CHARACTERIZING ECDYSTEROID TITER PROFILES AND THE
FUNCTIONAL ROLE OF ECDYSTEROIDS IN ADULT WORKER
HONEY BEES (APIS MELLIFERA)

BY

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A Dissertation Submitted to the Graduate Faculty of
WAKE FOREST UNIVERSITY GRADUATE SCHOOL OF ARTS AND SCIENCE
in Partial Fulfillment of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY
Biology
May 2016
Winston-Salem, North Carolina

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Acknowledgements

I cannot properly thank my advisor, Dr. Susan Fahrbach. Susan has been not only a mentor but a role model for the type of scientist, teacher, and mother I hope to be. She welcomed me into her lab and inspired a love of honey bees that will follow me the rest of my life. She often had confidence in my own abilities that I doubted and has pushed me to be a better writer, researcher, and teacher. In my six years at Wake Forest University I’ve brought news to Susan of personal events, both good and trying, that at times took my time and attention away from the lab. She never mistook those moments as a lack of dedication to my work or aspirations. I cannot thank you enough for the support of both my professional and personal life. It has been an honor to be a part of your lab.

I also want to thank the members of my committee; Dr. David Anderson, Dr. Bill Conner, Dr. Wayne Pratt and Dr. Olav Rueppell. Your questions, guidance and support in the completion of this dissertation have been invaluable. I would also like to thank Dr. Erik Johnson who often opened his lab to me for use of equipment and was always open to questions, as well as Dr. Dan Johnson, who encouraged my love of teaching and never once questioned one of my crazy ideas or suggestions. Our conversations in lab prep and your teaching courses will stay with me as I continue this journey in to the world of teaching. I’d also like to thank the members of the Southern Appalachian Honey Bee Research Consortium. The guidance, support and advice from the members has been so influential to my research.

The members of the Fahrbach lab: Scott, Rodrigo, Byron, Stephanie, Erika, James, Janeth, Simone, David, Katherine, Tatianaide, Chrissy, Rachel, Anissa (and anyone else I may have forgotten); thank you for your time, feedback, laughter and support. I consider myself lucky to have come to a lab with a surplus of truly friendly and kind people. Trips to the zoo and Brushy Mountain, lunch dates, bee painting parties, random animals in the lab, these are some of my fondest memories of my time at Wake Forest.

My family and friends, thank you for reminding me that life goes on outside the academic world. Jessica, Megan, and my Island girls for being my sounding board and understanding of my often disappearances into this crazy world of research. My siblings; Amie, Justin, and Savannah, you’ve been some of my greatest champions and have always been able to bring me back down to size. Most especially my parents, for loving and supporting me. You taught me to question the world around me and that I could do anything I set my mind to, for that I will always be grateful.

My beautiful, amazing son Everett. You showed up a little later in this journey and while you made things more complicated at times you have also made them infinitely more rewarding. You’ve reminded me that the impact factor of the most prestigious journal pales in comparison to the impact of Good Night Moon and The Very Quiet Cricket. You are my greatest accomplishment and I love you to the moon and back.

Finally and most of all, my husband, Andy. From the moment I asked you to start this crazy journey to North Carolina, to leave your family, friends and job for me, you were in whole heartedly. You’ve had more faith in me through this entire process then I’ve ever had in myself. Your love for me is a constant motivator and I will never be able to repay you for the strength and support you have given me. You are my home and I love you endlessly.
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List of Abbreviations

20-hydroxyecdysone (20E)

Ecdysteroids (E)

Ecdysone receptor (EcR)

Glucose oxidase (GOX)

Immunocompetence (IC)

Juvenile hormone III (JH)

Naturally mated (NM)

Queenless (QL)

Queen mandibular pheromone (QMP)

Queenright (QR)

Reproductive ground plan hypothesis (RGPH)

Single Drone Inseminated (SDI)

Vitellogenin (Vg)
The reproductive ground plan hypothesis (RGPH) suggests the coordination of social behaviors in honey bees emerged from pathways that regulate reproduction in solitary species. I examined possible mechanisms of the RGPH in honey bees through analysis of the ecdysteroid profiles of adult worker honey bees (*Apis mellifera* L.). Ecdysteroids have been thought to play little role in the physiology of adult worker honey bees outside of reproduction because little was known about ecdysteroid production across the lifespan of individual workers. The data reported here reveal that significant and dynamic production of ecdysteroids occurs in adult worker honey bees, typically in the absence of morphological signs of ovarian development, and that queen mandibular pheromone (QMP) regulation of ecdysteroid production is dependent upon social context. Additionally I explored novel roles of ecdysteroids in behavior and physiology. Comparison of observations in the first days of foraging revealed that 20-hydroxyecdysone (20E) treated bees displayed higher levels of foraging activity than vehicle-treated controls, suggesting a novel role for the ecdysteroids present in adult worker bees. I further demonstrated preliminary evidence of a modulatory effect of QMP on hemocytes in worker bees and a potential enhanced recovery from a decrease in hemocytes by treatment of 20E. I also provide evidence that bees held in queenless conditions may have unique immunocompetence profiles, specifically reducing the production of glucose oxidase (GOX). The results presented here provide an updated view of ecdysteroid profiles of adult bees and present an initial survey of the role of ecdysteroids and pheromone exposure in the physiology and behavior of adult worker honey bees.
CHAPTER 1

INTRODUCTION

A perennial question in biology asks how complex phenotypes evolve. Eusocial species such as the honey bee (*Apis mellifera*), with their intricate social organization and reproductive castes, have provided instructive but puzzling models for the study of such questions. Our current understanding of eusociality – defined by Wilson (1971) in terms of reproductive castes, cooperative brood care, and overlapping generations – was initially informed by the concepts of kin selection and inclusive fitness (Hamilton, 1972). Contemporary perspectives, however, recognize that other factors, such as construction of a permanent nest, are also important in promoting the evolution of societies in which some individuals do not reproduce (Nowak *et al.*, 2010; Avila and Fromhage, 2015; Olejarz *et al.*, 2015).

Another important level of analysis involves the physiological mechanisms that support the transition to eusociality. West-Eberhard (1987; 1996) proposed a mechanistic explanation for the evolution of social systems that include a non-reproducing female worker caste by positing that physiological coordination of social behaviors emerged from the pathways that regulate reproduction in solitary species. A modern formulation of this concept is called the reproductive ground plan hypothesis (RGPH), and considerable evidence has been presented in support of the RGPH in honey bees (Amdam *et al.*, 2004; Amdam *et al.*, 2007; Guidugli *et al.*, 2005; Toth and Robinson, 2007; Amdam and Page, 2010). An example of this is the recognition that vitellogenin serves as
both a yolk protein precursor and as a source of nutrients used in the synthesis of brood food proteins in the hypopharyngeal glands of worker bees (Amdam et al., 2003).

I sought to examine possible mechanisms supporting the RGPH in honey bees through analysis of the endocrine profiles of adult worker honey bees. I focused on the presence of the ecdysteroids in adult workers because their assigned function in adult female insects is regulation of protein yolk precursor synthesis and yolk deposition into eggs. The results presented here provide a substantially updated view of the ecdysteroid profiles of adult worker honey bees, describe new evidence of the modulatory effects of queen mandibular pheromone (QMP) on ovarian steroidogenesis, and begin to define novel roles of ecdysteroids in adult worker physiology and behavior.

1.1 Ecdysteroids

Steroids are fat-soluble organic molecules that share a 4-ring, 17-carbon core structure (Norris, 2007). There are numerous naturally occurring steroids, including sterols, bile acids, and the brassinosteroids of plants. Some steroids serve as signaling molecules that coordinate development, reproduction, and responses to environmental stressors. Here I restrict my considerations to steroids produced by animals. Among the metazoans, vertebrates are remarkable because they synthesize five different classes of steroids that function as hormones: estrogens, androgens, progestins,

**Figure 1.** Schematic of generalized ecdysteroid. Ecdysone, X = H, Y = OH; 20-hydroxyecdyson, X = OH, Y = OH
mineralocorticoids, and glucocorticoids. By contrast, invertebrate phyla, including the arthropods, produce only a single class of steroid hormones called ecdysteroids (Klowden, 2007) (Fig 1).

Steroid hormones partner with intracellular receptors to function as transcription factors. The structure of canonical steroid hormone receptors is strikingly conserved in metazoans (Markov and Laudet, 2011) (Fig. 2). All steroid hormone receptors have distinct DNA-binding and ligand-binding domains. Steroid hormone receptors are members of a larger family of nuclear receptors that can be recognized on the basis of this conserved structure. Genome projects have allowed the number of nuclear receptors present in different species to be estimated, primarily through the application of bioinformatics methods that recognize the conserved DNA-binding domain in sequence databases (e.g. King-Jones and Thummel, 2005).

The insect receptor for ecdysteroids is a canonical nuclear receptor (Fahrbach et al., 2012). The gene encoding the receptor is named ecdysone receptor. The gene symbol is EcR, with a prefix that indicates species: for example, DmEcR refers to the ecdysone receptor gene of Drosophila melanogaster, and AmEcR refers to the ecdysone receptor gene of Apis mellifera. The mechanisms of ecdysteroid action in metamorphosis have been extensively investigated in both holometabolous and hemimetabolous insects (Riddiford and Truman, 1993; Thummel, 1995). Pulses of ecdysteroid synthesis regulate all molts and a sustained high level of ecdysteroids drives adult development after
pupation. Larval and pupal ecdysteroid synthesis and secretion occur in the prothoracic glands in response to secretion of the brain neuropeptide, prothoracicotropic hormone (Gilbert et al., 1997). The primary product of the prothoracic glands is ecdysone, a 27 carbon steroid. The active ecdysteroid in most arthropods is 20-hydroxyecdysone (20E), metabolized at its target tissues from ecdysone to 20E by specific cytochrome P450 enzymes (Feldauer et al., 1985; Feyereisen, 1999). In holometabolous insects, including honey bees, the prothoracic glands degenerate after the molt to the adult stage (Dai and Gilbert, 1997; Snodgrass, 1956). This is unsurprising given that the primary function of ecdysteroids is to regulate molting, and adult insects do not molt.

In some adult female insects, the locus of ecdysteroid synthesis shifts to the ovaries. In these species, ovarian ecdysteroids are released into the hemolymph. The fat body, the source of a yolk protein precursor called vitellogenin (Vg), is the best-characterized target of ovarian ecdysteroids (Raikhel, 1992). The best known example of ecdysteroid action on the fat body comes from the yellow fever mosquito, Aedes aegypti. In this species, a blood meal is required to initiate oogenesis and ecdysteroid biosynthesis. After a blood meal, brain neurosecretory cells release ovarian ecdysteroidogenic hormone, which induces ovarian follicle cells to synthesize ecdysone (Hagedorn et al., 1975; Dhara et al, 2013; Gulia-Nuss et al., 2012). Circulating ecdysone is taken up by the fat body, converted to 20E, and then regulates the transcription of Vg by binding to the canonical ecdysone receptor (Raikhel et al., 2002).

Studies in the dipteran Drosophila melanogaster revealed that nuclear receptors, including the ecdysone receptor, are expressed in multiple tissues during all life stages, including the adult stage in both males and females (Burtis et al., 1990; Bayer et al.,
1996; Koelle et al., 1991; King-Jones and Thummel, 2005). Although the finding was initially surprising, it is now known that nuclear receptors for ecdysteroids are abundantly expressed in the adult central nervous system of several insect orders in addition to Diptera, including Hymenoptera, Lepidoptera, and Orthoptera (Riddiford et al., 1999; Wang et al., 2000; Velarde et al., 2006, 2009; Nemoto and Hara, 2007; Velarde et al., 2009; Fahrbach et al., 2012). However, the prevailing view is that, with the exception of females with active ovaries, titers (circulating levels in the hemolymph) of ecdysteroids are low to nonexistent in adult insects and therefore play little role in the regulation of adult physiology (Robinson et al, 1991; Hartfelder et al., 2002). This view predicts the absence of ecdysteroids in sterile adult workers in eusocial insects such as the honey bee.

What does it mean when the receptor for a hormone is abundantly expressed (as it is in the brains of adult worker honey bees) yet the circulating hormone is absent? Cases of mismatch between receptor and ligand raise several possibilities. It is possible that the receptor is a relic of earlier developmental or evolutionary phases and truly plays no role in adult life. Alternatively, perhaps the receptor responds to unique or undiscovered ligands or, in the case of nuclear receptors, the possibility remains that the unliganded receptor regulates transcription (Truman & Riddiford, 2002; Baker et al., 2003). Another possibility is that the existing hormone data are incomplete and that the production of ecdysteroids in adults is significantly underestimated.

Challenging the dogma regarding hormone profiles can lead to exciting discoveries. While ecdysteroid titers have been determined for several species, insect endocrinology is a field plagued with problems related to the small size of the experimental subjects. For example, the small volume of hemolymph that can be
obtained from a single insect often forces researchers to pool samples from multiple individuals, resulting in the masking of variation between individuals; it is also rare for repeated samples to be available from a single individual, as the process of sampling typically results in the death of the insect. Additionally, once subjects are beyond defined developmental transitions that can be synchronized by reference to external markers, transient peaks will inevitably be missed. Another intriguing possibility is that circulating levels of ecdysteroids are irrelevant to actions on the central nervous system, because nervous tissue itself is the source of a ligand. This would be similar to the ability of the vertebrate brain to synthesize behaviorally-relevant steroids (e.g. London et al., 2006; Schlinger 2015).

Why raise this question? In vertebrate physiology, it has been long understood that steroid hormones have significant actions in the central nervous system (reviewed by McEwen, 2001; Mong and Pfaff, 2004; Lee and Pfaff, 2008; Hara et al., 2015). Steroid hormones have been shown both to facilitate and suppress behavioral responses. For example, the ovarian hormones estradiol and progesterone, in addition to facilitating sex behavior, can alter mood, cognition, and sensory responses (Smith, 2004). If we do not consider a possible regulatory role for steroid hormones in the regulation of brain structure and function (including behavior) in adult insects, as is seen in vertebrates, we risk overlooking a major mechanism for integration of behavior and physiology during this life stage (Fahrbach et al., 2016).

Steroid hormones also act on many peripheral targets, such as the effect of estradiol on the vascular system (Mendelsohn, 1999) or the reproductive tract of vertebrates, which in turn may feed back onto the nervous system in simple or complex
signaling loops. Simultaneous actions of steroid hormones on central and peripheral targets are important because they coordinate physiology with behavior. Ongoing research in the Fahrbach laboratory focuses on direct actions of ecdysteroids on the central nervous system of the European honey bee (*Apis mellifera*). This research was inspired by the bioinformatics-based discovery of the expression of *AmEcR* in the brains of adult honey bees (Velarde *et al.*, 2006). The goal of my research was to build on these discoveries to understand the broader endocrinology of ecdysteroid signaling in adult honey bees. The worker honey bee provides an excellent model for studies of ecdysteroids in a non-reproductive context, as worker honey bees only rarely reproduce but still have ovaries, the most likely source of ecdysteroids in adult females. The honey bee model also offers a within-species comparative approach, as workers can be compared with queens.

1.2 The Honey Bee Model

Honey bees are eusocial insects that live in persisting colonies of up to 100,000 individuals. Each colony consists of a single mated, egg laying female (the queen), varying numbers of male drones, and tens of thousands of female workers that are facultatively sterile (Winston, 1987; Jackson *et al.*, 2011; Maisonnasse *et al.*, 2010; Smith *et al.*, 2013). Honey bees are widely used in studies of the evolution of sociality, behavioral ecology, and insect learning. Honey bees are also well studied because they are economically significant pollinators. For example, in the United States honey bees are responsible for the pollination of at least 95 different agricultural crops with an estimated economic value of $15.12 billion (Calderone, 2012).
All female eggs laid by a honey bee queen have the potential to develop as either a fully reproductive queen or a facultatively sterile worker. Sex determination in honey bees is expressed through a haplodiploid system: fertilized (diploid) eggs usually are females that develop into workers or queens, and unfertilized (haploid) eggs develop into drones. A primary role for the state of zygosity of a gene called *csd* (*complementary sex determiner*) has been demonstrated: all individuals heterozygous at this locus differentiate as females, while homozygous and hemizygous individuals differentiate as males (Beye, 2004). As a consequence, all female honey bees are diploid while males can be either haploid or diploid (Beye, 2004). Uncommon adult castes, such as worker-laid males, sometimes occur naturally and can also be experimentally induced (Poirie *et al.*, 1992).

By contrast to sex determination, which is genetically based and occurs in the embryo, caste determination in female honey bees is determined early in larval life by environmental factors such as size of the cell in which the egg is laid and the quantity and quality of the diet fed to developing larvae. A diet rich in royal jelly is fed to future queens, and this diet in turn induces changes in gene expression that organize irreversible changes in anatomy, physiology, and behavior (Evans and Wheeler, 1999; Evans and Wheeler, 2001; Wheeler *et al.*, 2006). Most female larvae, however, are fed a lower quality diet and develop as workers in terms of anatomy, physiology, and behavior (Lyko *et al.*, 2010; Shi *et al.*, 2011; Ashby *et al.*, 2016). Differential feeding affects a gene network sensitive to nutritional status, induces changes to DNA methylation and results in changes in hormone titers during development (Patel *et al.*, 2007; Kucharski *et al.*, 2008; Lyko *et al.*, 2010; Hartfelder and Engels, 1998). This results in the queen/worker
polyphenism, as well as variation in the number of ovarioles (the egg tubes that form the ovaries) within the worker caste (Jackson et al., 2011).

A benefit of a reproductive caste system is that individuals can be specialized for their role in the colony (Wilson, 1971). Many of the structures related to behaviors performed only by workers, such as foraging, are absent or reduced in the queen. For example, compared with workers, queens have a shortened proboscis and a reduced number of facets in the compound eyes. By contrast, the ovaries of queens are enormous compared with the ovaries of the worker. While most workers have 2-12 ovarioles, queens are born with 150-180 (Jackson et al., 2011). Queens can produce enormous numbers of eggs during a reproductive lifespan of up to three years, and it has been estimated that many queens lay a million eggs or more. Queens also have very large, well developed mandibular glands and use these glands to produce pheromone signals.

I will now review the specific aspects of queen and worker physiology relevant to my dissertation. I will first discuss the evidence that active ovaries are associated with ecdysteroidogenesis in honey bee queens. Next, I will describe the established effects of queen pheromones on worker ovaries and other tissues. I will then describe the reproductive physiology and hormonal regulation of behavioral maturation in workers.

1.3 Queen Physiology

While ecdysteroids are considered a female reproductive hormone in many insect species, the endocrine profiles of adult honey bee queens have been rarely studied and the resulting data are conflicting. A study in the 1980s isolated active ecdysteroids from the honey bee queen ovaries (Feldlaufer et al., 1986.) Only two subsequent studies have
examined honey bee queen ecdysteroid titers in the hemolymph. One study showed elevated ecdysteroids, relative to worker bees, when using pooled samples collected from mated and virgin queens ranging in age from 2-14 months (Robinson et al., 1991). Another study reported similar ecdysteroid titers in queens compared with workers and found no difference between mated and virgin queens (Hartfelder et al., 2002). In the primitively eusocial species *Bombus terrestris* (the buff-tailed bumblebee), endocrine titers have a broader role in shaping the social hierarchy within the colony. In this species, mated queens have significantly higher ecdysteroid titers than virgin queens and workers in the hive (Bloch et al., 2000). Further, in bumblebees, the ascension of a new queen, which can be any worker in the colony, is closely correlated with a sudden rise in the ecdysteroid titer (Bloch et al., 2000). While the bumblebee scenario presents an example of endocrine signaling co-opted from a reproductive role to organize social hierarchy, it provides little guidance for understanding the fully eusocial honey bees in which workers cannot become queens.

### 1.4 Queen Mandibular Pheromone

The term QMP refers to the queen pheromone produced in the mandibular glands (QMP = queen mandibular pheromone) (Fig 3). The main component of QMP is the compound 9-oxo-2-decenoic acid (9-ODA). QMP serves many purposes within the colony, from attracting young workers to tend the queen and suppressing the rearing of new queens to inhibiting egg laying by workers (Naumann, 1991a; Bortolotti and Costa, 2014; Traynor et al., 2014). QMP is dispersed throughout the colony through trophallaxis, antennation, and cuticular contact between workers. As a result, even those
workers that do not contact the queen directly are affected by the QMP she produces (Naumann et al., 1991b).

