates were pooled for further analysis. There were no significant differences between queenless treatments for sucrose consumption (ANOVA $F_{3, 140} = 0.146$, $P = 0.932$).

In the queenright environment, there were significant differences found between replicates for sucrose consumption (ANOVA $F_{3, 290} = 9.760$, $P < 0.0001$). However, there was no significant replicate x treatment interaction (GLM, $F_{15, 270} = 1.001$, $P = 0.454$). For this reason, replicates were pooled for further analysis. There were no significant differences between queenright treatments for sucrose consumption per bee (ANOVA $F_{5, 288} = 1.466$, $P = 0.201$).

In the queenless environment, no significant differences in water consumption were found between treatments. Water consumption was significantly different between replicates (ANOVA, $F_{2, 141} = 3.436$, $P = .035$). However, there was no significant replicate by treatment effect (GLM, $F_{6, 132} = .342$, $P = .914$). Therefore, replicates were pooled for further analysis. No significant differences in water consumption per bee were found between treatments (ANOVA, $F_{3, 140} = 1.206$, $P = .310$).

In the queenright environment, there were significant differences found between replicates for water consumption (ANOVA $F_{3, 290} = 16.017$, $P = 0.0001$). There was a significant replicate by treatment interaction (GLM, $F_{15, 270} = 2.503$, $P = 0.002$). For this reason replicates were analyzed separately. No significant differences occurred between treatments in 3 out of 4 replicates (ANOVA, $F_{5, 66} = 2.192$, $P = 0.066$; $F_{5, 66} = 2.117$, $P = .074$; $F_{5, 72} = .902$, $P = .484$). Significant differences occurred between water consumption of bees in the control compared with all queenright treatments in 1 of the 4 replicates (ANOVA, $F_{5, 66} = 4.934$, $P = .001$).

In the queenless environment, there was no significant difference between treatments for pollen consumption per bee. There were no significant differences between replicates (ANOVA $F_{2, 69} = 0.818$, $P = 0.446$) and there were no significant replicate by treatment interactions (GLM, $F_{2, 66} = .917$, $P = .405$). Therefore replicates were pooled for further analysis. There were no significant differences in diet consumption per bee (ANOVA $F_{1, 70} = 0.000$, $P = 0.998$).

In the queenright environment, there was no significant difference between treatments for pollen consumption. There was no significant difference between replicates for pollen consumption (ANOVA $F_{3, 143} = 0.647$, $P = 0.586$) and no significant replicate by treatment interactions (GLM $F_{6, 135} = .516$, $P = .795$). Replicates were pooled for further analysis. No significant treatment effect was found for pollen consumption (ANOVA $F_{2, 144} = 0.395$, $P = 0.674$).

Hypopharyngeal gland protein was normalized by a natural log transformation. Replicates were significantly different in the queenless ($F_{2, 947} = 25.850$, $P < 0.0001$) and

the queenright ($F_{3, 1863} = 7.117$, $P < 0.0001$) environments; however, the treatment differences were unidirectional. As a consequence replicates were pooled for further analysis. The overall worker hypopharyngeal gland mean extractable protein in the queenless and queenright rearing environments are shown in Figures 1 and 2 respectively. Letters indicate significant differences in hypopharyngeal gland extractable protein (ANOVA, $P < 0.05$).

In the queenless rearing environment (Fig. 1), control bees had significantly less extractable hypopharyngeal gland protein than bees in the BP treatment (ANOVA, $F_{1, 472} = 5.841$, $P = 0.016$). However, bees in the BP + pollen treatment did not have significantly greater hypopharyngeal gland protein content than bees provisioned pollen alone (ANOVA, $F_{1, 474} = 2.768$, $P = 0.097$). Bees in the BP + pollen treatment had significantly greater hypopharyngeal gland protein content than bees in the BP treatment (ANOVA, $F_{1, 472} = 92.613$, $P < 0.0001$), while bees in the pollen treatment had significantly greater hypopharyngeal gland protein content than the control (ANOVA, $F_{1, 474} = 86.891$, $P < 0.0001$). Overall, bees provisioned pollen diet had significantly greater extractable hypopharyngeal gland protein content than those without pollen diet (ANOVA, $F_{1, 948} = 179.445$, $P < 0.0001$).

In the queenright environment (Fig. 2), there was no significant difference in extractable hypopharyngeal gland protein between bees in the QMP – pollen treatment and the control (ANOVA, $F_{1, 630} = 2.986$, $P = 0.084$) or between bees in the QMP – pollen treatment and bees in the QMP + BP – pollen treatment (ANOVA, $F_{1, 614} = 0.436$, $P = 0.509$). Overall, bees provisioned pollen had significantly greater hypopharyngeal

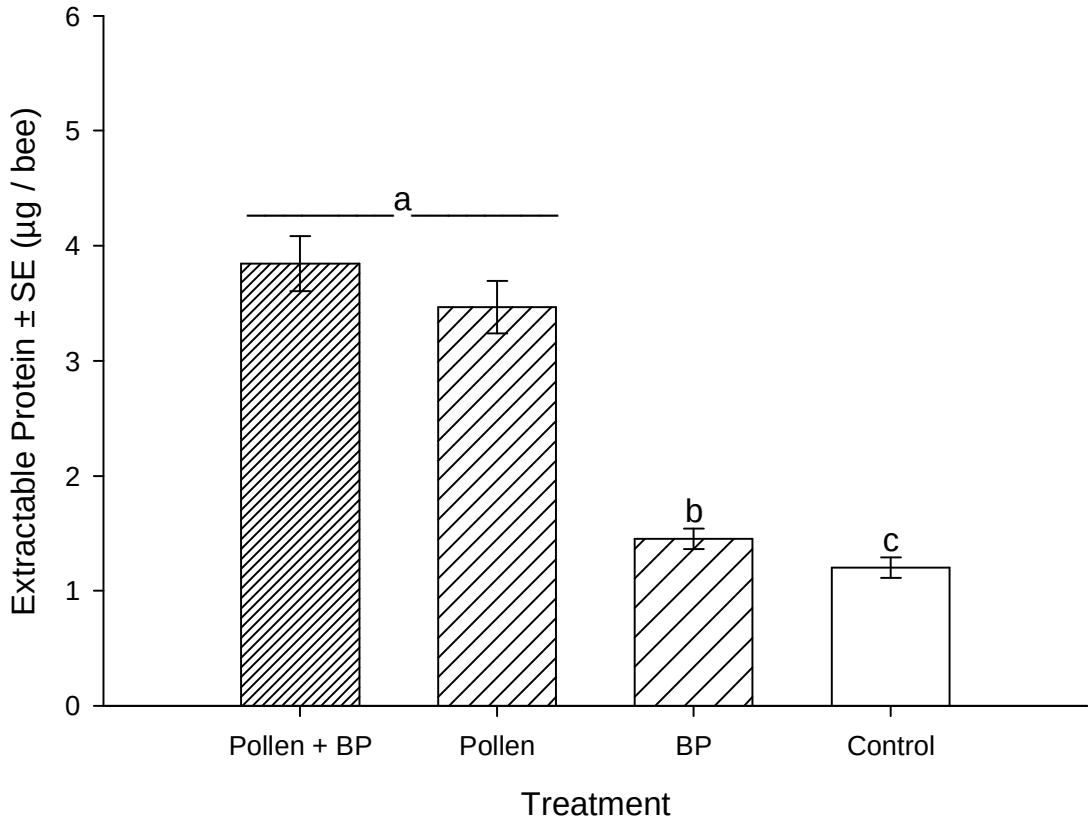

Figure 1. Honey bee hypopharyngeal gland extractable protein in a queenless environment. Letters indicate significant differences in hypopharyngeal gland extractable protein (ANOVA, $P < 0.05$).

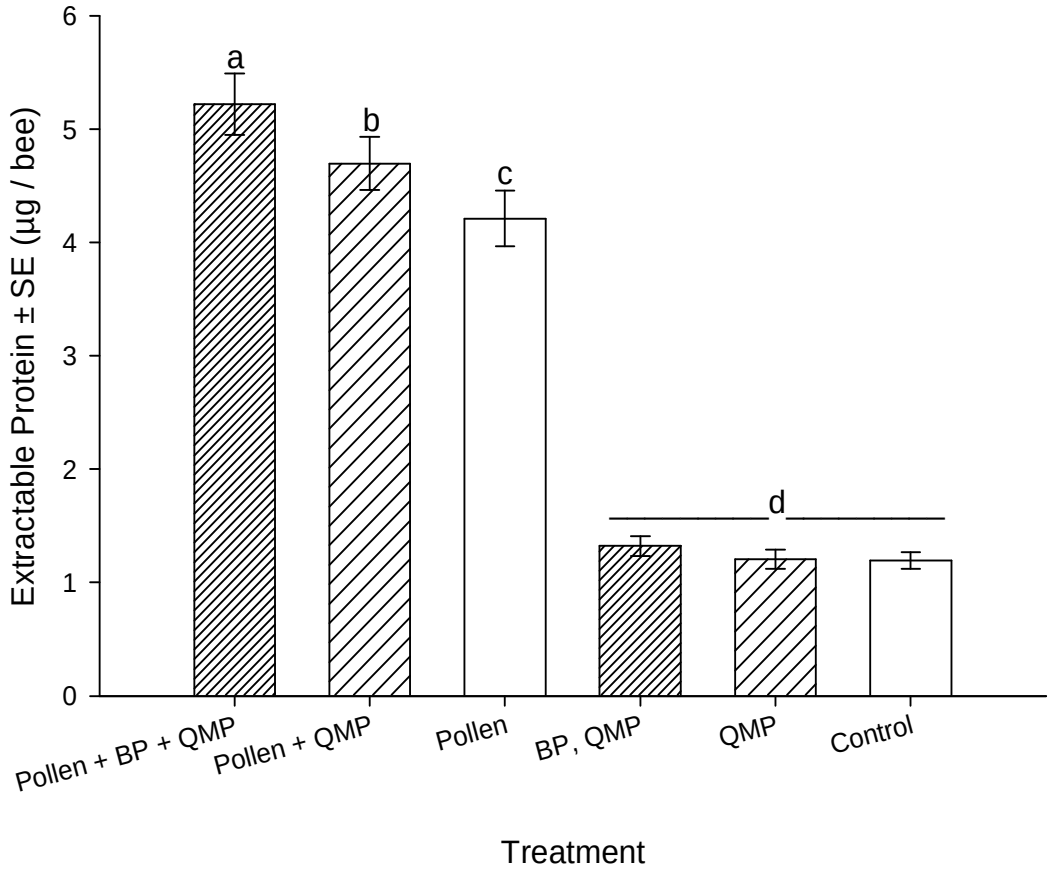

Figure 2. Honey bee hypopharyngeal gland extractable protein in a queenright environment. Letters indicate significant differences in hypopharyngeal gland extractable protein (ANOVA, $P < 0.05$).

gland protein content than bees in the non-pollen treatments (ANOVA, $F_{1, 1865}$ = 653.683, $P < 0.0001$). Hypopharyngeal glands of bees in the QMP – pollen treatment had significantly less extractable protein than those of bees in the QMP + pollen treatment (ANOVA, $F_{1, 618} = 260.883$, $P < 0.0001$). In addition, hypopharyngeal glands of bees in the QMP + BP – pollen treatment had significantly less extractable protein than those of the QMP + BP + pollen treatment (ANOVA, $F_{1, 609} = 226.142$, $P < 0.0001$). Hypopharyngeal glands of bees in the pollen treatment were significantly less than bees in the QMP + pollen treatment (ANOVA, $F_{1, 622} = 5.238$, $P = 0.022$). There were no significant differences between QMP + pollen and QMP + BP + pollen (ANOVA, $F_{1, 616} = 0.250$, $P = 0.618$). Finally, in the queenright environment, the QMP + BP + pollen treatment had significantly greater extractable protein than bees in the pollen treatment (ANOVA $F_{1, 625} = 7.490$, $P = 0.006$). Hypopharyngeal gland extractable protein content declined as bees aged in the queenless environment ($R^2 = 0.9106$, $P = 0.0117$; Fig. 3 (SigmaPlot, 2006)) and in the queenright environment ($R^2 = 0.8460$, $P = 0.0269$; Fig. 3).

Figures 4 and 5 depict the worker mandibular gland extractable protein means of bees sampled on days 3, 6, 9, and 12 in the queenless and queenright environments respectively. Mandibular gland protein amounts were significantly different by replicate in the queenless environment ($F_{2, 948} = 4.333$, $P = 0.013$) and in the queenright environment ($F_{3, 1889} = 27.760$, $P < 0.0001$); however, the differences were unidirectional. As a consequence replicates were pooled for further analysis. In the queenless environment, bees in the pollen treatment had significantly greater mandibular

gland protein content than bees in the control (ANOVA, $F_{1, 474} = 35.769$, $P < 0.0001$; Fig. 4). In addition, bees in the BP + pollen treatment had significantly greater mandibular gland protein amount than bees in the pollen treatment (ANOVA, $F_{1, 472} = 6.186$, $P = 0.013$; Fig. 4). Overall, in the queenless rearing environment, bees provisioned pollen had significantly greater protein content than bees given no pollen ($F_{1,949} = 107.652$, $P < 0.0001$; Fig. 4).

In the queenright environment, bees in the pollen treatment differed significantly from the control (ANOVA, $F_{1,634} = 65.269$, $P < 0.0001$; Fig. 5). Bees given QMP + BP + pollen did not have significantly greater mandibular protein than bees given only pollen (ANOVA, $F_{1, 624} = 3.851$, $P = 0.0502$; Fig. 5). Overall, bees in the queenright environment provisioned pollen diets had significantly greater extractable mandibular gland protein than bees in treatments without pollen (ANOVA, $F_{1,1891} = 237.506$, $P < 0.0001$; Fig. 5).

Mandibular gland size was also measured. In the queenless rearing environment, bees in the control had significantly less area than those given BP + pollen (ANOVA, $F_{1, 478} = 7.935$, $P = 0.005$; Fig. 6). In the queenright treatments, bees provisioned with a pollen diet had significantly greater area than bees not provisioned with a pollen diet (ANOVA, $F_{1, 1908} = 52.738$, $P < 0.0001$; Fig. 7). In addition, bees had significantly greater mandibular gland area on the QMP + BP + pollen treatment than bees on the pollen treatment (ANOVA, $F_{1, 633} = 4.029$, $P = 0.045$; Fig. 7).

Mandibular gland extractable protein content and size decreased with age. Mandibular gland extractable protein of bees in the queenless environment declined

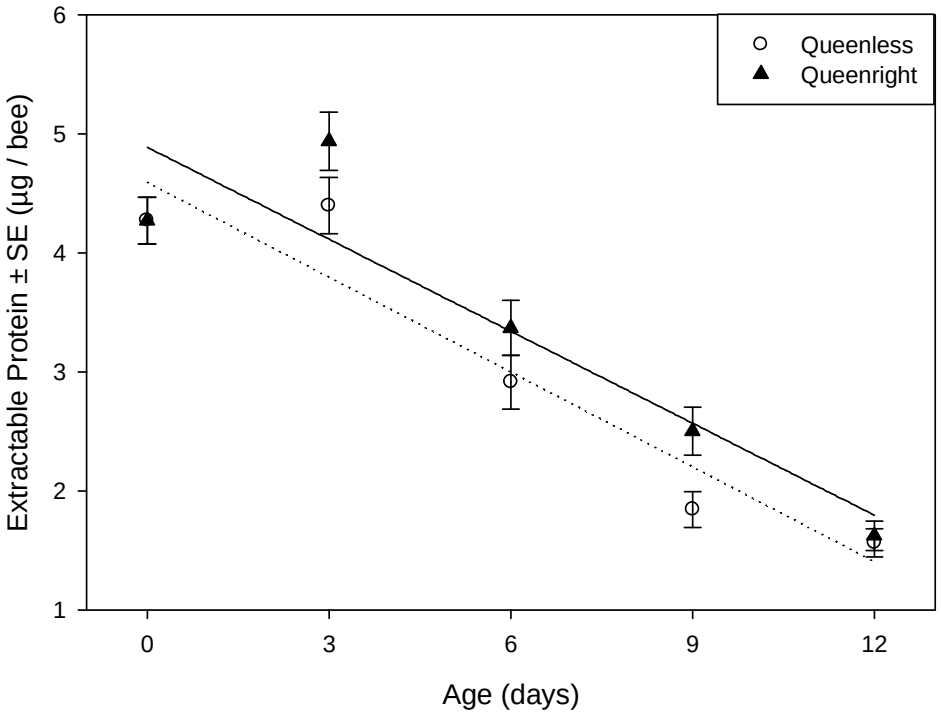

Figure 3. Honey bee hypopharyngeal gland extractable protein declined with age in both the queenless environment (R^2 = 0.9106, P = 0.0117) indicated by the dotted line, and the queenright environment (R^2 = 0.8460, P = 0.0269) indicated by the solid line.

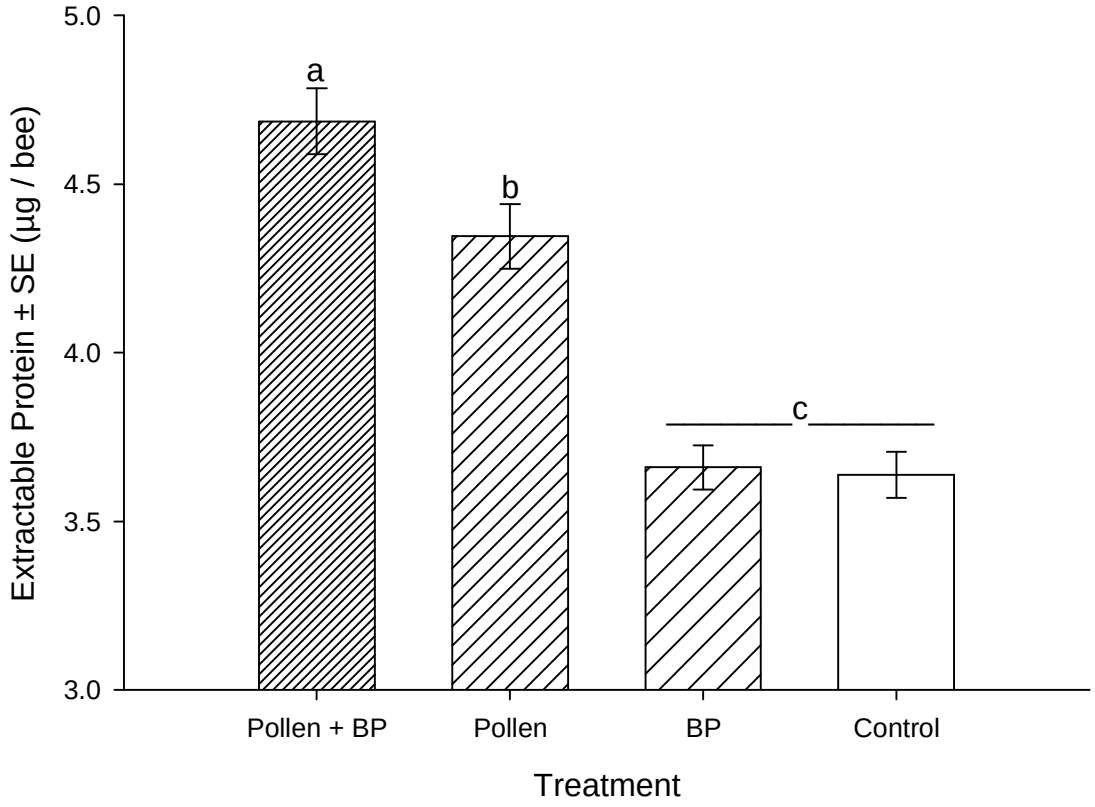

Figure 4. Mean of honey bee mandibular gland extractable protein collected on days 3, 6, 9, and 12 in a queenless environment. Letters indicate significant differences in mandibular gland extractable protein (ANOVA, $P < 0.05$).

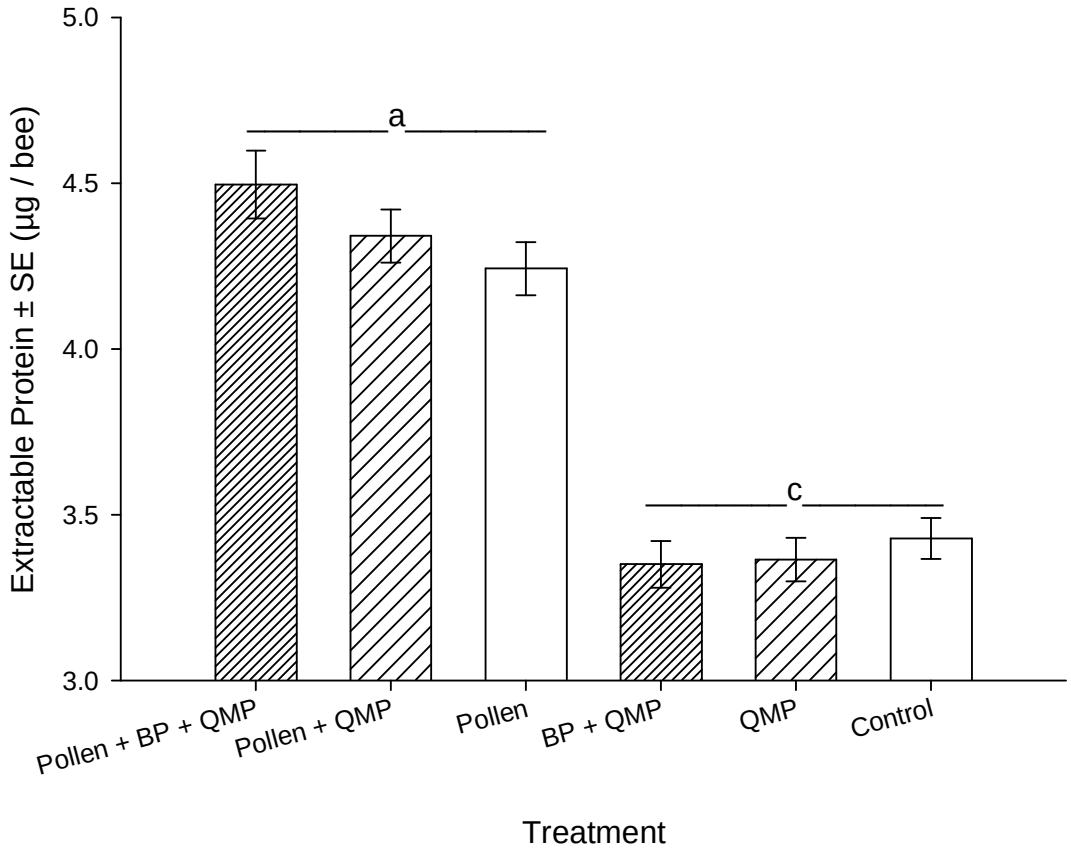

Figure 5. Mean honey bee mandibular gland extractable protein collected on days 3, 6, 9, and 12 in a queenright environment. Letters indicate significant differences in mandibular gland extractable protein (ANOVA, $P < 0.0001$).

from a mean max of 1.5220 ± 0.0185 to a minimum of 1.2850 ± 0.0117 in the queenless environment (R^2 = 0.9926, P = 0.0003; Fig. 8). A similar decline was observed in the queenright environment (maximum: 1.5220 ± 0.0185; minimum 1.2742 ± 0.0114; R^2 = 0.8618, P = 0.0228; Fig. 8). Mandibular gland size also decreased with age in both the queenless environment, (R^2 = 0.8580, P = 0.0238; Fig. 9), as well as in bees reared in the queenright environment (R^2 = 0.8536, P = 0.0249; Fig. 9).

