ABSTRACT

FUSSNECKER, BRENDON LOUIS. Analysis of Molecular and Physiological Pathways Regulating Social Behavior in Honey Bees: Elucidating the Role of *Kr-h1* and cGMP. (Under the direction of Christina M. Grozinger and James W. Mahaffey).

Kruppel homolog 1 (Kr-h1) is a zinc finger transcription factor whose brain expression levels are associated with foraging behavior and are regulated by queen mandibular pheromone (QMP) in honey bees. We used behavioral, physiological, and genomic approaches to investigate the factors which regulate expression of *Kr-h1* in honey bees, and employed a comparative genomics approach to begin to characterize the molecular function of this protein. We demonstrated that brain expression of *Kr-h1* is associated with permanent physiological changes that occur during behavioral maturation from nursing to foraging, rather than the acute expression of foraging behavior. Furthermore, we demonstrated that *Kr-h1* expression is modulated by cGMP, a key regulator of behavior maturation, and identified a potential cGMP response element in the promoter of *Kr-h1*. We then characterized the interactions between cGMP and QMP. cGMP inhibited behavioral and physiological responses to QMP, and partially inhibited expression changes of QMP-responsive genes in the brain. Treatment with these factors specifically altered expression of genes associated with GTPase regulator activity, phototransduction, and positive regulation of antibacterial peptide biosynthetic process. However, cGMP did not affect the expression of *AmOr11*, an odorant receptor specific for a major chemical component of QMP, suggesting that cGMP might work centrally rather than peripherally to modulate the response to QMP. Finally, we elucidated changes in gene expression that resulted from the absence of *Kr-h1* expression during *D. melanogaster* development and discovered an associated motif in

the promoter of the majority of the significantly expressed genes, which may mediate the effects of *Kr-h1*. Further analysis, however, showed that this motif is unlikely to be biologically relevant. These studies have demonstrated the ability of physiological factors to modulate responsiveness to social cues, and provided further information about the role of *Kr-h1* in regulating behavior in bees.

Analysis of Molecular and Physiological Pathways Regulating Social Behavior in Honey Bees: Elucidating the Role of *Kr-h1* and cGMP

by

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DEDICATION

To my wife, Kristen, without her support I would never have completed this journey.

BIOGRAPHY

Brendon Louis Fussnecker was born in Dayton, Ohio on October 20, 1982. He graduated from Archbishop Alter High School in 2001 and then graduated with a B.S. in Zoology and a minor in Molecular Genetics from The Ohio State University in 2005. During his undergraduate studies, Brendon worked in two research labs. He completed several research projects focusing on learning and memory and the role of biogenic amines in locomotion in honey bees with Dr. Brian H. Smith. One of his projects was published in *The Journal of Insect Physiology* in 2006. He also worked in the transplant immunology laboratory of Dr. Ginny Bumgardner in the Ohio State University Medical Center. Brendon began his graduate studies at North Carolina State University under the direction Dr. Christina M. Grozinger as a Master's student in the Department of Entomology before transferring to a PhD program in the Department of Genetics in 2006. He was named a Foundation Scholar by the Foundation for the Preservation of Honey Bees and published his first paper from his doctoral research in *Insect Molecular Biology* in 2006. He will graduate with a PhD in Genetics from North Carolina State University in December 2009. Brendon will be employed at Flywheel Partners in New York City following the completion of his degree. There, he will develop marketing and training content for the pharmaceutical industry.

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CHAPTER 1

Modulation of responses to pheromones in insects

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INTRODUCTION

All organisms must communicate with conspecifics in order to designate territories, call attention to predators, locate and court mates, and form and organize social groups. This communication can take many forms, including auditory, visual or behavioral, and chemical signals, such as pheromones. Pheromones can trigger both short-term (releaser) behavioral changes and long-term (primer) responses, in which the behavior and/or physiology of the receiving individual is altered (Bossert and Wilson 1963; Wyatt 2003). Pheromones are detected by the olfactory or gustatory sensory systems, and exposure to pheromones can cause changes in brain gene expression which are associated with downstream behavioral or physiological changes (Grozinger, Sharabash et al. 2003; Alaux, Le Conte et al. 2009). Pheromones are used in a variety of species for locating mates and courtship, but the pheromones of social insects are particularly elaborated, and are used for reproduction, division of labor, establishing dominance hierarchies, facilitating aggregation, and aiding in colony defense (Vander Meer 1998). Pheromonal communication has often been characterized as highly stereotyped. However, recent studies have demonstrated that both production of and responses to pheromones can be quite plastic, and modified by genotype, physiological factors, and environmental conditions. This represents a shift in the way we look at pheromones, from a static sender-receiver system to that of a dynamic and varied communication system. Below we highlight examples in which pheromonal communication

systems in insects are modulated by genetic, physiological, and environmental factors, and discuss the implications for our understanding of chemical communication.

Overview of insect chemosensory systems

Peripheral detection

The neurophysiological structures and mechanisms for detecting olfactory cues have been largely conserved across vertebrates and invertebrates (Hildebrand 1995; Hildebrand and Shepherd 1997; Krieger and Breer 1999; Rutzler and Zwiebel 2005). In insects, peripheral detection of olfactory cues occurs primarily via the antenna, which is located on the dorsal surface of the insect head. The antennae are covered in fine porous hairs, called sensillae, which contain two cell types, olfactory receptor neurons and support cells. The olfactory receptor neurons (ORNs) are the primary sensory cells and each sensillum may contain 1-4 of these cells. The ORNs extend their dendrites into the center of the sensillum, where they are bathed in sensillar lymph. These dendrites are covered in olfactory receptors (ORs), a family of transmembrane receptors that bind odorant ligands and lead to the activation of intracellular signaling cascades that may eventually result in an action potential (Jacquin-Joly and Merlin 2004). Interestingly, one highly conserved OR, Or83b, in *Drosophila melanogaster* must be co-expressed with other Ors in order to elicit a response to an olfactory cue (Larsson, Domingos et al. 2004). Orthologs of this OR have found in many insect taxa including silkmoths (Nakagawa, Sakurai et al. 2005) and honey bees (Wanner, Nichols et al. 2007). The cell bodies of the ORNs are embedded in a layer of support cells.

The major role of the support cells is to secrete proteins such as odorant binding proteins (OBPs) into the sensillar lymph (Pelosi and Maida 1995). OBPs can act as shuttles that carry odorant molecules from where they enter the sensillum to the surface receptors on the dendrites of the ORNs, or they can be involved in scavenging and removing excess odorant molecules (Gong, Pace et al. 2009).

Electrophysiological and neuroanatomical studies have demonstrated that there are dedicated pheromone-responsive neurons in the olfactory system (Steinbrecht and Gnatzy 1984; Baker, Ochieng et al. 2004; Hillier and Vickers 2007), and recently, specific pheromone receptors and pheromone binding proteins (PBPs) have also been identified (Krieger, Grosse-Wilde et al. 2005; Nakagawa, Sakurai et al. 2005). Interestingly, phylogenetic analysis suggests that the pheromone receptors in moths evolved as a distinct lineage, compared to the other olfactory receptors (Krieger, Grosse-Wilde et al. 2005; Robertson and Wanner 2006; Patch, Velarde et al. 2009). This suggests that pheromone detection systems evolved independently from the general olfactory system at the neural and molecular level.

Chemical cues are also detected through the gustatory system (Mitchell, Itagaki et al. 1999; Ebbs and Amrein 2007). Some pheromones, such as those involved in *Drosophila* courtship, are detected through gustatory receptors (Bray and Amrein 2003; Miyamoto and Amrein 2008). The gustatory system is organized similarly to the olfactory system in insects, but it is more widely distributed across chemosensory organs, including the maxillary palps and legs. Furthermore, the sensory neurons can contain multiple receptors that detect

different gustatory cues (Wang, Singhvi et al. 2004; Scott 2005). The gustatory receptors are also seven-transmembrane receptors, but form a distinct evolutionary lineage (Robertson and Wanner 2006).

Central processing

Processing of the olfactory signals occurs in the central brain (Lei and Vickers 2008). The axons of the ORNs extend into the antennal lobe (AL) where their synapses coalesce into structures known as glomeruli. Each glomerulus is made up of a large mass of axon terminals from both the ORNs and from other central brain regions along with neurites from local projection neurons. Axonal projections from ORNs that carry the same receptor converge into a single glomerus, and each glomerulus received input from only a single receptor type. However, since a single type of odor molecular can activate different receptors, each odor will stimulate multiple glomeruli - leading to a combinatorial code programmed into the antennal lobe. Many of these glomeruli are tuned for general odors, but some are specifically tuned for pheromone sensation. The macroglomerular complex (MGC) is a male-specific sensory pathway in the antennal lobe devoted solely to the reception of female pheromone components. This structure is most well-characterized in cockroaches and moths. Gustatory receptors project to the subesophageal ganglion (SOG) (Ignell and Hansson 2005; Inoshita and Tanimura 2006) which has a less-well organized glomerular structure when compared to the AL. However, like the AL, receptors that respond to a particular class of odors project to a similar region of the SOG.

Afferent connections from the AL and SOG connect into central brain structures known as the mushroom bodies, as well as the lateral horn (Touhara and Vosshall 2009). The mushroom bodies are widely considered the centers of learning and memory of the insect brain (Heisenberg 1998) and receive inputs from both the olfactory and optical systems (Abel, Rybak et al. 2001; Gronenberg 2001). Studies tracing the pheromone responsive neural pathways suggest that these project primarily to the laternal horn (Yamagata, Nishino et al. 2006; Jefferis, Potter et al. 2007; Datta, Vasconcelos et al. 2008).

Modulation of response to pheromones

Pheromone receptors

The simplest mechanism for modulating responses to pheromones is to alter signaling processes in the pheromone-responsive olfactory neurons. Expression differences in the pheromone receptor or downstream signaling pathways can alter responsiveness of the neuron, while genetic differences in the receptor can alter the specificity for a particular chemical. While there are examples demonstrating that changes in neuronal sensitivity of these receptors are associated with changes in behavioral responsiveness to pheromone blends (Baker, Ochieng et al. 2004; Domingue, Musto et al. 2007; Domingue, Haynes et al. 2009), the associated molecular changes have not been characterized. Molecular and genomic studies have thus far focused on simply identifying pheromone-responsive receptors in insects. Future research will undoubtedly reveal changes in expression levels or genetic variation linked to variation in behavioral responses to pheromones.

Bombykol was the first chemically-characterized pheromone (Butenandt, Groschel et al. 1959). This pheromone is released by female silkmoths (*Bombyx mori*) in order to attract male conspecifics, whose antennae are highly tuned to this pheromone. It has the unique distinction of being a one-component pheromone that is capable of eliciting the full array of mating behaviors (Butenandt, Groschel et al. 1959). Bombykal, another chemical released by females does not play a role in the male-orienting behavior classic of the bombykol response. Sakurai et al. (Sakurai, Nakagawa et al. 2004) have discovered and characterized the bombykol-specific pheromone receptor. This receptor, BMmOR-1, has male-specific expression and is a transmembrane receptor like other odorant receptors (as discussed in olfactory system overview). In their study, Sakurai et al. studied the response of *BmOR-1* in both heterologous and homologous systems. First, they expressed this receptor in *Xenopus laevis* oocytes and exposed these oocytes to bombykol. This exposure led to a dose-dependent shift in current, suggesting an activation of this receptor and its downstream targets. However, this observation was only seen for about 10-15% of the oocytes. Also, high concentrations of pheromones were necessary to elicit this response. Next, they used a baculovirus expression system to express *BmOR-1* in female silkmoths. Antennae of these females showed an electrophysiological response to bombykol. BmOR-1 was the only male-specific OR to show any response to bombykol. This, along with their results from both the heterologous and homologous systems, is strong evidence that BmOR-1 is the bombykol receptor. However, there are other components necessary that were missing from this system that explain the unexpected results of the heterologous system experiments. A follow-up

experiment performed by Nakagawa et al. (Nakagawa, Sakurai et al. 2005) clearly demonstrates that a second ORN, BmOR-2, is necessary for the full response to bombykol and in fact is almost always coexpressed with Bm-OR1. BmOR-2 is in a similar family of ORs as Or83b.

Several pheromone receptors have been identified in *Drosophila melanogaster*. OR67d is specific for 11-*cis*-vaccenyl acetate (VA), an aggregation pheromone (Ha and Smith 2006). Notably, an OBP called *lush*, is also necessary for the response to VA (Xu, Atkinson et al. 2005). This OBP seems to modulate the neurophysiology of Or67d by affecting the basal firing rate of the ORN. Another pheromone receptor, Gr68a, is actually a gustatory receptor. This receptor is expressed only in the forelegs of males and deletion or reduced expression of this receptor results in low courtship performance by males (Bray and Amrein 2003). Miyamoto and Amrein (2008) found that mutations in Gr32a resulted in strong male courtship behavior towards both females and males, suggesting that this receptor might function as an inhibitory receptor against the male courtship pheromone. Recently, a receptor for queen pheromone was identified in honey bees (Wanner, Nichols et al. 2007). AmOR11 responds to 9-oxo-2-decenoic acid (9-ODA), a major component of the pheromone released by honey bee queens. 9-ODA serves as a mating pheromone in that it attracts male (drone) bees to the queen, but it also plays an important role in regulating worker bee behavior (Slessor, Kaminski et al. 1988). Male (drone) bees have significantly more 9-ODA responsive sensilla than worker bees, suggesting that they should express higher levels of the 9-ODA receptor. Wanner et al. developed a custom chemosensory microarray to compare

expression levels of these genes in the antennae of workers and drones. Drones expressed significantly higher levels of AmOr11 as well as three other ORs. AmOr11 was the only one of the four to show a physiological response to any of the components of queen pheromone using a cell-based assay. This study demonstrates the utility of using high-throughput expression platforms to identify candidate pheromone receptors.

Though studies of peripheral perception have not yet focused on population/individual genetic variation, this will likely be an important component of variation in behavioral responses. In humans, it has been shown that genetic variation in odorant receptors may contribute to the observed differences in odorant perception among individuals (Keller, Zhuang et al. 2007).

Biogenic amines

Neurophysiological processing in the olfactory system is modulated by several biogenic amines, which alter the firing activity of ORNs and the response to olfactory stimuli in many studies. Octopamine (OA) has been implicated in several studies as a neuromodulator of pheromone sensitivity and ORN firing rate. Linn and Roelofs (1984; 1986) discovered that OA treatment results in a generalized over-sensitivity to an olfactory cue. This study was bolstered by later studies of Linn et al. (1992; 1996), in which they observed that OA treatment decreased the pheromone dose needed to elicit a behavioral response and improved discrimination of pheromone blends. These behavioral studies were later followed up on by Pophof (2000) in which an electrophysiological approach was taken.

Pophof found that injection of OA increased the nerve impulse frequency in the presence of pheromone blends. OA also increased the background firing rate of ORNs in a more general way in a study by Grosmaitre et al. (2001). The increased firing of ORNs treated with OA resulted in ORNs that were more responsive to olfactory stimuli, suggesting that OA is necessary for olfactory stimulation in general. This is further supported by Zhukovskaya and Kapitsky (2006), who discovered that OA increased the both the background firing rate and firing rate in response to pheromones of ORNs in cockroaches. OA has also been implicated in pheromone-mediated stimulation of foraging behavior in honey bees. Brood pheromone is a mixture of cuticular hydrocarbons released by honey bee larvae (Le Conte, Arnold et al. 1989; Le Conte, Arnold et al. 1990) and brood pheromone exposure leads to increased foraging activity throughout the honey bee colony (Pankiw, Page Jr et al. 1998; Pankiw and Page 2003). Barron et al. (2002) demonstrated that OA increased responsiveness to brood pheromone in terms of increasing foraging activity but, importantly, did not increase other behavioral activities regulated by brood pheromone. Thus, in this case, OA specifically modulated one behavior and did not generally increase activity.

Serotonin (5HT), another biogenic amine, is typically thought to function antagonistically to OA, though the relationship may be more complex. Linn and Roelofs (1986) found that 5HT did not affect the sensitivity to pheromones, but instead affected the time at which individuals were sensitive to pheromones. However, other research shows that serotonin may indeed act in an opposite fashion to that of OA. Harris and Woodring (1999) examined the effect of 5HT treatment on the response to honey bee alarm pheromone.

Honey bees release alarm pheromone in response to a disturbance to the colony or after a sting in order to recruit or alert other colony members to the disturbance. Its major component is isopentyl acetate, and 5HT treatment drastically increased the time it took for individual honey bee workers to respond to this component, while also lowering the maximum frequency of buzzing normally associated with the response to alarm pheromone. Also, Grosmaitre et al. (2001) examined the effect of 5HT on ORN firing and found that 5HT caused an inhibition of ORN firing, though this effect was reversible. These findings suggest that 5HT may play a modulatory role in the sensitivity of ORNs to an olfactory stimulus, a finding well-supported by the 5HT literature. 5HT is known to affect olfactory sensitivity (Mercer and Menzel 1982; Kloppenburg, Ferns et al. 1999), enhance neuronal sensitivity in the AL to both pheromonal and electrical activity (Kloppenburg and Hildebrand 1995; Kloppenburg, Ferns et al. 1999) and, in neurons cultured from the AL, cause an increase in spike number and broaden action potentials (Mercer, Kloppenburg et al. 1996). Furthermore, a study by Gatellier et al. (Gatellier, Nagao et al. 2004) has also clearly demonstrated the modulatory effect of serotonin in the AL. By treating the moth brain with many different concentrations of serotonin, they were able to achieve a spectrum of responses. Some treatment concentrations resulted in increased sensitivity, while others resulted in decreased sensitivity. It may be that serotonin plays a specific role in the insect AL, one that is markedly different from its role in the ORNs within the insect sensillae. Hill et al. (2002) discovered a pair of unique neurons that innervate the AL and appear to be immunoreactive to serotonin. These neurons have also been identified in the ALs of other insect species

(Schürmann and Klemm 1984; Karla, Sally et al. 1987; Rehder, Bicker et al. 1987; Breidbach 1990; Salecker and Distler 1990). Currently, the exact function of these neurons is unknown. Biogenic amines are prime candidates for the modulation of pheromone response in insects. Their roles as neurotransmitters and neuromodulators along with their tight associations with the insect olfactory system provide ample opportunity to perform in this manner. Biogenic amines do modulate responses to olfactory cues, but the exact mechanisms by which they function and how levels of biogenic amines are regulated remains to be determined.

Hormone levels

The physiological state of an organism can have profound effects on its response to pheromones. Juvenile hormone (JH) is a major hormone in insects that is known to regulate metamorphosis, reproductive state, and behavior (Riddiford 2008). JH has been shown to play an integral role in the modulation of sex pheromone response in the male black cutworm (*Agrotis ipsilon*). Previous research showed that as JH biosynthesis increases with age, male moths become increasingly behaviorally responsive to the female sex pheromone (Gadenne, Renou et al. 1993; Duportets, Dufour et al. 1998). Anton and Gadenne (1999; 2000) demonstrated that pheromone-responsive AL interneurons become more sensitive after JH exposure and that the ALs of males surgically deprived of JH are significantly less responsive to the sex pheromone. A later study by Jarriault et al. (2009) shows that JH alone is not enough to elicit a behavioral response to the female sex pheromone, but that

octopamine is also necessary, demonstrating the potentially complex interactions between hormones and biogenic amines in the insect olfactory system.