One of the primary roles of QMP is to inhibit reproduction by workers by maintaining the ovaries in a morphologically undeveloped (“inactive”) state (Winston et al., 1989, Winston et al., 1990, Traynor et al., 2014). While QMP plays a pivotal role in this inhibition, it is now understood that QMP has synergistic effects with other pheromones within the colony in suppressing ovarian activation (Willis et al., 1990; Hoover et al., 2003, Traynor et al., 2014). Additionally, QMP impacts worker behavior through instigation of the queen retinue response (Slessor et al., 1988; Kaminski et al., 1990). The retinue response is a dynamic circle of young workers that follows the queen. The members of the retinue feed and groom the queen between her bouts of egg laying (Winston, 1987).

As might be expected given its effects on behavior, QMP has also been shown to have significant effects on gene expression in the brains of worker bees. 9-ODA is detected by the odorant receptor, AmOr11, located on the antenna of honey bees (Wanner et al., 2007). Calcium imaging studies in drones have shown that QMP is then processed in an enlarged glomerulus in the antennal lobe (Sandoz, 2006; Jarriault and Mercer, 2012). Newly emerged bees held in cages in a laboratory with or without exposure to QMP for 3 days show significantly altered gene expression in the brain (Grozinger et al., 2003). Using microarrays and mRNA quantification by qRT-PCR, Grozinger and

Figure 3. Five primary components of QMP: (A) 9-ODA, (B) +9-HDA, (C) -9-HDA, (D) methyl p-hydroxybenzoate and (E) 2-(4-hydroxy-3-methoxy-) phenyl ethanol
colleagues showed that exposure to QMP transiently regulates the expression of several hundred genes and had a chronic effect on expression of 19 different genes.

1.5 Worker Physiology

What is the evidence that ecdysteroids are not present in adult worker honey bees? Several studies have used the technique of radioimmunoassay to define ecdysteroid profiles of honey bee workers and queens. These studies have focused on the concentration of ecdysteroids present in the hemolymph. This is equivalent to measurements of steroid concentrations in vertebrate blood. The few published studies are contradictory, with reports of titers in adult non-reproductive workers ranging from transient peaks of 15 pg/µl to levels too low to be detectable and assumed to be zero with notable (but ignored) outliers as high as those of workers actively laying eggs (Robinson et al., 1991, Hartfelder et al., 2002). These results have led to the view – which over time hardened into a dogma – that ecdysteroids do not play a central role in the physiology of adult honey bees. The prevalence of this view discouraged investigators from exploring a further role for ecdysteroids in most adult insects, including honey bees. The exceptions to this lack of interest in ecdysteroids in adult worker honey bees are a few studies focused on a small pulse of ecdysteroids detectable in worker hemolymph on the third day of adult life (Velarde et al., 2009, Velarde et al., 2006; Amdam et al., 2010; Hartfelder et al., 2002). This peak of ecdysteroids on the 3rd day of life induces the expression of several genes in the Kenyon cells (mushroom body neurons) of the honey bee brain, including AmEcR and AmE75 (Velarde et al., 2009), and it has been speculated that these ecdysteroid pulses might regulate plasticity in the brains of young adult honey bees (Fahrbach et al., 2016).
When the Kenyon cells of the honey bee mushroom body are grown in short-term primary culture and treated with 20E these neurons are more likely to extend a branched process than controls not exposed to the steroid (Velarde et al., 2010). Furthermore, this growth and the upregulation of AmE75, an early gene in the ecdysteroid response cascade, can be attenuated through the use of RNAi interference and dsRNA targeting EcR, suggesting a causal relationship between ecdysteroid signaling and neuronal plasticity in adults (Velarde and Fahrbach, 2011). Such a relationship is further supported by an in vivo study that showed that injection of ecdysteroids into one day old honey bees accelerated dendritic growth in protocerebral neurons (Ford et al., 2011).

As previously noted, worker ovaries vary in size. The size of honey bee ovaries is typically expressed in terms of the total number of ovarioles present, with worker honey bees typically having between 2 and 12 ovarioles (Michener, 2007) (Fig 3). Variation in ovary size in workers has been correlated with attributes such as age of first foraging and foraging specialization on nectar or pollen (Amdam et al., 2006; Wang et al., 2010). The number of ovarioles is established prior to completion of metamorphosis (Evans and Wheeler 1999; Evans and Wheeler 2001; Wheeler et al., 2006), and therefore does not change during adult life.
In a typical colony, a mated, egg-laying queen is present for the duration of a worker’s life. This colony condition is referred to as queenright (QR). As noted, egg laying by workers is suppressed primarily by exposure to QMP (Naumann, 1991a; Tsuruda et al., 2008). Additional suppression of ovarian morphological development in workers reflects exposure to a pheromone produced by the developing larvae of the colony called brood pheromone (Hoover et al., 2003). Under certain circumstances, however, the limited number of ovarioles present in adult workers are said to be “activated.” A stereotyped sequence of morphological development is associated with the laying of haploid eggs and an increase in ecdysteroid titers (Robinson et al., 1991).

Colonies that have lost their queen are referred to as queenless (QL) colonies. Following the loss of their queen, colonies attempt to rear existing larval brood as queens. In hopelessly QL colonies (those that lack suitable brood therefore will not be able to raise a new queen), a subset of workers will begin to lay haploid eggs that develop as males. It takes approximately 12-24 hours for a colony to recognize the loss of a queen (Bortolotti and Costa, 2014) and 14-30 days for a subset of workers to begin laying (Winston, 1987). Although small numbers of workers in QR colonies may possess fully

**Figure 4.** Drawing of typical ovaries in (A) facultatively sterile worker bee ovaries, (B) laying workers with ovaries displaying swelling and distinct oocytes and (C) fully functional ovaries of a mated queen.
developed ovaries, this percentage is minimal, typically less than 1% (Page and Erickson, 1988; Ratnieks, 1993). A positive correlation between increased ovariole number and the propensity to have developed ovarian follicles has been suggested on the basis of worker genotype (Makert et al., 2006). The genetic predisposition of workers, specifically patriline, is possibly a significant indicator of which individuals will become laying workers in a hopelessly QL colony (Martin et al., 2004; Makert et al., 2006).

1.6 Hormonal Regulation of Behavioral Maturation

The behavior of worker honey bees in a QR colony is characterized primarily by age polyethism, defined as division of labor on the basis of worker age. Workers perform a progression of tasks over their life span. This temporal behavioral caste structure has the property of simultaneously being predictable and responsive to colony conditions. The vast majority of young adult workers perform tasks within the hive. At 2 – 3 weeks of age, most workers stop performing in-hive tasks and become foragers (Seeley, 1995). The regulation of the transition from in-hive tasks to foraging has been extensively studied and numerous factors identified. Some of these include nutritional status, changes to colony demography, pheromone exposure, and changes in biogenic amine levels (Schulz and Robinson, 2001; Toth et al., 2005; Pankiw et al., 1998; Huang and Robinson, 1992).

One important factor is Vg. Vg levels are high in young workers and peak during the nurse period, a period during 5 to 15 days of age characterized primarily by brood care (Seeley 1982; Crailsheim 1992). In support of the RGPH, this behavioral group correlates with the reproductive role of Vg in females of solitary species, in which high levels of Vg characterize the period of caring for developing brood. The subsequent
switch to foraging tasks is accompanied by a decrease in the circulating levels of Vg (Bloch et al. 2002, Amdan and Omhold 2003; Guidugli et al. 2005). Recently, the RGPH has extended to explain distinctions not only between queens and workers but also within the foraging behavioral caste. Foraging specialization (whether an individual forager prefers pollen or nectar), has been shown to be correlated with number of ovarioles in workers (Amdam et al., 2006).

The transition to foraging is also influenced by juvenile hormone (JH) from the corpora allata glands. JH is a sesquiterpenoid molecule synthesized by the corpora allata through most of adult honey bee life (Gade et al., 1997). Young workers, working primarily as nurses, have significantly lower levels of JH than older foragers (Robinson, 1987; Robinson et al., 1989). Treating young bees with JH results in early or precocious foraging in a subset of treated bees, a result that suggested that a naturally-occurring increase in endogenous JH might regulate the shift from in-hive to field tasks (Robinson, 1985; Robinson et al., 1989; Sasagawa et al., 1989). However, while increased JH titers are strongly associated with a shift to foraging tasks, JH is not required for a worker to become a forager, as shown by a study that induced foraging in bees that had their corpora allata removed at the end of metamorphosis (Sullivan et al., 2000).

The critical aspect of JH in the regulation of the behavioral transition to foraging in worker honey bees appears to involve a link between JH and Vg. The interaction between the hormone and protein can be described as one of double repression, with Vg repressing JH and JH repressing Vg (Guidugli et al., 2005). This relationship contrasts with the role of JH and Vg observed in most insects, as JH typically drives increases in Vg synthesis (Robinson and Vargo, 1997). This, and other evidence in support of the
RGPH, has led us to examine new roles for classically defined components of adult physiology and behavior in the honey bee. From the ecdysteroid perspective, this changes everything. Perhaps adult worker ovaries are significant components of adult physiology and behavioral maturation process because they are the site of ecdysteroid biosynthesis, and ecdysteroids regulate the synthesis of Vg in honey bees?

1.7 Hormonal Regulation of Immunocompetence

Unlike vertebrates, which defend against pathogens with both innate and acquired (antibody-based) immunity, insects rely exclusively on their innate or non-specific immune responses. The initial annotation of the honey bee genome revealed that honey bees have roughly 1/3 as many genes in the 17 primary gene families involved in insect immunity (Evans et al., 2006). This is interpreted as a reflection of the high level of sociality exhibited by this species (Evans et al., 2006). The primary barrier to immune challenges are the cuticle and digestive tract, however when pathogens breach the hemocoel, additional defenses are necessary (Klowden, 2002). The innate immune system has two components, the hemocytic and humoral responses (Lavine & Strand, 2008). The hemocytic responses (also referred to as cell-mediated immunity) are based on hemocytes, macrophage-like cells resident in the hemolymph that can induce phagocytosis, cell aggregation, and nodulation (Lavine & Strand 2002). Honey bees produce several types of hemocytes, including plasmatocytes and granulocytes, the major hemocyte types involved in phagocytic responses (Klowden, 2002). Humoral responses include the release of antimicrobial and antifungal peptides from fat body and the release of enzymes that initiate melanization.
In addition to hemocyte and humoral defenses against pathogens, honey bees also display a phenomenon referred to as social immunity. The best characterized example of this is the addition of secretions to larval food and honey that results in the widely known antiseptic properties of those substances (White et al., 1963). An example of such a substance is glucose oxidase (GOX), a product of the hypopharyngeal glands of the worker honey bee (Alaux et al., 2010).

Similar to their role in behavioral maturation, JH and VG influence the immunocompetence (IC), the ability of an organism to mount an immune response, of honey bees (Amdam et al., 2004; Amdam et al., 2005). During adult life, worker honey bees have been reported to display a pattern of immunosenescence, or a reduction of immune efficiency, correlated with age and worker role in the colony (Amdam et al., 2005). As worker bees transition to foraging, the number of circulating hemocytes declines (Rutz et al., 1974; Wille and Rutz, 1975; Amdam et al., 2004). This decline can be reversed if workers are forced to revert to performing tasks inside the hive. The reversal is accompanied by a drop in JH and an increase in circulating Vg suggesting a potential regulatory role of hormones on the immune system (Amdam et al., 2004; Amdam et al., 2005). Further, laying workers, which have elevated Vg and 20E titers, have been shown to have lower viral loads then their age matched sisters (Cardoen et al., 2011). This suggests a potential regulatory role of ecdysteroids in the regulation of IC in honey bees.
1.8 Ecdysteroids in Other Adult Insects

Scattered reports have described the presence and possible function of ecdysteroids in adults of other insect species. When injected with 20E, male Egyptian cotton leaf worms, *Spodoptera littoralis*, upregulated nuclear receptors for ecdysone in a dose-dependent manner (Bigot *et al.*, 2012). The injections were also sufficient to induce the ecdysone signaling pathway in the antennae, suggesting ecdysone may play a role in hormonal regulation of olfactory processing. A limited number of *in vitro* studies have shown that ecdysteroids have direct growth promoting effects on some groups of neurons in the hawkmoth *Manduca sexta*, the silk moth *Bombyx mori*, and the fruit fly *Drosophila melanogaster* (Park *et al.*, 2003; Kraft *et al.*, 1998; Prugh *et al.*, 1992). In contrast, ecdysteroids were found to decrease new neuron numbers in distinct brain regions of a small number of Gryllidae (cricket) species (Cayre *et al.*, 1994; Cayre *et al.*, 2000; Malaterre *et al.*, 2003).

Further evidence that ecdysteroids regulate adult physiology and behavior is found in a small number of recent studies of fruit flies. Under some circumstances, ecdysteroids can influence neuron excitability: specifically, ecdysone-deficient mutants showed reduced synaptic strength (Li *et al.*, 2001). A 2009 study reported that reduced availability of 20E rendered adult male fruit flies defective in their ability to form long term memories related to courtship events five days after a seven hour training period (Ishimoto *et al.*, 2009). Additional studies showed that male *Drosophila* with reduced ecdysteroid receptor activity or 20E availability due to a targeted temperature sensitive mutation displayed male-to-male courtship instead of typical heterosexual courtship (Ganter *et al.*, 2007; Dalton *et al.*, 2009; Ganter *et al.*, 2011). Ecdysteroid titers also
affect wakefulness patterns in male and female *Drosophila*, with ecdysteroid deficient mutants showing reduced sleep-bout durations (Ishimoto & Kitamoto, 2010). Sleep-bout durations increased when the mutants were treated with exogenous 20E. Most recently, ecdysteroids were shown to regulate behavioral plasticity in *Drosophila* during a courtship conditioning assay (Ishimoto & Kitamoto, 2013). One possible link that may unite these diverse reports is that learning, courtship, and sleep-bouts have all been shown to depend upon the normal function of the mushroom bodies of the fruit fly brain, the same region of the brain shown in honey bees to respond to 20E with changes in gene expression and dendritic branching. Ecdysteroids in adult *Drosophila* have also been linked to stress resistance, longevity, and the circadian clock (Simon et al., 2003; Kumar et al., 2014; Uryo et al., 2015), although the relevant signaling pathways have not been defined. Reviews of ecdysteroids in adult fruit flies can be found in Shwedes and Carney (2012) and Uryo et al. (2015).

1.9 Goals of This Dissertation

My focus was to define the ecdysteroid profile of adult worker honey bees so that I could ask, in meaningful, naturally occurring contexts, if natural and experimental changes in ecdysteroid titers modulate adult worker physiology and behavior. The first goal of this dissertation was to elucidate, using modern research tools, the modulatory effects of QMP on ecdysteroid titers in adult honey bee workers reared in field and laboratory conditions. I predicted our results would expand the potential roles of ecdysteroids in normal adult physiology and provide support for the RGPH.
The next goal was to test the hypothesis that ecdysteroids play a functional physiological role in adult worker honey bees by modulating timing of initiation of foraging in adult worker honey bees. Originally, the transition to foraging from in hive worker tasks was thought to be controlled primarily by JH (Robinson, 1985). However, recent studies have led to formulation of a model that suggests that size of the worker ovary and vitellogenin are critical components of the endocrine feedback loop that initiates and maintains foraging (Amdam and Omholt, 2003; Guidugli et al., 2005). This in turn suggests that room exists in the current model for inclusion of an ecdysteroid effect (as modeled in Fig. 5). Using colonies containing worker honey bees injected early in life with ecdysteroids, I asked if early ecdysteroid exposure had significant effects on foraging behavior later in life. I predicted that increased early ecdysteroid exposure would result in early foraging patterns in adult worker bees.

The final goal of my dissertation was to test the hypothesis that ecdysteroids play a functional physiological role in adult worker honey bees by regulating IC. In addition to

Figure 5. Model of known circulating regulators (solid lines) of foraging and questions (dotted lines) addressed in this dissertation. JH, produced by the corpora allata, increases with the transition to foraging. It represses, and is repressed, by Vg, which decreases with the transition to foraging. The production of ecdysteroids by the ovaries is explored in chapter 2, effects on foraging behaviors are explored in chapter 3.
elevated Vg levels, laying workers have been shown to have significantly lower viral loads than non-reproductive workers from the same colony (Cardoen et al., 2011). This suggests that workers with elevated ecdysteroids, as are typically observed in laying workers, may have a stronger, more readily activated immune system. Because an increase of Vg is often associated with an increase in ecdysteroid titers, I predicted that ecdysteroids add another layer to the regulation of the immune system of the adult worker honey bee. I predicted that an increase in ecdysteroid titers would lead to an increase in IC as measured by increased number of hemocytes (a measure of individual immunity) and increased GOX synthesis (a measure of social immunity).

The honey bee is well known for its striking reproductive castes and complex societal structure. Research has recently supported the view that a sterile worker’s reproductive biology is a pivotal regulator of her physiology and behavior (Amdam et al., 2007). While some studies have focused on the evolution of the honey bee colony as a "superorganism," it is apparent that underlying reproductive mechanisms of the individual have played a direct role in the evolution of eusocial societies (West-Eberhard 1987; West-Eberhard 1996; Amdam and Page, 2010; Toth and Robinson, 2007). The studies presented in this dissertation continue to elucidate this mechanism with a specific focus on the role of ecdysteroids.
1.10 LITERATURE CITED


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CHAPTER 2

PHEROMONE MODULATION OF ECDYSTEROID PRODUCTION IN ADULT WORKER HONEY BEES (*APIS MELLIFERA*)

2.1 ABSTRACT

Insect C27 steroids (ecdysteroids) have been thought to play little role in the physiology of adult worker honey bees (*Apis mellifera* L.), in part because little was known about ecdysteroid production across the lifespan of individual workers. We used a sensitive enzyme immunoassay to measure ecdysteroids in tissues of individual adult worker honey bees reared under various conditions, including presence or absence of queen mandibular pheromone (QMP). Production of ecdysteroids was significantly higher in caged 12-day-old workers exposed to QMP than in caged 12-day-old workers reared with no exposure to QMP. Ecdysteroid titers at day 12 in queenright (QR) colonies were similar to those observed in QMP+ cages but were lower than those seen in queenless (QL) colonies. Neither workers exposed to QMP in cages nor workers sampled from QR colonies showed morphological evidence of ovarian development. These and other data reported here reveal that significant and dynamic production of ecdysteroids occurs in adult worker honey bees, typically in the absence of morphological signs of ovarian development, and that QMP regulation of ecdysteroid production in workers is dependent upon social context.
2.2 INTRODUCTION

Insects produce a single class of C27 steroid hormones, the ecdysteroids (Feyereisen, 1999; Lafont et al., 2005; Niwa, 2014). The source of ecdysteroids during larval and pupal stages is the prothoracic gland (Gilbert et al., 2002). This gland degenerates after the onset of metamorphosis (Snodgrass, 1956; Dai and Gilbert, 1991; 1997). In adult females of many species, the locus of steroid synthesis shifts to ovarian follicle cells (Brown et al., 2009). The primary steroid produced by the ovaries is ecdysone (C_{27}H_{44}O_{6}). Ecdysone (E) is taken up by peripheral tissues such as fat body and converted to 20-hydroxyecdysone (20E) by CYP314A1, a mitochondrial P450 20-hydroxylase (Petryk et al., 2003). One of the primary reproductive actions of 20E in adult female insects is transcriptional regulation of the gene encoding the yolk protein precursor vitellogenin (Vg) in fat body cells (Hagedorn et al., 1975; Raikhel et al., 2002; Xu et al., 2010). Circulating Vg is subsequently internalized by developing oocytes (Tufail and Takeda, 2009).

The regulation of transcription by ecdysteroids in developmental and reproductive contexts is mediated by a canonical nuclear receptor, EcR (Fahrbach et al., 2012). The gene encoding EcR is abundantly expressed in the central nervous system of many adult insects, suggesting that ecdysteroids might regulate adult behavior and physiology (Fahrbach and Velarde, 2009). Recent studies have explored roles for ecdysteroids in adult insects in functions as diverse as learning, memory, courtship, and sleep, primarily in Drosophila melanogaster (Ishimoto and Kitamoto, 2011; Schwedes and Carney, 2012). A smaller number of studies have focused on adult honey bees (Velarde et al.,
2009; Geddes et al., 2013). These studies have begun to elucidate the neuroendocrine actions of ecdysteroids in adult insects, although the tissue source of the steroids is rarely specified.