Discussion

I hypothesized that pollen would increase protein content of both mandibular and hypopharyngeal glands and size of mandibular glands of adult honey bees. Amounts of extractable protein from hypopharyngeal and mandibular glands significantly increased with pollen diet. This strongly suggests that pollen provides essential nutrients that affect gland protein content (Figs. 1, 2, 4, and 5). To some degree, mandibular gland size also increased with pollen (Figs. 6 and 7). In previous studies, it has been observed that poor hypopharyngeal gland development and a shorter life span resulted from insufficient pollen consumption early in adult life (Maurizio, 1950). Although Haydak (1935), did not directly measure the glands of nurse bees, he did report a loss in nitrogen content of nurse bee heads in colonies without pollen. Haydak (1935), also observed brood could only be reared for one week without pollen.

Consumption did not differ between treatments except in one replication in which water consumption differed between the control and QMP treatments. There is no explanation for this one inconsistency. Mohammedi et al., (1996) also found that no

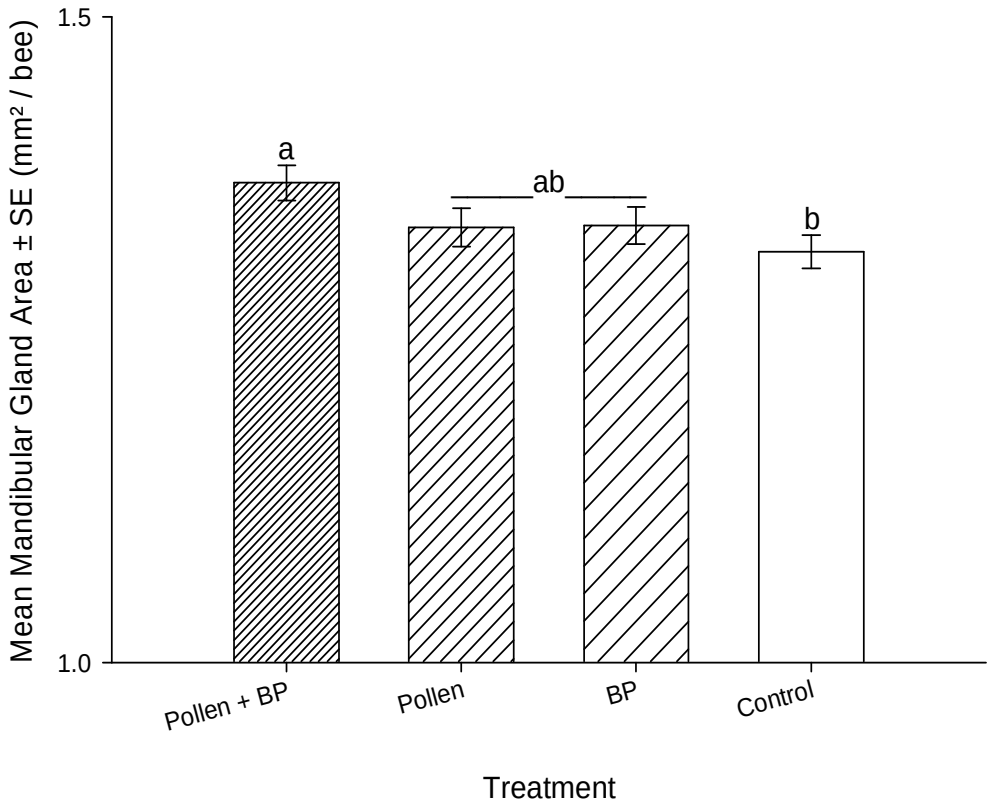

Figure 6. Mean honey bee mandibular gland size in a queenless environment. Letters indicate significant differences in mandibular gland extractable protein (ANOVA, $P < 0.05$).

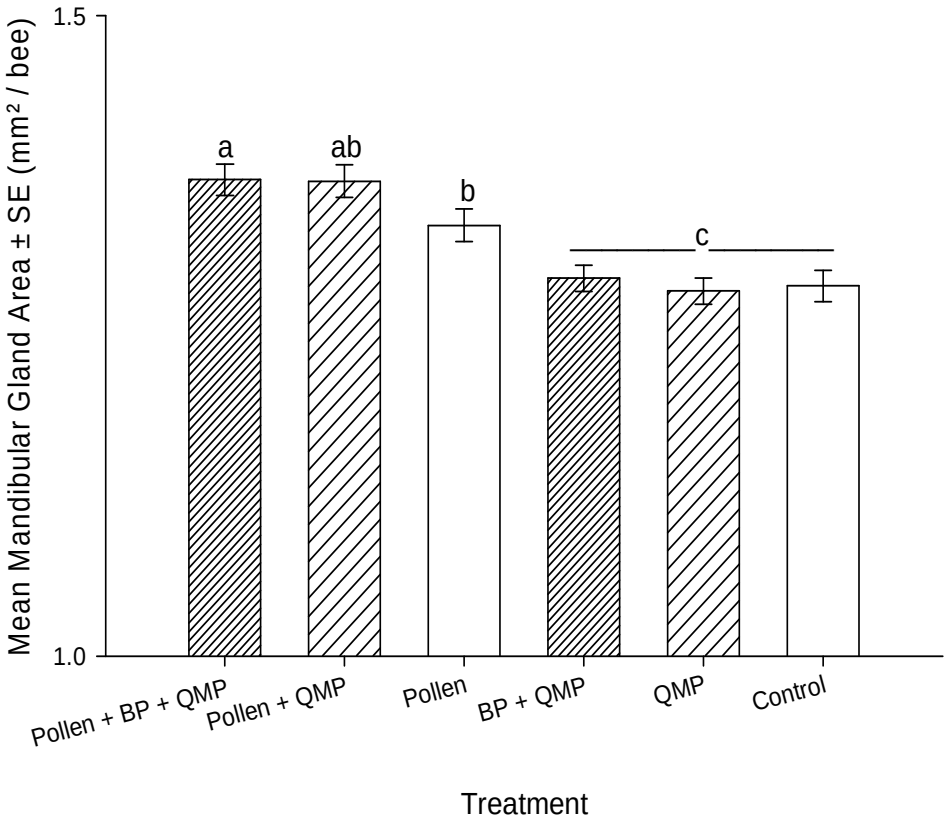

Figure 7. Honey bee mandibular gland size in a queenright environment. Letters indicate significant differences in mandibular gland extractable protein (ANOVA, $P < 0.05$).

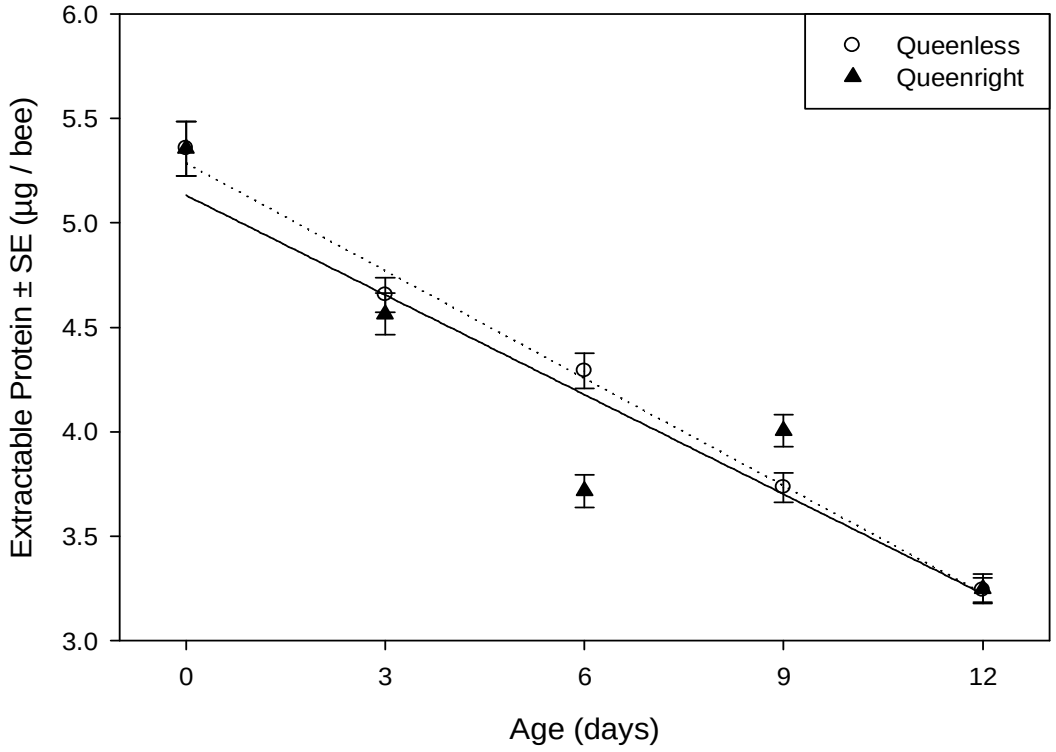

Figure 8. Honey bee mandibular gland extractable protein declined with age in a queenless environment (R^2 = 0.9926, P = 0.0003) indicated by the dotted line, and in the queenright environment (R^2 = 0.8618, P = 0.0228) indicated by the solid line.

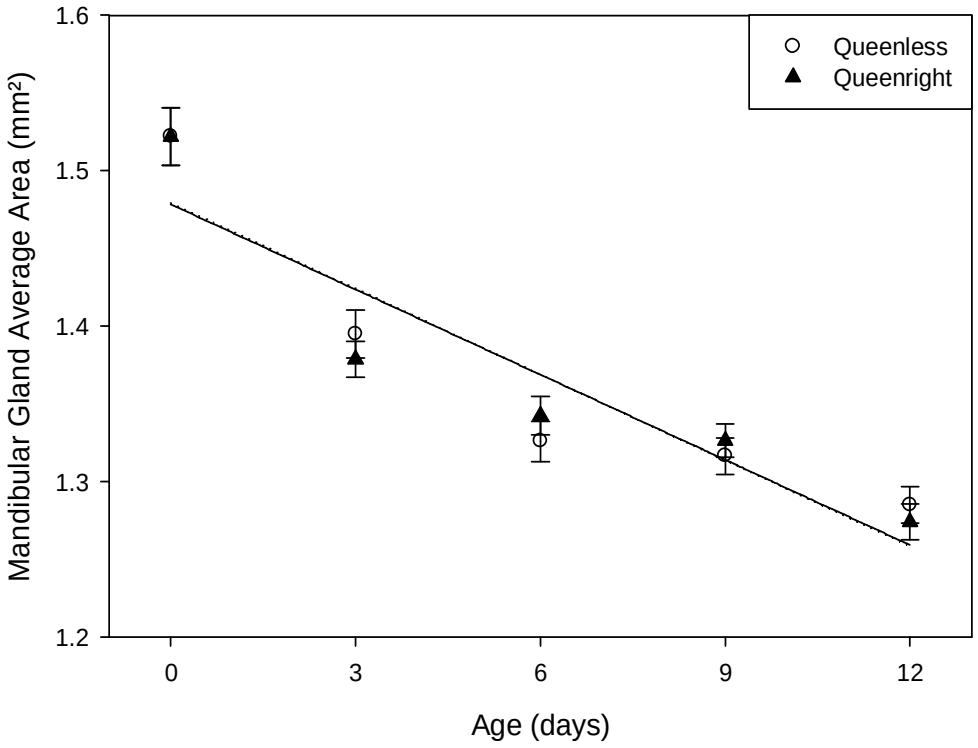

Figure 9. Honey bee mandibular gland average area declined with age in a queenless environment (R^2 = 0.8580, P = 0.0238), and in the queenright environment (R^2 = 0.8536, P = 0.0249). The line shown on the graph represents the linear regression of both the queenless and queeright environments.

significant differences in pollen consumption occurred between treatments with and without brood pheromone. He concludes that the esters provided in the brood pheromone are not simply phagostimulants, but are prompting the increase of bee hypopharyngeal glands without an increase in pollen consumption.

I hypothesized that BP would increase protein content of both hypopharyngeal and mandibular glands because brood presence has previously been shown to increase hypopharyngeal acini diameter (Hrassnigg and Crailsheim, 1998), and hypopharyngeal gland activity (Brouwers, 1982, 1983; Huang and Otis, 1989; Huang et al., 1989). Also, brood pheromone has previously been shown to increase hypopharyngeal gland protein content even in the absence of a pollen diet (Mohammedi et al., 1996). My results supported this hypothesis for hypopharyngeal glands from the queenless rearing environment in the absence of pollen (Fig. 1), as well as mandibular gland protein content in the presence of pollen (Fig. 5), and mandibular gland size in the presence of BP + pollen (Fig. 6).

In contrast with Mohammedi's (1996) results, hypopharyngeal gland protein content did not significantly increase in bees reared with BP + pollen when compared with bees on the pollen diet alone (Fig. 1). However, my study was shorter than that of Mohammedi (1996) and it is possible that greater differences could have been found in bees aged 14 and 25 d.

Mandibular gland protein content and size were not significantly different in BP and control environments (Figs. 4 and 6). Nurses rearing larvae in colonies regularly secrete the contents of their mandibular glands as part of brood food or royal jelly. If

bees are not able to expel glandular protein naturally, amount of extractable protein may reach an asymptote. A feedback mechanism may be functioning to inhibit additional protein biosynthesis when glandular protein content reaches threshold amounts. Crailsheim and Stolberg (1989) measured the acini size of hypopharyngeal glands to be significantly less in cage reared bees than bees reared in a colony.

I hypothesized that QMP would decrease protein content and size of mandibular glands and have no effect on hypopharyngeal glands. However, no differences were found between bees in the pheromone environments where pollen was not in the diet (Figs. 2, 5, and 7). Interestingly, when both QMP and BP were present in addition to pollen, hypopharyngeal gland protein content and mandibular gland size increased significantly as compared to pollen alone (Figs. 2 and 7). This suggests that both primer pheromones are necessary for the greatest amount of extractable protein and gland size.

In this experiment, where bees were reared in cages, hypopharyngeal gland extractable protein declined with age (Fig. 3). Hypopharyngeal glands follow a secretory cycle as bees age (Deseyn and Billen, 2005). It has been shown that bees reared in cages develop faster than those in the colony (Crailsheim and Stolberg, 1989; Lass and Crailsheim, 1996). Crailsheim et al., (1992) found that hypopharyngeal glands increased in acini diameter until day 9 in colonies while Haydak (1957) noted a degeneration in the glands occurring by age 15 d in colony reared bees. My observations are supported by the results of Crailsheim and Stolberg showing decline in hypopharyngeal gland acini diameter after day 3 in caged bees (Crailsheim and Stolberg, 1989).

Mandibular gland extractable protein and size also declined with age (Figs. 8 and 9). Crewe and Moritz (1989) studied *Apis mellifera intermissa* and Simon et al. (2001) studied *Apis mellifera capensis* both finding that, in general, fatty acid production of mandiblar glands increase as bees age. Vallet et al. (1991) found that as bees age mandibular gland increases occur in size and secretion of 2-heptanone per headspace sample. It is possible that as the amount of fatty acid within glands increases, the amount of protein content decreases. However, because gland size also decreased with age it is possible that caging bees has adverse effects on these glands.

Pankiw et al., (1998b) stated that the distribution method of brood pheromone is unknown and when applied to glass may last for a limited time, possibly only a few hours. Glass plates were replaced daily in our experiment in which bees were observed to lick the glass plates; however, this method of distributing the pheromone to the bees may not be sufficient to maintain gland state. It is possible bees require a physical stimulation such as the larvae cuticle. It is also possible that the synthetic blends and pollen diet are not sufficient to maintain the physiological state of the gland.

CHAPTER III

EFFECTS OF DILUTED POLLEN DIETS ON MANDIBULAR AND HYPOPHARYNGEAL GLAND PROTEIN CONTENT

Introduction

Pollen is the sole source of dietary protein as well as some lipids, vitamins, minerals and minimal amounts of carbohydrates (Herbert, 1992). A honey bee pollen forager collects pollen from the anthers of a plant, carries it on her corbiculae to the hive, and deposits the load of pollen in wax comb cells usually located near the brood (Winston, 1987). Pollen is further packed into the cell with the addition of a glandular secretion, thought to originate from both mandibular and hypopharyngeal glands, and is topped with a small cover of honey (Herbert, 1992; Winston, 1987). The glandular additions stop pollen grain germination over a two day period and begin the digestive processes .

Pollen is crucial for colonies as the sole source of protein for brood rearing. The pollen foraging effort of a colony must constantly be adapted to the requirements of the brood and, like foraging for nectar, must rapidly adapt to a changing foraging environment. Unlike honey, pollen is not hoarded in amounts up to 137 kg. A few days of inclement weather can significantly diminish the amount of stored pollen (Schmickl et al., 2003). Protein is consumed primarily by larvae through nurse bee food gland secretions and is therefore stored in cells near the brood rearing area in the center of the nest. The second greatest consumers of pollen are newly emerged adults followed by

nurse bees. There is a direct correlation between amount of stored pollen and amount of brood successfully reared (Allen and Jeffree, 1956).

Adult bees begin consuming pollen one to two hours after emergence. Mass consumption begins when workers are from 42 to 52 hrs old and reaches a maximum around day five (Haydak, 1935; Haydak, 1970). Protein consumption is necessary for complete development of muscles, and hypopharyngeal, mandibular, and wax glands (Herbert, 1992). On average, pollen consumption decreases at eight to ten days (Haydak, 1935; Haydak, 1970). Following development, adult bees rely on a carbohydrate rich diet for energy and metabolic processes (Haydak, 1970; Kunieda et al., 2006).

Nurse bees consume and digest stored pollen and then convert it into proteinaceous secretions (Brouwers, 1982; Crailsheim, 1990; Crailsheim et al., 1992). Crailsheim, (1998), found that brood food and royal jelly are distributed among adults. Queens similarly receive royal jelly. Latter instar larvae also receive pollen directly provisioned by nurses (Brouwers et al., 1987; Schmickl et al., 2003). Once bees have moved on to other tasks, they usually no longer perform nursing duties. However, if necessary, they can revert back to this protein-producing state by again consuming pollen (Ohashi et al., 2000).

A deficiency of protein leads to developmental failures (Jay, 1963). If colonies are prevented from increasing amounts of incoming pollen, then more stored pollen is consumed and amount of brood rearing is adjusted to match pollen stores (Fewell and Winston, 1992; Filmer, 1932; Free and Racey, 1968; Pankiw et al., 2004). Once pollen

stores are depleted, nurse bees convert their body reserves for brood food production (Haydak, 1970). Additionally nursing intensity is adjusted (Schmickl et al., 2003) and, finally cannibalism of young larvae may occur to sequester nutrients for young (Schmickl and Crailsheim, 2001).

In order to digest pollen, bees must break down 2 resistant walls of pollen grains to access the cytoplasmic nutrients. Nutrients found in pollen include protein, nitrogen, amino acids, starch, sterols, and lipids (Roulston and Cane, 2000). Ten amino acids necessary to ensure normal honey bee development include arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine (DeGroot, 1953; Herbert, 1992). Most pollens contain all common amino acids (Johri and Vasil, 1961); however, essential amino acids tryptophan and phenylalanine are sometimes lacking (Solberg and Remedios, 1980). Ways (Human and Nicolson, 2003) and extent of pollen digestibility is variable (Crailsheim et al., 1992). It has been estimated that a honey bee colony has a nitrogen assimilation efficiency of 77-83% ingested nitrogen (Schmidt and Buchmann, 1985).

In foraging assays, both Schmidt (1982) and Pernal and Currie (2001), found bees could not discriminate among pollen sources containing varying protein content. Pernal and Currie (2001) concluded that bees choose pollen based on the efficiency with which it can be collected. Peng and Jay (1976) performed a cage experiment comparing aged pollen with added nutrients to fresh pollen. Using a gland development scale of 1 to 4, they concluded that pollen quality can have a significant effect on hypopharyngeal gland development. The cage study of Pernal and Currie (2000) also led to a positive

correlation of hypopharyngeal gland protein content with calculated protein consumed. Pernal and Currie concluded that hypopharyngeal gland development is a good indication of pollen quality. Hypopharyngeal glands are used to convert pollen into proteinaceous secretions mostly provisioned directly to larvae, but also fed to adults. The quality of pollen may therefore influence overall rate of colony growth.

The objectives of this experiment were to measure the effect of diets containing varying amounts of pollen on worker bee mass, extractable lipids from adult bees, worker hypopharyngeal and mandibular gland extractable protein, and consumption of diet.

Methods

Pollen used was purchased from Glory Bee Foods (item #78-23063; Eugene, OR. USA), and α-cellulose from Sigma-Aldrich (C-8002, lot 111K0080; St. Louis, MO. USA). Newly emerged bees from 1 source colony were caged in one of 5 plexiglass/ wiremesh cages (15 cm x 11 cm x 8 cm). Bees were reared in cages for 7 days in an incubator maintained at hive conditions (32 °C, 50% RH). The experiment was replicated 4 times.

Daily, each cage of 250 bees was provisioned 30 ml of a 30% sucrose solution (wt:wt), 30 ml of distilled H_2O, and treatment diet. There were 5 treatment diets: (1) 1:1 pollen: cellulose (vol:vol), (2) 1:2 pollen: cellulose (vol:vol), (3) 1:3 pollen: cellulose (vol:vol), (4) pure cellulose, and (5) pure pollen. Cages received 5.0 ± 0.1 g of treatment diet per day. After each 24 h period, remaining sucrose and water volumes as well as

treatment diet were recorded. Subsamples of 20 bees per treatment were collected on days 3 and 7 for lipid and protein analysis.

Ten bees were used for lipid analysis. Bees were cold anesthetized and stored at -20 °C prior to desiccation. Bees were desiccated in 1.5 ml microcentrifuge tubes in a drying cabinet at 50 °C (LEEC model F1; Kitchener, ON, Canada). Next, carcasses were weighed to the nearest 0.1 mg. Individual carcasses were then moved to a 4 ml glass vial. Vials were filled with chloroform (no. 319988-4L; Sigma-Aldrich, St. Louis, MO. USA). Next, a glass marble (1 inch diameter) was placed on top of each vial to slow evaporation. A total of 3 24 h baths of chloroform were performed, with chloroform removed and replaced at the 24 h interval. Following the third bath, the marble and remaining chloroform were removed. Vials containing a single bee were desiccated as above and then weighed. The difference in dessication weight was an estimate of lipid weight (Behmer et al., 2002).