As mentioned previously, honey bees use alarm pheromone to alert the colony to a disturbance or threat. Robinson (1987) found that honey bees that had been treated with a JH analog, methoprene, had a lower behavioral threshold response to alarm pheromone. Another study by Robinson (1987) demonstrated that JH is a hormonal regulator of the age polyethism that occurs in honey bee colonies. He proposes that changing JH titers as honey bee workers age result in different "tunings" necessary for the specific tasks. For example, a young bee performing nursing behavior should be less responsive to alarm pheromone as this caste of bees is not responsible for colony defense. JH may also affect the expression of genes normally regulated by pheromones. One such gene is *Krüppel Homolog-1* (*Kr-h1*). *Kr-h1* encodes a zinc-finger transcription factor and is stably down-regulated by honey bee queen mandibular pheromone (QMP) (Grozinger, Sharabash et al. 2003). Treatment of worker honey bees with methoprene prevented the down-regulation of *Kr-h1* expression usually associated with QMP treatment (Grozinger and Robinson 2007), and also inhibited the QMP-induced decrease in sucrose sensitivity (Grozinger, Fischer et al. 2007).

Response to queen pheromone are also altered by treatment with cGMP, which stimulates the activity of *Amfor,* a 3', 5'-cyclic guanosine monophosphate (cGMP)-dependent protein kinase (PKG). Foragers have higher expression of this gene when compared to nurses and treatment with cGMP results in precocious, or early, foraging (Ben-Shahar, Robichon et al. 2002). cGMP treatment has also been shown to modulate short- and long-term behavioral

and physiological responses to QMP, including QMP-sensitive gene expression (Fussnecker and Grozinger 2008).

Hormones are known regulators of a suite of physiological and behavioral processes. This role positions them as potentially key modulators of the response to pheromones, as this response is dependent on age and other physiological factors.

Developmental factors

Responses to pheromones can also be altered by developmental factors, which can subsequently cause changes in adult physiology. For example, there is considerable individual variation in worker responses to queen pheromone, even if workers are reared in common environments and are genetically similar (Pankiw, Winston et al. 1994). Though there are undoubtedly genetic factors involved in this variation since the response is heritable and selectable (Pankiw, Winston et al. 1994). It also appears that this adult behavioral variation is linked to differences in ovariole number, a trait which is set up during larval development (Kocher et al. in review). Bees with more ovarioles are less likely to be attracted to queen pheromone in a caged assay, and also have significantly different brain expression patterns associated with this reduced response. Thus, environmental or genetic factors that act during developmental stages can have profound effects on adult behavioral responses to social stimuli.

Modulation by Sender

Changes in the chemical composition of the pheromone blend can also result in changes in behavioral or physiological responses of the receiving individual. Changes to an individual's social context, immune system, or diet can result in changes in the function or expression of key biosynthetic enzymes involved in pheromone production, resulting in differences in pheromone composition and signaling. Modulation of pheromone signaling as a consequence of the health or fertility of the individual is also an important component of theories related to sexual conflict. For example, production of a pheromone may be costly, and thus only healthy individuals can produce the best quality pheromone. This would be an "honest signaling" mechanism, for putative mates to assess the fitness of the individual (Dor, Katzav-Gozansky et al. 2005).

Stimulation of the immune system can dramatically change the quality of the pheromone blend an individual produces. Rantala et al. (2002) show that female mealworm beetles prefer the pheromones produced by healthier, more immunocompetent males. This preference correlates with the ability of the male to quickly encapsulate antigens and high levels of phenoloxidase, an enzyme used to recognize and defend against microbial infections, in the haemolymph. A later study performed by Sadd et al. (2006) shows that male mealworm beetles increase their pheromone production when they are immune-challenged. Increasing the intensity of their pheromone production makes these males more attractive to females. This is an excellent example of a dishonest signal. In contrast to these

studies, Rantala et al. (2003) demonstrate that male mealworm beetles that are nutrient-deprived produce pheromones that are less attractive to females, an honest signal. These results clearly show that different physiological states affected by different challenges can result in outcomes varying in honesty and receiver response.

Another strong driver of physiological state is that of mating. Mating induces substantial physiological changes in female insects. In the case of honey bees, the females will mate with an average of 13 males (drones) during a short period in their young adult life (Tarpy and Nielsen 2002), after which their behavior and physiology dramatically changes (Winston 1987). The pheromone composition of queens also changes after mating (Plettner, Slessor et al. 1993), but it was unclear if mating quality, specifically mating number also affected queen physiology and, in turn, pheromone composition. Studies have shown that genetically diverse colonies headed by multiply-mated queens produce colonies with greater fitness and productivity (Mattila and Seeley 2007) and higher resistance to disease (Seeley and Tarpy 2007). Thus, it would be beneficial for workers to distinguish between well- and poorly mated queens. Richard et al. (2007) used honey bee queens that had been differentially mated and examined differences in pheromone composition and worker response to pheromone. They found that mating number has a significant effect on the blend of the pheromone released by the honey bee queen, and that worker honey bees prefer the pheromone of queens who had been inseminated with semen from 10 drones vs a single drone. These results suggest that honey bee queen pheromone is an honest signal that accurately presents the mating state of the queen to the workers.

CONCLUSION

Pheromone signaling is often thought to be a stereotyped, static process. However, an increasing number of studies demonstrate that responses to pheromones and production of pheromones are dynamic, and modulated by genetic, developmental, physiological and environmental contexts. These systems will require further characterization to determine if this modulation of response is adaptive and if specific mechanisms exist to change signaling according to the distinct circumstances. Furthermore, with the development of more sensitive chemical analyses and improved feasibility of genome-wide analysis, including high throughput sequencing or microarray expression studies, it should be possible to begin to characterize the molecular mechanism regulating this variation

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CHAPTER 2

Dissecting the role of *Kr-h1* brain gene expression in foraging behavior in honey bees

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Dissecting the role of *Kr-h1* brain gene expression in foraging behavior in honey bees (*Apis mellifera*)

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Abstract

Expression of *Krüppel homolog-1* (*Kr-h1*) in the honey bee brain is strongly associated with foraging behavior. We performed a series of studies to determine if *Kr-h1* expression correlates with specific aspects of foraging. We found that *Kr-h1* expression is unaffected by flight experience in male bees. Expression was unaffected by behavioral reversion of workers from foraging to brood care, suggesting that expression is not associated with the active performance of foraging, but rather with stable physiological changes. *Kr-h1* expression is increased by cGMP treatment in workers, and the *Kr-h1* promoter contains a conserved potential cGMP response element. Since cGMP treatment causes precocious foraging, our results suggest that *Kr-h1* expression is associated with cGMP-mediated changes in the brain that occur early in the transition to foraging behavior.

Keywords: gene expression, honey bee, behavior, foraging, brain, *Krüppel homolog-1*.

Introduction

Honey bees are an excellent model for behavioral research due to their rich behavioral repertoire and plasticity. With the recent sequencing of the honey bee genome (Honey Bee Genome Sequencing Consortium, 2006), the honey bee can serve as an excellent model for linking genes with complex behavior. Arguably, one of the most complex honey bee behaviors is that of foraging; the culmination of behavioral development that occurs throughout a honey bee worker's life (Winston, 1987). Upon emergence, a worker honey bee begins the task of 'nursing' behavior, where she is responsible for the feeding and care of the queen and brood. She then transitions through a series of in-hive behaviors until reaching the foraging state. Forager honey bees exhibit a wide array of complex and fascinating behaviors, including orientation and navigation, higher-order learning and memory, and the ability to translate the location of food sources into a symbolic dance language. Identifying genes involved in producing foraging behavior is challenging due to pleiotropic effects of genes, complex gene networks, and genotype by environment interactions (Robinson *et al.*, 2005).

While foragers and nurses differ dramatically in brain gene expression with more than 3000 differentially expressed genes (Whitfield *et al.*, 2003), thus far only two genes, *malvolio* (*Ammvl*) and the *foraging* gene (*Amfor*) have been demonstrated to be strongly linked to foraging behavior. *Amfor* encodes a 3′,5′-monophosphate (cGMP)-dependent protein kinase (PKG) which has been associated with food-related locomotion in many organisms including fruit flies (Pereira & Sokolowski, 1993), honey bees (Ben-Shahar *et al.*, 2002), *C. elegans* (Fujiwara *et al.*, 2002), mouse (Morley *et al.*, 1996; Morley *et al.*, 1999), bumble bees (Grozinger *et al.*, unpublished data), and harvester ants (Ingram *et al.*, 2005). Forager honey bees have higher expression of *Amfor* than nurse bees, and increased activity of the PKG enzyme using treatment of 8-Br-cGMP results in precocious, or early, foraging (Ben-Shahar *et al.*, 2003). *Malvolio*, a manganese transporter, was discovered in a screen for sucrose responsiveness in *Drosophila melanogaster* (Rodrigues *et al.*, 1995). *Ammvl* has been shown to be associated with increased sucrose responsiveness, and treatment of young honey bees with manganese results in precocious foraging (Ben-Shahar *et al.*, 2004).

Krüppel-homolog 1 (*Kr-h1*) is a third candidate gene whose expression is strongly associated with foraging behavior and which has been studied in more detail. Expression of the transcription factor *Krüppel-homolog 1* (*Kr-h1*) in the honey bee brain was found to be significantly higher in foragers than in nurses (Whitfield *et al.*, 2003) and

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is higher in foragers than in all other behavioral groups (Grozinger & Robinson, 2007). Additionally, queen mandibular pheromone (QMP) slows the transition from nursing to foraging behavior (Robinson *et al.*, 1998; Pankiw & Page, 2003) and down-regulates expression of genes that may be associated with foraging including *Kr-h1* (Grozinger *et al.*, 2003) (Grozinger *et al.*, 2007; Grozinger & Robinson, 2007). However, since foragers are very different in almost every aspect from their younger siblings in terms of age, physiology, neuroanatomical structure, and behavior (Robinson & Ben-Shahar, 2002), *Kr-h1* expression may not be involved in directly producing foraging behavior per *se*, but may be differentially expressed simply as a byproduct of one of these other factors.

Some of the potential factors that may regulate *Kr-h1* expression, such as age and physiological differences, have already been examined. Grozinger & Robinson (2007) showed that in a colony of same-aged bees, forager bees still had greater *Kr-h1* expression, suggesting that *Kr-h1* expression is not regulated by age. In terms of physiological factors, the transition to foraging is regulated by circulating juvenile hormone (JH) and brain octopamine levels (Schulz *et al.*, 2002). JH titers are highest in foragers compared to other behavioral groups, and treatment of young bees with methoprene, a juvenile hormone analog, increases the rate at which honey bees transition to foraging (Schulz *et al.*, 2002). Furthermore, *Kr-h1* expression is increased by JH treatment during pupal development in *Drosophila* (Minakuchi *et al.*, 2008). However, treatment with methoprene did not increase *Kr-h1* expression in adult bees, though it did reduce the effect of queen mandibular pheromone on decreasing *Kr-h1* expression (Grozinger & Robinson, 2007). Octopamine is a biogenic amine associated with many behaviors in invertebrates, including locomotion (Saraswati *et al.*, 2004; Fussnecker *et al.*, 2006) and associative learning (Hammer & Menzel, 1998; Schwaerzel *et al.*, 2003). Brain levels of octopamine are also higher in foragers than in-hive bees, and treatment with octopamine can accelerate the transition to foraging in young bees (Schulz *et al.*, 2002). However, octopamine had no effect on *Kr-h1* expression (Grozinger & Robinson, 2007), suggesting that other factors are involved.

Here, we use behavioral manipulations and pharmacological treatments to better understand what particular aspect of foraging is associated with increased *Kr-h1* expression. We have focused on three aspects of foraging: flight experience (i.e. the act of taking repeated flights outside of the colony), foraging behavior vs. relatively stable physiological changes associated with the foraging state, and PKG activity. To test these three factors, we first examined the association of *Kr-h1* expression with flight experience in male bees (drones). Secondly, we determined if *Kr-h1* expression changes in response to behavioral reversion from foraging to nursing behavior. Finally, we determined if *Kr-h1* expression is associated with increased PKG activity in the brain.

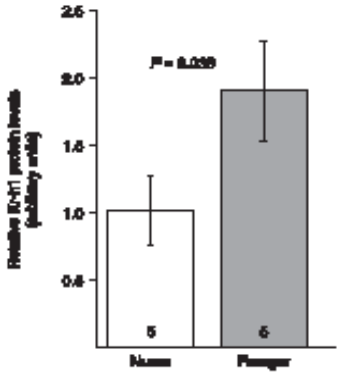

Figure 1. Protein levels of Kr-h1 are higher in foragers than nurses. Protein levels of Kr-h1 in nurse and forager whole brains were quantified using Kodak Molecular Imaging Software to measure pixel density of Kr-h1 bands. Five blots were performed from five different source colonies. Forager brains contained significantly higher Kr-h1 protein levels than nurses (two-tailed *t*-test: $P < 0.038$). *Data* represent mean values ± standard error. The number of samples for each behavioral group is shown in the base of each bar.

Results

Western immunoblotting of nurse and forager brains

Previous results (Grozinger *et al.*, 2003) demonstrated that *Kr-h1* RNA levels are higher in forager brains when compared to nurse brains. To determine if protein levels are also higher in foragers, we used a Western blot analysis. We found that Kr-h1 protein was expressed significantly higher in forager brains compared to nurse brains, almost a two-fold difference in expression (Fig. 1, two-tailed *t*-test: $P = 0.038$; see Supporting Information Fig. S1 for example of Western blot).

Flight experience

To determine if *Kr-h1* expression is associated with flight experience we examined *Kr-h1* levels in drone (male) honey bees since drones take mating flights but do not forage. We used qRT-PCR to monitor *Kr-h1* levels in the brains of 14 day old drones which were permitted to take mating and orientation flights, and those that had no flight experience (Fig. 2). There was no significant effect of flight experience (ANOVA: behavior: $P = 0.6829$, trial: $P = 0.9634$, trial*behavior: $P = 0.9792$), suggesting that flight experience does not affect *Kr-h1* expression in drones.

Reverted foragers

When all the nurse bees are removed from a colony, foragers can undergo behavioral reversion and perform nursing

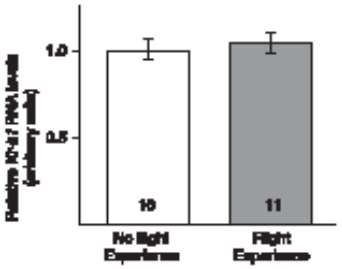

Figure 2. Brain *Kr-h1* expression is not affected by flight experience. Drones were collected from within the 'no-flight' colony or upon return from attempted mating flights from the 'flight' colony at 14 days of age. *Kr-h1* levels were quantified with qRT-PCR. Two trials were completed using bees derived from two different source colonies. Flight experience did not affect *Kr-h1* levels (ANOVA: behavior: $P = 0.8829$, trial: $P = 0.9634$, flight*behavior: $P = 9792$). *Data* represent mean values ± standard error. The number of individual brains used in each sample is shown in the base of each bar.

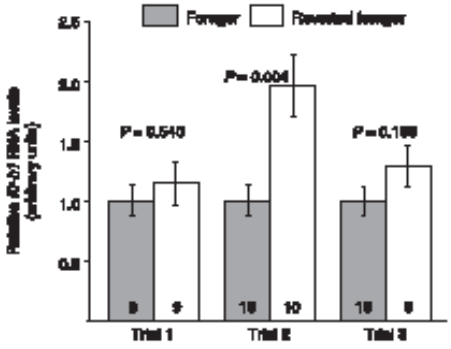

Figure 3. *Kr-h1* expression is not higher in foragers compared to reverted foragers performing nursing behavior. Foragers and reverted foragers were collected 5 days after reversion. *Kr-h1* levels were quantified with qRT-PCR. Expression levels were not significantly different in two out of three trials (two-tailed *t*-test: $P = 0.540$ and $P = 0.190$, respectively), but significantly higher in reverted foragers in the second trial ($P = 0.004$). There was no obvious difference between the two groups when all three trials were combined and analyzed with an ANOVA ($P = 0.3337$). *Data* represent mean values ± standard error relative to the non-reverted forager sample. The number of individual brains used in each sample is shown in the base of each bar.

behavior, but several physiological aspects remain unchanged, including lipid levels and neuroanatomy (Fahrbach *et al.*, 2003; Toth & Robinson, 2005). To determine if *Kr-h1* expression is associated with the performance of foraging behavior or an associated physiological aspect, we monitored expression in reverted foragers using qRT-PCR (Fig. 3). No significant difference between reverted and non-reverted foragers was observed for two out of the three trials (two-tailed *t*-test, $P = 0.540$ and $P = 0.190$) with only trial 2 showing a significant difference, with higher expression in reverted foragers ($P = 0.004$). A mixed model ANOVA shows no effect of

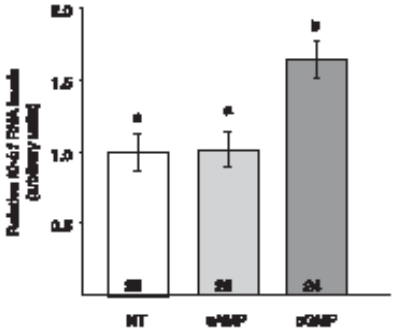

Figure 4. cGMP treatment increases *Kr-h1* expression in worker brains. One day old bees were treated for 4 days with sucrose only (NT), sucrose with 8-Br-cAMP (1 mM), or sucrose with 8-Br-cGMP (500 μM). *Kr-h1* levels were quantified with qRT-PCR. Differences in expression levels were analyzed using an ANOVA, followed by pairwise comparisons with Tukey adjustments. *Letters* denote significant differences between samples ($P < 0.01$). *Data* represent mean values ± standard error relative to the sucrose only (NT) sample. The number of individual brains used in each sample is shown in the base of each bar.

reversion when all three trials are combined ($P = 0.3337$), suggesting that behavioral reversion does not generally effect *Kr-h1* expression.

cGMP treatment

Treatment of caged bees with 8-Br-cGMP has been shown to cause increased phototaxic behavior and precocious foraging (Ben-Shahar *et al.*, 2003). To determine if this treatment also results in increased brain *Kr-h1* expression, we performed qRT-PCR on whole brains following treatment (Fig. 4). There was a significant effect of treatment (ANOVA: treatment: $P < 0.001$, trial: $P = 0.2125$, treatment*trial: $P = 0.2742$). Pairwise comparisons with Tukey adjustments found significant differences between 8-Br-cGMP treatment and cAMP ($P = 0.0158$) and control ($P = 0.0132$) treatments, reflecting a 1.75-fold increase in *Kr-h1* expression as a result of 8-Br-cGMP treatment. cAMP was used to control for non-specific effects of cyclic nucleotide treatment. No difference in expression was observed between the control and cAMP treatments ($P = 0.9562$).

cGMP response element in Kr-h1 *promoter*

Due to the apparent connection between cGMP treatment and increased *Kr-h1* expression, we examined the *Kr-h1* gene for the presence of a cGMP response element. Previously, Hum *et al.* (2004) characterized a cGMP response element in mammals, whose presence in the promoter region of genes would cause increased expression of those genes in the presence of cGMP. A similar element was found 715 bp upstream of the predicted *Kr-h1 Apis mellifera* (GenBank Accession number NM_001011566) start site. This sequence shares 77.8% sequence with the mammalian cGMP

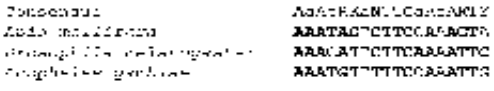

Figure 5. Alignment of putative cGMP response elements. A consensus sequence described by Hum *et al.* (2004) was used to search for similar elements in the promoters of *Kr-h1* orthologs in several insect species. Lowercase letters in the consensus represent sites not tested by Hum *et al.* Also, R (A or G), K (G or T), N (all), and Y (T or C) represent sites that can have different nucleotides. Letters in bold represent sites that exactly match the proposed consensus sequence.

response element (Hum *et al.*, 2004). Similar cGMP response elements were found in *Kr-h1* for *D. melanogaster* and *Anopheles gambiae* (Fig. 5). In *An. gambiae*, the element is found 516 bp upstream of the ATG start of the ORF that includes the predicted Kr-h1 protein (GenBank ID: AGAP009662-PA). In *D. melanogaster*, the *Kr-h1* gene codes for three transcripts with different N-terminal exons. The Kr-h1α transcript is most similar to the *Ap. mellifera Kr-h1* gene (Grozinger *et al.*, 2003). A putative cGMP response element was identified 6404 bp upstream of the ATG start site for Kr-h1 α, and 4800 bp downstream of the identified transcription start site for the Kr-h1α. Note that this sequence is also downstream of the first exon of the Kr-h1β transcript. Thus, though it is quite distant from the Kr-h1α protein coding sequence, this putative response element appears to be within the 5′UTR of the Kr-h1 α transcript. It is possible that the addition of extra exons resulted in an elongation of the promoter compared to the *Ap. Mellifera* and *An. gambiae Kr-h1* genes. Finally, no apparent response element was found in the *Tribolium castaneum Kr-h1* ortholog (GenBank Accession Number NW_001092838).

