THE EFFECTS OF CAFFEINE ON LEARNING AND LOCOMOTOR BEHAVIOR IN THE HONEY BEE (*Apis mellifera)*

By

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LIST OF ABBREVIATIONS

^{3}H-caffeine – Tritiated Caffeine

CPG – Central Pattern Generator

CS – Conditioned Stimulus

DA – Dopamine

dMF – Dimethylformamide

DMTS – Delayed-Match-to-Sample

FTLP – Feeding Table Learning Paradigm

PDE – Phosphodiesterase

PER – Proboscis Extension Reflex

S+ – Sucrose Target

S- – Water Target

US – Unconditioned Stimulus

VUM_{mx1} – Ventral Unpaired Neuron No. 1 of the Maxillary Neuromere

Alana S. Malechuk

THE EFFECTS OF CAFFEINE ON LEARNING AND LOCOMOTOR BEHAVIOR IN THE HONEY BEE *(Apis mellifera)*

Thesis under the direction of Susan E. Fahrbach, Ph.D., Reynolds Professor of Developmental Neuroscience.

Caffeine affects learning and memory in several different species, including the honey bee, *Apis mellifera*. The purpose of this research was to (1) ask if caffeine could enhance the ability of honey bees to learn a task involving foraging in their natural environment and (2) test for effects of caffeine on motor activity. I tested bees using a new adaptation of a place learning feeding table paradigm in which forager bees treated topically on the abdomen with 100 mM caffeine were trained to distinguish two feeding targets containing either sucrose or water. After training, the sucrose was replaced with water, and the forager was tested in a ten minute extinction test to ask if she had developed a memory of the location of the sucrose during training. The results of this study do not allow me to make any definitive conclusions on the effects of caffeine on learning in this assay due to an unexpected vehicle effect. Young honey bees were treated orally with caffeine and tested in a simple walking assay to ask if caffeine affects locomotor behavior in bees. Low doses of caffeine increased locomotor behavior but high doses did not, as has been demonstrated in mammals.

INTRODUCTION

These studies investigated the effects of acute exposure to caffeine on performance in a place learning assay and the effects of chronic and acute exposure to caffeine on locomotor activity. This introduction provides the context and rationale for my studies by describing the use of the honey bee as a model for the study of learning and by reviewing the known effects of caffeine on insect behavior, including locomotor behavior.

The Honey Bee as a Model for the Study of Learning

The honey bee (*Apis mellifera*) has served as a useful model for studying learning at the behavioral and ecological levels for many decades (von Frisch, 1967). More recently, the honey bee has been used to investigate the neural and molecular mechanisms underlying learning and behavior (Giurfa, 2003, Menzel, 2001). In this context, the techniques of behavioral pharmacology have been applied to the honey bee to study the effects of psychoactive drugs and neurotransmitters on olfactory and visual learning and motor behavior (Giurfa, 2003, Maze *et al.*, 2006, Menzel *et al.*, 1988, Scheiner *et al.*, 2002, Si *et al.*, 2005). For example, the effect of caffeine on visual and olfactory learning in honey bees was assessed using two different laboratory assays (Si *et al.*, 2005). Other research has focused on the effects of ethanol on olfactory appetitive learning and odor discrimination (Mustard *et al.*, 2008) and locomotor behavior in honey bees, also studied in a laboratory setting (Maze *et al.*, 2006).

While the fruit fly, *Drosophila melanogaster,* is the leading insect model for the study of learning and behavior (Bainton *et al.*, 2000, Shaw *et al.*, 2000), it can be argued that the honey bee serves as a significant alternative because so many different levels of

analysis are available. As with the fruit fly, neural correlates of learning in the honey bee can be tested by behavioral tests of learning in the laboratory, while the relatively large honey bee brain is then probed by electrophysiological, anatomical, biochemical, and molecular means (Fahrbach and Robinson, 1995). An example of such multi-level analysis involves the olfactory conditioning assay, the proboscis extension reflex, PER, (Menzel, 2001). In this task, which can be used to study both the rate of acquisition of an association and the duration of the memory for that association, harnessed bees are exposed to an odor (designated the conditioned stimulus or CS), followed by a reward of a drop of sucrose (unconditioned stimulus or US) delivered to their antennae, which in turn elicits extension of the proboscis, a slender, tubular feeding appendage (Bitterman *et al.*, 1983, Giurfa, 2003, Menzel, 2001, Takeda, 1961): when a honey bee has learned the association, the odor alone can elicit proboscis extension (Giurfa, 2003). The PER has been used to show how bees discriminate odors (Bitterman *et al.*, 1983), and the memories formed during PER training have been shown to correspond to the memories that bees form while foraging at flowers (Menzel, 1990). Also, using the PER task, a single modulatory neuron in the honey bee brain, the ventral unpaired neuron no. 1 of the maxillary neuromere, VUM_{mx1}, the activation of which serves as the neural correlate of the presence of the US in olfactory learning, was identified using intracellular stimulation (Hammer, 1993, Hammer, 1997, Menzel, 2001). By identifying the VUM_{mx1} neuron, researchers were able to link a specific neurotransmitter, octopamine, to learning (Menzel, 2001).

Another advantage of using the honey bee as a model to study learning and behavior is that honey bees have a rich behavioral repertoire (Menzel, 2001) that reflects

task specialization and division of labor based on worker age. Thus, learning and behavior can be studied within the bee's own social and ecological context (Fahrbach and Robinson, 1995). As noted previously, learning in honey bees is most typically studied in a laboratory setting (Giurfa, 2003, Maze *et al.*, 2006, Menzel, 2001, Menzel *et al.*, 1988, Mustard *et al.*, 2008, Scheiner *et al.*, 2002, Si *et al.*, 2005), but studying honey bees in the field, in their natural environment allows one to study how bees learn when performing a behavior that is part of their normal repertoire. An example of such naturally occurring learning comes from the study of place and position learning in honey bees during a foraging task designed as a field experiment known as the feeding table learning paradigm, FTLP (Huber *et al.*, 1994).

Feeding Table Learning Paradigm

The FTLP is a task in which honey bees foraging for nectar are trained to fly to a test table where they are presented with a choice of two different feeding options, typically either a sucrose solution of a defined concentration or water. A recruitment station, which is used to obtain nectar foragers for testing, is set up in a different location, away from both the hive and the test table, and consists of a feeder containing a lower concentration solution of sucrose in water than is presented at the test table. In the course of their normal foraging activity, nectar foragers eventually locate the recruitment station and reliably begin to forage there. It has been previously shown that, although most workers collect both pollen and nectar on each foraging trip, some are dedicated to one type of foraging task at a time, either nectar collection or pollen collection (Hunt *et al.*, 1995, Page *et al.*, 2000). During performance of the specific foraging task, both types of foragers show constancy for nectar or pollen collection during multiple consecutive visits

(Winston, 1987). It is therefore predictable that workers identified at a recruitment station will return directly to the hive to unload their nectar load, and then immediately return to the recruitment station. Workers at the recruitment station therefore constitute a pool of nectar foragers from which test bees can be selected. Note that all honey bee workers are female, and are hereafter referred to using the feminine pronoun.

Once a forager is chosen at random from the many foragers at the recruitment station to be a test bee, she is collected and transported to the feeding test table. The test table is initially set up with a single target in the center of the table that contains a single drop of sucrose solution that is more concentrated (and presumably more rewarding) than the sucrose at the recruitment station. The forager is placed on the single sucrose target and is allowed to drink until repletion. Worker bees are exquisitely sensitive to sucrose concentration, and are reliably able to discriminate more and less rewarding sucrose solutions (higher and lower sucrose concentrations) using pre-ingestive gustatory mechanisms including chemosensory receptors on the antennae and proboscis (Pankiw *et al.*, 2001, Scheiner, 2004, Wright *et al.*, 2007).

After she has filled her crop (the first chamber of the alimentary canal, which serves as a nectar storage organ), the test bee then typically flies directly back to her hive. Here she unloads her crop. She then typically returns to the recruitment station, where she can be re-identified by the experimenter and re-transported to the test table. After three to four experimenter-initiated transports on average, a test bee will almost always return to the feeding table on her own and forage from the sucrose on the single target. When the test bee returns for the first time to the test table on her own, she is allowed to drink until

repletion and then return to the hive. At this point, the acquisition phase of the FTLP is set up.

During the acquisition phase, two identical targets are placed at the center of the test table separated by a carefully measured distance determined by the requirements of the individual experiment. One target contains a drop of 50% sucrose solution (this is the reward or S+ target) and the other contains a drop of water (this is the non-reward or S- target). For a nectar forager, water, which is likely detected by dedicated TRP channel hygroreceptors on the antennae and mouthparts (Liu *et al.*, 2007), is considered an aversive stimulus (Huber *et al.*, 1994, Menzel, 1990) especially when she is expecting a sucrose solution (Menzel, 1990).

When the forager next returns to the test table, she encounters the new foraging situation. She must learn to distinguish the S+ target from the S- target to benefit from her foraging bouts at the test table. Because the targets are identical in appearance and presented against a uniform background, she can make this discrimination only on the basis of distal visual cues. After completion of the acquisition phase of the experiment, during which all interactions of the forager with the S+ and S- targets are carefully recorded (the number of acquisition trials is determined by the requirements of the individual experiment), her formation of a memory for the location of the S+ target is assessed using a ten minute extinction test. In the extinction test, both targets contain a drop of water. The number of contacts (landing on the target and/or probing the drop of water with the proboscis) the forager makes with each target is recorded. The extinction test therefore asks if the forager has developed a memory of the location of the S+ target during acquisition. A significant preference for the S+ target over the S- target during the

extinction test is taken as evidence that the location has been learned. This is conceptually similar to the extinction tests used to study learning and memory in other animal models, including mammals (Wade and Tavris, 2005).

The FTLP has been previously used to provide direct evidence of place and position learning in honey bees (Huber *et al.*, 1994). Foragers developed a significant preference for the S+ target when the S+ and S- targets were separated by 40 cm during a 16-trial acquisition phase (Huber *et al.*, 1994). Thus, dependent on the separation distance of the targets, the foragers were able to learn the place of the sucrose target; consequently these results are evidence of place learning in the honey bee. Place learning is not based on recognition or discrimination of the target, but rather recognition of the environment surrounding the target. Most likely, the bees used landmarks in the surrounding environment to help them learn the place of the S+ target. In contrast to those results, when the S+ and S- targets were separated by 10 cm, there was no significant preference for S+ during the extinction test (Huber *et al.*, 1994). Foragers appeared unable to learn the location of the sucrose target when the targets were separated by a shorter distance, implying that, when the targets were separated by a greater distance, the bees responded to the targets based on contextual stimuli, which was not different enough when the targets were closer together (Huber *et al.*, 1994).

After this demonstration of place learning in the honey bee, foragers were tested in an intermediate task in which the targets were still separated by 10 cm but their absolute, not relative, positions on the table were varied during acquisition. With this set up, the foragers did develop a significant preference for S+ during the extinction test (Huber *et al.*, 1994). Therefore, these results suggest a basis for position learning in

honey bees. Position learning occurs because the foragers had to adopt a common orientation (or position) to the targets to learn which contained the sucrose reward. Most likely, the flight pattern of the forager when returning to the test table was such, that her position in relation to the S+ target was the same throughout the acquisition phase in this version of the FTLP.

The FTLP was adapted in the present study to test the effects of caffeine on learning. The simpler version of the task in which the positions of the targets do not vary during testing was used. I selected this task because the degree of difficulty of the FTLP can be altered by either systematically varying the distance between the two targets or by varying the number of training trials during acquisition. My goal was to produce a task of intermediate difficulty so that I could ask if my pharmacological manipulations either improved or impaired performance. My purpose for using the FTLP task in this research project was to gauge performance of foraging honey bees under natural conditions, not to address the issue of whether or not bees are place learners or how they learn the task. The advantages of using this task are that it is a simple, robust, highly relevant reflection of the natural foraging behavior of worker honey bees and that the foragers performing the task are unlikely to be stressed. Also, prior to training, foragers can be easily dosed with pharmacological treatments while filling their crop at the recruitment station. The main disadvantage of this task is that it samples primarily nectar foragers, which differ from pollen foragers on a number of subtle measures, although both have been shown to be capable of learning olfactory associations in laboratory training paradigms (Page *et al.*, 2006).

Caffeine as an Enhancer of Learning

Caffeine is one of the most common psychostimulant drugs used worldwide, and its impact on alertness and general performance in humans is widely acknowledged (Daly and Fredholm, 1998, Fredholm, 1995, Fredholm *et al.*, 1999, Si *et al.*, 2005, Smith, 2002). Caffeine consumption tends to increase alertness and vigilance in individuals, especially in fatiguing situations (Si *et al.*, 2005, Smith *et al.*, 1999). There are numerous reports on the arousal effects of caffeine in vertebrates, but this effect has also been seen in the fruit fly *Drosophila melanogaster:* when *Drosophila* were treated with caffeine, they showed a dose-dependent decrease in resting behaviors (Shaw *et al.*, 2000).

