Sublethal Effects of Heavy Metal and Metalloid Exposure in Honey Bees:

Behavioral Modifications and Potential Mechanisms

by

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ABSTRACT

Neurotoxicology has historically focused on substances that directly damage nervous tissue. Behavioral assays that test sensory, cognitive, or motor function are used to identify neurotoxins. But, the outcomes of behavioral assays may also be influenced by the physiological status of non-neural organs. Therefore, toxin induced damage to non-neural organs may contribute to behavioral modifications. Heavy metals and metalloids are persistent environmental pollutants and induce neurological deficits in multiple organisms. However, in the honey bee, an important insect pollinator, little is known about the sublethal effects of heavy metal and metalloid toxicity though they are exposed to these toxins chronically in some environments. In this thesis I investigate the sublethal effects of copper, cadmium, lead, and selenium on honey bee behavior and identify potential mechanisms mediating the behavioral modifications. I explore the honey bees' ability to detect these toxins, their sensory perception of sucrose following toxin exposure, and the effects of toxin ingestion on performance during learning and memory tasks. The effects depend on the specific metal. Honey bees detect and reject copper containing solutions, but readily consume those contaminated with cadmium and lead. And, exposure to lead may alter the sensory perception of sucrose. I also demonstrate that acute selenium exposure impairs learning and long-term memory formation or recall. Localizing selenium accumulation following chronic exposure reveals that damage to non-neural organs and peripheral sensory structures is more likely than direct neurotoxicity. Probable mechanisms include gut microbiome alterations, gut lining damage, immune system activation, impaired protein function, or aberrant DNA

methylation. In the case of DNA methylation, I demonstrate that inhibiting DNA methylation dynamics can impair long-term memory formation, while the nurse-to-forager transition is not altered. These experiments could serve as the bases for and reference groups of studies testing the effects of metal or metalloid toxicity on DNA methylation. Each potential mechanism provides an avenue for investigating how neural function is influenced by the physiological status of non-neural organs. And from an ecological perspective, my results highlight the need for environmental policy to consider sublethal effects in determining safe environmental toxin loads for honey bees and other insect pollinators.

DEDICATION

This work is dedicated to my family and friends, whose love, encouragement, and support made it possible for me to complete this degree. In particular, I dedicate this to my mother, Doris Yeatts, who was always there for me and encouraged me to persist through the difficult days. She frequently discussed my work with me, providing thoughtful and innovative ideas to help me find solutions for the problems I faced with the research and writing.

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CHAPTER 1

INTRODUCTION

This thesis is a contribution to the field of neurotoxicology. Neurotoxicology is the study of how toxic substances alter the anatomical & physiological integrity of the nervous system and consequentially modify the behavior of the animal. With my work I show that sublethal exposure to toxic heavy metals and metalloids has a significant effect on honey bee behavior, and I identify potential mechanisms mediating these toxic effects.

BACKGROUND

The field of neurotoxicology has predominantly focused on direct damage to the nervous system caused by toxin exposure. Though not readily accepted at first, the behavioral response to toxin exposure has become a routine method for identifying neurotoxins and characterizing their overall effects on the animal (Tilson, 2000). However, the focus on the action of toxins only in neural tissue as an explanation for toxin-induced behavioral modifications may be too restrictive in light of recent studies showing the effects of non-neural physiological status on cognitive function. For example, several studies have reported that disturbance of the gut microbiome has a significant impact on learning and memory performance, anxiety and depression-like behaviors, in rodents (Cryan and O'Mahony, 2011; Desbonnet et al., 2015; Foster and

Mcvey Neufeld, 2013; Fröhlich et al., 2016; Gareau et al., 2011; Li et al., 2009; Luczynski et al., 2016). And, immune activation has been reported to alter rodents' performance on a learning and memory task (Donzis and Tronson, 2014; Huang et al., 2013; Mallon et al., 2003; Yirmiya and Goshen, 2011). These studies highlight the connection between the physiological status of the whole body and the functionality of the nervous system. Similarly, toxin-induced damage to non-neural tissues could alter the connection or communication between these peripheral tissues and the brain and consequently influence neural function. Toxins that have diffuse target sites and affect multiple biochemical processes in the body and also cause behavioral modifications or cognitive impairments are likely candidates for this indirect neurotoxicity.

Heavy metals and metalloids: Toxicology

Heavy metals and metalloids are naturally occurring elements that can be toxic to animals. At low concentrations some heavy metals and metalloids – including selenium, copper, zinc, manganese, and iron – are important trace nutrients, required for the proper function of biochemical processes throughout the body (Fraga, 2005; Torres-Vega et al., 2012; Wright and Baccarelli, 2007). They function as cofactors of enzymes, components of antioxidant proteins, and as free ions in cellular signaling cascades (Baly et al., 1985; Battin and Brumaghim, 2009; Fontecave and Pierre, 1998; Gacheru et al., 1990; McCall et al., 2000; Tamano and Takeda, 2011; Torres-Vega et al., 2012). However, when an animal is exposed to high concentrations, these substances exert a toxic effect (Fraga, 2005; Torres-Vega et al., 2012; Wright and Baccarelli, 2007). Other heavy metals and metalloids – such as cadmium, lead, mercury, and arsenic – have no

known physiological function in animals and are toxic even in small quantities (Neathery et al., 1975; Wright and Baccarelli, 2007).

The toxic effects of heavy metal and metalloid overexposure are associated with dysfunction and deterioration in multiple organ systems and behavioral modifications. Toxicity compromises the functional and structural integrity of organs that are the avenue of exposure for the heavy metal or metalloid (e.g. skin, lungs and gastrointestinal tract) or that accumulate the toxin (e.g. bone, kidneys, liver, and brain), and it increases the risk of developing cancer in the affected organs (Crossgrove and Zheng, 2004; Farrar et al., 1994; Hass et al., 1964; Hughes, 2002; Lilis et al., 1968; Martelli et al., 2006; Tsunoda et al., 2000; Vogiatzis and Loumbourdis, 1998). Sublethal exposure to toxic levels of metals and metalloids – including mercury, lead, cadmium, selenium, zinc, and copper – causes sensory impairments, neuromuscular dysfunction, learning and memory deficits, and mood disorders (Neathery et al., 1975; Torres-Vega et al., 2012; Wright and Baccarelli, 2007; Yang et al., 2013). Exposure to these heavy metals and metalloids is also associated with the occurrence of neurodevelopmental and neurodegenerative diseases like Autism spectrum disorder, Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (Adams et al., 2009; Deas et al., 2016; Dusek et al., 2015; Huang et al., 1999; Mutter et al., 2005; Roos et al., 2013; Vinceti et al., 2014, 2012; Wright and Baccarelli, 2007). These neural, neuromuscular, and sensory system impairments and multiple forms of behavioral dysfunction highlight the neurotoxic components of these elements' effects.

Though many of the details of the pathophysiology of metal and metalloid toxicity have not yet been elucidated, some common physiological mechanisms are

known to underlie the toxic effects of multiple heavy metal and metalloid toxins. These include mimicking the physiological action of or replacing other metals or metalloids essential to normal physiological function, causing oxidative damage, and disrupting DNA methylation. The primary mechanism underlying a toxic effect depends on the specific metal and the molecular form of the toxin.

When there are similarities in the ions' size and charge, heavy metals and metalloids can replace or mimic the physiological role of another metal or metalloid and alter protein function or molecular signaling cascades. Free metal ions may act as antagonists or agonists of the physiological role of other metal or metalloid ions (Tamano and Takeda, 2011). For example, inorganic lead ions mimic calcium ions in cellular processes involved with neurotransmitter release and calcium-dependent intracellular signaling cascades (Gorkhali et al., 2016; Jadhav et al., 2000). Toxic metals and metalloids that interact with or replace the native metals or metalloids in metalloenzymes and other metalloproteins can either inactivate or over-activate the protein through alterations in the shape of and charge distribution within the biomolecule (Dudev and Lim, 2014; Torres-Vega et al., 2012). In isolated mouse, *Escherichia coli* and viral DNA, DNA repair proteins that contained zinc finger motifs were found to substitute other heavy metals (cadmium, copper, cobalt, mercury, and nickel) in the place of the native zinc ion (Asmuss et al., 2000; O'Connor et al., 1993). In addition to replacing an essential metal or metalloid in a protein, toxic metals or metalloids can also bind to novel sites on a protein and allosterically modulate the function in a similar manner as the essential metal or metalloid. Zinc is co-released with glutamate at some synapses and allosterically inhibits the binding of glutamate to N-

methyl-D-aspartate receptors in the brain (Fayyazuddin et al., 2000; Rachline et al., 2005; Traynelis et al., 1998). Lead ions mimic this inhibitory action of zinc ions in immature animals, though evidence suggests that lead ions are binding to a separate allosteric site on the receptor protein than are the zinc ions (Gilbert and Lasley, 2007; Lasley and Gilbert, 1999; Omelchenko et al., 1997).

Oxidative stress is a mechanism of action common among toxic metals and metalloids, especially when they are present at high concentrations. Oxidative stress is the result of the generation of free radicals and peroxides in excess of the body's antioxidant capabilities (Valko et al., 2006, 2005). These free radicals damage DNA, proteins, lipids, and other biomolecules, disrupting their structural integrity and impairing their function (Valko et al., 2006, 2005). Heavy metals and metalloids are very effective catalysts for the formation of free radicals, especially when these elements are present in excess (Valko et al., 2006, 2005). Selenium and copper are two examples of the many metals and metalloids that are known to cause oxidative damage. At physiological concentrations selenium and copper are important antioxidants that protect the body against oxidative damage (Atif et al., 2008; Glaser et al., 2010; Kim et al., 2001; Qazzaz et al., 2013; Sanchez et al., 2003). However, excess selenium and copper begin functioning as prooxidants, participating in the generation of free radicals (Drake, 2006; Lee et al., 2002; Mézes and Balogh, 2009; Sokol et al., 1989).

Heavy metals or metalloids may exert their toxic effect by disrupting DNA methylation or impairing the enzymes that catalyze the maintenance, addition, and removal of genomic DNA methylation. The metals nickel, cadmium, and lead and the metalloids arsenic and selenium are known to cause alteration in either global DNA

methylation or the methylation status of specific genes or other genetic elements (Brocato and Costa, 2013; Davis et al., 2000; Hughes, 2002; Kippler et al., 2013; Sanders et al., 2014; Schneider et al., 2013; Senut et al., 2014). For example, Cadmium toxicity has been shown to induce initial DNA hypomethylation followed by global hypermethylation in human cells and multiple organs in rats and birds through aberrant DNA methyltransferase activity (Jiang et al., 2008; Takiguchi et al., 2003; Zhang et al., 2009). The inhibition of DNA methylation was sufficient to alleviate the symptoms of cardiac depression induced by chronic cadmium exposure in mice, indicating that there are some DNA methylation mediated gene expression alterations associated with this cadmium-induced cardiac anomaly (Turdi et al., 2013). Lead exposure also causes abnormal DNA methylation patterns in human embryonic stem cells through the hypomethylation of some regions in the genome and hypermethylation of other regions (Hanna et al., 2012; Pilsner et al., 2009; Senut et al., 2014). However, it is not known whether these heavy metals and metalloid directly alter the activity of enzymes that maintain or alter genomic DNA methylation or if they affect general developmental or carcinogenic mechanisms that then trigger the observed hyper- and hypomethylation.

Heavy metals and metalloids: Environmental contamination

Exposure to heavy metals and metalloids is a concern since there is widespread environmental contamination with many of these elements (Nriagu et al., 2016). Though volcanic activity and weathering of rock release them into the environment (Adamo et al., 2003; Buat-Menard and Arnold, 1978; Quantin et al., 2001), anthropogenic sources also release high quantities into concentrated areas that are often

near population centers or agricultural regions (Besser et al., 2015; Chabukdhara and Nema, 2013; Xia et al., 2011). Major sources of metal and metalloid contamination are process that break up metal-containing rock beds – such as metal ore and coal mining and refining, phosphate extraction for fertilizer manufacturing, and hydraulic fracturing – and heavy irrigation or fertilization of agricultural ground (Besser et al., 2015; Chen et al., 2013; Järup, 2003; W. Li et al., 2014; Z. Li et al., 2014; Savci, 2012; Vengosh et al., 2014). Heavy metals and metalloids are used in many industrial products and are components of household products, so industrial and household waste often contain significant quantities of these toxic substances (Chabukdhara and Nema, 2013; Guo et al., 2012; Hasselriis and Licatab, 1996; Islam et al., 2015; Järup, 2003; Mehdi et al., 2013). For example, selenium and cadmium are components of pigments in paint and glass or ceramics, of solar panels, and of some plastics (Mehdi et al., 2013; Méndez-Armenta and Ríos, 2007). Copper is used in many construction applications and automotive (e.g. wiring, pipes, and fittings), is a component of some metal alloys, and is used in some fungicides (Soler-Rovira et al., 2013; Stern et al., 2007). In the past lead was also used widely in paint and in plumbing pipes and fittings (Gidlow, 2004; Riva et al., 2012). Subsequent recognition of lead's toxicity resulted in its removal from these products and reduced exposure levels. Fossil fuels can contain heavy metal and metalloids and combustion of these fuels in power plants, industries, and vehicles emits airborne heavy metal and metalloid particles in the exhaust (Järup, 2003; Johansson et al., 2009; Meij and Te Winkel, 2007; Pacyna et al., 2007). Prior to the 1980s, tetraethyl lead was added to automobile and airline fuel as an antiknock agent, which caused high

levels of contamination in urban and roadside environments (Järup, 2003; Riva et al., 2012).

Heavy metals and metalloids can move readily through the environment and animals living in or near contaminated area have a high risk of exposure. Airborne particles emitted from volcanoes, fossil fuel combustion, and burning of municipal waste can potentially disperse the contaminants over significant distances downwind of the source (Archibald and Crisp, 1983; Buat-Menard and Arnold, 1978; Hynninen, 1986; Meij and Te Winkel, 2007; Sheppard et al., 2007; Yemets et al., 2014). Under the right conditions (e.g. pH and organic matter content), heavy metals and metalloids dissociate from soil particles, are leached from the soil or solid wastes through heavy precipitation or agricultural irrigation, move into the surface water and groundwater reservoirs, travel down through the watershed, and re-deposited in soils surrounding the waterways and in irrigated agriculture fields (Brown, Jr. et al., 1999; Chen et al., 2013; Jacob et al., 2013; Xiao et al., n.d.; Zhang et al., 2014). Plants that grow in contaminated soil or water take up the bioavailable forms of these contaminants and incorporate them into their tissues (Angelova et al., 2004; Banuelos et al., 2011; Hladun et al., 2015, 2013b; Pickering et al., 2003; Quinn et al., 2011; Salt et al., 1995). Animals that feed on or pollinate the plants consume these toxicants along with the plant material (Quinn et al., 2011; Roberts and Johnson, 1978; Vickerman et al., 2004). In addition, organisms living in the water column and soil may absorb metals and metalloids from the water or by feeding on the organic material in the soil or water column (Desouky, 2006; Kennette et al., 2002; Phibbs et al., 2011; Samecka-Cymerman and Kempers, 1996).