Discussion

Here, we use behavioral and physiological manipulations to uncouple different aspects of a complex behavioral trait to find underlying associations with brain gene expression. We confirm that Kr-h1 protein levels track previous RNA expression data in that foragers have higher Kr-h1 protein levels in the brain than nurse bees. We determined that *Kr-h1* expression is not affected by flight experience in drones, and that *Kr-h1* levels in the brains of reverted foragers are not lower than levels in non-reverted foragers; thus high *Kr-h1* expression is not specifically associated with the active production of foraging behavior but may be associated with some other physiological change associated with the foraging behavioral state, such as neuroanatomical changes in the brain. We show that *Kr-h1* RNA expression levels are associated with PKG activation and the *Kr-h1* promoter contains a potential cGMP-response element that is relatively conserved. Since cGMP treatment leads to increased phototaxis and an earlier transition to foraging in young bees, PKG may be involved in organizing the transition to foraging (Ben-Shahar *et al.*, 2003), suggesting that *Kr-h1* may also be involved in the early stages of the transition to foraging. Furthermore, since *Kr-h1* is a transcription factor and regulates expression of other genes, it may coordinate the large scale differences in brain gene expression observed between foragers and nurses (Whitfield *et al.*, 2003).

Like worker bees, drone honey bees exhibit behavioral maturation in that the first part of their life takes place within the colony before they begin to take short orientation flights and eventually perform mating flights, which require complex navigation and orientation abilities. We found that flight experience had no effect on *Kr-h1* expression in 14 day old drones.

We have also demonstrated that *Kr-h1* expression does not change as a result of behavioral reversion from foraging to nursing. It is possible that the rather artificial conditions of these experiments (i.e. very small colonies with unnatural age structures and no queens) could have created conditions which masks the role of *Kr-h1* in foraging behavior. For example, stressful conditions may raise *Kr-h1* levels in all bees. However, we did observe normal behavior in these bees, suggesting that they were fully capable of performing their nursing and forager tasks. It is important to note that though there was no overall effect on *Kr-h1* expression across three trials, in one trial *Kr-h1* levels were actually *increased* in reverted foragers that were performing nursing behavior. It is unclear what factor caused this change in expression in this trial, but this experiment demonstrates that high *Kr-h1* expression is not uniformly associated with the active performance of foraging or flight behavior. While juvenile hormone levels are lower in reverted foragers (Robinson *et al.*, 1989), several aspects of forager physiology are not altered as a result of behavioral reversion including lipid levels (Toth & Robinson, 2005) and forager associated-neuroanatomical changes (Fahrbach *et al.*, 2003). Previous work has shown that the mushroom body neuropil of honey bees increases just prior to the transition to foraging, and increases in response to foraging experience (Fahrbach *et al.*, 1998). Mushroom body neuropil volume remains the same size even if foragers revert to nursing behavior (Fahrbach *et al.*, 2003). Interestingly, *Kr-h1* appears to be associated with neuronal morphology during *D. melanogaster* development (Shi *et al.*, 2007). Overexpression of *Kr-h1* inhibits axon branching in *D. melanogaster* mushroom body neurons, but knocking out *Kr-h1* globally is lethal in pre-adult stages and has no obviously morphological effect when it is knocked out in small groups of mushroom body neurons (Shi *et al.*, 2007). Thus the endogenous function of *Kr-h1* relating to neuronal morphology is unclear, but it is possible that *Kr-h1* expression is associated with these permanent neuroanatomical changes that occur during the transition to foraging.

The effect of 8-Br-cGMP treatment on *Kr-h1* expression is robust. Treatment of 8-Br-cGMP has been shown to be

sufficient to induce precocious foraging in young bees (Ben-Shahar *et al*., 2002; Ben-Shahar *et al*., 2003), suggesting that PKG/*Amfor* may be a master regulator of a foraging network that includes *Kr-h1*. The PKG pathway also could affect the function of the Kr-h1 protein through phosphorylation or other post-translational modifications, though this has not been tested. Given that the *Kr-h1* promoter has a potential cGMP response element, it suggests that PKG/cGMP directly regulates expression of *Kr-h1*, though studies with cell cultures will be necessary to determine if this is the case. Furthermore, the promoters of some *Kr-h1* orthologs in other insect species have this potential cGMP response element as well, suggesting a conserved link between *Kr-h1* and PKG across insect species. However, previous studies of the effects of queen pheromone on brain gene expression demonstrated a robust connection with *Kr-h1* expression levels, but no effect on *Amfor* RNA expression levels (Grozinger *et al*., 2003): thus, queen pheromone may operate through a different mechanism to regulate *Kr-h1* expression, perhaps by modulating biological activity of the *Amfor* protein.

We still know relatively little about the molecular and cellular function of *Kr-h1* in adult honey bees. During developmental stages in *D. melanogaster*, there is evidence suggesting that *Kr-h1* may modulate the response to juvenile hormone (Minakuchi *et al*., 2008) and that it is associated with neuronal morphology (Shi *et al*., 2007) and bristle number (Minakuchi *et al*., 2008). Also, there is evidence that *Kr-h1* modulates the response to ecdysone during pre-pupal development (Pecasse *et al*., 2000). However, these responses may be both species- and stage-specific, and the role of *Kr-h1* in honey bees may be remarkably different. In adult honey bees, *Kr-h1* does not appear to be directly regulated by juvenile hormone, based on the results of feeding methoprene, a juvenile hormone analog, to caged adult bees (Grozinger & Robinson, 2007). However, this lack of effect may be due to other factors, such as high baseline levels of juvenile hormone in caged bees, or non-specific effects of long-term methoprene treatment. *Kr-h1* is unlikely to be regulated by ecdysone due to the low levels of ecdysone found in adult honey bees (Hartfelder *et al*., 2002), but adult honey bees do express the ecdysone receptor (Velarde *et al*., 2006), so this possibility can not be ruled out at this time.

In conclusion, our studies suggest that *Kr-h1* expression is regulated by PKG in the brains of honey bees, and may be associated with permanent physiological changes that occur when bees transition to foraging and/or flight behavior. Given that Kr-h1 protein is relatively well-conserved across species (Grozinger *et al*., 2003) and that *Kr-h1* orthologs in *Anopheles* and *Drosophila* have a conserved putative cGMP regulatory element in their promoter regions, it suggests that molecular function of *Kr-h1* is conserved in solitary insects as well. However, due to pleiotropic effects on larval and pupal development, the function of *Kr-h1* in adult *D. melanogaster* is unclear, and further comparative studies will be necessary to determine the exact molecular function of *Kr-h1*. Indeed, there has been increasing evidence that many genes and molecular pathways involved in controlling behavior in solitary insects have been co-opted for complex social behaviors (Toth & Robinson, 2007), highlighting the importance of comparative behavioral genomics studies for developing a complete understanding of the molecular mechanisms underlying behavior.

Experimental procedures

General bee rearing

Worker *Apis mellifera carnica* bees (Glenn Apiaries, Fallbrook, CA) were derived either from queens that were instrumentally inseminated with semen from a single, different drone, according to established procedures (Laidlaw, 1987), or with naturally mated queens (Weaver Apiaries, Navasota, TX). Sources of bees will be noted in their respective sections below: inseminated colonies will be denoted with an 'SI', while naturally mated colonies will be denoted with an 'NM'. Colonies were maintained at the North Carolina State University Honey Bee Research Laboratory according to standard commercial procedures.

Brain dissection

Whole bee heads were partially lyophilized to facilitate brain dissection (Schulz *et al*., 1999). Dissections were performed over dry ice so tissue never thawed. Because ocelli and the subesophageal ganglion frequently fractured during dissection, these were removed during all dissections, while the remainder of the brain was included.

Western immunoblotting

Worker honey bees from naturally mated queens were collected onto dry ice. Foragers were identified by pollen loads on their hind legs, while nurse bees were collected off the brood frame, while feeding young larvae (Ben-Shahar *et al*., 2004). Western blot analysis was used to determine differences in relative protein level in honey bee brains. Five blots were performed using honey bees from five different source colonies (NM1, NM2, NM3, NM4, and NM5) and each blot contained one nurse sample and one forager sample; thus five biological replicate comparisons of nurses and foragers were performed. For each sample, total protein was extracted from 10 whole brains and pooled. Protein quantity was determined using the Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE). 50 μg of total protein was used for each sample. Protein samples were separated using a 10% Tris-glycine gel run at 150 V for 45 min. (BioRad, Hercules, CA). The gel was then transferred to the nitrocellulose membrane (BioRad) at 200 mA for 1 h. The blot was blocked at 4 °C overnight in a 5% BSA/PBST with 1% goat serum solution. The blot was probed with rabbit anti-Kr-h1 primary antibody at 1:1000 for 3 h at room temperature (Biosynthesis Incorporated, Lewisville, TX). The anti-Kr-h1 antibody was raised against a peptide corresponding to amino acids 132–151 in the *Ap. mellifera* Kr-h1 sequence: KENLSVHR-RIHTKERPYK and was not affinity purified. The antibody only recognizes a single band in the correct molecular weight range; the specificity of the anti-Kr-h1 antibody was verified by demonstrating

that incubation with excess peptide blocked binding to the Kr-h1 protein band (data not shown). After washing off unbound primary antibody, the blot was incubated with a goat anti-rabbit secondary (Sigma-Aldrich, St. Louis, MO) antibody conjugated with horseradish peroxidase at 1:10 000 for 2 h at room temperature. Blots were developed using a DAB metal-enhanced substrate (Pierce, Rockford, IL) and stable peroxide substrate buffer (Pierce) and were allowed to develop for 15 min. Kodak Molecular Imaging Software (Kodak, Rochester, NY) was used to visualize blots and quantify pixel density. The pixel intensity for the Kr-h1 band was quantified for each nurse/forager sample on each blot. Data was analyzed using a two-tailed *t*-test assuming equal variances.

Quantification of Kr-h1 *RNA levels by real-time quantitative RT-PCR (qRT-PCR)*

Total RNA was extracted from whole bee brains (dissected as above) using the RNAeasy kit (Qiagen, Valencia, CA). 150 ng of total RNA from each sample were reverse-transcribed using random oligo-dT primers. Reaction mix included 10X buffer (Ambion, Austin, TX), RNAseOUT (Invitrogen, Carlsbad, CA), and Array Script (Ambion). Real-Time qRT-PCR was performed with an ABI Prism 7900 sequence detector and the SYBR green detection method (Applied Biosystems, Foster City, CA). *eIF-S8*, a housekeeping gene that did not vary in expression level in previous microarray experiments (Grozinger *et al*., 2003), was used as a loading control. Quantification was performed using a standard curve with genomic DNA. A negative control containing no cDNA was used to check for genomic contamination of the RNA samples. Also, a dissociation step was added to the qRT-PCR to ensure primer fidelity. Data were normalized to one group for presentation purposes. The sequences for the primers (5′ TO 3′) are as follows:

Kr-h1	FWD	GCACTGGCAGTGACAAGGAA
	REV	CGTGGAGTGTTATCGTAAGTAGCAA
eIF-S8	FWD	TGAGTGTCTGCTATGGATTGCAA
	REV	TCGCGGCTCGTGGTAAA

Drone flight

To provide drones of known age, frames containing drone brood were removed from a source colony and placed in an incubator to emerge at 33 °C and 30% relative humidity. Two trials were completed using drones from two different source colonies (NM 6 and NM 7). Drones were collected 16 h after eclosion and paint-marked on the thorax with Testor's paint. Equal numbers of drones from the same source colony (~50) were then placed into two experimental five-frame colonies that consisted of two frames of honey/pollen, two frames of brood, and one empty frame. The entrance of one colony was modified with a grating to prevent the drones from leaving the colony, but allowing the smaller worker bees to leave unimpeded. Drones were collected from inside the colonies for the 'no-flight' group, or they were collected at the entrance of the colony upon return from presumed attempted mating flights for the 'flight' group. Since drones begin taking orientation flights around 6–8 days of age and gradually shift into mating flights (Gilray & Robinson, 1996; Fahrbach *et al*., 1997), we collected paint-marked drones into liquid nitrogen when they were 14 days old, to ensure that drones in the 'flight' group had to opportunity to take multiple flights. Relative brain *Kr-h1* levels were determined using above qRT-PCR procedure with approximately 8 individual brains for each timepoint for each trial (see Fig. 2 for exact numbers of bees in each samples). Data was log transformed and normalized to the mean of the non-flight group. Data was analyzed in SAS (SAS Institute, Cary, NC), using a generalized linear model with experience, trial, and trial*experience as variables.

Forager reversion

Reversion experiments were performed as in Fahrbach *et al*. (1998). Several hundred foragers were collected from the hive entrance of a source colony. Three trials were completed using foragers from three different source colonies (NM9, NM10, and NM11). Approximately 300 foragers were then placed into each of two colonies containing one frame of young, uncapped brood and one frame of honey and pollen. Since no other bees were present in these colonies, a queen mandibular pheromone strip (Pherotech, Delta, British Columbia) was used to simulate the presence of a queen. The colony entrances were sealed with wire mesh for the first 2 days to prevent foragers from flying and returning to their original source colonies. After 5 days, reverted foragers (which were performing nursing behavior) and non-reverted foragers were collected into liquid nitrogen. Relative brain *Kr-h1* levels were determined using above qRT-PCR procedure with approximately 8 individual brains for each group for each trial (see Fig. 3 for exact number of bees for each sample). Two-tailed *t*-tests assuming equal variances were performed on each trial to determine differences between the means of individual trials. An experiment-wise mixed model ANOVA was also performed in SAS (Cary, NC) with behavior as a fixed factor, and with trial and behavior*trial as variables.

cGMP treatment

To provide bees of known age, honeycombs containing late-stage pupae were removed from source colony and placed in an incubator to emerge (33 °C, 30% relative humidity). This was repeated for three trials, using bees from a different source colony for each replicate (SI1, SI2, and SI3). For cage studies, bees were allowed to eclose for 1 day and then placed in small ($10 \times 10 \times 7$ cm) Plexiglas cages. Cages were kept in an incubator (33 °C, 30% relative humidity) in the dark. All treatments and feedings were performed under red light. 35 1-day-old bees were placed in the above Plexiglas cages and provided with 50% sucrose, replaced daily. The three experimental groups include 50% sucrose only, sucrose with 8-bromo-cAMP (1 mM), and sucrose with 8-bromo-cGMP (500 μM); as used by Ben-Shahar *et al*. (2002). 8-Br-cAMP and 8-Br-cGMP were purchased from Sigma-Aldrich. Treatment was replaced daily for 4 days, and on the fifth day, bees were collected into liquid nitrogen. Mortality for sucrose- and cAMP-fed bees was < 1%, while treatment with cGMP resulted in mortality < 10%; dead bees were removed from cages. Relative brain *Kr-h1* levels were determined using qRT-PCR with approximately 8 individual brains per treatment group per trial (see Fig. 4 for exact number of individual bees in each sample). Data was analyzed in SAS (proc mixed) using a mixed model ANOVA setting treatment as a fixed factor with trial and treatment by trial as random factors. A Tukey-Kramer adjustment on the lsmeans was performed for pairwise comparisons.

cGMP response element in Kr-h1 *promoter*

A sequence for a cGMP response element (Hum *et al*., 2004) was used in a motif search in GeneQuest (DNASTAR, Madison, WI). The *Ap. mellifera Kr-h1* gene (GenBank Accession number NM_001011566) was localized to a genomic contig (GenBank Accession number NW_001260508). The ATG transcription start site was found to be at position 8004 of the contig (on the minus

strand). The potential cGMP response element was 715 bp upstream of this start site. The same procedure was used to find potential cGMP response elements in *Kr-h1* for *D. melanogaster* and *An. gambiae* (Fig. 5). In *An. gambiae*, the element is found 516 bp upstream of the ATG start of the ORF that includes the predicted Kr-h1 protein (GenBank ID: AGAP009662-PA) or 696 bp upstream of the start of this annotated Kr-h1 protein (note that the annotated protein is shorter than the predicted ORF). The *D. melanogaster Kr-h1* gene has three transcripts, one of which (*Kr-h1α*, GenBank Accession number NM_058119) most closely matches the honey bee *Kr-h1* (Grozinger *et al.*, 2003). The gene is found on the 2 L chromosome (GenBank Accession number NT_033779.4). A putative cGMP response motif was identified 6404 bp upstream of the ATG start site of *Kr-h1α*. Note this motif was 4800 bp downstream of the identified transcription start site for the *Kr-h1α* and thus appears to be in the 5′UTR of the transcript. The *An. gambiae Kr-h1* gene (GenBank Accession number AGAP009662-PA) was localized to a genomic contig (GenBank Accession number NT_045834). The ATG transcription start site was found to be at position 2566 of the contig (on the plus strand). The potential cGMP response element was 696 bp upstream of this start site. The *T. castaneum Kr-h1* ortholog (GenBank Accession number NW_001092838) was also searched in this manner, but no potential cGMP element was found.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Kr-h1 protein levels are higher in foragers than nurses. Representative western blot showing 3 out of 5 colonies used for experiment shown in Fig. 1. Molecular weight marker in kiloDaltons (kD) is shown on right side of blot. Each lane contains one pooled sample of either forager (for) or nurse protein. The expected size band for Kr-h1 is approximately 63 kD.