Ten bees were used for gland protein analysis. Bees were cold anesthetized and stored at -20 °C until dissected. For each dissection the bee was first decapitated. Next, the head was pinned to a wax mount and dissected under a Stereo Zoom Binocular microscope body (CO-SZ-600 on Boom Stand, Sciencescope, Chino, CA. USA), objective: 10x. Both hypopharyngeal glands were removed and placed into a 1.5 ml microcentrifuge tube containing Tris buffer (0.1 M Tris-HCl, pH 7.9). Both mandibular glands were removed. Glandular tissue was separated from the mandibles. The mandibular gland tissue was placed into a 1.5 ml microcentrifuge tube containing Tris

buffer, (0.1 M Tris-HCl, pH 7.9). Glands were stored at -20 °C prior to protein content measurement using the Bradford assay.

The Bradford assay methods followed Sagili et al. (2005) detailed in Ch 2 p. 20-21. The 500-0202 Quick Start Bradford Protein Assay Kit 2 was used (Bio-Rad Laboratories, CA, and U.S.A.) containing all necessary reagents and dyes. Absorbency values for each sample were measured at 595 nm against a blank reagent using a Milton Roy Spectronic UV/VIS model 1201 (Ivyland, PA. USA). A linear regression equation was calculated from each standard curve and samples were fitted to the equation to estimate micrograms of extractable protein. Protein quantity was statistically analyzed using analysis of variance (ANOVA) (Sokal and Rohlf, 1995; SPSS, 2007).

Results

There was no significant treatment effect on sucrose consumption (ANOVA $F_{4, 135} = 0.298$, $P = 0.879$). Sucrose consumption was significantly different between replicates (GLM, $F_{3, 136} = 7.188$, $P = 0.000$). However, there was no significant replicate by treatment interaction (GLM, $F_{12, 120} = 0.293$, $P = 0.990$). Therefore replicates were pooled for further analysis.

There was no significant treatment effect on water consumption (ANOVA, $F_{4, 135} = 1.525$, $P = 0.198$). Water consumption was significantly different between replicates (GLM, $F_{3, 12} = 14.168$, $P < 0.0001$); however there was no significant replicate by treatment interaction (GLM $F_{12, 120} = 0.549$, $P = 0.878$). For this reason, replicates were pooled for further analysis.

Overall, caged bees consumed significantly less pure pollen diet than any of the other treatment diets (ANOVA, $F_{1, 138}$=30.473, $P < 0.0001$; Fig. 10). There was a significant effect of replicate on pollen diet consumption (GLM, $F_{3, 12} = 9.833$, $P = 0.001$); however, there was no significant treatment by replicate interaction (GLM, $F_{12, 120} = 0.958$, $P = 0.493$). Therefore, replicates were pooled for further analysis. There were significant differences found between treatments (ANOVA, $F_{4, 135} = 8.363$, $P < 0.0001$; Fig. 10). Excluding the pure pollen diet, there was no significant difference in consumption (ANOVA, $F_{3, 108} = 0.954$, $P = 0.418$).

Overall, caged bees provisioned a pure pollen diet were significantly heavier than bees provisioned any other treatment diet (ANOVA, $F_{1, 398} = 95.705$, $P < 0.0001$; Fig. 11). There was a significant effect of replicate on bee mass (GLM, $F_{3, 12} = 12.422$, $P = 0.001$) and significant replicate by treatment interaction (GLM, $F_{12, 380} = 2.063$, $P = 0.019$). However, the treatment differences found were unidirectional for all replicates. For this reason, all replicates were pooled for further analysis. Significant differences occurred between the mean dry mass of bees provisioned different treatment diets (ANOVA, $F_{4, 395} = 36.865$, $P < 0.0001$; Fig. 11). Caged bees provisioned 1:1 pollen: cellulose diet were significantly heavier than bees provisioned treatment diets with a greater ratio of cellulose (ANOVA, $F_{1, 318} = 38.035$, $P < 0.0001$). Caged bees provisioned 1:2 pollen: cellulose diet were significantly heavier than bees provisioned a cellulose diet (ANOVA, $F_{1, 158} = 8.136$, $P = 0.005$). Caged bees provisioned 1:3 pollen: cellulose diet were not significantly different from caged bees provisioned 1:2 pollen:

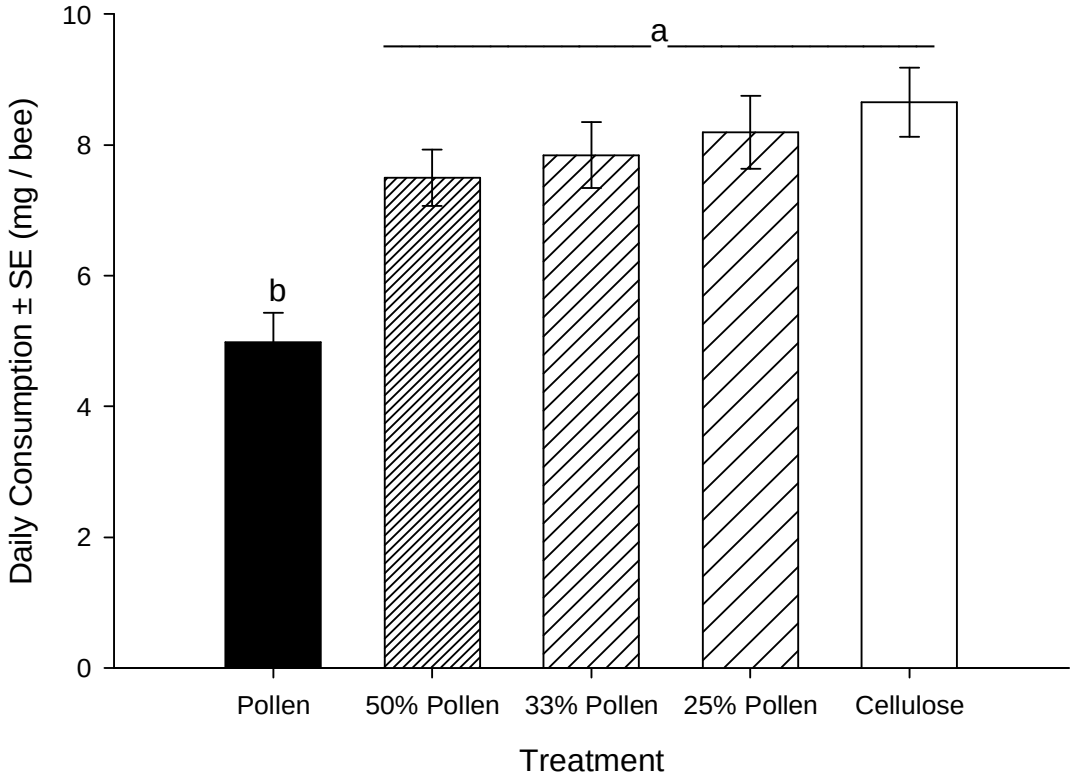

Figure 10. Mean daily consumption for each treatment. Letters indicate significant differences between treatments (ANOVA, $P < 0.05$).

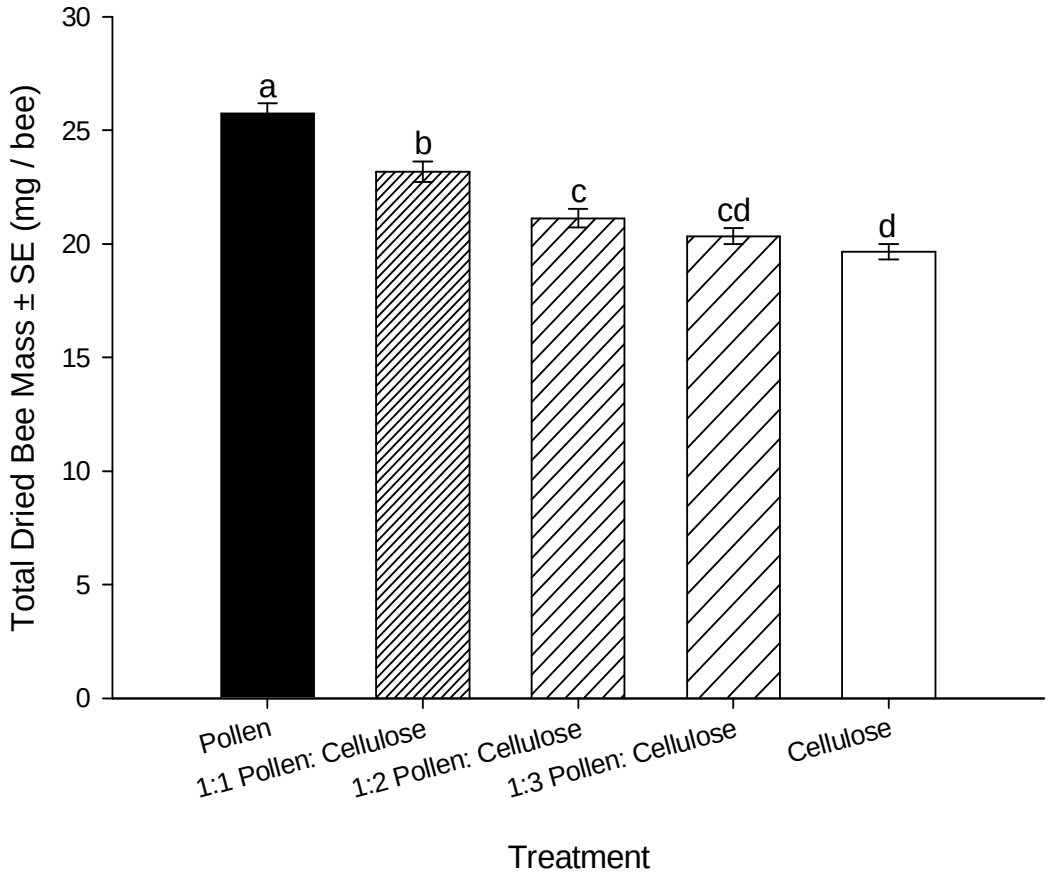

Figure 11. Mean dry bee mass of bees. Letters indicate significant differences between treatments (ANOVA, $P < 0.05$).

cellulose diet (ANOVA, $F_{1, 158} = 2.103$, $P = 0.149$) or from bees provisioned pure cellulose diet (ANOVA, $F_{1, 158} = 1.989$, $P = 0.160$).

Overall, caged bees provisioned pure pollen diet had significantly more lipids than bees provisioned any of the other treatment diets (ANOVA, $F_{1, 398} = 198.069$, $P < 0.0001$; Fig. 12). Significant differences occurred between replicates (GLM, $F_{3, 12} = 10.471$, $P = 0.001$) and significant interactions were found between replicates and treatments (GLM, $F_{12, 380} = 1.779$, $P = 0.0497$). However, the differences between treatments were unidirectional for all replicates. For this reason, all replicates were pooled for further analysis. Caged bees provisioned a 1:1 pollen: cellulose treatment diet had significantly greater lipids than treatment diets with a greater ratio of cellulose (ANOVA, $F_{1, 398} = 48.793$, $P < 0.0001$). Caged bees provisioned a 1:2 pollen: cellulose treatment diet did not have significantly greater lipids than caged bees given a 1:3 pollen: cellulose treatment diet (ANOVA, $F_{1, 158} = 3.792$, $P = 0.053$). Caged bees provisioned a 1:2 pollen: cellulose and a 1:3 pollen: cellulose treatment diet had significantly greater lipids than caged bees provisioned a pure cellulose treatment diet (ANOVA, $F_{2, 237} = 11.560$, $P < 0.0001$). Differences were found between treatments for the mean amount of bee lipids (ANOVA, $F_{4, 395} = 67.699$, $P < 0.0001$).

Overall, caged bees provisioned with a pure pollen diet had significantly more hypopharyngeal gland protein content than bees provisioned any of the other treatment diets (ANOVA, $F_{1, 397} = 128.141$, $P < 0.0001$; Fig. 13). No significant differences occurred between replicates for hypopharyngeal gland protein content (GLM, $F_{3, 12} =$

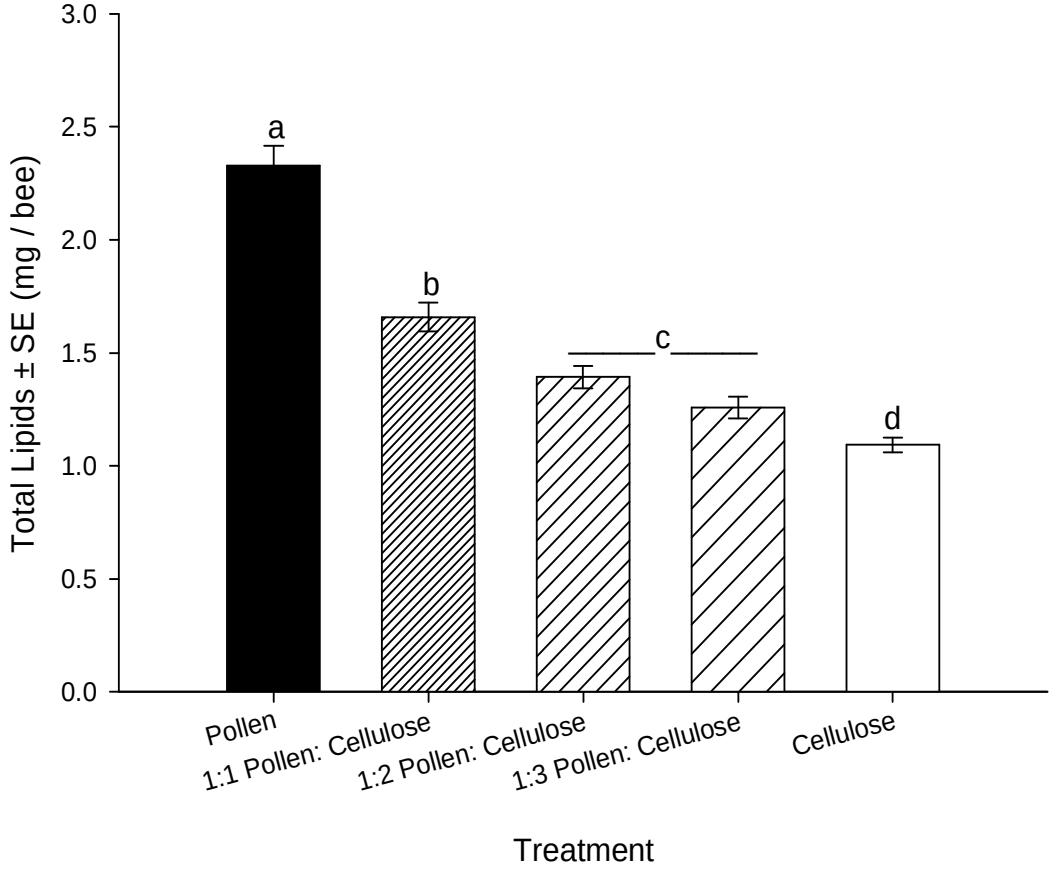

Figure 12. Mean total lipids per bee. Letters indicate significant differences between treatments (ANOVA, $P < 0.05$).

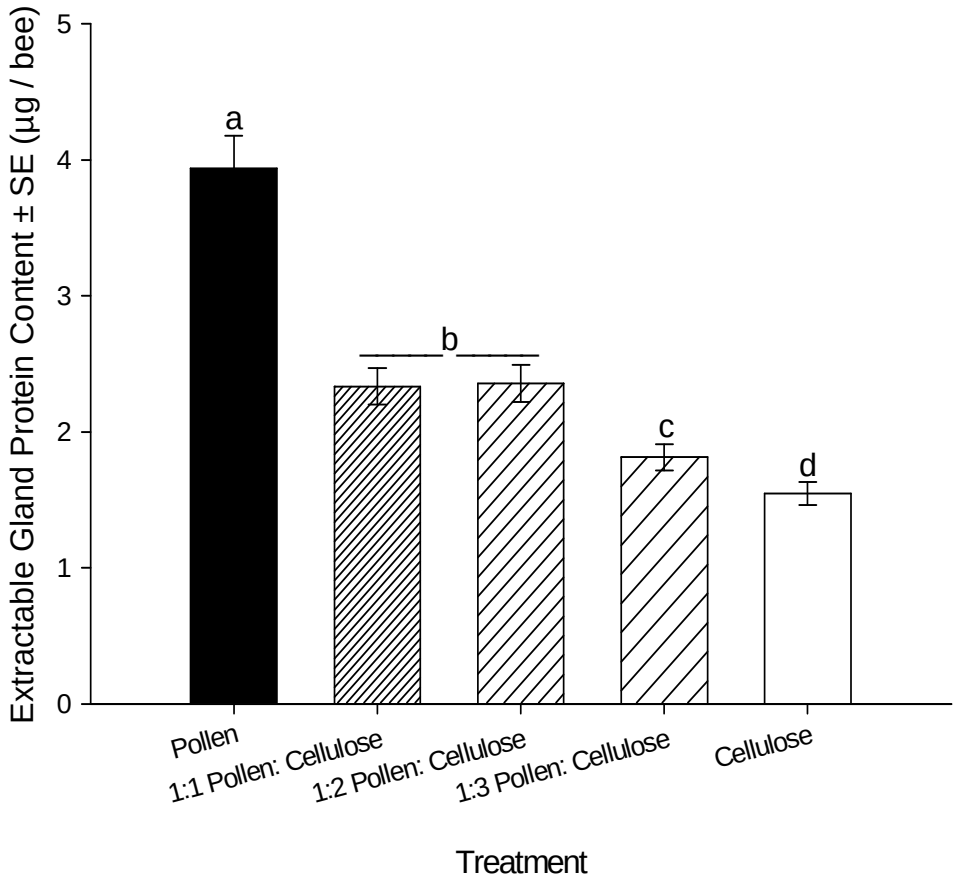

Figure 13. Mean bee hypopharyngeal gland extractable protein. Letters indicate significant differences between treatments (ANOVA, $P < 0.05$).

1.247, $P = 0.336$). Significant interactions were found between replicates and treatments (GLM, $F_{12, 379} = 5.616$, $P < 0.0001$). However, effect of treatment on protein extracted was unidirectional between replicates. Therefore, replicates were pooled for further analysis. Significant differences occurred between hypopharyngeal gland protein content for bees in all treatments (ANOVA, $F_{4, 394} = 38.979$, $P < 0.0001$; Fig. 13). Caged bees provisioned 1:1 pollen: cellulose and 1:2 pollen: cellulose treatment diets did not have significantly different extractable hypopharyngeal gland protein (ANOVA, $F_{1, 157} = 0.013$, $P = 0.909$; Fig. 13), but were both significantly greater than caged bees provisioned 1:3 pollen: cellulose treatment diet (ANOVA, $F_{2, 236} = 6.212$, $P = 0.002$; Fig. 13) and caged bees provisioned pure cellulose (ANOVA, $F_{2, 236} = 14.645$, $P < 0.0001$; Fig. 13). Caged bees provisioned 1:3 pollen: cellulose treatment diet had significantly greater extractable hypopharyngeal gland protein than caged bees provisioned pure cellulose (ANOVA, $F_{1, 158} = 4.447$, $P < 0.037$; Fig. 13).

Bees provisioned pure pollen had significantly greater extractable mandibular gland protein only from bees provisioned pure cellulose (ANOVA, $F_{1, 118} = 6.867$, $P = 0.010$; Fig. 14). Significant differences occurred between replicates (GLM, $F_{3, 12.371} = 78.642$, $P < 0.0001$), and significant interactions were found between treatments and replicates (GLM, $F_{12, 329} = 3.179$, $P < 0.0001$). However, overall, replicates showed unidirectional changes for each treatment. Therefore, replicates were pooled for further analysis. The total number of bees differed between treatments (pollen: n = 60; 1:1 pollen: cellulose: n = 80; 1:2 pollen: cellulose: n = 69; 1:3 pollen: cellulose: n = 80; and cellulose: n = 60). Caged bees provisioned a 1:1 pollen: cellulose treatment diet had

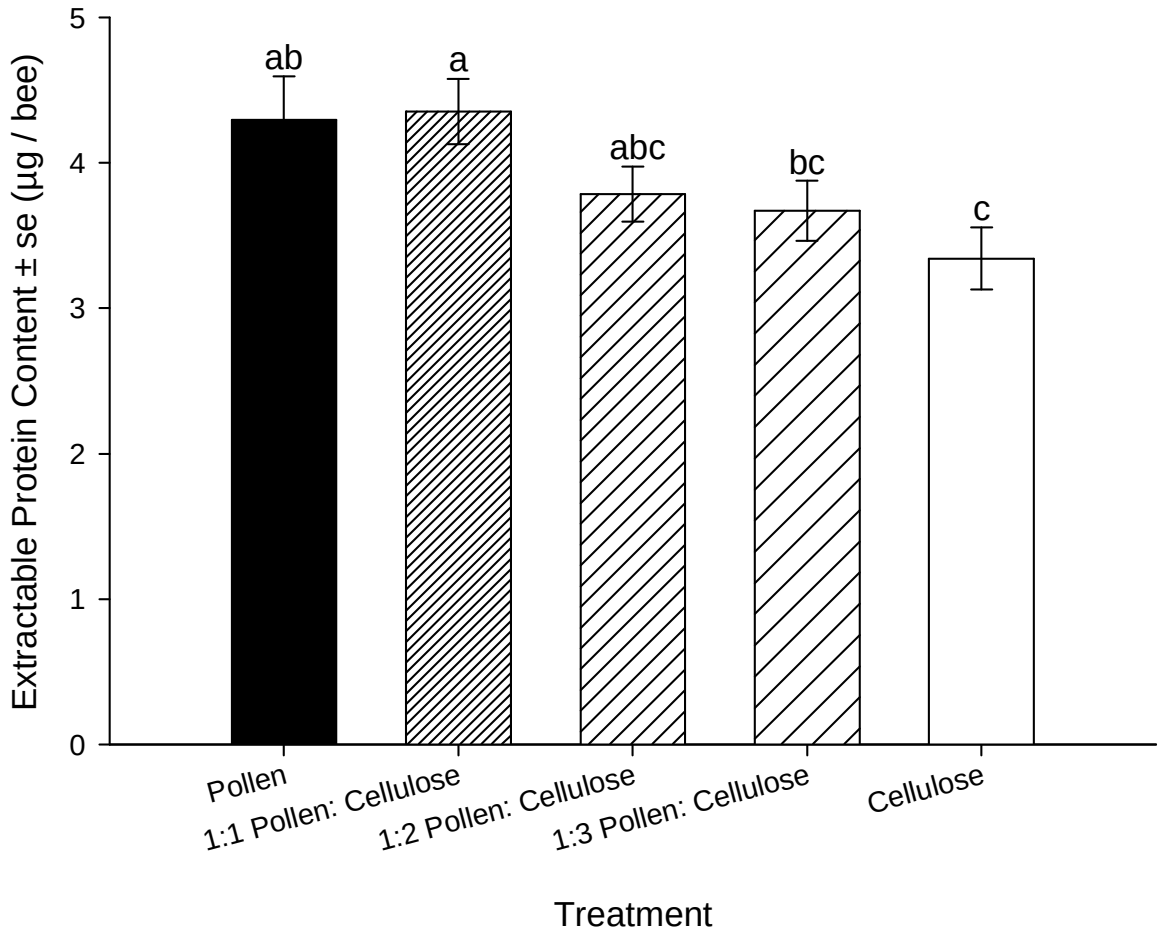

Figure 14. Mean bee mandibular gland extractable protein for each treatment. Total number of bees differed between treatments. Letters indicate significant differences between treatments (ANOVA, $P < 0.05$).

significantly greater extractable mandibular gland protein than caged bees provisioned a 1:3 pollen: cellulose treatment diet (ANOVA, $F_{1, 158} = 5.023$, $P = 0.026$) as well as greater extractable mandibular gland protein content from bees provisioned pure cellulose (ANOVA, $F_{1, 138} = 10.053$, $P = 0.002$).