Caffeine has also been shown to enhance encoding of new information in humans (Smith, 2002, Smith *et al.*, 1999). To determine if encoding of new information could be enhanced in invertebrates as well, the effects of caffeine on olfactory and visual learning and memory have been studied using the honey bee as a model (Si *et al.*, 2005). Si *et al.* (2005) used the PER task to investigate the effects of caffeine on olfactory associative learning and long-term olfactory associative memory in young honey bees. Si *et al.* (2005) found that a single topical treatment of bees with caffeine (100 mM caffeine dissolved in dimethylformamide, dMF, administered on the thorax) of newly emerged worker honey bees significantly reduced the minimum age at which an olfactory association could be reliably stored in long-term memory (in this study long-term memory was defined as 24 hours); however, once bees were six days old, an effect of early caffeine exposure on performance in the olfactory association task was no longer detectable in that control bees now performed as well as caffeine-treated bees. This study revealed that early caffeine exposure could accelerate certain aspects of development, but

did not test the effect of having caffeine present at the time learning occurred or when memory was tested.

Si *et al.* (2005) also examined the effects of caffeine on performance in a more complex cognitive task, the delayed-match-to-sample (DMTS) task, which was used to assess working memory and the ability of adult foragers of unknown age to learn concepts such as sameness and difference. Caffeine-treated and vehicle control bees were trained to fly through a 1 m long tunnel, which contained a sample stimulus at the entrance. *"Following the 1-2 s time delay caused by the flight through the tunnel, bees entered a decision chamber, where they encountered two choice stimuli, one of which was identical to the sample stimulus. If the bee picked the matching choice stimulus, it would enter a reward chamber with a feeder containing sugar solution"* (Si *et al.*, 2005). In the DMTS task, Si *et al.* (2005) found that adult foragers of unknown age treated one time topically on the thorax with 100 mM caffeine after they had learned to fly through the maze and find the feeder, but before being trained with any stimuli, matched the choice stimulus to the sample stimulus a significantly higher percentage of the time for a single trial compared to the vehicle control bees. Two sets of DMTS experiments were performed using different sets of foragers in each experiment; the same results were obtained in each trial (Si *et al.*, 2005).

Table I provides a summary of the tests conducted in honey bees treated with psychoactive drugs and the time elapsed between drug administration and training or testing in all published studies of honey bees in which dMF was used as the vehicle for topical delivery of a drug presumed to exert its effects via actions on the central nervous

system. This information is presented so that the results obtained in the present study can be readily compared with the results of previously published studies.

Table I: Summary of tests of honey bees with different conditions of psychoactive drug treatment and the time elapsed between drug administration and training or testing in honey bees. For all tests except for the locomotor test, bees were treated topically and dMF was used as the drug vehicle. For the locomotor test, the drug was administered orally in 50% sucrose solution.

			Caffeine Present		
Type of Test/Drug	Bee Age at Testing	Time From Drug Treatment to Testing	One Day Old	Acute	Chronic
PER[1]/caffeine	2-7 days	2-7 days	X		
DMTS[1]/caffeine	Foragers	1 hour		X	
Sucrose Responsiveness[2]/cocaine	Foragers	1 hour		X	
FTLP[3]/caffeine	Foragers	15 minutes		X	
Locomotor[3]/caffeine	1-7 days	Variable	X	X	X

[1]Si *et al.* (2005); [2]Barron *et al.* (2009); [3]Present study.

Taken together, the results of Si *et al.* (2005) indicate that treatment with caffeine appears to enhance performance of honey bees trained in olfactory and visual association tasks in a laboratory setting. However, honey bees reach a learning plateau in the proboscis extension task once they reach 6 days old, at which point their performance in the task can no longer be enhanced. In the proboscis extension task, caffeine was administered early in development, at emergence, and then acquisition and testing occurred several days later. In this way, the long term developmental effects of caffeine were investigated, but nothing can be said about the acute effects of caffeine on learning. By contrast, in the DMTS task, caffeine was administered one hour before acquisition to bees that had not received developmental exposure to caffeine; therefore caffeine was present both during acquisition and testing such that only the acute effects of caffeine exposure were tested in the DMTS task.

The focus of the research described below is to study the effects of caffeine on learning of a behavior that is normally performed by honey bees, specifically, foraging, in

a natural setting, using the FTLP. My first goal was to look at the acute effects of caffeine when it is present during both acquisition and testing, rather than its long term developmental effects. The acute effects of caffeine are more relevant to the way that humans use caffeine, and also, if effects can be found, may permit the development of caffeine as a tool to study the neuropharmacology of the honey bee. Specifically, caffeine's purported actions via dopaminergic systems (see below) could make it a safer research alternative to other drugs known to regulate dopamine-signaling, such as cocaine.

Mechanism of Caffeine Action and Locomotor Effects

Caffeine has been shown to cause inhibition of phosphodiesterase (PDE) (Fredholm, 1995, Fredholm *et al.*, 1999, Si *et al.*, 2005), and a possible mechanism of memory enhancement comes from the inhibition of PDE (Blokland *et al.*, 2006). The inhibition of PDE by caffeine could possibly account for the results obtained from the PER and DMTS tasks by Si, *et al.*, 2005. The only caveat is that inhibition of PDE by caffeine occurs at extremely high levels that are not typically reached during normal human consumption (Fredholm, 1995, Fredholm *et al.*, 1999, Si *et al.*, 2005). Thus, it is probably not possible that a high enough dose of caffeine penetrates the cuticle via topical application in honey bees; therefore the high levels of caffeine required to cause inhibition of PDE are likely not reached in the bee brain.

At doses comparable to those reached with human dietary intake, it is well-established that caffeine acts as an antagonist of adenosine A_1 and A_{2A} receptors in mammals (Cauli and Morelli, 2005, Daly and Fredholm, 1998, El Yacoubi *et al.*, 2000, Fredholm, 1995, Fredholm *et al.*, 1999, Garrett and Griffiths, 1997, Howell *et al.*, 1997, Snyder *et al.*, 1981, Svenningsson *et al.*, 1997) leading to a cascade of events involving

activation or inhibition of adenylyl cyclase and cAMP, depending on which receptor subtype is antagonized, as well as changes in protein phosphorylation and gene transcription (Fredholm, 1995, Fredholm *et al.*, 1999, Kucharski and Maleszka, 2005). Caffeine is a more powerful antagonist at the A_{2A} receptors than the A_1 receptors in humans (Daly and Fredholm, 1998). The selective adenosine A_{2A} receptor antagonist, SCH 58261, has been shown to increase locomotor activity in rats in the same way as caffeine (Svenningsson *et al.*, 1997). Also, studies using adenosine A_{2A} receptor knock out mice have shown that motor stimulant effects elicited by acute caffeine in rodents are mainly mediated by adenosine A_{2A} receptor blockade (El Yacoubi *et al.*, 2000). There is a consistent decrease in locomotor activity of the A_{2A} receptor knock out mice treated with varying doses of caffeine compared with wild-type controls, which show a biphasic response to caffeine (increased locomotion at low-moderate caffeine doses and decreased locomotion at high caffeine doses) (El Yacoubi *et al.*, 2000), providing further evidence that the mechanism of action of caffeine in mammals occurs through the A_{2A} adenosine receptor.

At the neurotransmitter level, a number of systems might be affected by adenosine receptor antagonism, including dopaminergic and cholinergic transmission (Cauli and Morelli, 2005, Fredholm, 1995, Fredholm *et al.*, 1999, Garrett and Griffiths, 1997, Kucharski and Maleszka, 2005, Schwarzschild *et al.*, 2002). For example, it has been shown that adenosine A_{2A}-receptor stimulation decreases the affinity of the dopamine D_2 receptors for dopamine in striatal membrane preparations (Cauli and Morelli, 2005, Ferré *et al.*, 1991, Fredholm, 1995, Fredholm *et al.*, 1999, Garrett and Griffiths, 1997, Hillion *et al.*, 2002, Kudlacek *et al.*, 2003). Therefore, when caffeine

blocks adenosine A_{2A}-receptors, the effectiveness of endogenous dopamine at D_2 receptors would increase (Daly and Fredholm, 1998, Ferré *et al.*, 1991, Fredholm, 1995, Fredholm *et al.*, 1999). A prevalent model for caffeine actions in the central nervous system proposes that increased dopamine signaling mediates the behavioral effects of caffeine (Fredholm *et al.*, 1999, Garrett and Griffiths, 1997). For a summary of the actions of dopamine, adenosine, and caffeine in the central nervous system see Figure 1.

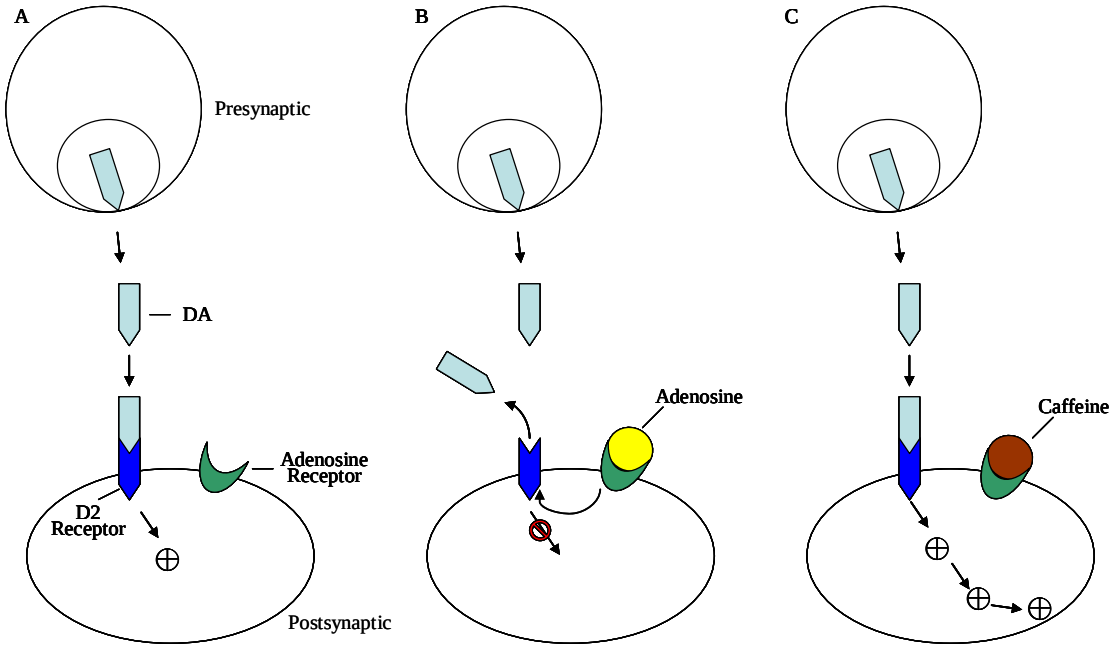

Figure 1: Summary of the actions of dopamine (DA), adenosine, and caffeine in the central nervous system: (A) DA binds the D2 dopamine receptor increasing DA signaling; (B) adenosine binding in a neuron decreases the affinity of the D2 dopamine receptor for DA decreasing DA signaling; (C) caffeine antagonizes adenosine receptors which increases the affinity of D2 dopamine receptors for DA increasing DA signaling. Figure modified from Stahl (2008).

Studies conducted in humans show that caffeine produces subjective and behavioral effects that are similar to those of typical psychomotor stimulant drugs (e.g., amphetamine and cocaine) known to be mediated by dopamine receptors (Garrett and Griffiths, 1997). It is thought that caffeine's dopamine agonist-like effects are due to an indirect action on dopamine receptors through the antagonistic effects of caffeine at adenosine receptors (Garrett and Griffiths, 1997), in part because the adenosine A_{2A}-

receptors are found primarily in dopamine-rich regions of the brain (Fredholm, 1995, Fredholm *et al.,* 1999). The adenosine A_{2A} receptors and the dopamine D_2 receptors are colocalized and form heteromers in neuronal cells (Hillion *et al.*, 2002).

Several studies have examined the behavioral effects of caffeine and dopamine antagonism to investigate further the role that dopamine plays in mediating the effects of caffeine. Caffeine stimulates motor activity in rodents and in non-human primates in a dose-dependent non-linear fashion (Cauli and Morelli, 2005, Daly and Fredholm, 1998, Fredholm *et al.*, 1999, Howell *et al.*, 1997, Svenningsson *et al.*, 1997). Low to intermediate doses of caffeine produce increases in spontaneous locomotor activity; whereas high doses produce depressant effects (Daly and Fredholm, 1998, Garrett and Griffiths, 1997, Nehlig *et al.*, 1992). The locomotor effects stimulated by caffeine can be blocked by selective D_1 and D_2 dopamine receptor antagonists (Fredholm *et al.*, 1999, Garrett and Griffiths, 1997, Garrett and Holtmann, 1994, Kuribara and Uchihashi, 1994, Mukhopadhyay and Poddar, 1995). Locomotor activity has been measured in mammals as spontaneous locomotion, open-field locomotor activity, rotation, or vertical rearing frequency (Daly and Fredholm, 1998, Fredholm *et al.*, 1999, Mukhopadhyay and Poddar, 1995).