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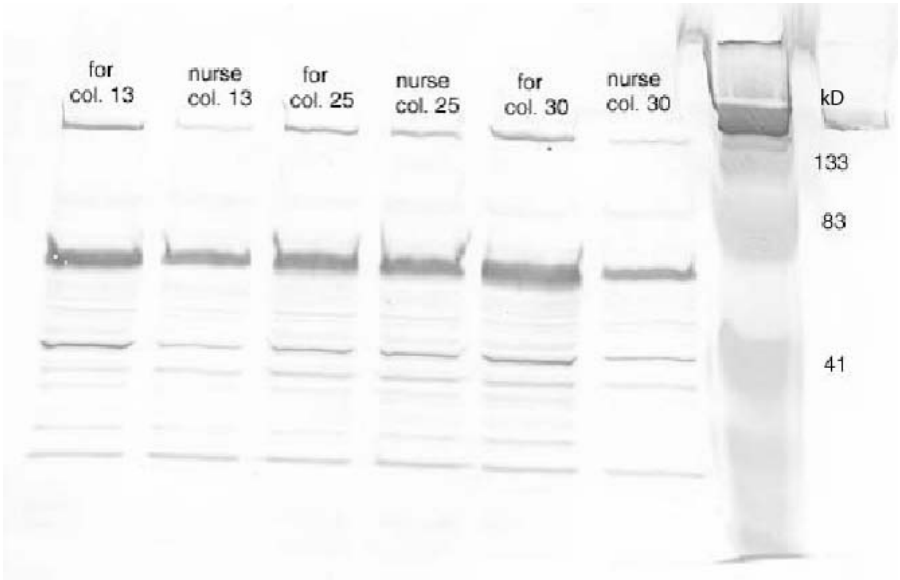

Figure 2.1 *Kr-h1 protein levels are higher in foragers than nurses*. Representative western blot showing 3 out of 5 colonies. Molecular weight marker in kiloDaltons (kD) is shown on right side of blot. Each lane contains one pooled sample of either forager (for) or nurse protein. The expected size band for Kr-h1 is approximately 63 kD.

CHAPTER 3

The role of cGMP in modulating behavior, physiology, and brain gene expression in response to queen mandibular pheromone in honey bees

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ABSTRACT

There is increasing evidence that responses to social cues, such as pheromones, can be modified by a number of factors. We examined the role that cGMP-mediated processes played in modulating responses to QMP. Treatment with a cGMP analog, 8-Br-cGMP, resulted in significant reductions in both releaser and primer responses to QMP in young caged workers. Treatment significantly reduced the retinue response and blocked QMP-mediated increase in *vitellogenin* levels in the fat bodies of worker bees (high *vitellogenin* levels are associated with the nurse behavioral state, and with increased lipid levels). However, 8-Br-cGMP treatment does not affect levels of a pheromone receptor that is responsive to the main component in QMP, suggesting that cGMP treatment is modulating pheromone response by changing central brain processing of this social signal, rather than perception of the signal in the peripheral nervous system. Principal component analysis showed a large effect of cGMP treatment on brain gene expression, as well as demonstrating a lesser, but significant effect of QMP treatment. Interestingly, the third PCA demonstrated that QMP has specific effects in the presence of cGMP, suggesting that some responses to pheromones may be specific to the physiological state. There were significant over-representations of several GO categories in the significant gene list, including GTPase regulator activity, phototransduction, positive regulation of antibacterial peptide biosynthetic process, and carboxylic acid transmembrane transporter activity. Our data suggest that, of the known physiological factors that regulate the transition to foraging, only 8-Br-cGMP appears to be involved in globally shifting responsiveness to QMP.

INTRODUCTION

Pheromones are important regulators of animal behavior and are used to mediate social interactions between members of the same species such as mating behaviors, social hierarchies, and coordinating defense of nests or colonies (Wyatt 2003). The effects of pheromones can be divided into two major functional categories, release and primer. Releaser pheromones result in an immediate behavioral response in the receiver, while primer pheromones cause long-term physiological and behavioral changes in the receiver, which may be mediated by changes in gene expression. Typically, pheromones are thought of as fixed chemical blends that cause stereotyped behavioral and physiological responses. However, there is increasing evidence that there can be modulation of pheromonal responses by any number of internal or external factors. For example, biogenic amines can alter the sensitivity of antennal pheromone receptor neurons in moths (Linn 1984; Linn, Campbell et al. 1986; Linn, Campbell et al. 1992; Linn, Campbell et al. 1996; Pophof 2000) and cockroaches (Zhukovskaya and Kapitsky 2006), while increasing juvenile hormone (JH) titers increases behavioral responses to alarm pheromone in honey bees (Robinson 1987) and to sex pheromones in moths (Gadenne, Renou et al. 1993; Gadenne and Anton 2000). Furthermore, the primer effects of a pheromone can persist even in the absence of any releaser effect and vice versa (Grozinger and Robinson 2007), demonstrating that the two major divisions of pheromone action can be uncoupled, therefore adding even another layer of complexity to the potential plasticity of the pheromone response. Though the neural

networks that process pheromone cues are well-characterized (Touhara and Vosshall 2009), little is known about the underlying molecular pathways that translate the detection of these cues to behavioral responses or how responses to pheromones are modulated at a molecular level.

Honey bee (*Apis mellifera*) queen mandibular pheromone (QMP) is arguably the best characterized pheromone in honey bees, and serves as an important means of communication between the honey bee queen and her workers (Slessor, Winston et al. 2005). QMP is one example of a pheromone with both primer and releaser effects. QMP is a five-component blend comprised of: (E)-9-oxodec-2-enoic acid (9-ODA), (R,E)-(-)-9-hydroxy-2-enoic acid (9-HDA), (S,E)-(+)-9-HDA, methyl-p-hydroxybenzoate (HOB), and 4-hydroxy-3-methoxy phenylethanol (HVA) (Slessor, Kaminski et al. 1988). Though QMP does not represent the honey bee queen's full pheromone blend, these five components can elicit many of the responses produced by a live queen. QMP's primary releaser effect is that it attracts young workers, or nurse bees, to the queen, so that they can attend and care for her, a behavior known as the retinue response (Slessor, Kaminski et al. 1988). Secondly, it is used to attract male honey bees (drones) during mating flights (Gary 1962). As a primer pheromone, QMP delays the worker transition from nursing to foraging behavior (Robinson, Winston et al. 1998) increases brain expression of nurse-related genes (Grozinger, Sharabash et al. 2003), and causes young caged bees to develop nurse-like phenotypes in regards to higher lipid levels and higher *Vitellogenin* (*Vg*) expression (Fluri, Lüscher et al. 1982; Toth, Kantarovich et al. 2005; Fischer and Grozinger 2008). Nurse bees are usually the youngest bees in the

colony and are responsible for feeding and caring for the queen and brood. As they age, workers will transition through a series of in-hive tasks until they become foragers (Winston 1987). Foraging honey bees are responsible for leaving the hive to collect nectar and pollen, which they bring back to the colony (Winston 1987).

Interestingly, forager bees are unresponsive to QMP, in terms of the retinue response and brain gene expression (Pham-Delegue, Bailez et al. 1993; Grozinger and Robinson 2007), suggesting that there is some physiological change in these workers that modulates their responsiveness to this pheromone. There are many factors that contribute to foraging behavior that may also modulate their response to QMP or other honey bee pheromones. For example, there are more than 3000 differentially expressed genes in the brains of nurses and foragers (Whitfield, Cziko et al. 2003). Juvenile hormone regulates worker behavioral ontogeny (Robinson 1987) and also modulates the primer response to QMP (Grozinger and Robinson 2007). Treatment of honey bees with the biogenic amine octopamine causes precocious, or early, foraging in honey bee colonies (Schulz and Robinson 2001) and modulates the response of foragers to brood pheromone (a pheromone that promotes foraging behavior) (Barron, A. et al. 2002).

Another key factor in the regulation of the behavioral maturation process is the foraging gene, *Amfor*. *Amfor* is a 3', 5'-cyclic guanosine monophosphate (cGMP)-dependent protein kinase (PKG) that has been associated with food-related locomotion in many organisms including fruit flies (Pereira and Sokolowski 1993), *C. elegans* (Fujiwara, Sengupta et al. 2002), mouse (Morley, Kumar et al. 1996; Morley, Alshaher et al. 1999),

bumble bees (G. Bloch, H. Patch and C.M. Grozinger, unpublished data), harvester ants (Ingram, Oefner et al. 2005), and honey bees (Ben-Shahar, Robichon et al. 2002). In addition, PKG has also been associated with learning and memory (Kaun, Hendel et al. 2007; Kaun, Riedl et al. 2007; Mery, Belay et al. 2007; Wang, Pan et al. 2008) and sucrose responsiveness (Scheiner, Sokolowski et al. 2004; Belay, Scheiner et al. 2007) in *Drosophila melanogaster*. This gene is more highly expressed in the brains of honey bee foragers than nurses, and treatment of young workers with 8-Bromo-cGMP (8-Br-cGMP, an analog of cGMP) leads to increased PKG activity, increased phototaxic behavior, and accelerates the transition from nursing to foraging behavior (Ben-Shahar, Robichon et al. 2002; Ben-Shahar, Leung et al. 2003). 8-Br-cGMP treatment has also been shown to affect brain gene expression, by up-regulating expression of genes associated with the foraging behavioral state (Whitfield, Ben-Shahar et al. 2006; Fussnecker and Grozinger 2008). Thus, PKG-regulated pathways may be key regulators of foraging behavior, in honey bees and other species.

QMP serves to slow down the behavioral maturation process, resulting in bees with more nurse-like brain gene expression patterns and physiological characteristics. 8-Br-cGMP treatment accelerates behavioral maturation, and causes young bees to have forager-like brain gene expression patterns. Thus, we predict that 8-Br-cGMP treatment will reduce the effect of QMP on honey bee workers' behavior, physiology, and brain gene expression. To test this prediction, we examined whether 8-Br-cGMP treatment reduces the retinue response to QMP, results in forager-like physiological traits such as low lipid and *Vg* levels, and affects

QMP-induced changes in *Vg* levels. Finally, we determined if 8-Br-cGMP treatment altered brain gene expression responses to QMP.

MATERIALS AND METHODS

General Bee Rearing

Honey bee colonies were maintained at the North Carolina State University Honey Bee Laboratory according to standard commercial procedures. Worker bees were derived either from queens that were instrumentally inseminated with semen from a single, different drone (SDI, *Apis mellifera carnica*, Glenn Apiaries, CA), according to established procedures (Laidlaw 1987), or with naturally mated queens, which are typically inseminated by multiple drones (MDI) (Buckfast-SMR bees, Weaver Apiaries, TX). Sources of bees will be noted in their respective sections below. To provide bees of known age, honeycombs containing late-stage pupae were removed from a source colony and placed in an incubator to emerge (33^{o}C, 30% relative humidity). Bees less than 24 hours old were collected off the frames and placed into Plexiglas cages (10x10x7cm). Bees were provided with 50% sucrose and ground pollen; sucrose was changed daily, while pollen was changed every 3 days. Cages were maintained in an incubator under red light at 33^{o}C, 30% relative humidity for the duration of the experiments. Details about specific experiments are below.

Pheromone Treatment

Experimental cages were treated with either synthetic QMP (0.1 queen equivalents in 10µL of 1% water/isopropanol; Pherotech, Canada) or control slide containing solvent only (1% water/isopropanol) as in (Grozinger, Sharabash et al. 2003; Grozinger and Robinson 2007; Fischer and Grozinger 2008). Each treatment was administered by placing 10µL of either

QMP or solvent onto a microscope coverslip, which was allowed to evaporate before being placed in its respective cage. Fresh treatment was applied daily, at the same time each day. Number of days of treatment for each experiment will be described in their respective sections below.

8-Br-cGMP Treatment

Experimental cages were treated with 8-Br-cGMP (Sigma Aldrich, St. Louis, MO) or a sucrose control, as in (Ben-Shahar et al. 2002; Fussnecker and Grozinger 2008). 8-Br-cGMP was fed to bees as a 500μM solution in 50% sucrose. Treatment was replaced daily. Number of days of treatment for each experiment will be described in their respective sections below.

Retinue Response

The effect of 8-Br-cGMP treatment on retinue responses to QMP was assessed in three trials, using bees from a SDI and two naturally mated source colonies in the different trials (SDI6, MDI20, and MDI21). Bees were reared as described above, with 20 bees/cage, 4 cages/treatment group in each trial. There were four experimental treatments: sucrose only with solvent (NN), sucrose only with QMP (NQ), 8-Br-cGMP with solvent (GN), and 8-Br-cGMP with QMP (GQ). Retinue counts were performed at the same time each day by counting the number of bees contacting (antennating or licking) the QMP or solvent slide every 5 minutes for 15 minutes each day. This was repeated for 8 days post-emergence. Data was analyzed in JMP 7 (SAS, Cary, NC) using an a standard least squares ANOVA with treatment set as a fixed factor and colony, day, cage, colony by treatment, day by treatment,

and cage by treatment set as random factors. A post-hoc Tukey HSD was performed in order to determine significant differences for each day of treatment.

Lipid Weight Analysis

In order to determine if 8-Br-cGMP affected lipid levels in the abdominal fat bodies, bees were assayed using a naturally mated source colony (MDI5). Bees were reared in cages as described above, with 20 bees/cage, 4 cages/treatment group. There were two experimental treatments: sucrose only with solvent (NN) and 8-Br-cGMP with solvent (GN). Treatment was replaced daily for four days, and on the fifth day, bees were collected into liquid nitrogen. Fat body lipid levels were analyzed following the protocol in (Toth, Kantarovich et al. 2005). Abdomens were dissected to remove the organs from the cuticle and associated fat bodies. The resulting carcasses were lyophilized, weighed, and then extracted overnight in 5mL of 2:1 chloroform/methanol solution. The abdomens were reweighed to calculate the weight of the extracted lipids. Two abdomens were weighed simultaneously to improve precision. Data analysis was performed using a Student's t-test in JMP 7 (SAS, Cary, NC).

Vitellogenin *Quantification by Real-Time qRT-PCR*

In order to determine if 8-Br-cGMP affected *vitellogenin* expression levels in abdominal fat bodies, bees were assayed in two trials, using either a SDI or a naturally mated colony (SDI83 and MDI5). Bees were placed in cages, 20 bees/cage, 4 cages/treatment. Bees were

treated with either sucrose only or sucrose with 8-Br-cGMP for 4 days. Bees were then collected into liquid nitrogen on the 5th day after emergence. Abdomens were dissected as above and total RNA was extracted from carcasses (with fat bodies) using the RNAeasy kit (Qiagen, Valencia, CA). 150ng of total RNA from each sample were reverse-transcribed using random primers. Reaction mix included 10X ArrayScript buffer (Ambion, Austin, TX), RNAseOUT (Invitrogen, Carlsbad, CA), and ArrayScript RT enzyme (Ambion, Austin, TX). Real-Time qRT-PCR was performed with an ABI Prism 7900 sequence detector and the SYBR green detection method (Applied Biosystems, Foster City, CA). *eIF-S8*, a housekeeping gene that did not vary in expression levels in a previous study (Grozinger, Fischer et al. 2007), was used as a loading control. Individual sample/primer sets were used in triplicate reactions. Quantification was performed using a standard curve with genomic DNA. A negative control containing no cDNA was used to check for genomic contamination of the RNA samples. Also, a dissociation step was added to the qRT-PCR to ensure primer fidelity. Data was normalized to one group for presentation purposes. The sequences for the primers (5' TO 3') are as follows:

Vg	FWD	TTGACCAAGACAAGCGGAACT
	REV	AAGGTTCGAATTAACGATGAA
eIF-S8	FWD	TGAGTGTCTGCTATGGATTGCAA
	REV	TCGCGGCTCGTGGTAAA

Quantification was based on the number of PCR cycles (C_T) required to cross a threshold of fluorescence intensity (ABI User Bulletin 2) described in Bloch et al 2001. Data was analyzed in JMP 7 (SAS, Cary, NC) using a standard least squares ANOVA with treatment as a fixed factor and colony and treatment by colony set as random factors.

Collections for Microarray Analysis

Bees from a single source colony, headed by a naturally mated queen (MDI5), were used for the microarray collections. Bees were reared as described above, with 20 bees/cage and 4 cages/treatment. There were four experimental treatments: sucrose only with solvent (NN), sucrose only with QMP (NQ), 8-Br-cGMP with solvent (GN), and 8-Br-cGMP with QMP (GQ). Bees were treated as above, but were treated over 9 days and collected on day 10 into liquid nitrogen. Retinue response was measured as above, using all the cages. *Vitellogenin* was measured as described above, using 15 bees from 1 NN cage and 16 bees from 1 GN cage. For analysis of the brain expression levels, heads of bees were freeze-dried to facilitate dissection. Brains were dissected on dry ice. Brains were pooled in groups of 4, with 6 pools per treatment. These pools were made from the two cages of each treatment with the highest retinue response.

AmOr11 *Quantification by Real-Time qRT-PCR*

Antennae were collected on dry ice from bees treated as in microarray experiment. Eight antennae were pooled to form one sample (corresponding to four bees); pooled antennae

were from bees used in microarray experiment. Samples were extracted and analyzed as described for *vitellogenin*. *AmOr2* was used as a loading control because it is a known ortholog to *Or83b* (Robertson and Wanner 2006), an odorant receptor that is expressed in all olfactory neurons (Touhara and Vosshall 2009). The sequences for the primers (5' TO 3') are as follows:

AmOr11	FWD	TGCTCTCAAACGCATTATGG
	REV	ACAATTTAATCTTCGTCCTG
AmOr2	FWD	GGACATGGATCTTCGAGGGAT
	REV	TTGAACGTCATTCCAGCAGTT

Data was analyzed in JMP 7 (SAS, Cary, NC) using a Student's t-test.

Microarray extraction and hybridization

RNA extractions from brains were performed using the Qiagen RNAeasy Isolation kit (Qiagen, Valencia, CA). 500 ng RNA was amplified using the Amino Allyl MessageAmp II aRNA Amplification Kit (Ambion, Austin, TX). 5 μg of amplified RNA was labeled with Cy3 or Cy5 dye (Kreatech, Amsterdam, Netherlands). Two sets of labeled probe were then hybridized to the whole-genome oligonucleotide arrays supplied from the laboratory of Dr. Gene Robinson (University of Illinois, Urbana-Champaign). Brains were hybridized using a

loop design with dye-swaps incorporated (n = 6 for each group, 24 arrays total). Arrays were scanned using the Axon Genepix 4000B scanner (Molecular Devices, Sunnyvale, CA) using GENEPIX software (Agilent Technologies, Santa Clara, CA).