Unlike organic toxins, which eventually biodegrade, heavy metals and metalloids persist in the environment. Therefore, to reclaim areas contaminated with toxic levels of heavy metals and metalloids, these substances must be intentionally removed from the soil and water or immobilized in a non-bioavailable form. Phytoremediation aims to remove metals from the soil and water through growing plant species that sequester metals and/or metalloids in their tissues within contaminated areas (Bhargava et al., 2012; El Mehdawi and Pilon-Smits, 2012; Gaur et al., 2014; Raskin et al., 1997). These plants are then harvested and processed to reclaim the metals and metalloids. Similarly, a species of fungus (*Clitocybe maxima*) was shown to able to extract the toxic elements from the soil and could provide additional methods for bioremediation of polluted soils (Liu et al., 2015). With phytoremediation and similar processes the bioavailability of the metals or metalloids in the soil determines the effectiveness of the process, so factors like soil pH and composition can make a significant difference in the amount of extraction possible (Parisien et al., 2016). The use of metal hyperaccumulating plants or fungi for remediation also increases the risk of exposing animals living in the area to the bioavailable metals or metalloids by consuming contaminated plant or fungal tissues (Parisien et al., 2016). In situ chemical immobilization involves the addition of a chemical that reduces the mobility and bioavailability of the accumulated metals and metalloids (McGowen et al., 2001; Zhang et al., 2013). These chemicals include alkalizing agents, which raise the pH to the point where the metals or metalloids are bound to soil particles rather than dissolved in the water, and phosphates, which forms a precipitate with the metals or metalloids (McGowen et al., 2001; Zhang et al., 2013). Biosorption is the use of porous substances

– like coconut husks or a combination of oyster shells and steel slag – to absorb the metals or metalloids (Gaur et al., 2014; Hasany and Ahmad, 2006; Moon et al., 2015). A similar method, biomineralization, uses bacteria that absorb the metals and precipitates them as stable crystalline structures in their cell walls to reduce the bioavailability of these elements (Li et al., 2013). And, bioleaching and electrokinetic remediation involves acidifying the soil to mobilize the metals and metalloids and then using an electrode draw those elements out of the soil (Dong et al., 2013; Maini et al., 2000). Regardless of the method employed, reclaiming contaminated areas is a long process and in light of this time requirement and of continued the heavy metal and metalloid release into the environment, understanding the effects these toxins have on the normal behavior and neural function of animals living in the contaminated environments is ecologically relevant.

The honey bee: An ecologically relevant study organism

The European honey bee (*Apis mellifera*) is an excellent study species for investigating the behavioral effects of heavy metal and metalloid toxicity. Several behaviors within the repertoire of honey bee workers have been well studied and provide sensitive assays for testing the effects of various treatments on the disruption of the ecologically relevant behavior, such as foraging for food and caring for developing brood. I employ two of these behaviors, the proboscis extension reflex and the nurse-to-forager transition, in my research.

The proboscis extension reflex (PER) is particularly useful in assessing the effects of different kinds of treatments on the feeding behavior and cognitive function

of the honey bee (Giurfa and Sandoz, 2012; Hladun et al., 2012; Smith and Burden, 2014). PER is a behavioral response in which a honey bee reflexively extends its proboscis to feed when the antennae are stimulated with sucrose or pollen (Frost et al., 2012). The bees readily perform this behavior in the lab, while restrained in harnesses, making PER a useful basis for behavioral assays (Bitterman et al., 1983). PER can be used to test the bees' motivation or ability to respond to olfactory, gustatory, or tactile stimuli and their willingness to feed (e.g. de Brito Sanchez, 2011; Erber et al., n.d.; Guerrieri et al., 2005; Pankiw and Page, 2003). PER has also been employed as the basis of olfactory learning tasks for over fifty years to study the neural and molecular mechanisms of learning and memory (Giurfa and Sandoz, 2012). In associative learning experiments the honey bees learn to exhibit PER in response to a conditioned stimulus through repeated pairing of the conditioned stimulus with an appetitive stimulus (Bitterman et al., 1983). The bees form long-term memories of the association between the conditioned stimulus and the appetitive stimulus (Menzel, 1999). Coupling this type of learning experiment with heavy metal or metalloid exposure can reveal whether exposure to these toxins is able to disrupt learning and/or memory.

Another behavior that can be manipulated in an experimental setting is the nurse-to-forager transition exhibited by worker honey bees. For approximately 3 weeks, young worker honey bees remain inside the nest, performing nest maintenance, nursing brood, and caring for the queen (Robinson, 1987). Then, the bee switches from in-nest "nursing" tasks to flying out to forage for the nectar, pollen, water, and plant resins the colony needs to survive (Robinson, 1987). The genetic and physiological bases of this transition have been well studied, and its timing can be manipulated by altering the

nurse or forager population or nutritional status of the colony and early the life experience of worker bees (e.g. Ben-Shahar, 2005; Calderone and Page, 1996; Robinson, 1987; Siegel et al., 2013; Toth et al., 2005). Coupling the manipulation of this transition with treatments to disrupt the underlying physiological mechanisms – including heavy metals and metalloids or substances acting on similar mechanisms – may reveal how the treatments may affect the timing and stability of the transition.

The honey bee also provides a good platform for identifying the pathophysiology of the effects of heavy metal and metalloid toxicity on behavior. They are small enough to scan the entire animal for accumulation of the toxic elements in order to identify the organs that potentially are subjected to toxic damage. Normal sensory and cognitive function and the physiology of several non-neural organs likely to accumulate the toxins have been well studied in the honey bee (e.g. Amdam et al., 2004; de Brito Sanchez, 2011; Engel et al., 2012; Even et al., 2012; Giurfa and Sandoz, 2012; Hori et al., 2006; Kuterbach et al., 1982; Moran et al., 2012; Seehuus et al., 2006; Wang et al., 2012) , so it is possible to identify physiological abnormalities and potentially relate them to behavioral modifications.

For the honey bee, the effect of heavy metal and metalloid toxicity on behavior and neural function is an ecologically relevant issue. Honey bees in or near areas contaminated with heavy metals and metalloids are exposed to these toxins through the pollen and nectar they collect from flowers growing in contaminated soils, the water they collect from contaminated sources, and from airborne particles that adhere to their bodies (Lakin, 1972; Negri et al., 2015; Quinn et al., 2011; Van Der Steen et al., 2011). The young in the colony are exposed to heavy metals and metalloids as these toxins

build up in the nest (Aghamirlou et al., 2015; Conti and Botrè, 2001; Hladun et al., 2016; Pohl, 2009; Solayman et al., 2016). At sufficiently high levels, exposure to heavy metals and metalloids is lethal to honey bees (Di et al., 2016; Hladun et al., 2016, 2013a). And, in some wild bee species, proximity to a source of metal contamination is correlated with reduced species diversity and abundance (Moroń et al., 2014). At sublethal levels, some metals and metalloids reduce larval growth rate and increase mortality in both honey bee larvae and adult workers (Di et al., 2016; Hladun et al., 2013a). There is evidence that some heavy metals affect honey bee behavior, since they alter foraging behavior both in honey bees and other bee species (Meindl and Ashman, 2013). However, which behaviors are affected, how that might affect honey bees' ability to collect resources for the colony, and the underlying pathology of these toxic effects is not well understood.

In addition to the toxic effect of individual heavy metals and metalloids to which the honey bees are exposed, there is evidence of synergistic interactions between different toxins and between the toxins and other environmental challenges. In honey bees, exposure to some combinations of insecticides, herbicides, or fungicides and their metabolites exhibit synergistic interactions, increasing their effect on mortality (Aufauvre et al., 2014; Iwasa et al., 2004; Meled et al., 1998). Combined exposure to pesticides and the parasite *Nosema ceranae* results in synergistic increase in mortality and a decrease in immune system function and gut tissue integrity in the honey bee (Alaux et al., 2010; Aufauvre et al., 2014; Gregorc et al., 2016). Exposure to one combination of insecticides results in improve learning performance in honey bees even though the one of individual toxins impaired this behavior, indicating that synergistic

effects may not always be negative (Williamson et al., 2013). In the nematode *Caenorhabditis elegans*, exposure to multiple combinations of heavy metals and metalloids has synergistic effects on mortality and in stress tests (Chu et al., 2002). So, it is likely that metals and metalloids have synergistic effects in combination with other toxins – like pesticides or waste chemicals – and environmental pressures – like climate change or altered habitat structure – in honey bees as well (Goulson et al., 2015; Ricketts et al., 2008).

The ability of heavy metal and metalloid toxicity to modify honey bee behavior has ecological and economic implications. Many wild insect pollinator species inhabit the same ecosystems as honey bees and consequently are also exposed to toxins, including heavy metals and metalloids (Hladik et al., 2016; Meindl and Ashman, 2014; Moroń et al., 2014; Quinn et al., 2011). Indeed, some of these species have experienced population decline or become extinct in recent decades as a result of natural habitat destruction, toxin exposure, urbanization, and other anthropogenic and natural factors, leading to concerns regarding their continued survival (Kosior et al., 2007; Potts et al., 2010a). A loss in wild pollinators could lead to reduced wildflower abundance and/or diversity as well as some reduction in crop pollination (Biesmeijer et al., 2006; Potts et al., 2010a).

Though the number of colonies has increased globally through expansion of apiculture in Asia, Africa, and some parts of Europe, managed honey bee populations have declined over recent decades in the United States and regions of Europe through increased colony loss and a dwindling number of beekeepers in these regions (Ellis et al., 2010; Moritz and Erler, 2016; Pettis and Delaplane, 2010; Potts et al., 2010b;

vanEngelsdorp and Meixner, 2010; Vanengelsdorp et al., 2008). Concerns over this decline center around the need for insect pollinators, especially honey bees, to maintain high productivity in approximately 70% of commercial crops, which account for 35% of global food production (Klein et al., 2007). A recent estimate places the value of this global pollination industry, in terms of increased crop productivity and produce quality, at approximately €153 billion ($212 billion US; Gallai et al., 2009; vanEngelsdorp and Meixner, 2010). The combination of the decline in pollinator populations and an increasing need for pollinators in agricultural systems has prompted the question of whether there may be shortage of pollinators in the future if these trends continue and spread (Aizen and Harder, 2009; Calderone, 2012; Goulson et al., 2015; Potts et al., 2010a). This has lead to greater efforts to identify causes of the increase in colony loss. Both experimental and modeling approaches to identifying potential causal factors of the population decline repeatedly point to actions of multiple stressors – including habitat fragmentation, multiple parasites and diseases, exposure to pesticides and other chemicals, and current colony management practices – that weaken the colonies and lead to their demise, (Berenbaum, 2014; Breeze et al., 2011; Dainat et al., 2012; Dennis and Kemp, 2016; Exley et al., 2015). It is conceivable that exposure to toxic heavy metals and metalloids, even at sublethal levels, is another factor contributing to this phenomenon (Bryden et al., 2013).

SIGNIFICANCE

Though behavioral assays frequently screen for substances that act directly on neural tissue, behavioral “neurotoxic” effects may also be due to more diffuse

mechanisms acting on non-neural tissues. The connection between the nervous system and the rest of the body has recently received more attention, as reviewed above, because of evidence that the status of non-neural organs has a greater ability to alter neural function than previously considered (Cryan and O'Mahony, 2011; Desbonnet et al., 2015; Donzis and Tronson, 2014; Foster and Mcvey Neufeld, 2013; Fröhlich et al., 2016; Gareau et al., 2011; Huang et al., 2013; Li et al., 2009; Luczynski et al., 2016; Mallon et al., 2003; Yirmiya and Goshen, 2011). Because the physiological status of non-neural organs and even the gut microbiome appear to alter neural signaling in the central nervous system and, consequently, behavior, a better understanding of the mechanisms and neural pathways involved is of interest. From an ecological perspective, a better understanding of the communication between the nervous systems and non-neural organs may reveal additional strategies through which an animal is able to detect changes and challenges in its environment and then respond with adaptive behaviors.

As discussed above, assessing the effects heavy metal and metalloid toxicity on honey bee behavior is ecologically relevant in light of the contamination levels in and surrounding industrial, urban, and agricultural areas and the population decline of several insect pollinator species. My research assesses the level of toxin exposure required to begin affecting the cognitive function of honey bees. The concentrations of metals and metalloids I have used are lower than the doses at which mortality begins to increase. My results indicate that acceptable environmental contamination levels, which would eliminate negative affects on honey bee and other insect pollinator health and behavior, may be significantly lower than what is currently accepted as the standards.

PURPOSE

The purpose of my research is to show how sublethal heavy metal and metalloid exposure affects honey bee behavior and identify potential mechanisms mediating the underlying impairments in neural function. I assess how heavy metals affect sensitivity in food recognition and valuation and whether ingestion of the toxins alters the perception of a food reward. I also provide an assessment of the risk of exposure to these toxins through the honey bees' willingness to consume contaminated food. I determine how selenium ingestion interferes with normal neural function through assessing learning performance and the integrity of memory formation. I also identify potential mechanisms mediating these toxic effects, which provides some direction for avenues of future research on the pathophysiology of heavy metal and metalloid toxicity in honey bees. One potential mechanism mediating the effects of heavy metal and metalloid toxicity is DNA methylation. I establish a protocol that can serve as a basis of and reference group for the effects of heavy metal and metalloid exposure on DNA methylation or the enzymes mediating alterations in DNA methylation.

APPROACH

In my research, I use three heavy metals – cadmium, copper, and lead – and a metalloid – selenium – to investigate the effect of sublethal metal and metalloid exposure on honey bee behavior and neural function. As prevalent environmental contaminants these metals are an important focus for the effect of heavy metal and metalloid pollution on the behavior and neural function of organisms living in contaminated areas (Chen et al., 2013; Fishbein, 1983; Holmgren et al., 1993; Lakin,

1972; Roberts and Johnson, 1978; Wuana and Okieimen, 2011). In vertebrates, exposure to toxic quantities of these elements induces neurological symptoms including learning and memory impairments, sensory system deficits, and neuromuscular dysfunction (Neathery et al., 1975; Torres-Vega et al., 2012; Wright and Baccarelli, 2007; Yang et al., 2013). Therefore, these toxins have the potential to cause aberrant honey bee behavior and neural dysfunction.

To assess the effect of sublethal metal exposure on honey bee sensitivity to and perception or valuation of food, I use a series of assays involving antennal and proboscis exposure to metal contaminated sucrose and a test of sucrose sensitivity following pretreatment with a metal (See Chapter 2). Antennal stimulation with metal-contaminated sucrose provides an avenue to discover if the bees can detect and reject the contaminant through receptors on their antennae or if their ability to detect the sucrose is altered by the presence of the metal. Proboscis stimulation with metal-contaminated sucrose allows me to assess whether the metal is unpalatable to the bees or makes the sucrose seem less valuable, causing them to refuse to feed on the contaminated sucrose. Pretreatment with metal-contaminated sucrose followed by a test of sucrose sensitivity allows me to determine if ingestion of the metals changes the bees' motivation to feed or their perception of the sucrose solutions used in the assay.