The study of ovarian ecdysteroids in eusocial insects is complicated by the presence of two female castes. A typical honey bee colony contains a single queen with hundreds of ovarioles filled with oocytes and tens of thousands of female workers with inactive, threadlike ovaries, each containing no more than a dozen ovarioles (Snodgrass, 1956; Jackson et al., 2011). The difference in size of the ovaries is one of the changes in adult phenotype that occurs as a result of the regulation of gene expression by differential larval nutrition (Evans and Wheeler, 1999; Evans and Wheeler, 2001; Wheeler et al., 2006). Despite their small number of ovarioles, worker ovaries are capable of producing unfertilized eggs that develop as haploid drones (Page and Erickson, 1988). The function of the ovaries in workers is suppressed in part by exposure to queen pheromones (Hoover et al., 2003). Other factors, including larval brood pheromones, assist in the suppression of worker ovaries (Winston et al., 1989; Winston et al., 1990; Traynor et al., 2014), but worker ovaries containing eggs are rare in queenright (QR) honey bee colonies, confirming the importance of signals from the queen (Maisonnasse et al., 2010).

If ovarian production of ecdysteroids in honey bees, as in other species of insects, is coupled with reproduction, then queens and laying workers from queenless (QL) colonies but not workers from QR colonies are predicted to produce ecdysteroids. The literature on this topic is sparse but in general conforms to this expectation (Robinson et al., 1991; Hartfelder et al., 2002). One prior study noted that a few outlier samples pooled from foragers with undeveloped ovaries (two out of seven samples tested) had elevated
ecdysteroids, with hemolymph titers equal to those of laying workers from a QL colony (Robinson et al., 1991).

The initial aim of the present study was to re-examine the capacity of adult worker honey bees to produce ecdysteroids in light of the mismatch between abundant expression of EcR in the central nervous system and low-to-undetectable levels of steroid in adult insect tissues. Based on the assumption that ecdysteroid titers reflect ovarian development, we predicted that reducing worker exposure to queen pheromones would promote ovarian development and enhance ecdysteroid production.

Conditions within QL or QR colonies were simulated in the laboratory by housing young workers in cages with or without synthetic queen mandibular pheromone (QMP), a blend of five compounds that regulates behavior, physiology, and gene expression in worker honey bees (Bortolotti and Costa, 2014). The initial results revealed that, contrary to expectation, hemolymph ecdysteroid titers were elevated in a significant subset of workers sampled from cages in which QMP was present (simulation of the QR condition) and low in caged workers not exposed to QMP (simulation of the QL condition). This unexpected result, made possible by application of a sensitive enzyme immunoassay to samples from individual workers, served as the basis of further investigations of QMP modulation of ecdysteroid production. Based on the prior literature, our hypotheses were that (1) ecdysteroid titers are correlated with an increase in ovarian morphological development, (2) the effects of QMP on worker physiology are age-dependent, (3) the ovaries are the primary source of ecdysteroids in adult workers, and (4) that QMP effects on workers are modulated by social context.
2.3 METHODS

2.3.1 Honey bees

Honey bees (*A. mellifera*) were collected from apiaries maintained by Wake Forest University in Forsyth County, NC, USA (36° 5' 59" N and 80° 14' 39" W). Unless otherwise noted, colonies were headed by commercially-obtained, naturally-mated queens, and large scale genetic variation can be assumed to be present among workers sampled from a single colony and among workers sampled across colonies. Combs of capped late worker pupae were removed from colonies and held overnight in custom-built frame boxes in a laboratory incubator (33ºC). Newly-emerged adult workers less than 12 h post-emergence were collected directly from their natal frames and re-housed in either cages or field colonies according to the requirements of the individual experiments.

2.3.2 Hemolymph and tissue collections

Honey bees were immobilized by chilling in glass vials on ice for 10 min. A size 4 insect pin was used to create a small opening in the intersegmental membrane posterior to one of the anterior abdominal sterna. Hemolymph was collected at this point by insertion of a 75 mm glass microcapillary tube (Drummond Scientific Company, Broomall, PA). Sample volumes obtained ranged from 2-15 µL. Samples visibly contaminated with fat body or gut contents were discarded. Clear samples were immediately dispensed into siliconized microcentrifuge tubes (Thermo Fisher Scientific, Waltham, MA) containing 100 µL of HPLC grade methanol (Sigma-Aldrich, St. Louis, MO). Samples were stored at -40ºC prior to assay. Other tissue samples were obtained after hemolymph collection according to the requirements of the study. For determination
of ovarian status, whole ovaries were removed and rinsed with honey bee saline (Bicker 1996), then assessed for activation based on a widely-used 5 point scale (Pernal and Currie 2000) in which 0 indicates ovarioles with no signs of swelling, 4 indicates queen-like ovarioles with elongated eggs, and intermediate scores are assigned based on degree of swelling and the presence of constrictions between adjacent follicles. Both ovaries were inspected, and a score given based on the most advanced development observed. Then, one ovary was selected at random and the individual ovarioles were counted. Inspections of ovaries and counts of ovarioles were conducted at a magnification of 40X. For ecdysteroid assay, whole ovaries were rinsed with honey bee saline and stored individually in 100 µL of methanol at -40ºC; whole brains were removed, freed from any adherent hypopharyngeal glands, rinsed with honey bee saline, and stored in 100 µL methanol, also at -40ºC.

2.3.3 Enzyme immunoassay of ecdysteroids

The enzyme immunoassay (EIA) used to detect ecdysteroids was previously used for assay of ecdysteroids in whole-body extracts of Drosophila melanogaster (Ishimoto et al., 2009). The polyclonal rabbit IgG antibody on which this EIA is based was raised against 20E and is commercially available (Item No. 482202, Cayman Chemical, Ann Arbor, MI). Although the antibody recognizes 20E, it is possible that it may also bind to related steroids, some of which may be present in A. mellifera. The literature suggests that those possibly present are makisterone A (C_{28}H_{46}O_{7}), and the 20E precursor, ecdysone. Both 20E and makisterone A are known ligands for EcR. Whether or not the precursor ecdysone is also a ligand is unclear (Wang et al., 2000). Other steroids, such as 20, 26-dihydroxyecdysone and 20-deoxymakisterone, may be present, although their
presence in honey bee hemolymph has not been conclusively demonstrated (Redfern, 1984; Warren et al., 1998). Given this uncertainty, we conservatively interpreted results obtained using this EIA as a measure of total ecdysteroids.

Stored samples (hemolymph or other tissues) were thawed, vortexed, and held at room temperature for 1 h immediately prior to running the EIA. Non-hemolymph samples were twice homogenized in 200 µL methanol for 30 sec with a handle pestle prior to the 1 h incubation period. Extracted samples were spun at 13,000g for 15 min to remove cellular debris. Supernatants were each transferred to a new siliconized microcentrifuge tube, then dried using a centrifugal evaporator (Integrated Speedvac ISS110) at room temperature. Dried samples were resuspended using EIA buffer (Item No. 400060, Cayman Chemical), and the assay was run using a conjugate of 20E and acetylcholinesterase (Item No. 482200, Cayman Chemical) following the manufacturer’s protocol. Signal was detected at 450 nm after a 2 h incubation using a PerkinElmer EnSpire 2300 Multilabel Reader. Standard curves for calibration were prepared using 20E (H5142; Sigma-Aldrich, St. Louis, MO). Results were expressed as pg 20E/µL sample. Analyses were performed in duplicate for each sample and averaged.

**2.3.4 Treatments**

*Experiment 1:* Newly-emerged worker honey bees < 12 h post-emergence were transferred to acrylic cages (Plexiglas®, 10 cm x 8 cm x 10 cm) similar in design to traditional Pain cages (Pain, 1966). Each cage contained 25-30 honey bees and was randomly assigned to a treatment group: QMP- (control), QMP+ (whole QMP strip), QMP 0.75 (3/4 QMP strip) QMP 0.5 (1/2 QMP strip), or QMP 0.25 (1/4 QMP strip). The QMP condition cages contained a single 50 mm, 5-component synthetic pheromone strip.
(Phero Tech Inc., Delta, BC, Canada), which mimics exposure to natural QMP, defined as a mixture of 9-oxo-2-decenoic acid (9-ODA), the enantiomers of 9-hydroxydec-2-enoic acid (9-HDA), methyl p-hydroxybenzoate (HOB), and homovanillyl alcohol (HVA). Each QMP strip contains 10 queen equivalents of QMP. QMP is not volatile. It is obtained and dispersed by honey bee workers from both queens and QMP strips via trophallaxis, antennation, and cuticular contact (Seeley 1979; Naumann et al. 1992). Release of QMP from QMP strips is therefore gradual as direct contact of bees with the strip is required. QMP strips were suspended from the back wall of the cage, offering the opportunity for ad libitum exposure. QMP- cages contained a single strip of plastic polymer that matched the size and shape of a full-size QMP strip.

All cages were maintained in a laboratory incubator (28ºC, 40% relative humidity) in constant darkness to mimic conditions in a hive. Cages were supplied with ad libitum deionized water, 50% sucrose (w/v) dissolved in deionized water, and a 1:1 mix of pollen (Brushy Mountain, Moravian Falls, NC) and honey collected from Wake Forest University-maintained hives; pollen was provided to ensure that lack of nutrients did not preclude worker ovarian development independent of pheromone signals (Hoover et al., 2006). Any honey bees that died were removed daily from cages to mimic the daily removal of dead honey bees from the hive by undertaker bees (Trumbo et al. 1997). Daily feeding and cleaning tasks were performed using red light, to which honey bees are insensitive (Peitsch et al., 1992). On day 12 after the cages were established (day 12 of adult life), hemolymph samples were obtained for EIA and development of ovaries was assessed as previously described.
Experiment 2: Newly-emerged worker honey bees were randomly assigned to QMP+ and QMP- cages and housed as described in Experiment 1. On day 12, hemolymph samples, brains, and ovaries were collected for EIA. Only individuals from which all three tissues could be collected were included in subsequent analyses.

Experiment 3: Newly-emerged worker honey bees were marked on the dorsal thorax with a small dot of enamel paint (Testors, Vernon Hills, IL) and transferred to a large, QR field colony. On days 0, 5, 12 and 20, samples of marked workers of known age were collected from the field colony and placed into cages as described in Experiment 1. Workers were randomly assigned to QMP+ and QMP- treatments. After 5 days, hemolymph samples were obtained for EIA. Development of the ovaries was assessed as previously described.

Experiment 4: Newly-emerged worker honey bees were randomly assigned to different treatment cages: QMP+, QMP-, QMP+ and Q (naturally mated queen present), QMP- and Q, QMP+ and SDI Q (single-drone-inseminated queen present, hereafter referred to as a SDI queen; obtained from Honey Bee Insemination Service, Washington State University, Pullman, WA), QMP- and SDI Q. Naturally mated queens were collected from colonies with eggs present in the brood nest. SDI queens were collected after being observed laying. On day 12, hemolymph samples were obtained from workers for EIA, and development of the ovaries was assessed.

Experiment 5: Newly-emerged worker honey bees were randomly assigned to one of two treatment cages: QMP+ Comb, QMP- Comb. Sections of drawn wax comb approximately 60 mm x 35 mm were fixed to the back of the cages so that bees were able to access the comb from both sides. All comb was previously used in colonies but was
free of food or brood when used in this experiment. On day 12, hemolymph samples were obtained for EIA, and development of the ovaries was assessed.

Experiment 6: Worker honey bees used in this experiment were obtained from a QR colony established by a SDI queen. The use of an SDI queen reduces genetic variation among workers in a colony (all workers are full sisters) and is associated with increased incidence of partial ovarian development in workers (Mattila et al., 2012). Newly-emerged honey bees were collected from this SDI colony and assigned to QMP+ and QMP- cages. An additional 1500 one day old honey bees from the SDI colony were painted on the dorsal thorax and assigned to one of three field colony treatments: the natal SDI colony, an unrelated QR colony, or an unrelated QL colony. Workers were collected for sampling from cages and field colonies on days 5, 12, and 20. All workers sampled from field colonies were collected from frames; marked workers were typically found on frames of the brood nest. Workers from colony treatments were chilled in the field to reduce the possibility that transport stress would alter ecdysteroid titer; all honey bees were sampled as previously described for ecdysteroid production and development of the ovaries.

2.3.5 Data analysis

The effects of QMP on ecdysteroid production, ovariole counts and development of the ovaries were analyzed with two-way ANOVAs (age and treatments) followed by Tukey-Kramer post hoc tests for pairwise tests. Ecdysteroid data in all experiments were transformed using a standard log transformation to correct for skew (Zar, 2010). In experiment 1 a hierarchical cluster analysis was performed on untransformed ecdysteroid titers using Ward’s method to separate individuals exposed to QMP into clusters. Ward’s
method joins cases into clusters such that the variance within each cluster is minimized (Everitt, 1993). In experiments 1 and 2, Pearson’s coefficient was used to determine correlation (IMB SPSS Statistics 22). In experiment 5, a student’s t test assuming unequal variance was used for the pairwise comparison. Graphical representation of data displays untransformed values for ease of analysis.

2.4 RESULTS

2.4.1 QMP exposure increases ecdysteroids in young worker honey bees

In contrast to previous studies that relied upon samples pooled from many individuals to measure ecdysteroids present in honey bee tissues, the use of a sensitive EIA allowed the routine determination of hemolymph titers in individual worker honey bees. Experiment 1 examined effects of dosage of QMP on ecdysteroid titers. Length of the QMP strip, which is directly related to the surface area available for contact, had a significant effect on ecdysteroid titers (Fig. 1; $F_{4, 70} = 8.26, p < 0.0001$). On day 12, mean ecdysteroid titers were significantly elevated in workers exposed to a full QMP strip ($75.99 \pm 16.60$ pg/µL, $n = 18$) compared with the QMP- treatment ($10.97 \pm 3.95$ pg/µL, $n = 17; p < 0.0001$). Hemolymph titers of ecdysteroids were significantly elevated in all three intermediate-length QMP conditions relative to the QMP- condition ($1/4, n = 14, p < 0.01; 1/2, n = 11, p < 0.05; 3/4, n = 16, p < 0.01$). Ecdysteroid titers were significantly elevated in the QMP+ group relative to all three intermediate-length treatments ($1/4, p < 0.05; 1/2, p < 0.05, 3/4, p < 0.05$). There were no morphological changes in the ovaries (QMP+, 0.0 ± 0.0; 3/4, 0.0 ± 0.0; 1/2, 0.02 ± 0.10; 1/4, 0.12 ± 0.18; QMP- 0.0 ± 0.0) or differences in ovariole counts (QMP+, 4.16 ± 0.41; 3/4, 3.18 ± 0.31; 1/2, 3.00 ± 0.33; 1/4, 3.86 ± 0.35; QMP- 2.26 ± 0.18). There was no significant correlation between ovariole
number and ecdysteroid titer in any group (QMP+, $r = -0.01$; 3/4, $r = 0.55$; 1/2, $r = 0.59$; 1/4, $r = 0.247$; QMP-, $r = -0.23$; $p > 0.05$ all groups). Hierarchical cluster analysis using Ward’s method revealed two distinct subgroups within all samples exposed to QMP. We called these groups responders (individuals sorted into a group with a mean ecdysteroid titer of 201.8 ± 32.32 pg/µl, $n = 12$ when exposed to QMP) and non-responders (18.24 ± 2.33 pg/µl, $n = 40$).

**Figure 1.** Experiment 1 Ecdysteroid titers (pg/µL) (untransformed data) in dose response trial after 12 days caged in laboratory conditions. Different letters represent significant difference ($p < 0.05$). Box: 25-75%, solid line: median; dotted line: mean; whiskers: min – max; open squares: outliers. $n = 17$ (QMP-), 14 (1/4), 11 (1/2), 16 (3/4), and 18 (QMP+).
2.4.2 Worker ovaries have high ecdysteroids without growth after exposure to QMP

Based on the existing literature, we expected the ovaries to be the most significant post-metamorphic source of ecdysteroids. Nervous tissue, however, is a site of steroid production in vertebrates (e.g. Schlinger and Arnold 1991; Schumacher et al. 2015), and a previous report showed that genes encoding two of the cytochrome P450 enzymes required for ecdysteroid synthesis are expressed in the worker honey bee brain (Yamazaki et al. 2011). In Experiment 2, hemolymph ecdysteroids were found to be significantly ($p < 0.01$) elevated in QMP+ conditions (115.25 ± 39.9 pg/µL, $n = 27$) compared with QMP- conditions (17.71 ± 5.76 pg/µL, $n = 28$). Ecdysteroid levels differed significantly between tissue sources in these same workers (Fig. 2; $F_{1,142} = 36.88$, $p < 0.00001$). In both QMP+ (102.3 ± 21.2 pg/µL, $n = 36$) and QMP- groups (69.53 ± 14.5 pg/µL, $n = 32$), ecdysteroids were higher in ovaries relative to levels in the brain (14.35 ± 3.5, $n = 39$; 20.47 ± 3.79 pg/µL, $n = 38$; $p = 0.00001$). Neither the QMP+ nor QMP- groups displayed significant morphological signs of ovary activation (QMP+, 0.035 ± 0.19; QMP-, 0.0 ± 0.0) nor significant differences in ovariole count (QMP+, 2.72 ± 0.20; QMP-, 2.26 ± 0.18). Brain ecdysteroids did not correlate with hemolymph titers in either the QMP+ or the QMP- condition (Fig 3A; $r = 0.33$, $n = 28$, $p > 0.05$; Fig 3C; $r = -0.13$, $n = 29$, $p > 0.05$). Ovarian ecdysteroid levels also did not correlate with hemolymph titers in either the QMP+ or QMP- conditions (Fig. 3B; $r = 0.15$, $n = 29$, $p > 0.05$; Fig3D; $r = -0.21$, $n = 31$, $p > 0.05$).
Figure 2. Experiment 2 Mean ± standard error ecdysteroid levels (pg/μL) from whole brain and ovaries in QMP+ and QMP- conditions. * denotes significant differences, $p < .01$. $n = 39$, 38, 36, 32
Figure 3. Experiment 2 Matched tissue and circulating ecdysteroid titers from worker bees caged in QMP+ and QMP- conditions. 

a. Individual ecdysteroid level (pg/µL) in brain tissue and circulating hemolymph in QMP+ conditions, n = 28. 

b. Individual ecdysteroid level (pg/µL) in ovary tissue and hemolymph samples in QMP+ conditions, n = 29. 

c. Individual ecdysteroid level (pg/µL) in brain tissue and circulating hemolymph in QMP- conditions, n = 29. 

d. Individual ecdysteroid level (pg/µL) in ovary tissue and circulating hemolymph in QMP- conditions, n = 31
2.4.3 Age-dependence of QMP-induced ecdysteroid production

Experiment 3 investigated the role of age at exposure to QMP on ecdysteroid production. There was a significant interaction of age with treatment (Fig 4; \( F_{3, 183} = 5.519, p < 0.01 \)). QMP+ workers caged on the first day of adult life immediately after adult emergence had significantly higher hemolymph ecdysteroids (101.2 ± 35.39 pg/µL, \( n = 33 \)) compared with their QMP- counterparts (15.92 ± 4.97 pg/µL, \( n = 31, p < 0.01 \)). No other groups responded to QMP with elevated hemolymph ecdysteroids (5 day QMP+, \( n = 28 \); QMP-, \( n = 32 \); 12 day QMP+, \( n = 12 \); QMP-, \( n = 16 \); 20 day QMP+, \( n = 16 \); QMP-, \( n = 18 \)). Neither age (\( F_{3, 183} = 1.411, p > 0.05 \)) nor QMP treatment (\( F_{1, 183} = 0.25, p > 0.05 \)) independently impacted ecdysteroid titers. There were no morphological

Figure 4. Experiment 3 Effect of age when caged in QMP+ and QMP- conditions. Mean ± standard error ecdysteroid titer (pg/µL) after 5 days of caged laboratory conditions. * denotes significant differences, \( p < 0.0001 \). \( n = 31 \) and 33 (Day 0), 32 and 28 (Day 5), 16 and 17 (Day 12), and 18 and 16 (Day 20).
signs of ovary development or differences in ovariole counts in any of the workers sampled. Data are given in S1 (Section 2.6).