Microarray Data Analysis

Any spots with an intensity of less than 300 in two or more arrays were removed from the analysis. Also, spots present on less than 10 arrays were excluded from the data set as well. Expression data was log-transformed and normalized using a mixed-model ANOVA (proc MIXED, SAS, Cary, NC) with the following model:

$$Y = \mu + dye + array + block + dye*array + array + block + \epsilon$$

where Y is expression, dye is a fixed effect, and array, block, and their interactions are random effects. Genes with significant expression differences between groups were detected by using a mixed-model ANOVA with the model:

$$Y = \mu + treatment + dye + array + dye*array + \epsilon$$

where Y represents the residual from the previous model. The fixed effects are behavior and dye and the random effects are array and dye*array. P-values were corrected for multiple testing using a false discovery rate < 0.01 (proc MULTTEST, SAS). Hierarchical clustering

and principal components analysis were performed in JMP 7 (SAS, Cary, NC). Gene ontology analysis was performed using DAVID (Dennis, Sherman et al. 2003; Huang da, Sherman et al. 2009) with a cutoff of $p < 0.05$. Analysis of overlap between the significantly regulated genes in our study and previous array studies was performed using statistical significance of the overlap between two groups of genes (Dr. Jim Lund, University of Kentucky, http://elegans.uky.edu/MA/progs/overlap_stats.html)

RESULTS

8-Br-cGMP decreases response to QMP in retinue assay

Honey bees were reared with and without QMP, and with and without 8-Br-cGMP. Responses to the QMP or solvent control were recorded daily, starting at day 3 of the experiment. There was an overall significant effect of treatment (ANOVA, $p<0.0001$, Figure 1a). Untreated and 8-Br-cGMP treated bees were not attracted to the solvent lure. Untreated bees were significantly more attracted to a QMP lure (the NQ group) than 8-Br-cGMP treated bees by day 4 of the experiment, and this difference continued throughout the rest of the experiment (Tukey HSD) (Figure 3.1A). We repeated this experiment for the bees used in the microarray analysis and found, once again, a significant effect of treatment on the retinue response (ANOVA, $p<0.0001$, Figure 3.1B). However, the NQ treatment group was only significantly different from the other treatment groups on day 7, 9 and 10 (Tukey HSD) (Figure 3.1B). Note that the number of bees attracted to the QMP lure was substantially lower in this trial. These bees were from a different colony than those used in Figure 3.1A, but did demonstrate responsiveness to QMP for lipid levels and *vitellogenin* levels (see results below). Previous studies have found that there can be substantial effects of both genotype and season on retinue response (Pankiw, Winston et al. 1994).

8-Br-cGMP treatment reduces abdominal lipid levels in worker bees

To determine if 8-Br-cGMP treatment affects lipid levels of young bees, thereby causing them to develop forager-like physiological characteristics, we measured lipid levels as in Toth et al. (2005). We found that 8-Br-cGMP treatment significantly reduces lipid levels in young caged honey bees (t-test, $p=0.0228$) (Figure 3.2).

Vitellogenin *expression results*

To determine if 8-Br-cGMP treatment affects *Vg* expression, we performed qRT-PCR on RNA extracted from the fat bodies of young workers after treatment. 8-Br-cGMP treatment significantly reduced *Vg* expression (t-test, $p=0.0207$, Figure 3.3A). We repeated this experiment with the four treatment groups used in the microarray experiment and found that *Vg* expression is significantly affected by treatment (ANOVA, $p<0.0001$, Figure 3.3B). Expression is significantly higher in bees exposed to QMP (but not treated with 8-Br-cGMP) than any other group (Tukey HSD $p>0.05$). 8-Br-cGMP treated bees have slightly lower levels than untreated controls, as do bees treated with 8-Br-cGMP and QMP.

8-Br-cGMP treatment does not affect AmOR11 expression levels

The AmOR11 odorant receptor is responsive to 9-ODA (Wanner, Nichols et al. 2007), a major component of QMP. To determine if 8-Br-cGMP affects the expression of *AmOr11*, we performed qRT-PCR on RNA extracted from pools of antennae from workers after treatment. 8-Br-cGMP treatment had no significant effect on *AmOr11* expression (t-test, $p=0.319$, Figure 3.4).

Brain gene expression profiles results

Both QMP exposure and 8-Br-cGMP treatment significantly affect brain expression patterns. QMP exposure causes workers to display nurse-like brain expression patterns (Grozinger, Sharabash et al. 2003), while 8-Br-cGMP treatment causes workers to develop forager-like trends in expression patterns (Whitfield, Ben-Shahar et al. 2006). Statistical analysis showed that 379 transcripts out of the 13,229 transcripts included in the analysis were differentially expressed in our treatment groups (n=6, all groups, FDR < 0.01). Hierarchical clustering analysis grouped NN and NQ together and GN and GQ together, suggesting that 8-Br-cGMP treatment is the major driver of brain gene expression in this study (Figure 3.5). Principal component analysis was used to detect any underlying expression patterns across all four groups (Figure 3.6). The first principal component accounts for 43% of the variation and again shows a substantial effect of 8-Br-cGMP treatment. The second principal component explains 30% of the variation and demonstrates that the NQ group is markedly different from the NN group, demonstrating the effect of QMP on brain gene expression. Lastly, the third principal component comprises 27% of the variation and shows the effect of an interaction between the QMP and 8-Br-cGMP treatments.

Gene ontology analysis was performed using DAVID (Dennis, Sherman et al. 2003). This analysis shows a significant over-representation of genes involved in carboxylic acid transmembrane activity (p=0.00328), GTPase regulator activity (p=0.00612),

phototransduction (p=0.00411), and sensory perception (p=0.00520), among others (Table 3.1).

There was less overlap than expected by chance between significant genes in our study and previous studies examining the effects of QMP (Grozinger, Sharabash et al. 2003) (representation factor = 0.3) or 8-Br-cGMP treatment (Whitfield, Ben-Shahar et al. 2006) (representation factor = 0.2) on brain gene expression. The representation factor is the number of overlapping genes divided by the expected number of overlapping genes drawn from two independent groups, where a representation number > 1 indicates more overlap than expected by chance. However, 18 genes were both significantly regulated in our study and previously found to be down-regulated by QMP treatment, and in this group there was a significant overrepresentation of genes associated with GTPase regulator activity ($p = 3.4e^{-02}$). Additionally, 5 genes were both significantly regulated in our study and previously shown to be associated with the foraging behavioral state, and in this group there was a significant overrepresentation of genes associated with kinase activity ($p = 1.0e^{-02}$).

DISCUSSION

We found that 8-Br-cGMP treatment reduced multiple responses to QMP. While QMP treatment elicited the retinue response, increased lipid levels and *Vg* expression, 8-Br-cGMP treatment effectively inhibited these responses. These results are recapitulated at the gene expression level, as our PCA shows a strong effect of 8-Br-cGMP. QMP treatment also had a large effect, but explained less of the variation than 8-Br-cGMP. Our third principal component indicated that there may be a set of QMP-responsive genes that are primarily affected in 8-Br-cGMP treated bees. This suggests that some transcriptional responses to QMP are specific to the physiological state produced by 8-Br-cGMP treatment. However, there were no significant GO categories associated with the genes that were significantly different between the 8-Br-cGMP-QMP (GQ) and 8-Br-cGMP (GN) treatment groups (data not shown). Future work needs to be done in order to determine if QMP causes specific behavioral or physiological changes in 8-Br-cGMP-treated bees which are associated with these transcriptional effects. For example, exposure to QMP will activate pollen foraging behavior in forager bees in newly established colonies (Jaycox 1970; Heather A. Higo 1992), and since 8-Br-cGMP-treated bees are more forager-like, this behavioral change may be associated with the transcriptional effects we have observed.

Changes in the peripheral or central nervous system can alter perception and responses to pheromonal cues. At the level of the peripheral nervous system, changes can occur in expression or activity levels of pheromone receptors, pheromone binding proteins,

or properties of the olfactory receptor neurons located in the antennae. For example, the properties of pheromone receptors can be affected by pheromone binding proteins (Xu, Atkinson et al. 2005) while the electrochemical properties of the olfactory receptor neurons may be modulated by biogenic amines (Linn 1984; Linn, Campbell et al. 1986; Linn, Campbell et al. 1992; Linn, Campbell et al. 1996; Pophof 2000). If a single receptor system regulates all downstream behavioral and physiological response, changes in this system should result in inhibition of most or all pheromone responses. Changes in central processing could also alter the downstream behavioral and physiological responses to pheromones, though in this case responses might be modulated separately. For example, Grozinger et al. (2007) demonstrate that two components of QMP that do not elicit the retinue response still alter brain gene expression. In our studies, we did not find changes in antennal expression levels of *AmOr11*, a known receptor for the main QMP component 9-ODA (Wanner, Nichols et al. 2007). These results suggest that, at least for 9-ODA, the modulation of the response to QMP is likely not occurring peripherally. Thus, our results suggest that 8-Br-cGMP treatment is modulating the response to QMP at the central processing level, though further studies using electrophysiological approaches would be necessary to verify this for the full QMP blend.

Results of our expression analyses are consistent with the effects of 8-Br-cGMP on insects, and of QMP on honey bee workers. Significantly regulated genes contained an over-representation of multiple gene ontology categories, including genes involved in phototransduction, lipid metabolism, GTPase regulator activity, and positive regulation of

antibacterial peptide biosynthetic process. Modulation of genes associated with phototransduction processes is expected, since 8-Br-cGMP increases phototaxic behavior in young bees (Ben-Shahar, Leung et al. 2003). Our data show that lipid levels are affected by both QMP and 8-Br-cGMP, so the GO term of lipid metabolic process was not surprising. PKG has been shown to activate at least one GTPase in an *in vitro* study (Hou, Ye et al. 2004), and it may be performing a similar role in the honey bee brain, as evidenced by the significant GO category of GTPase regulator activity. The GO category of positive regulation of antibacterial peptide biosynthetic process is interesting in that honey bee (Amdam, Simoes et al. 2004) and bumble bee (Konig and Schmid-Hempel 1995) foragers have fewer haemocytes than nurse bees. Haemocytes are cellular components of the defense response involved in encapsulation and phagocytosis of pathogens. Thus, our results suggest that immune responses may be another forager-related physiological phenotype that is regulated by 8-Br-cGMP and/or QMP.

While there was less overlap than expected by chance between our study and previous analyses of the effects of QMP and 8-Br-cGMP, the genes that did overlap were in important functional categories. Transcripts significantly regulated in our study which were previously shown to be down-regulated by QMP treatment were significantly associated with GTPase regulator activity, a component of the PKG pathway (Hou, Ye et al. 2004). Those significantly regulated transcripts which also overlapped with foraging-associated transcripts from previous studies were significantly associated with kinase activity. The lack of a significant overlap between our study and these previous studies is possibly due to the use of

different array platforms. Previous studies used double-stranded DNA arrays, while we used oligo arrays. Also, our experiments were performed under different experimental conditions: previous studies were performed with 4-5 day old caged bees, while we used 10 day old bees. Modulation of the pheromone response is quite prevalent in honey bees. As noted previously, there are genotypic differences in how workers respond to QMP (Pankiw, Winston et al. 1994), while physiological factors can also alter responsiveness to QMP, alarm and brood pheromone (Robinson 1987; Barron, A. et al. 2002; Grozinger and Robinson 2007; Grozinger, Fischer et al. 2007). Modulating the pheromone response at a number of different levels may play an important role in maintaining and regulating the division of labor in honey bee colonies, leading to greater colony efficiency. Changes in response to these social cues appear to be linked most closely with changes in behavioral task: thus, nurses and foragers have different responses to QMP (Jaycox 1970; Heather A. Higo 1992; Pham-Delegue, Bailez et al. 1993; Grozinger and Robinson 2007). The physiological differences between nurses and foragers appear to trigger these changes in responsiveness. However, since exposure to QMP will keep bees in the nursing state longer, workers must somehow become unresponsive to QMP or decrease their exposure to QMP in order to advance their behavioral development.

How do these bees become unresponsive? It could be due to age-related changes in physiological factors, or it could be due to social factors such as spending less time near the queen which then triggers changes in these physiological factors. Thus, there is interplay between how the receiver's own behavior and physiology modulates the response to the

pheromone, while simultaneously the pheromone modulates the receiver's behavior and physiology.

Of the known physiological factors that regulate the transition to foraging, only 8-Br-cGMP appears to be involved in globally shifting responsiveness to QMP. Octopamine treatment, though a strong promoter of foraging behavior (Schulz and Robinson 2001), has no effect on the retinue response or QMP-mediated gene expression (Grozinger and Robinson, unpublished data). QMP reduces levels of another biogenic amine, dopamine, in the honey bee brain (Beggs, Glendining et al. 2007), but dopamine treatment has no obvious effect on the retinue response (Fussnecker and Grozinger, unpublished data). JH, another strong driver of foraging behavior, inhibits the primer responses to QMP but not the retinue responses (Grozinger and Robinson 2007; Grozinger, Fischer et al. 2007). However, assays with JH and OA were performed with younger bees (4-5 days old) and thus the effects of long-term treatment need to be further investigated.

There is increasing evidence that responses to social cues, such as pheromones, can be modified by a number of factors. In honey bees, genetic (Pankiw, Winston et al. 1994), developmental (Kocher et al., in review, 2009), physiological (Robinson 1987; Barron, A. et al. 2002), and environmental (Heather A. Higo 1992) factors all play a role. Thus, the response to pheromones appears to be quite plastic and labile. This may be adaptive, particularly in animal societies, where all possible social cues may be present at any given time, but only a subset of the individuals should respond to these cues in order for the colony to function optimally.

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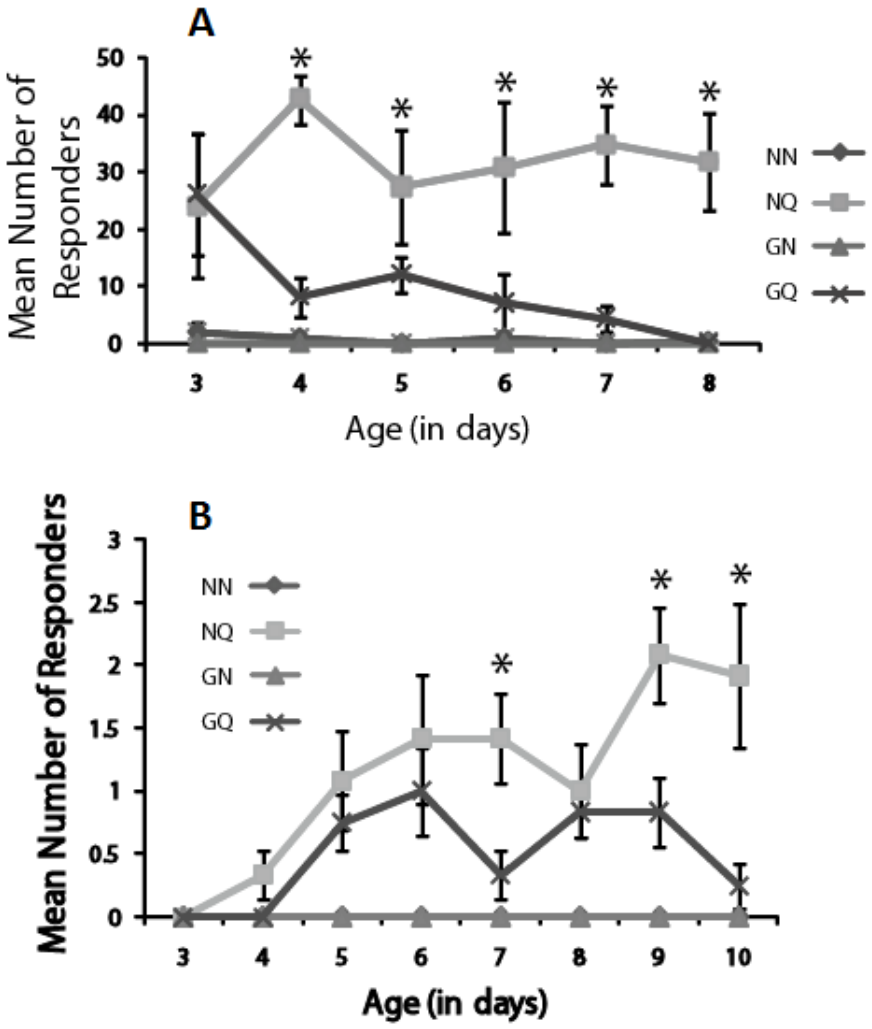

Figure 3.1. (A) *8-Br-cGMP treatment reduces the response to QMP in a retinue assay.* One day old bees were treated for 8 days with one of four treatments: 50% sucrose and solvent slide (NN), 50% sucrose and QMP slide (NQ), 50% sucrose and QMP slide (NQ), 500 μM 8-Br-cGMP and solvent slide (GN), and 500 μM 8-Br-cGMP and QMP slide (GQ). Differences in retinue response were analyzed using an ANOVA with Tukey HSD. Asterisks denote days in which a significant difference in treatment was observed. (B) *Retinue responses of bees collected for microarray analysis.* These bees were treated as for 1A. Significant differences in retinue response between the NQ group and three other treatment groups were observed on days 7, 9, and 10. Differences in retinue response were analyzed using an ANOVA with Tukey HSD. Asterisks denote days in which a significant difference in treatment was observed.

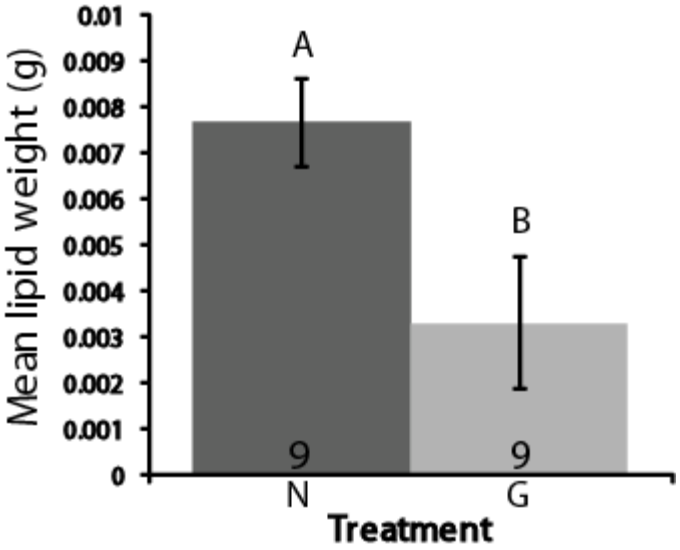

Figure 3.2. *8-Br-cGMP treatment reduces lipid levels in worker bees.* One day old bees were treated for 5 days with either 50% sucrose (N) or 500μM 8-Br-cGMP (G). Data were analyzed using a Student's t-test and analysis showed a significant reduction of lipid levels as a result of the 8-Br-cGMP treatment ($p=0.0228$). Two abdomens were weighed simultaneously in order to increase precision and the number of samples for each treatment group (9 samples=18 abdomens) is shown in the base of each bar. Different letters denote significant differences ($p<0.05$).