I determine the effect of acute selenium exposure on the honey bee performance on an olfactory discrimination learning and memory task (See Chapter 3). The bees' performance during a discrimination learning assay provides insight into whether selenium ingestion affected their ability to learn the associations between the two odors (sucrose-reinforced odor and unreinforced odor) and the presence or absence of a

sucrose reward. The degree to which the bees discriminate between the sucrose-reinforced and unreinforced odors also allows me to assess the effect of selenium on their ability to differentiate between the olfactory cues. A short-term memory test, given 30 minutes following the learning task, verifies how well the bees' learned the task and determines whether selenium treatment impaired the formation of short-term olfactory memories. And, a long-term memory test, given 24 h following the learning task, allows me to assess how selenium affects the formation of long-term olfactory memories.

In order to identify potential mechanisms underlying the learning and memory impairments exhibited by selenium-treated bees, ascertaining which organs or tissues accumulate selenium is vital. I localize and characterize the accumulation of selenium in honey bees chronically exposed to selenium contaminated food (See Chapter 4). The cyclotron based micro scanning x-ray fluorescence microscopy (μ-SXRF) technique allows me to map the locations of selenium accumulation in whole honey bees with high spatial resolution and high sensitivity (Korbas et al., 2008; Mogren et al., 2013; Quinn et al., 2011). I then characterize the selenium accumulation by determining the molecular forms of selenium in these deposits using micro x-ray absorbance near edge spectroscopy (μ-XANES; Akabayov et al., 2005; Andrahennadi et al., 2007; Mogren et al., 2013; Pickering et al., 2006; Polette et al., 2000).

Finally, I investigate the effect of inhibiting DNA methyltransferases (DNMTs), some of the enzymes that modulate DNA methylation, on honey bee behavioral modifications (See Chapter 5). I assess how injection of a DNMT inhibitor alters olfactory learning and long-term memory formation and recall. And, I determine whether DNMT inhibitor treatment is sufficient to alter the timing of the nurse-to-

forager transition. Because DNA methylation is altered by heavy metal and metalloid exposure, the learning assay establishes a potential basis of and reference group for studies investigating the effect of heavy metals and metalloids on DNA methylation patterns that are important in honey bee behavior.

CHAPTER 2

HEAVY METAL EXPOSURE MODIFIES FEEDING BEHAVIOR

INTRODUCTION

In multiple regions around the world, the soil and water reservoirs are contaminated with heavy metals, especially within and surrounding urbanized and industrialized areas (Bai et al., 2012; Bjerregaard, 1982; Jacob et al., 2013; Lakin, 1972; W. Li et al., 2014; Pilarczyk et al., 2015; Varol and Şen, 2012; Zhang et al., 2014). In addition to the release of heavy metals through natural weathering processes, substantial quantities of these elements are released through mining and fossil fuel extraction processes, industrial and electronic waste, fossil fuel dependent power plant and motorized vehicle emissions, and sewage disposal (Chabukdhara and Nema, 2013; Durán et al., 2013; Guo et al., 2012; Kabir et al., 2012; Z. Li et al., 2014; Vengosh et al., 2014; Zhang et al., 2014). Heavily used agricultural areas also are a source of environmental contamination as irrigation runoff carries the heavy metal components of fertilizers and some fungicides into surrounding lowlands and water reservoirs (Chen et al., 2013; Jacob et al., 2013).

Many of these heavy metals are taken up by the plants growing in contaminated soil and accumulate to high levels in plant tissues (Hladun et al., 2015, 2013a; Lakin, 1972; Meindl and Ashman, 2015, 2014; Quinn et al., 2011). In addition to affecting plant productivity and survival, this contamination exposes the herbivores and pollinators that depend on these plants to potentially toxic levels of the metals. Studies

have shown a reduction in species diversity, brood growth, and survival of wild and managed pollinator species in areas known to have high levels of metal contamination (Exley et al., 2015; Moroń et al., 2014). Additionally, high metal content of flowers has been shown to decrease in the frequency of visits by pollinators (Meindl and Ashman, 2014, 2013; Moroń et al., 2014). Other metals and metalloids – such as selenium and aluminum – however, do not to appear to deter pollinators from visiting flowers with high concentrations of these elements even though they have been shown to be toxic to several pollinator species (Exley et al., 2015; Quinn et al., 2011).

Understanding how environmental pollution with heavy metals affects one pollinator species, the European honey bee (*Apis mellifera*), is of special concern since they are important for the pollination of approximately 70% of food crops (Klein et al., 2007). Previous studies have shown that honey, propolis, and wax in colonies around the world contain multiple toxic substances – including insecticides, fungicides, herbicides, and heavy metals (Mullin et al., 2010; Pettis et al., 2013). However, the sensitivity and vulnerability of this pollinator species to many of these toxins is not well understood.

Honey bees are particularly at risk to toxins that they are unable to detect or that they are unable to recognize as harmful. Honey bees can detect some toxic substances through receptors on their antennae and proboscis (Wright et al., 2010). The toxins may be recognized as harmful substances through the way the honey bee perceives the "taste" of the substance. Bees have been shown to reject sucrose contaminated with quinine and concentrated sodium chloride upon stimulation of the antennae or proboscis, presumably because of an unpalatable "taste" (Wright et al., 2010). Some of

these toxins have been shown to activate receptors on the honey bee proboscis differentially from sucrose stimulation (de Brito Sanchez, 2011; Wright et al., 2010). However there are some toxins that honey bees do not appear to be able to detect through these sensory structures. For example, selenium, a metalloid that is toxic at high concentrations, does not appear to be detected through stimulation of receptors on the antennae or the proboscis (Hladun et al., 2012). And, honey bees readily consume sucrose contaminated with even lethal concentrations of selenium (Hladun et al., 2012). Honey bees also may be able to recognize a substance as harmful through the induction of a malaise-like state following ingestion of the toxin (Ayesteran et al., 2010; Hurst et al., 2014). The animals then may associate the sensory perception of the toxin with the malaise-like state, and through conditioned taste aversion the animal learns to avoid the substance in the future (Ivanova and Bures, 1990; Wright, 2011). But, not all toxins may induce this malaise-like state at the concentrations the bees are exposed to in the environment. Investigating the likelihood that honey bees will readily feed on metal contaminated resources helps determine level of threat a toxin poses to the bee population. If the bees are able to detect and reject the toxin in their food and water sources through the negative sensory experience with the toxin or learning to avoid it via conditioned taste aversion, that toxin poses a somewhat lower risk to the foraging bees and their colony.

Three heavy metals often detected at high levels in the environment are cadmium, copper, and lead, all of which have been shown to bioaccumulate in adult and larval honey bees and the colony’s honey, wax, and propolis supplies (Di et al., 2016; Hladun et al., 2016). These metals all have significant negative effects on individual

honey bee health and survival and on the whole colony (Di et al., 2016; Hladun et al., 2016). However, it is still not known if bees are able to detect or reject toxic levels of these metals. Consequently, at this time it is difficult to assess the exposure risk of these metals for honey bees living near contaminated areas.

We tested honey bees' likelihood of rejecting toxic levels of heavy metals in sucrose to determine the degree of risk environmental contamination with these metals pose to honey bee health and survival based on the likelihood of the bees rejecting contaminated food. We used antennal and proboscis stimulation with the contaminate sucrose to determine if they are able to reject contaminated food prior to ingestion. And, we investigate the possibility of post-ingestional rejection of the contaminated food based on the induction of a malaise-like state.

METHODS

Animals

Worker honey bees from colonies with open-mated New World Carniolan (Cobey 1999) queens were used for all experiments. Queens were purchased from commercial bee breeders in northern California. We collected only pollen foragers at the colony entrance as they returned from foraging flights. The use of only pollen foragers reduced the between subject variability in sucrose responsiveness, since pollen foragers generally have a high sucrose response threshold. All animals were briefly anesthetized on ice and restrained in custom harnesses, which allowed unrestricted movement of the antennae and proboscis. Upon recovery from the anesthetization, the

animals were fed to satiation with 1 M sucrose and housed in a humidified plastic tub for approximately 24 h.

Heavy Metal Toxicants

The heavy metals used in these experiments were cadmium (II) chloride, copper (II) chloride, and lead (II) chloride, which are major contaminants in the soil and water surrounding urbanized and industrialized locations and near mining and hydraulic fracturing sites (Chabukdhara and Nema, 2013; Durán et al., 2013; Kabir et al., 2012; Vengosh et al., 2014). The metals were found to accumulate the floral tissues of plants grown in soil contaminated with these metals (Hladun et al., 2015). For cadmium chloride and lead chloride, the metal concentrations used were 0.001mg/l, 0.01mg/l, 0.1mg/l, 1mg/l, 10mg/l. For copper, the metal concentrations used were 0.002 mg/L, 0.02 mg/L, 0.2 mg/L, 2 mg/L, and 20 mg/L. These concentrations are comparable to and less than the concentrations of these metals found in contaminated environments and measured in the floral parts of plants grown in contaminated soils (Hladun et al., 2015). And, these concentrations were shown to be sublethal, though still potentially toxic, to honey bees (Di et al., 2016).

Antennal response assay

We tested the bees' responsiveness to antennal stimulation with heavy metal contaminated sucrose solutions. Approximately 50 min prior to beginning the assay, the bees were feed 30 µl 1 M sucrose and were placed in the humidified box for 20 min. Then the bees were feed to satiation with water and placed in the humidified box for an

additional 30 min. During the assay the bees' antennae were briefly stimulated with the following series of stimuli: deionized water, 1M sucrose, deionized water, 1 M sucrose + metal. This series was repeated 5 times. The concentration of metal in the contaminated sucrose solution was increased with each repetition. The presence or absence of the proboscis extension reflex (PER) in response to antennal stimulation was recorded for each trial. At no point during the assay were the bees allowed to feed.

Proboscis response assay

We tested the bees' responsiveness to proboscis stimulation with metal contaminated sucrose solutions. Approximately 30 min prior to beginning the assay, the bees' were fed 30 µl 1 M sucrose. During the assay, the bee's antennae were stimulated with 0.6 µl 1M sucrose to elicit PER. If the bee extended its proboscis, it was fed 0.6 µl of one of the following series of stimuli: 1 M sucrose, deionized water, 1 M sucrose + metal. The small volume fed during each trial ensured that the bees would not become satiated during the assay. If the bee consumed the entire droplet of the test solution offered, its response for the trial was recorded as a "1". If it did not consume the droplet its response for the trial was recorded as a "0". Independent treatment groups were used for each concentration of metal in the contaminated sucrose stimuli.

Sucrose response threshold assay

We examined the effect of ingesting metal contaminated food on the bees' sucrose response threshold. Approximately 2 h prior to beginning the assay, 6 groups of bees were feed 20 µl 1M sucrose or 1 M sucrose + metal for all metal concentrations

listed above. Immediately prior to beginning the assay, the bees were feed to satiation with deionized water. During the assay, the bees' antennae were briefly stimulated with increasing concentrations of sucrose (0.1%, 0.3%, 1%, 3%, 10%, 30%). Prior to each of the sucrose stimulations the bees' antennae were briefly stimulated with deionized water, to serve as a control for sensitization. The presence or absence of PER was recorded for each water and each sucrose trial. At no time during the assay were the bees allowed to feed on the solutions used for stimulation.

Statistical Analysis

All statistical analyses were completed in IBM SPSS version 23. The results of the antennal response assay and the proboscis response assay were analyzed using a binary logistic regression analysis adjusted for repeated measures: Logistic generalized estimating equations (Logistic GEE). This analysis evaluates the differences in the probability of a PER response to stimulation with sucrose and to stimulation with metal contaminated sucrose over each of the metal concentrations tested. The percentage of bees responding to water was not included in the analysis. When indicated by the data structure, second-order interaction terms between the test SOLUTIONS (Sucrose or Sucrose + Metal) and the metal CONCENTRATION were included in the analysis. If the interaction term was not significant, it was removed from the model and the main effects model was used. When indicated, *post hoc* pair-wise comparisons with a Bonferroni correction for multiple comparisons were used to determine which concentrations of metal significantly altered the probability of the exhibition of PER following stimulation.

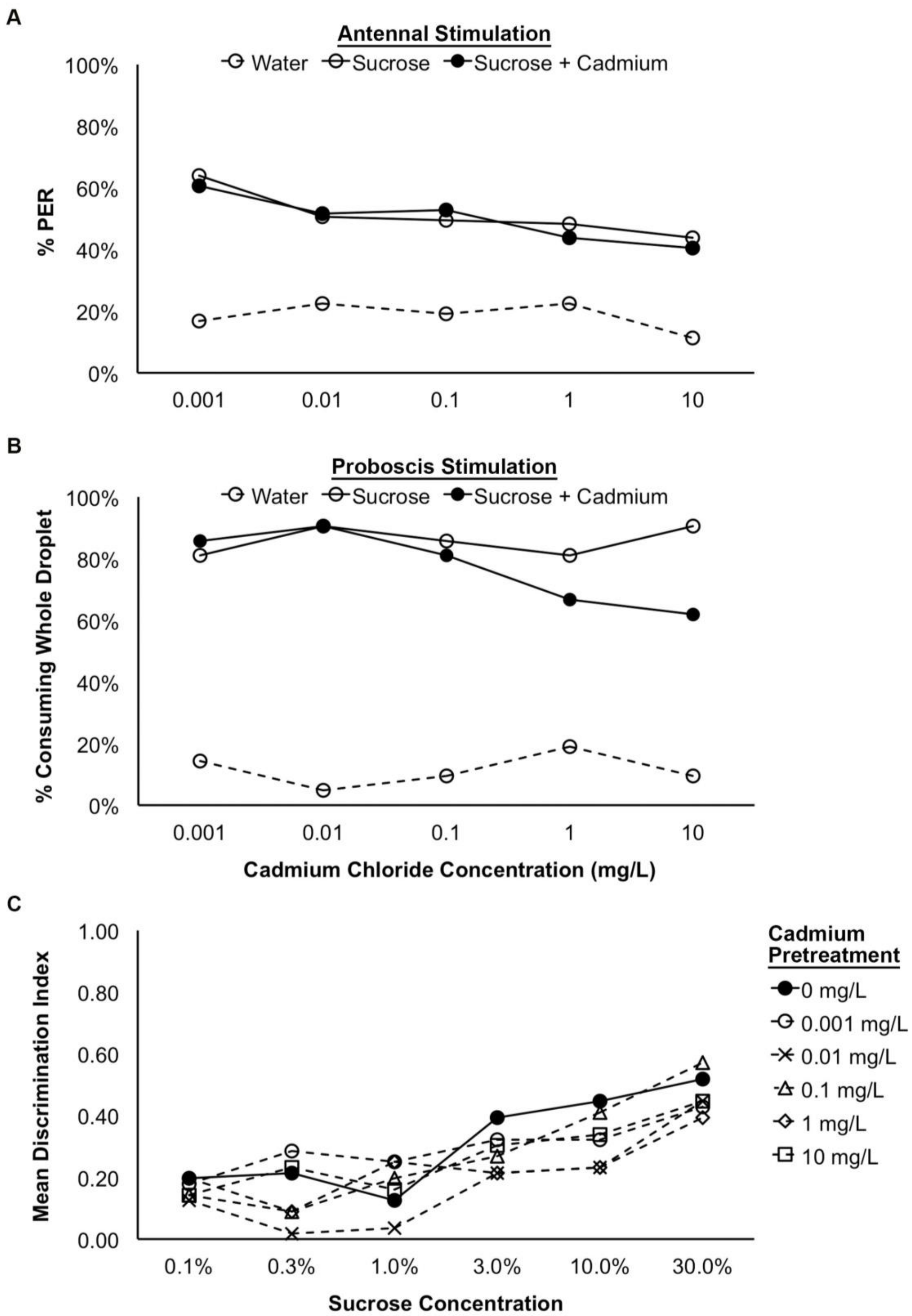
The results of the sucrose response threshold assay were analyzed as a series of discrimination index (DI) scores for each pair of water and sucrose trials. The DI score describing a bee's response to each pair of trials was calculated using the following formula: DI = response to sucrose stimulation − (response to water stimulation / 2). This generated unique DI scores for individuals that responded to sucrose stimulation only (DI = 1), individuals that responded to water stimulation only (DI = −0.5), individuals that responded to both water stimulation and sucrose stimulation (DI = 0.5), and individuals that did not respond to either water stimulation or sucrose stimulation (DI = 0). The differences in the probabilities of each of these outcomes occurring were analyzed for each sucrose concentration tested using a multinomial logistic regression analysis adjusted for repeated measures: Multinomial logistic generalized estimating equations (MultLog GEE).