A study performed by Mukhopadhyay and Poddar (1995) examined locomotor activity as measured by vertical rearing frequency of caffeine-treated rats and caffeine-treated rats that were also administered a dopamine antagonist. They found that rats treated with a single exposure to low doses of caffeine only (10-40 mg/kg of body weight, oral administration), 30 minutes prior to testing, had a significant increase in their locomotor activity as indexed by vertical rearing frequency. Mukhopadhyay and Poddar

(1995) also found that giving non-caffeine treated rats a dopaminergic agonist mixture (L-Dopa + carbidopa) induced an increase in locomotor activity that was dose-dependent; while the dopaminergic antagonist, haloperidol, significantly reduced the locomotor activity of caffeine treated rats in a dose-dependent manner.

In light of this literature, another goal of my research was to investigate the chronic, developmental, and acute effects of caffeine on locomotor activity. My objective for assessing locomotor effects of caffeine in honey bees was to determine if a biphasic response to caffeine is present in invertebrates, as it is in mammals. My studies are the first to investigate the effects of caffeine on locomotor activity in the honey bee. This information will be helpful in future studies to determine the mechanism of action of caffeine in invertebrates. It should be noted, however, that, unlike the FTLP studies described above, for practical reasons these studies used young bees comparable in age to those used by Si *et al.* (2005). Locomotor activity was assessed using a walking assay as opposed to a flying assay because in nature, young bees perform in-hive tasks which require walking, unlike older bees that perform out-of-hive tasks, such as foraging, which require flying; thus, because young bees were used for the locomotor assay, walking was a more appropriate measure of locomotor activity than flying.

Dopamine in the Honey Bee Brain

The long-term goal of this research program is to understand the function of the mushroom bodies, the most prominent protocerebral structure in the insect brain. The biogenic amine, dopamine, has been identified in the honey bee brain using high-performance liquid chromatography studies (Brandes *et al.*, 1990, Fuchs *et al.*, 1989, Harris and Woodring, 1992, Harris and Woodring, 1995, Mercer *et al.*, 1983, Schäfer and

Rehder, 1989, Schürmann *et al.*, 1989, Taylor *et al.*, 1992, Wagener-Hulme *et al.*, 1999). It has been well established that dopamine D_1- and D_2-like receptors, known as AmDOP1 and AmDOP2, are expressed in the adult honey bee brain, specifically, in the mushroom bodies as revealed by *in situ* hybridization of mRNA from *Amdop1* and *Amdop2* (Blenau *et al.*, 1998, Humphries *et al.*, 2003, Kokay and Mercer, 1997, Kurshan *et al.*, 2003). Because the mushroom bodies integrate olfactory, visual, and mechanosensory information and are essential for certain forms of association learning and spatial navigation, this localization suggests that, in addition to octopamine, dopamine signaling modulates learning and memory (Fahrbach, 2006). In insects, it appears that dopamine is responsible for memory recall (Unoki *et al.*, 2005). In a visual learning assay using crickets (Unoki *et al.*, 2006) and olfactory learning assays using crickets (Unoki *et al.*, 2005) and honey bees (Vergoz *et al.*, 2007), researchers found that aversive learning is modulated by dopamine and that blockade of dopamine receptors suppresses visual and olfactory aversive learning (Unoki *et al.*, 2005, Unoki *et al.*, 2006, Vergoz *et al.*, 2007).

To ask if the dopaminergic system is a target for caffeine in honey bees, Kucharski and Maleszka (2005) used high-throughput cDNA microarray and quantitative real-time PCR analyses to look for changes in gene expression in the brains of honey bees that had been treated with caffeine. They found 25 genes with significantly altered RNA expression levels in the honey bee brain following caffeine treatment (100 mM, administered topically to the thorax). Eighteen of the 25 expressed sequence tags encoded *"highly conserved polypeptides with well-characterized functions in other species providing a glimpse of the pathways that are affected by caffeine treatment in the honey bee brain"*, some of which are involved in neurotransmission, including the dopamine

D_2-like receptor (Kucharski and Maleszka, 2005). Their findings provide the first evidence that caffeine may work through the dopaminergic system in honey bees, suggesting that blocking dopamine could reverse the effects of caffeine on learning and locomotion.

Hypotheses and Summary

Hypothesis 1: Treatment with caffeine immediately prior to training (independent variable) will increase, relative to vehicle-treated controls, the percentage of nectar foragers (dependent variable) able to form a memory of the location of a rewarding sucrose solution.

Hypothesis 2: Treatment with caffeine will alter the walking behavior of young bees in a dose-dependent manner.

> Hypothesis 2a: Chronic, developmental, and acute treatment with **low** doses of caffeine (independent variable) will increase, relative to untreated controls, the number of times walking bees cross the center line of a dish during a short observation period (dependent variable).
>
> Hypothesis 2b: Chronic, developmental, and acute treatment with **high** doses of caffeine (independent variable) will decrease, relative to untreated controls, the number of times walking bees cross the center line of a dish during a short observation period (dependent variable).

MATERIALS AND METHODS

Overview

For my research, I initially replicated the studies of Huber *et al.* (1994) to ensure that my implementation of the FTLP is a valid assay for studying learning in free-flying honey bees. I then varied the difficulty of the FTLP to establish test parameters that would prevent honey bees from developing a preference for the S+ target. This permitted me to design an experiment to test Hypothesis 1. Based on the extensive literature on the cognition-enhancing effects of caffeine in mammals and the studies in honey bees of Si *et al.* (2005), I predicted that caffeine would improve the performance of bees in the FTLP. My study is the first to examine the effects of caffeine on learning in free-flying foragers performing a task that is native to that species in its natural environment. The FTLP, a field assay that investigates the role of learning required in foraging situations, has been used to show that honey bee foragers are able to learn the place and position of foraging targets, even if other cues such as color and odor are not present. Thus, the feeding table learning paradigm can serve as an important and useful assay for studying the effects of psychostimulants on learning because foraging is a normal behavior performed by honey bees in their natural environment. Also, as I have noted, the degree of difficulty of this learning paradigm can be varied so that I am able to detect both improvement and impairment in performance.

Caffeine produces a biphasic locomotor effect in mammals. In the present study, I developed a simple line crossing assay to assess locomotor activity of bees that were treated chronically, developmentally, and acutely via oral administration of caffeine to determine if there is a similar caffeine effect on locomotor behavior in invertebrates. I

predicted that caffeine would have a similar effect on locomotor behavior in bees as has been shown in mammals: low doses of caffeine would increase activity and high doses of caffeine would depress activity. Several studies have investigated the role of other psychoactive agents such as ethanol on locomotor behavior in bees (Maze *et al.*, 2006), however, this study is the first to explore a possible role of caffeine on locomotor behavior in bees.

There are several options for drug administration in insects including oral administration, drug injection, and topical application of the drug to either the thorax or the abdomen. It has been shown, using the drug octopamine, that topical administration, both on the thorax and the abdomen in honey bees, is as effective as injection or oral administration of the drug (Barron *et al.*, 2007). This finding suggests that the question(s) being asked and the test arena should dictate which drug treatment method is best for a particular experiment. For the FTLP, it was important to ensure that bees were being exposed to the same amount of caffeine, so the oral route of administration of caffeine would not have allowed me to control the amount of caffeine each individual bee was ingesting; and because these tests were performed in a field setting using free-flying bees, drug injection was not possible because of the precision required for this method of drug administration. I, therefore, chose to use the abdominal topical application method of drug administration for the FTLP.

Several studies have used a topical method of delivery for caffeine (Kucharski and Maleszka, 2005, Si *et al.*, 2005), but the thorax was the point of caffeine application. To ensure that caffeine was reaching the brain via abdominal topical administration, I used liquid scintillation counting to quantify the percent of caffeine recovery from the

brains of bees that had been treated topically on the thorax or the abdomen with tritiated-caffeine (^{3}H-caffeine). To develop a defined treatment procedure, I chose to compare the topical application of caffeine on the thorax and the abdomen to ask which area is superior for drug administration. I predicted that more caffeine would reach the brain via the abdomen because the abdominal cuticle is thinner than the thoracic cuticle, thus more caffeine will penetrate the abdomen than the thorax.

Collection of Subjects

Adult foragers of unknown age were obtained from a typical colony containing a commercially multi-mated queen (obtained from Brushy Mountain Bee Farm, Moravian Falls, North Carolina) and a population of ~10,000 workers of a mixture of European subspecies of *Apis mellifera*. The hive was kept in the same location throughout testing in a residential neighborhood in a partially wooded area (Forsyth County, North Carolina). Foragers were collected as they fed from an artificial feeder (recruitment station) containing a 20% w/v sucrose solution that was located approximately 18 meters from the hive entrance in a wooded and landscaped area and was not immediately visible from the hive entrance.

Feeding Table Learning Paradigm

The FTLP was used to assess learning in nectar foraging honey bees that were trained to distinguish between two feeding options. Upon completion of the FTLP, administration of an extinction test permitted each forager tested to be classified as either a learner or a non-learner.

FTLP Series I: Replication. I first replicated the studies of Huber *et al.* (1994), using the same testing parameters developed by these investigators to show that the FTLP

is an appropriate assay for studying learning in free-flying honey bees; thus, I can be certain that any improvement or impairment resulting from caffeine is real and not because the assay does not work for investigating learning. Following the methods of Huber *et al.* (1994), the feeding table was set up with the S+ and S- targets separated by 40 cm and test bees were allowed 16 training trials during the acquisition phase. For this initial study, however, several proximal landmarks were placed on the test table to help bees find their way back. The acquisition phase of the FTLP began after a test bee returned to the table on her own to drink from the single sucrose target. When she returned to the hive, the table was set up with the two targets, S+ and S-, separated by a specified distance determined by the needs of the individual experiment. During acquisition, the test bee was allowed to fly back to the table and drink from either target. If she chose S- on the first attempt for any trial during acquisition, she was allowed to correct herself and drink from S+. She drank for as long as she wanted, and then was allowed to fly back to the hive. A trial was completed when the test bee left the test table and was observed entering the hive. The 16 trial acquisition phase was immediately followed by a 10 minute extinction test. Twelve bees were tested in this version of the FTLP. See Figure 2 for an illustration of the test table used in this version of the FTLP.

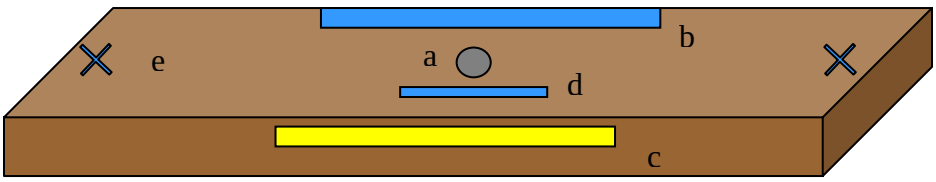

Figure 2: Diagram of the test table with the proximal landmarks used during the first sets of FTLP tests: (a) single target spray painted gray placed at the center of the table, (b) 61.5 cm long piece of blue tape (Fisherbrand Labeling Tape, 1", Fisher Scientific, USA) placed at center on the back of the table, (c) 61.5 cm long piece of yellow tape placed at center on the front edge of the table, (d) 10 cm long blue plastic rod taped 20 cm above the front edge of the table (the target was 15 cm above the blue rod), and (e) blue pieces of tape in the shape of an "X" on the sides of the table.

In a second series of trials, I separated the targets by 10 cm. Again, 16 training trials were given during acquisition, and the same proximal table landmarks were set up and 12 bees were tested using this version of the FTLP.

FTLP Series II: Development of a Test Procedure to Prevent Preference for S+. Evidence of learning was detected during the replication phase of my FTLP project; therefore, to make the test more difficult and establish testing parameters that did not result in a preference for the S+ target, I took away the proximal table landmarks and repeated the FTLP, but this time I used 10 cm separation distance with only 10 training trials during acquisition. Again, evidence of learning was detected (significant preference for S+), so I continued modifying the procedure to produce a task that would not produce a significant preference for the S+ target. The testing parameters that did not result in a preference for S+ are the following: the S+ and S- targets being separated by 10 cm, 5 training trials during acquisition, and no proximal table landmarks. This procedure was followed in trials using caffeine treatment to avoid problems of interpretation of drug effects that result from a performance ceiling, which in behavioral studies refers to a lack of an effect in performance because the test subject is already performing at its maximum level (Reber and Reber, 2001).

FTLP Series III: Test of Hypothesis 1. A recruitment station was set up to attract nectar foragers to be used as test bees in the FTLP. The recruitment station was a round stool 113 cm diameter spray painted blue approximately 23 cm off the ground that held a feeder of sucrose. The feeder was a pint sized Mason jar marked with yellow tape (Fisherbrand Labeling Tape, 1", Fisher Scientific, USA) on two opposite sides (see

Figure 3). The colors, blue and yellow, were chosen because bees are able to learn colors from yellow to ultraviolet (Gould, 1986, von Frisch, 1967).