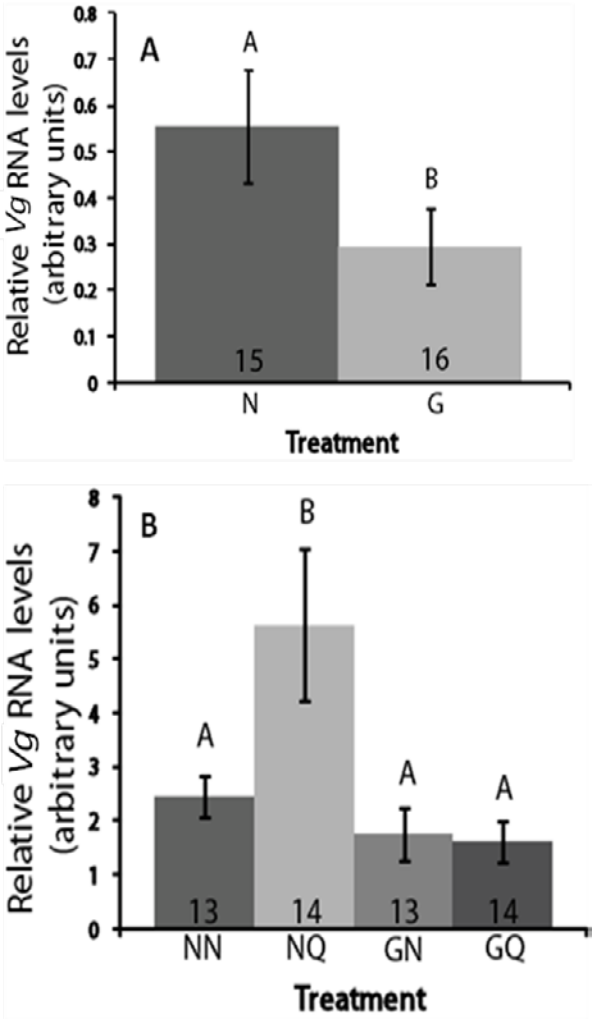

Figure 3.3. (A) *8-Br-cGMP treatment significantly reduces* Vg *expression in the fat bodies of young bees.* One day old bees were treated for 5 days with either 50% sucrose only (N) or 500μM 8-Br-cGMP (G). 8-Br-cGMP treatment signicantly reduced *Vg* expression levels (ANOVA, $p=0.0207$). Number of individual abdomens used is shown at the base of each bar. Different letters denote significant differences ($p<0.05$). (B) *8-Br-cGMP reduces effect of QMP exposure on* Vg *levels*. One day old bees were treated for 9 days with 50% sucrose and a solvent slide (NN), 50% sucrose with a QMP slide (NQ), 500μM 8-Br-cGMP with a solvent slide (GN), or 500μM 8-Br-cGMP with a QMP slide (GQ). 8-Br-cGMP treatment signicantly reduced *Vg* expression levels in the fat bodies in GQ treatment compared to the NQ treatment (ANOVA, Tukey HSD). Number of individual abdomens used is shown at the base of each bar. Different letters denote significant differences ($p<0.05$).

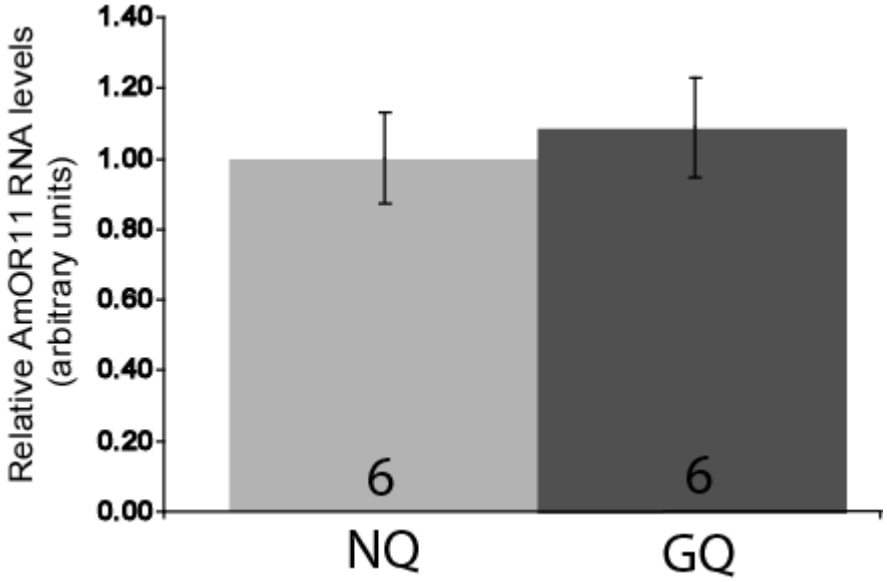

Figure 3.4. *8-Br-cGMP treatment does not affect* AmOr11 *expression levels*. Pools of eight antennae were collected from honey bees used in the microarray experiment from the 50% sucrose with a QMP slide (NQ) and 500µM 8-Br-cGMP with a QMP slide (GQ) treatment groups. 8-Br-cGMP treatment did not affect expression levels of *AmOR11* (t-test, p=0.319). Number of pools of antennae used is shown at the base of each bar.

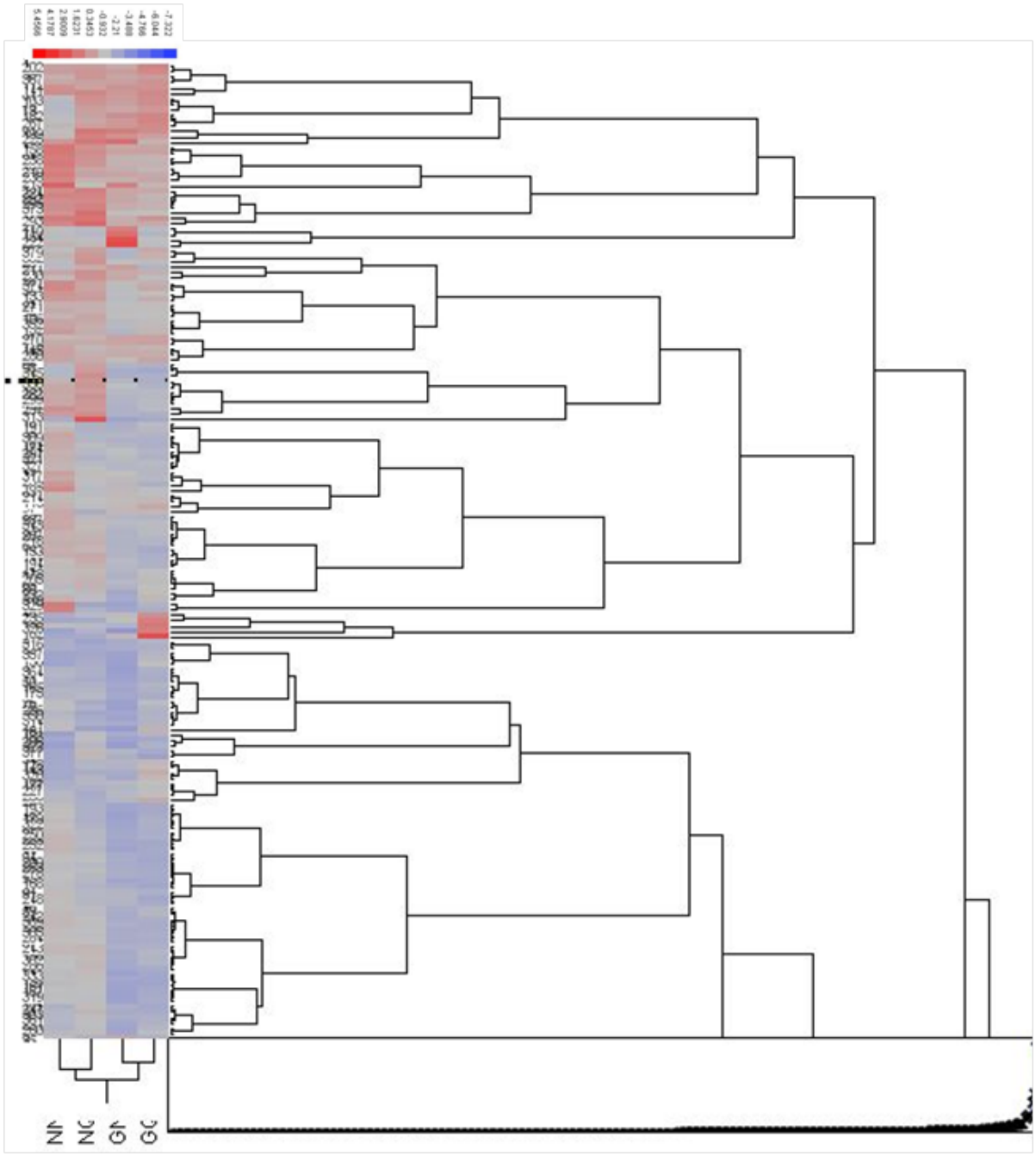

Figure 3.5. *Hierarchical clustering analysis*. Hierarchical clustering analysis using the significantly regulated genes shows that treatment with 8-Br-cGMP had a greater global effect on gene expression levels than QMP.

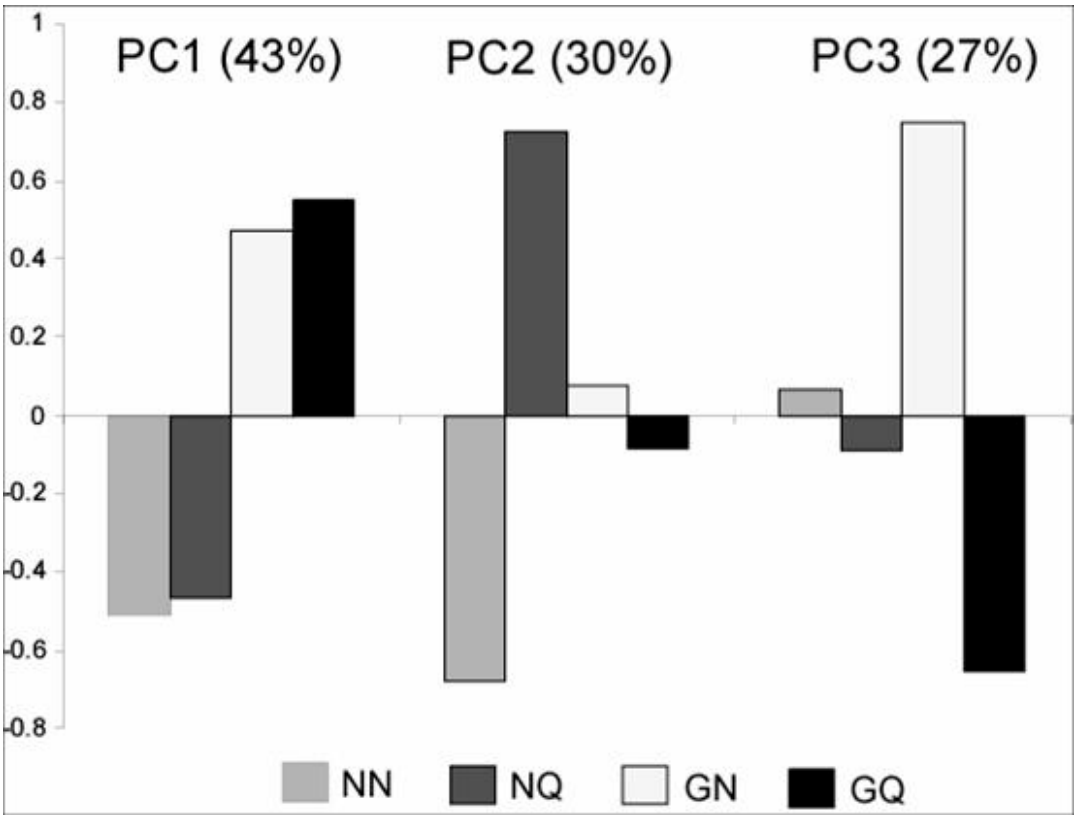

Figure 3.6. *Principal components analysis of brain expression patterns*. The first principal component accounts for 43% of the variation and shows a substantial effect of 8-Br-cGMP treatment. The second principal component explains 30% of the variation and demonstrates that the NQ group is markedly different from the NN group, demonstrating the effect of QMP on brain gene expression. Lastly, the third principal component comprises 27% of the variation and shows the effect of the interaction between the QMP and 8-Br-cGMP treatments.

Table 3.1. Gene Ontology Analysis: Overrepresented Biological Processes in the Brain

GO Biological Process	Count	%	p-value
carboxylic acid transmembrane transporter activity	6	2.44%	0.003281
GTPase regulator activity	11	4.47%	0.006121
intracellular signaling cascade	21	8.54%	0.03662
lipid metabolic process	16	6.50%	0.011274
molecular transducer activity	29	11.79%	0.002841
phototransduction	6	2.44%	0.004113
positive regulation of antibacterial peptide biosynthetic process	4	1.63%	0.006214
response to abiotic stimulus	11	4.47%	0.001931
sensory perception	12	4.88%	0.005203
transmembrane receptor activity	17	6.91%	0.004284

CHAPTER 4

Analysis of *Drosophila melanogaster* larval gene expression in the absence of *Kr-h1*

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ABSTRACT

Comparative studies have demonstrated that genes which are involved in basic physiological processes, including metabolism and reproduction, have been utilized in novel fashions to coordinate social behavior. *Kr-h1*, a zinc-finger transcription factor, has roles in both insect development and social behavior. We compared whole-body gene expression patterns from heterozygote and homozygote *Kr-h1* p-element mutant lines in order to find genes affected by the absence of *Kr-h1*expression. We analyzed samples from the wandering larvae stage, since this is a significant period in larval development, and the homozygous animals die shortly thereafter. Significant GO terms over-represented in this study were chromatin remodeling and chromatin assembly/disassembly. Many of the significantly expressed genes were associated with development of the nervous system. A motif of 20 nucleotides, which matched the motifs of three known developmental transcription factors, was found in the 5' UTR's in 66 of the 87 significantly expressed genes using MEME, but further analysis suggested that this motif is unlikely to be biologically relevant. There was no significant overlap with QMP-regulated or behaviorally associated genes from adult honey bees, which is not surprising given the differences in developmental state. Here, we have demonstrated that expression of genes associated with development, in particular of the nervous system, is significantly altered by loss of *Kr-h1* expression.

INTRODUCTION

With the development of new genomic tools and techniques, it is becoming possible to address the evolution of complex phenotypes, including social behavior, at the molecular level. Comparative studies have demonstrated that genes which are involved in basic physiological processes, including metabolism and reproduction, have been utilized in novel fashions to coordinate social behavior (Toth and Robinson 2007). For example, the transcription factor, *Kruppel homolog 1* (*Kr-h1*), plays a critical role in pre-adult developmental processes in solitary insects such as *Drosophila melanogaster* and *Tribolium castaneum* (Pecasse, Beck et al. 2000; Beck, Pecasse et al. 2004; Parthasarathy, Tan et al. 2008), but is also linked to social behavior in adult honey bees (Grozinger, Sharabash et al. 2003; Grozinger and Robinson 2007; Fussnecker and Grozinger 2008). In this study, we take advantage of the powerful genetic tools available in *D. melanogaster* to begin to characterize the transcriptional processes which are regulated by this KR-H1.

Complete metamorphosis is a complex developmental process which occurs in some insect species, in which larval tissues must be replaced with adult tissues. Some of these tissues do not undergo complete replacement, but instead undergo extensive remodeling, such as the nervous system (Truman 1996; Consoulas, Duch et al. 2000; Weeks 2003). The process of metamorphosis requires both terpenoid hormones, such as juvenile hormone (JH), and steroid hormones, such as ecdysone (Nijhout 1994). Ecdysone is responsible for controlling the regulatory cascades necessary for molting and metamorphosis (Riddiford 1993). JH works

together with ecdysone to ensure that metamorphosis results in a full transition to the next developmental stage (Flatt, Tu et al. 2005). Interestingly, both ecdysone and JH also play significant roles in the regulation of reproduction and other important functions in adult insects (Robinson 1992; Flatt, Tu et al. 2005; Bogomolova, Adonyeva et al. 2009). JH, in particular, regulates worker division of labor in honey bees (Robinson 1987).

Kruppel-homolog 1 (*Kr-h1*), a zinc-finger transcription factor, has been shown to play a role in regulating transcriptional responses to ecdysone during embryogenesis and metamorphosis in *D. melanogaster* and is required during the prepupal ecdysone response (Pecasse, Beck et al. 2000; Beck, Pecasse et al. 2004). Without it, flies are unable to complete prepupariation and die during this stage. Overexpression of *Kr-h1* results in defects in axon pathfinding (Kraut, Menon et al. 2001), suggesting that *Kr-h1* may play a role in neuronal remodeling during morphogenesis. Also, ecdysone has been shown to be important in the neuronal remodeling of the mushroom bodies (Kraft, Levine et al. 1998; Lee, Marticke et al. 2000), centers of learning and memory integration in the insect brain (Davis 2005). The association between ecdysone and *Kr-h1* suggested that *Kr-h1* may play a role in the development of the mushroom bodies. Furthermore, changes in Kr-h1 expression levels in adult honey bee brains are linked with changes in mushroom body architecture, suggesting that Kr-h1 may play a role in synaptic plasticity in adult insect mushroom bodies as well (Fussnecker and Grozinger 2008). Shi et al. (2007), however, demonstrate that *Kr-h1* is not necessary for proper neuronal morphogenesis in the mushroom bodies: neurons in which Kr-h1 expression has been eliminated through genetic mosaic develop normally. However,

overexpression of *Kr-h1* results in neurons with reduced axonal branching, suggesting that *Kr-h1* may negatively modulate the morphogenesis of neurons in the mushroom bodies.

Kr-h1 expression during development appears to be regulated by JH. In *D. melanogaster* and *T. castaneum*, treatment with a JH analog during specific developmental stages results in increased *Kr-h1* expression (Minakuchi, Zhou et al. 2008; Parthasarathy, Tan et al. 2008; Minakuchi, Namiki et al. 2009). The correlation between JH levels (both natural and manipulated) and *Kr-h1* expression is also observed in both bumble bees (Shpigler et al, submitted) and honey bees (Grozinger and Robinson 2007). However, the association between JH and *Kr-h1* in honey bees is complicated and seems to be dependent on the presence (Grozinger and Robinson 2007) or absence (Fussnecker and Grozinger, unpublished observations; Fussnecker and Grozinger 2008) of the honey bee queen.

In order to better understand the molecular function of this transcription factor, we examined the effects of perturbations of *Kr-h1* expression levels on transcriptional programs during development in *D. melanogaster*, using whole-genome microarrays.