RESULTS

Rejection of metal contaminated sucrose following antennal and proboscis stimulation

The honey bees did not exhibit any significant rejection of the sucrose solutions contaminated with cadmium chloride following antennal stimulation or proboscis stimulation. During the antennal stimulation assay, there was no difference in the percentage of bees responding to the sucrose and the sucrose + cadmium solutions for any of the concentrations of cadmium tested (Fig 2.1A; Logistic GEE: CONCENTRATION: 0.001 mg/L vs. 0.01 mg/L: $\chi^2 = -0.460$, $p = 0.013$, 0.001 mg/L vs. 0.1 mg/L: $\chi^2 = -0.460$, $p = 0.013$, 0.001 mg/L vs. 1 mg/L: $\chi^2 = -0.663$, $p = 0.001$, 0.001 mg/L vs. 10 mg/L: $\chi^2 = -0.822$, $p < 0.001$; SOLUTION: $\chi^2 = 0.055$, $p = 0.535$).

During the proboscis stimulation assay, there was a reduction in the percentage of bees consuming the sucrose + cadmium solution at the two highest concentrations (1 mg/L and 10 mg/L), but this trend did not reach significance (Fig 2.1B; Logistic GEE: CONCENTRATION: 0.001 mg/L vs. 0.01 mg/L: $\chi2 = 0.265$, $p = 0.628$, 0.001 mg/L vs. 0.1 mg/L: $\chi2 = -0.290$, $p = 0.586$, 0.001 mg/L vs. 1 mg/L: $\chi2 = -0.378$, $p = 0.413$, 0.001 mg/L vs. 10 mg/L: $\chi2 = -0.290$, $p = 0.586$; SOLUTION: $\chi2 = 0.292$, $p = 0.128$).

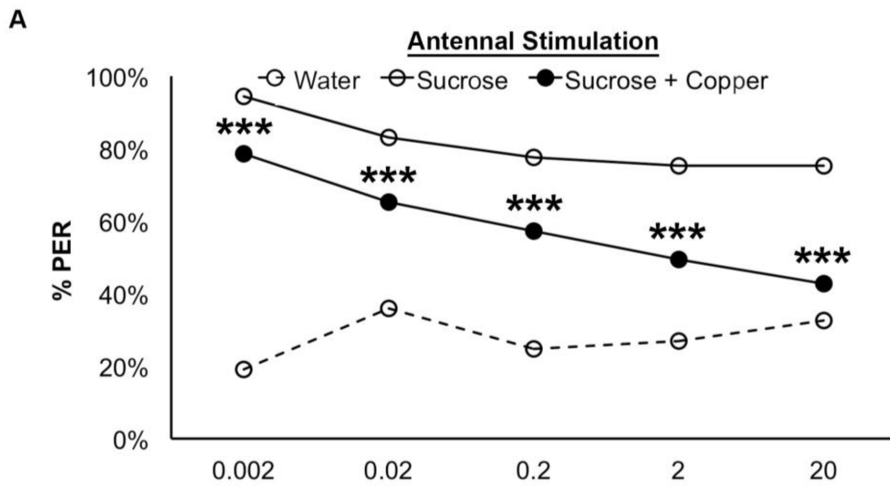


A
Antennal Stimulation
Water
Sucrose
Sucrose + Cadmium
100%
80%
60%
40%
20%
0%
% PER
0.001
0.01
0.1
1
10
B
Proboscis Stimulation
Water
Sucrose
Sucrose + Cadmium
% Consuming Whole Droplet
Cadmium Chloride Concentration (mg/L)
C
Mean Discrimination Index
1.00
0.80
0.60
0.40
0.20
0.00
0.1%
0.3%
1.0%
3.0%
10.0%
30.0%
Sucrose Concentration
Cadmium Pretreatment
0 mg/L
0.001 mg/L
0.01 mg/L
0.1 mg/L
1 mg/L
10 mg/L

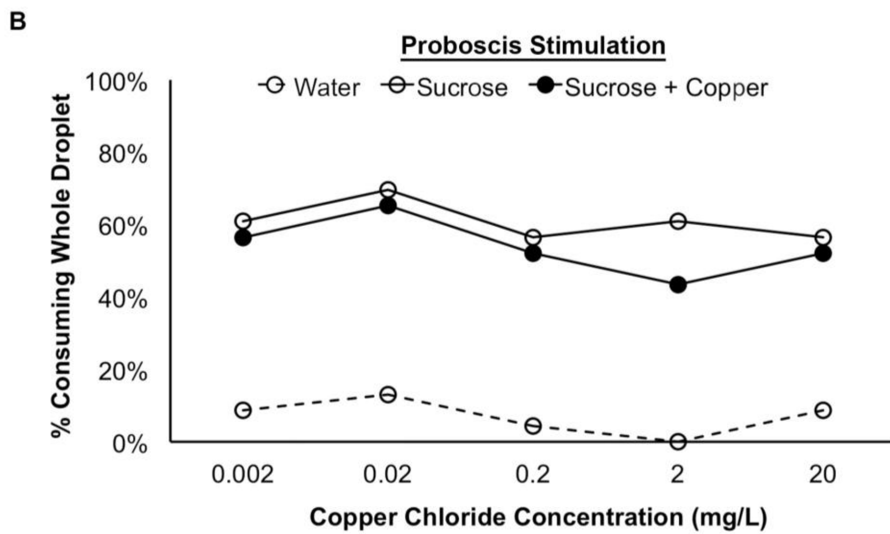
Figure 2.1. The responsiveness of worker honey bees to antennal (A; n = 89) and proboscis (B; n = 21 / treatment group) stimulation with water, sucrose, and sucrose contaminated with cadmium chloride and the effect of pretreatment with cadmium chloride on the sucrose response threshold in treated and control bees (C; n = 28/treatment group). For the assay testing the bees' responsiveness to antennal stimulation with cadmium-contaminated sucrose solutions and the assay testing the sucrose response threshold following cadmium pretreatment, the percentages bees exhibiting the proboscis extension reflex (% PER) are shown. For the assay testing the bees' responsiveness to proboscis stimulation with cadmium-contaminated sucrose solutions, the percentages bees that consumed the whole droplet of each test solution (% consuming whole droplet) are shown.

The presence of copper chloride in the sucrose solution did affect the percentage of bees exhibiting PER during the antennal stimulation assay. There was a significant reduction in the percentage of bees exhibiting PER to all solutions containing copper as compared to the uncontaminated sucrose solution (Fig 2.2A; Logistic GEE: CONCENTRATION: 0.002 mg/L vs. 0.02 mg/L: $\chi^2 = -0.844$, $p < 0.001$, 0.002 mg/L vs. 0.2 mg/L: $\chi^2 = -1.193$, $p < 0.001$, 0.002 mg/L vs. 2 mg/L: $\chi^2 = -1.433$, $p < 0.001$, 0.002 mg/L vs. 20 mg/L: $\chi^2 = -1.586$, $p < 0.001$; SOLUTION: $\chi^2 = 1.165$, $p < 0.001$). And, the magnitude of this reduction increased with the increasing concentration of copper in the solution. There was, however, no effect of copper on the bees' willingness to consume the contaminated sucrose solution for any of the concentrations of copper

A



B



C

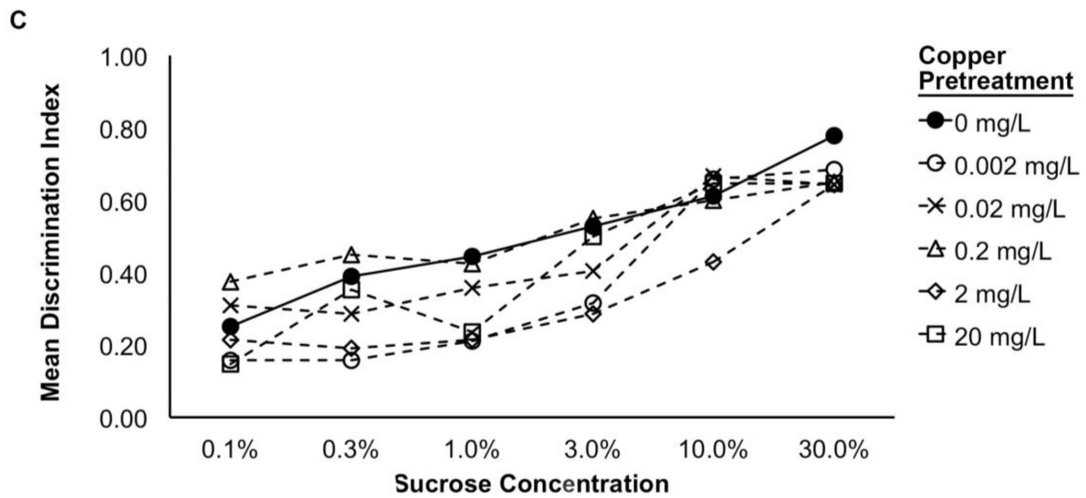


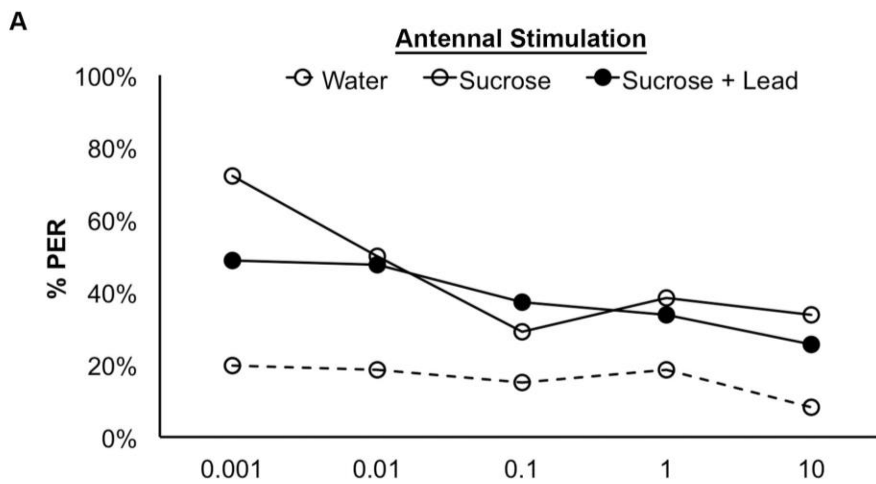
Figure 2.2. The responsiveness of worker honey bees to antennal (A; n = 89) and proboscis (B; n = 23 / treatment group) stimulation with water, sucrose, and sucrose contaminated with copper chloride and the effect of pretreatment with copper chloride on the sucrose response threshold in treated and control bees (C; n = 17-21 / treatment group). For the assay testing the bees' responsiveness to antennal stimulation with copper-contaminated sucrose solutions and the assay testing the sucrose response threshold following copper pretreatment, the percentages bees exhibiting the proboscis extension reflex (% PER) are shown. For the assay testing the bees' responsiveness to proboscis stimulation with copper-contaminated sucrose solutions, the percentages bees that consumed the whole droplet of each test solution (% consuming whole droplet) are shown.

tested following proboscis stimulation (Fig 2.2B; Logistic GEE: CONCENTRATION: 0.002 mg/L vs. 0.02 mg/L: $\chi2 = 0.265$, $p = 0.628$, 0.002 mg/L vs. 0.2 mg/L: $\chi2 = -0.290$, $p = 0.586$, 0.002 mg/L vs. 2 mg/L: $\chi2 = -0.378$, $p = 0.413$, 0.002 mg/L vs. 20 mg/L: $\chi2 = -0.290$, $p = 0.586$; SOLUTION: $\chi2 = -0.292$, $p = 0.128$).