2.4.4 Interaction of QMP with other queen signals

Experiment 4 examined effects of the presence of a queen and queen mating condition on worker responses to QMP. There was a significant effect of queen type (Fig 5; $F_{2,78} = 8.57, p < 0.0001$) on ecdysteroid titers. Bees caged with an SDI queen had significantly lower ecdysteroids in the QMP+ (5.61 ± 2.94 pg/µL, $n = 12$) and QMP- (4.13 ± 0.69 pg/µL, $n = 14$) conditions compared with workers housed with naturally mated queens in QMP+ (172.77 ± 40.54 pg/µL, $n = 14$) and QMP- (154.57 ± 50.27 pg/µL, $n = 19$) conditions. Workers caged with naturally mated queens did not differ significantly from cages without queens in either treatment. There were no morphological

![Figure 5. Experiment 4 Effect of queen condition when caged in QMP + and QMP– conditions. Mean ± standard error ecdysteroid titer (pg/µL) after 12 days of caged laboratory conditions, * denotes significant differences, $p <0.0001$ $n = 10, 14,$ and 12 (QMP+), and 10, 19, and 14 (QMP–)](image-url)
signs of ovary activation or differences in ovariole count in any of the workers sampled. Data are given in S1 (Section 2.6).

**2.4.5 Presence of comb does not modulate QMP-induced ecdysteroid production**

*Experiment 5* investigated the role of comb on ecdysteroid titers in caged conditions. The presence of comb did not affect the trends previously displayed in caged conditions. QMP+ titers were significantly elevated (67.89 ± 24.19 pg/µL) compared with QMP- titers (19.28 ± 4.36 pg/µL) in the presence of comb (\( p < 0.05, t = 1.98 \)). There were no morphological signs of ovary activation or differences in ovariole count in any of the workers sampled. Data are given in S1 (Section 2.6).

**2.4.6 Social modulation of QMP-induced ecdysteroid production**

*Experiment 6* compared ecdysteroid production in workers produced by an SDI queen reared in two different cage environments (QMP+ and QMP-) and three different colony environments (their natal SDI colony, a full size QR colony, and a full size QL colony). There was a significant effect of treatment (Fig 6A; \( F_{3, 281} = 10.95, p < 0.0001 \)) and age (\( F_{2, 281} = 115.203, p < 0.0001 \)) on ecdysteroid titers in QMP+ vs. QMP- cages and QR vs. QL colonies. Additionally, there was a significant effect of interaction between treatment and age (\( F_{6, 281} = 19.98, p < 0.0001 \)). In contrast to the results obtained in *Experiment 3*, no significant difference was found between hemolymph ecdysteroids of caged QMP+ and QMP- workers sampled on Day 5 (18.82 ± 4.12 pg/µL, \( n = 18 \) vs. 44.82 ± 18.68 pg/µL, \( n = 27 \)). Hemolymph ecdysteroids for both cage treatment groups were elevated on Day 5 relative to those in workers reared in field colonies under both QR (0 ± 0 pg/µL, \( n = 24, p < 0.0001 \)) and QL (2.77 ± 0.58 pg/µL, \( n = 17, p < 0.001 \)) conditions.
Ecdysteroid titers were significantly higher in workers reared in QMP+ cages (38.44 ± 9.15 pg/µL, \( n = 32 \)) on Day 12 compared with titers of workers reared in QMP- cages (29.36 ± 7.73 pg/µL, \( n = 44 \); \( p = 0.05 \)). Workers housed in the QR colony did not differ significantly from either cage treatment when sampled on Day 12. By contrast, ecdysteroids were significantly elevated on day 12 in the QL colony (549.72 ± 102.78 pg/µL, \( n = 14 \)) relative to all other day 12 day old workers sampled in this study (\( p < 0.0001 \)). There was a significant effect of treatment (Fig 6B; \( F_{3, 279} = 25.896, p < 0.0001 \)) with age (\( F_{2, 279} = 25.316, p < 0.0001 \)) on ovarian development. The 12 day old QL group contained the only workers sampled in these studies with substantial morphological signs of ovary activation (1.5 ± 0.29; \( p < 0.0001 \)). All other groups studied had a mean level of 0.5 ovary development scores or less across all sampling time points. Across all treatments, however, no groups differed significantly in ovariole count, including the 12 day olds housed in the QL colony described here. Full data are given in S1 (Section 2.6).

On day 20, ecdysteroid titers were found to be significantly decreased (\( p < 0.0001 \)) in all cage and colony groups, independent of exposure to pheromone (QMP+: 1.25 ± 0.85 pg/µL, \( n = 15 \); QMP-: 1.89 ± 0.83 pg/µL, \( n = 20 \); QR: 0.96 ± 0.28 pg/µL, \( n = 12 \); QL: 1.08 ± 0.30 pg/µL, \( n = 33 \)). There was no difference between treatment groups (\( p > 0.05 \)).
Figure 6. Experiment 6 Effect of age and treatment in field and laboratory conditions, $n = \text{QMP+}: 18, 32, 15; \text{QMP-}: 27, 44, 20; \text{QR}: 24, 25, 12; \text{QL} 17, 14, 33$. a. Comparative hemolymph ecdysteroid titers in daughters of an SDI queen in laboratory and field conditions. Mean ± standard error ecdysteroid titer (pg/µL) by experimental treatment. Zero readings were below threshold of the assay, <0.48 pg/ul. Different letters denote significant difference, comparisons across treatments by day, $p < 0.05$. b. Mean ± standard error ovary activation by treatment, * denotes significant difference, $p < .05$. 
Workers raised in their natal SDI colony displayed a trend not seen in any other sampling condition studied. Age had a significant effect on ecdysteroid titers in the QR SDI colony (Fig 7A; $F_{2, 57} = 16.96, p < 0.0001$). Ecdysteroid levels decreased from day 5 (56.20 ± 7.88 pg/µL, $n = 19$) to day 12 (15.36 ± 6.40 pg/µL, $n = 15$; $p < 0.0001$). The elevation of hemolymph ecdysteroids on day 5 was unique to workers sampled from the colony headed by the SDI queen. Hemolymph ecdysteroids of SDI workers did not change between days 12 and days 20 (13.05 ± 4.12 pg/µL, $n = 23$). Although post hoc tests revealed no significant differences between treatment groups, there was a general effect of age on morphological development of the ovaries (Fig 7B; $F_{2, 57} = 3.368, p < 0.05$), indicating that SDI workers tended to have higher scores with age. Despite this trend, no groups sampled had scores above 0.5 or significant differences in ovariole count.
Figure 7. Experiment 6 a. Comparative hemolymph ecdysteroid titers in daughters of an SDI queen reared in SDI natal colony. Mean ± standard error ecdysteroid titer (pg/µL). n = 19, 15, 23. b. Mean ± standard error ovary activation. * denotes significant difference, p < 0.0001. n = 19, 15, 23
2.5 DISCUSSION

Our current incomplete view of the role of ecdysteroids in adult worker physiology reflects the limitation that an individual honey bee can provide only a tiny volume of hemolymph for analysis, typically less than 10 μl. And she can provide that sample only once! This has resulted in a literature based on analysis of pooled samples; subtle but biologically relevant individual differences are often masked as a result of pooling. We overcome this limitation in our present study by modification of an EIA for analysis of samples from individuals. Studies in many species (vertebrate and invertebrate) have demonstrated that transient hormone pulses, often small in magnitude, are critical to normal physiology (e.g. Riddiford, 1976; Valk et al., 1980; Pfaff et al., 2004; Campos and Herbison, 2014). In particular, pooling of samples from unsynchronized populations or sampling at inappropriate times may have led to physiologically-significant pulses being overlooked.

The highly variable conditions found in field colonies are another cause of incomplete endocrine data in honey bees. Despite their disadvantages, cages allow for a constant, controlled source of nutrition (e.g. a steady protein supply); unvarying and close proximity to a stable QMP source; and the control of many highly variable environmental and social influences. By pairing a sensitive EIA with the use of cages, we found that continuous exposure to QMP from the first day of adult life significantly elevated endogenous ecdysteroids in a subset of worker bees. Although the magnitude of this increase varied across replicate experiments, an increase in ecdysteroid production was reliably induced by QMP exposure across several field seasons and many different worker genotypes.
In QMP+ groups, a subset of individuals exhibited ecdysteroid titers as much as 10 times higher than the lowest observed values within the same treatment group. We suggest that responders (defined as subset of individuals that show elevated ecdysteroid titers in the presence of QMP without morphological signs of ovarian development) and non-responders (subset of individuals that do not show elevated titers in the presence of QMP) possibly represent different genotypes but may also reflect as yet to be defined differences in epigenetic factors. Pheromone response threshold has been previously shown to be influenced by individual differences such as ovary size, social behavior and age of exposure (Traynor et al., 2014; Amdam et al 2009; Pankiw and Page 1999, 2001; Vergoz et al. 2007, 2009). Our experimental design provides clarity by controlling two variables commonly cited as influencing variability amongst individuals, adult nutritional status and age of pheromone exposure.

Hemolymph ecdysteroids are not predicted by or predictors of ovarian status

The assumed role of ecdysteroids in the reproductive physiology of honey bees is based primarily on studies of insects from other orders, primarily dipterans (Schwartz 1989; Kozlova and Thummel 2000). Elevated ecdysteroids were previously reported for both honey bee queens and laying worker bees; the ovaries of the laying workers, however, were not examined in this study (Robinson et al., 1991). Here we show that, in adult workers, elevated ecdysteroids are not consistently associated with reproduction. We found that workers with elevated ecdysteroid titers on day 12 in QR treatments did not exhibit morphological signs of ovarian activation. This violates a commonly made assumption but is in accord with results reported by Hartfelder et al. (2002), who reported no correlation between ecdysteroid titers in worker honey bees and morphological
development of the ovaries. This is also consistent with observations from another hymenopteran, the European paper wasp (*Polistes dominula*), showing lack of correlation between ecdysteroid titers and length of the terminal oocyte, a measure of ovarian activation in this species (Roseler *et al.* 1985).

We have also shown that ecdysteroid titers (hemolymph concentration) are not an accurate predictor of ovarian ecdysteroids. These results are similar to those reported for the buff-tailed bumblebee (*Bombus terrestris*): ecdysteroid levels in the ovary and hemolymph showed no correlation in queens and only a very weak correlation in workers (Geva *et al.* 2005). While we do not show a correlation between hemolymph titers and ovarian levels, we regard the ovaries as the most likely candidate source of hemolymph ecdysteroids given that all brain samples included in our studies contained significantly lower levels. This is in contrast to the suggestion of Yamazaki *et al.* (2011), who used gene expression analyses to investigate various honey bee organs to determine which have the potential to synthesize ecdysteroids. These investigators suggested that the brain may be the primary source of ecdysteroids in adult worker bees because they detected upregulation of mRNAs for CYP306A1 and CYP302A1, two cytochrome P450 enzymes involved in the biosynthesis of steroids (Feyereisen, 1999). Based on our assays, it is unlikely that ecdysteroid levels observed in the brain compared with those observed in the ovaries indicate sufficient ecdysteroidogenesis to support the brain as the likely source for circulating ecdysteroids, although neural ecdysteroids may have a local signaling role.

*Hemolymph ecdysteroids are not predicted by ovarian size*

We did not prescreen colonies for ovarian size, which in honey bees is defined as the total number of ovarioles. Ovariole number is determined in larvae by genotypic and
nutritional factors and remains constant in adulthood (Evans and Wheeler 1999; Evans and Wheeler 2001; Wheeler et al., 2006). There is a reported positive association between worker ovariole number and the propensity to show signs of morphological activation of the ovaries (Traynor et al. 2014; Amdam et al. 2006; Tsuruda et al. 2008; Page et al., 2006, 2012). We did not find any correlation between ovariole number and ecdysteroid titer or between morphological signs of activation and ecdysteroid titer. This suggests some level of separation between ecdysteroid production and reproduction in these non-laying workers.

The omission of QMP from cages mimics the queenless state, so it is notable that we did not see significant levels of ovarian activation, as measured by the swelling of ovarioles and presence of oocytes, in any caged group in our study. This disparity between our results and others that have reported significant ovarian activation in caged workers (e.g. Hoover et al. 2003; Tsuruda et al. 2008; Traynor et al. 2014) might reflect the relatively small size of the ovaries in the unselected colonies we used. Mean ovariole counts in this study range from 1.5 ± 0.27 to 4 ± 0.32 ovarioles per side while studies reporting significant numbers of activated worker ovaries have reported ovariole counts as high as 6 ± 3.6 and >8 (Tsuruda et al. 2008; Traynor et al. 2014). Our small ovary size and small range of variation is instead consistent with the study of Oldroyd and Beekman (2008), who reported lower levels of ovarian activation and smaller ovary size overall in workers from naturally mated queens collected from field colonies. Because we work with commercially available lines that are not heavily selected for specific traits, we suggest our results reflect the more typical condition of ovaries in worker bees. Genetic sources of variation, such as directional behavioral selection for pollen-hoarding (Amdam
et al. 2010) or patriline selection for traits such as anarchy (Thompson et al. 2008), have been shown to increase the propensity of workers to activate their ovaries (Backx et al. 2012). Heavy selection for behavioral traits may result in the loss or masking of the more subtle aspects of physiological regulation that are apparent in individuals in this study.

We further note that many studies reporting ovarian activation typically report very low scores, rarely exceeding a 1 (slight swelling of the ovarioles) or 2 (presence of distinct cells leading to moderate swelling of the ovarioles) on the Pernal and Currie (2000) scale. While ovaries with scores of 1 or 2 are often considered to be activated, other investigators have taken a more conservative approach in classifying only ovarioles with developed oocytes and clear signs of yolk deposition as activated (Malka et al. 2007). We suggest that ovarian activation measured as swelling alone is not a predictor of an individual worker’s commitment to reproduction.

Age and social context modulate QMP-induced ecdysteroid production

QMP-induced production of ecdysteroids in workers appears to be strikingly age dependent. We showed that workers needed to be held in close proximity to a QMP source from the first day of adult life to induce an elevated ecdysteroid response. A benefit to cage studies is our confidence that all sampled individuals have had consistent contact with QMP since shortly after emergence. Age-based changes in responsiveness has also been shown for brood pheromone, a pheromone produced by developing brood within the colony that stimulates foraging activity in older bees and delays foraging in younger bees (Le Conte et al., 2001; Pankiw, 2004; Pankiw & Page 2001). The basis of these age-related changes is unknown, in part because the mechanism of action of QMP
on worker honey bees is unknown. 9-ODA, a primary component of QMP which also acts as a sex pheromone, is detected by the odorant receptor, AmOr11, located on the antenna of honey bees (Wanner et al., 2007). Calcium imaging studies in drones have shown that QMP signals are processed in an enlarged glomerulus, an area where sensory neurons synapse with projection and local neurons, in the antennal lobe of the brain, but the location of QMP processing in the antennal lobes of worker bees has yet to be identified (Sandoz, 2006; Jarriault and Mercer, 2012). It has been suggested that pheromone signals processed in the brain lead to changes in function of neurosecretory cells, such as those found in the corpora allata (Jarriault and Mercer, 2012). This suggests that early exposure to QMP could alter neural pathways in the brains of young adult worker bees, culminating in the age-related responsiveness of bees to QMP. This is supported by investigations that have shown that the attractiveness of QMP changes as workers age: young workers are attracted to QMP while older workers are repelled (Vergoz et al. 2007, Vergoz et al. 2009). It has also been shown that levels of expression of dopamine receptor, Amdop1, increase in the antenna and brains of bees exposed to QMP early in life compared with those who deprived of early exposure (Beggs et al. 2007; Vergoz et al. 2009). Using microarrays and mRNA quantification by qRT-PCR, Grozinger and colleagues (2003) showed that exposure to QMP differentially regulated the expression of several hundred genes in young nurse bees versus older foragers. This accords with our results, which show a clear effect of age of exposure to QMP in terms of ecdysteroid production.

*Social modulation of responses to QMP*
We found that production of ecdysteroids induced by QMP was significantly modulated by social context. QL colonies produced the highest levels of ovary activation, with a subset of individuals showing developed oocytes, and elevated ecdysteroid levels, consistent with prior views of the reproductive role of ecdysteroids (Robinson et al. 1991). Ecdysteroid titers on day 12 in QR colonies were similar to those observed in QMP+ cages but were lower than those seen in QL colonies. In cages containing an SDI queen, ecdysteroid titers of workers were low regardless of exposure to QMP. Naturally mated queens induced the same titer trend as seen with synthetic QMP exposure. Unique ecdysteroid titer patterns as well as a trend towards increased ovarian activation over time were observed in our field colonies headed by a SDI queen. This in accordance with findings by Matille and colleagues (2012) showing higher levels of ovarian activation in SDI headed colonies. In contrast, similar studies have shown workers are less likely to develop their ovaries in colonies headed by SDI queens (Peso et al. 2012; Hoover et al. 2003).

It is difficult to compare SDI queens to naturally mated queens as there are marked differences in their early handling (i.e. insemination process). Instead, it might be beneficial to examine differences between single instrumentally inseminated and multiple inseminated queens (Cobey, 2007). Live queens produce multiple pheromones (QMP, tergal pheromones, Dufour’s gland secretions) as well as additional compounds not currently considered primary components of queen pheromones (Slessor et al. 2005; Hoover et al. 2003; Peso et al. 2013). It is possible that minor QMP components differentially produced by SDI queens are responsible for ecdysteroid titer and ovarian
activation differences elicited in our workers (Richard et al. 2007; Peso et al. 2009; Kocher et al. 2009).

*Interpretation within the reproductive ground plan hypothesis framework*

The reproductive ground plan hypothesis emerged from the idea that social behaviors evolved through the pathways of reproductive mechanisms already present in solitary species that were coopted for the regulation of complex social behaviors (West-Eberhard 1987; West-Eberhard 1996; Amdam et al., 2004). Our data suggest that early QMP exposure has differential effects on individual worker honey bees, resulting in downstream differences on ecdysteroid titers in the hemolymph creating distinct groups (i.e. responders and non-responders). While these results have not yet been linked directly to behavioral differences, the suggestion that pheromone exposure modulates ovarian ecdysteroid production provides further support for understanding the complex relationships between reproductive physiology and complex social behaviors that have been shown to be linked to endocrine titers. It is of interest to investigate what predisposes adult workers to be QMP responders or non-responders, specifically if QMP responders or non-responders are more widely represented in highly selected lines such as pollen or nectar foragers.

This study has provided significant evidence that QMP is modulating ecdysteroid titers in young adult worker bees. In terms of our initial hypotheses, it has been shown that ecdysteroid titers in QR cage and colony conditions are not correlated with an increase in ovarian morphological development; that the effects of QMP on worker physiology are strongly age-dependent; and that the ovaries are likely the primary source of ecdysteroids in adult workers. Finally, it is evident from our results that QMP effects
on workers are modulated by social context, specifically queen presence and mating condition.
### 2.6 SUPPLEMENTAL DATA

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<th>QMP</th>
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**Table I** *Experiment 3* mean ± std error of ovariole count and morphological activation score, age represents age bees were first placed in caged conditions.

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<td>No Queen</td>
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<td>0.5 ± 0.26</td>
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**Table II** *Experiment 4* mean ± std error of ovariole count and morphological activation score

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**Table III** *Experiment 5* mean ± std error of ovariole count and morphological activation score
Table IV Experiment 6 mean ± std error of ovariole count and morphological activation score, age represents age at sampling

<table>
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<th>Treatment</th>
<th>Age</th>
<th>Ovariole Count</th>
<th>Morphological Activation Score</th>
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<tr>
<td>QMP+</td>
<td>5</td>
<td>3.0 ± 0.24</td>
<td>0.22 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>2.43 ± 0.22</td>
<td>0.21 ± 0.74</td>
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<td></td>
<td>20</td>
<td>2.87 ± 0.36</td>
<td>0.47 ± 0.13</td>
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<td>QMP-</td>
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<td>0.07 ± 0.05</td>
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<td>12</td>
<td>2.84 ± 0.22</td>
<td>0.19 ± 0.06</td>
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<td></td>
<td>20</td>
<td>3.20 ± 0.21</td>
<td>0.35 ± 0.11</td>
</tr>
<tr>
<td>Queenright</td>
<td>5</td>
<td>3.04 ± 0.23</td>
<td>0.08 ± 0.06</td>
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<tr>
<td></td>
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<td>3.04 ± 0.20</td>
<td>0.12 ± 0.06</td>
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<tr>
<td></td>
<td>20</td>
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<td>0.08 ± 0.73</td>
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<tr>
<td>Queenless</td>
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<td>12</td>
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<td>1.5 ± 0.29</td>
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<td>20</td>
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<td>0.64 ± 0.11</td>
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Table V Experiment 6 mean ± std error of ovariole count and morphological activation score of bees raised in a colony headed by an SDI queen.