Figure 3: A photograph of the recruitment station located in a wooded area, Forsyth County, North Carolina. The feeder consisted of a glass Mason jar with yellow tape in the shape of an "X" on opposite sides, inverted on a Plexiglas dish with 8 mm slots cut into it. Peppermint extract (McCormick and Co., Inc., Hunt Valley, MD) was put on the round filter paper to alert the foragers that the feeder had been set out. Peppermint extract was used simply because bees learn odors paired with a reward quickly (Gould, 1986, von Frisch, 1967), and this was the most efficient method for attracting nectar foragers to the artificial feeder.

Once the feeder was filled with sucrose, it was turned upside down on a Plexiglas dish and placed on the blue stool. The Plexiglas dish had 8 mm slots cut into it so that the foragers could get the sucrose from the feeder. The concentration of sucrose in the feeder was varied as the summer field season progressed depending on the number of foragers at the recruitment station and the availability of natural nectar. Typically, the sucrose concentration in the feeder was high (20% w/v) at the beginning of the summer, then, as the natural nectar flow started to decrease, the concentration of sucrose at the feeder was decreased to prevent overcrowding. Overcrowding occurred because once a forager

found the recruitment station, she was loyal to it and also likely recruited other foragers to the feeder. The lowest concentration of sucrose in the feeder was 6.25% w/v during the summer 2007 field season. Similar seasonal trends have been reported by other bee researchers (Mujagic and Erber, 2009).

A forager of unknown age was chosen at random from the many foragers at the recruitment station to be a test bee. She was marked with a dot of paint (Testors, Rockford, IL) on her thorax (see Figure 4) to identify her throughout the rest of the experiment and was gently collected into a small 20 ml glass scintillation vial (Wheaton Science Glass Liquid Scintillation Vials, Fisher Scientific, USA) while she drank at the feeder.

Figure 4: A photograph of the foragers feeding at the recruitment station, marked with different colors of paint on the thorax.

The vial was immediately capped and the test bee was then immediately transported in the vial to the feeding test table, which was set up approximately 3.5

meters in front of the hive. The test table (74 cm × 183 cm × 76 cm) used during the 2007 field season had a dark brown, wood grain laminate top, while the table used during the 2008 field season had a gray resin top with the same dimensions and was covered during tests with brown packing paper so the color matched that from the previous year, but had no discernible features.

At the start of training, the feeding table was set up with a single target in the center of the table. The target was a 5.5 cm diameter plastic Petri dish, spray painted gray on the inside and placed on the table such that the bottom of the dish was facing upward. The target was painted gray following the methods of Huber *et al.* (1994). A single drop of 50% w/v sucrose solution (~ 200 µl) was pipetted onto the center of the target. The scintillation vial containing the test bee was placed on the single sucrose target, over the drop of sucrose, and the forager was allowed to drink (see Figure 5).

Figure 5: A photograph showing the test table used during the 2007 summer field season set up for FTLP training with a single target in the center of the table containing a large drop of 50% w/v sucrose. The glass scintillation vial with a test bee inside represents how foragers were collected from the recruitment station and transported to the feeding table. The test bee almost always made her way to the bottom of the vial to find the drop of sucrose and begin drinking.

A forager was allowed a maximum of 2 minutes to find the drop of sucrose and begin drinking. Once the forager began to drink, the vial was carefully removed and she was allowed to feed until repletion. It was rare that a forager did not discover the drop and drink.

After the forager had filled her crop and stopped drinking, she typically engaged in an orientation flight (flight consisting of repeated circles gradually increasing in diameter around a point of interest, such as the hive entrance or a food source (Winston, 1987) around the test table, and then flew the short distance directly back to the hive to empty her crop. Typically, a test bee was transported three or four times to the test table before she returned on her own; however, if more than six transports were required, that bee was no longer used in the FTLP. When the test bee returned to the test table of her

own volition and landed on the single sucrose target and drank, then this forager had officially started the FTLP.

Once the returned forager left the test table to return to the hive after her first self-initiated visit, the table was immediately set up for the acquisition phase of the experiment. At this point, two identical targets were placed at the center of the table separated by 10 cm measuring from the inside edge of one target to the inside edge of the other target (see Figure 6). One target contained a 100 µl drop of 50% w/v sucrose solution (this was the reward or S+ target) and the other contained a 100 µl drop of water (this was the non-reward or S- target).

Figure 6: A photograph showing the feeding table set up for the acquisition phase of the experiment with two identical targets separated by 10 cm in the center of the table. One target contained a 100 µl drop of 50% w/v sucrose (S+) while the other target contained a 100 µl drop of water (S-). The white structure in the background is the hive. The feeding table was 3.5 m in front of the hive.

When the forager returned to the test table, she encountered the new acquisition phase foraging situation. Because the targets were identical in appearance and the table was featureless, we infer that she could learn the location of the sucrose only on the basis

of distal visual cues. The forager was allowed 5 training trials during the acquisition phase of the experiment. She was allowed to drink to repletion and then she would fly back to the hive to empty her crop. When the forager left after each trial, the drop of sucrose was replenished to ensure that the S+ and S- targets had the same volume of liquid and maintained the same appearance. The targets were not moved, but the remaining sucrose on S+ was pipetted off and then refilled with a fresh 100 µl drop of sucrose. Some insects, like the honey bee and bumble bee, deposit repellent pheromones from their mandibular or tarsal glands on flowers that have been previously visited and depleted of nectar to improve foraging efficiency; yet, there is also some evidence to suggest that honey bees may deposit attractant pheromones on flowers that indicate rewarding food sources to other foragers (Stout and Goulson, 2001). Because I did not change out the targets after each trial during acquisition, I cannot be certain that test bees were not marking the S+ target with an attractant pheromone to help them to know which target contained the sucrose; however, I am confident that any attractant pheromones that may have been deposited on the S+ target did not affect the results of these studies because test bees visited both the S+ and S- targets during acquisition, with S- being visited even during later trials, and the S+ target was wiped clean before the extinction test which would have presumably removed any chemical substance left by the test bee on the target.

After completion of the acquisition phase of the experiment with the fifth training trial, learning was assessed using a 10 minute extinction test. For the extinction test, the targets remained in the same position on the feeding table and the sucrose and water were pipetted from the targets. The sucrose target was wiped clean to remove any traces of

sucrose solution, and both targets were then replenished with a 100 µl drop of water. When the forager returned to the feeding table, the 10 minute extinction test began during which the total number of landing and probing contacts the forager made with each target was recorded, binned in 30-second intervals. Landing consisted of the forager landing directly on the target, not within the vicinity of the target. Probing refers to extension of the proboscis and touching (tasting) the drop. The extinction test was performed to ask if the forager had developed a memory of the location of the S+ target during acquisition. During a typical extinction test, the test bee would return to the table and after a brief orientation flight, she would land on one of the targets and probe the drop of water. Sometimes the test bee would continue probing the same drop, while others immediately flew to the other target and probed that drop. Not all test bees continued returning to the table for the entire 10 minute extinction test period. A preference for the S+ target (number of interactions with S+ significantly > number of interactions with S-) during the extinction test was taken as evidence that the discrimination had been learned. The significance of any differences in the number of landings and probes was assessed using the Wilcoxon Signed-Rank Test (see below). Landings and probes were summed rather than looked at separately because I wanted to have a global measure of contacts. Furthermore, if a test bee landed on a target, she would usually also probe the drop of water (personal observation). These observations were binned in 30-second intervals based on the methods of Huber *et al.* (1994) and were then used to create mean cumulative response curves for each group tested to characterize differences between versions of the FTLP (see below). If the discrimination had been learned (significant preference for S+) those foragers were classified as learners; however, if the

discrimination had not been learned (no significant preference for S+) the foragers were classified as non-learners.

To answer the question does caffeine enhance the ability of a forager to learn the place of a foraging target in the same foraging situation that did not result in learning, a 2 µl drop of 100 mM caffeine (Sigma, St. Louis, MO) in dMF (Sigma, St. Louis, MO) was applied to the abdomen of a forager while she drank at the recruitment station using a 2 µl glass microcap (Drummond Scientific, Broomall, PA), as shown in Figure 7.

Figure 7: A photograph showing the topical drug application method. A 2 µl glass microcap was used to administer caffeine, dMF, or water (only during the 2008 summer field season) to the abdomen of a test bee while she drank from the feeder.

The forager of unknown age was chosen at random from the recruitment station and marked with a dot of paint on the thorax to identify her throughout the experiment. This method of drug treatment was based on the published procedure of Barron *et al.* (2007). dMF is routinely used in topical treatments of the hydrophobic insect cuticle because it can easily penetrate the cuticle (Barron *et al.*, 2007). Topical application

allows rapid delivery of a drug while bees are stationary at a sucrose feeder; however, this method of drug delivery is not as precise as injection, but topical application is less stressful for the animal and poses less risk of infection than injection (Barron *et al.*, 2007, Kucharski and Maleszka, 2003).

Any forager that was used for the caffeine portion of the experiment had not been previously tested in any other part of the FTLP or treated with any other chemicals (this was possible because a record was kept of the colors of paint used on each test day). Foragers were paint marked before being treated with caffeine and paint marking typically occurred in the morning. The caffeine-treated foragers were trained in the FTLP as described above, with training initiated 15 minutes post-treatment. Between being treated with caffeine and the beginning of training in the FTLP, test bees would typically forage at the recruitment station or return to the hive to unload their crop.

As a vehicle control for the caffeine treatment, another group of foragers was treated with 2 µl of dMF and trained in the FTLP. During the 2007 field season, I was cognizant of the treatment each test bee received; however, in the 2008 field season, I was blind to the treatment that each test bee received and whenever possible, a topical water control, vehicle control (dMF), and caffeine-treated bee were tested on the same day in a randomized fashion. *In lieu* of a no-treatment control, a topical water control was used in the 2008 field season to control for the physical touch (light tap of the microcap on the abdomen) received by a test bee during the drug treatment phase. The sample size for the caffeine and dMF vehicle control groups was 12 with two replicates each. The no-treatment control group was tested only during the 2007 field season with a sample size

of 12 bees. The water control group was tested only during the 2008 field season with a sample size of 12 bees.

Locomotor Assay

A locomotor assay was designed to ask if caffeine enhanced the locomotor activity of honey bees. Locomotor activity was assessed using a simple line crossing assay in which the number of times walking bees crossed a center line was counted and the total line crosses for all bees were used as a measure of locomotor activity. Newly-emerged honey bees were collected from a brood frame that was kept in an incubator at 33-34ºC and 85-95% RH on day one of the locomotor assay. Five to eight newly emerged bees and a small piece of comb (~25 mm^2) from their natal colony were placed in a Petri dish for observation (90 mm diameter, 10 mm deep, with an 8 mm hole drilled in the lid for feeding tube access). An inverted 1.5 ml polypropylene microcentrifuge tube with three holes punched in the tip with an 18 gauge needle was placed in the hole in the top of the Petri dish filled with the appropriate solution for feeding. For the overall design of the locomotor assay, see Table II.

Table II: Locomotor assay experimental design. All oral treatments were started on day one (set up) when all bees were < 24 hours old. Bees were allowed to drink their respective solution *ad libitum*.

Treatment Group	Oral Treatment	# of Dishes	Bees/dish	Days Received Treatment
Sucrose	50% Sucrose	15	6-8	1-6
Chronic 2.5	2.5 mg/ml Caffeine	10	8	1-6
Chronic 5	5.0 mg/ml Caffeine	12	5-8	1-4*
Developmental 2.5	2.5 mg/ml Caffeine	10	8	1
Developmental 5	5.0 mg/ml Caffeine	10	8	1

***The bees in this group did not survive after day five.**

All oral caffeine treatments were prepared in 50% w/v sucrose and all feeding tubes were changed daily through day 6 of the experiment. The bees in the 5.0 mg chronic caffeine group did not survive past day 5. For both developmental treatments,

bees received caffeine only on day 1; on days 2 through 6, these bees received 50% w/v sucrose. Day 1 was defined as the day of emergence and the set up day, and locomotor testing occurred on days 1 through 3, and 6. On day 6 of the experiment, the bees in the sucrose group were given 5.0 mg/ml caffeine in 50% sucrose *in lieu* of sucrose only AFTER locomotor activity had been assessed on day 6. Day 7 was referred to as the "switch" day on which locomotor activity was assessed 24 hours after the bees in the sucrose group received their caffeine treatment. This switch day was used to assess any acute effect from the caffeine treatment.