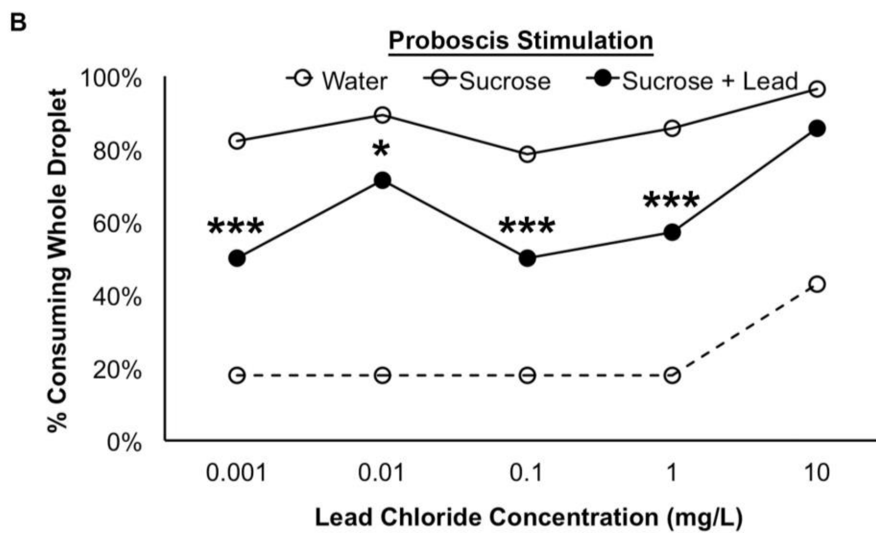
Lead chloride contaminated solutions elicited a different pattern of responses from the bees than the other two metals. During the antennal stimulation assay, there was a significant effect of lead concentration on the percentage of bees exhibiting PER to the sucrose stimulation and the sucrose + lead solutions (Fig 2.3A; Logistic GEE: CONCENTRATION: 0.001 mg/L vs. 0.01 mg/L: $\chi^2 = -0.047$, $p = 0.841$, 0.001 mg/L vs. 0.1 mg/L: $\chi^2 = -0.477$, $p = 0.038$, 0.001 mg/L vs. 1 mg/L: $\chi^2 = -0.629$, $p = 0.018$,

0.001 mg/L vs. 10 mg/L: $\chi^2 = -1.021$, $p < 0.001$; SOLUTION: $\chi^2 = 0.996$, $p = 0.001$; CONCENTRATION × SOLUTION: 0.001 mg/L vs. 0.01 mg/L: $\chi^2 = -0.903$, $p = 0.013$, 0.001 mg/L vs. 0.1 mg/L: $\chi^2 = -1.364$, $p < 0.001$, 0.001 mg/L vs. 1 mg/L: $\chi^2 = -0.794$, $p = 0.030$, 0.001 mg/L vs. 10 mg/L: $\chi^2 = -0.604$, $p = 0.118$). At the lowest concentration of lead tested (0.001 mg/L) the percentage of bees exhibiting PER to the lead contaminated sucrose solution was less than the percentage of bees responding to the uncontaminated sucrose solution. However, across the range of lead concentrations we tested, the percentage of bee exhibiting PER to the uncontaminated sucrose solution decreased by 38% while the percentage responding to the sucrose + lead solution decreased by only 23%, leading to a significant interaction of the effects of the metal concentration and the solution tested (Sucrose or Sucrose + Metal). This resulted in there being no significant difference between the percentage of bees responding to the lead contaminated sucrose solution and the uncontaminated sucrose solution at the highest concentrations of lead tested. During the proboscis stimulation assay, the concentration of lead in the contaminated sucrose solution also had a significant effect on the percentage of bees consuming the test solutions (Fig 2.3B; Logistic GEE: CONCENTRATION: 0.001 mg/L vs. 0.01 mg/L: $\chi^2 = 0.809$, $p = 0.139$, 0.001 mg/L vs. 0.1 mg/L: $\chi^2 = -0.088$, $p = 0.855$, 0.001 mg/L vs. 1 mg/L: $\chi^2 = 0.276$, $p = 0.579$, 0.001 mg/L vs. 10 mg/L: $\chi^2 = 1.768$, $p = 0.007$; SOLUTION: $\chi^2 = 1.403$, $p < 0.001$). For the lower concentrations of lead, the percentage of bees that consumed the sucrose + lead solution was significantly lower than the percentage of bees that consumed the uncontaminated sucrose solution. However, the difference between the consumption of the lead contaminated sucrose and the uncontaminated sucrose decreased as the

A



B



C

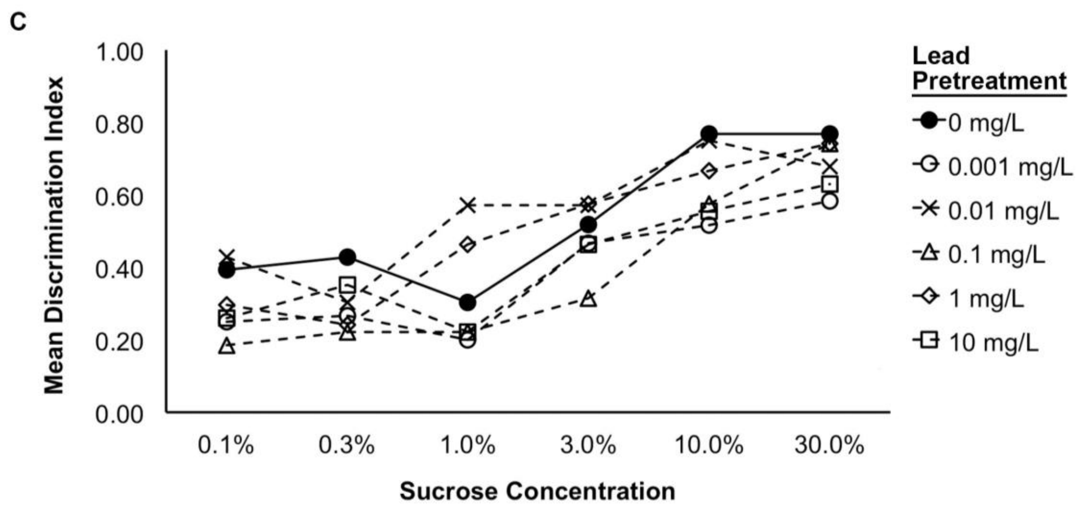


Figure 2.3. The responsiveness of worker honey bees to antennal (A; n = 86) and proboscis (B; n = 28 / treatment group) stimulation with water, sucrose, and sucrose contaminated with lead chloride and the effect of pretreatment with lead chloride on the sucrose response threshold in treated and control bees (C; n = 27-30 / treatment group). For the assay testing the bees' responsiveness to antennal stimulation with lead-contaminated sucrose solutions and the assay testing the sucrose response threshold following lead pretreatment, the percentages bees exhibiting the proboscis extension reflex (% PER) are shown. For the assay testing the bees' responsiveness to proboscis stimulation with lead-contaminated sucrose solutions, the percentages bees that consumed the whole droplet of each test solution (% consuming whole droplet) are shown.

concentration of lead in the contaminated sucrose increased. At the highest concentration of lead tested (10 mg/L) there was no significant difference between the percentage of bees consuming the lead contaminated sucrose and those consuming the uncontaminated sucrose.

The effect of ingestion of metal contaminated sucrose on the sucrose response threshold

There was no significant effect of cadmium chloride pretreatment on the bees' ability to discriminate between sucrose and water or in the overall responsiveness over the series of trials (Fig 2.1C; Table 2.1). All groups showed an approximately equal increase in responsiveness to increasing concentrations of sucrose.

Table 2.1. The p-values from the MultiLog GEE analysis of sucrose response thresholds in honey bees pretreated with cadmium chloride.

	---	0 mg/L $CdCl_2$[a]	0.001 mg/L $CdCl_2$	0.01 mg/L $CdCl_2$	0.1 mg/L $CdCl_2$	1 mg/L $CdCl_2$	10 mg/L $CdCl_2$
---		---	0.962	0.229	0.598	0.881	0.579
0.1% Sucrose[a]	---	---	---	---	---	---	---
0.3% Sucrose	0.926	---	0.455	0.336	0.414	0.251	0.265
1% Sucrose	0.262	---	0.182	0.890	0.331	0.228	0.341
3% Sucrose	0.058	---	0.658	0.938	0.594	0.266	0.939
10% Sucrose	0.021	---	0.314	0.753	0.813	0.097	0.995
30% Sucrose	0.002	---	0.539	0.486	0.330	0.452	0.886

a. This category is the reference category to which all other categories are compared

* Indicates a significant relationship between the responses to 1% sucrose and the lead treatment group

For bees pretreated with copper chloride, all treatment groups exhibited an increased percentage of bees exhibiting PER over increasing sucrose concentrations and an increasing discrimination between sucrose trials and water trials during the assay (Fig

2.2C; Table 2.2). There was, however, no effect of copper pretreatment on the percentage of bees responding to each concentration of sucrose tested.

Table 2.2. The p-values from the MultiLog GEE analysis of sucrose response thresholds in honey bees pretreated with copper chloride.

	---	0 mg/L $CuCl_2$[a]	0.002 mg/L $CuCl_2$	0.02 mg/L $CuCl_2$	0.2 mg/L $CuCl_2$	2 mg/L $CuCl_2$	20 mg/L $CuCl_2$
---		---	0.707	0.713	0.320	0.872	0.357
0.1% Sucrose[a]	---	---	---	---	---	---	---
0.3% Sucrose	0.187	---	0.248	0.233	0.624	0.137	0.502
1% Sucrose	0.074	---	0.189	0.349	0.357	0.133	0.805
3% Sucrose	0.036	---	0.462	0.273	0.428	0.135	0.496
10% Sucrose	0.027	---	0.639	0.969	0.438	0.414	0.386
30% Sucrose	0.004	---	0.981	0.413	0.190	0.655	0.877

a. This category is the reference category to which all other categories are compared

* Indicates a significant relationship between the responses to 1% sucrose and the lead treatment group

Ingestion of lead contaminated sucrose resulted in a small yet significant increase in sucrose sensitivity in bees treated with 0.01 mg/L and 1 mg/L lead chloride (Fig 2.3C;

Table 2.3). All treatment groups showed an increasing percentage of bees responding to the increasing sucrose concentrations and an increasing ability to discriminate between the sucrose and water trials as the sucrose concentration of the test solutions increased.

Table 2.3. The p-values from the MultiLog GEE analysis of sucrose response thresholds in honey bees pretreated with lead chloride.

	---	0 mg/L $PbCl_2$[a]	0.001 mg/L $PbCl_2$	0.01 mg/L $PbCl_2$	0.1 mg/L $PbCl_2$	1 mg/L $PbCl_2$	10 mg/L $PbCl_2$
---		---	0.262	0.727	0.093	0.542	0.344
0.1% Sucrose[a]	---	---	---	---	---	---	---
0.3% Sucrose	0.640	---	0.813	0.148	0.920	0.291	0.652
1% Sucrose	0.242	---	0.483	0.041*	0.435	0.032*	0.760
3% Sucrose	0.191	---	0.528	0.971	0.845	0.367	0.659
10% Sucrose	0.002	---	0.547	0.732	0.860	0.870	0.566
30% Sucrose	<0.001	---	0.945	0.356	0.162	0.776	0.925

a. This category is the reference category to which all other categories are compared

* Indicates a significant relationship between the responses to 1% sucrose and the lead treatment group

The bees pretreated with 0.01 mg/L and 1 mg/L lead exhibited a significantly higher responsiveness and discrimination to the 1% sucrose test trials than the control or the other treatment groups indicating a slightly higher sensitivity to sucrose and a better ability to discriminate sucrose trials from water trials for this sucrose concentration.

DISCUSSION

The buildup of heavy metals in the nectar and pollen of flowering plants growing near sources of contamination can have a significant effect on pollinator health and survival. The risk a metal poses to the pollinator population can be linked to how readily the pollinator species detects and rejects the substance as harmful. This can occur through the sensory structures of the antennae and proboscis or through induction of a malaise-like state following ingestion of the heavy metal. In our study, pollen foragers exhibited some limited rejection of metal contaminated sucrose solution, which depended on the metal being tested and the sensory system stimulated during the assay.

We show that pollen forager honey bees did not exhibit any significant rejection of cadmium contaminated sucrose solutions at the concentrations we tested. Either they were not able to detect these concentrations of cadmium in the sucrose or it was not perceived as harmful. For the highest concentrations of cadmium we tested, there was a decrease in the percentage of bees consuming the contaminated sucrose, though it did not reach significance. This may be indicative of the ability to detect cadmium via receptors on the proboscis but that there is a higher threshold for detection than the concentrations we tested. The ingestion of cadmium-contaminated sucrose also did not alter the sucrose response threshold of the animals. Di, et al (2016) showed that

cadmium ingestion decreases the amount of sucrose consumed by adult bees 24 and 48 h following exposure but only at concentrations 5 to 10 times higher than the highest dose we used. The higher doses of cadmium Di, et al (2016) used are likely inducing a malaise like state. So, either the doses of cadmium we tested were not high enough to induce a malaise-like state or we tested sucrose sensitivity of the bees before sufficient time had passed to be able to detect any behavioral alteration.

The bees lack of rejection of cadmium contaminated food is especially interesting since Di, et al (2016) showed that cadmium is highly toxic to the honey bee, even at the concentrations we tested. In foragers, concentrations similar to those we used significantly increased adult mortality (Di et al., 2016). Larvae, which are more sensitive to toxins, exhibited increased mortality at cadmium concentrations similar to the low and moderate concentrations we tested (Di et al., 2016; Hladun et al., 2016). Though the foraging honey bees appear to be tolerant to the concentrations of cadmium we tested and not likely to reject food contaminated with these concentrations, larvae do suffer significant negative effects from these levels of exposure. Foragers would likely not discriminate between uncontaminated nectar or pollen and those contaminated with these lower concentrations of cadmium and would bring the contaminated resources back to the colony. This could potentially have significant negative repercussions, especially as the metal accumulated within the nest over time.

The bees did show a significant dose dependent rejection of copper contaminated sucrose via stimulation of antennal receptors. Copper ions may be altering the responsiveness of sucrose receptors found on the antennae through competitive or noncompetitive inhibition or there may be receptors that are able to detect the presence

of copper independently of sensing sucrose. However, the mechanisms in the antennae that sense copper appear to not be present on the proboscis or they are non-functional or less sensitive since the bees readily consumed copper contaminated sucrose following stimulation of receptors on the proboscis. An examination of the electrophysiological responses of the receptors on the antennae or proboscis following stimulation with copper would help clarify the reason for the differential behavioral responses of these sensory structures to copper. The ingestion of copper contaminated sucrose also did not induce any change in the bees' sucrose response thresholds, indicating that the concentrations of copper we tested did not induce a malaise-like state within the timeframe of our experiment.

Honey bees in areas contaminated with copper may be able to avoid the contaminated food sources through the avoidance response we demonstrated in this study if they also have access to uncontaminated resources. Even if the environmental contamination with copper is low, it may still pose a significant threat to honey bee survival since they can build up in the nest over time. And, very low concentrations of copper (as little as 0.32 mg/L) can cause significant increases in larval mortality(Di et al., 2016; Hladun et al., 2016). So, though adult honey bees may be able to tolerate and collect resources from an area with low level contamination, the negative effect of copper toxicity on brood survival may still be significant.

When presented with lead contaminated sucrose, the bees exhibited a pattern of responses that indicates there may be an interaction between perception of lead and perception of sucrose upon stimulation of the antennae or proboscis and following ingestion of the metal. The percentage of bees exhibiting PER to antennal stimulation

with lead contaminated sucrose remained fairly constant. But, the percentage of bees responding to the sucrose only trials decreased over the trials, as the antennae were stimulated with higher concentrations of lead during the sucrose + metal trials. It may be that repeated sensory exposure to lead was altering the function of the sensory receptors in later trials. Interestingly with proboscis stimulation, the initial responses to low concentrations of lead in the contaminated sucrose were significantly lower than the uncontaminated sucrose trials. However, increasing the concentration of lead in the contaminated sucrose resulted in an increase in the percentage of bees consuming the contaminated sucrose. In humans, lead is reported to taste "sweet" (Pawlowski, 2011; Smith and Margolis, 1999), so it is conceivable that lead contamination may be similarly perceived by honey bees or may alter the taste perception of the contaminated sucrose. In another study, honey bees were not willing to consume sucrose contaminated with very high concentrations (≥400 mg/L) of lead (Di et al., 2016). Therefore, only a narrow range of lead concentrations may be perceived as "sweet" or may increase the apparent value of the food source, while lower and higher concentrations have the opposite effect. Ingestion of low or moderate doses of lead caused an increase in the sucrose sensitivity with antennal stimulation, indicating that ingestion of lead may be altering sensory perception during subsequent feeding bouts as well.