Discussion

In this experiment I measured the effect of quality of pollen diet on worker bee mass, amount of extractable lipids from adult bees, amount of protein extractable from hypopharyngeal and mandibular glands, and consumption of diet. It is apparent that quality of pollen diet does affect the factors measured as significant differences due to dilution were found. In fact, there is a threshold of significance between pure pollen and 1:1 pollen: cellulose. Bees on the pollen diet were significantly different from bees on the 1:1 pollen: cellulose diet in every measure except mandibular gland protein content (Figs. 10 - 14). This included greater mean dry mass, greater total lipids, greater hypopharyngeal gland protein content, and less pollen consumption. From these results it is evident that significantly less consumption of the most concentrated diet is required to achieve the greatest adult bee mass, greatest adult bee extractable lipids, greatest extractable hypopharyngeal gland protein in comparison with diluted pollen diets.

Because quality of pollen diet affected diet consumption, it may be hypothesized that bees engage in compensatory consumption to meet nutritional needs. However, because bees were unable to achieve the same mean dry mass, total lipids, and hypopharyngeal gland protein content, diet consumption appears not to be compensatory

in nature. There is a significant threshold in each measured factor besides mandibular gland protein content between pollen diet and 1:1 pollen: cellulose, it may be possible that this dilution was too great to measure compensatory feeding mechanisms in bees. It may also be possible that bees do not engage in compensatory feeding at the colony level. Dussutour et al., (2007) found that caterpillars at the collective level chose between two food sources at random and became stuck at the chosen food source, despite nutritional balance, for 24 h due to trail following. Pernal and Currie (2001) found that in honey bees, no effect of stored pollen nitrogen quality could be found on the nitrogen quality of forager collected pollen.

It is surprising that mandibular gland protein content of bees on the pollen diet did not have the greatest extractable protein content and in fact only differed significantly from bees given pure cellulose (Fig. 14). It has previously been shown that pollen quality can have a significant effect on hypopharyngeal gland size (Peng and Jay, 1979) and protein content (Pernal and Currie, 2000). It is possible that because queens cannot be reared without worker mandibular glands (Peng and Jay, 1977), these glands are less affected by environmental circumstances than are the hypopharyngeal glands. The robustness of this gland would benefit *Apis mellifera* in the emergency rearing of queens.

CHAPTER IV

CONCLUSIONS

The thesis presented novel data on the effects of honey bee primer pheromones on worker mandibular gland food producing ability as well as data verifying the necessity of pollen for glandular protein content. The results in the preceding chapters accessed the extractable protein content of mandibular and hypopharyngeal glands in the presence of two primer pheromones, namely QMP and BP.

In the first experiment, it was shown that pollen was the main factor contributing to significant increases in extractable gland protein content and size strongly suggesting the necessity of pollen for the greatest amount of extractable protein content and gland size. BP increased hypopharyngeal glands when pollen was absent; increased mandibular gland protein content in treatments with pollen; and mandibular gland size in the queenless environment when combined with pollen indicating that the effects of BP are more complex than those of pollen. Future studies should include quantification of BP effects on gland activity as well as comparison with colonies containing live brood.

No differences were found between bees in the QMP and non-QMP environments in the absence of pollen. It can be concluded that the synthetic blend of QMP failed to regulate both food producing glands, including the mandibular gland, a key gland in royal jelly production. Most noteworthy was the significant increase of hypopharyngeal gland protein content and mandibular gland size of bees on treatment QMP + BP + pollen.

These results suggest that both primer pheromones are necessary for the greatest amount of extractable protein and gland size.

In this experiment, it was also noted that hypopharyngeal and mandibular gland extractable protein declined with age. This decline in hypopharyngeal glands conforms with previous cage studies and is indicative of an increased speed of bee development within the cages (Crailsheim and Stolberg, 1989; Lass and Crailsheim, 1996). It is possible that a physical stimulation such as the larvae cuticle is required to maintain gland protein content. It is also possible that the synthetic blends and pollen diet are not sufficient to maintain gland protein content.

The second experiment measured effects of varying pollen dilutions on hypopharyngeal and mandibular gland protein content, as well as bee mass and lipid content. In this experiment, bees on the pollen diet were significantly greater than bees on all other diluted diets in measurements of hypopharyngeal gland protein content, lipid content, and mass. Bees on the pollen diet also consumed significantly less diet than bees on all other diets. Consumption differences were not compensatory to meet nutritional needs. This conclusion derived from the inability of bees on diluted or pure cellulose diets to achieve the same adult bee mass, extractable lipids, and extractable gland protein as those of bees given pure pollen.

Bees on the pollen diet had a mandibular gland protein content significantly greater only from pure cellulose. It is possible that due to the mandibular gland's significance in royal jelly production (Lensky and Rakover, 1983; Peng and Jay, 1977, 1979) that this gland may be more robust that than of the hypopharyngeal gland. From

this experiment it was concluded that the most concentrated diet promoted the greatest worker bee mass, extractable lipids, and hypopharyngeal gland extractable protein content.

Overall, the results of these two experiments strongly suggest that for hypopharyngeal and mandibular glands to reach maximum extractable protein, a concentrated pollen diet combined with both QMP and BP should be utilized.

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EFFECT OF POLLEN DIET AND HONEY BEE (*Apis mellifera* L.) PRIMER PHEROMONES ON WORKER BEE FOOD PRODUCING GLANDS

A Thesis

by

LIZETTE ALICE PETERS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2008

Major Subject: Entomology

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ABSTRACT

Effect of Pollen Diet and Honey Bee (*Apis mellifera* L.) Primer Pheromones on Worker Bee Food Producing Glands. (December 2008)

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Chair of Advisory Committee: Dr. Tanya Pankiw

This thesis examines three factors that may influence the change in protein content and size of the brood food glands in honey bees. Effects on the mandibular gland, involved in the production of brood food and in royal jelly, have not been examined in relation to primer pheromones while effects on the hypopharyngeal glands, also involved in the production of brood food, have not been examined in relation to queen mandibular pheromone. This thesis provides preliminary insight into how these pheromones affect the extractable protein content of brood food glands.

The first study in this thesis assessed the effects of brood pheromone (BP), queen mandibular pheromone (QMP), and pollen presence on the protein content of hypopharyngeal and mandibular glands of the honey bee. In this study, newly emerged bees were caged for 12 days in one of eight treatments: Queenless state: 1) control (no pollen + no pheromone), 2) pollen, 3) BP, 4) BP + pollen; Queenright state: 1) QMP, 2) QMP + pollen, 3) BP + QMP, 4) BP + QMP + pollen. This study indicated that regardless of pheromone treatment, the most influential factor on gland protein content and size was pollen.

The second experiment examined effects of varying pollen dilution on hypopharyngeal and mandibular gland protein content, bee mass, and lipid content of the honey bee. In this experiment, newly emerged bees were caged for 7 days and fed one of five treatments: pollen, 1:1 pollen: cellulose (vol:vol), 1:2 pollen: cellulose (vol:vol); 1:3 pollen: cellulose (vol:vol), and cellulose. This study indicated that bees on the pollen diet were significantly greater than all other diluted diets in measurements of hypopharyngeal gland protein content, lipid content, and mass with significantly less consumption. However, mandibular gland protein content of bees on the pollen diet was significantly greater only from pure cellulose.

DEDICATION

I dedicate this thesis to my father who steadfastly supported and encouraged my education. It is by his constant recounting of his enjoyable experiences that I strived for a degree at Texas A&M University, by chance or fate, within the same building he achieved his doctorate.

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CHAPTER I

INTRODUCTION

This thesis is divided into four chapters. Chapter I is an introduction to honey bee colony division of labor, brood rearing, pheromones, and lists the objectives. Chapter II reports a study on effects of QMP, BP and pollen on hypopharyngeal and mandibular gland protein content, mandibular gland size, and pollen consumption. Chapter III describes a study on pollen diet dilution effects on mandibular and hypopharyngeal gland protein content. Chapter IV is an overall summary and conclusions pertaining to each chapter.

The honey bee is a eusocial insect, a term used to designate the highest level of social organization where there is reproductive division of labor, overlapping generations, cooperative care of young, and irreversible castes (Crespi and Yanega, 1995; Wilson, 1976). Reproductive division of labor in the honey bee is such that there is only one queen heading the colony as the only mated female and principal egg layer. Honey bee queens mate once in a lifetime usually in their second week of adult life with 5 to 20 or more males (Tarpy and Neilsen, 2003; Tarpy and Page, 2000). As a consequence of polyandry worker relatedness within colonies ranges from 0.25 to 0.75 (Page and Erber, 2002). There may be from 0 to 1000's of males living in the colony depending on time of year. Males called drones are haploid developing from

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This thesis follows the style of the Journal of Insect Physiology.

unfertilized eggs. The reproductive castes do no work and are solely engaged in individual reproduction. All the work associated with colony survival, growth, brood care, and colony-level reproduction is performed by the worker caste comprised of overlapping generations of 1000's of semi-sterile diploid females capable of laying unfertilized eggs but not of mating.

A primary characteristic of eusocial life is an age-related division of labor. The temporal patterning of behavior is known as temporal, or age, polyethism (Hölldobler and Wilson, 1990; Jeanne, 1991; Robinson et al., 1992) and is expressed as apparent changes in probabilities that workers perform different behavioral tasks. In general, as bees age they make transitions from performing tasks in the center of the nest to performing tasks at the periphery, and finally they leave the nest to forage. Centrally located tasks include cell cleaning and tending to the needs of the brood and the queen. Medial tasks include comb building and food processing and storage. Peripheral tasks include receiving nectar from foragers, removing dead bodies, constructing comb, and guarding the colony entrance (Seeley, 1995; Winston, 1987). Progression from working in the nest to foraging marks a major transition in a worker honey bee's life. When workers are in about their third week of life they cease performing tasks within the nest and begin foraging outside for pollen, nectar, water, and propolis (a resinous material collected from plants used in nest construction). Once workers begin foraging, they seldom revert to perform within-nest tasks.

The timing of these behavioral transitions is not fixed; workers do not perform all possible tasks or necessarily develop into foragers. Factors such as genotype, the

demographic structure of the immature and adult worker population, and pheromones that communicate demographic structure affect behavioral development trajectories. For example, in the absence of older bees, worker bees will initiate foraging behavior at younger ages. In the absence of young bees, old bees may revert to performing within-nest tasks like feeding larvae (Huang and Robinson, 1996). Pheromones extracted from the surface of young non-foraging and foraging workers exert similar effects on foraging ontogeny suggesting that bees use pheromones to estimate amount of young and old bees and adjust their development accordingly (Leoncini et al., 2004; Pankiw, 2004c).

Honey bee queen mandibular pheromone also exerts similar effects; colonies given supplemental doses of synthetic pheromone show delayed onset of foraging relative to non-supplemented control colonies (Pankiw et al., 1998a). This is in addition to other effects of queen mandibular pheromone as a sex attractant, a releaser of retinue behavior and an inhibitor of queen-rearing behavior (Winston and Slessor, 1992). Nurse bees come into contact with the queen most frequently leading Pankiw et al. (1998a) to hypothesize that exposure to queen mandibular pheromone can extend the duration of the nursing phase to ensure more efficient brood rearing. The addition of larvae or their pheromones, called brood pheromone, to colonies also changes rate of behavioral development that is dose-dependent (Le Conte et al., 2001; Pankiw, 2004b). Additions of relatively small amounts of brood pheromone accelerate foraging ontogeny (Le Conte et al., 2001; Pankiw, 2004b; Pankiw et al., 2004; Sagili, 2007). Conversely, additions of relatively large amounts of brood pheromone delays foraging ontogeny and thus extends the duration a worker performs nursing duties (Le Conte et al., 2001; Pankiw, 2004b).

The principal function of nurses is to progressively provision larvae food produced from two glands found in the head, namely the hypopharyngeal and mandibular glands. Nurse bees feed on stored pollen as their sole source of protein and on recently collected nectar or stored honey as their sources of carbohydrate. The glands produce proteinaceous secretions deposited in a pool surrounding each larva. The ratio of hypopharyngeal to mandibular gland secretion deposited is varied depending on larva age, sex, and caste. Female larvae chosen to be reared as queens are mass provisioned nearly 100% mandibular gland secretion during the first 3 days of larval life followed by a 1:1 ratio of mandibular to hypopharyngeal gland secretion over the final 2 days as a larva (Beetsma, 1979; Brouwers et al., 1987). The mixture fed to queen larvae is commonly called "royal jelly" (Winston, 1987). Proteins belonging to the major royal jelly protein family constitute 90% of total royal jelly proteins (Santos et al., 2005; Scarselli et al., 2005). Worker larvae are progressively provisioned "brood food" in a 2:9:3 ratio of mandibular to hypopharyngeal gland secretions to pure pollen (Beetsma, 1979). Fourth and fifth instar worker larvae are given some honey inducing a phagostimulatory response, as well as pollen to accommodate the rapid rate of growth in these latter instars (Brouwers et al., 1987). Male larvae are provisioned food of lower protein quality than that provisioned to workers but of greater quantity due to their larger size and longer time of larval development (Brouwers et al., 1987; Winston, 1987).

As workers age and transition from performing nursing tasks to tasks found in more peripheral regions of the nest, hypopharyngeal and mandibular gland activity and function may also change. For example, the hypopharyngeal glands in young nurse bees

are large and well developed producing primarily proteins. The function then switches in middle-aged bees engaged in food processing to produce α-glucosidase used to hydrolyze the sucrose of nectar into glucose and fructose (Deseyn and Billen, 2005; Kubo et al., 1996; Ohashi et al., 1999). Finally, the gland atrophies in foragers (Robinson, 1987; Sasagawa et al., 1989). The mandibular gland also changes with worker age related behaviors; however, the range of change is greater and more complex than what is currently known of the hypopharyngeal gland.

Like the hypopharyngeal gland, the mandibular gland has food producing activity among nurse bees in a colony with a queen (queenright) laying fertilized eggs that develop into worker bees. However as the worker ages in a queenright colony, the gland permanently switches to alarm pheromone production, principally 2-heptanone (Kerr et al., 1974). In a queenless colony or one in which the queen is no longer laying fertilized eggs, reproductive division of labor lines begin to blur and a form of social anarchy ensues where some workers grow well-developed ovaries and become egg layers (Oldroyd and Ratnieks, 2000). The mandibular glands of egg laying workers become queen-like, even producing queen-like mandibular gland pheromone, a blend of fatty acids and some aromatic compounds (Plettner et al., 1993). Two factors are associated with the loss of social cohesion in the honey bee, 1) the loss of the queen and, 2) the loss of diploid larvae which are communicated to the colony through queen mandibular gland (QMP) and brood (BP) primer pheromones, respectively. Primer pheromones produced by the queen and larvae affect worker bee endocrine, physiological and neurobiological systems (Pankiw, 2004b). Primers exert changes gradually and changes are permanent

even after the pheromone is no longer detectable and absent (Hölldobler and Wilson, 1990; Pankiw, 2004b).

The regulation of reproduction and cooperative brood care are critically important to eusocial species survival. As a consequence, pheromone mediated reproduction and cooperation systems regulating the worker caste have evolved. The first chemically characterized social insect primer pheromone is queen mandibular pheromone (Slessor et al., 1988). QMP induces workers to feed and groom the queen called retinue behavior, a releaser response (Slessor et al., 1988). QMP has a wide range of primer effects including the inhibition of queen rearing, regulation and timing of colony-level reproduction (swarming), partial inhibition of worker ovariole development, regulation of comb-building, regulation of foraging ontogeny, and modulation of worker brain dopamine function (Beggs et al., 2007; Hoover et al., 2003; Ledoux et al., 2004; Melathopoulos et al., 1996; Pankiw and Garza, 2007; Pankiw et al., 1998a; Pettis et al., 1995; Winston et al., 1991; Winston et al., 1990). The focal primer effect of QMP in this study is the regulation of worker mandibular gland size and amount of extractable protein because it is a key gland used for the production of royal jelly in queen rearing.

For social insect colonies, colony-level reproduction is the principal sources of fitness. As such, much of individual worker and colony behaviors are ultimately related to colony reproduction. Honey bee colonies reproduce through a process of colony budding, commonly referred to as swarming. In general, swarming is such that about half of the adult workers leave the parental nest with the old mother queen to initiate a

new nest elsewhere. Inheriting the parental nest is a new young daughter queen and the remaining workers to begin the colony life cycle anew. Colonies begin to rear queens approximately 10 days prior to swarming. That is, natural swarming does not occur without first initiating the process of queen rearing. Intra-colony factors that inhibited queen rearing are released and new queens are reared in the presence of the old queen (Winston and Slessor, 1992). One important queen rearing inhibitor is queen mandibular pheromone (Melathopoulos et al., 1996; Pettis et al., 1997; Winston et al., 1991; Winston et al., 1990).

In general, QMP communicates queen presence to the colony. One queen equivalent (QEq) of QMP extractable from the paired mandibular glands of a mated, egg laying queen is approximately, 200 µg 9-keto2-(E)-decenoic acid (9-ODA), 100 µg 9-hydroxy-2(E)-decenoic acid [88%R-(-) and 12% S-(+)] (9-HDA), 20 µg methyl p-hydroxybenzoate (HOB), and 2 µg 4-hydroxy-3-methoxyphenylethanol (HVA) (Pankiw et al., 1996; Slessor et al., 1988). While some honey bee pheromones are transmitted by diffusion through the air, many are non-volatile, and are transmitted by contacts between bees. Queen mandibular pheromone is considered non-volatile having a very short volatile space of approximately 12 mm, thus transmission throughout the nest is principally through a series of contacts between the queen and retinue bees, and retinue bees with other bees (Naumann, 1991; Naumann et al., 1993; Naumann et al., 1992; Naumann et al., 1991). Retinue response is characterized as a dynamic group of workers surrounding the queen or source of QMP, frequently antennating, licking, grooming and sometimes feeding (Kaminski et al., 1990; Naumann, 1991; Pankiw et al., 1994). QMP

is dispersed throughout the nest in part by the movement of the queen, who remains in the brood nest area, and through serial worker-to-worker transmissions (Naumann, 1991; Naumann et al., 1993; Naumann et al., 1992). As colonies grow in worker number, the amount of QMP reaching individuals decreases due to a simple dilution effect and due to colony crowding that obstructs transmission (Watmough et al., 1998). Once QMP reaching individuals drops below detectable levels, estimated to be from 10^{-5} to 10^{-7} QEq (Slessor et al., 1988), queen rearing may be initiated.

Female caste development in the honey bee is triggered solely by the diet of the larvae. Larvae fed royal jelly throughout their larval life become queens; those that are not become workers. Proteinaceous secretions of the nurse bee mandibular gland are a major component of royal jelly. While the role of QMP is well understood for the initiation of queen rearing and the regulation and timing of colony-level reproduction, effects of QMP on the mandibular gland of workers are not understood, even at the most rudimentary level. One objective of this thesis is to compare the effect of QMP and non-pheromone rearing environments on worker mandibular gland size and extractable protein.

Colony-level reproduction is not the only context in which queens are reared. Queens are reared to replace dead, injured, old, or sick queens (Winston, 1987). Emergency queen rearing takes place when a queen is killed or removed consequently placing the colony in a sudden ‘queenless’ state (Winston, 1987). Within about 20 min after a queen is removed from a colony or killed, workers become agitated, and about 2 hrs later queen rearing is initiated (Seeley, 1985). The loss of a queen triggers

emergency queen rearing because colonies have approximately 6 days to choose larvae from among the remaining larvae that are 3 days old or younger to rear queens (Pettis et al., 1997; Winston, 1987; Winston and Slessor, 1992). If colonies do not successfully rear a queen to replace the dead queen, it is placed in a "hopelessly queenless" state and the colony eventually dies. In addition to QMP, the communication of larva sex, age, and caste of larvae clearly play a critical role in the regulation of nurse bee food producing glands.

Pettis et al. (1997) demonstrated that the presence of young larvae (1-3 d) may play a role in the regulation of queen rearing. The first of three experiments was designed to examine the effects of synthetic queen mandibular gland pheromone and the addition of young larvae plus QMP on the number of queens reared and time to queen rearing in queenless colonies (emergency queen rearing). The number of queens reared and timing were significantly negatively correlated with amount of young larvae added to colonies (Pettis et al., 1997). In this experiment addition of QMP confounded the effects of larvae on queen rearing, however results suggested larvae play a role in regulating queen rearing. In a second experiment queenless colonies received either young larvae (1-3 d) or old larvae (3-5 d) over a 3 day period (Pettis et al., 1997). In colonies treated with young larvae, significantly fewer queens were initiated, and significantly fewer queen cells were prepared. Only in colonies containing young larvae was no queen rearing observed in the first 24 hr period of the experiment (Pettis et al., 1997). In a third experiment colonies with queens were manipulated such that 1) young larvae were removed and thus contained mostly old larvae, 2) colonies contained mostly

young larvae, and 3) controls with no larva manipulation. Adult population growth was controlled by allowing equal amounts of pupae to emerge in colonies. Colonies were measured for amount of queen rearing over a 14 day period. Colonies containing mostly old larvae reared significantly more queen cells in a significantly shorter period of time compared to colonies with mostly young larvae and controls (Pettis et al., 1997). Combined, these experiments strongly suggest that larvae play a key role in regulating the timing and amount of queen rearing. Pettis et al. (1997) concluded that colonies perceive the presence of young larvae as a queen fecundity cue that feeds back on worker queen rearing behaviors, even in queenless colonies.