The locomotor assay was performed in a room kept at 28.2-29.8ºC and 31-37% RH with the lights on. The dishes were removed one at a time from the incubator and placed on a table over a circle drawn on a piece of white bench paper with a line drawn down the middle. After a 3 minute period of acclimation to placement on the table, the locomotor activity of the bees was measured by counting the total number of line crosses for all bees in the dish during 3 minutes. Before the locomotor assays began, mortality was assessed for each dish. If a dish did not contain at least two living bees, then the test was not performed and that dish was no longer used; however, the previous data generated by that dish was still used in the analysis. To the degree possible, all locomotor assays and feedings occurred at the same time of day for each group tested, which ranged from approximately 10:00 AM until 2:00 PM. No testing occurred on days 4 and 5 of the test period, but feeding tubes were changed on both of these days. I chose to use walking to assess locomotor behavior because young bees typically perform in-hive tasks which require walking, not flying, unlike older bees that perform foraging tasks. By using young bees, I was able to have a more parallel measure of the effects of caffeine as described by

Si *et al.* (2005), who also used young bees to investigate the effects of caffeine. Furthermore, walking is one of the forms of locomotion that is commonly used in vertebrate studies to assess locomotor activity (Daly and Fredholm, 1998, Fredholm *et al.*, 1999).

Tritiated Caffeine Recovery

Tritiated caffeine was used to ensure that the caffeine was getting to the brain using the topical abdominal treatment method and to determine the most effective site of application (abdomen versus thorax). The treatments were performed following the methods of Barron *et al*. (2007). Twenty pollen or nectar foragers of unknown age were collected from the hive entrance in 20 ml scintillation vials and chilled on ice. A fresh solution of 100 mM caffeine in dMF was prepared and used to make a 100 mM ^{3}H-caffeine solution. The ^{3}H-caffeine was obtained from American Radiolabeled Chemicals, Inc., St. Louis, MO (specific activity 50 Ci/mmol) dissolved in ethanol under argon at a concentration of 1 mCi/ml.

Foragers were separated into 5 groups with 4 bees per group: control (no treatment), 15 minute topical abdomen, 60 minute topical abdomen, 15 minute topical thorax, and 60 minute topical thorax. The 15 and 60 minute time points were chosen because during the FTLP, training began 15 minutes post-drug treatment, and on average, each test bee had completed the FTLP in 60 minutes. Depending on the group, each test bee was treated topically on either the abdomen or the thorax with 2 µl of 100 mM ^{3}H-caffeine using a 2 µl glass microcap. Each individual bee in the 15 minute thorax and abdomen groups was placed in a 2 ml glass scintillation vial and allowed to regain mobility. The 4 bees in the 60 minute topical thorax group were marked with different

colors of paint on the abdomen and placed in a plastic cage supplied with sucrose for feeding and allowed to regain mobility. The 4 bees in the 60 minute topical abdomen group were marked with different colors of paint on the thorax and placed in a different plastic cage supplied with sucrose for feeding and allowed to regain mobility.

Bees in both 15 minute groups were immobilized 15 minutes post-treatment by brief chilling. Each brain was dissected and placed in its own plastic scintillation vial containing 2.5 ml ScintiVerse ™ BD Scintillation Cocktail (Fisher, Pittsburgh, PA). Bees in both 60 minute groups were immobilized 60 minutes post-treatment by chilling; each brain was dissected and placed in its own plastic scintillation vial with 2.5 ml of scintillation cocktail.

Tritium was quantified using a Beckman LS 6500 Liquid Scintillation Counter. To consider background radioactivity, I used a blank consisting of 2.5 ml of scintillation cocktail only. The percent of caffeine recovered from the brain tissue was estimated for a single bee using the counts recovered from an equivalent amount of ^{3}H-caffeine added directly to scintillation cocktail. The percent recovery from the 4 bees in each group was averaged to get a final percent recovery of caffeine in the brain for each treatment group. The ^{3}H-caffeine recovery was replicated twice; however, the second replicate did not include the control brains for analysis.

Statistical Analyses

Feeding Table Learning Paradigm. The discrimination between S+ and S- was analyzed using the Wilcoxon Signed-Rank Test to determine if there was a preference for S+ for each group that was tested. If a significant preference for S+ was found, it was concluded that learning had occurred.

Locomotor Assay. When the assumptions of normality and homogeneity were met, a one-way ANOVA using a *post hoc* Newman-Keuls multiple comparisons test was performed to analyze the locomotor assays. When the ANOVA assumptions were not met, data were analyzed with the Kruskal-Wallis Test using a *post hoc* Dunn's multiple comparisons test. A t-test was used for the analysis of the acute effects of caffeine on locomotor activity.

Tritiated Caffeine Recovery. To determine if caffeine was getting to the brain using the topical abdominal method of delivery, the percent recovery of caffeine in the brain was compared using a t-test between groups of honey bees that had been treated topically on the thorax and topically on the abdomen with ^{3}H-caffeine, 15 and 60 minutes post-treatment.

For all analyses, an alpha level of less that 0.05 was considered significantly different. All data are represented as mean ± S.E.M. Statistical analyses were carried out using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA).

RESULTS

Feeding Table Learning Paradigm

FTLP Series I: Replication. The first tests completed with the FTLP were for the purpose of repeating the tests using the same parameters as Huber *et al.* (1994). One reason to do this was that it was impossible to replicate the Hawaiian location of the published studies. I found that, when the S+ and S- targets were separated by 40 or 10 cm, 16 training trials were given during acquisition, and the test table was set up with proximal landmarks, the bees showed a significant preference for the S+ target during the

extinction test. I therefore concluded that learning did occur under these acquisition phase conditions (Wilcoxon Signed-Rank Test: 40 cm separation distance: W = 78.00, p = 0.0025, Fig. 8A; 10 cm separation distance: W = 58.00, p = 0.03, Fig. 8B).

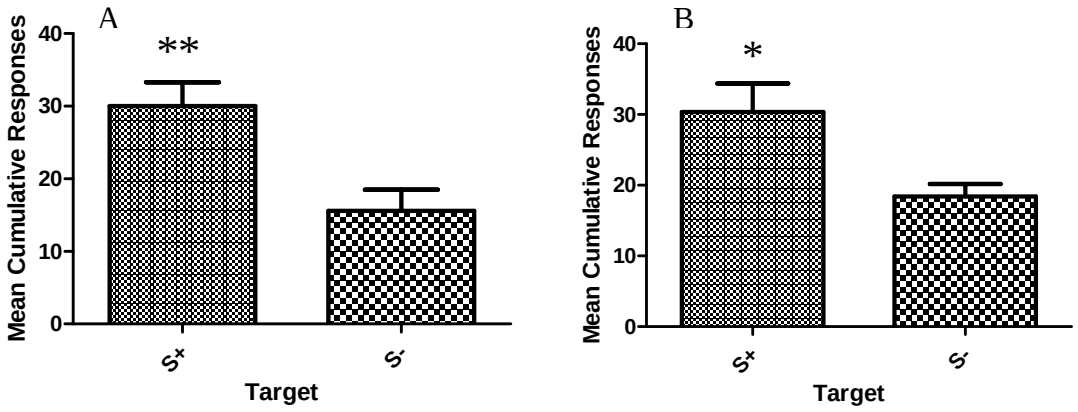

Figure 8: Mean cumulative responses (summed landing and probing) of control bees (no drug or vehicle treatment) at S+ and S- during the extinction test of the FTLP. Targets were separated by 40 cm (A) or 10 cm (B). Bees were allowed 16 training trials and proximal table landmarks were present. There was significant preference for S+ for both versions of the FTLP; learning did occur. Error bars represent SEM, n = 12. ** p < 0.01, * p < 0.05.

The proportion of bees in the 40 cm, 16 training trial version of the FTLP that had a preference (responses to S+ minus responses to S- > 0) for S+ was 12 of 12. The proportion of bees in the 10 cm, 16 training trial version of the FTLP that had a preference for S+ was 8 of 12. The mean cumulative response curves generated from the extinction test for these two versions of the FTLP are shown in Figure 9.

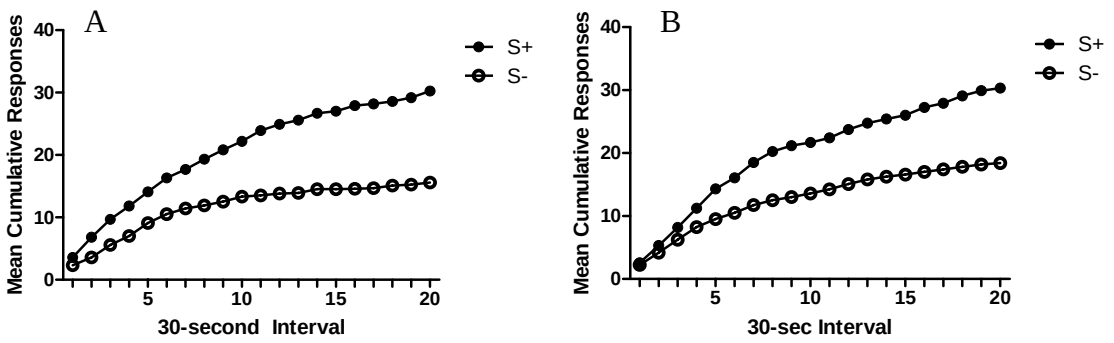

Figure 9: Mean cumulative response curves in the 10 minute extinction test of the 40 cm, 16 trial FTLP experiment (A) and the 10 cm, 16 trial FTLP experiment (B). The proportion of bees that preferred S+ to S- was 12 of 12 (A) and 8 of 12 (B); these preferences were significant. Error bars are omitted from the cumulative response curves for clarity and because the statistical analysis is based only on the total cumulative responses at the end of the 10 minute observation period.

In an attempt to make the FTLP more difficult, I separated the targets by 10 cm but reduced the number of trials during the acquisition phase to 10 and took away all proximal table landmarks. I still found that bees had a significant preference for the S+ target (Wilcoxon Signed-Rank Test: W = 72.00, $p = 0.005$, Fig. 10).

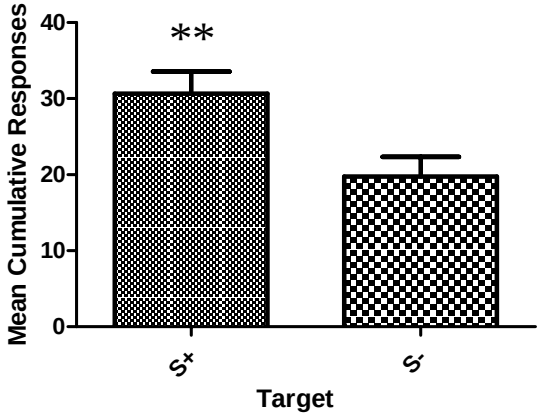

Figure 10: Mean cumulative responses (summed landing and probing) of control bees (no drug or vehicle treatment) at S+ and S- during the extinction test of the FTLP. Targets were separated by 10 cm and bees were allowed 10 training trials with no proximal table landmarks present. There was significant preference for S+; learning did occur. Error bars represent SEM, n = 12. ** $p < 0.01$.

The proportion of bees in the 10 cm, 10 training trial version of the FTLP that had preference for S+ was 10 of 12. The mean cumulative response curve generated from the extinction test for this version of the FTLP is shown in Figure 11.

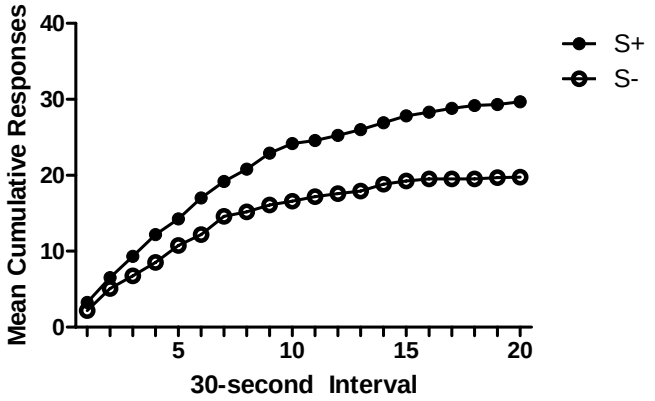

Figure 11: Mean cumulative response curve in the 10 minute extinction test of the 10 cm, 10 training trial FTLP experiment. The proportion of bees that preferred S+ to S- was 10 of 12; this preference was significant. Error bars are omitted from the cumulative response curves for clarity and because the statistical analysis is based only on the total cumulative responses at the end of the 10 minute observation period.

FTLP Series II: Development of a Test Procedure to Prevent Preference for S+.

After several sets of FTLP tests in which bees developed a significant preference for S+, I was able establish more difficult test parameters that inhibited bees from displaying a significant preference for S+. I found that when the S+ and S- targets were separated by 10 cm, only 5 training trials were given during acquisition, and no proximal table landmarks were present, there was no difference in the responses given to the S+ and S- targets during the extinction test (Wilcoxon Signed-Rank Test: 2007 No-treatment Control: W = 34.00, p = 0.14, Fig. 12A; 2008 Water Control: W = 36.00, p = 0.12, Fig. 12B).