<table>
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<th>Age (days)</th>
<th>Ovariole Count</th>
<th>Morphological Activation Score</th>
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<tr>
<td>5</td>
<td>3.2 ± 0.26</td>
<td>0.11 ± 0.07</td>
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<td>12</td>
<td>2.93 ± 0.23</td>
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<td>2.91 ± 0.30</td>
<td>0.39 ± 0.12</td>
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2.7 LITERATURE CITED


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CHAPTER 3

EARLY ECDYSTEROID TREATMENT ACCELERATES FORAGING IN HONEY BEES (APIS MELLIFERA)

3.1 ABSTRACT

The transition to foraging in honey bees is regulated by multiple factors. We asked if ecdysteroids, which have been previously shown to modify gene expression and cytoarchitecture in an area of the brain that supports the cognitive tasks involved in foraging (the mushroom bodies), affect the timing of the transition to foraging. We treated newly emerged bees with 20-hydroxyecdysone and introduced them to single cohort colonies. Bees treated with ecdysteroids foraged significantly earlier than age-matched, vehicle-treated controls. Comparison of foraging after the first 3 days of behavioral observation revealed that ecdysteroid treated bees displayed higher levels of foraging activity than vehicle-treated controls. We found no evidence that ecdysteroid treatment influenced ovarian development. We also found no indication that individual reproductive physiology, i.e. ovary size, influenced the initiation of foraging. This study suggests a novel role for the ecdysteroids present in adult worker bees.
3.2 INTRODUCTION

Colony life in honey bees is characterized by temporal polyethism, the division of labor on the basis of worker age (Seeley, 1995). This results in a general progression where the youngest workers perform in-hive jobs such as cell cleaning, brood and queen care, and food storage, transitioning after two to three weeks to foraging (Seeley, 1995). This transition is accompanied by physiological as well as behavioral changes. These changes have been shown to be strongly influenced by endocrine signals.

As honey bees age, juvenile hormone (JH) titer increases in parallel to the age related changes in behavior (Fluri et al., 1982; Huang et al., 1991, 1994; Robinson, 1987; Robinson, 1992). JH increases as bees transition to foraging, remains low in individuals forced to remain in the hive as nurses, and decreases in foragers forced to return to nursing (Huang and Robinson, 1996). However, experiments in which bees had their corpora allata removed at the end of metamorphosis (thus removing the source of JH), have shown that JH is not required for the transition to foraging (Sullivan et al., 2000) although its absence slightly delays its onset.

The switch to foraging tasks is also accompanied by a decrease in the circulating levels of yolk protein precursor vitellogenin (Vg) (Amdan and Omhold, 2003; Guidugli et al., 2005). The link between JH and Vg has been described by a double repressor model, in which Vg represses JH and JH represses Vg (Guidugli et al., 2005). Since the initial description of this mutually inhibitory relationship between Vg and JH, these signals have been widely viewed as critical components of an endocrine feedback loop that initiates and maintains the behavioral switch to foraging.
Despite the importance of the Vg-JH interaction, the transition to foraging likely involves multiple signals. For example, treatment with the biogenic amine octopamine increases the likelihood of initiation of foraging in younger than typical foragers from single-cohort colonies and in workers from larger colonies (Schulz and Robinson, 2001). Also, both the naturally occurring reduction in abdominal lipids observed in young adult workers and experimental lipid depletion induced an early transition to foraging (Toth and Robinson, 2005; Toth et al., 2005). Other signals influence the resources sought by foragers: for example, increased exposure of workers to brood pheromone resulted in increases in the ratio of pollen to nectar foragers (Pankiw et al., 1998). In general, worker honey bees are highly sensitive to cues related to colony demography, as manipulation of the number of typical age foragers in a colony can induce young worker bees to forage at a younger age than usual: conversely, foragers will revert to brood care when young workers are removed from the hive (Robinson 1992, Huang and Robinson 1992). We now ask if ecdysteroids, hormones primarily characterized for their roles in metamorphosis, contribute to this complex regulatory system.

Current scenarios explaining the regulation of behavioral maturation in adult workers include no role for ecdysteroids, a reflection of the wide acceptance of the view that ecdysteroids are unimportant during adulthood in this caste. Previously, ecdysteroids had been described as having primarily reproductive roles in adult honey bees. Our recent studies have shown that, in colonies with a laying queen (queenright conditions), morphologically undeveloped ovaries are a significant source of circulating ecdysteroids in a subset of workers (Chapter 2, this dissertation). Other investigators have shown that
foraging specialization (preference for pollen or nectar) is influenced by an individual’s reproductive morphology, particularly size of the ovaries (Amdam et al., 2006).

Additional evidence suggests ecdysteroids impact gene expression, structure, and function of adult insect brains. Nuclear receptors for ecdysteroids are abundantly expressed in the adult central nervous system of several other insect orders, including Hymenoptera, Lepidoptera, and Orthoptera, suggesting a possible regulatory role of ecdysteroids in adult behavior (Riddiford et al., 1999; Wang et al., 2000; Velarde et al., 2006, 2009; Nemoto and Hara, 2007; Fahrbach et al., 2012). The mushroom bodies, the most prominent structure in the protocerebrum of the insect brain, are required for several forms of learning and memory in the honey bee (Fahrbach, 2006). This structure, which comprises approximately a third of the neurons in the honey bee brain, is uniquely tied to foraging behavior on the basis of its foraging-associated structural plasticity. Foraging honey bees have larger mushroom bodies compared with same age individuals who lack foraging experience (Withers et al., 1993; Durst et al., 1994; Gronenberg et al., 1996; Ismail et al., 2006). The growth of this area in honey bees is at least in part the result of an increase in length and branching of dendritic arborizations of the Kenyon cells, the intrinsic neurons of the mushroom bodies (Farris et al., 2001). Ecdysteroids have also been shown to induce gene expression and dendritic growth in the Kenyon cells; in vivo studies have provided evidence that ecdysteroid treatments accelerated dendritic growth (Velarde et al., 2009; Velarde et al., 2011; Ford et al., 2011). Taken together, these results suggest there may be room for an expansion of the prevailing model for the transition to foraging to modulation by ecdysteroids.
The primary goal of this study was to examine the effects of early exposure to elevated ecdysteroids on foraging in single cohort colonies. Single cohort colonies are composed solely of workers of the same age plus a queen. This induces early onset of foraging in a subset of young workers, which are referred to as precocious foragers (Robinson, 1992). Young bees treated with JH in the first 24 hours of adult life are more likely to become precocious foragers than controls, a result that provided evidence that JH modulates the shift from in-hive tasks to foraging in honey bees (Robinson, 1985; Robinson et al., 1989; Sasagawa et al., 1989). In this study we tested a parallel hypothesis that newly emerged workers treated with ecdysteroids in the first 24 hours of adult life will make an early transition to foraging. This hypothesis was based on the strong effects of ecdysteroids on gene expression in the mushroom bodies and the growth-promoting effects of ecdysteroids on the Kenyon cells. We reasoned that exposure to ecdysteroids would produce a configuration of the mushroom bodies similar to that observed in experienced foragers. This hypothesis requires an assumption that the experience-dependent plasticity of the mushroom bodies supports the more efficient foraging typical of experienced foragers (Dukas and Visscher, 1994).

3.3 METHODS

3.3.1 Honey bees

Honey bees (A. mellifera) were collected from apiaries maintained by Wake Forest University in Forsyth County, NC, USA (36° 5' 59" N and 80° 14' 39" W). Colonies were headed by commercially-obtained, naturally-mated queens, and large scale genetic variation can be assumed to be present among workers sampled from a single
colony and among workers sampled across colonies. Combs of capped late worker pupae were removed from colonies and held overnight in custom-built frame boxes in a laboratory incubator (33°C). Newly-emerged adult workers less than 12 h post-emergence were collected directly from their natal frames and re-housed in field colonies. The experiment was run three times with colonies set up in August 2013, June 2014, and July 2014.

3.3.2 Ecdysteroid Treatment

Honey bees were injected with 20-hydroxyecdysone (20E) (AG Scientific, A7344) dissolved first in isopropanol and then diluted in sterile bee saline (130 mM NaCl, 6 mM KCl, 4 mM MgCl₂, 135 mM CaCl₂, 160 mM sucrose, 25 mM glucose, 10 mM 4-(2-hydroxyethyl)-1-piperazine ethane-sulfonic acid in dH₂O; Richard et al., 2008) to a final concentration of 0.04 µg/µl solution, 20% isopropanol. This dose was selected based on prior studies of the effects of 20E injections on gene expression in newly emerged workers (Velarde et al., 2009). Control treatments consisted of 20% isopropanol in sterile bee saline (vehicle). Honey bees were chilled briefly on ice for 60 sec prior to injection to immobilize. A microinjection system (Micro4, World Precision Instruments, Sarasota, FL, USA) was used to inject 1 µl of each treatment for a final dose of 0.04 µg 20E or vehicle control into the lateral abdomen. The injection tip (34 gauge, NanoFil Injection, World Precision Instruments, Sarasota, FL, USA) was inserted through the lateral intersegmental membrane above the third abdominal tergite. Fluid was injected at a rate of 200 nl/s. To prevent backflow of hemolymph, the injection tip was not withdrawn until 30 s post injection. Honey bees that displayed visible bleeding or
backflow of injection fluid or did not quickly regain mobility after treatment were discarded.

Honey bees that received injections were transferred to acrylic cages (Plexiglas®, 10 cm x 8 cm x 10 cm) similar in design to traditional Pain cages (Pain, 1966). Each cage contained a total 25-30 treated honey bees. Bees were marked on the dorsal thorax with a small dot of enamel paint (Testors, Vernon Hills, IL). All cages were maintained in a laboratory incubator (28°C, 40% relative humidity) in constant darkness to resemble conditions in the hive for 24 hours. Cages were supplied with *ad libitum* deionized water, 50% sucrose (w/v) dissolved in deionized water, and a 1:1 mix of pollen (Brushy Mountain, Moravian Falls, NC) and honey collected from Wake Forest University-maintained hives. After 24 hours, injected bees were transferred to a single cohort colony.

### 3.3.3 Single Cohort Colonies

Newly emerged honey bees were collected and painted on the dorsal thorax as previously described. Approximately 1500 bees were marked as background bees and installed in a five frame nucleus colony with empty comb, developing brood comb, food stores, and a naturally mated, laying queen. An additional 70 - 100 control injected and 70 - 100 20E injected bees were added. Twenty four hours later an additional 500 - 800 painted background bees were added to the colony. This resulted in single cohort colonies of approximately 2200 individuals within 24-36 hours of the same age. After the final
addition of honey bees, the colony was kept closed and held indoors for 48 hours before being transferred to the field site. The same field site was used for all trials.

![Timeline]

**Figure 1.** Timeline. Honey bees were between 6 and 12 h post-emergence at the time of injection. Background bees were no more than 24 h post-emergence when collected. All three trials followed this timeline.

### 3.3.4 Behavioral Observations

Behavioral observations began 24 h after colonies had been moved to their field location in Winston-Salem, NC. Entrance counts were conducted to measure activity of focal and background bees. The hive entrance was fitted with a metal hardware cloth screen that slowed but did not block the entrance of honey bees into the colony, allowing time for an observer to note thorax color and assess behavior. The following information was recorded for each honey bee that returned to the hive entrance during the observation period: performance of a large, circular flight pattern around the hive entrance (orientation flight; Winston, 1987) or a direct, focused flight to the hive entrance (foraging trip; Robinson, 1987). It was also noted if foragers carried pollen. Observations occurred twice daily for a 30 min period between 1000 and 1100 h Eastern daylight time (EDT) and 1300 and 1500 h EDT and continued for 9-10 days. This sampling scheme was modeled on that used in Robinson (1987), Sullivan *et al.* (2000), and many other studies of precocious foraging.
3.3.5 Colony Census and Ovary Dissection

Census data were collected at the conclusion of each trial to check for differential mortality among focal groups. At the conclusion of behavioral observations, the colony entrance was closed 1 h after sunset. This ensures the most accurate population count as foragers return to the colony at dusk. The entire sealed colony was then placed in a freezer (-40°C) for 48 h. All injected individuals were then collected, counted, and placed in ethanol to store for later ovary dissection. A sample of 20-30 background individuals were also collected and placed in ethanol for ovary dissection. Queens were retrieved on all trials, indicating that all hives were queenright during the entire period of observation.

For assessment of ovarian status, whole ovaries were removed and then scored for activation on a widely-used 5 point scale (Pernal and Currie, 2000) in which 0 indicates typical worker ovarioles with no signs of swelling, 4 indicates queen-like ovarioles with elongated eggs, and intermediate scores, assigned on the basis of degree of swelling and constrictions between adjacent follicles, indicate partial development. Both ovaries were inspected, and a score awarded based on the most advanced development observed. Then, one ovary was selected at random and individual ovarioles were counted. Inspections of ovaries and counts of ovarioles were conducted at a magnification of 40X.

3.3.6 Statistics

Chi-square tests were used to determine differences in foraging initiation and intensity by observation period, following a well-established procedure used by Robinson (1987), Huang et al., 1998, Schulz and Robinson (2001), and Barron et al. (2002), among others. Where noted data from all three trial colonies was pooled (Wagener-Hulme et al., 1999). A student’s t test assuming equal variance was used to determine differences in
pooled retrieval rates (IMB SPSS Statistics 22). A one way analysis of variance was used to determine differences in ovariole number and morphological development.

3.4 RESULTS

20E treatment increased precocious foraging

Over all colonies tested, workers treated with 20E on day 0 were significantly more likely to engage in precocious foraging compared with vehicle treated workers. Over the first three days of foraging (days 3 - 5), 20E treated workers foraged in significantly greater numbers than control treated bees in pooled colony data ($\chi^2 = 9.64, p < 0.01$) (Fig 2).
Figure 2. Pooled foraging trips of all three colonies of vehicle and 20E injected bees over first 9 days of foraging. Chi-square tests revealed significantly more foraging ($p < 0.01$) of the first three days of foraging by 20E bees compared with vehicle bees.

The difference between experimental and control honey bees was observed in the August colony ($\chi^2 = 6.89, p < 0.01$) on days 3 – 5 and in the June colony ($\chi^2 = 6.0, p < 0.05$) on days 3 – 6. Due to rain (which inhibits foraging) on days 2 and 6, this trend was first observed on days 4 – 7 in the July colony ($\chi^2 = 4.95, p < 0.05$) (Fig 3).
treated bees foraged more than vehicle treated bees

Honey bees treated with 20E completed significantly more foraging trips (230) over the entire nine days of observation than vehicle treated bees (157) across all trials ($\chi^2 = 13.77, p < 0.001$) (Fig 4). To control for earlier foraging by 20E treated bees, we also examined foraging over days 7 through 9. The trend of increased foraging by 20E bees was still observed even when correcting for “early” foraging days. Bees treated with 20E continued to forage in higher numbers (137 foraging trips) on days 7 through 9 compared with vehicle treated bees (107 foraging trips; $\chi^2 = 3.689, p = 0.05$), suggesting a sustained increase in foraging numbers by 20E treated bees.

**Figure 3.** Foraging trips of vehicle and 20E treated honey bees by colony. Chi-square tests revealed significantly more foraging by 20E treated bees as compared with vehicle bees in the first three days of foraging in the August colony ($p < 0.05$) and the first four days in the June colony ($p < 0.05$). Due to rain on days 2 and 6 significant foraging differences were not observed in the July colony until days 4–7 ($p < 0.05$).

*20E treated bees foraged more than vehicle treated bees*

Honey bees treated with 20E completed significantly more foraging trips (230) over the entire nine days of observation than vehicle treated bees (157) across all trials ($\chi^2 = 13.77, p < 0.001$) (Fig 4). To control for earlier foraging by 20E treated bees, we also examined foraging over days 7 through 9. The trend of increased foraging by 20E bees was still observed even when correcting for “early” foraging days. Bees treated with 20E continued to forage in higher numbers (137 foraging trips) on days 7 through 9 compared with vehicle treated bees (107 foraging trips; $\chi^2 = 3.689, p = 0.05$), suggesting a sustained increase in foraging numbers by 20E treated bees.
Foraging was not affected by ovarian size or morphological development

Differences in reproductive physiology have been previously linked to differences in foraging preference and intensity among bees from high and low pollen-hoarding strains (Amdam et al., 2006). We found no difference in mean ovariole count between any group (20E treated bees: 2.03 ± 0.14; vehicle treated bees: 2.3 ± 0.19; non-injected background bees: 2.17 ± 0.14) (F2, 142 = 0.61, p = 0.55). Additionally, ecdysteroid treatment did not induce morphological activation in treated groups based on the Pernal and Currie (2001) scale. No morphological changes were observed in any group of bees.

Figure 4. Total foraging trips of all treatment bees over 3 replicate colonies. 20E treated bees foraged significantly more than vehicle treated bees over the first 9 days of foraging (p < 0.001).
across all trials (20E treated bees: 0.36 ± 0.08; vehicle treated bees: 0.54 ± 0.09; non-injected background bees: 0.32 ± 0.05) \((F_{2,142} = 2.58, p = 0.08)\).

Colony census and focal bee retrieval

We found no difference in survival rate, based on retrieval rates of individuals during the colony census, between 20E treated and vehicle and treated bees \((p > 0.05)\) (Fig 5). Over three trials, 35.14% of 20E treated bees and 38.83% of vehicle treated bees were collected at the conclusion of behavioral observations. Recovery rates varied across trials (June colony: 8.6% 20E, 14.3% vehicle; July colony: 38.8% 20E, 40% vehicle; August colony: 65.4% 20E; 67.9% vehicle). We also noted that eggs, larvae and capped

**Figure 5.** Percentage of focal bees retrieved at each colony census. There was no difference in retrieval rates between treatment groups in any of the replicate colonies \((p > 0.05)\).
brood were present in each of the experimental colonies, confirming that colony conditions did not vary across trials.

3.5 DISCUSSION

The results of this study demonstrate that early ecdysteroid exposure can induce precocious foraging in young worker honey bees housed in single cohort field colonies. Treatment with 20E resulted in earlier foraging compared with vehicle treated controls, and more foraging trips overall compared with vehicle treated controls.

Ecdysteroids have been shown to have significant effects in honey bees on the mushroom bodies, a multisensory integration center of the insect brain comprising several hundred thousand small neurons called Kenyon cells and two associated neuropils, the calyces and lobes (Fahrbach, 2006). The mushroom bodies play a significant role in learning, memory and sensory integration: particularly in Hymenoptera, the mushroom bodies receive significant inputs from the optic lobes, consistent with a pivotal role in foraging (Gronenberg, 2001; Ehmer & Gronenberg, 2002; Robinson et al., 1997; Fahrbach, 2006). In honey bees, the final volume of the mushroom body neuropils is extremely plastic: maximal volumes are achieved only after individuals accrue more than a week of foraging experience (Fahrbach, 2006).