The fatty acid esters extractable from the surface of larvae induce the greatest number of known primer pheromone responses in honey bees (Pankiw, 2004b). The ten fatty acid esters of honey bee larvae that have been reported as pheromonal are methyl and ethyl esters of linoleate, linolenate, oleate, palmitate, and stearate of male or drone larvae (LeConte et al., 1990), as well as queens and workers (Trouiller, 1993; Trouiller et al., 1994; Trouiller et al., 1991). Total amount of esters are reported to change with larval instar for all castes. In general, total amount of detectable esters increase with age (Le Conte et al., 1994; Trouiller et al., 1994; Trouiller et al., 1991). Although weight and surface area measures have not been considered, increased size with age is likely in part due to increased total amount of esters. Proportion of ethyl to methyl esters also changes with age. "Young" larvae (estimated as 3rd to 4th instars) secrete about 64% ethyl esters (ratio of 1.7 ethyl to methyl esters) and "old" larvae (estimated as 5th instar to prepupa), about 69% methyl esters (ethyl: methyl ratio of 0.4; Le Conte et al. (1994)).

Mature drone larvae have greater proportions of methyl and ethyl palmitate than queens or workers. Workers have greater proportions of methyl stearate and linolenate and, queens have greater proportions of methyl and ethyl oleate (Trouiller et al., 1994). For all sexes and castes, total extractable esters peak for several hours prior to and after pupation, triggering cell capping by adults (Le Conte et al., 1994; Trouiller et al., 1994; Trouiller et al., 1991). Larval esters regulate worker hypopharyngeal gland development and protein biosynthesis of nurse bees in cages (Mohammedi et al., 1996) as well as in colonies (Pankiw et al., 2004). The 10-component blend is as follows; 1% ethyl linoleate, 13% ethyl linolenate, 8% ethyl oleate, 3% ethyl palmitate, 7% ethyl sterarate, 2% methyl linoleate, 21% methyl linolenate, 25% methyl oleate, 3% methyl palmitate and, 17% methyl stearate. Brood pheromone acts as a releaser of multiple individual forager behaviors and primer on foraging behavior development (Le Conte et al., 2001; Pankiw, 2004a, b; Pankiw and Page, 2001a; Pankiw et al., 1998b; Pankiw et al., 2004; Pankiw and Rubink, 2002). Nurse bees, aged from around 7 to14 days consume pollen and convert it into proteinaceous secretions provisioned to larvae. In this way larvae consume pollen via nurse bees. Larval cues and pollen are necessary for hypopharyngeal gland development, activity and, protein production (Brouwers, 1982, 1983; Hrassnigg and Crailsheim, 1998; Huang and Otis, 1989; Huang et al., 1989; Mohammedi et al., 1996). Larvae or their esters stimulate hypopharyngeal gland development even in the absence of a pollen diet (Mohammedi et al., 1996). However a protein source is necessary for glandular protein biosynthesis resulting in greater amounts of extractable protein (Brouwers, 1983; Huang and Otis, 1989; Huang et al.,

1989; Mohammedi et al., 1996). Larvae and larval esters have clearly been demonstrated to prime hypopharyngeal gland development and, pollen provides the protein source fueling brood-food production. Addition of larval esters to average colonies also increases amount of protein extractable from hypopharyngeal glands even in the winter when few to no larvae are being reared in colonies (Pankiw et al., 2004; Pankiw et al., 2008).

This review reveals that changes in amounts and proportions of larval esters (ester profile) can result in changes in adult behavioral responses. Despite what seems like a wealth of information on honey bee larval esters, important primer effects on nurse bee mandibular glands are unknown. Additionally, a more integrative approach has not been previously examined measuring the effects of QMP + BP on worker food producing glands. The objectives of this thesis were to 1) analyze effects of QMP, BP, and pollen on hypopharyngeal and mandibular gland protein content, mandibular gland size, and pollen consumption; and 2) evaluate the effects of pollen dilutions on honey bee hypopharyngeal and mandibular gland extractable protein.

CHAPTER II

EFFECTS OF PRIMER PHEROMONES AND POLLEN ON BROOD FOOD GLANDS

Introduction

Primer pheromones induce long term effects on endocrine, reproductive, and neurobiological systems of worker bees ultimately affecting individual bee behavior (Pankiw, 2004c; Pankiw and Page, 2003). Two primers: queen mandibular pheromone (QMP), produced by the mandibular glands of mated, egg laying queens (Pankiw et al., 1996; Slessor et al., 1988); and brood pheromone (BP), extractable from the cuticle of larva (Le Conte et al., 1989; Mohammedi et al., 1996; Trouiller et al., 1991), were used with a pollen diet to measure effects on amount of extractable protein on brood food producing glands and size of the mandibular glands.

QMP aids in colony organization through both its releaser and primer effects. Primer effects of QMP include partial inhibition of worker ovary development (Hoover et al., 2003; Lin and Winston, 1998; Willis et al., 1990), delayed foraging onset (Pankiw et al., 1998a), and inhibition of queen rearing (Melathopoulos et al., 1996; Pettis et al., 1995; Winston et al., 1991; Winston et al., 1990). Colonies with a queen are termed queenright, those without are termed queenless. Workers in a queenless colony (Melathopoulos et al., 1996) or in a highly congested queenright colony (Watmough et al., 1998) no longer inhibited by QMP may initiate queen rearing. Both hypopharyngeal and mandibular

glands produce necessary components of royal jelly fed to queen larvae (Lensky and Rakover, 1983).

Royal jelly is comprised of carbohydrates (11.9 ± 0.7), lipids (6.1 ± 0.4), proteins (12.7 ± 0.8), and moisture content (68.3 ± 1.4) (Takenaka and Takenaka, 1996). The proteins comprising royal jelly are not entirely known (Schonleben et al., 2007). What is known is that a series of major royal jelly proteins or MRJPs of which there are five main members comprise 82-90% of royal jelly proteins . These main proteins are identical to those found in worker jelly fed to worker larvae (Schmitzova et al., 1998). Most of the proteins are synthesized by the hypopharyngeal glands and secreted in royal jelly (Hanes and Simuth, 1992; Kubo et al., 1996; Santos et al., 2005). Mandibular glands also produce some proteins present within royal jelly (Lensky and Rakover, 1983) as well as royal jelly acid or 10-hydroxy-dec-2-enoic acid (Barbier, 1981).

Because nurse bees without mandibular glands are unable to rear queens (Peng and Jay, 1977, 1979), it is likely that treatments without QMP will have greater amounts of extractable protein from mandibular glands as well as a potentially larger gland. If the reverse is found, then QMP will have failed to suppress a key gland used to produce royal jelly. Because hypopharyngeal glands produce the majority of worker brood food protein content (Brouwers, 1982; Lensky and Rakover, 1983), it was expected that QMP would not regulate the extractable protein from this gland. However, because royal jelly is a 1:1 mandibular to hypopharyngeal gland secretion, then it is possible that treatments without QMP would have greater amounts of extractable hypopharyngeal glands. Regardless of

the outcome, the findings of this study will be novel: there is currently no data on primer pheromone effects on worker mandibular gland food producing ability.

Brood pheromone (BP) induces effects that stimulate brood care. BP aids QMP by inducing partial inhibition of worker ovariole development (Arnold et al., 1994; Mohammedi et al., 1998; Pankiw and Garza, 2007). There is a direct correlation in the amount of stored pollen and the amount of brood successfully reared (Allen and Jeffree, 1956). BP has been shown to increase colony number of pollen foragers (Pankiw, 2004a, c; Pankiw and Garza, 2007; Pankiw and Page, 2001b; Pankiw et al., 1998b; Pankiw et al., 2004; Schulz et al., 2002), increase the forager returning load weights of pollen (Pankiw, 2004a), and increase the number of trips an individual pollen forager takes per unit time (Pankiw, 2007). All of these effects help ensure that the brood are tended to properly. It is therefore not surprising that brood stimulate hypopharyngeal gland development even in the absence of a pollen diet (Mohammedi et al., 1996) resulting in an increased amount of extractable hypopharyngeal glands (Pankiw et al., 2004). The mandibular glands aid in brood food production (Barker et al., 1959; Lensky and Rakover, 1983); therefore, it is likely that brood pheromone will increase mandibular gland size and protein content.

Protein is necessary for development of larvae. Adult worker bee hypopharyngeal glands produce the protein component of brood food (Patel et al., 1960). The rate of protein synthesis peaks at 8-16 days of age (Knecht and Kaatz, 1990). A protein source is necessary for glandular protein biosynthesis resulting in greater amounts of extractable protein (Brouwers, 1983; Huang and Otis, 1989; Huang et al.,

1989; Mohammedi et al., 1996). However, adult honey bees will temporarily utilize proteins from their own bodies to feed brood if pollen is not available (Haydak, 1970). Poor gland development and a shorter length of life can result from insufficient pollen consumption early in adult life (Haydak, 1970; Maurizio, 1950); therefore, I hypothesized that pollen would increase the amount of extractable protein content of both the mandibular and hypopharyngeal glands.

In this study, I measured effects of QMP, BP, and pollen on amount of extractable protein from hypopharyngeal and mandibular glands as well as mandibular gland size. Pollen diet consumption was recorded. This study is the first to measure the effects of a synthetic pheromone environment on mandibular gland protein content and to measure the effects of synthetic QMP on hypopharyngeal glands of honey bees.

Methods

Combs containing pupae about to emerge as adult bees were placed inside an incubator (32°C, 50% RH) for 24 h. Three hundred (300) newly emerged bees from multiple colony sources were placed in plexiglass/wiremesh cages (15 cm x 11 cm x 8 cm). Bees were reared for 12 days in an incubator maintained at hive conditions (32°C, 50% RH) with one of the following pheromone treatments: control, QMP, BP, or QMP + BP. Each pheromone treatment was replicated with and without pollen diet resulting in a total of 8 treatments (see Table 1). Due to logistical constraints and availability of newly emerged bees, only 4 cages were reared in the incubator at a time. Treatments were replicated 3 times. The third replicate contained 50 additional older bees collected

directly from the brood nest area to compensate for mortality in this replicate. These 50 bees were paint-marked for distinction. A fourth replicate was conducted with 6 cages in the incubator: control, pollen, QMP, QMP + pollen, QMP + BP, and QMP + BP + pollen.

Every 24 h, each cage was provisioned 30 ml of 30% sucrose solution and 30 ml of distilled H_2O. Pollen (#78-23063) was obtained from Glory Bee Foods (Eugene, OR. USA) and sucrose from the Imperial Sugar Company (Sugar Land, TX. USA). To make a homogenized pollen diet, 200 g of powdered pollen was blended with 40 ml of 30% sucrose (13 g sucrose, 32 ml distilled H_2O) using an Artisan Series Tilt-Head Stand Mixer (KitchenAid, St. Joseph, MI. USA). Pollen diet was provisioned in a 15 mm x 10 mm (depth x radius) plastic test tube cap placed in the bottom of the rearing cage. Treatments without pollen received an empty plastic cap. After each 24 h period, remaining sucrose and water volumes were recorded. Pollen diet consumption was measured by subtracting post-feeding weight from pre-feeding weight.

The blend of brood pheromone used here was as follows; 1% ethyl linoleate, 13% ethyl linolenate, 8% ethyl oleate, 3% ethyl palmitate, 7% ethyl stearate, 2% methyl linoleate, 21% methyl linolenate, 25% methyl oleate, 3% methyl palmitate, and 17% methyl stearate (Sigma-Adrich, St. Louis, MO, USA). Isopropanol was used as the solvent to formulate daily doses of 560 ng/µl per bee. Brood pheromone was delivered on a glass plate (7.5 cm x 8 cm) and suspended from the center of each plexiglass/ wiremesh cage. Cages with no BP received a glass plate rinsed in isopropyl alcohol.

Table 1. Cages received one of the following treatments: control, QMP, BP, or QMP + BP. Each pheromone treatment was conducted both with and without pollen resulting in a total of 8 treatments. Treatments were replicated at least 3 times at the cage level. A fourth replicate included: control, pollen, QMP, QMP + pollen, QMP + BP, and QMP + BP + pollen.

Treatment	QMP[1]	BP[2]	Pollen
Control	-	-	-
Pollen	-	-	+
BP	-	+	-
BP + pollen	-	+	+
QMP	+	-	-
QMP + pollen	+	-	+
QMP + BP	+	+	-
QMP + BP + pollen	+	+	+

[1] +QMP is queenright, –QMP is queenless

[2] +BP is broodright, -BP is broodless

Bee Boost® (Pherotech International Inc., Delta, B.C. Canada) was the source of QMP. Bee Boost® delivers approximately 1 queen equivalent (QEq) of QMP per day, (200 µg 9-keto-2 (*E*)-decenoic acid (ODA), 80 µg for 9-hydroxy-2-(E)-decenoic acid (HDA), 20 µg methyl *p*-hydroxybenzoate and 2 µg homovanillyl alcohol (HVA) (Pankiw et al., 1996)). Bee Boost® is loaded with 30 QEq of QMP. Treatments receiving QMP simulated colonies with a queen and were therefore termed "queenright," while those not receiving QMP simulated colonies without a queen present were therefore termed "queenless." One Bee Boost® stick was placed in each QMP + treatment cage for the duration of the experiment (Table 1).

Sub-samples of 20 bees per treatment were collected every 3 days from each cage, cold anesthetized, and stored at -20 °C until dissected for protein quantification of hypopharyngeal and mandibular glands and mandibular gland measurement. For each dissection the bee was first decapitated. Next, the head was pinned on a wax mount and dissected under a Stereo Zoom Binocular microscope body (CO-SZ-600 on Boom Stand, Sciencescope, Chino, CA. USA), objective: 10x. The head was dissected from right to left. An incision was made to separate the right compound eye and ommatidia from the rest of the head. Then, the right hypopharyngeal gland was removed, rinsed in distilled H_2O, and placed into a vial containing 20 µl Tris buffer, (0.1 M Tris-HCl, pH 7.5). Following the removal of the hypopharyngeal gland, the mandible and all attached glandular tissue were removed to measure width and length of the gland using a microscope reticle. The mandible with gland attached was rinsed with distilled H_2O and placed into a second vial of 20 µl Tris buffer, (0.1 M Tris-HCl, pH 7.5). This procedure

was repeated to remove glands on the left. The vials, each containing 2 hypopharyngeal or 2 mandibular glands were stored at -20 °C prior to protein quantification using the Bradford assay (Bradford, 1976).

The Bradford assay methods were after Sagili et al. (2005). Glands were homogenized using a homogenizer that tightly fitted in 1.5 µl microcentrifuge tubes used to store the glands. Next, tubes were centrifuged at 10,000 rpm for 5 min. Supernatant from each tube was used for analysis. The 500-0202 Quick Start Bradford Protein Assay Kit 2 was used (Bio-Rad Laboratories, CA, and U.S.A.) containing all necessary reagents and dyes. The dye reagent was prepared by adding 1 part Coomassie Brilliant Blue G-250 dye reagent to 4 parts distilled water. Subsequently, 2 µl or 5 µl aliquots were added from each sample to be analyzed to a microcentrifuge tube containing 1 ml Bradford reagent. Tubes were vortexed to homogenize the contents, and then incubated for 10 min at room temperature (approximately 24° C). Standard-curves were prepared using bovine serum albumin (BSA). Protein absorbance was measured at 595 nm against blank reagent using a Thermo Genesys 10 UV Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA). A standard curve was generated by plotting the known weight of BSA against the corresponding absorbance values measured (SPSS, 2007). Protein extracted from the glands was estimated using the linear regression equation generated from the BSA standard curve assay.

Protein quantity was statistically analyzed using analysis of variance (ANOVA) (Sokal and Rohlf, 1995; SPSS, 2007). The hypopharyngeal gland data were natural log transformed prior to analysis to normalize the distribution (Sokal and Rohlf, 1995). To

reduce the probability of a type 1 error (a false positive result), the Bonferroni post-hoc test was used to analyze differences between treatments (Sokal and Rohlf, 1995).

Results

In the queenless environment, there were no significant differences between replicates for sucrose consumption per bee (ANOVA $F_{2, 141} = 0.593$, $P = 0.554$). Therefore, replicates were pooled for further analysis. There were no significant differences between queenless treatments for sucrose consumption (ANOVA $F_{3, 140} = 0.146$, $P = 0.932$).

In the queenright environment, there were significant differences found between replicates for sucrose consumption (ANOVA $F_{3, 290} = 9.760$, $P < 0.0001$). However, there was no significant replicate x treatment interaction (GLM, $F_{15, 270} = 1.001$, $P = 0.454$). For this reason, replicates were pooled for further analysis. There were no significant differences between queenright treatments for sucrose consumption per bee (ANOVA $F_{5, 288} = 1.466$, $P = 0.201$).

In the queenless environment, no significant differences in water consumption were found between treatments. Water consumption was significantly different between replicates (ANOVA, $F_{2, 141} = 3.436$, $P = .035$). However, there was no significant replicate by treatment effect (GLM, $F_{6, 132} = .342$, $P = .914$). Therefore, replicates were pooled for further analysis. No significant differences in water consumption per bee were found between treatments (ANOVA, $F_{3, 140} = 1.206$, $P = .310$).

In the queenright environment, there were significant differences found between replicates for water consumption (ANOVA $F_{3, 290} = 16.017$, $P = 0.0001$). There was a significant replicate by treatment interaction (GLM, $F_{15, 270} = 2.503$, $P = 0.002$). For this reason replicates were analyzed separately. No significant differences occurred between treatments in 3 out of 4 replicates (ANOVA, $F_{5, 66} = 2.192$, $P = 0.066$; $F_{5, 66} = 2.117$, $P = .074$; $F_{5, 72} = .902$, $P = .484$). Significant differences occurred between water consumption of bees in the control compared with all queenright treatments in 1 of the 4 replicates (ANOVA, $F_{5, 66} = 4.934$, $P = .001$).

In the queenless environment, there was no significant difference between treatments for pollen consumption per bee. There were no significant differences between replicates (ANOVA $F_{2, 69} = 0.818$, $P = 0.446$) and there were no significant replicate by treatment interactions (GLM, $F_{2, 66} = .917$, $P = .405$). Therefore replicates were pooled for further analysis. There were no significant differences in diet consumption per bee (ANOVA $F_{1, 70} = 0.000$, $P = 0.998$).

In the queenright environment, there was no significant difference between treatments for pollen consumption. There was no significant difference between replicates for pollen consumption (ANOVA $F_{3, 143} = 0.647$, $P = 0.586$) and no significant replicate by treatment interactions (GLM $F_{6, 135} = .516$, $P = .795$). Replicates were pooled for further analysis. No significant treatment effect was found for pollen consumption (ANOVA $F_{2, 144} = 0.395$, $P = 0.674$).

Hypopharyngeal gland protein was normalized by a natural log transformation. Replicates were significantly different in the queenless ($F_{2, 947} = 25.850$, $P < 0.0001$) and

the queenright ($F_{3, 1863} = 7.117$, $P < 0.0001$) environments; however, the treatment differences were unidirectional. As a consequence replicates were pooled for further analysis. The overall worker hypopharyngeal gland mean extractable protein in the queenless and queenright rearing environments are shown in Figures 1 and 2 respectively. Letters indicate significant differences in hypopharyngeal gland extractable protein (ANOVA, $P < 0.05$).

In the queenless rearing environment (Fig. 1), control bees had significantly less extractable hypopharyngeal gland protein than bees in the BP treatment (ANOVA, $F_{1, 472} = 5.841$, $P = 0.016$). However, bees in the BP + pollen treatment did not have significantly greater hypopharyngeal gland protein content than bees provisioned pollen alone (ANOVA, $F_{1, 474} = 2.768$, $P = 0.097$). Bees in the BP + pollen treatment had significantly greater hypopharyngeal gland protein content than bees in the BP treatment (ANOVA, $F_{1, 472} = 92.613$, $P < 0.0001$), while bees in the pollen treatment had significantly greater hypopharyngeal gland protein content than the control (ANOVA, $F_{1, 474} = 86.891$, $P < 0.0001$). Overall, bees provisioned pollen diet had significantly greater extractable hypopharyngeal gland protein content than those without pollen diet (ANOVA, $F_{1, 948} = 179.445$, $P < 0.0001$).

In the queenright environment (Fig. 2), there was no significant difference in extractable hypopharyngeal gland protein between bees in the QMP – pollen treatment and the control (ANOVA, $F_{1, 630} = 2.986$, $P = 0.084$) or between bees in the QMP – pollen treatment and bees in the QMP + BP – pollen treatment (ANOVA, $F_{1, 614} = 0.436$, $P = 0.509$). Overall, bees provisioned pollen had significantly greater hypopharyngeal

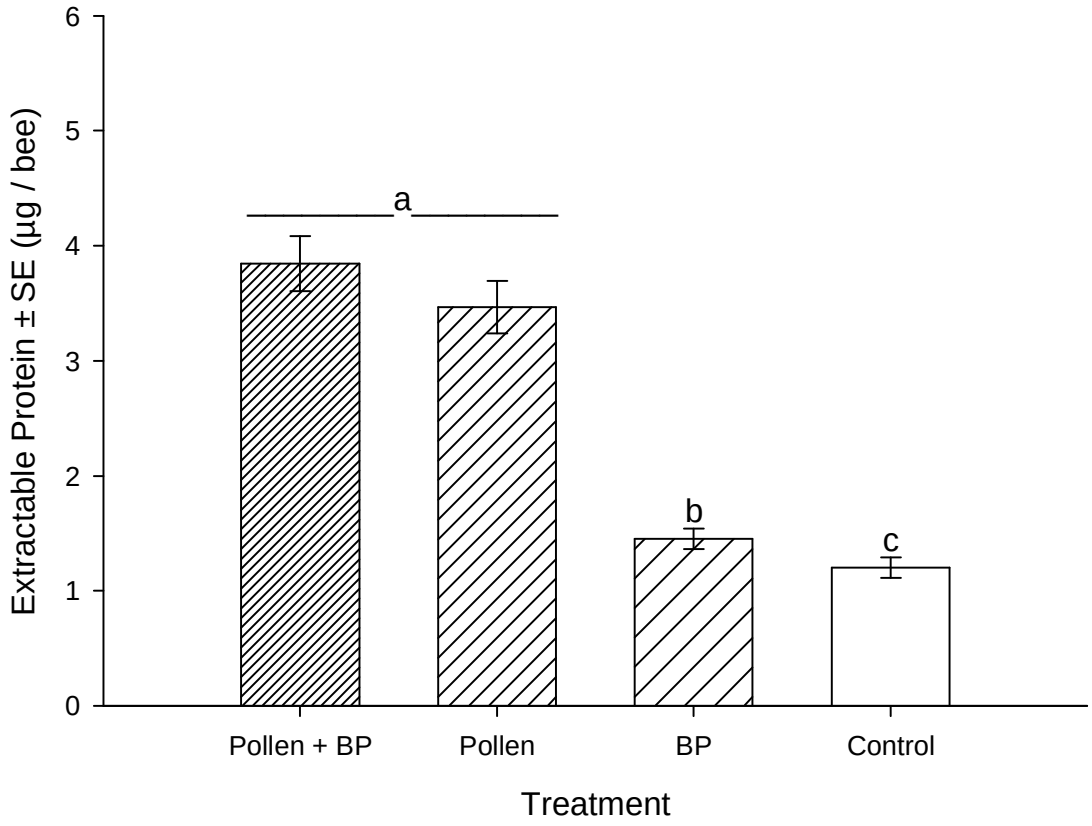

Figure 1. Honey bee hypopharyngeal gland extractable protein in a queenless environment. Letters indicate significant differences in hypopharyngeal gland extractable protein (ANOVA, $P < 0.05$).