MATERIALS AND METHODS

Lines and collections

The fly strains used in this study were w1118 (WT) and *Kr-h1[1]/CyO-GFP* (Beck et al. 2004) and were maintained at the University of Massachusetts, Worcester by L. Shi and T. Lee. All larval collections were also performed at the University of Massachusetts, Worcester. *Kr-h1[1]/Krh1[1]* mutant larvae (MT) were produced by crossing males and females from the *Kr-h1[1]/CyO-GFP* line. Homozygous mutant larvae (MT) that result from this cross do not express GFP. WT and non-GFP expressing larvae were collected shortly after entering the wandering larvae stage. Note that attempts to make temperature-controlled *Kr-h1* expression lines were unsuccessful, possibly due to negative feedback mechanisms which reduced levels of KR-H1 protein, even under transcriptionally stimulated conditions (Shi and Lee, unpublished results).

Approximately 200 adult flies were raised in bottles and fed with yeast paste on a grape juice agar medium. Larvae were genotyped between the first and second instar using fluorescent microscopy. ~80 mutant larvae were transferred into a single vial, and multiple vials were prepared. All larvae were raised at 25^{o}C and the developmental stages were determined by the size and number of spiracles. Third instar (wandering) larvae were collected from both the MT and WT line. *Kr-h1* expression levels were not significantly different between 2nd instar and wandering larvae (data not shown), but since mortality increased greatly as larvae

matured (Shi and Fussnecker, personal observations), we selected the wandering larval stage for our microarray studies in order to examine the effects of expression differences in a relatively healthy background.

PCR validation of fly line

DNA was extracted from a single wandering larva using the Qiagen DNeasy extraction kit (Qiagen, Valencia, CA). 10µg DNA was used for PCR reaction. The PCR was run for 35 cycles with a 94^{o}C denaturing step for 2 min, an annealing step at 60^{o}C for 1 min, an extension at 72^{o}C for 30 sec, and a final extension at 72^{o}C for 2 min during the last cycle. Primers used were as follows:

Kr-h1	FWD	5' AGCGCGAAGGTTGTCGACGAGT 3'
	REV	5' CGCACTAGCCAATGACCAATTTAC 3'

A 1% agarose gel was run at 100mV for 45 minutes. The GeneRuler 1kb Plus DNA Ladder (Fermentas Inc., Glen Burnie, MD) was used to determine molecular weights. Kodak Molecular Imaging Software (Kodak, Rochester, NY) was used to visualize the gel.

Microarray extraction and hybridization

RNA extractions from pools of 5 wandering larvae were performed using the Qiagen RNAeasy Isolation kit (Qiagen, Valencia, CA). 500 ng RNA was amplified using the Amino

Allyl MessageAmp II aRNA Amplification Kit (Ambion, Austin, TX). 5 ug of amplified RNA was labeled with Cy3 or Cy5 dye using a Kreapure kit (Kreatech Diagnostics, Amsterdam, Netherlands). Two sets of labeled probes were then hybridized to the whole-genome oligonucleotide arrays (DGRC-2) supplied from *The Drosophila Genome Research Center* (DGRC, Bloomington, IN). Individual pools of larval RNA were hybridized using a loop design with dye-swaps incorporated (n = 4 for each group). Arrays were scanned using the Axon Genepix 4000B scanner (Molecular Devices, Sunnyvale, CA) using GENEPIX software (Agilent Technologies, Santa Clara, CA).

Microarray Data Analysis

All spots were kept in the analysis because their intensity was well over the background intensity. Any spots present on less than 4 arrays were excluded from the data set. In total, 15,158 spots on the arrays were used in the analysis (SAS, SAS Institute, Cary, NC).
Expression data was log-transformed and normalized using a mixed-model ANOVA (proc MIXED, SAS) with the following model:

$$Y = \mu + dye + array + block + dye*array + array*block + \epsilon$$

where Y is expression, the fixed effect is dye, and the random effects were array, block, and their interactions. Significance for differential expression on residuals was detected by using a mixed-model ANOVA with the model:

$Y = \mu + group + dye + array + dye*array + \epsilon$

where Y represents the residuals from the previous model. The fixed effects are group and dye and the random effects are array and dye*array. P-values were corrected for multiple testing using a false discovery rate < 0.05 (proc MULTTEST, SAS). Hierarchical clustering was performed in JMP 7 (SAS). Gene ontology analysis was performed using DAVID (Dennis, Sherman et al. 2003; Huang da, Sherman et al. 2009). k-means clustering analysis was performed using Genesis (Graz, Austria). The number of clusters used in this analysis was selected because these clusters demonstrated the greatest differentiation between lines and the closest similarity to the hierarchical clustering. *De novo* motif search was performed using the 5'UTR's (Flybase.org) of the 87 significantly expressed genes in the MEME online software (Bailey and Elkan 1994). TOMTOM (Gupta, Stamatoyannopoulos et al. 2007) was used to compare any found motifs against a database of known motifs. GOMO (Boden and Bailey 2008) was used to find GO terms associated with this motif.

RESULTS

PCR validation of fly line

A 200bp band was present in WT, but not in MT larvae (Figure 4.1). This band is part of the first exon of *Kr-h1*, which is disrupted by the p-element present in the MT larvae. No band was present in the sample containing no template DNA.

Gene expression profiles

Statistical analysis showed that 87 out of the 9,309 transcripts used in the analysis were differentially expressed in our two groups (n=4 each group, FDR < 0.05). Hierarchical clustering grouped samples by WT or MT, suggesting that the major driver of gene expression in this experiment was the presence or absence of *Kr-h1* (Figure 4.2).

Gene ontology analysis was performed using DAVID (Dennis et al. 2003). This analysis showed a significant over-representation of genes involved in chromatin assembly or disassembly ($p = 0.0248$, Table 4.1, denoted by an asterisk). Four genes made up this significant GO cluster.

Genes associated with the absence of *Kr-h1* in development could also be associated with changing *Kr-h1* levels in adult honey bees. To test this, we compared our significant gene list to those genes which are significantly regulated by honey bee queen pheromone, which

includes *Kr-h1* (Grozinger, Sharabash et al. 2003). We found no overlap between genes significantly expressed in our study and this previous study.

k-means clustering

k-means clustering analysis resulted in four distinct clusters (Figure 4.3, Table 4.1). Clusters 1 and 2 showed fairly minor expression differences between the two groups. Cluster 3 contains transcripts up-regulated in WT when compared to MT. These transcripts included genes with known interactions from BioGRID (Stark, Breitkreutz et al. 2006) with genes involved in leg, wing, and eye development, and also axonogenesis (see Table 4.1 for complete list). Cluster 4 included transcripts up-regulated in MT when compared to WT. Transcripts in this cluster had known BioGRID interactions with genes involved in motor axon guidance, brain development, and the *wnt* pathway.

Promoter motif analysis

A *de novo* motif search in the promoters of the significantly regulated genes was performed using MEME (Bailey and Elkan 1994). A common motif with a length of 20 nucleotides (Figure 4.4) was found in the UTRs of 66 of the 87 significantly expressed transcripts (e-value = $1.4e^{-12}$, see Table 4.1, in bold). We compared this motif to known motifs using Tomtom (Gupta, Stamatoyannopoulos et al. 2007), and found significant similarity to three other known transcription factor binding motifs: *extradenticle*, *hunchback*, and *broad* (Table

4.2). GOMO (Boden and Bailey 2008) was used to find GO terms associated with this motif. Protein kinase CK2 regulator activity (e-value = 5.875e-03) and protein-DNA complex (e-value = 3.819e-02) were two of the significant categories found by this program. However, additional analyses demonstrated that this motif unlikely to be biologically relevant. We randomized 20 sets of 87 genes from the 9,309 genes used in our microarray analysis. Then, we performed a *de novo* motif search in MEME (Bailey and Elkan 1994) as above for each of the 20 random sets. The median motif discovery e-value for the 20 sets of random genes used in this analysis was $1.95e^{-22}$, suggesting that the e-value of 1.4^{-12} for our significant gene set is far below what we would expect to get from a random set of genes.

DISCUSSION

We show that the absence of *Kr-h1* expression alters expression of genes associated with transcriptional and developmental processes in *D. melanogaster* wandering larvae. However, it is somewhat surprising that we found only 87 genes significantly expressed in our study, given that *Kr-h1* is a transcription factor required for proper metamorphosis. Our study used whole larvae, so it is possible that subtle changes in gene expression in specific tissues, such as the central or peripheral nervous systems, were not detected. Also, due to the high mortality of later developmental changes, we chose to use third instar larvae. Though expression of *Kr-h1* is present in third instar larvae (Pecasse, Beck et al. 2000), the phenotypic effects associated with the loss of *Kr-h1* expression typically occur later during development, and thus we may have observed only the initial changes in the transcriptional programs. We found no overlap between our study and a previous study examining the regulation of *Kr-h1* expression in honey bees (Grozinger, Sharabash et al. 2003), suggesting that the transcriptional pathways associated with high vs low *Kr-h1* expression may differ between *Drosophila* development and adult honey bees. However, given that these studies utilized different array platforms, (oligo vs spotted) and tissue types (whole body vs bee brain); it is still possible that we would find common elements under different conditions.

Among the significantly regulated transcripts, there was a significant overrepresentation of the GO category of chromatin assembly or disassembly. This is consistent with the observation that the Kr-h1 protein is associated with the polytene chromosomes during the prepupal transition (Beck Y., unpublished data). The polytene

chromosomes are regions of dynamic chromatin modulation during development and many regions undergo endoreduplication (Daneholt 1975). *Kr-h1*, or genes regulated by it, may play a role in this process, however, it is important to note that overrepresentation of GO categories does not imply functional significance.

Our k-means clustering analysis identified groups of transcripts up-regulated in the WT larvae compared to the MT larvae. Many transcripts contained in this cluster had known interactions with other *D. melanogaster* genes (BioGRID) with roles in leg, wing, and eye development, and axonogenesis. These interactions represent potential avenues for future research and may not be biologically significant. Specifically, *bric-a-brac 2* (*bab2*) was contained in this cluster. This gene is a transcription factor necessary for normal development and has a GO biological process of eye-antennal disc morphogenesis in its annotation. It is implicated in sex comb patterning, specifically bristle pattern and joint formation of the forelegs (Godt, Couderc et al. 1993). Also, *bab2* has shown to be necessary for ovarian development (King 1970; Godt and Laski 1995; Sahut-Barnola, Godt et al. 1995). A second gene in this cluster was *JIL-1*. *JIL-1* is a tandem kinase that phosphorylates histone H3 *in vitro*, is localized to chromosomes during embryonic development, and its expression correlates with increased expression of the male chromosome (Jin, Wang et al. 1999; Jin, Wang et al. 2000; Wang, Zhang et al. 2001).

A second k-means cluster contained transcripts up-regulated in the MT group compared to the WT group. The *C15* gene is a homeodomain protein and a homolog to the human oncogene, *Hox11*, and has been shown to repress several genes involved in tarsal

development (Campbell 2005). Other transcripts in this cluster had known interactions with other *D. melanogaster* genes (BioGRID) that had roles in motor axon guidance, brain development, dendrite development, and interactions with the *wnt* pathway. The *wnt* pathway has been implicated in embryogenesis, adult tissue homeostasis, and cell fate specification and differentiation (Logan and Nusse 2004).

We used MEME (Bailey and Elkan 1994) for *de novo* identification of motifs present in the 5'UTRs of our significant gene list. A 20 nucleotide motif was found in 66 of the 87 significant genes in our study. A secondary analysis, based on analyses of promoter regions of random sets of unregulated genes from our arrays, demonstrates that our motif was no more likely to be significant than motifs found in random sets of genes. However, it did match known binding motifs for three transcription factors: *extradenticle*, *hunchback*, and *broad* (Tomtom, (Gupta, Stamatoyannopoulos et al. 2007)). All three have known roles in development. *Broad* is part of the *Broad-complex*, a family of zinc finger transcription factors (DiBello, Withers et al. 1991) shown to have a significant role in metamorphosis (Kiss, Beaton et al. 1988; DiBello, Withers et al. 1991; Karim, Guild et al. 1993). *Broad* has also been shown to be important for the response to ecdysone during this process (Karim, Guild et al. 1993; von Kalm, Crossgrove et al. 1994; Sempere, Sokol et al. 2003) and has been linked to *Kr-h1* expression in several studies (Beck, Dauer et al. 2005; Riddiford 2008). *Hunchback* is a maternally expressed gap gene important for embryogenesis and proper development (Papatsenko and Levine 2008). *Extradenticle* is a transcription factor shown to be important for regulating, and being regulated by, the expression of HOX genes (Pearson,

Lemons et al. 2005). Also, it has been implicated in leg (Kojima 2004) and eye development (Pappu and Mardon 2004). Determining the extent and functionality, if any, of any interactions KR-H1 may have with these other transcription factors could lead to important insights into the function of KR-H1 during development.

With the development of new high-throughput genomic tools, it is becoming increasingly feasible to perform comparative genomic studies in order to identify conserved molecular pathways. *Kr-h1* is an interesting candidate gene for these analyses, since it plays an important role in development in *D. melanogaster* and *T. castaneum*, but also has been linked to complex social behavior in honey bees and bumble bees (Grozinger, Sharabash et al. 2003, Shpigler submitted). Furthermore, KR-H1 is widely conserved across all insects with sequenced genomes (Shpigler, submitted). Here, we have demonstrated that expression of genes associated with development, in particular of the nervous system, is significantly altered by the loss of *Kr-h1* expression. Further studies using more precise methods to control *Kr-h1* expression, both during development and in adults, in *D. melanogaster* and other insects will allow us to continue to dissect the function of this fascinating protein.

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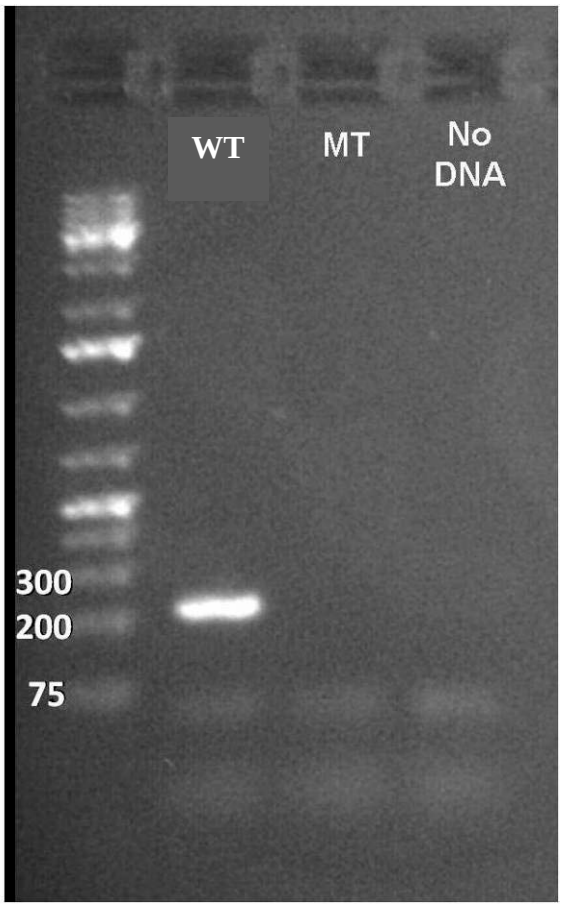

Figure 4.1. *PCR validation of line used in the experiment.* DNA was extracted from single wandering larva, and 10µg DNA was used for PCR reaction. A 200bp band was present in HT, but not in MT larvae. This band is part of the first exon of *Kr-h1*, which is disrupted by the p-element present in the MT larvae. No band was present in the sample containing no template DNA. The DNA ladder units are in base pairs.

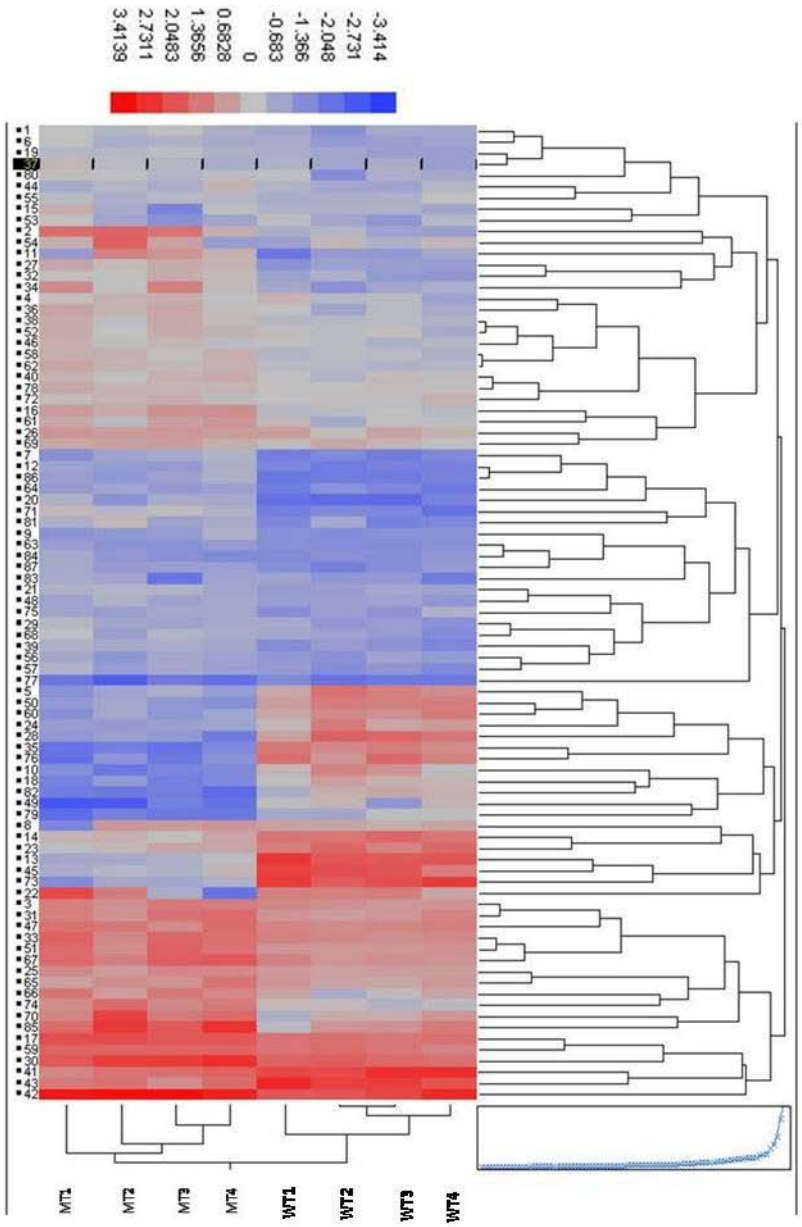

Figure 4.2. *Hierarchical clustering analysis*. Hierarchical clustering analysis was performed in JMP 7 using the 87 significant transcripts. This clustering demonstrates that grouping of transcripts was based on presence or absence of *Kr-h1* expression.