The complex array of responses to exposure to lead contamination may be due to lead causing some type of interference with sensory transduction or an alteration the bees' perception of the sucrose content of the solution. In other organisms, lead has been reported to inhibit calcium signaling, which is a vital component to sensory

transduction and neurotransmission (Audesirk, 1985; Bressler and Goldstein, 1991). Lead has also been documented to interfere with acetylcholine, GABA, and dopamine release, all of which are involved in sensory processing and reward valuation in the honey bee (Bressler and Goldstein, 1991). Stimulation of the antennae or proboscis exposes the sensory cells to dissolved lead ions. The repeated stimulation of the antennae may have allowed the lead ions to interact with the sensory receptor proteins or intracellular targets during the initial trial, which may have altered the bees' responses to subsequent stimulations with both sucrose + lead solutions and uncontaminated sucrose. When ingested, lead may be taken up by cells in the central nervous system and be more directly altering neural signaling in the neuromodulatory circuits involved with reward valuation (Bressler and Goldstein, 1991).

The complex pattern of the alteration in sensory detection and perception of food sources caused by lead exposure makes it difficult to determine the likelihood of foraging honey bees rejecting a contaminated food source during a foraging excursion. It is possible that foraging honey bees could either not differentiate between lead contaminated and uncontaminated food or even prefer moderately contaminated resources. Though adult honey bees are tolerant to substantial amounts of lead contamination, honey bee larvae are very sensitive to lead toxicity. Concentrations of lead as low as 0.1 mg/L significantly increased larval mortality (Di et al., 2016; Hladun et al., 2016). Therefore, even the collection of small amounts of lead contaminated sucrose could have significant effects on colony health and survival.

We show that exposure to toxic levels of three different heavy metals elicits three very different response profiles in forager honey bees. The bees' response profiles

for copper and lead showed strong dose dependence. The bees' responses to cadmium were less dose dependent but may become more so at higher concentrations of the metal. These response profiles have implications for the level of threat these metals have to honey bees.

Those metals that honey bees are able to detect preingestionally are more likely to be avoided if the bees have an alternative food source that is not contaminated. Other metals have also been shown to elicit an avoidance of the contaminated food by pollinator species. For example, studies investigating the effect of nickel contamination on pollinator visits to contaminated flowers showed that higher metal content reduced the rate of visits by generalist pollinators, indicating an avoidance response to the contaminated food (Meindl and Ashman, 2014, 2013).

The metals that are not detected preingestionally at the concentrations present in contaminated environments are more likely to be readily consumed and brought back to the nest. Metals and metalloids – like cadmium, aluminum, and selenium – that are readily consumed at concentrations toxic to honey bees pose a significant threat to the health and survival of the colony (Hladun et al., 2012; Meindl and Ashman, 2013). Selenium, however, has been shown to cause a reduced state of feeding motivation and learning performance, likely from long-term post-ingestional malaise. Through conditioned taste aversion the bees learn to associate the malaise with the sensory cues from that food source and avoid the contaminated food in the future (Ayesteran et al., 2010; Wright et al., 2010).

Metals – like lead – that alter the sensory detection or perception of sucrose and other important food sources can have a wide array of consequences to the honey bee.

Not only do they alter the foragers' feeding and resource gathering behaviors, but they also may have broader effects on neural function if they affect cellular mechanisms central to neural signaling throughout the brain. For example, exposure to toxic levels of manganese impaired navigation in honey bees and reduced the number of effective foraging trips they were able to make before dying (Søvik et al., 2015). To determine if these alterations in neural function are due to direct impairment of neural signaling or due to peripheral damage altering responsiveness to sensory cues, the post-ingestional targets of these metals must be identified.

Though honey bees are able to reject food contaminated with some toxic heavy metals, the toxic levels of metals and metalloids in the environment still poses a significant risk to pollinators. We have shown that worker bees are still willing to consume contaminated food, if the toxin concentration is sufficiently low. This still allows the toxin to build within the hive and cause reductions in brood survival and reduce worker health and survival. Not only is colony survival significantly impacted, individual health and normal behavior are also altered by even sublethal toxin exposure. The high probability that contaminated areas contain high levels of multiple metals and other toxins is also problematic, since it is very likely that these toxins act synergistically on pollinator health. Therefore, investigating the behavioral and physiological effects of sublethal exposure to these environmental toxins individually and in mixtures is of value.

CHAPTER 3

EFFECTS OF ACUTE SELENIUM EXPOSURE ON LEARNING AND MEMORY

INTRODUCTION

With the global decline in honey bee (*Apis mellifera*) populations, there is heightened interest in the factors that influence their survival. In addition to the normal challenges of predators and natural environmental dynamics – such as weather and resource availability – honey bees face a multitude of human-generated factors – such as toxin release into the environment – that negatively affect their health. In order to understand how to better manage our honey bee populations in the face of these human-generated factors, we need to know the effects of each individual toxin on honey bee health and behavior at sublethal, as well as lethal, levels.

One of the challenges honey bees are currently facing is the accumulation of naturally occurring toxic chemicals, such as selenium, in the environment. In addition to being released into the environment by the natural weathering of rocks, selenium is released in larger quantities from metal ore during metal extraction, from coal and petroleum during burning, and from phosphate containing rocks that are used to manufacture agricultural fertilizer (Lakin, 1972). High soil concentrations of selenium have been found in areas contaminated with runoff from heavily used agricultural areas, industrial waste sites, and mining waste dumps (Mehdi et al., 2013). Selenium contamination from agricultural runoff is widespread across the western United States,

affecting approximately 1.5 million acres across 8 states (Brown, Jr. et al., 1999). In areas contaminated with toxic levels of selenium, plants can accumulate high levels of selenium in nectar and pollen, which is then collected by foraging pollinators, like honey bees, and fed to the young in the colony (El Mehdawi and Pilon-Smits, 2012; Hladun et al., 2013a, 2012; Quinn et al., 2011).

In vertebrates, trace amounts of selenium are known to be essential for proper normal development, antioxidant protein and enzyme function, and hormone regulation (Letavayova et al., 2006). These essential functions are mainly mediated through its participation in antioxidant activities when selenium is incorporated into selenoproteins. However, when ingested at high concentrations, selenium becomes toxic. Excess selenium catalyzes the production of reactive oxygen species, causing oxidative damage, that can result in developmental abnormalities, neurological impairment, and death (Letavayova et al., 2006). In comparison little is know about the requirement and functions of selenium and the mechanisms of selenium toxicity in invertebrates.

In adult honey bees, a single dose of selenium greater than 60 mg/L causes a significant increase in mortality within 5 days of exposure (Hladun et al., 2012). Yet, honey bees do not appear to be able to taste the presence of even lethal concentrations of selenium in a sucrose solution with antennal or proboscis stimulation (Hladun et al., 2012). They therefore readily consume the highly contaminated food. Foragers will also bring the contaminated nectar and pollen back to the hive, which exposes the rest of the bees to toxic levels of selenium.

Even sublethal concentrations may have a significant effect on honey bee health and behavior. The accumulation of sublethal concentrations of selenium in the hive may

impair forager bees' ability to efficiently gather resources for the colony and nurse bees' ability to maintain the hive and care for the brood and the queen. This would further compromise colony health even before selenium accumulates to a lethal concentration and increase the colony's susceptibility to other toxins, disease, or infestation by pests or parasites. However, the effect of sub-lethal selenium exposure on honey bee behavior is still largely unknown.

In this study, we used a discrimination conditioning paradigm and memory tests coupled with acute sublethal selenium exposure to explore the possibility of selenium induced impairments in honey bee behavior. Hladun, et al. (2012) described a reduction in some honey bee feeding behaviors and survival following consumption of selenium contaminated food (Hladun et al., 2013a, 2012). However these assays are not sensitive enough to resolve some of the more subtle behavioral effects sublethal selenium exposure such as learning and memory impairments. Conditioning the proboscis extension reflex (PER) in honey bees is a more sensitive measure for the influence of toxic compounds on neural function and behavior (Smith and Burden, 2014). PER tests can also provide information about potential mechanisms for how sublethal selenium toxicity influences honey bee behavior.

We hypothesized that acute exposure to sublethal levels of selenium would reduce honey bees' performance during conditioning and the recall tests. The impaired performance would likely be seen as a reduced proportion of bees responding to the rewarded odor, especially during the long-term recall test. This would indicate impairments in the ability or the motivation to respond to olfactory stimuli, or it may be

attributed to a disruption of processes required for effective learning and memory consolidation.

METHODS

Animals & Selenium Exposure

For this study, bees were collected from 3 colonies with open-mated New World Carniolan queens (Cobey 1999). The queens were purchased from commercial bee breeders in northern California. Returning foragers were captured at the entrance of the hive in the morning. Only bees not carrying a pollen load were collected. The bees were briefly cold anesthetized and restrained in custom harnesses that left their proboscis and antennae free to move normally. After they recovered from the anesthetization, the bees were divided into treatment groups.

In a first set of experiments, the bees were then fed 3 µl of either 0.5 M sucrose solution or 0.5 M sucrose + selenium 3 hours prior to beginning conditioning. All bees were able to consume the whole dose of selenium-contaminated sucrose. The two selenium compounds used were sodium selenate (BioXtra, Sigma-Aldrich, Saint Louis, MO) and methylseleno-L-cysteine, 98% (Acros Organics, Pittsburgh, PA). Sodium selenate and methylseleno-L-cysteine are the predominant forms found in many flower parts, including nectar and pollen, of several plant species (Hladun et al., 2013a; Quinn et al., 2011). The concentrations of both selenium compounds used in this study were 0.6mg/L (1.8 ng), 6mg/L (18 ng), or 60mg/L (180 ng). These concentrations were shown to be sublethal following a single acute exposure and are comparable to and lower than the ranges of selenium concentrations found in nectar of plants grown in

selenium-contaminated greenhouse or natural environments (Hladun et al., 2013a, 2012).

In a second set of experiments, the bees were fed 3 µl 0.5 M sucrose without selenium before conditioning. They were then fed 3 µl of either 0.5 M sucrose or 6 mg/L selenium in 0.5 M sucrose 3 hours before the beginning of a long-term recall test. For this second set of experiments, the 6mg/L concentration was chosen for the selenium treatment group since it had the greatest effect on honey bee behavior during the first set of experiments.

Following dosing, the animals were left undisturbed in a humidified plastic box for 3-4 h. Next, just prior to the beginning of olfactory conditioning, we performed a motivation test in which each bee was tested for proboscis extension reflex (PER) to antennal stimulation with a droplet of 1.5 M sucrose, which they were not allowed to consume. This test provided a measure of the reduction in motivation to feed following selenium ingestion. And, only bees that showed PER to sucrose stimulation were used in olfactory conditioning, as they were sufficiently motivated to learn the task.

Odor stimulation

The two odors used for olfactory conditioning and test trials were 2M 1-hexanol (Sigma-Aldrich, St. Louis, MO) and 2M 2-octanone (Fluka, Sigma-Aldrich, St. Louis, MO). These odors have been used in several previous experiments investigating odor perception and olfactory learning in honey bees (Thorn and Smith, 1997; Wright et al., 2009, 2005). Odors were diluted in heavy mineral oil (Sigma-Aldrich, St. Louis, MO).

Odor cartridges consisted of a glass 1 cc tuberculin syringe barrel (BD Medical, Franklin Lakes, NJ) with a short length of silicon tubing (Cole-Parmer, VernonHills, IL) as a constriction in the broad end. 10 µl of an odor solution was placed on a small strip of filter paper (Whatman 114, Sigma-Aldrich, St. Louis, MO) inside each odor cartridge. The odor cartridge was connected to the automated odor delivery system via tubing attached to the narrow end of the cartridge and placed so the broad end was approximately 2 cm from the bee's antennae when she was in the conditioning arena.

The odors were presented via an automated odor delivery system coordinated by a DirectLogic 05 programmable logic controller (Automation-Direct, Cumming, GA) that triggers the opening of a valve (The Lee Co., Westbrook, CT), re-directing an airstream (~400 ml/min) through the odor cartridge. During odor stimulation, the airstream was passed through the odor cartridge, pushing odor-laden air toward the bee's antennae. A continuous flow exhaust system, located approximately 5 cm behind the bee, removed the odor from the conditioning area after every trial to maintain temporally discrete odor exposure.

Olfactory conditioning

The animals were conditioned to discriminate between the two odors. Each bee was exposed to the two odors in a pseudorandomized sequence of 16 trials (+ − − + − + + − + − − + − + + −) or (− + + − + − − + − + + − + − − +), where '+' represents the sucrose-reinforced odor (CS+) and '−' represents the unreinforced odor (CS−) (Smith and Burden, 2014; Smith et al., 1991). The odor used as the CS+ was alternated with each repetition of the experiment. The conditioning paradigm allowed us to assess the

effect of selenium exposure on each bee's ability to discriminate the two odors, in addition to assessing the acquisition of the conditioned associations.

For each trial the bee was placed within the conditioning area and allowed to acclimate for a few seconds. During presentation of the odor stimulus the airstream was directed through the odor cartridge for 4 seconds. On CS+ trials, the odor stimulus was forward-paired with 0.6 µl 1.5 M sucrose. The sucrose was delivered 3 seconds after odor onset to allow for a 1 second overlap between the odor stimulus and the reward. On CS− trials, the odor stimulus was not paired with any reward. At the end of each type of trial the bee was left undisturbed in the conditioning area for a few seconds before she was removed and placed into a holding area. The inter-trial interval was 8 minutes.

Individual responses to each conditioning trial during the acquisition phase were recorded as binary yes/no responses. A positive response to the odor stimulus was defined as the presence of the proboscis extension reflex (PER) during the olfactory stimulus and before presentation of sucrose for the CS+ trials (Smith and Burden, 2014). PER was defined as the extension of the proboscis beyond an imaginary line drawn between the tips of the opened mandibles. The overall percentage of bees exhibiting PER to any given conditioning or recall test trial (% PER) was calculated and used as an overall measure of the bees' performance during conditioning and recall testing.

Short-term and long-term recall test trials

Approximately 30 min following the end of the acquisition phase, each bee was exposed to a single unreinforced test trial with each odor. The presence or absence of PER was once again recorded as a binary variable. Following the short-term recall test trials the bees were fed to satiation with 0.5 M sucrose and placed in a humidified box overnight. Then, 24 h later, the bees were exposed to a series of 3 unreinforced test trials of each odor presented in the same pseudorandomized sequence used during conditioning. The odor presented first during both short- and long-term recall test trials was alternated with each daily repetition of the experiment. The odor presented first during conditioning was presented second during the test trials.