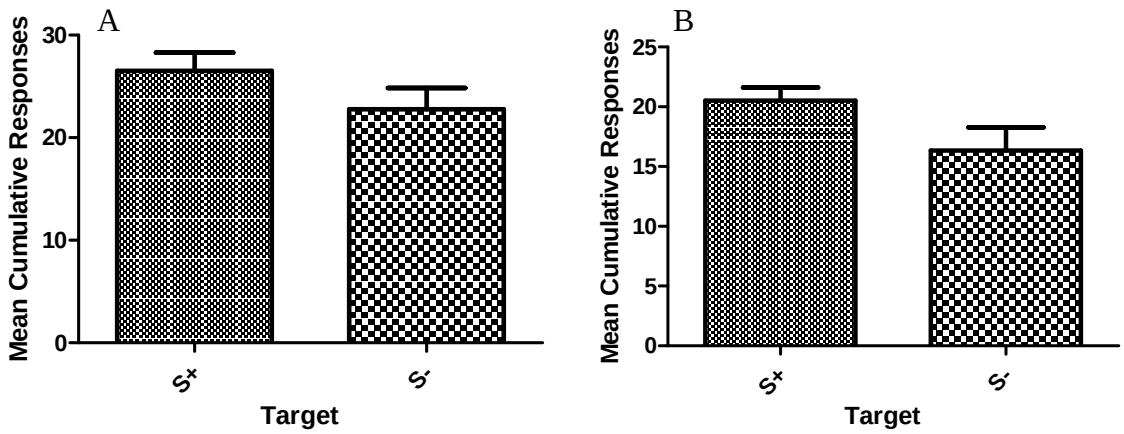

Figure 12: Mean cumulative responses (summed landing and probing) of control bees (no drug or vehicle treatment) at S+ and S- during the extinction test of the FTLP in 2007 (A) and 2008 (B). Targets were separated by 10 cm and bees were allowed 5 training trials during acquisition. There was no significant preference for S+; learning did *not* occur. Error bars represent SEM, n = 12.

The proportion of bees in the 10 cm, 5 training trial version of the FTLP that had a preference for S+ was 7 of 12 for both the no-treatment and the water control groups. The mean cumulative response curves generated from the extinction test for this version of the FTLP are shown in Figure 13.

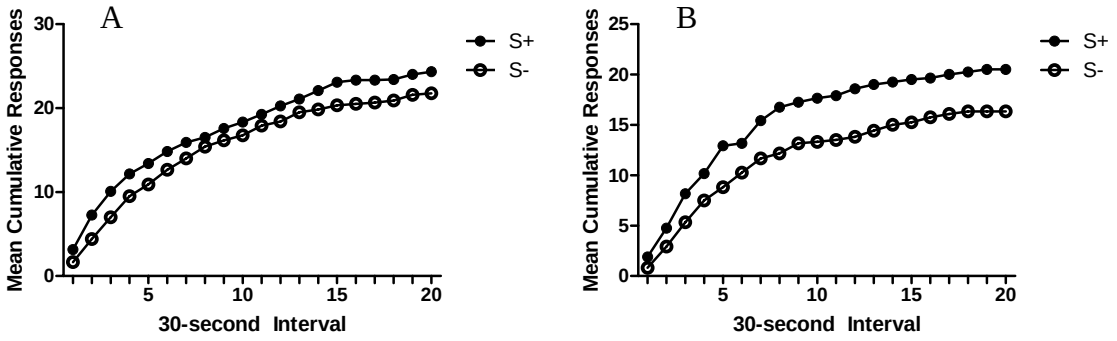

Figure 13: Mean cumulative response curves in the 10 minute extinction test of the 10 cm, 5 training trial FTLP experiment for the no-treatment control group (A) and the water control group (B). For both A and B, the proportion of bees that preferred S+ to S- was 7 of 12; these preferences were not significant. Error bars are omitted from the cumulative response curves for clarity and because the statistical analysis is based only on the total cumulative responses at the end of the 10 minute observation period.

FTLP Series III: Test of Hypothesis 1. To ask if caffeine could enhance learning in the FTLP using the same test parameters as for the control bees that did not show a significant preference for S+, 2 µl of 100 mM caffeine in dMF was applied topically on

the abdomen to a different set of bees that were then trained in the FTLP. Like the control bees, the caffeine-treated bees did not show a significant preference for the S+ target during the extinction test, thus by this criterion learning did not occur (Wilcoxon Signed-Rank Test: 2007 Caffeine Treatment: W = 42.00, p = 0.053, Fig. 14A; 2008 Caffeine Treatment: W = 38.00, p = 0.073, Fig. 14B).

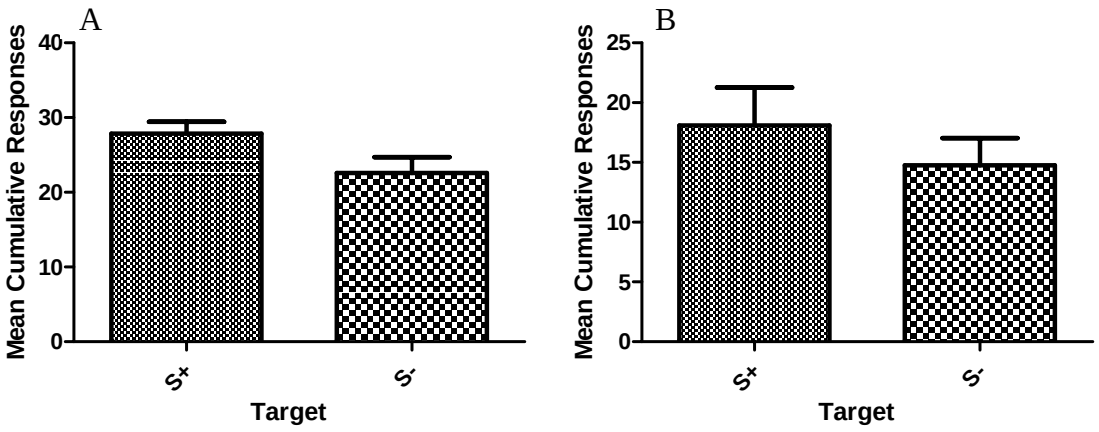

Figure 14: Mean cumulative responses (summed landing and probing) of caffeine-treated bees (2 µl of 100 mM caffeine in dMF) at S+ and S- during the extinction test of the FTLP in 2007 (A) and 2008 (B). There was no significant preference for S+; learning did *not* occur. Error bars represent SEM, n = 12.

The proportion of bees that were treated with caffeine in dMF that had a preference for S+ was 10 of 12 for the 2007 field season and 8 of 12 for the 2008 field season. The mean cumulative response curves generated from the extinction test for this version of the FTLP are shown in Figure 15.

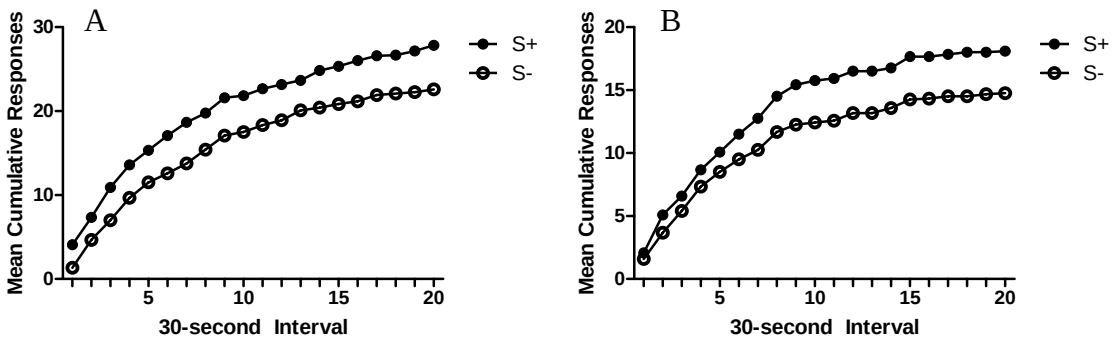

Figure 15: Mean cumulative response curves in the 10 minute extinction test of the 10 cm, 5 training trial FTLP experiment for the caffeine treatment groups in the 2007 summer field season (A) and the 2008 summer field season (B). The proportion of bees that preferred S+ to S- was 10 of 12 (A) and 8 of 12 (B). These preferences were not significant. Error bars are omitted from the cumulative response curves for clarity and because the statistical analysis is based only on the total cumulative responses at the end of the 10 minute observation period.

Vehicle control tests were performed where bees were treated topically on the abdomen with 2 µl of dMF (vehicle) only and then trained in the FTLP using the above parameters. These tests revealed that there was a significant preference for the S+ target during the extinction test, thus by this criterion, dMF enhanced learning in the FTLP (Wilcoxon Signed-Rank Test: 2007 dMF Controls: W = 66.00, p = 0.011, Fig. 16A; 2008 dMF Controls: W = 58.00, p = 0.03, Fig. 16B).

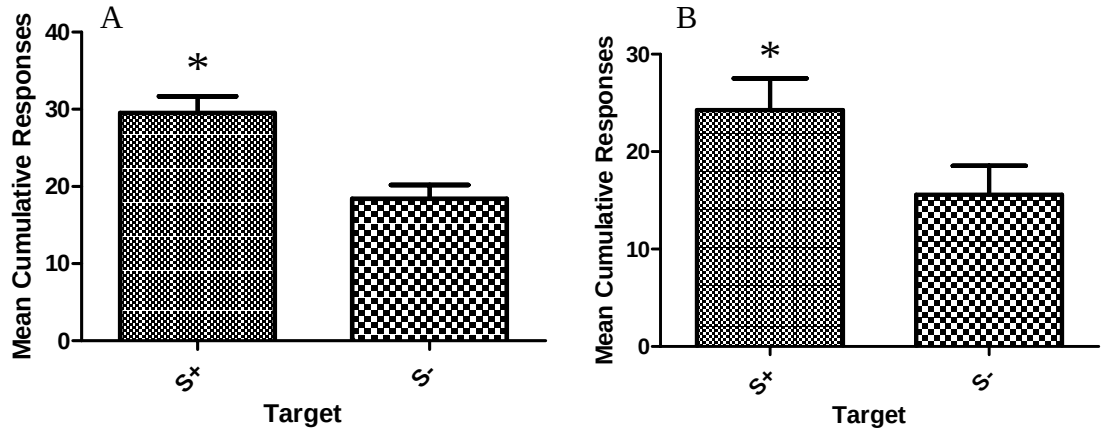

Figure 16: Mean cumulative responses (summed landing and probing) of dMF-treated bees (2 µl of dMF) at S+ and S- during the extinction test of the FTLP in 2007 (A) and 2008 (B). There was a significant preference for S+; dMF did enhance learning. Error bars represent SEM, n = 12. * p < 0.05.

The proportion of bees that were treated with dMF that had a preference for S+ was 10 of 12 for the 2007 summer field season and 9 of 12 for the 2008 summer field season. The mean cumulative response curves generated from the extinction test for this version of the FTLP are shown in Figure 17.

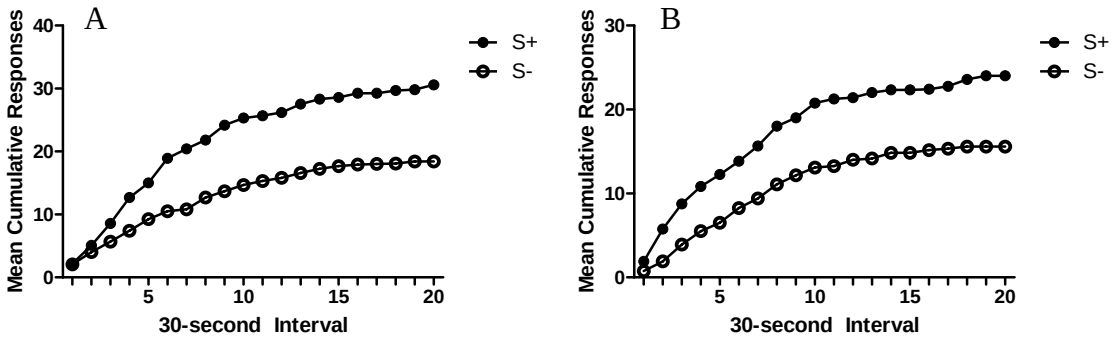

Figure 17: Mean cumulative response curves in the 10 minute extinction test of the 10 cm, 5 training trial FTLP experiment for the dMF vehicle control groups in the 2007 summer field season (A) and the 2008 summer field season (B). The proportion of bees that preferred S+ to S- was 10 of 12 (A) and 9 of 12 (B). These preferences were significant. Error bars are omitted from the cumulative response curves for clarity and because the statistical analysis is based only on the total cumulative responses at the end of the 10 minute observation period.

A summary of the FTLP Series II and III studies are shown in Figures 18 and 19.