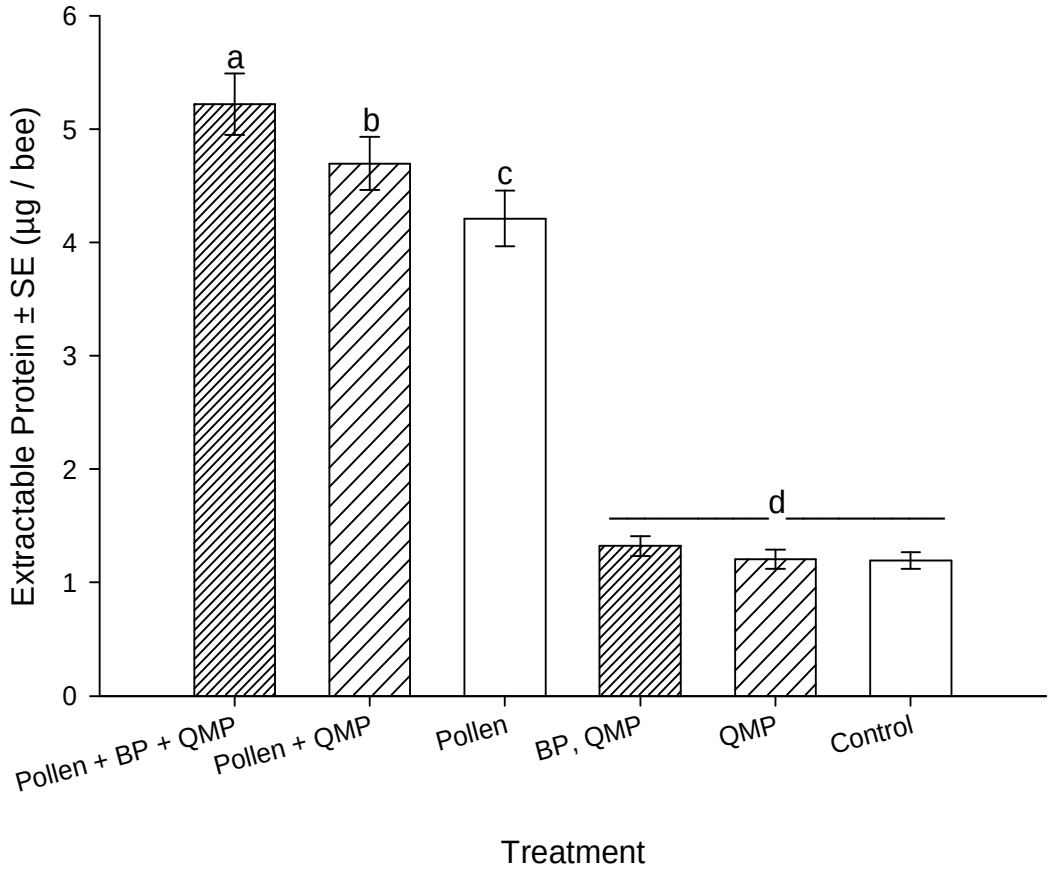

Figure 2. Honey bee hypopharyngeal gland extractable protein in a queenright environment. Letters indicate significant differences in hypopharyngeal gland extractable protein (ANOVA, $P < 0.05$).

gland protein content than bees in the non-pollen treatments (ANOVA, $F_{1, 1865}$ = 653.683, $P < 0.0001$). Hypopharyngeal glands of bees in the QMP – pollen treatment had significantly less extractable protein than those of bees in the QMP + pollen treatment (ANOVA, $F_{1, 618} = 260.883$, $P < 0.0001$). In addition, hypopharyngeal glands of bees in the QMP + BP – pollen treatment had significantly less extractable protein than those of the QMP + BP + pollen treatment (ANOVA, $F_{1, 609} = 226.142$, $P < 0.0001$). Hypopharyngeal glands of bees in the pollen treatment were significantly less than bees in the QMP + pollen treatment (ANOVA, $F_{1, 622} = 5.238$, $P = 0.022$). There were no significant differences between QMP + pollen and QMP + BP + pollen (ANOVA, $F_{1, 616} = 0.250$, $P = 0.618$). Finally, in the queenright environment, the QMP + BP + pollen treatment had significantly greater extractable protein than bees in the pollen treatment (ANOVA $F_{1, 625} = 7.490$, $P = 0.006$). Hypopharyngeal gland extractable protein content declined as bees aged in the queenless environment ($R^2 = 0.9106$, $P = 0.0117$; Fig. 3 (SigmaPlot, 2006)) and in the queenright environment ($R^2 = 0.8460$, $P = 0.0269$; Fig. 3).

Figures 4 and 5 depict the worker mandibular gland extractable protein means of bees sampled on days 3, 6, 9, and 12 in the queenless and queenright environments respectively. Mandibular gland protein amounts were significantly different by replicate in the queenless environment ($F_{2, 948} = 4.333$, $P = 0.013$) and in the queenright environment ($F_{3, 1889} = 27.760$, $P < 0.0001$); however, the differences were unidirectional. As a consequence replicates were pooled for further analysis. In the queenless environment, bees in the pollen treatment had significantly greater mandibular

gland protein content than bees in the control (ANOVA, $F_{1, 474} = 35.769$, $P < 0.0001$; Fig. 4). In addition, bees in the BP + pollen treatment had significantly greater mandibular gland protein amount than bees in the pollen treatment (ANOVA, $F_{1, 472} = 6.186$, $P = 0.013$; Fig. 4). Overall, in the queenless rearing environment, bees provisioned pollen had significantly greater protein content than bees given no pollen ($F_{1,949} = 107.652$, $P < 0.0001$; Fig. 4).

In the queenright environment, bees in the pollen treatment differed significantly from the control (ANOVA, $F_{1,634} = 65.269$, $P < 0.0001$; Fig. 5). Bees given QMP + BP + pollen did not have significantly greater mandibular protein than bees given only pollen (ANOVA, $F_{1, 624} = 3.851$, $P = 0.0502$; Fig. 5). Overall, bees in the queenright environment provisioned pollen diets had significantly greater extractable mandibular gland protein than bees in treatments without pollen (ANOVA, $F_{1,1891} = 237.506$, $P < 0.0001$; Fig. 5).

Mandibular gland size was also measured. In the queenless rearing environment, bees in the control had significantly less area than those given BP + pollen (ANOVA, $F_{1, 478} = 7.935$, $P = 0.005$; Fig. 6). In the queenright treatments, bees provisioned with a pollen diet had significantly greater area than bees not provisioned with a pollen diet (ANOVA, $F_{1, 1908} = 52.738$, $P < 0.0001$; Fig. 7). In addition, bees had significantly greater mandibular gland area on the QMP + BP + pollen treatment than bees on the pollen treatment (ANOVA, $F_{1, 633} = 4.029$, $P = 0.045$; Fig. 7).

Mandibular gland extractable protein content and size decreased with age. Mandibular gland extractable protein of bees in the queenless environment declined

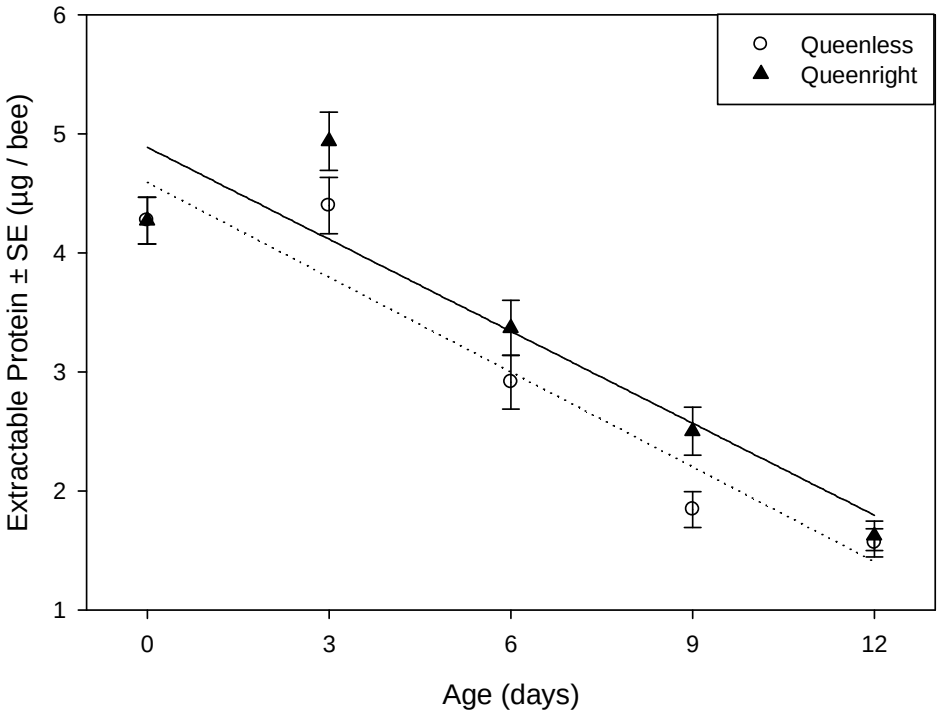

Figure 3. Honey bee hypopharyngeal gland extractable protein declined with age in both the queenless environment (R^2 = 0.9106, P = 0.0117) indicated by the dotted line, and the queenright environment (R^2 = 0.8460, P = 0.0269) indicated by the solid line.

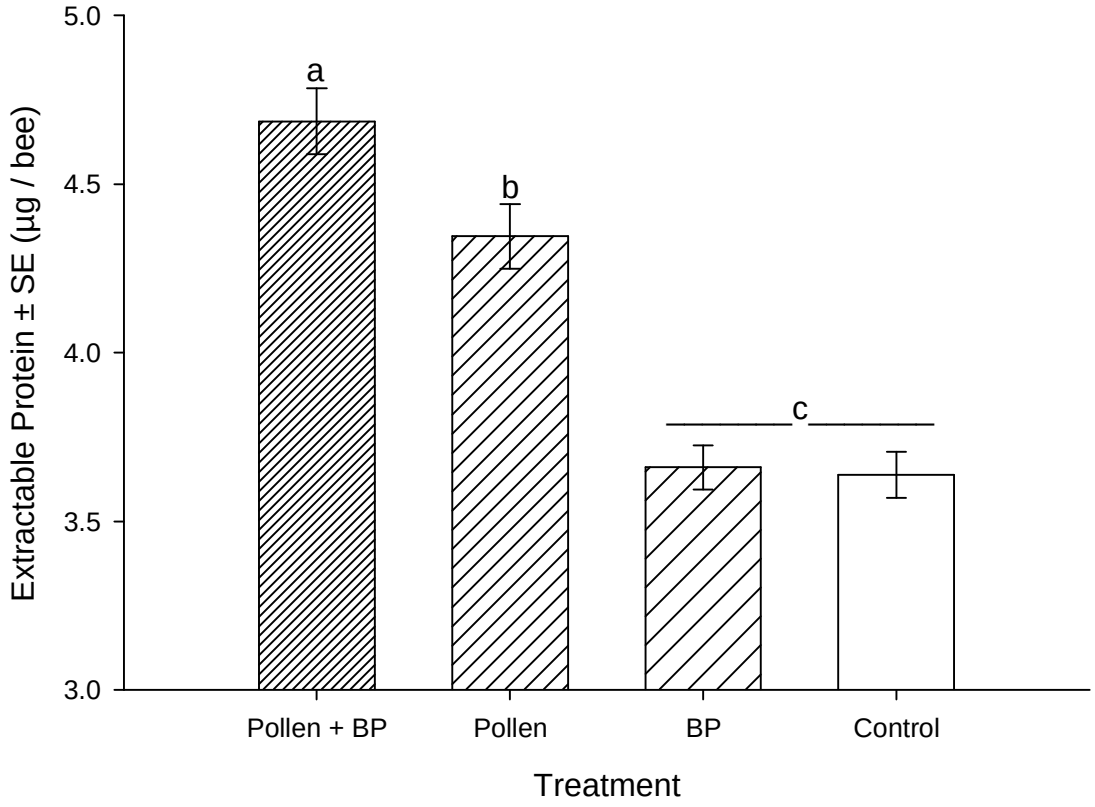

Figure 4. Mean of honey bee mandibular gland extractable protein collected on days 3, 6, 9, and 12 in a queenless environment. Letters indicate significant differences in mandibular gland extractable protein (ANOVA, $P < 0.05$).

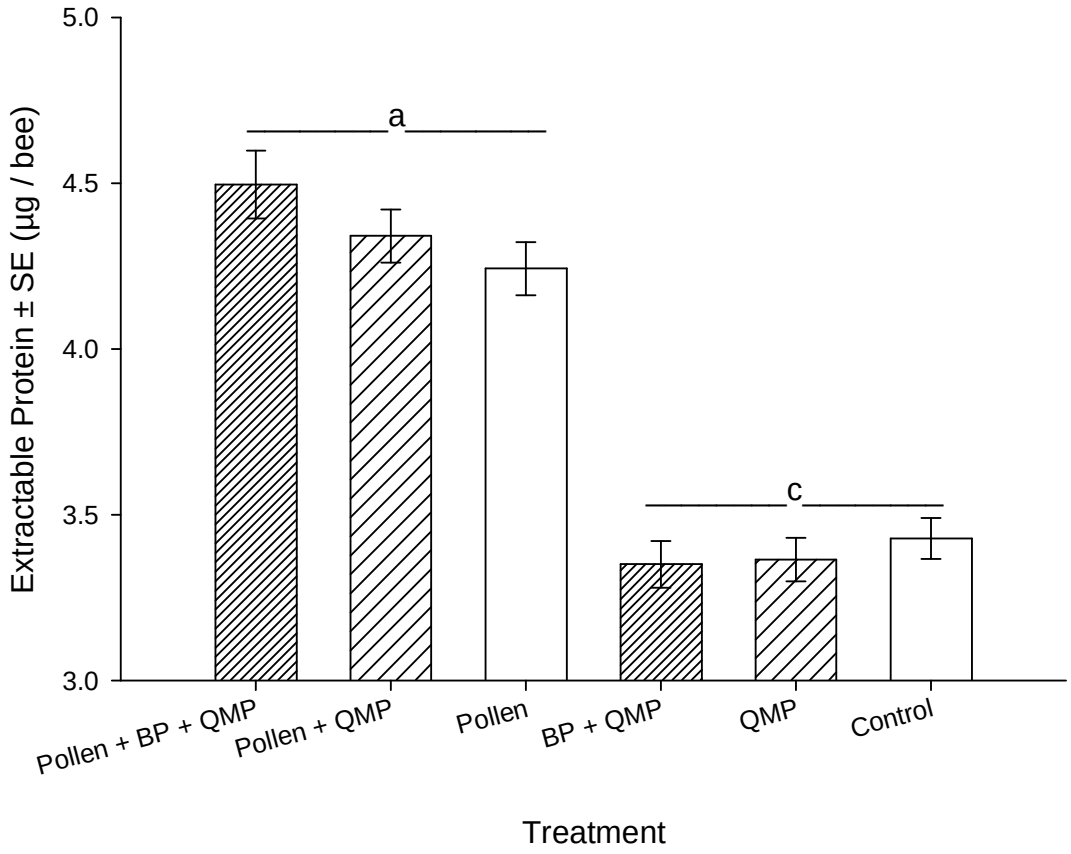

Figure 5. Mean honey bee mandibular gland extractable protein collected on days 3, 6, 9, and 12 in a queenright environment. Letters indicate significant differences in mandibular gland extractable protein (ANOVA, $P < 0.0001$).

from a mean max of 1.5220 ± 0.0185 to a minimum of 1.2850 ± 0.0117 in the queenless environment (R^2 = 0.9926, P = 0.0003; Fig. 8). A similar decline was observed in the queenright environment (maximum: 1.5220 ± 0.0185; minimum 1.2742 ± 0.0114; R^2 = 0.8618, P = 0.0228; Fig. 8). Mandibular gland size also decreased with age in both the queenless environment, (R^2 = 0.8580, P = 0.0238; Fig. 9), as well as in bees reared in the queenright environment (R^2 = 0.8536, P = 0.0249; Fig. 9).

Discussion

I hypothesized that pollen would increase protein content of both mandibular and hypopharyngeal glands and size of mandibular glands of adult honey bees. Amounts of extractable protein from hypopharyngeal and mandibular glands significantly increased with pollen diet. This strongly suggests that pollen provides essential nutrients that affect gland protein content (Figs. 1, 2, 4, and 5). To some degree, mandibular gland size also increased with pollen (Figs. 6 and 7). In previous studies, it has been observed that poor hypopharyngeal gland development and a shorter life span resulted from insufficient pollen consumption early in adult life (Maurizio, 1950). Although Haydak (1935), did not directly measure the glands of nurse bees, he did report a loss in nitrogen content of nurse bee heads in colonies without pollen. Haydak (1935), also observed brood could only be reared for one week without pollen.

Consumption did not differ between treatments except in one replication in which water consumption differed between the control and QMP treatments. There is no explanation for this one inconsistency. Mohammedi et al., (1996) also found that no

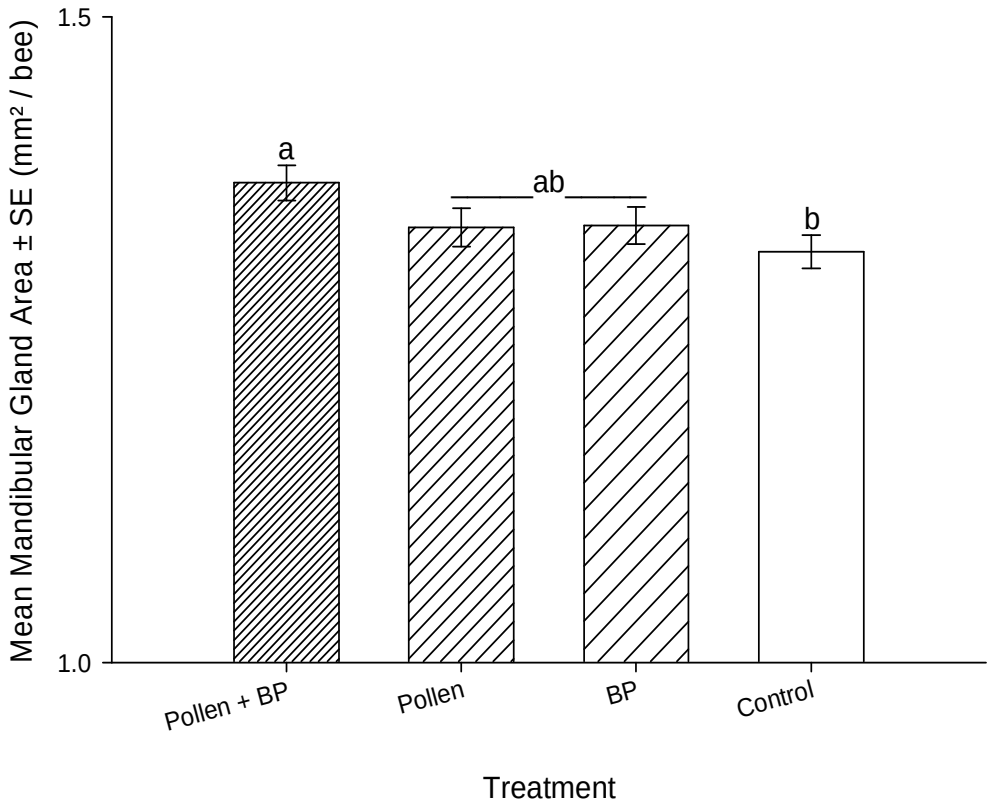

Figure 6. Mean honey bee mandibular gland size in a queenless environment. Letters indicate significant differences in mandibular gland extractable protein (ANOVA, $P < 0.05$).

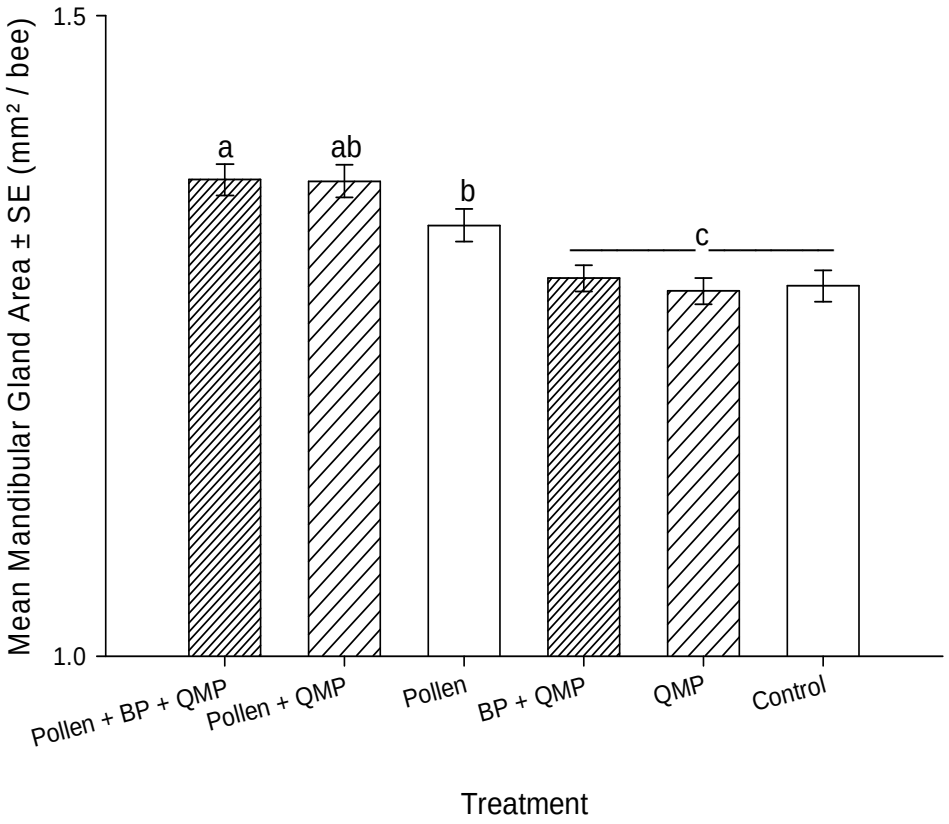

Figure 7. Honey bee mandibular gland size in a queenright environment. Letters indicate significant differences in mandibular gland extractable protein (ANOVA, $P < 0.05$).