Immediately following the short-term recall test trials and again following the long-term recall test trials, we stimulated the bees' antennae with a small droplet of 1.5 M sucrose. The presence or absence of PER in response to the stimulation was recorded as a binary yes/no variable. This sucrose responsiveness test was a measure of how motivated the bees were to feed and thus to respond to the olfactory stimulus. It also allowed us to assess whether the motor/feeding responses were affected by selenium.

Graphing and statistical analysis

The proportion of bees responding during the preconditioning motivation test and the presence/absence scoring for the conditioning trials, short-term recall test trials, and long-term recall test trials were plotted as the percentage of bees exhibiting PER to each trial (% PER).

All statistical analyses were performed in SPSS Statistics v.22. We used a Pearson's Chi-square test to determine if there was a significant difference between treatment groups in the number of bees exhibiting PER during the sucrose responsiveness tests before conditioning and following the short- and long-term recall test trials. The differences across treatment groups in the probability of the bees exhibiting PER during odor stimulation were analyzed using logistic regression via generalized estimating equations (Logistic GEEs) with Least Squares Difference *post hoc* pairwise comparisons. We assessed the bees' performance during conditioning, the short-term recall test, and the long-term recall test separately using models including main effects and all appropriate 2-way and 3-way interaction terms. When interaction terms were not significant they were removed from the model. The final reduced models are reported below. Predictors for the models included trial number (TRIAL), the square of each trial number ($TRIAL^2$) to account for nonlinear increase in % PER over the trials, the difference between the rewarded odor and the unreinforced odor during the experiments (ODOR), and the concentration of selenium the bees were exposed to (DOSE). ODOR and DOSE were entered into the models as categorical predictors. Possible correlations between the repeated measurements taken from individual bees were accounted for by a within-subject variable identifying the responses by each bee (BEEID). This variable does not appear in the models below as it was an internal parameter used to adjust the significance levels for each of the predictors mentioned above.

RESULTS

Sodium selenate

In the experiment where the bees were treated with sodium selenate prior to conditioning, we tested the bees' sucrose responsiveness just before beginning conditioning to determine whether selenium altered their motivation to feed. Approximately 90-95% of the bees in the control group and each treatment group responded during this preconditioning sucrose responsiveness test (Table 3.1). There was no significant effect of treatment with sodium selenate on the number of bees that responded with PER during the sucrose responsiveness test (Pearson's Chi-square: $\chi^2 = 1.603$, df = 3, p = 0.659).

Table 3.1. Numbers of bees collected, deaths prior to conditioning, and positive responses to the preconditioning sucrose responsiveness test from experiments where bees were treated with selenium 3 hours prior to beginning conditioning.

	Sodium selenate				Methylseleno-L-cysteine			
	0 mg/L	0.6 mg/L	6 mg/L	60 mg/L	0 mg/L	0.6 mg/L	6 mg/L	60 mg/L
Bees / Group	106	107	108	107	77	78	78	78
Died	2	2	2	3	0	1	0	0
Positive Response	100	101	103	98	76	74	77	77

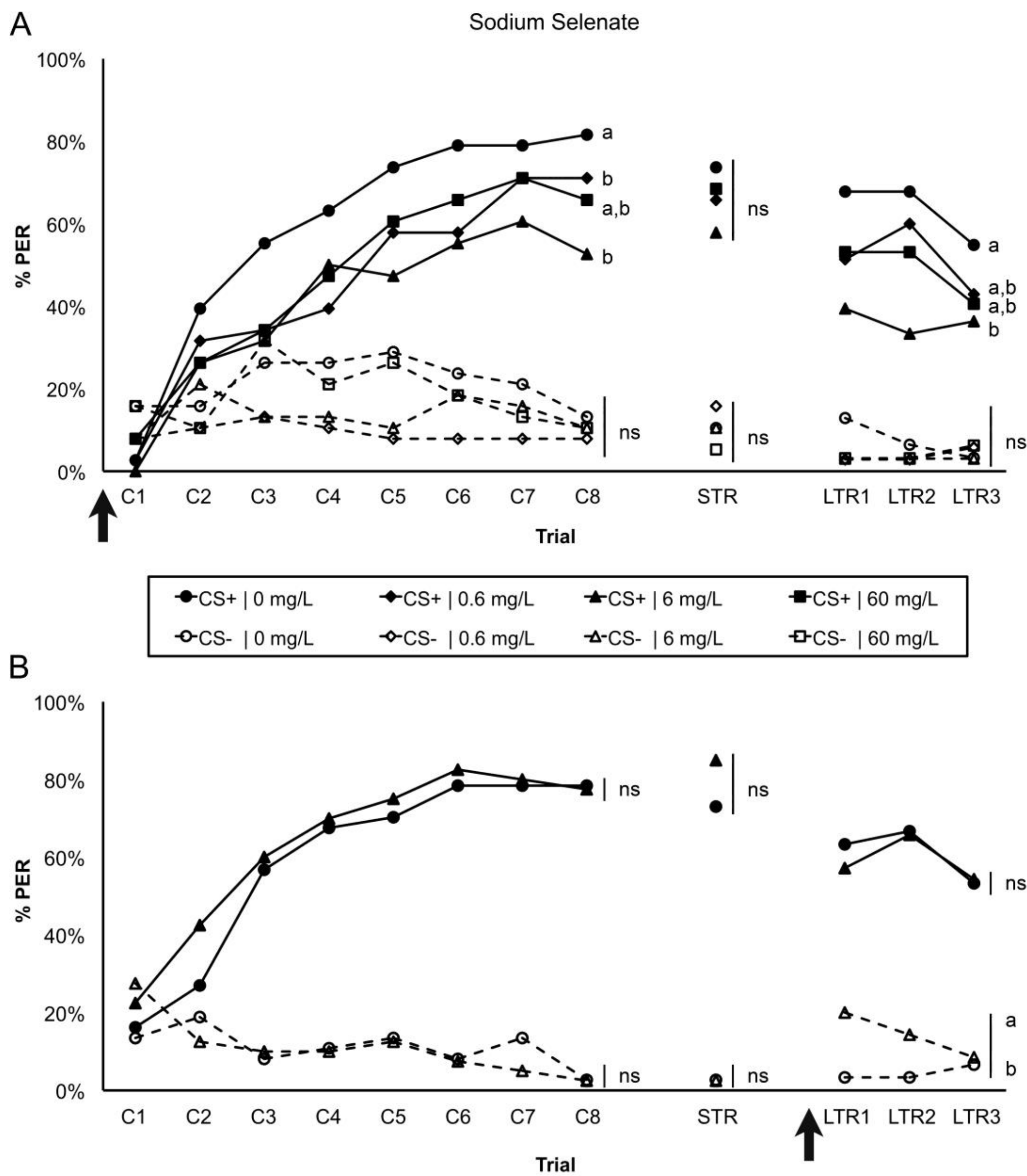


Figure 3.1. The percentage of sodium selenate treated bees exhibiting PER (% PER) to each trial during acquisition trials (C1-C8), test trials immediately following conditioning (STR), and test trials approximately 24 h following conditioning (LTR1-LTR3). Bees were either treated with sodium selenate 3 hours prior to conditioning (A; for C1-C8 & STR: n = 37-38, for LTR1-LTR3: n = 31-35) or 3 hours prior to the long-

term test trials (B; for C1-C8 & STR: n = 35-36, for LTR1-LTR3: n = 30-35). CS+ indicates the sucrose-reinforced odor, while CS− represents the unreinforced odor. The arrows indicate the timing of selenium treatment.

Table 3.2. Logistic Generalized Estimating Equations analysis of the bees' responses during conditioning, short-term recall, and long-term recall of bees treated with sodium selenate 3 hours before conditioning.

		Conditioning		Short-term Recall		Long-term Recall	
Predictor	Contrast	β[1] Wald χ^2	p-value	β[1] Wald χ^2	p-value	β[1] Wald χ^2	p-value
ODOR	CS− vs. CS+	1.75 218	0.000*	6.401 31.038	0.000*	3.110 132.437	0.000*
DOSE	0 mg/L vs. 0.6 mg/L	-0.718 6.43	0.011*	0.326 0.235	0.628	-0.531 1.855	0.173
DOSE	0 mg/L vs. 6 mg/L	-0.738 4.86	0.027*	0.262 0.138	0.710	-1.106 6.404	0.011*
DOSE	0 mg/L vs. 60 mg/L	-0.401 1.64	0.200	-0.560 0.598	0.439	-0.604 2.460	0.117
TRIAL		0.941 101	0.000*	- - -		0.423 0.616	0.433
$TRIAL^2$		-0.075 70.8	0.000*	- - -		-0.150 1.272	0.259

1. The parameter estimate indicates the relationship of the predictor to the percentage of bees responding during conditioning and recall.

* p-value ≤ 0.05

Bees in the control group and all treatment groups did learn the task and discriminate between the CS+ and the CS− during conditioning (Figure 3.1A, Table 3.2). Over the conditioning trials, the percentage of bees responding to the CS+ increased in all groups. And, there was a significant effect of ODOR on the probability of exhibiting PER, with the percentage of bees responding to the CS+ being significantly higher than the percentage responding to the CS− trials.

All of the selenate treated groups showed a reduction, relative to control, in their responses to the CS+ (Figure 3.1A, Table 3.2). Only the 0.6mg/L and 6mg/L sodium selenate treated groups showed a significantly lower percentage of bees exhibiting PER to the CS+ compared to the control group. Although the group treated with 60 mg/L showed a lower percentage of bees responding to the CS+ compared to controls, this decrease was not significant. There was not a significant difference between the percentages of bees responding to the CS− across the treatment groups.

Recall tests also showed differences between control and selenium treated groups. However, these differences were significant only for the long-term recall tests. During the short-term recall test, the bees in all groups discriminated between the CS+ and CS− odors (Figure 3.1A, Table 3.2). As in acquisition, the lowest response was in the group treated with 6 mg/L selenium. However, the difference in the percentage of bees exhibiting PER to unreinforced trials of the CS+ between treatment groups and the controls failed to reach significance for any comparison. There were also no significant differences in the percentages of bees responding to the CS− across the treatment groups.

During the long-term recall test trials, the bees also discriminated between the CS+ and the CS− (Figure 3.1A, Table 3.2). There was no significant effect of TRIAL on the probability of exhibiting PER, which indicates that there was little detectable extinction as a result of the unreinforced trials with the CS+. In these tests the group treated with 6mg/L sodium selenate exhibited a significantly lower percentage of bees exhibiting PER to the CS+. The groups treated with 0.6mg/L and 60mg/L sodium selenate exhibited smaller decreases in the percentage of bees responding to the CS+, although these decreases were not significant. There were no significant differences between the percentages of bees responding to the CS− across the treatment groups.

Following the short- and long-term recall trials, we tested the bees' motivation to feed and motor function by antennal stimulation with 1.5 M sucrose. The number of bees responding to stimulation was a measure of the bees' motivation to feed, and thus to respond to a feeding cue. Their ability to respond to the stimulation by extension of the proboscis was also an assessment of motor function. There was no difference in the percentage responding to the sucrose stimulation across all control and treatment groups for bees treated with sodium selenate (Table 3.3; Pearson's Chi-square: short-term recall $\chi^2 = 2.027$, df = 3, p = 0.567; long-term recall $\chi^2 = 3.264$, df = 3, p = 0.353).

Table 3.3. The responses to sucrose responsiveness tests following the short- and long-term recall tests. Reported are the numbers of bees exhibiting PER to the sucrose stimulation and, in parentheses, the total number of bees in each treatment group.

	Short-Term Recall				Long-Term Recall			
Selenium Exposure	0 mg/L	0.6 mg/L	6 mg/L	60 mg/L	0 mg/L	0.6 mg/L	6 mg/L	60 mg/L
Sodium selenate before conditioning	37 (*38*)	38 (*38*)	37 (*38*)	38 (*38*)	28 (*31*)	32 (*35*)	27 (*33*)	29 (*32*)
Sodium selenate before long-term recall	35* (*37*)	---	36* (*40*)	---	27 (*30*)	---	33 (*35*)	---
Methylseleno-L-cysteine before conditioning	30 (*30*)	28 (*29*)	31 (*31*)	30 (*30*)	24 (*29*)	25 (*28*)	26 (*30*)	24 (*28*)
Methylseleno-L-cysteine before long-term recall	25 (*26*)	---	30 (*30*)	---	24 (*26*)	---	26 (*30*)	---

* We are missing data from several bees for these data points. All bees that were tested showed positive sucrose responsiveness.

We trained a second set of bees using the same discrimination conditioning paradigm. However, instead of selenium exposure prior to conditioning, we exposed the treated group of bees to 6 mg/L sodium selenate 3 hours before the beginning of the long-term recall test.

Both the control group and the selenium-treated group increased their response to the CS+ over the course of conditioning and exhibited significant discrimination between the CS+ and the CS− (Figure 3.1B, Table 3.4). There was no significant

difference between the performance of the control and treated bees during conditioning, which is expected since they did not differ in terms of treatment during this phase.

We then tested short-term recall as before. Both groups discriminated well between the CS+ and CS− during the short-term recall test (Figure 3.1B, Table 3.4). There was no significant difference between the performance of bees in the control group and treatment group during the short-term recall test, as expected since the groups had been treated identically up to that point.

Table 3.4. Logistic Generalized Estimating Equations analysis of the responses during conditioning, short-term recall, and long-term recall of bees treated with sodium selenate 3 hours prior to beginning the long-term recall test.

		Conditioning		Short-term Recall		Long-term Recall	
Predictor	Contrast	β^1 Wald χ^2	p-value	β^1 Wald χ^2	p-value	β^1 Wald χ^2	p-value
ODOR	CS− vs. CS+	2.65 131	0.000*	5.06 39.1	0.000*	3.58 38.9	0.000*
DOSE	0 mg/L vs. 6 mg/L	0.155 0.275	0.600	0.603 1.39	0.238	1.31 4.90	0.027*
TRIAL		0.694 16.4	0.000*	- - -		0.682 0.685	0.408
TRIAL^2		-0.056 13.3	0.000*	- - -		-0.212 1.09	0.297
ODOR × DOSE		- - -		- - -		-1.41 4.48	0.034*

1. The parameter estimate indicating the relationship of the predictor to the percentage of bees responding during conditioning and recall.

* p-value ≤ 0.05

Following treatment with sodium selenate, we tested long-term recall. Both groups discriminated between the CS+ and CS− during the long-term recall test (Figure 3.1B, Table 3.4). There was no effect of TRIAL or $TRIAL^2$ on the percentage of bees exhibiting PER, indicating no significant extinction of the conditioned response. There was a significant effect of DOSE and the DOSE × ODOR interaction, indicating that bees treated with sodium selenate showed a significantly greater percentage responding to the CS− than the control bees, though there was no significant difference between the control and treatment groups' responses to the CS+.