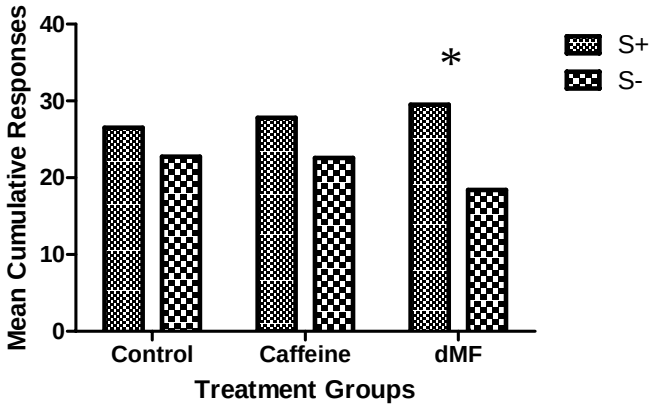

Figure 18: Mean cumulative responses for all treatment groups during the 2007 summer field season. Only the dMF vehicle-control group exhibited a significant preference for S+ during the extinction test, * $p < 0.05$.

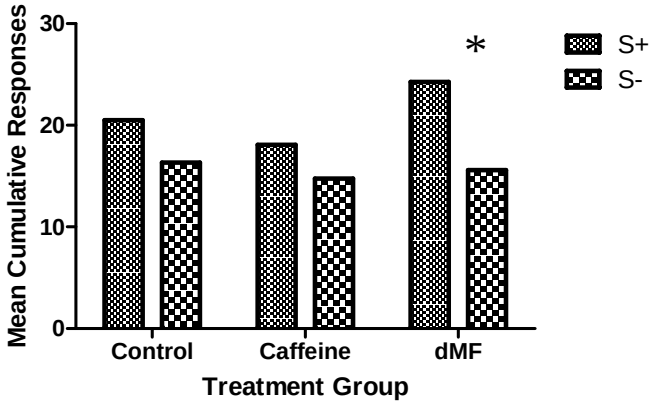

Figure 19: Mean cumulative responses for all treatment groups during the 2008 summer field season. Only the dMF vehicle-control group exhibited a significant preference for S+ during the extinction test, * $p < 0.05$.

Locomotor Assay

Bees receiving the low dose (2.5 mg/ml) of caffeine in the chronic treatment condition had a significant increase in the number of line crosses, while those receiving the high dose (5.0 mg/ml) of caffeine in the chronic treatment condition had a significant decrease in the number of line crosses (one-way ANOVA (df = 2, 102) F = 69.07; $p <$ 0.0001, Fig. 20). Based on Newman-Keuls *post hoc* comparison, the locomotor effects for all groups were significantly different (Fig. 20). Only the first three days of locomotor activity were compared in this analysis because the bees in the 5 mg chronic caffeine group did not survive past day 5.

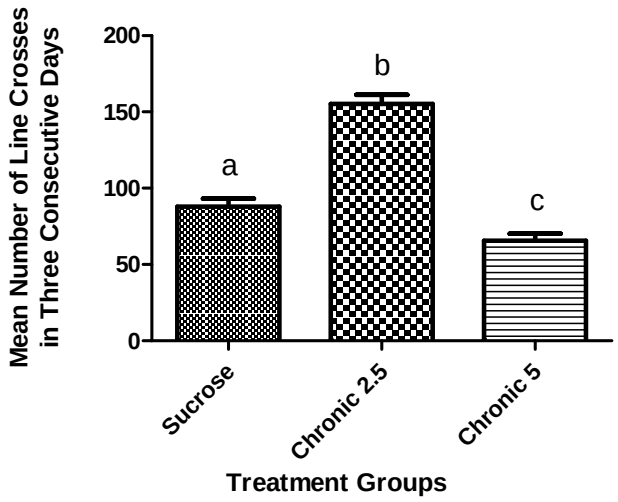

Figure 20: Mean total number of line crosses in three consecutive days of locomotor tests for the sucrose only control group and the two chronic caffeine groups. Only the first three days of locomotor tests were used for this analysis and the developmental treatment groups were excluded. A biphasic effect of caffeine on locomotor activity was observed. Letters above the bars indicated groups that differed significantly as indicated by *post hoc* comparison (Newman-Keuls test). The total number of viable dishes at the *beginning* of day 3 for the sucrose, chronic 2.5, and chronic 5 groups was 12, 12, and 10, respectively. Error bars represent SEM.

There were significant differences in the number of line crosses for all treatment groups over all test days (days 1 through 3 and 6) (Kruskal-Wallis Statistic = 57.54, $p < 0.0001$, Fig. 21). According to Dunn's *post hoc* comparison, there was no significant difference in the number of line crosses for the chronic or developmental caffeine groups at the 2.5 mg/ml dose; however, the 2.5 mg chronic *and* developmental caffeine groups had significantly greater average number of line crosses than the sucrose, 5 mg chronic caffeine, and 5 mg developmental caffeine groups (Fig. 21).

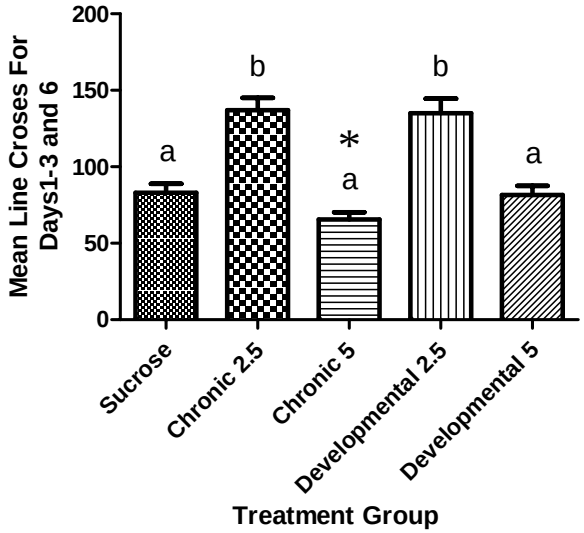

Figure 21: Mean total number of line crosses in the locomotor assay for test days 1 through 3 and 6 for all treatment groups. A biphasic effect of caffeine on locomotor activity was observed for the chronically and developmentally treated bees. Letters above the bars indicated groups that differed significantly as indicated by *post hoc* comparison (Dunn's test). Groups assigned the same letters are statistically similar to each other. Error bars represent SEM. The total number of viable dishes at the *beginning* of day 6 for the sucrose, chronic 2.5, chronic 5, developmental 2.5, and developmental 5 groups was 12, 9, 0, 7, and 9, respectively. *All bees in the 5 mg chronic caffeine group died by day 5, only the first 3 days of locomotor data were used for this comparison.

To test for an acute effect of caffeine on locomotor activity, the number of line crosses for day 6 and day 7 were compared for the sucrose group. After locomotor activity had been assessed on day 6, the sucrose group was offered 5.0 mg/ml of caffeine for 24 hours, and then locomotor activity was assessed again. The number of line crosses was higher on day 7 than on day 6, but the difference was not significant (t-test: t = 0.8788, (df = 21), $p = 0.19$, Fig. 22).

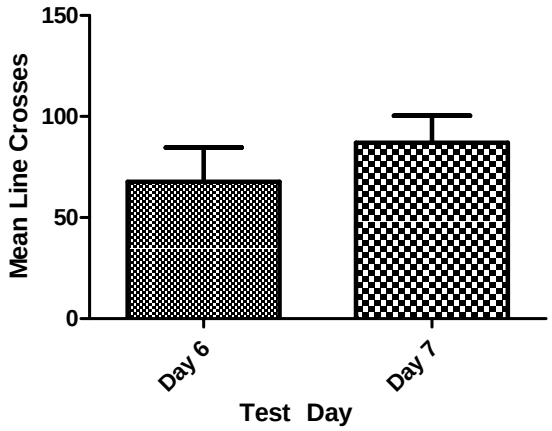

Figure 22: Mean number of line crosses in the locomotor assay for the sucrose group for days 6 and 7. No significant difference for locomotor activity was found, but an increase in locomotor activity was seen on day 7. Error bars represent SEM. The total number of viable dishes at the *beginning* of test days 6 and 7 was 12 and 11, respectively.

Tritiated Caffeine Recovery

Comparing topical thoracic drug application to topical abdominal drug application, I found no significant difference in the percent of ^{3}H-caffeine recovered from the brains 15 and 60 minutes post-treatment (t-test: 15 minutes: t = 1.24 (df = 6), p = 0.13; 60 minutes: t = 1.04 (df = 6), p = 0.17, Fig. 23).

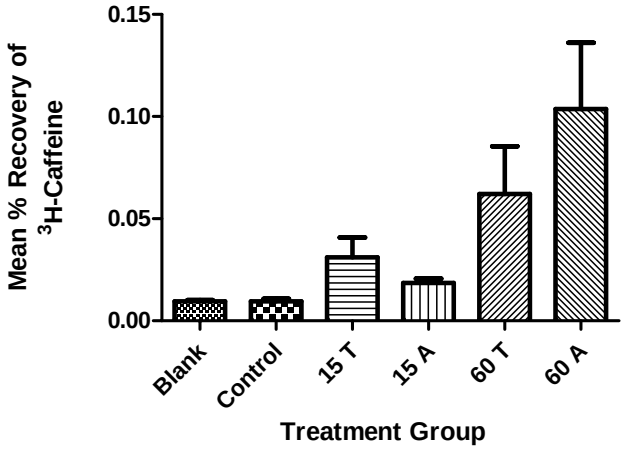

Figure 23: Average amount of ^{3}H-caffeine recovered from the brains of honey bees treated with ^{3}H-caffeine 15 and 60 minutes post-treatment. Bees were either treated topically on the thorax (T) or abdomen (A). The blank was scintillation cocktail only, and the control consisted of brains from bees with no topical drug treatments. Percentages were calculated by dividing counts recovered from the individual tissue sample by total counts of ^{3}H-caffeine. Error bars represent SEM, n = 4 per treatment group.

As shown in Figure 23, 15 minutes after thoracic treatment, more ^{3}H-caffeine was recovered from the brain; however, after 60 minutes, the honey bees treated on the abdomen had more ^{3}H-caffeine in their brains. The time points of 15 and 60 minutes were chosen based on the times at which the FTLP training had begun and, on average, was completed following drug treatment, respectively. Because there was no significant difference in the amount of the drug reaching the brain between the two topical application methods and more of the drug was recovered by the average completion point of the FTLP (60 minutes) from bees treated topically on the abdomen, I elected to treat test bees using the abdomen as the point of drug application for the FTLP because it removed the possible confounding factor of treating the surface of bees already marked with a dot of paint. Similar results were found for the second set of bees tested using liquid scintillation counting (data not shown).

DISCUSSION

Feeding Table Learning Paradigm

By repeating the experiments of Huber *et al.* (1994), I have shown that the FTLP is a robust assay for studying learning in free-flying honey bees and can be implemented in various locations. When the S+ and S- targets were separated by 40 and 10 cm, 16 training trials were given during acquisition, and proximal table landmarks were present, the foragers developed a significant preference for the S+ target in the FTLP. A possible explanation for why my results are different from the results of Huber *et al.* (1994), who found that bees could not solve the 10 cm task, is that the location in which the FTLP tests were carried out for this study offered more distal landmarks than the original

Hawaiian location of these test, such that the bees were able to use these landmarks to better distinguish the two targets even when separated by only 10 cm. The results of my studies make it clear that this assay is generally useful, but that specific parameters must be adjusted to each location. I found that performance in the FTLP is dependent upon the separation distance of the targets and the number of training trials during acquisition. When the targets were separated by a distance of 10 cm and 10 training trials were given during acquisition, a significant preference for S+ was still exhibited; however, by decreasing the number of training trials to 5, I developed an assay that prevented foragers from developing a preference for S+. Because the FTLP can be made more or less difficult by these simple variations, a learning plateau will not be reached and this test can be used to study the effects of performance-enhancing drugs.

Caffeine Treatment

My results were surprising: the effect of caffeine on learning in the FTLP cannot be assessed because the vehicle control, dMF, unexpectedly and independently enhanced learning. dMF is used by many researchers who have studied drug effects in honey bees (Barron *et al.*, 2007, Barron *et al.*, 2009, Barron *et al.*, 2007, Kucharski and Maleszka, 2005, Si *et al.*, 2005). It is commonly used because it is a strong, polar organic solvent that easily dissolves organic and inorganic chemicals including caffeine and octopamine. There have been no reports of dMF affecting learning in honey bees in any prior studies.

A potential explanation for the dMF effect seen in the FTLP, is that dMF caused changes in the response of treated bees to sucrose that interacted with the expected place learning. In a recently published study, natural populations of nectar foragers were found that varied in their responses to sucrose solutions. Bees were designated as *insensitive*

(less sensitive) to sucrose if they only collected sucrose with a concentration greater than 10% (Mujagic and Erber, 2009). This finding suggests that some foragers have a response to a sucrose food source that is concentration-dependent, and that such foragers will find sucrose solutions highly rewarding. By contrast, other foragers were considered to have extreme *sensitivity* (be more sensitive) to sucrose if they collected water and any offered concentration of sucrose, including very low concentrations (0.1%, 0.3%, 1%, 3%, 10%, 30%, and 50%) (Mujagic and Erber, 2009). The response of these sucrose-*sensitive* foragers to a food source was not concentration-dependent, and it was proposed that these foragers will find sucrose solutions less rewarding than their *insensitive* sisters. I do not have data on the sucrose sensitivity of the foragers in my study; however, one hypothesis is that dMF makes all treated bees insensitive to concentration differences in sucrose solutions, leading to enhanced perception of the concentration-dependent rewarding properties of sucrose. dMF-treated bees would therefore be more responsive to higher concentrations of sucrose than untreated bees. Possibly this enabled the dMF-treated foragers to develop a preference for the 50% sucrose S+ target during the FTLP. As counterintuitive as it seems, especially given the confusing terminology introduced by Mujagic and Erber (2009), this insensitivity to sucrose concentration may have increased the rewarding properties of sucrose, which may in turn have facilitated learning. One caveat with this explanation is that it assumes that the addition of caffeine to dMF counteracted the reduction in sucrose-sensitivity.