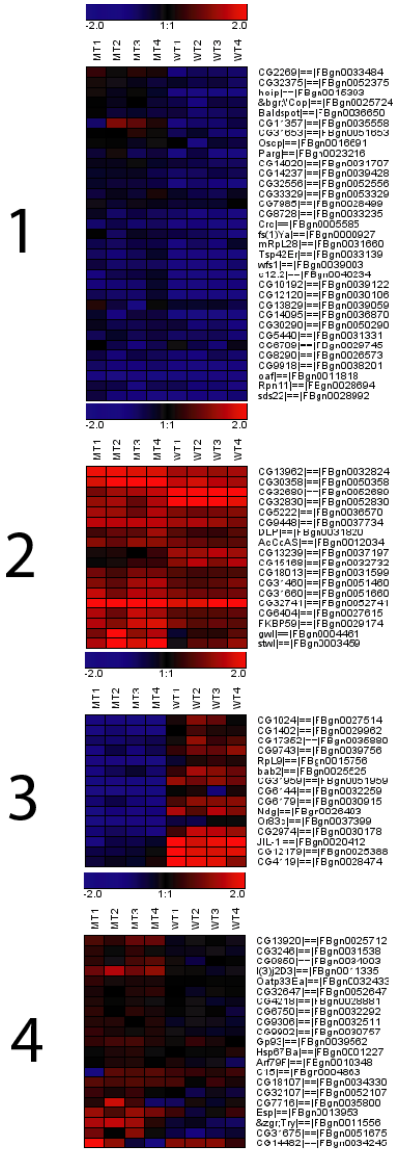

Figure 4.3. *k-means clustering of significant transcripts*. k-means clusters were selected based on their differentiation and similarity to hierarchical clustering. This analysis shows that Clusters 1 and 2 showed minor expression differences. Cluster 3 contains transcripts up-regulated in HT when compared to MT. Cluster 4 shows transcripts up-regulated in MT when compared to HT

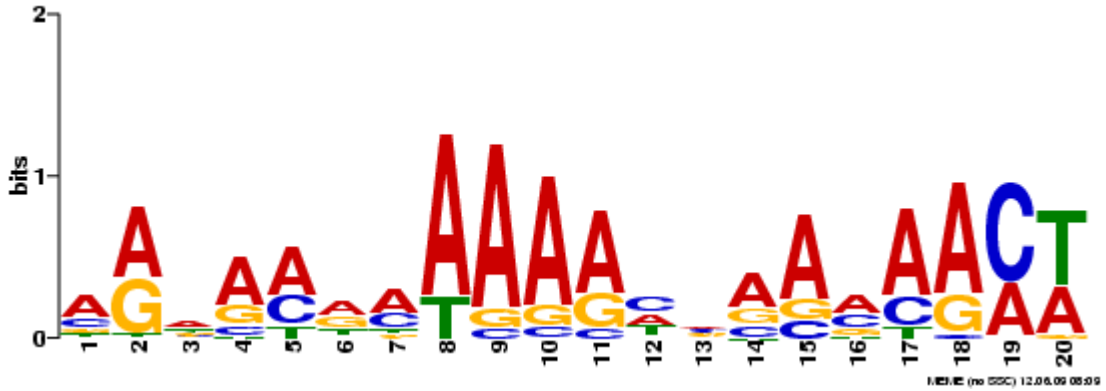

Figure 4.4. *de novo* motif discovery using MEME. A common motif with a length of 20 nucleotides (Figure 4.4) was found in the UTRs of 66 of the 87 significantly expressed transcripts. However, additional analyses demonstrated that this motif unlikely to be biologically relevant.

Table 4.1. Significant gene list.

Flybase ID	NAME	GO Biological Process	Interac-tion #	Interaction example	Interaction example GO Biological Process
CLUSTER 1					
FBgn0033484	CG2269				
FBgn0015393	hoi-polloi	nuclear mRNA splicing, via spliceosome	7	cnn	central nervous system development
FBgn0025724	beta'-coatomer protein	ER to Golgi vesicle-mediated transport			
FBgn0036650	Baldspot	mediated transport	5	Bx42	eye-antennal disc development
FBgn0035558	CG11357	protein amino acid glycosylation			
FBgn0016691	Oligomycin sensitivity-conferring protein	proton transport			
FBgn0023216	Poly(ADP-ribose) glycohydrolase*	carbohydrate metabolic process	1	CG5027	protein disulfide isomerase activity
FBgn0031707	CG14020				
FBgn0039428	CG14237				
FBgn0052556	CG32556				
FBgn0053329	Serine-peptidase 212	proteolysis			
FBgn0028499	CG7985	carbohydrate metabolic process			
FBgn0033235	CG8728	protein processing			
FBgn0005585	Calreticulin	central nervous system development			
FBgn0000927	female sterile (1) Young arrest*	nuclear division	1	RpLP0	DNA repair
FBgn0031660	mitochondrial ribosomal protein L28	translation			
FBgn0033139	Tetraspanin 42Er				
FBgn0039003	wolfram syndrome 1				
FBgn0040234	c12.2				
FBgn0039059	CG13829				
FBgn0036870	CG14095				
FBgn0050290	CG30290				

Table 4.1. Continued

FBgn0031331	CG5440				regulation of protein metabolic process
FBgn0029745	CG6789				
FBgn0026573	CG8290				
FBgn0038201	CG9918				G-protein coupled receptor protein signaling pathway
FBgn0011818	out at first				eclosion
FBgn0028694	Rpn11	proteolysis	2	Mov34	ubiquitin-dependent protein catabolic process
FBgn0028992	sds22				
CLUSTER 2					
FBgn0032824	CG13962		4	exd	brain development; leg disc pattern formation
FBgn0050358	CG30358				
FBgn0052830	CG32830				
FBgn0036570	CG5222	mRNA cleavage			
FBgn0037734	CG9448				
FBgn0031820	Daxx-like protein	regulation of apoptosis			
FBgn0012034	Acetyl Coenzyme A synthase	metabolic process			
FBgn0037197	CG13239				
FBgn0032732	CG15168				
FBgn0031599	Psf2	DNA strand elongation during DNA replication			
FBgn0051460	CG31460				
FBgn0051660	CG31660	metabotropic glutamate receptor signaling pathway			
FBgn0052741	CG32741	phospholipid metabolic process			
FBgn0027615	CG6404	respiratory chain complex IV assembly			
FBgn0029174	FK506-binding protein FKBP59	peripheral nervous system development			
FBgn0003459	stonewall	regulation of transcription			

Table 4.1. Continued

CLUSTER 3					
FBgn0027514	CG1024				
FBgn0029962	CG1402	one-carbon compound metabolic process			
FBgn0035880	CG17352				
FBgn0039756	CG9743	lipid metabolic process			
FBgn0015756	Ribosomal protein L9	translation	9	eyeless	eye-antennal disc morphogenesis
FBgn0025525	bab2*	eye-antennal disc morphogenesis	5	CG3731	mitochondrial processing peptidase activity
FBgn0051959	CG31959				
FBgn0032259	CG6144				
FBgn0030915	CG6179	negative regulation of catalytic activity	1	l(3)IX-14	brain development; centrosome duplication
FBgn0026403	Nidogen/entactin	bioluminescence	3	cpb	wing disc development
FBgn0037399	Odorant receptor 83c	sensory perception of smell			
FBgn0030178	CG2974				
FBgn0020412	JIL-1*	establishment or maintenance of chromatin architecture	1	lola	axonogenesis, axon guidance
FBgn0025388	CG12179	regulation of alternative nuclear mRNA splicing, via spliceosome			
FBgn0028474	CG4119				

Table 4.1. Continued

CLUSTER 4					
FBgn0025712	CG13920				
FBgn0031538	CG3246				
FBgn0034903	CG9850	cell proliferation	3	E5	regulation of transcription, DNA dependent
FBgn0011335	lethal (3) j2D3		14	lim3	nervous system development
FBgn0032433	Organic anion transporting polypeptide 33Ea	organic anion transport			
FBgn0052647	CG32647	methylation	9	cad	gastrulation involving germ band extension
FBgn0032292	CG6750				
FBgn0032511	CG9306	mitochondrial electron transport, NADH to ubiquinone	7	surf6	ribosome biogenesis and assembly
FBgn0030757	CG9902		24	toy	eye-antennal disc development
FBgn0039562	Glycoprotein 93	protein folding			
FBgn0001227	Heat shock gene 67Ba	response to heat	36	RfaBp	wnt receptor signaling pathway
FBgn0010348	ADP ribosylation factor 79F	protein amino acid ADP-ribosylation	6	Df31	establishment or maintenance ofchromatin architecture
FBgn0004863	C15	regulation of transcription, DNA-dependent	47	E2f2	DNA endoreduplication
FBgn0034330	CG18107				
FBgn0052107	CG32107				
FBgn0035800	CG7716	microtubule nucleation	6	Bap60	neuron development, dendrite morphogenesis
FBgn0013953	Epidermal stripes and patches	sulfate transport			
FBgn0011556	zetaTrypsin	proteolysis	2	alphaPS5	cell-matrix adhesion
FBgn0051675	CG31675				
FBgn0034245	CG14482	mitochondrial electron transport, ubiquinol to cytochrome c			

Table 4.2. Significant tomtom motif similarities.

Gene Name	p-value	Biological Processes
extradenticle	0.0041	brain development; leg disc proximal/distal pattern formation; salivary gland boundary specification
hunchback	0.0073	anatomical structure development; anterior/posterior axis specification; embryonic pattern specification
broad	0.0087	organ morphogenesis; sensory organ development; response to hormone stimulus; eclosion

CHAPTER 5

Conclusions and Future Directions

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Regulation of behavior in honey bees and other eusocial organisms is integral for their success and survival. A complex interplay between physiological factors, social cues, and gene expression contributes to behavioral phenotypes and provides multiple targets for which behavior may be modulated. *Kr-h1* was proposed as a candidate for foraging behavior in honey bees due to the timing of its expression and the modulation of its expression by exposure to QMP, which also delays the transition to foraging behavior. In addition to its potential role in adult honey bee social behavior, *Kr-h1* has known roles in development and metamorphosis in other insects. Our studies addressed the role of *Kr-h1* in foraging behavior and also attempted to elucidate the molecular function of this gene using a comparative approach. Furthermore, we examined the role of cGMP, a potential regulator of *Kr-h1* expression, in the global modulation of the response to QMP. Though we have learned much, the exact role of *Kr-h1* in honey bee social behavior is still unclear. However, our studies have provided numerous new insights and elucidated potential avenues for future research.

In Chapter 2, *Dissecting the role of* Kr-h1 *brain gene expression in foraging behavior honey bees*, we determined that *Kr-h1* expression was not affected by flight experience in male honey bees. Flight experience is a major component of foraging behavior and this experience leads to changes in the neuroanatomical structure of the mushroom bodies. *Kr-*

h1, due to its role in development of the nervous system, may contribute to these neuroanatomical changes. Our data suggests that this is not the case for flight experience, but *Kr-h1* may still play a role in the neuroanatomical changes that occur during the transition to foraging behavior. In order to determine if *Kr-h1* is associated with these structural changes, future studies could culture dissociated neurons from the honey bee mushroom bodies during the transition from nursing to foraging. These cells could then be treated with known promoters of axonal growth, such as ecdysone, or inhibitors of growth, such as brefeldin A, and then be assayed for expression of *Kr-h1*. This study would demonstrate whether *Kr-h1* is associated with the promotion or inhibition of axonal growth/branching. In addition, these cells could also be treated with siRNAs to down-regulate *Kr-h1* expression, allowing visualization of any global effects of *Kr-h1* knock-down on axonal branching. I predict, based on its role in *D. melanogaster*, that *Kr-h1* will be associated with inhibition of this axonal growth/branching.

We also demonstrated in this study that *Kr-h1* expression does not change as a result of behavioral reversion from foraging to nursing. This experiment uncouples foraging behavior and *Kr-h1* expression and is further evidence that *Kr-h1* may not be a direct regulator of foraging behavior, but instead is one of a number of genetic or physiological factors that undergo permanent changes during this behavioral transition. A better understanding of the exact time course of *Kr-h1* expression is needed to fully support this

prediction. This study would necessitate the use of marked worker bees (in order to track age) and highly detailed behavioral observations in order to determine the transition to foraging. However, this study would be highly difficult due to the plasticity of honey bee behavior and the inexact timing of foraging, making it nearly impossible to determine the exact timing of this behavioral transition.

Our final experiment in this study demonstrated that treatment with 8-Br-cGMP, a known promoter of foraging behavior, increases *Kr-h1* expression. However, several questions remain. How is this regulation occurring? Is the cGMP/PKG pathway directly regulating *Kr-h1* expression or is this pathway indirectly affecting expression through other pathways? We partially addressed this question by finding a potential cGMP response element in the promoter of honey bee *Kr-h1*, as well as in two other insect orthologs. However, this is not functional evidence, so more in-depth studies will need to be done in order to determine the exact relationship between cGMP and *Kr-h1*. Cell culture studies will be invaluable in the pursuit of the answer to this question. Specifically, I could add cGMP to primary cell cultures of honey bee mushroom body neurons to see if this effect can be seen in individual cells. Also, I could link the *Kr-h1* promoter to a reporter such as GFP or luciferase and place this construct in mammalian cell lines to determine if there is a functional link between cGMP and the *Kr-h1* promoter. Also, it is possible that the cGMP/PKG pathway makes post-translational modifications to the KR-H1 protein, such as phosphorylation. These types of modifications can be visualized using Western blots in

conjunction with reagents that affect these modifications, such as an alkaline phosphatase. Determining the nature of the link between cGMP/PKG and *Kr-h1* expression would further support the prediction that *Kr-h1* is up-regulated during the transition to foraging, since 8-Br-cGMP treatment promotes this transition.

Chapter 3, *The role of cGMP in modulating behavior, physiology, and brain gene expression in response to queen mandibular pheromone in honey bees*, examined the role of cGMP in modulating responses to QMP. QMP promotes nurse-like phenotypes in regards to behavior, physiology, and gene expression. We predicted that 8-Br-cGMP treatment would promote forager-like phenotypes and inhibit the effects of QMP. Our data show that cGMP treatment is a global modulator of the QMP response, and is the only physiological factor known to affect such a large number of pheromone responses in honey bees. In addition, we demonstrated that both 8-Br-cGMP and QMP treatments affected brain gene expression.

Many questions remain, however, about how the pheromone response is actually being modulated. Is this modulation occurring at the peripheral level (the antennae) or at a more central level? There are many opportunities for modulation at both. In order to determine whether modulation of the pheromone response to QMP by cGMP is occurring peripherally, an electroantennogram (EAG) could be performed. This would show any differences in the electrochemical response to QMP that occurred as a result of 8-Br-cGMP treatment. It is also reasonable to expect that 8-Br-cGMP treatment might affect the

expression of genes important for the peripheral pheromone response. We looked at one such example, *AmOr11*. This odorant receptor responds to 9-ODA, a major component of QMP. Our data demonstrate that 8-Br-cGMP treatment does not affect the expression of this receptor, but it is possible that differences in responses could be due to other components of the pheromone response in the antennae including pheromone binding proteins and the properties of the olfactory receptor neuron. All of these could contribute to the modulation we observe as a result of 8-Br-cGMP treatment. Additional electrophysiological studies could also be performed to examine whether 8-Br-cGMP treatment acts centrally to modulate the pheromone response. Calcium imaging could be used to view brain regions responsive to QMP and if/how 8-Br-cGMP affects this response. Determining the location at which 8-Br-cGMP modulates the response to QMP at a peripheral or central level may lead to insight into how worker honey bees undergo their transition from nursing to foraging behavior. A hallmark of this behavioral transition is the change from responsiveness to QMP by nurses to the unresponsiveness to QMP of foragers. We know many physiological factors that contribute to foraging behavior and many of the genes differentially expressed between nurses and foragers, but we do not have a firm grasp on how these components interact and how they result in the observed complex behavioral phenotypes. Our data demonstrate that 8-Br-cGMP globally modulates the response to QMP. Further studies examining how and

where this modulation occurs will elucidate mechanisms both underlying pheromone response and behavioral plasticity in honey bees.

Chapter 4, *Analysis of* Drosophila melanogaster *larval gene expression in the absence of* Kr-h1, is a microarray study with the aims of finding potential targets of KR-H1 and elucidating a potential KR-H1 DNA binding motif. This study faced numerous obstacles, as *Kr-h1* homozygous mutant organisms fail to pupate and, in fact, have increased mortality with age, even in prepupal stages. Temperature controlled expression lines fail to effectively regulate *Kr-h1* expression, which could be due to autoregulation of *Kr-h1* or redundancy with other transcription factors. We also attempted to use a RNAi line, but, again, this was unsuccessful. Genes expressed in the nervous system are notoriously difficult to silence using RNAi, and in hindsight, we should have attempted to co-express Dicer in the nervous system of this line. Finally, we settled on using a p-element line previously used in numerous other *Kr-h1* studies. This line contains a CyO-GFP balancer in order to maintain this line and simplify larval collections. In this study, we found a number of transcripts with known roles in development. Additionally, we found a potential KR-H1 binding motif using an online *de novo* motif search and the 5'UTRs of our significant gene list. This motif is not statistically overrepresented, so most likely is not functionally significant. Once again, a cell culture approach would be most appropriate.

We still know very little about the molecular function of *Kr-h1* and this is partly due to the limitations of the two model systems we've employed thus far. Branching out into a new, more pliable model system would greatly benefit *Kr-h1* research. One possible alternative model system is *Tribolium castaneum*. These organisms are extremely amenable to RNAi and this would allow us to examine the role of *Kr-h1* both bees and flies. In addition, adult *T. castaneum* beetles perform a type of foraging behavior when searching for a new food source. It would be relatively easy to knock-down expression of *Kr-h1* in these animals and examine the effect on social behaviors. Also, silencing *Kr-h1* at multiple developmental stages will lead to new insights into the exact role of *Kr-h1* in development and metamorphosis.

Our data strongly suggest that *Kr-h1* is associated with permanent physiological changes that occur during the transition to foraging behavior. This transition is accelerated by 8-Br-cGMP treatment, which also increases *Kr-h1* expression. Future research should examine the connection between cGMP/PKG and *Kr-h1* and how they might influence or modulate the foraging transition. Also, more work most be done to elucidate the molecular function of *Kr-h1*. In order to do this, cell culture studies will probably have the highest rate of success and provide the most insights. However, other types of studies will also prove invaluable. Variations of immunoprecipitation techniques such as protein-complex immunoprecipitation or chromatin immunoprecipitation will elucidate protein-protein

interactions of KR-H1 (if any exist) and DNA regions that KR-H1 interacts with, respectively. We still need a better understanding of where *Kr-h1* is expressed in the honey bee brain, and we could do this using either *in situ hybridization* or immunohistochemical techniques. Lastly, the utilization of a new model to study *Kr-h1*, *T. castaneum*, will lead to a new understanding of the role of *Kr-h1* in development and how it may contribute to adult foraging behavior. Though its role in honey bee foraging is still not completely understood, *Kr-h1* still stands as an example of a gene with important roles in development that has been potentially co-opted for a role in adult social behavior. Understanding how a gene such as *Kr-h1* functions and how it is regulated in solitary vs social systems will lead to insights into the origins of eusociality.