The mechanisms that drive this foraging-dependent plasticity have not yet been clearly defined. Previous studies have demonstrated that foraging stimulates the growth of Kenyon cell dendrites (Farris et al., 2001; Ismail et al., 2006). Additional evidence has shown that treatment of appropriately aged non-foragers with the muscarinic agonist pilocarpine increased the volume of the mushroom bodies and increased dendritic
complexity of the Kenyon cells in a similar fashion to the changes observed in bees with foraging experience (Ismail et al., 2006; Dobrin et al., 2011). Conversely, treatment with a muscarinic antagonist, scopolamine, enhanced dendritic pruning in these same cells (Dobrin et al., 2011) suggesting that muscarinic signaling may be a mechanism for foraging-dependent plasticity. It has also been speculated that an early pulse of ecdysteroids on the 3rd day of adult life and the subsequent gene cascade it induces may regulate the plasticity observed in the mushroom bodies (Velarde et al., 2009, Velarde et al., 2006; Fahrbach et al., 2016). Additional in vivo and in vitro results also provide support. Kenyon cells treated with ecdysteroids in culture respond with increased length and branching of the dendrites of the calyces as is seen in the brains of foragers (Farris et al., 2001). Further, one day old bees injected with low doses of ecdysteroids showed accelerated dendritic growth when compared with vehicle treated controls (Ford et al. 2011). Our results provide evidence that early exposure to 20E can accelerate the transition to foraging in small experimental colonies. We speculate that the behavioral changes observed in this study were accompanied by (and possibly driven by) 20E-induced growth of the mushroom bodies.

While it is likely that 20E is having direct effects on the brain, it is also possible that effects of 20E on the ovaries could also modulate foraging behavior. The ovaries of worker bees have been related to adult behavioral development. Prior reports have described a positive correlation between ovary size and increased behavioral progression, both in wild type and strains selected for foraging behavior (Amdam et al., 2006; Amdam et al., 2007; Wang et al., 2009). Honey bees with more ovarioles (i.e. larger ovaries) tend to forage earlier and collect larger and more frequent loads of pollen. In another
hymenopteran, the paper wasp *Polistes gallicus*, removal of the ovaries retarded behavioral development and decreased ecdysteroid titers (Roseler *et al.*, 1985). In honey bees, surgery to increase the mass of the ovaries in individual workers had similar effects; a greater ovarian mass was associated with a decrease in the time to foraging (Wang *et al.*, 2010). While Wang and colleagues did not identify the signal from the ovary resulting in the behavioral change, it is possible that ecdysteroids are this unknown signal, as we have shown in this study that ecdysteroids appear to have a causal relationship with acceleration of this behavioral transition. Due to the design of our study (in which we marked treatment groups instead of individual workers), it was not possible to examine correlations between individual ovarian size and foraging pattern. However, we did not find differences in ovary size or activation between treatment groups, nor a wide range of variation within groups, and therefore cannot explain our results on the basis of differences in size of the ovaries or stage of activation. We note that ovary size alone only partially addresses this issue, as our previous studies have shown that ecdysteroid levels in the ovaries or hemolymph titers do not correlate with ovarian size or development assessed using the Pernal and Currie scale (Chapter 2, this dissertation). If 20E treatments resulted in an increase of production of endogenous ecdysteroids by the ovaries, this effect would not be detectable through a morphological approach alone.

Consideration of Vg titers may resolve the difference between previous reports and the present lack of a correlation between ovary size and foraging onset. There is a positive correlation between early Vg levels and ovary size (Tsuruda *et al.*, 2008). In contrast, Wang and colleagues did not show an increase in Vg transcripts with surgical increases in the size of the ovaries in workers (Wang *et al.*, 2010). They did show
however an increase in the putative ecdysteroid response gene HR46 with increased ovary size (Wang et al., 2010; Wang et al., 2012). A homolog of this gene, HR3, has been shown to correlate with ecdysteroid levels and is involved in vitellogenesis in A. aegypti ovaries (Kapitskaya et al., 2000). Increased ovary size is positively correlated with an increased propensity to become a laying worker and oogenesis requires an upregulation of Vg titers (Makert et al., 2006); however, a direct link of 20E with ovary size is not clearly evident in our data.

JH is established as one of the regulators of the transition to foraging (Robinson, 1986; Sullivan et al., 2000), but we do not yet know if 20E and JH work in tandem to coordinate this change. JH titers are elevated coincident with increases in the volume of the mushroom bodies (Robinson, 1992; Fahrbach & Robinson, 1996), yet there is no evidence of any direct effect of JH on the size or organization of this brain structure. While JH has been shown to have direct effects on neural populations in the mushroom bodies in other insects (i.e. crickets), this hormone does not appear to be necessary for the development of the mushroom bodies in honey bees (Fahrbach et al 2003; Sullivan et al., 2000). JH treatment changes gene expression in the brain to a pattern similar to that observed in foragers (Whitfield et al., 2006), and both JH and ecdysteroids modulate expression of an overlapping group of genes in the mushroom bodies and other tissues (Jindra et al., 2013; Dubrovsky & Bernardo, 2014). In particular, both JH and 20E upregulate expression of AmUSP, the downregulation of which in the fat body has previously been associated with a delay in maturation as a forager (Ament et al., 2012). It is possible that crosstalk between both hormones underlies the behavioral effect seen in our study; this is an area where much work remains to be done.
There were substantial differences in recovery numbers of focal bees between colonies. What is interesting is that these individuals likely died outside of the hive as they were not recovered in the colony census. Stress responses, as would be expected after handling and injection, could have resulted in unanticipated physiological changes or developmental changes that resulted in foragers being unable to find their way back to the natal colony. However, due to the fact that retrieval numbers varied between colonies, and not between treatments, we find it more likely that variation in recovery rates most likely reflected environmental conditions. Foragers could have been accepted into a nearby colony or died during foraging trips. Weather varied notably across trials. Periods of intense and heavy rain resulted in significant decreases in the number of observation flights and foraging trips, as observed in the July colony, and could have contributed to higher levels of mortality. It is also possible that dead bees were removed by undertakers during the foraging period, although this was not directly observed (Trumbo et al. 1997). Despite differences in retrieval rates, the total number of foraging trips by focal bees was similar across colonies, suggesting that focal bees in colonies with low retrieval rates were lost gradually over the period of behavioral observations.
This study contributes to a growing body of literature supporting a role for ecdysteroids in the reproductive ground plan hypothesis (RGPH). The RGPH is based on the idea that pathways which were once based solely in reproduction were co-opted for the regulation of complex social behaviors as organisms evolved higher levels of sociality (West-Eberhard 1987; West-Eberhard 1996; Amdam et al., 2004). We have previously shown that a subset of workers in queenright colonies referred to as responders display elevated levels of ecdysteroids prior to the typical transitioning age to foraging (Chapter 2, this dissertation). The current study shows that early ecdysteroid treatment can accelerate the onset of foraging. While reproductive physiology, in the terms of gross differences in ovary size produced by genetic selection or surgical manipulation of the ovaries, have been linked with division of labor, we speculate that it is these covert groups of responders and non-responders, and their subsequently different

**Figure 6.** Model of the hormonal control of the initiation of foraging with inclusion of a potential role of ecdysteroids. Non-responders represent the classic regulation (as described in Guidugli et al., 2005), JH and Vg in a double repressor relationship as modulated by colony cues and reproductive physiology. We speculate that earlier foragers are also responders (as defined in chapter 2 of this dissertation). This represents an additional level of regulation through the modulation of ecdysteroid titers of sensitive individuals by pheromonal cues within the colony. The increased synthesis of 20E, by the ovaries, acts on the brain decreasing the age of transition to foraging.
ecdysteroid profiles, that are the more subtle typical mechanism underlying the division of labor in typical, unselected populations (as modeled in Fig 6). A current challenge is to identify responders without terminal sampling techniques to evaluate if the effects of endogenous pulses are causing similar acceleration of behavioral transitions as exogenous treatments. Our results nevertheless suggest that ecdysteroids have a role to play in the regulation of the transition to foraging.
3.6 LITERATURE CITED


CHAPTER 4  
HORMONAL REGULATION OF CELLULAR IMMUNITY IN WORKER HONEY BEES (APIS MELLIFERA)

4.1 ABSTRACT

Insects rely on an entirely innate immune system for protection against pathogens. While vertebrate literature has extensively explored the role of steroids in immunocompetence, this area has lagged in insect studies. We asked if ecdysteroids and exposure to queen mandibular pheromone are modulating two facets of innate insect immunity, hemocyte concentrations and glucose oxidase (GOX) levels in adult worker honey bees. We demonstrate preliminary evidence of a modulatory effect of queen mandibular pheromone (QMP) on hemocytes in 12 day old worker bees, in the form of a correlation between elevated ecdysteroid titers in responders and an increase in hemocyte concentration. We show potential enhanced recovery from a decrease in hemocytes by treatment of 20E in 5 day old bees independent of pheromone exposure. Finally, we also provide evidence that bees held in queenless conditions may have unique immunocompetence profiles, specifically reducing the production of GOX. These findings present an initial survey of the role of ecdysteroids and pheromone exposure in the immunocompetence of adult worker honey bees.
4.2 INTRODUCTION

Much evidence supports a regulatory role of steroid hormones and nuclear hormone receptors in animal defenses against pathogens. In mammals steroid receptors, such as the estrogen and glucocorticoid receptor, have been linked with regulation of the innate immune system (Smoak and Cidlowski, 2004; Beagly and Gockel, 2003). Androgens have also been suggested to play a role in vertebrate immunocompetence (Martin, 2000; Chen & Parker, 2004) Far less is known about the role steroid hormones have on the defensive systems of insects. Unlike vertebrates, which defend against pathogens with both innate and acquired (antibody-based) immunity, insects rely exclusively on their innate, non-specific immune responses.

There is preliminary evidence that suggests that ecdysteroids, well characterized for their role in metamorphosis and reproduction, regulate specific aspects of insect immune responses. For example, in the fruit fly Drosophila melanogaster, ecdysteroids are required for development of the hematopoietic organs called lymph glands: ecdysteroids have also been shown to increase the phagocytic activity of hemocytes in fruit fly larvae (Sorrentino et al., 2002; Lanot et al., 2001). Ecdysteroids have also been shown to induce expression of antimicrobial peptide genes in Drosophila, such as Diptericin, Cecropin, and Attacin and pattern recognition receptors, such as PGRP-LC (Flatt et al., 2008; Rus et al., 2013). Additionally 20-hydroxyecdysone (20E) has been shown to enhance hemocyte motility, encapsulation, and nodulation in fruit fly larvae (Regan et al., 2013; Sampson et al., 2013). In the silk moth Bombyx mori, elevated 20E during an immune response resulted in an enhancement of the response, as assessed by survival rates and clearance rate of FITC-labeled bacteria in silk worms injected with
bacterial cultures (Sun et al., 2016). While this literature is sparse, it is growing, and taken together these results suggest a potential role for ecdysteroids in insect defenses against pathogens.

In comparison to other insects, honey bees (Apis mellifera) have roughly 1/3 as many genes in the 17 primary gene families involved in insect immunity (Evans et al., 2006). This is interpreted as a reflection of the high level of sociality exhibited by this species, which may be reflected in social behavior-based barriers to disease transmission (Evans et al., 2006). As is the case in other insects, the immune system of honey bees has two major components - hemocytic and humoral – but as noted also adds the third component of social immunity. The hemocytic responses are produced by the hemocytes, macrophage-like cells that can perform phagocytosis, encapsulate parasites, and form nodules around any foreign particles in the hemocoel (Lavine & Strand, 2008). Humoral responses center on the release of antimicrobial and antifungal peptides from fat body and the release of enzymes that initiate melanization in response to the breach of the hemocoel. The third component, social immunity, includes individual behaviors that decrease disease and parasite levels on a colony-wide basis, (Cremer et al., 2007; Simone-Finstrom & Spivak, 2010). An example of social immunity is the production of the antimicrobial peptide glucose oxidase (GOX) by the hypopharyngeal glands and its addition to larval food (White et al., 1963; Oashi et al., 1999; Alaux et al., 2010).

During adult life, worker honey bees have been reported to display a pattern of immunosenescence, or a reduction of immune efficiency, correlated both with age and worker role in the colony (Amdam et al., 2005). As worker bees transition, typically at around 2-3 weeks of adult life, to tasks such as foraging that take them outside of the
hive, the number of circulating hemocytes declines (Rutz et al., 1974; Wille and Rutz, 1975; Amdam et al., 2004). This decline can be reversed if workers are forced to revert to performing tasks inside the hive. The reversal is accompanied by a drop in juvenile hormone (JH) and an increase in circulating vitellogenin (Vg). JH, a sesquiterpinoid hormone, and Vg, a yolk protein precursor, have been shown to have a mutually inhibitory relationship in which JH represses Vg production by the fat body and Vg represses JH production by the corpora allata (Amdam and Omholt 2003). These two signals are thought to be the primary determinants of the timing of the transition to foraging. Therefore, their modulation during an experimentally-induced reversal of immunosencence suggests a potential regulatory role of these hormones on the immune system (Amdam et al., 2004; Amdam et al., 2005).

In addition to regulating the transition of foraging, Vg plays a more typical role in the reproductive ability of worker bees. In a queenright colony (QR) a mated, egg-laying queen is present and produces queen mandibular pheromone (QMP) throughout the duration of a worker’s life. One role of QMP is to suppress egg laying by workers (Naumann, 1991; Tsuruda et al. 2008). When a colony is without a queen, referred to as a queenless colony (QL), a subset of workers will begin to lay haploid eggs. An increase of Vg is associated with morphological development of ovaries required for egg laying (Makert et al., 2006). Workers with one or more fully formed oocytes, an indicator that workers have or will begin laying, had significantly lower viral loads than non-reproductive workers of the same age from the same colony (Cardoen et al., 2011). In addition to elevated Vg levels, laying workers typically display elevated levels of the steroid hormones, ecdysteroids (Robinson et al., 1991; Chapter 2, this dissertation). This
suggests that workers with elevated ecdysteroids, may have a stronger, more readily
activated immune system (Robinson et al., 1991, Hartfelder et al., 2002, Chapter 2, this
dissertation).

Our recent studies (Chapter 2, this dissertation) have revealed that laying workers
are not the only workers within a hive with elevated ecdysteroids. Exposure to QMP from
the day of adult emergence, which is typical in QR colonies, reveals two distinct
subgroups of workers. Responders are individuals characterized by elevated ecdysteroid
titers at approximately the 12th day of adult life, and non-responders are individuals with
significantly lower levels of ecdysteroids at this time (Chapter 2, this dissertation). The
raised levels of ecdysteroids in hive bees likely has downstream effects on worker
behavior and physiology, but at present these are only beginning to be characterized.

Taken together, this evidence suggests that ecdysteroids may modulate some
aspects of resistance to pathogens in honey bees. We asked if ecdysteroid treatment had a
direct effect on two different measures of immunocompetence: traditional hemocyte-
based innate immunity and honey bee-specific social immunity. We predicted that
ecdysteroids would increase the number of circulating hemocytes and increase the
activity of GOX produced by the hypopharyngeal glands. We further asked if ecdysteroid
treatment modulates response to an active immune challenge, measured by an increase in
available hemocytes, through the injection of lipopolysaccharides (LPS), the major outer
surface membrane found on most gram-positive bacteria. LPS is known to act as a strong
innate immune system stimulant in both arthropods and vertebrates (Alexander &
Rietschel, 2001). Finally, we characterized the immune qualities ecdysteroid titers of bees
from a naturally overwintered, queenless (QL) colony and asked if morphological ovarian
development modulated immunocompetency as measured by changes in hemocyte and GOX levels.

4.3 METHODS

4.3.1 Honey bees

Honey bees (A. mellifera) were collected from apiaries maintained by Wake Forest University in Forsyth County, NC, USA (36° 5' 59" N and 80° 14' 39" W). Colonies were headed by commercially-obtained, naturally-mated queens. As a result, large scale genetic variation can be assumed to be present among workers sampled from a single colony and among workers sampled across colonies. Combs of capped late worker pupae were removed from colonies and held overnight in custom-built frame boxes in a laboratory incubator (33°C). Newly-emerged adult workers less than 12 h post-emergence were collected directly from their natal frames and re-housed in cages (Plexiglas®. 10 cm x 8 cm x 10 cm) according to the requirements of the experiments.

4.3.2 Injections

Honey bees were chilled briefly on ice for 60 s prior to injection to immobilize individuals. A microinjection system (Micro4, World Precision Instruments, Sarasota, FL, USA) was used to inject 1 μl of each treatment (20E or LPS) into the lateral abdomen. The injection tip (34 gauge, NanoFil Injection, World Precision Instruments, Sarasota, FL, USA) was inserted through the lateral intersegmental membrane above the third abdominal tergite. One μl of fluid was injected at a rate of 200 nl/s. To prevent backflow of hemolymph, the injection tip was withdrawn 30s post injection. Honey bees that displayed visible bleeding, loss of injection fluid or did not regain mobility after injection were discarded.
4.3.3 Hemolymph and tissue collections

Honey bees were immobilized by chilling in glass vials on ice for 10 min. A size 4 insect pin was used to create a small opening in the intersegmental membrane posterior to one of the anterior abdominal sterna. Hemolymph for hormone assay was collected at this point by insertion of a 75 mm glass microcapillary tube (Drummond Scientific Company, Broomall, PA). Sample volumes obtained ranged from 2-15 µL. Samples visibly contaminated with fat body or gut contents were discarded. Clear samples were immediately dispensed into siliconized microcentrifuge tubes (Thermo Fisher Scientific, Waltham, MA) containing 100 µL of HPLC grade methanol (Sigma-Aldrich, St. Louis, MO). Samples were stored at -40ºC prior to assay. Other tissue samples were obtained after hemolymph collection according to the requirements of the study.

For determination of ovarian status, whole ovaries were removed and rinsed with honey bee saline (Bicker 1996), then assessed for activation based on a 5 point scale (Pernal and Currie 2000) in which 0 indicates undeveloped ovarioles with no signs of swelling, 4 indicates queen-like ovarioles with elongated eggs, and intermediate scores indicating partial development are assigned on the basis of degree of swelling and constrictions between adjacent follicles. Both ovaries were inspected, and a score was given based on the most advanced development observed. Then, one ovary was selected at random and the individual ovarioles were counted. Inspections of ovaries and counts of ovarioles were conducted at a magnification of 40X.

For the hemocyte assay, a 1 µl sample of hemolymph was collected as previously described. This was combined with 9 µl of deionized water and placed onto a “Neubauer Improved” hemocytometer (Fisher Scientific) (Wilson-Rich et al, 2008). Samples were
immediately analyzed under a light microscope (100X) using phase contrast for hemocyte quantification (average of 5 chambers per bee). In cases where both hemocytes and hemolymph ecdysteroids were assayed, the sample used for the hemocyte count was obtained first. For GOX analysis whole heads were removed, flash frozen in liquid nitrogen and placed into pre-chilled microcentrifuge tubes on dry ice. Samples were stored at -40°C prior to analysis.

4.3.4 Enzyme immunoassay of ecdysteroids

An enzyme immunoassay (EIA) used to detect ecdysteroids was previously used for assay of ecdysteroids in *Apis mellifera* (Chapter 2, this dissertation). The polyclonal rabbit IgG antibody on which this EIA is based was raised against 20E and is commercially available (Item No. 482202, Cayman Chemical, Ann Arbor, MI). Although the antibody recognizes 20E, it is possible that it may also bind to related steroids (i.e. makisterone A (C_{28}H_{46}O_{7}), and the 20E precursor, ecdysone). Given this uncertainty, we conservatively interpreted results obtained using this antibody as a measure of total ecdysteroids.

Stored hemolymph samples were thawed, vortexed, and held at room temperature for 1 h immediately prior to running the EIA. Extracted samples were spun at 13,000g for 15 min to remove cellular debris. Supernatants were each transferred to a new siliconized microcentrifuge tube, then dried using a centrifugal evaporator (Integrated Speedvac ISS110) at room temperature. Dried samples were resuspended using EIA buffer (Item No. 400060, Cayman Chemical), and the assay was run using a conjugate of 20E and acetylcholinesterase (Item No. 482200, Cayman Chemical) following the manufacturer’s protocol. Signal was detected at 450 nm after a 2 h incubation using a PerkinElmer
EnSpire 2300 Multilabel Reader. Standard curves for calibration were prepared using 20E (H5142; Sigma-Aldrich, St. Louis, MO). Results were expressed as pg 20E/µL sample. Analyses were performed in duplicate for each sample and averaged.

Glucose oxidase levels were determined using a commercially available kit (Biovision K788-100) following the procedure of Alaux et al. (2010). Briefly, whole heads were homogenized in 200 µL assay buffer using a beadmill (Qiagen TissueLyser CT) and 5 mm steel beads (69989; Qiagen). Samples were then centrifuged at 13,000 g for 10 min. Supernatant was removed to complete the GOX assay. Absorbance was read over 30 minutes at 570 nm after a 5 min incubation at 37°C using a PerkinElmer EnSpire 2300 Multilabel Reader. Standard curves for calibration were prepared using supplied standards. Analyses were performed in duplicate for each sample and averaged.