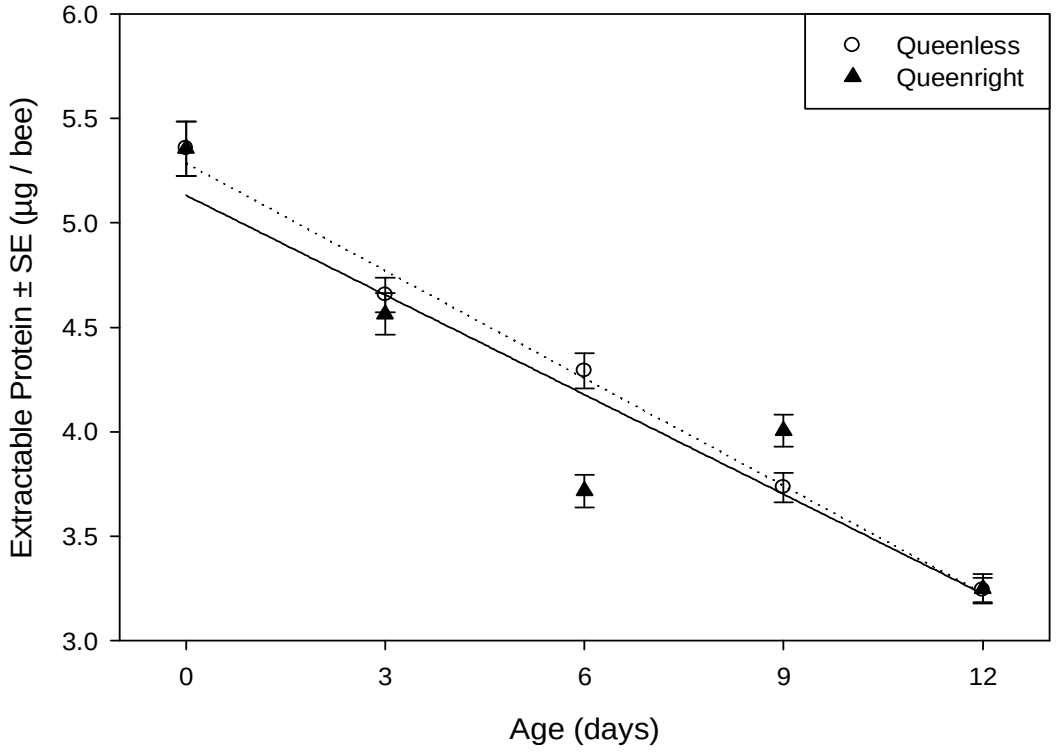

Figure 8. Honey bee mandibular gland extractable protein declined with age in a queenless environment (R^2 = 0.9926, P = 0.0003) indicated by the dotted line, and in the queenright environment (R^2 = 0.8618, P = 0.0228) indicated by the solid line.

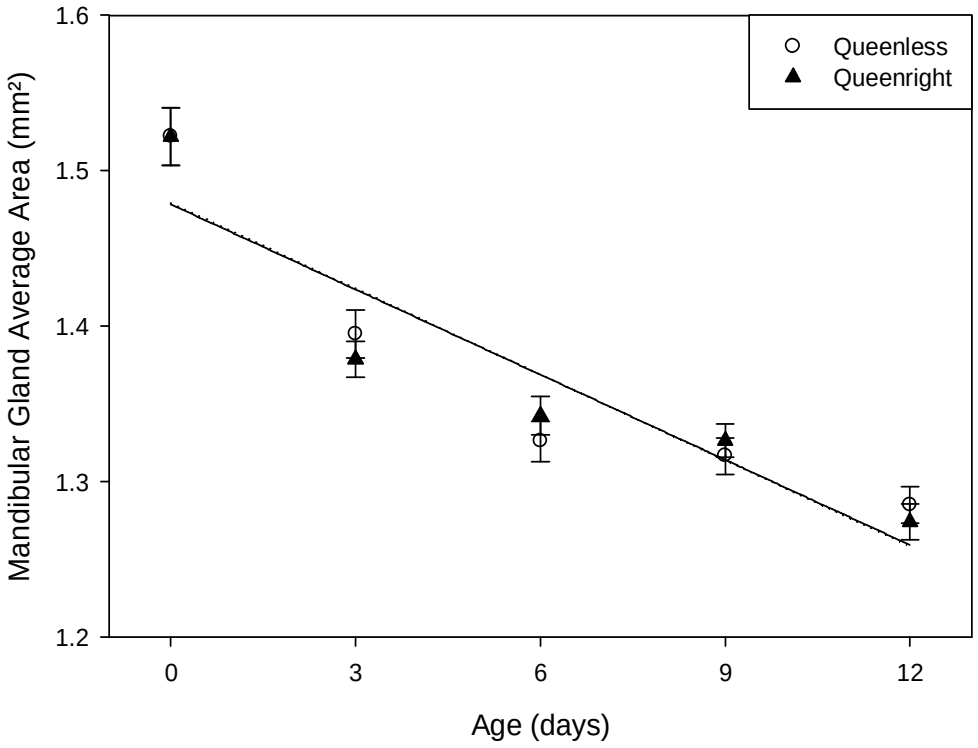

Figure 9. Honey bee mandibular gland average area declined with age in a queenless environment (R^2 = 0.8580, P = 0.0238), and in the queenright environment (R^2 = 0.8536, P = 0.0249). The line shown on the graph represents the linear regression of both the queenless and queeright environments.

significant differences in pollen consumption occurred between treatments with and without brood pheromone. He concludes that the esters provided in the brood pheromone are not simply phagostimulants, but are prompting the increase of bee hypopharyngeal glands without an increase in pollen consumption.

I hypothesized that BP would increase protein content of both hypopharyngeal and mandibular glands because brood presence has previously been shown to increase hypopharyngeal acini diameter (Hrassnigg and Crailsheim, 1998), and hypopharyngeal gland activity (Brouwers, 1982, 1983; Huang and Otis, 1989; Huang et al., 1989). Also, brood pheromone has previously been shown to increase hypopharyngeal gland protein content even in the absence of a pollen diet (Mohammedi et al., 1996). My results supported this hypothesis for hypopharyngeal glands from the queenless rearing environment in the absence of pollen (Fig. 1), as well as mandibular gland protein content in the presence of pollen (Fig. 5), and mandibular gland size in the presence of BP + pollen (Fig. 6).

In contrast with Mohammedi's (1996) results, hypopharyngeal gland protein content did not significantly increase in bees reared with BP + pollen when compared with bees on the pollen diet alone (Fig. 1). However, my study was shorter than that of Mohammedi (1996) and it is possible that greater differences could have been found in bees aged 14 and 25 d.

Mandibular gland protein content and size were not significantly different in BP and control environments (Figs. 4 and 6). Nurses rearing larvae in colonies regularly secrete the contents of their mandibular glands as part of brood food or royal jelly. If

bees are not able to expel glandular protein naturally, amount of extractable protein may reach an asymptote. A feedback mechanism may be functioning to inhibit additional protein biosynthesis when glandular protein content reaches threshold amounts. Crailsheim and Stolberg (1989) measured the acini size of hypopharyngeal glands to be significantly less in cage reared bees than bees reared in a colony.

I hypothesized that QMP would decrease protein content and size of mandibular glands and have no effect on hypopharyngeal glands. However, no differences were found between bees in the pheromone environments where pollen was not in the diet (Figs. 2, 5, and 7). Interestingly, when both QMP and BP were present in addition to pollen, hypopharyngeal gland protein content and mandibular gland size increased significantly as compared to pollen alone (Figs. 2 and 7). This suggests that both primer pheromones are necessary for the greatest amount of extractable protein and gland size.

In this experiment, where bees were reared in cages, hypopharyngeal gland extractable protein declined with age (Fig. 3). Hypopharyngeal glands follow a secretory cycle as bees age (Deseyn and Billen, 2005). It has been shown that bees reared in cages develop faster than those in the colony (Crailsheim and Stolberg, 1989; Lass and Crailsheim, 1996). Crailsheim et al., (1992) found that hypopharyngeal glands increased in acini diameter until day 9 in colonies while Haydak (1957) noted a degeneration in the glands occurring by age 15 d in colony reared bees. My observations are supported by the results of Crailsheim and Stolberg showing decline in hypopharyngeal gland acini diameter after day 3 in caged bees (Crailsheim and Stolberg, 1989).

Mandibular gland extractable protein and size also declined with age (Figs. 8 and 9). Crewe and Moritz (1989) studied *Apis mellifera intermissa* and Simon et al. (2001) studied *Apis mellifera capensis* both finding that, in general, fatty acid production of mandiblar glands increase as bees age. Vallet et al. (1991) found that as bees age mandibular gland increases occur in size and secretion of 2-heptanone per headspace sample. It is possible that as the amount of fatty acid within glands increases, the amount of protein content decreases. However, because gland size also decreased with age it is possible that caging bees has adverse effects on these glands.

Pankiw et al., (1998b) stated that the distribution method of brood pheromone is unknown and when applied to glass may last for a limited time, possibly only a few hours. Glass plates were replaced daily in our experiment in which bees were observed to lick the glass plates; however, this method of distributing the pheromone to the bees may not be sufficient to maintain gland state. It is possible bees require a physical stimulation such as the larvae cuticle. It is also possible that the synthetic blends and pollen diet are not sufficient to maintain the physiological state of the gland.

CHAPTER III

EFFECTS OF DILUTED POLLEN DIETS ON MANDIBULAR AND HYPOPHARYNGEAL GLAND PROTEIN CONTENT

Introduction

Pollen is the sole source of dietary protein as well as some lipids, vitamins, minerals and minimal amounts of carbohydrates (Herbert, 1992). A honey bee pollen forager collects pollen from the anthers of a plant, carries it on her corbiculae to the hive, and deposits the load of pollen in wax comb cells usually located near the brood (Winston, 1987). Pollen is further packed into the cell with the addition of a glandular secretion, thought to originate from both mandibular and hypopharyngeal glands, and is topped with a small cover of honey (Herbert, 1992; Winston, 1987). The glandular additions stop pollen grain germination over a two day period and begin the digestive processes .

Pollen is crucial for colonies as the sole source of protein for brood rearing. The pollen foraging effort of a colony must constantly be adapted to the requirements of the brood and, like foraging for nectar, must rapidly adapt to a changing foraging environment. Unlike honey, pollen is not hoarded in amounts up to 137 kg. A few days of inclement weather can significantly diminish the amount of stored pollen (Schmickl et al., 2003). Protein is consumed primarily by larvae through nurse bee food gland secretions and is therefore stored in cells near the brood rearing area in the center of the nest. The second greatest consumers of pollen are newly emerged adults followed by

nurse bees. There is a direct correlation between amount of stored pollen and amount of brood successfully reared (Allen and Jeffree, 1956).

Adult bees begin consuming pollen one to two hours after emergence. Mass consumption begins when workers are from 42 to 52 hrs old and reaches a maximum around day five (Haydak, 1935; Haydak, 1970). Protein consumption is necessary for complete development of muscles, and hypopharyngeal, mandibular, and wax glands (Herbert, 1992). On average, pollen consumption decreases at eight to ten days (Haydak, 1935; Haydak, 1970). Following development, adult bees rely on a carbohydrate rich diet for energy and metabolic processes (Haydak, 1970; Kunieda et al., 2006).

Nurse bees consume and digest stored pollen and then convert it into proteinaceous secretions (Brouwers, 1982; Crailsheim, 1990; Crailsheim et al., 1992). Crailsheim, (1998), found that brood food and royal jelly are distributed among adults. Queens similarly receive royal jelly. Latter instar larvae also receive pollen directly provisioned by nurses (Brouwers et al., 1987; Schmickl et al., 2003). Once bees have moved on to other tasks, they usually no longer perform nursing duties. However, if necessary, they can revert back to this protein-producing state by again consuming pollen (Ohashi et al., 2000).

A deficiency of protein leads to developmental failures (Jay, 1963). If colonies are prevented from increasing amounts of incoming pollen, then more stored pollen is consumed and amount of brood rearing is adjusted to match pollen stores (Fewell and Winston, 1992; Filmer, 1932; Free and Racey, 1968; Pankiw et al., 2004). Once pollen

stores are depleted, nurse bees convert their body reserves for brood food production (Haydak, 1970). Additionally nursing intensity is adjusted (Schmickl et al., 2003) and, finally cannibalism of young larvae may occur to sequester nutrients for young (Schmickl and Crailsheim, 2001).

In order to digest pollen, bees must break down 2 resistant walls of pollen grains to access the cytoplasmic nutrients. Nutrients found in pollen include protein, nitrogen, amino acids, starch, sterols, and lipids (Roulston and Cane, 2000). Ten amino acids necessary to ensure normal honey bee development include arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine (DeGroot, 1953; Herbert, 1992). Most pollens contain all common amino acids (Johri and Vasil, 1961); however, essential amino acids tryptophan and phenylalanine are sometimes lacking (Solberg and Remedios, 1980). Ways (Human and Nicolson, 2003) and extent of pollen digestibility is variable (Crailsheim et al., 1992). It has been estimated that a honey bee colony has a nitrogen assimilation efficiency of 77-83% ingested nitrogen (Schmidt and Buchmann, 1985).

In foraging assays, both Schmidt (1982) and Pernal and Currie (2001), found bees could not discriminate among pollen sources containing varying protein content. Pernal and Currie (2001) concluded that bees choose pollen based on the efficiency with which it can be collected. Peng and Jay (1976) performed a cage experiment comparing aged pollen with added nutrients to fresh pollen. Using a gland development scale of 1 to 4, they concluded that pollen quality can have a significant effect on hypopharyngeal gland development. The cage study of Pernal and Currie (2000) also led to a positive

correlation of hypopharyngeal gland protein content with calculated protein consumed. Pernal and Currie concluded that hypopharyngeal gland development is a good indication of pollen quality. Hypopharyngeal glands are used to convert pollen into proteinaceous secretions mostly provisioned directly to larvae, but also fed to adults. The quality of pollen may therefore influence overall rate of colony growth.

The objectives of this experiment were to measure the effect of diets containing varying amounts of pollen on worker bee mass, extractable lipids from adult bees, worker hypopharyngeal and mandibular gland extractable protein, and consumption of diet.

Methods

Pollen used was purchased from Glory Bee Foods (item #78-23063; Eugene, OR. USA), and α-cellulose from Sigma-Aldrich (C-8002, lot 111K0080; St. Louis, MO. USA). Newly emerged bees from 1 source colony were caged in one of 5 plexiglass/ wiremesh cages (15 cm x 11 cm x 8 cm). Bees were reared in cages for 7 days in an incubator maintained at hive conditions (32 °C, 50% RH). The experiment was replicated 4 times.

Daily, each cage of 250 bees was provisioned 30 ml of a 30% sucrose solution (wt:wt), 30 ml of distilled H_2O, and treatment diet. There were 5 treatment diets: (1) 1:1 pollen: cellulose (vol:vol), (2) 1:2 pollen: cellulose (vol:vol), (3) 1:3 pollen: cellulose (vol:vol), (4) pure cellulose, and (5) pure pollen. Cages received 5.0 ± 0.1 g of treatment diet per day. After each 24 h period, remaining sucrose and water volumes as well as

treatment diet were recorded. Subsamples of 20 bees per treatment were collected on days 3 and 7 for lipid and protein analysis.

Ten bees were used for lipid analysis. Bees were cold anesthetized and stored at -20 °C prior to desiccation. Bees were desiccated in 1.5 ml microcentrifuge tubes in a drying cabinet at 50 °C (LEEC model F1; Kitchener, ON, Canada). Next, carcasses were weighed to the nearest 0.1 mg. Individual carcasses were then moved to a 4 ml glass vial. Vials were filled with chloroform (no. 319988-4L; Sigma-Aldrich, St. Louis, MO. USA). Next, a glass marble (1 inch diameter) was placed on top of each vial to slow evaporation. A total of 3 24 h baths of chloroform were performed, with chloroform removed and replaced at the 24 h interval. Following the third bath, the marble and remaining chloroform were removed. Vials containing a single bee were desiccated as above and then weighed. The difference in dessication weight was an estimate of lipid weight (Behmer et al., 2002).

Ten bees were used for gland protein analysis. Bees were cold anesthetized and stored at -20 °C until dissected. For each dissection the bee was first decapitated. Next, the head was pinned to a wax mount and dissected under a Stereo Zoom Binocular microscope body (CO-SZ-600 on Boom Stand, Sciencescope, Chino, CA. USA), objective: 10x. Both hypopharyngeal glands were removed and placed into a 1.5 ml microcentrifuge tube containing Tris buffer (0.1 M Tris-HCl, pH 7.9). Both mandibular glands were removed. Glandular tissue was separated from the mandibles. The mandibular gland tissue was placed into a 1.5 ml microcentrifuge tube containing Tris

buffer, (0.1 M Tris-HCl, pH 7.9). Glands were stored at -20 °C prior to protein content measurement using the Bradford assay.

The Bradford assay methods followed Sagili et al. (2005) detailed in Ch 2 p. 20-21. The 500-0202 Quick Start Bradford Protein Assay Kit 2 was used (Bio-Rad Laboratories, CA, and U.S.A.) containing all necessary reagents and dyes. Absorbency values for each sample were measured at 595 nm against a blank reagent using a Milton Roy Spectronic UV/VIS model 1201 (Ivyland, PA. USA). A linear regression equation was calculated from each standard curve and samples were fitted to the equation to estimate micrograms of extractable protein. Protein quantity was statistically analyzed using analysis of variance (ANOVA) (Sokal and Rohlf, 1995; SPSS, 2007).

Results

There was no significant treatment effect on sucrose consumption (ANOVA $F_{4, 135} = 0.298$, $P = 0.879$). Sucrose consumption was significantly different between replicates (GLM, $F_{3, 136} = 7.188$, $P = 0.000$). However, there was no significant replicate by treatment interaction (GLM, $F_{12, 120} = 0.293$, $P = 0.990$). Therefore replicates were pooled for further analysis.

There was no significant treatment effect on water consumption (ANOVA, $F_{4, 135} = 1.525$, $P = 0.198$). Water consumption was significantly different between replicates (GLM, $F_{3, 12} = 14.168$, $P < 0.0001$); however there was no significant replicate by treatment interaction (GLM $F_{12, 120} = 0.549$, $P = 0.878$). For this reason, replicates were pooled for further analysis.

Overall, caged bees consumed significantly less pure pollen diet than any of the other treatment diets (ANOVA, $F_{1, 138}$=30.473, $P < 0.0001$; Fig. 10). There was a significant effect of replicate on pollen diet consumption (GLM, $F_{3, 12} = 9.833$, $P = 0.001$); however, there was no significant treatment by replicate interaction (GLM, $F_{12, 120} = 0.958$, $P = 0.493$). Therefore, replicates were pooled for further analysis. There were significant differences found between treatments (ANOVA, $F_{4, 135} = 8.363$, $P < 0.0001$; Fig. 10). Excluding the pure pollen diet, there was no significant difference in consumption (ANOVA, $F_{3, 108} = 0.954$, $P = 0.418$).

Overall, caged bees provisioned a pure pollen diet were significantly heavier than bees provisioned any other treatment diet (ANOVA, $F_{1, 398} = 95.705$, $P < 0.0001$; Fig. 11). There was a significant effect of replicate on bee mass (GLM, $F_{3, 12} = 12.422$, $P = 0.001$) and significant replicate by treatment interaction (GLM, $F_{12, 380} = 2.063$, $P = 0.019$). However, the treatment differences found were unidirectional for all replicates. For this reason, all replicates were pooled for further analysis. Significant differences occurred between the mean dry mass of bees provisioned different treatment diets (ANOVA, $F_{4, 395} = 36.865$, $P < 0.0001$; Fig. 11). Caged bees provisioned 1:1 pollen: cellulose diet were significantly heavier than bees provisioned treatment diets with a greater ratio of cellulose (ANOVA, $F_{1, 318} = 38.035$, $P < 0.0001$). Caged bees provisioned 1:2 pollen: cellulose diet were significantly heavier than bees provisioned a cellulose diet (ANOVA, $F_{1, 158} = 8.136$, $P = 0.005$). Caged bees provisioned 1:3 pollen: cellulose diet were not significantly different from caged bees provisioned 1:2 pollen:

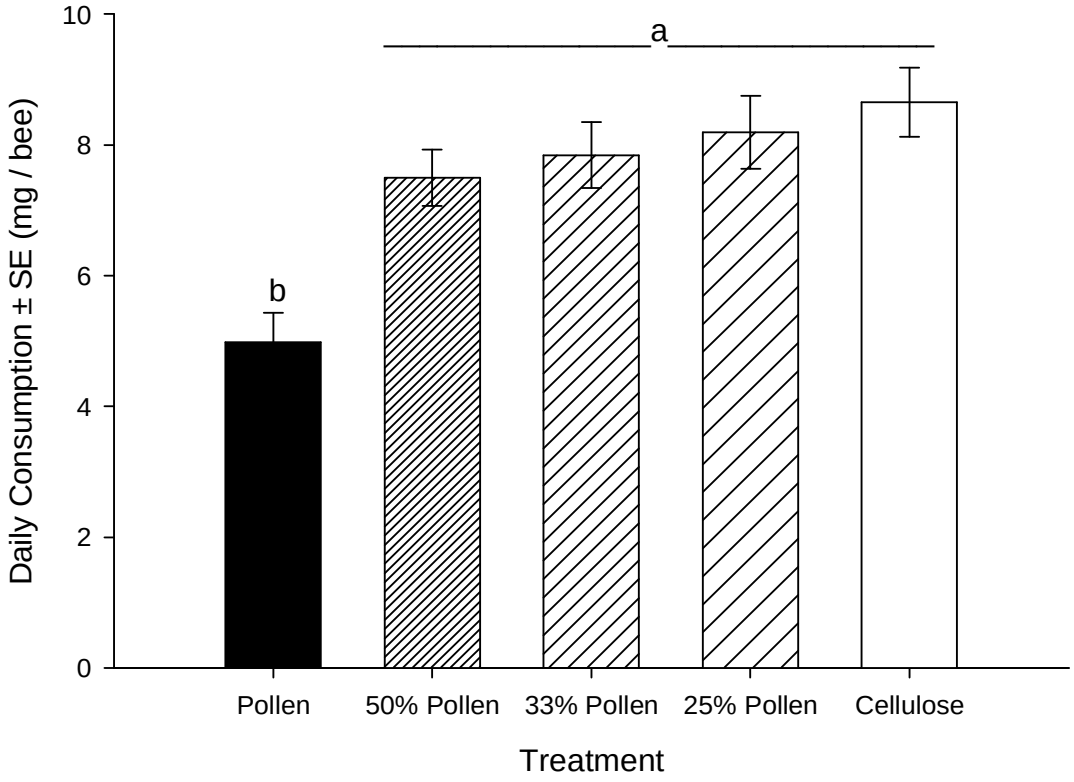

Figure 10. Mean daily consumption for each treatment. Letters indicate significant differences between treatments (ANOVA, $P < 0.05$).