This line of reasoning is particularly interesting because, as previously noted, the antagonistic effects of caffeine on A_{2A} adenosine receptors cause an increase in dopamine signaling (Daly and Fredholm, 1998, Ferré *et al.*, 1991, Fredholm, 1995, Fredholm *et al.*,

1999, Garrett and Griffiths, 1997). It has been shown that dopamine injected into the thorax of foraging honey bees significantly decreases sucrose responsiveness (Scheiner *et al.*, 2002). These two results taken together may explain why bees treated with caffeine did not develop a preference for the S+ target. The caffeine-induced increase in dopamine signaling could have resulted in the caffeine-treated bees responding as much to the S- target, which contained water, as to the S+ target during extinction (similar to the sucrose *sensitive* bees of Mujagic and Erber, 2009).

At this point, such considerations are purely speculative, but many studies of learning in many species have shown the importance of the rewarding properties of sucrose on performance in learning assays. It should be noted that, in the present task, the individual bees were foraging to meet the needs of their colony and not their individual nutritional needs; I therefore predict that any effect of dMF on sucrose sensitivity would involve the responses of peripheral gustatory receptors and/or the central processing of gustatory information rather than metabolic signals.

The hypothesis that dMF makes honey bees *insensitive* to sucrose, leading to enhanced perception of the concentration-dependent rewarding properties of sucrose and therefore, very, very responsive, to higher concentrations of sucrose can be investigated by testing foragers that have been treated with dMF using an assay developed by Mujagic and Erber (2009). Free-flying foragers would be offered a choice of two different sucrose concentrations to drink. If dMF-treated bees preferred the higher concentration of sucrose compared with their non-treated counterparts, this may point to dMF increasing sucrose insensitivity and responsiveness. Also, foragers that will drink water and lower concentrations of sucrose (sensitive foragers) could be treated with dMF and then offered

low and high concentrations of sucrose to drink. If dMF increases insensitivity to sucrose, then one would expect these foragers to drink more often from the higher concentrations of sucrose. Because sucrose sensitivity tests are typically performed using harnessed bees in the PER task (Barron *et al.*, 2009, Mujagic and Erber, 2009), the PER should also be used to investigate a dMF effect on sucrose sensitivity.

Locomotor Activity

Mukhopadhyay and Poddar (1995) showed that acute caffeine treatment causes a biphasic effect on locomotor activity in mammals such that with low to intermediate doses of caffeine, locomotor activity is increased while high doses of caffeine cause a decrease in locomotion. Using a laboratory assay of walking behavior, I have shown that caffeine alters locomotor activity of bees in a dose-dependent fashion. The results of the locomotor assays showed a distinct biphasic caffeine effect in honey bees for both the bees in the chronically treated group and for the bees treated only once upon emergence (developmental group).

This biphasic effect on locomotor activity for both the chronic and developmental groups was seen in honey bees that had been treated with the low dose, 2.5 mg/ml, of caffeine only. The high dose, 5.0 mg/ml, of caffeine produced locomotor behavior that was statistically indistinguishable from that of the sucrose only group; the high dose also led to increased mortality of the bees. The locomotor results of the developmental group suggest that caffeine exposure early in life causes developmental effects in honey bees, as was suggested by the results of the study of Si *et al*. (2005). One limitation to the interpretation of these locomotor results is that caffeine may have caused either faster walking or more persistent walking; however, my present observations using small arenas

in which total line crosses were recorded do not permit me to uncouple these two aspects of activity.

Although acute caffeine treatment did not significantly increase locomotor activity in the sucrose group that was tested after 24 hours of caffeine exposure, locomotor activity trended slightly higher after caffeine exposure. These results are similar to those of Mukhopadhyay and Poddar (1995), who found that locomotor activity in rats was significantly increased 30 minutes after a single exposure to caffeine (acute effect). Mukhopadhyay and Poddar (1995) also showed that a dopamine antagonist, haloperidol, decreased the caffeine-induced locomotor activity of rats. This finding further implicates dopamine as one of the neurotransmitters involved in the actions of caffeine, at least in vertebrates.

As mentioned earlier, locomotor activity has been studied in honey bees that were fed different concentrations of ethanol solutions (Maze *et al.*, 2006). The locomotor effects of ethanol treatment on walking consisted of shorter walking duration and less frequent bouts of walking behavior at moderate and high doses of ethanol consumption (Maze *et al.*, 2006). Walking is a behavior that *"proceeds from rhythmic neural activity produced by central pattern generators (CPGs) in the central nervous system"* (Delcomyn, 2005, Marder *et al.*, 2005, Marder and Calabrese, 1996, Maze *et al.*, 2006). Maze *et al.* (2006) concluded that because locomotion is affected by ethanol, ethanol may disrupt CPGs underlying rhythmic behavior. Neuromodulators, such as, GABA, biogenic amines, or neuropeptides, are involved in *"controlling the timing and feedback"* of CPGs (Alford *et al.*, 2003, Marder and Bucher, 2001, Maze *et al.*, 2006); therefore, ethanol may affect CPGs by interference with one or all of these neuromodulators (Maze *et al.*, 2006).

We know that caffeine's actions likely occur through the dopaminergic system via secondary antagonism of adenosine receptors in vertebrates (Garrett and Griffiths, 1997), and because I have shown that caffeine affects locomotor behavior in honey bees, it is possible that caffeine disrupts the CPGs that are responsible for rhythmic behavior due to its interaction with dopamine which may be involved in the control of these neural circuits. Also, the microarray and quantitative real-time PCR studies of Kucharski and Maleszka (2005) have shown that dopamine neurotransmission is one pathway that may be affected by caffeine treatment in honey bees. Based on the evidence of a developmental locomotor effect from caffeine found in this study, it is possible that caffeine affects the DA rich regions of the brain early in development to produce long term changes in the CPGs responsible for walking behavior. To explore further the mechanism of action of caffeine in invertebrates, future studies could use the simple locomotor assay developed in this study to assess locomotor activity for bees that have been treated with both caffeine and a dopamine antagonist. If caffeine is working through the dopaminergic system in honey bees, I would predict that the caffeine-induced increase in locomotor activity would be reduced by a dopamine antagonist.

Tritiated Caffeine Recovery

There are many different drug administration methods for insects including topical application such that the drug is absorbed through the cuticle, injection directly into the brain, and feeding or oral drug administration (Barron *et al.*, 2007). The type of administration method depends largely on the question being asked and the test environment. For the FTLP, I needed a drug treatment method that would allow me to treat a small number of bees very quickly while they fed at a sucrose feeder and would

cause as little stress as possible to the individual; therefore, I elected to use topical application. I feel confident that caffeine was reaching the brains of the bees tested in the FTLP because I was able to recover ^{3}H-caffeine from the brains of the bees as assessed by liquid scintillation counting of dissected brains. I can therefore be sure that delivering the drug via topical application is not preventing the drug from reaching the intended target organ. As previously reported by Si *et al.* (2005), Kucharski and Maleszka (2005), and Barron *et al.* (2007), a drug applied topically to honey bees can just as effectively reach the brain as a drug that has been injected into the median ocellus (brain) of a honey bee or consumed orally, and this drug treatment method is less invasive than drug injection and the amount of drug delivered is more easily controlled than with oral drug administration. Also, topical application is particularly useful in field studies of unrestrained bees.

Oral administration of drugs is a widely used drug treatment method in insect research. For honey bees, oral drug administration is particularly useful when a large number of individuals require treatment, for example, whole colonies of honey bees, or for bees contained in cages or dishes as was used in the locomotor assays for this research. If bees do not drink sucrose solution for more than 24 hours, they will die (Ismail *et al.*, 2006, Lorenz *et al.*, 2001), so one can assume that all surviving bees have consumed the provided solution. Thus, I am confident that there was a true effect from caffeine on the locomotor activity of the bees in the locomotor assays.

Summary and Future Directions

The findings of Si *et al.* (2005) are important for beginning to understand the role of psychostimulants in learning in invertebrates, which in turn will lead to an

understanding of the central pathways involved in learning in the insect brain. Their results suggest that psychostimulants can be used as tools to understand the neuropharmacology of learning. At this point, however, we still cannot definitively say that caffeine affects learning rather than performance on specific tasks until its effects on learning have been examined outside of the laboratory.

While the FTLP is a sensitive and efficient assay for studying learning in free-flying honey bees, I cannot definitively say that caffeine improves or impairs learning in this assay due to the unexpected dMF effect. Figure 24 shows the chemical structure of dMF.

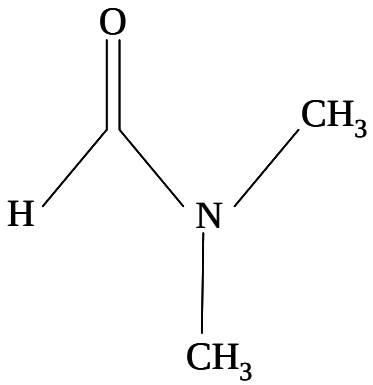

Figure 24: Chemical structure of dimethylformamide, dMF.

dMF is commonly used in insect neuropharmacology studies as a vehicle because it is a polar, organic solvent, but based on the results obtained from this study, extreme caution is recommended when using this solvent as a vehicle in insect studies. dMF is a potent solvent that when not handled properly is toxic to the liver and can lead to birth defects. Using a radiolabeled tracer, I have shown that caffeine penetrates the cuticle of the honey bee and can be reliably recovered from the brain of bees using either the thorax or the abdomen as the point of application. This is in accord with previously published studies (Barron *et al.*, 2007).

As shown in Table I, other studies that have investigated the effects of psychoactive drugs in honey bees using dMF as the vehicle and topical application as the method of drug delivery waited 30 or 60 minutes post-treatment before administering the learning or behavioral assays and none of these studies found evidence of a vehicle effect (Barron *et al.*, 2009, Si *et al.*, 2005). In the FTLP, only 15 minutes elapsed between topical caffeine administration and training in the assay. Also, I have shown from the results of the tritiated caffeine studies, that 60 minutes post-treatment, a higher percentage of caffeine was recovered from the brains of bees treated topically on the abdomen than from the thorax; where as, at 15 minutes post-treatment, a higher percentage of caffeine was recovered from the brains of bees treated topically on the thorax. Thus, if the abdomen is to be used as the point of caffeine application, a future goal might be to repeat the FTLP using caffeine but wait 60 minutes before beginning the training procedure.

A possible way of avoiding difficulties with organic solvents is to treat bees in the FTLP orally with caffeine. This could be done during the training phase of the FTLP by introducing caffeine in the 50% sucrose drop on the single target; however, I have concerns with this treatment method for the FTLP. Using the oral route of administration creates multiple variables that would make data interpretation difficult. For instance, honey bees do not forage for their own metabolic needs, but rather for the needs of the colony; therefore, the nectar collected by a forager is stored in her crop until she returns to the hive where the nectar is then unloaded (Winston, 1987). Thus, it would be difficult to determine if any caffeinated sucrose was being digested and reaching the brain. Secondly, if one could ensure that the caffeinated sucrose that was collected was in fact

being digested, it would be difficult to control the amount of caffeine that each forager ingests unless the foragers were restrained and fed a known amount of caffeinated sucrose. However, restraining a test bee in the FTLP would detract from the original goal of the assay to use free-flying honey bees to investigate the effects of caffeine on learning.

In a short-term measure of activity based on walking, caffeine had both a chronic and developmental biphasic effect on locomotor activity in young honey bees, but an acute caffeine effect was not found. This result may have occurred because only a single high dose of caffeine was used for this acute treatment condition. Based on the result that caffeine caused an increase in locomotor activity for the chronic and developmental groups at the lower caffeine dose and that the higher dose caused increased mortality, an acute caffeine effect on locomotor activity should be assessed using the low dose of 2.5 mg/ml of caffeine. The acute effects of caffeine should continue to be explored as this treatment condition most resembles the way that humans use caffeine.

As mentioned above, future studies should further investigate the role of dopamine in the mechanism of caffeine's actions on locomotor effects in invertebrates. Future efforts should also focus on developing a flying locomotor assay to test the locomotor effects of caffeine in foragers. It would be interesting to see if a developmental effect from caffeine treatment still exists at this later stage in a honey bee's life and also determine if the chronic effects of caffeine seen in younger bees is present in older bees.

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