4.3.5 Treatments

Experiment 1: Newly-emerged worker honey bees < 12 h post-emergence were transferred to acrylic cages (Plexiglas®, 10 cm x 8 cm x 10 cm) similar in design to traditional Pain cages (Pain, 1966). Each cage contained 25-30 honey bees and was randomly assigned to a treatment group: control (QMP-) and QMP+. The QMP condition contained a single 50 mm, 5-component synthetic pheromone strip (Phero Tech Inc., Delta, BC, Canada), which mimics exposure to natural QMP, defined as a mixture of 9-oxo-2-decenoic acid (9-ODA), the enantiomers of 9-hydroxydec-2-enoic acid (9-HDA), methyl p-hydroxybenzoate (HOB), and homovanillyl alcohol (HVA). Each QMP strip contains 10 queen equivalents of QMP. QMP is obtained and dispersed by honey bee workers via trophallaxis, antennation, and cuticular contact (Seeley 1979; Naumann et al. 1992). Release of QMP is gradual as direct contact of bees with the strip is required.
QMP strips were suspended from the back wall of the cage, offering the opportunity for *ad libitum* exposure. Control cages contained a strip of plastic polymer that matched the size and shape of a full-size QMP strip, attached in the same position to the back wall of the cage.

All cages were maintained in a laboratory incubator (28°C, 40% relative humidity) in constant darkness to resemble conditions in the hive. Cages were supplied with *ad libitum* deionized water, 50% sucrose (w/v) dissolved in deionized water, and a 1:1 mix of pollen (Brushy Mountain, Moravian Falls, NC) and honey collected from Wake Forest University-maintained hives; pollen was provided to ensure that lack of nutrients did not preclude worker ovarian development independent of pheromone signals (Hoover et al. 2006). Any honey bees that died were removed daily from cages to mimic the daily removal of dead honey bees from the hive by undertaker bees (Trumbo et al. 1997). Daily feeding and cleaning tasks were performed using red light, to which honey bees are insensitive (Peitsch et al. 1992). On day 12 after the cages were established (day 12 of adult life), hemolymph samples were obtained for EIA and hemocyte analysis. Development of ovaries was assessed with the widely-used 5 point scale of Pernal and Currie (2000).

**Experiment 2:** Newly-emerged worker honey bees were randomly assigned to treatment groups [Control (non-injected), vehicle injected and 20E injected)]. 20E treated bees were injected with 20E (AG Scientific, A7344) dissolved first in isopropanol (HPCL Grade) and then diluted in sterile bee saline (130 mM NaCl, 6 mM KCl, 4 mM MgCl₂, 135 mM CaCl₂, 160 mM sucrose, 25 mM glucose, 10 mM 4-(2-hydroxyethyl)-1-piperazine ethane-sulfonic acid in dH₂O; Richard *et al.*, 2008) to a final concentration of
0.04 µg/µl solution, 20% isopropanol. This dose was previously used in studies of gene expression in the honey bee brain, neuroanatomical studies of honey bee dendritic growth, and in behavioral studies (Velarde et al., 2009; Ford et al., 2011; Chapter 3, this dissertation). Vehicle treatments consisted of 20% isopropanol in sterile bee saline. Non-injected bees were chilled and handled. Injected honey were transferred to acrylic cages (Plexiglas®, 10 cm x 8 cm x 10 cm) and randomly assigned to the QMP+ or QMP- condition. Each cage contained 25-30 treated honey bees. All cages were maintained as described in experiment 1. On the fifth day after the cages were established (day 5 of adult life), hemolymph samples were obtained for EIA and hemocyte analysis. Whole heads were frozen in liquid nitrogen for GOX analysis. Development of ovaries was assessed using the 5 point scale of Pernal and Currie (2000).

**Experiment 3:** Newly emerged bees were randomly assigned to QMP+ and QMP- cages and housed as described in experiment 1. On day 5 bees were prepared for injection and were randomly assigned to a treatment: control (non-injected), vehicle treatment, LPS treatment, 20E and LPS treatment. LPS is the major outer surface membrane found on most gram-positive bacteria. It acts as a strong innate immune system stimulant in arthropods and vertebrates. LPS treatment consisted of a 1 µl injection of 500 ng/µl LPS (from *Escherichia coli* serotype 055:B5, Sigma) dissolved in a 4:1 solution of bee saline to isopropanol. Ecdysone-LPS treatments consisted of a 1 µl injection of 500 ng/µl LPS and 0.04 pg/µl 20E dissolved in a 4:1 solution of bee saline to isopropanol. Dose of LPS was based on a study by Richard and colleagues (2008). Vehicle treatments consisted of a 1 µl injection of a 4:1 solution of bee saline to isopropanol. Non-injected bees were chilled and handled. All bees were transferred to acrylic cages (Plexiglas®, 10 cm x 8 cm
x 10 cm) and held in an incubator (28°C, 40% relative humidity) for three hours prior to sampling. Following this incubation period hemolymph samples were obtained for EIA and hemocyte analysis. Development of ovaries was assessed using the 5 point scale of Pernal and Currie (2000).

Experiment 4: Honey bees were collected from an established colony in March 2016. All individuals present were overwintered workers. Due to a lack of queen laid brood this colony was considered hopelessly queenless (QL). The colony was QL for a period of time sufficient to allow for significant numbers of worker-laid eggs to accumulate and develop as drone brood. Workers used in this study were collected from drone comb. Workers from colony treatments were chilled in the field to reduce the possibility that transport stress would alter ecdysteroid titers. Hemolymph samples were obtained for EIA and hemocyte analysis. Development of ovaries was assessed using the 5 point scale of Pernal and Currie (2000). Whole heads were flash frozen for GOX analysis.

4.3.6 Statistics

The effects of QMP on ecdysteroid production (log transformation used to correct for skew) and hemocytes in experiment 1 was analyzed using a student’s t test assuming unequal variance. The effects of QMP on ecdysteroid production, hemocytes and GOX production were analyzed with two-way ANOVAs (pheromone exposure and treatments) followed by Tukey-Kramer post hoc tests for pairwise tests. In experiment 1 a hierarchical cluster analysis was performed on untransformed ecdysteroid titers using Ward’s method to separate individuals exposed to QMP into clusters. Ward’s method joins cases into clusters such that the variance within each cluster is minimized (Everitt,
1993). Ecdysteroid and hemocyte data in experiments 2 and 3 were transformed using a standard log transformation to correct for skew prior to analysis (IMB SPSS Statistics 22). Graphical representation of data displays untransformed values for ease of analysis. Student t-test assuming equal variance was used to compare hemocyte and GOX data in experiment 4. A student’s t-test assuming unequal variance was used to compare ecdysteroid titers in experiment 4. Differences in ovariole number and morphological development were examined with student’s t test assuming equal variance and a two-way ANOVA where appropriate.

4.4 RESULTS

Experiment 1 compared ecdysteroid and hemocyte levels of 12 day old worker bees held in cages with or without continuous exposure to QMP. Ecdysteroid titers were not significantly higher in the QMP+ group (49.93 ± 28.39 pg/µl, n = 14) compared with the QMP- group (13.51 ± 6.3 pg/µl n = 18) (p = 0.11)(Fig 1A). However, consistent with Chapter 2 of this dissertation there was a large variation in the QMP+ group, with a percentage of individuals (responders) showing individually very high levels of ecdysteroids. Hemocyte counts were significantly higher in the QMP+ group (3400 ± 414.73 mm$^3$, n = 21) compared with the QMP- group (2478.3 ± 275.12 mm$^3$, n = 23)(p < 0.05, t = 1.88, df =42) (Fig 1B). There was a strong positive correlation within responders in the QMP+ group with hemocyte counts (r = 0.8876, p = 0.05, n = 4). This sample size represents the average number of responders seen in QMP+ cages (approximately 30%, as identified by a hierarchical cluster analysis using ward’s method) but is a relatively small size to attempt such correlations on. Non-responders did not show a correlation within the QMP+ group (r = -0.3376, p > 0.05, n = 10). There was a
moderate positive correlation in the QMP- group (no responders present) \( (r = 0.5274, p < 0.05, n = 17) \). There were no signs of morphological development of the ovaries (QMP+ 0.23 ± 0.09, QMP- 0.35 ± 0.2) or difference in ovariole count (QMP + 2.1 ± 0.23; QMP- 2.7 ± 0.23, \( n = 21, 23 \)).

**Figure 1.** *Experiment 1* (A) Mean ecdysteroid titers (pg/µl) of 12 day old bees in QMP+ and QMP- conditions, \( n = 14, 18 \). (B) Mean hemocyte counts (mm\(^3\)) of 12 day old bees held in QMP+ and QMP- conditions, \( n = 21, 23 \). * indicates significant difference, \( p < 0.05 \).
Experiment 2 compared ecdysteroid titers, hemocyte count and GOX levels of 5 day old worker bees that had been treated with ecdysteroids on day 1 and held in QMP+ and QMP- conditions. A trend of elevated ecdysteroid titers was observed in the non-injected QMP+ group (108.17± 64.7 pg/µl, n = 18) compared with non-injected bees in QMP- conditions (27.12 ± 10.03 pg/µl, n = 25) (p = 0.06) (Fig 2). In the vehicle injected group there were elevated ecdysteroid levels in QMP- conditions (81.18 ± 32.1 pg/µl, n = 9) compared to QMP+ conditions (4.38 ± 1.08 pg/µl, n = 15) (p < 0.05). There was no significant difference between groups when injected with 20E (QMP+ 20E 39.66 ± 18.68 pg/µl, n = 7; QMP- 20E 100.48 ± 80.4 pg/µl, n = 9).

Figure 2. Experiment 2 Mean ecdysteroid titers (pg/µl) of 5 day old bees in QMP+ and QMP- conditions, treated on day 0, n = 18, 25,15, 9, 7, 9. * denotes significant difference, p < 0.05.
There was a significant effect of injection on hemocytes in the QMP+ condition \((F_{2,58}= 20.06 \ p < 0.0001)\) (Fig 3A). Hemocytes were significantly elevated in the control condition \((6950 \pm 233.21 \ \text{mm}^{-3}, \ n = 24)\) as compared with the vehicle injection \((3105.09 \pm 720.52 \ \text{mm}^{-3}, \ n = 17)(p < 0.0001)\). Hemocytes were significantly lower in the 20E treatment \((4366.67 \pm 428.17 \ \text{mm}^{-3}, \ n = 18)\) compared with the control \((p < 0.01)\). Hemocytes were significantly higher in the 20E treatment as compared with the vehicle \((p <0.01)\). There was no significant effect of injection on GOX levels \((F_{2,38} = 0.470, \ p > 0.05)\) (Fig 3B) in the QMP+ condition. There were no signs of morphological development \((\text{QMP+} \ 0 \pm 0, \ \text{Vehicle} \ 0.2 \pm 0.11, \ 20E \ 0 \pm 0)\) or difference in ovariole count \((\text{QMP+} \ 3.55 \pm 0.26, \ \text{Vehicle} \ 3 \pm 0.31, \ 20E \ 3.29 \pm 0.29)\) \((n = 18, 15, 7)\).
There was a significant effect of injection on hemocytes in the QMP- condition \( (F_{2,36} = 13.77, p < 0.0001) \) (Fig 4A). Control hemocytes \((7208.7 \pm 2251.85 \text{ mm}^3, n = 23)\) were significantly higher than those in vehicle injected group \((3275 \pm 579.33 \text{ mm}^3, n = 8)\) \((p < 0.0001)\). The vehicle group had significantly lower hemocyte counts than the 20E injected group \((5666.67 \pm 635.96 \text{ mm}^3, n = 7)\) \((p < 0.0001)\). The control and 20E group did not differ. There was a significant effect of treatment on GOX values in the QMP-
condition \( (F_{2,38} = 22.54, p < 0.0001) \) (Fig 4B). GOX levels were significantly lower in the 20E injected group \( (3.51 \pm 1.36, n = 13) \) compared with the control \( (10.39 \pm 1.12 \text{ mU/ml, } n = 13) \) and vehicle injected group \( (13.44 \pm 0.59 \text{ mU/ml, } n = 13) \) \( (p < 0.0001) \). There were no signs of morphological development of the ovaries \( (\text{QMP- } 0.08 \pm 0.05; \text{ Vehicle } 0.11 \pm 0.11; \text{ 20E } 0.11 \pm 0.11) \) or difference in ovariole count \( (\text{QMP- } 3.24 \pm 0.3, \text{ Vehicle } 3.22 \pm 0.6, \text{ 20E } 4.33 \pm 0.24) \) \( (n = 25, 9, 9) \).
Experiment 3 compared the immediate effects of an immune challenge, injection of LPS, on 5 day old bees held in QMP+ and QMP- conditions. In the QMP+ group, there was no significant effect of treatment on ecdysteroids ($F_{3,38} = 0.690, p > 0.05$) or hemocytes ($F_{3,51} = 1.758, p > 0.05$). As seen previously, there was a trend of elevated ecdysteroid levels QMP+ ($63.15 \pm 35.39$ pg/µl, $n = 11$) compared to those in QMP- conditions ($12.75 \pm 6.80$ pg/µl, $n = 13$).

Figure 4. Experiment 2 (A) Mean hemocyte counts (mm$^{-3}$) of 5 day old bees in QMP- conditions, treated on day 0, $n = 23, 8, 7$. (C) Mean GOX (mU/ml) levels in 5 day old QMP- bees, $n = 13$ all groups. Letters denote significantly different groups, $p < 0.05$. 

Experiment 3 compared the immediate effects of an immune challenge, injection of LPS, on 5 day old bees held in QMP+ and QMP- conditions. In the QMP+ group, there was no significant effect of treatment on ecdysteroids ($F_{3,38} = 0.690, p > 0.05$) or hemocytes ($F_{3,51} = 1.758, p > 0.05$). As seen previously, there was a trend of elevated ecdysteroid levels QMP+ ($63.15 \pm 35.39$ pg/µl, $n = 11$) compared to those in QMP- conditions ($12.75 \pm 6.80$ pg/µl, $n = 13$).
There was a significant effect of treatment on ecdysteroids in the QMP- group (F_{3, 40} = 4.305, p < 0.05). Control titers (12.75 ± 6.80 pg/µl, n = 13) were significantly lower than the vehicle (26.09 ± 4.86 pg/µl, n = 10)(p < 0.05) and LPS group (47.27 ± 9.18 pg/µl, n = 6 )(p < 0.01). Control titers did not differ from the 20E LPS group (26.6 ± 8.27 pg/µl, n = 12).

Figure 5. Experiment 3 (A) Mean hemocyte counts (mm^{-3}) of 5 day old bees housed in QMP+ conditions 3 hours after treatment, n = 14, 9, 16, 11. (B) Mean hemocyte counts (mm^{-3}) of 5 day old bees housed in QMP- conditions 3 hours after treatment, n = 9, all groups.
There was no significant effect of treatment on hemocytes in the QMP- group ($F_{3,49} = 2.078, p > 0.05$). There was a significant effect of pheromone exposure on hemocytes across all treatments ($F_{1,101} = 6.675, p < 0.05$)(Fig 5). Higher levels of hemocytes were observed across all QMP- treatments as compared to QMP+ treatments.

Experiment 4 described ecdysteroid titers, hemocyte counts and GOX units in bees collected from an overwintered, hopelessly QL colony. There was a significant positive correlation between ovariole number and morphological development of the ovaries ($r = 0.578, p < 0.0005, n = 33$). Ecdysteroid titers were low in all collected bees. There was no significant difference in ecdysteroid titers in bees with no morphological development ($2.044 \pm 1.96 \text{ pg/µl}, n = 25$) compared with bees with morphological development greater than 0 ($0.0 \pm 0.0 \text{ pg/µl}, n = 7$)(Fig 6A). There was no significant difference in hemocyte count in bees with no morphological development ($7232 \pm 998.7 \text{ mm}^{-3}, n = 25$) with bees with morphological development greater than 0 ($5628.57 \pm 1103.6 \text{ mm}^{-3}, n = 7$)(Fig 6B). There was no difference in GOX units in bees with no morphological development ($1.873 \pm 0.016 \text{ mU/ml}, n = 22$) with bees with morphological development greater than 0 ($1.87 \pm 0.013 \text{ mU/ml}, n = 6$)(Fig 6C).
Figure 6. *Experiment 4* (A) mean ecdysteroids (pg/µl) of overwintered bees from a QL colony, *n* = 25, 7. (B) mean circulating hemocytes (mm$^{-3}$) of overwintered bees from a QL colony, *n* = 25, 7. (C) mean GOX units (mU/ml) of overwintered bees from a QL colony, *n* = 22, 6.
4.5 DISCUSSION

Immunocompetence in 12 day old worker bees

Our results suggest that ecdysteroids and pheromone exposure may modulate immunocompetence, although the overall pattern of responses is complex and requires further confirmation. We found that QMP exposure resulted in increased ecdysteroid titers and an increase in circulating hemocyte concentrations in 12 day old worker bees. However, it is unclear if high levels of hemocytes are a reliable indicator of increased immunocompetence. Amdam and colleagues (2004) have suggested that decreased hemocyte levels correlate with a loss of immunocompetence; alternatively, a high level of circulating hemocytes may indicate the presence of active pathogens, which may in turn indicate that other defenses have failed. Hemolymph concentrations of vitellogenin, a potent zinc carrier in oviparous organisms, decrease as honey bees begin to forage, typically around 2 - 3 weeks of age (Amdam et al., 2004; Mitchell and Carlisle, 1991). Zinc is thought to inhibit the pyknosis (shrinkage, often a precursor to cell death) of hemocytes. It has been proposed that, as vitellogenin titers decrease, the amount of zinc in the hemolymph decreases, resulting in a decrease in the proportion of functional (non-pyknotic) hemocytes. In contrast, Alaux and colleagues (2010) have suggested that lower levels of hemocytes may be due to individuals investing more metabolic activity in the production of small numbers of more metabolically costly subtypes of hemocytes, such as granulocytes. Indeed, bees fed a diet deficient in protein display higher circulating levels of hemocytes then those fed a rich protein source (Alaux et al., 2010). Because we did not differentiate between hemocyte types in the present study it is difficult to assess if decreased hemocyte concentrations was due to a general decrease in hemocytes or in
specific subtypes of hemocytes. This hypothesis could be further explored using methods that allowed for identification of hemocyte type as an alternative to total hemocyte counts, as was done by Manfredini et al. (2008).

We found preliminary evidence to suggest a positive correlation in responders (bees that have elevated ecdysteroid titers after exposure to QMP), between ecdysteroid titers and circulating hemocyte concentrations. This suggests a scenario where high ecdysteroid concentrations may be inducing an overall increase in immunocompetence. However, this sample size was very small due to the presence of on average 30% of individuals being responders in a sample (Chapter 2 of this dissertation). While knowledge of the effects of 20E on immunity in honey bees is limited, recent studies performed in the silk moth Bombyx mori provide support for this hypothesis. Injection of silk worms with low doses of 20E increased the transcription of antimicrobial peptide and pattern recognition receptor genes (Xu et al., 2012). In another study, treatment with 20E increased survival rates of silk worms injected with live bacterial cultures and increased the clearance rate of FITC-labeled bacteria, suggesting 20E resulted in an enhancement of immunocompetence (Sun et al., 2016). These methods could be adapted for use in honey bees.

*Immunocompetence in 5 day old worker bees*

We also examined hemocyte concentrations in younger honey bees. Interestingly, 5 day old bees had higher circulating levels of hemocytes in all treatments in both QMP+ and QMP- conditions compared with concentrations observed in 12 day old bees from either condition. This is in accord with studies that have shown high levels of circulating
hemocytes in young adult honey bees that subsequently decrease with age (Alaux et al., 2010; Schmid et al., 2008; Amdam et al. 2004). A conflicting report, however, points to lower hemocyte concentrations in newly emerged bees compared with foraging bees (Wilson-Rich et al., 2008). It is possible that newly emerged honey bees begin adult life with very low concentrations of hemocytes and rapidly increase these numbers in the first few hours or days post emergence. It will be necessary to sample hemocytes in developing adults and pharate adults to test this scenario.