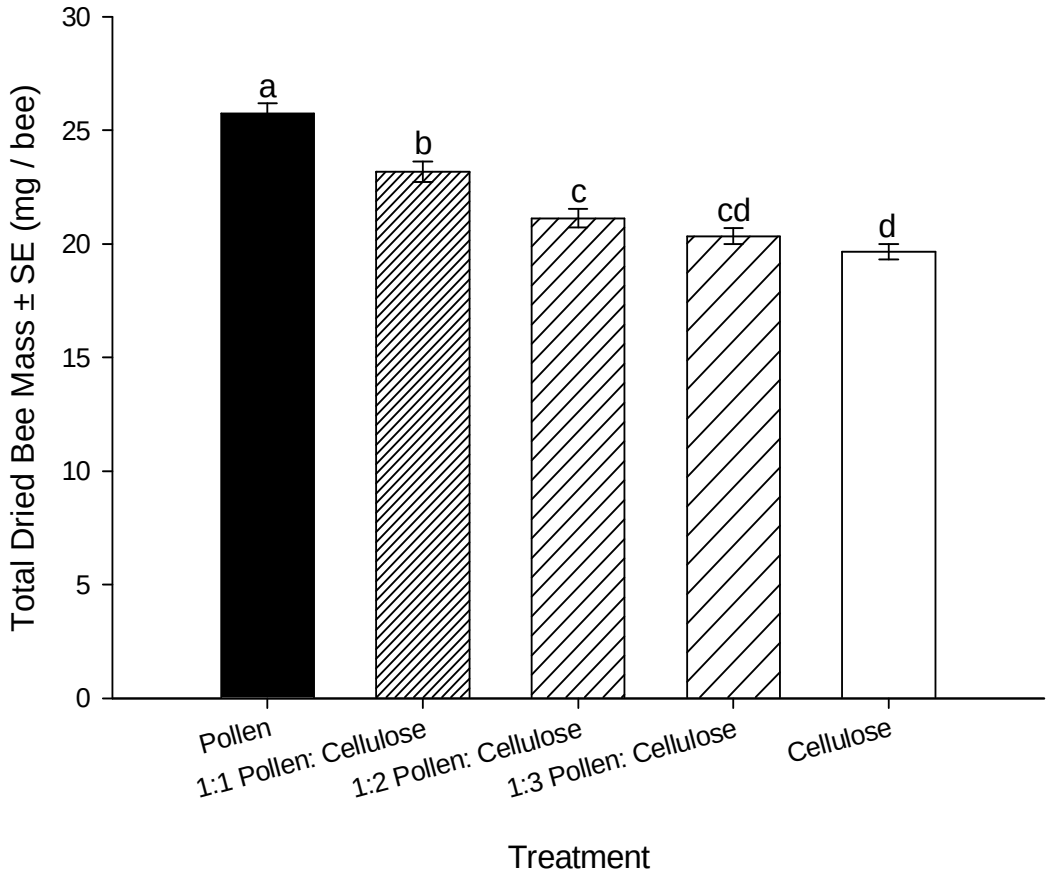

Figure 11. Mean dry bee mass of bees. Letters indicate significant differences between treatments (ANOVA, $P < 0.05$).

cellulose diet (ANOVA, $F_{1, 158} = 2.103$, $P = 0.149$) or from bees provisioned pure cellulose diet (ANOVA, $F_{1, 158} = 1.989$, $P = 0.160$).

Overall, caged bees provisioned pure pollen diet had significantly more lipids than bees provisioned any of the other treatment diets (ANOVA, $F_{1, 398} = 198.069$, $P < 0.0001$; Fig. 12). Significant differences occurred between replicates (GLM, $F_{3, 12} = 10.471$, $P = 0.001$) and significant interactions were found between replicates and treatments (GLM, $F_{12, 380} = 1.779$, $P = 0.0497$). However, the differences between treatments were unidirectional for all replicates. For this reason, all replicates were pooled for further analysis. Caged bees provisioned a 1:1 pollen: cellulose treatment diet had significantly greater lipids than treatment diets with a greater ratio of cellulose (ANOVA, $F_{1, 398} = 48.793$, $P < 0.0001$). Caged bees provisioned a 1:2 pollen: cellulose treatment diet did not have significantly greater lipids than caged bees given a 1:3 pollen: cellulose treatment diet (ANOVA, $F_{1, 158} = 3.792$, $P = 0.053$). Caged bees provisioned a 1:2 pollen: cellulose and a 1:3 pollen: cellulose treatment diet had significantly greater lipids than caged bees provisioned a pure cellulose treatment diet (ANOVA, $F_{2, 237} = 11.560$, $P < 0.0001$). Differences were found between treatments for the mean amount of bee lipids (ANOVA, $F_{4, 395} = 67.699$, $P < 0.0001$).

Overall, caged bees provisioned with a pure pollen diet had significantly more hypopharyngeal gland protein content than bees provisioned any of the other treatment diets (ANOVA, $F_{1, 397} = 128.141$, $P < 0.0001$; Fig. 13). No significant differences occurred between replicates for hypopharyngeal gland protein content (GLM, $F_{3, 12} =$

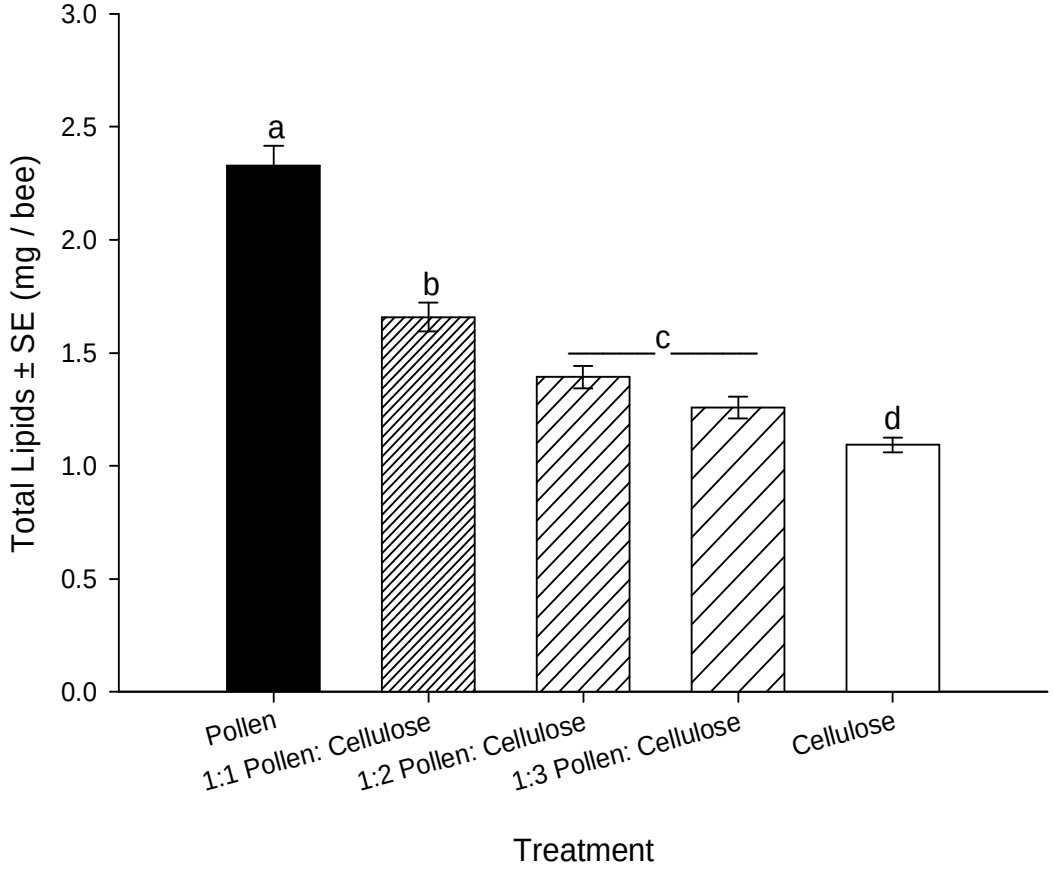

Figure 12. Mean total lipids per bee. Letters indicate significant differences between treatments (ANOVA, $P < 0.05$).

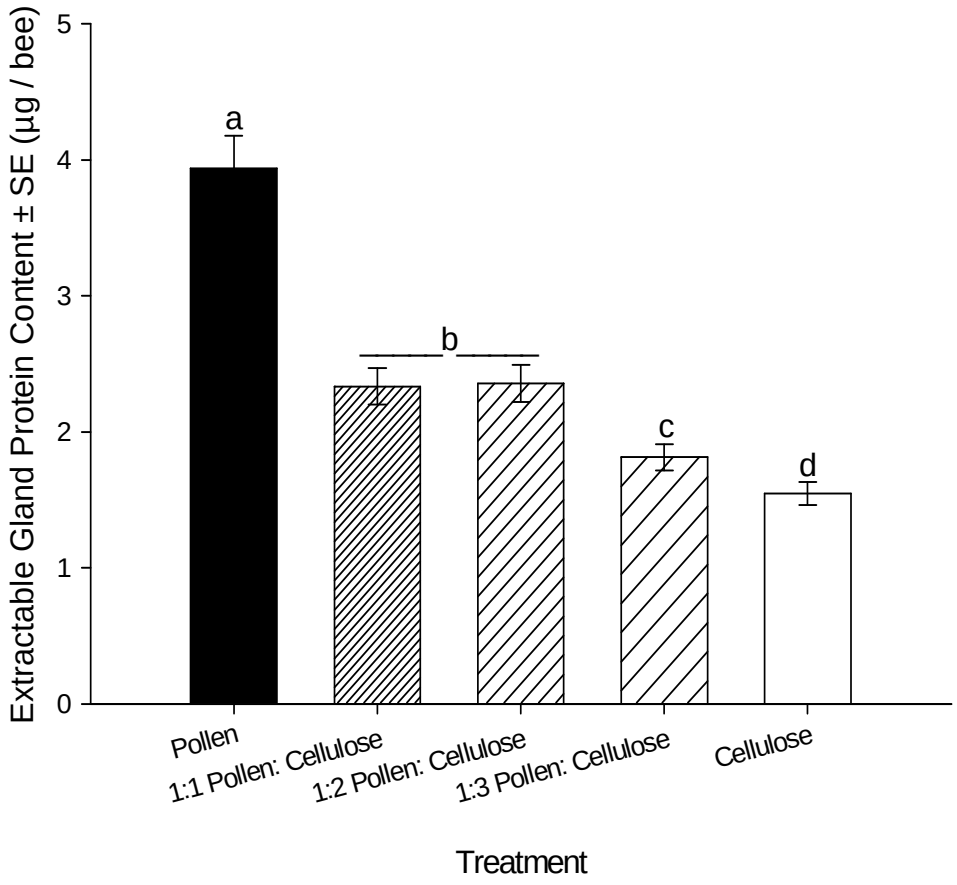

Figure 13. Mean bee hypopharyngeal gland extractable protein. Letters indicate significant differences between treatments (ANOVA, $P < 0.05$).

1.247, $P = 0.336$). Significant interactions were found between replicates and treatments (GLM, $F_{12, 379} = 5.616$, $P < 0.0001$). However, effect of treatment on protein extracted was unidirectional between replicates. Therefore, replicates were pooled for further analysis. Significant differences occurred between hypopharyngeal gland protein content for bees in all treatments (ANOVA, $F_{4, 394} = 38.979$, $P < 0.0001$; Fig. 13). Caged bees provisioned 1:1 pollen: cellulose and 1:2 pollen: cellulose treatment diets did not have significantly different extractable hypopharyngeal gland protein (ANOVA, $F_{1, 157} = 0.013$, $P = 0.909$; Fig. 13), but were both significantly greater than caged bees provisioned 1:3 pollen: cellulose treatment diet (ANOVA, $F_{2, 236} = 6.212$, $P = 0.002$; Fig. 13) and caged bees provisioned pure cellulose (ANOVA, $F_{2, 236} = 14.645$, $P < 0.0001$; Fig. 13). Caged bees provisioned 1:3 pollen: cellulose treatment diet had significantly greater extractable hypopharyngeal gland protein than caged bees provisioned pure cellulose (ANOVA, $F_{1, 158} = 4.447$, $P < 0.037$; Fig. 13).

Bees provisioned pure pollen had significantly greater extractable mandibular gland protein only from bees provisioned pure cellulose (ANOVA, $F_{1, 118} = 6.867$, $P = 0.010$; Fig. 14). Significant differences occurred between replicates (GLM, $F_{3, 12.371} = 78.642$, $P < 0.0001$), and significant interactions were found between treatments and replicates (GLM, $F_{12, 329} = 3.179$, $P < 0.0001$). However, overall, replicates showed unidirectional changes for each treatment. Therefore, replicates were pooled for further analysis. The total number of bees differed between treatments (pollen: n = 60; 1:1 pollen: cellulose: n = 80; 1:2 pollen: cellulose: n = 69; 1:3 pollen: cellulose: n = 80; and cellulose: n = 60). Caged bees provisioned a 1:1 pollen: cellulose treatment diet had

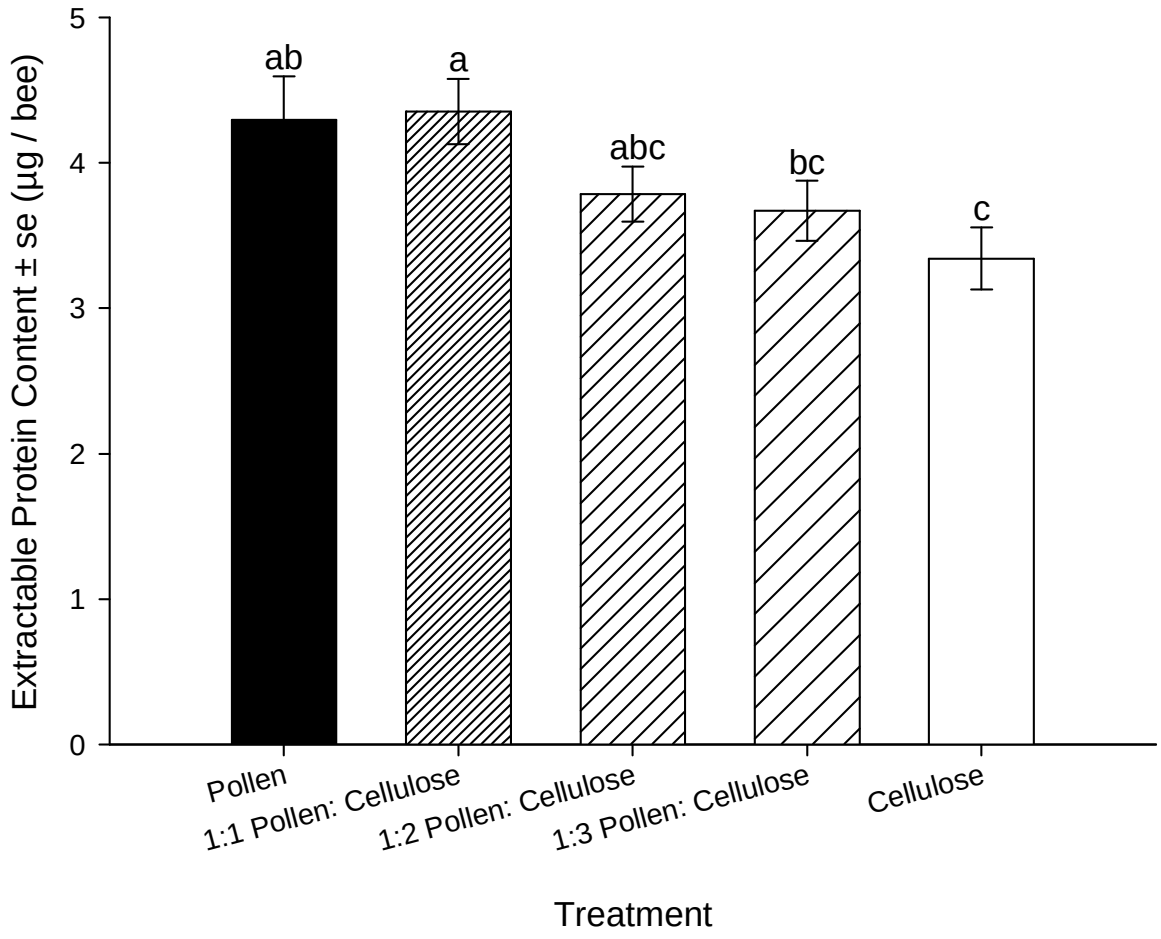

Figure 14. Mean bee mandibular gland extractable protein for each treatment. Total number of bees differed between treatments. Letters indicate significant differences between treatments (ANOVA, $P < 0.05$).

significantly greater extractable mandibular gland protein than caged bees provisioned a 1:3 pollen: cellulose treatment diet (ANOVA, $F_{1, 158} = 5.023$, $P = 0.026$) as well as greater extractable mandibular gland protein content from bees provisioned pure cellulose (ANOVA, $F_{1, 138} = 10.053$, $P = 0.002$).

Discussion

In this experiment I measured the effect of quality of pollen diet on worker bee mass, amount of extractable lipids from adult bees, amount of protein extractable from hypopharyngeal and mandibular glands, and consumption of diet. It is apparent that quality of pollen diet does affect the factors measured as significant differences due to dilution were found. In fact, there is a threshold of significance between pure pollen and 1:1 pollen: cellulose. Bees on the pollen diet were significantly different from bees on the 1:1 pollen: cellulose diet in every measure except mandibular gland protein content (Figs. 10 - 14). This included greater mean dry mass, greater total lipids, greater hypopharyngeal gland protein content, and less pollen consumption. From these results it is evident that significantly less consumption of the most concentrated diet is required to achieve the greatest adult bee mass, greatest adult bee extractable lipids, greatest extractable hypopharyngeal gland protein in comparison with diluted pollen diets.

Because quality of pollen diet affected diet consumption, it may be hypothesized that bees engage in compensatory consumption to meet nutritional needs. However, because bees were unable to achieve the same mean dry mass, total lipids, and hypopharyngeal gland protein content, diet consumption appears not to be compensatory

in nature. There is a significant threshold in each measured factor besides mandibular gland protein content between pollen diet and 1:1 pollen: cellulose, it may be possible that this dilution was too great to measure compensatory feeding mechanisms in bees. It may also be possible that bees do not engage in compensatory feeding at the colony level. Dussutour et al., (2007) found that caterpillars at the collective level chose between two food sources at random and became stuck at the chosen food source, despite nutritional balance, for 24 h due to trail following. Pernal and Currie (2001) found that in honey bees, no effect of stored pollen nitrogen quality could be found on the nitrogen quality of forager collected pollen.

It is surprising that mandibular gland protein content of bees on the pollen diet did not have the greatest extractable protein content and in fact only differed significantly from bees given pure cellulose (Fig. 14). It has previously been shown that pollen quality can have a significant effect on hypopharyngeal gland size (Peng and Jay, 1979) and protein content (Pernal and Currie, 2000). It is possible that because queens cannot be reared without worker mandibular glands (Peng and Jay, 1977), these glands are less affected by environmental circumstances than are the hypopharyngeal glands. The robustness of this gland would benefit *Apis mellifera* in the emergency rearing of queens.

CHAPTER IV

CONCLUSIONS

The thesis presented novel data on the effects of honey bee primer pheromones on worker mandibular gland food producing ability as well as data verifying the necessity of pollen for glandular protein content. The results in the preceding chapters accessed the extractable protein content of mandibular and hypopharyngeal glands in the presence of two primer pheromones, namely QMP and BP.

In the first experiment, it was shown that pollen was the main factor contributing to significant increases in extractable gland protein content and size strongly suggesting the necessity of pollen for the greatest amount of extractable protein content and gland size. BP increased hypopharyngeal glands when pollen was absent; increased mandibular gland protein content in treatments with pollen; and mandibular gland size in the queenless environment when combined with pollen indicating that the effects of BP are more complex than those of pollen. Future studies should include quantification of BP effects on gland activity as well as comparison with colonies containing live brood.

No differences were found between bees in the QMP and non-QMP environments in the absence of pollen. It can be concluded that the synthetic blend of QMP failed to regulate both food producing glands, including the mandibular gland, a key gland in royal jelly production. Most noteworthy was the significant increase of hypopharyngeal gland protein content and mandibular gland size of bees on treatment QMP + BP + pollen.

These results suggest that both primer pheromones are necessary for the greatest amount of extractable protein and gland size.

In this experiment, it was also noted that hypopharyngeal and mandibular gland extractable protein declined with age. This decline in hypopharyngeal glands conforms with previous cage studies and is indicative of an increased speed of bee development within the cages (Crailsheim and Stolberg, 1989; Lass and Crailsheim, 1996). It is possible that a physical stimulation such as the larvae cuticle is required to maintain gland protein content. It is also possible that the synthetic blends and pollen diet are not sufficient to maintain gland protein content.

The second experiment measured effects of varying pollen dilutions on hypopharyngeal and mandibular gland protein content, as well as bee mass and lipid content. In this experiment, bees on the pollen diet were significantly greater than bees on all other diluted diets in measurements of hypopharyngeal gland protein content, lipid content, and mass. Bees on the pollen diet also consumed significantly less diet than bees on all other diets. Consumption differences were not compensatory to meet nutritional needs. This conclusion derived from the inability of bees on diluted or pure cellulose diets to achieve the same adult bee mass, extractable lipids, and extractable gland protein as those of bees given pure pollen.

Bees on the pollen diet had a mandibular gland protein content significantly greater only from pure cellulose. It is possible that due to the mandibular gland's significance in royal jelly production (Lensky and Rakover, 1983; Peng and Jay, 1977, 1979) that this gland may be more robust that than of the hypopharyngeal gland. From

this experiment it was concluded that the most concentrated diet promoted the greatest worker bee mass, extractable lipids, and hypopharyngeal gland extractable protein content.

Overall, the results of these two experiments strongly suggest that for hypopharyngeal and mandibular glands to reach maximum extractable protein, a concentrated pollen diet combined with both QMP and BP should be utilized.

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Peters, L. and T. Pankiw (2008). Effect of pollen diet and primer pheromones on worker honey bee mandibular glands. 9th Ecological Integration Symposium and TAMU Student Research Week. College Station, TX

Peters, L. and T. Pankiw (2008). Effect of pollen diet and honey bee (*Apis mellifera*) primer pheromones on worker bee mandibular glands. Southwest Branch Entomological Society of America. Ft. Worth, TX

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