Treatment of 5 day old honey bees with 20E in QMP+ conditions resulted in depressed hemocyte concentrations compared with non-injected controls. Hemocyte concentrations were augmented in the 20E treated group when compared with the vehicle injected group. Low circulating numbers of hemocytes in the vehicle treatment could be due to the puncturing of the hemocoel, a traumatic event that likely acts as an immune stressor (Adamo and Parsons, 2006). The midrange hemocyte concentration of hemocytes in the 20E treatment group suggests several possibilities. 20E could have exerted a protective, buffering effect on the stress of injection, preventing hemocyte levels from falling to those observed in the vehicle injected group. It is also possible that individuals in the 20E treatment were initially depressed to vehicle-like hemocyte counts but recovered more quickly in the presence of the hormone. It is difficult to identify the direction of the change without hemocyte counts from newly emerged honey bees. Finally, it is possible that hemocyte levels were low in all newly emerged bees at the time of injection (as reported by Wilson-Rich et al., 2008) and the rate of hemocyte addition was depressed in the vehicle group, normal in the control group and only partially depressed in the 20E group. A similar trend was observed in the QMP- group: depressed
hemocyte concentrations in the vehicle group with increased levels in control and 20E treatments.

In the QMP+ condition, GOX levels were unaffected by injection suggesting a lack of steroid effect on this measure of social immunity. This is somewhat surprising in light of the report from Alaux et al. (2010), who showed that GOX levels were significantly increased in honey bees offered a pollen source. Pollen is the primary source of sterols in the honey bee diet, sterols that are necessary for the production of steroid hormones. We did not show any modulatory effect in any treatment group in the QMP+ condition. However, in the QMP- condition GOX levels were significantly depressed in the 20E treatment group. This could suggest an interaction between the stress of QL conditions and the steroid. Interestingly, GOX levels were also low in all workers collected from the QL overwintered colony. It has been previously shown that workers with ovaries developed for egg laying, collected from hopelessly QL colonies, have lower viral loads then age matched sterile workers (Cardoen et al., 2011). This was interpreted as an indicator of increased immune function in laying workers. In contrast, our results suggest that workers from conditions more likely to favor increased ovary development (those from QMP- or QL conditions), have decreased levels of GOX. Active egg laying by worker bees is accompanied by an increase in ecdysteroid titers (Robinson, 1991; Chapter 2 of this dissertation). This increase, like ovarian activation, may be transient and only accompany the period of active laying, similar to the injections in our experiment. It is possible that those workers collected from the QL colony had previously experienced periods of laying and elevated 20E titers that had similar effects as those observed in the QMP- cage. GOX is considered a measure of social immunity as its addition to brood
food stores benefits the colony as a whole. Egg laying by workers most often occurs in hopelessly QL colonies as a last ditch attempt to proliferate the colony’s genes. This dichotomy, investing in immune measures that increase personal immunity while not investing in social immunity, may representative a tradeoff in overall colony health brought about by the stress of QL conditions. It will be profitable to explore this trade-off directly in future studies.

*LPS immune challenge*

Experiment 3 focused on the short term response of ecdysteroid titers and hemocyte concentrations to the direct immune challenge of an LPS injection in 5 day old bees. In the QMP- treatment, both vehicle injections and LPS injections induced an increase in ecdysteroid titers three hours after treatment. A moderate increase in ecdysteroid titers was observed in bees injected with a combination of LPS and 20E. Elevated circulating ecdysteroid titers has been shown to occur in insects undergoing stressful conditions such as starvation, elevated temperatures and sleep deprivation (Rauschenbach et al., 2000; Chen & Gu, 2006; Ishimoto & Kitamoto, 2010). It is possible that the elevated ecdysteroid titers observed here are the result of ecdysteroids functioning as a stress hormone to facilitate the immune response due to breach of the hemocoel, similar to the increase of glucocorticoids in vertebrates in response to stress (Busillo et al., 2011). While a response is evident in ecdysteroid titers, within the time course of our measurements it did not induce detectable changes to hemocyte concentrations. This could be due to the length of time between immune challenge and sample collection or due to 20E have more long term modulatory effects, such as the upregulation of immune related gene expression (Russ et al., 2013). Increases in
Ecdysteroid titers was not observed in the QMP+ group suggesting a potential pheromone dependent effect. It is possible that the QMP- condition, meant to mimic QL colonies, is inherently stressful, leaving these individuals more susceptible to elevated stress responses. While hemocyte levels were unaffected by treatment in both groups, hemocytes in the QMP- group were elevated in all treatments as compared to the QMP+ treatments.

*Overwintered QL bees*

The final area we focused on was immunocompetence in overwintered bees from a hopelessly QL colony. While summer bees typically live 30 days, overwintered bees can live for 6 to 9 months and undergo several physiological changes such as increased protein content in the fat body and well developed hypopharyngeal glands (Halberstadt, 1966). We confirmed a positive correlation between ovariole number and morphological development of the ovaries as reported in previous studies (Traynor et al., 2014; Amdam et al., 2006; Tsuruda et al., 2008; Page et al., 2006, 2012). However, ecdysteroid titers were low in both groups and did not correlate with either ovariole count or morphological development. It is important to note that while a subset of bees did display some signs of morphological development, only one sample showed significant oocyte development with others only showing intermediate levels of ovariole swelling. It is also possible that individuals had previously been active egg layers as ovarian development is a dynamic state (Malka et al., 2007). This is the first reported measure of ecdysteroid titers from true overwintered bees. These values were lower than those reported by Amdam and colleagues (2004) who took samples from bees less than 8 days in age housed in simulated overwinter conditions. Further, we found no differences in GOX activity.
between individuals with or without signs of morphological development. As previously described, GOX activity was low in all sampled winter bees. Prior studies have shown hemocyte levels in overwintered bees to be comparable to those observed in foragers (Fluri, 1977). We confirm that hemocyte levels were generally high and found no differences correlated with ovarian development.

The effects of ecdysteroids on innate insect immunity is a rapidly developing field of study. Here we provide preliminary evidence of a modulatory effect of QMP on hemocytes in 12 day old worker bees, in the form of a correlation between elevated ecdysteroid titers in responders and an increase in hemocyte concentration. We also showed a potential enhanced recovery from a decrease in hemocytes by treatment of 20E in 5 day old bees independent of pheromone exposure. We also present evidence that bees held in QL conditions may have unique immunocompetence profiles, specifically reducing the production of GOX. These findings make an initial contribution to basic research on honey bee health: many questions remain to be answered.
4.6 LITERATURE CITED


White, J. W., Subers, M. H., & Schepartz, A. I. (1963). The identification of inhibine, the antibacterial factor in honey, as hydrogen peroxide and its origin in a honey glucose-oxidase system. Biochimica et Biophysica Acta (BBA) - Specialized Section on Enzymological Subjects 73(1), 57–70.


CHAPTER 5
SUMMARY AND CONCLUSION

5.1 Synoptic Overview

My dissertation aimed to provide insight into a fundamental question in biology: how do complex phenotypes evolve? I specifically sought to provide answers to this question for eusocial insects under the umbrella of the reproductive ground plan hypothesis. I focused on hormones historically considered to regulate molting, metamorphosis, and reproduction, the ecdysteroids. Studies in the early 2000s revealed that other mechanisms once regarded as exclusively reproductive also function to regulate the physiology and behavior of non-reproductive worker honey bees. A well-known example is the reconceptualization of the yolk protein precursor, vitellogenin, as both a support for the production of brood food and a regulator of the transition from hive tasks to foraging (Amdam et al., 2003; Amdam et al., 2004; Guidugli et al., 2005).

In chapter 1 of this dissertation I posed the question of why receptors for a hormone assumed absent in the adult insect would be present throughout adult life in tissues such as the central nervous system. Previous research in this field has focused on pooled samples and a reliance on a radioimmunoassay based on a single antibody that is no longer available. This has resulted in a field based on a relatively small number of measurements and by and large blind to variation amongst individuals. I focused on the use of a commercially available and sensitive enzyme immunoassay to overcome this persistent obstacle to progress in insect endocrinology. My ability to discriminate
between individuals and to correlate circulating levels of hormones with other tissue samples from the same bee has provided invaluable information related to the ecdysteroid profile of adults in normal colony conditions. The data presented in chapter 2 led to a revision of the previously established view of developmental hormones in *Apis mellifera* (Fahrbach *et al.*, 2016) (Fig 1). I have shown that in normal colony conditions with an active queen present, adult worker honey bees display a bimodal response modulated by the presence and age of exposure to QMP. This results in distinct groups of responders (individuals that exhibit high levels of ecdysteroids in response to early continuous QMP exposure) and non-responders (individuals that do not exhibit high levels of ecdysteroids to early continuous QMP exposure) (Chapter 2, this dissertation). This reformulation of the expected ecdysteroid titers of adult worker honey bees is important to the continued development of this field and fills long standing gaps in our knowledge. I have also provided evidence that the ovaries of facultatively sterile worker bees represent a continued source of ecdysteroids in the adult worker. This has provided substantial insight into one of the questions that inspired this research: ecdysteroid receptors are present into adulthood because ecdysteroids are present. I have also demonstrated that so-called “morphologically inactive ovaries” can and do produce ecdysteroids.

Chapters 3 and 4 of this dissertation were designed to explore roles for ecdysteroids in worker honey bees. With the knowledge that both ecdysteroid receptors and the hormone were present in adult workers, I asked with confidence the question of what role was this hormone system playing in adult physiology and behavior? In chapter 3 I expanded upon previously established model of hormonal regulation in the transition to foraging (Huang and Robinson, 1996; Guidugli *et al.*, 2005). I provided evidence that
early ecdysteroid treatment can accelerate the transition to foraging. This integrates another facet of regulation into the suite of factors already implicated in regulating the timing of the transition to foraging.

While it has been previously shown that increased ovary size accelerated the transition to foraging, the factor that links the ovaries and the brain regions that control the behavior had not yet been identified (Wang et al., 2010). My studies extend this work by showing both that the undeveloped worker ovaries are a significant source of circulating ecdysteroid and that ecdysteroids can accelerate the transition to foraging. Continued research should focus on methods that would allow for identification of responders and non-responders without the use of terminal sampling techniques. Similar to the other known effects of ecdysteroids on the brain, fat body, and ovaries, perhaps the previously documented early ecdysteroid pulses prepare the brains of some individuals to transition to foraging more readily (Velarde et al. 2009, Wang et al., 2009, 2010; Amdam and Page, 2010).

Chapter 4 provided preliminary evidence for a role of ecdysteroids in modulation of adult immunocompetence. I showed a modulatory effect of QMP on hemocytes in 12 day old worker bees. I interpreted this as a potential ecdysteroid effect through a correlation between elevated ecdysteroid titers in responders (individuals in QMP+ conditions with elevated ecdysteroids) and an increase in hemocyte concentration. Through injection treatments we showed that a puncturing of the hemocoel is a traumatic stressor that appears to decrease hemocyte levels with a potential recovery effect by treatment with 20E. I also present evidence that bees held in QL conditions may have unique immunocompetence profiles, specifically affecting the production of GOX. These
studies, however, represent an area where significant research remains to be done. The parameters defining immunocompetence must be better defined in terms of concentration, availability and types of hemocytes present in individuals. A profile of typical patterns of hemocytes in adults would be highly beneficial to this area of research.

This chapter also extended our knowledge of the ecdysteroid titers in adult workers by making the first ecdysteroid measurements in overwintered individuals. Overwintered bees present a unique opportunity for this field of research as they allow examination of the impact of longevity on physiology.

5.2 Ecdysteroids and the reproductive ground plan hypothesis

This dissertation contributes to a growing body of literature that supports a role for ecdysteroids in the reproductive ground plan hypothesis. The reproductive group plan hypothesis is based on the idea that pathways that were once based solely in reproduction were co-opted for the regulation of complex social behaviors as organisms evolved higher levels of sociality (West-Eberhard 1987; West-Eberhard 1996; Amdam et al., 2004). Its original formulation for honey bees proposed that worker honey bees with more ovarioles and increased titers of Vg are more “reproductive” than other workers and bias their foraging towards pollen collection, resulting in a division of foraging tasks between workers of similar ages (Amdam et al., 2004, 2006; Page 2012). I now suspect that part of the underlying mechanism behind the bias seen toward pollen foraging may reflect the evolution of ecdysteroids as a regulator in the division of labor. This idea, the inclusion of our findings, and some of questions that remain to be answered are further outlined in Figure 2.
I chose in my research to focus on hormones, behavior, hemocyte populations, and enzyme expression rather than rely on analysis of gene expression. I thought this would keep my attention focused on robust phenomena as well as keep me from going down dead ends based solely on interpretation of slight differences in gene expression. In the end I am surprised at the subtle and slight manifestations of many of the effects I studied. But the data presented here should provide a strong foundation for further investigations into physiology of worker honey bees and possibly the mechanisms that keep insect societies running smoothly.
Figure 1. Upper panel shows an earlier view of hormone titers (JH and ecdysteroids) in adult worker honey bees. X-axis represents days of adult life with typical colony roles. Presented in the bottom panel is the updated view to reflect the possible bimodal ecdysteroid response of workers in a QR colony as established by chapter 2 of this dissertation.
Figure 2 (Next page). Broader context of endocrine ovarian activation in adult honey bee workers. Italics represent untested hypotheses. Data compiled from: Robinson (1985); Robinson et al., (1991); Hartfelder (2002); Amdam & Omholt (2003); Grozinger (2003); Grozinger et al., (2007); Chapter 2, this dissertation.
5.3 Literature Cited


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EDUCATION

Wake Forest University
Ph.D. Biology
Winston-Salem, NC
May 2016

Gettysburg College
B.S. Biology, Minor Neuroscience
Gettysburg, PA
May 2009

RESEARCH EXPERIENCE

Doctoral Dissertation
Wake Forest University
Winston-Salem, NC
Aug 2010 – May 2016

- Investigating the role of the steroid hormones, ecdysteroids, on the physiology and behavior of adult worker honey bees (Apis mellifera)

Biology Thesis Study
Gettysburg College
Gettysburg, PA
Sept 2008 - May 2009

- Studied the effects of cortisol on the behavior and adoptability of dogs housed in public shelters

Neuroscience Thesis
Gettysburg College
Gettysburg, PA
Sept 2008 - May 2009

- Examined the effects of perinatal exposure to environmental pollutant bisphenol A in rats on gender specific adult play behavior

PROFESSIONAL PRESENTATIONS


**MANUSCRIPTS IN PRESS**


**MANUSCRIPTS IN PREPERATION**

Trawinski, A. M. and Fahrbach, S. E. Pheromone Modulation of Ecdysteroid Production in Adult Worker Honey Bees (*Apis mellifera*). Apidologie.

Trawinski, A.M. and Fahrbach, S. E. Early Ecdysteroid Treatment Accelerates Foraging in Honey Bees (*Apis mellifera*).

**TEACHING EXPERIENCE**

**Undergraduate Research Mentor**  
Wake Forest University  
Winston-Salem, NC  
Aug 2011 - Present

- Facilitated undergraduate students in independent research projects spanning a single semester to year-long projects
- Taught experimental design, lab techniques, and data analysis
- Coached and supported students through their creation of scientific presentations, posters, and reports

**Guest Lecturer, AP Biology**  
Forsyth Country Day Upper School  
Lewisville, NC  
Feb 2014

- Designed and delivered interactive lecture on insect endocrinology and behavior.
Biology Teaching Assistant, Cell Biology Laboratory  
Winston-Salem, NC  
Wake Forest University  
Aug 2014 - Dec 2015

- Taught upper division cellular biology laboratory courses  
- Designed syllabus, assignments, grading rubric, quizzes, and projects  
- Employed online learning management system to administer quizzes, deliver and collect assignments, provide continual access to grades, and direct communication with students  
- Aided in laboratory setups  
- Taught critical science writing and evaluated laboratory reports

Biology Tutor  
Winston-Salem, NC  
Wake Forest University  

- Tutored subjects include: comparative physiology, biology for non-majors, cellular biology, microbiology, genetics, virology, molecular biology, and animal behavior  
- Facilitated students in targeting problem areas in scientific writing and lecture comprehension  
- Designed active study techniques  
- Created active learning based study guides

Lead Instructor, Project SEARCH Academy  
Winston-Salem, NC  
Wake Forest School of Medicine  
June 2015

- Taught week long science camp targeted at rising 9th graders.  
- Focused on concepts including experimental design and critical thinking.  
- Managed labs designed to reinforce hypothesis driven thinking.

Guest Lecturer, Animal Behavior  
Greensboro, NC  
Guilford College  
May 2015

- Designed and delivered interactive lecture on insect pheromones and eusociality

Guest Lecturer, Animal Behavior  
Greensboro, NC  
Guilford College  
Oct 2014

- Designed and delivered interactive lecture on insect endocrinology and behavior.

Assistant Instructor, Project SEARCH Academy  
Winston-Salem, NC  
Wake Forest School of Medicine  
July 2011 - 2014

- Teaching assistant for week long biomedical science camp targeted at rising 9th graders.  
- Taught concepts including experimental design, critical thinking, clinical chemistry, protein electrophoresis, and medical imaging.  
- Aided in executing labs designed to reinforce biomedical themes.
Biology Teaching Assistant, Comparative Physiology  
Winston-Salem, NC  
Wake Forest University  
Aug 2010 – May 2013

- Taught introductory level comparative physiology and biology laboratory course
- Created syllabus, assignments, quizzes, and projects
- Aided in laboratory setups
- Taught critical science writing, and graded laboratory reports

Guest Lecturer, Comparative Physiology  
Winston-Salem, NC  
Wake Forest University  
April 2012

- Designed and delivered interactive lecture covering endocrine control of human reproduction

Guest Lecturer, Comparative Physiology  
Winston-Salem, NC  
Wake Forest University  
Feb 2012

- Guided students through a case study designed to target understanding of the cardiac and pulmonary systems

PROFESSIONAL MEMBERSHIPS

National Association of Biology Teachers: Member  
2014 - Present

GRANTS

Wake Forest University Center for Molecular Signaling Grant  
2013

PROFESSIONAL EXPERIENCE

Aquarium Employee  
Point Pleasant Beach, NJ  
May 2006 - Aug 2010

- Interned in summer of 2006. Returned as both a full and part time employee
- Responsible for daily feeding, cleaning, enrichment, and caring for marine mammals, small primates, exotic birds, fish, amphibians, reptiles, and invertebrates
- Provided educational tours for children aged 3 – 18, and adults
- Assisted with workshops and labs for students aged 7-18
Chemist
MILSPRAY Military Technologies
Lakewood, NJ
Oct 2009 - Aug 2010

- Primary organic chemist in research and development laboratory
- Responsible for design and execution of quality and environmental tests
- Provided monthly summary reports of results, expectations, and product reviews
- Assisted in creation and modification of products designed for the US military

Child Care Assistant
Learning Tree Day Care
Gettysburg, PA
Sept 2008 - May 2009

- Assisted in care of 20 – 30 preschool aged children

Biology Department Assistant
Gettysburg College
Gettysburg, PA
Sept 2007 - May 2008

- Assisted department in the care of research animals.
- Aided in laboratory setups

VOLUNTEER SERVICE

Admitted Student Visitation Program
Wake Forest University
Winston-Salem, NC
March 2014 -2016

- Escorted and demonstrated lab techniques for 60 high school students

High School Visitation Program
Wake Forest University
Winston-Salem, NC
Sept 2011 – 2015

- Escorted and demonstrated lab techniques for 60 high school students

Stokes High School Science Outreach Program
Wake Forest University
Winston-Salem, NC
March 2014

- Escorted and demonstrated lab techniques for 60 high school students

High School Genetics Outreach
Mt. Tabor High School
Winston-Salem, NC
March 2013

- Aided in high school outreach, consisting of two sessions of approximately 60 students each.
- Taught concepts that included punnett squares, cladograms, and gene modification through hands-on activities.
Science Olympiad Judge
Atkins High School
Winston-Salem, NC
March 2013

- Aided in creating, judging and scoring biology themed events for middle and high school students.

Microscopy Demonstration and Lab
Kernersville Elementary School
Kernersville, NC
Dec 2010

- Science demonstration teaching microscopy skills to 6 – 9 year olds.