After the short- and long-term recall trials, we tested the bees' motivation and motor function by antennal stimulation with 1.5 M sucrose. For the short-term recall sucrose responsiveness test, all of the bees tested for sucrose responsiveness exhibited PER to the sucrose stimulation, so the Pearson's Chi-square statistic could not be calculated. As before, there was no difference in the percentage responding to the sucrose stimulation following long-term recall trials between the control group and sodium selenate treated group. (Table 3.3; Pearson's Chi-square: long-term recall $\chi^2 = 0.369$, $df = 1$, $p = 0.544$).

Methylseleno-L-cysteine

Prior to conditioning, we tested the bees' sucrose responsiveness to determine their motivation to feed. Approximately 95-98% of the bees in each treatment group responded during this preconditioning sucrose responsiveness test (Table 3.1). There was no significant effect of treatment with methylseleno-L-cysteine on the number of

bees that responded with PER to the sucrose responsiveness test (Pearson's Chi-square: $\chi^2 = 1.001$, df = 3, p = 0.801).

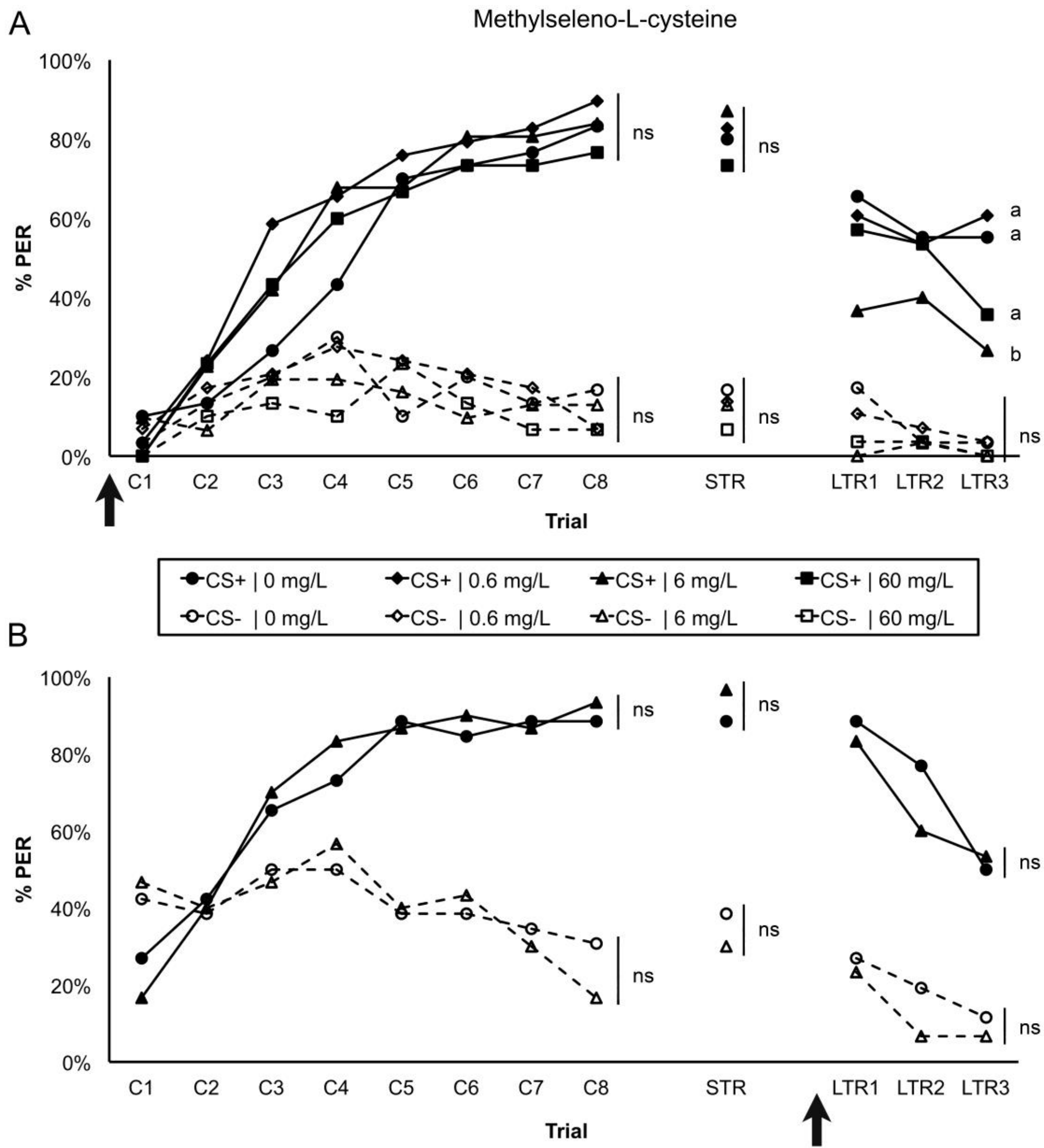


Figure 3.2. The percentage of methylseleno-L-cysteine treated bees exhibiting PER (% PER) to each trial during acquisition trials (C1-C8), test trials immediately following conditioning (STR), and test trials approximately 24 h following conditioning (LTR1-

LTR3). Bees were either treated with methylseleno-L-cysteine 3 hours prior to conditioning (A; for C1-C8 & STR: n = 28-31, for LTR1-LTR3: n = 28-30) or 3 hours prior to the long-term test trials (B; for C1-C8 & STR: n = 25-30, for LTR1-LTR3: n = 26-30). CS+ indicates the sucrose-reinforced odor, while CS− represents the unreinforced odor. The arrows indicate the timing of selenium treatment.

For all groups, the percentage of bees responding to the CS+ increased over the conditioning trials and the bees successfully discriminated between the CS+ and CS− odors, indicating the bees in all of the groups learned the task (Figure 3.2A, Table 3.5). Unlike bees treated with sodium selenate, there was no significant effect of methylseleno-L-cysteine on the percentage of bees exhibiting PER to the CS+ or the CS− during conditioning.

Following conditioning, we also performed recall tests. In the short-term recall test, the bees discriminated between the CS+ and CS− odors, but there was no significant effect of methylseleno-L-cysteine treatment on the percentage of bees responding to the CS+ and CS- (Figure 3.2A, Table 3.5). During the long-term recall test, all control and treatment groups exhibited discrimination between the rewarded and unreinforced odors. However, in contrast to the short-term tests, there was a significant decrease in the percentage of bees treated with methylseleno-L-cysteine responding to CS+ and to CS−. The group treated with 6 mg/L methylseleno-L-cysteine exhibited a significantly lower % PER to the CS+ test trials than the control group. Over the 3 long-

Table 3.5. Logistic Generalized Estimating Equations analysis of the bees' responses during conditioning, short-term recall, and long-term recall of bees treated with methylseleno-L-cysteine 3 hours prior to beginning conditioning.

		Conditioning		Short-term Recall		Long-term Recall	
Predictor	Contrast	β^1 Wald χ^2	p-value	β^1 Wald χ^2	p-value	β^1 Wald χ^2	p-value
ODOR	CS− vs. CS+	2.255 175.512	0.000*	3.444 97.999	0.000*	3.148 86.878	0.000*
DOSE	0 mg/L vs. 0.6 mg/L	0.384 1.155	0.282	-0.004 0.000	0.994	-1.179 0.651	0.420
DOSE	0 mg/L vs. 6 mg/L	0.113 0.105	0.746	0.129 0.061	0.805	-4.469 7.357	0.007*
DOSE	0 mg/L vs. 60 mg/L	-0.095 0.075	0.784	-0.633 1.394	0.238	-2.926 3.453	0.063
TRIAL		1.392 92.862	0.000*	- - -		-2.046 4.261	0.039*
$TRIAL^2$		-0.110 66.374	0.000*	- - -		0.409 3.123	0.077
DOSE × TRIAL	0 mg/L vs. 0.6 mg/L	- - -		- - -		0.961 0.433	0.511
DOSE × TRIAL	0 mg/L vs. 6 mg/L	- - -		- - -		3.742 4.678	0.031*
DOSE × TRIAL	0 mg/L vs. 60 mg/L	- - -		- - -		2.920 3.148	0.076
DOSE × $TRIAL^2$	0 mg/L vs. 0.6 mg/L	- - -		- - -		-0.168 0.242	0.623
DOSE × $TRIAL^2$	0 mg/L vs. 6 mg/L	- - -		- - -		-0.889 4.348	0.037*
DOSE × $TRIAL^2$	0 mg/L vs. 60 mg/L	- - -		- - -		-0.741 3.382	0.066

1. The parameter estimate indicating the relationship of the predictor to the percentage of bees responding during conditioning and recall.

* p-value ≤ 0.05

term recall trials, there was a significant decline in the percentage of bees responding to the trials, indicating a significant extinction of the conditioned response overall. Additionally, for the 6 mg/L treatment group, the interaction terms DOSE × TRIAL and DOSE × TRIAL^2 were significant. This interaction shows a significant reduction in the percentage of these bees responding to successive long-term recall trials and a further reduction in the responses to the first and third long-term recall trials in relation to the second trial, compared to the opposite trend seen in control bees.

Following the short-term and long-term recall tests we determined the bees' response levels to antennal stimulation with sucrose. There was no difference in the percentage responding to the sucrose stimulation across all treatment groups for bees treated with methylseleno-L-cysteine (Table 3.3; Pearson's Chi-square, short-term recall: $\chi^2 = 3.164$, df = 3, p = 0.367; long-term recall: $\chi^2 = 1.017$, df = 3, p = 0.797).

We exposed different groups of bees to methylseleno-L-cysteine (6mg/L) 3 hours before the beginning of the long-term recall test (Figure 3.2B, Table 3.6). During conditioning the bees were divided into two equally sized groups. Both groups showed increased percentage of responding to the CS+ over conditioning trials and discriminated between the CS+ and CS− odors. There was no significant difference between the two groups before treatment with methylseleno-L-cysteine, as was expected.

We then tested the bees' memory of the task with recall tests. During the short-term recall test there was no significant difference between the performances of two groups of bees (Figure 3.2B, Table 3.6). Both groups discriminated well between the CS+ and the CS−. Following exposure to methylseleno-L-cysteine, the bees still

discriminated well between the CS+ and the CS−. There was no difference between the methylseleno-L-cysteine treated bees performance on the long-term recall trials.

Following the short- and long-term recall tests we determined the bees' response levels to antennal stimulation with sucrose. There was no difference in the percentage responding to the sucrose stimulation between bees treated with methylseleno-L-cysteine and controls following the short- and long-term recall tests (Table 3.3; Pearson's Chi-square, short-term recall: $\chi^2 = 1.175$, df = 1, p = 0.278, long-term recall: $\chi^2 = 0.463$, df = 1, p = 0.496).

Table 3.6. Logistic Generalized Estimating Equations analysis of the responses during conditioning, short-term recall, and long-term recall of bees treated with methylseleno-L-cysteine 3 hours prior to beginning the long-term recall test.

		Conditioning		Short-term Recall		Long-term Recall	
Predictor	Contrast	β^1 Wald χ^2	p-value	β^1 Wald χ^2	p-value	β^1 Wald χ^2	p-value
ODOR	CS− vs. CS+	1.336 73.506	0.000*	3.231 35.872	0.000*	2.706 76.957	0.000*
DOSE	0 mg/L vs. 6 mg/L	0.071 0.017	0.897	-0.009 0.000	0.987	-0.411 1.006	0.316
TRIAL		0.861 34.189	0.000*	- - -		-1.408 2.516	0.113
$TRIAL^2$		-0.078 26.490	0.000*	- - -		0.159 0.536	0.464

1. The parameter estimate indicating the relationship of the predictor to the percentage of bees responding during conditioning and recall.

* p-value ≤ 0.05

DISCUSSION

Acute treatment with sublethal dosages of selenium affects a honey bee's performance during acquisition and/or recall of learned olfactory information. This reduction could occur because of interference with the ability to distinguish between the rewarded and unreinforced odors, or because of an overall reduction in the response to olfactory stimuli, especially seen in the response to the rewarded odor. The reduction may be due to impairment in sensory detection of the olfactory stimulus or through interference in one or more of the neural processes involved in learning, memory consolidation, and memory recall. It did not seem to be due to interference with motor processes involved in PER or to reduction in the motivation to feed. Bees showed normal behavioral responses to sucrose in spite of showing reductions in responses to conditioned odors.

The effect of selenium treatment depended on the phase of conditioning and testing as well as on the form of selenium. During the initial acquisition phase, bees that ingested a single dose of sodium selenate before the beginning of the conditioning trials exhibited a decrease in their responsiveness to the CS+, and sometime to the CS−, during olfactory discrimination conditioning. Bees ingesting a dose of methylseleno-L-cysteine, a reportedly less toxic form of selenium (Quinn et al., 2011), did not show this impairment during conditioning. Interestingly, there was no significant difference between selenium treated bees and controls during the short-term recall test for either form of selenium, which could indicate at least some recovery 30 min after acquisition. In spite of this apparent early recovery, the largest effect of selenium treatment – for

either form – was during the long-term recall test. The bees treated with moderately high doses (6 mg/L) of either form of selenium prior to conditioning exhibited decreased performance during the long-term recall test.

The absence of a treatment effect during the short-term recall test, and the emergence of an effect during the long-term test, may be because more time is required for selenium to be absorbed and exert its toxic effect than the 3 hours between selenium exposure and conditioning and the short-term recall test. By the time of the long-term recall test, sufficient damage from the toxic levels of selenium may have occurred to alter the bees' behavior. Alternatively, the mechanisms targeted by selenium toxicity may be those involved in long-term memory consolidation or recall, leaving short-term memory relatively unimpaired.

Unexpectedly, for both sodium selenate and methylseleno-L-cysteine, the bees fed the highest dose (60 mg/L) did not show significant deficits in their performance on either conditioning trials or the short- and long-term recall trials. This unusual type of u-shaped dose-response curve has been identified in previous neurotoxicology studies, though the underlying mechanisms remain elusive (Bleecker et al., 1997; Davis and Svendsgaard, 1990; Davis et al., 1990). Consequently, any interpretation of the u-shaped dose response curve must be given with caution. One possible explanation, however, is that honey bees may have some type of physiological compensatory mechanism that either combats or masks the effects of selenium toxicity at this high yet sublethal dosage.

Following the short- and long-term recall trials, we performed a sucrose responsiveness assay to determine if the performance of bees exposed to selenium could

be attributed to reduced motivation or ability to respond with PER to the odor stimulus. We did not observe a decreased responsiveness to sucrose stimulation in selenium treated animals compared to controls; however, we used a relatively high sucrose content stimulus, which typically elicits very strong PER. The conditioned response of PER to test trial odor stimulation is a sensitive measure of motivation and thus could be detecting a moderate decline in motivation or the ability to respond to sucrose stimulation that could not be resolved with the sucrose responsiveness test following the test trials.

Our data clearly show an effect of sublethal dosages of selenium on a behavior that is important for colony performance. Exposure to sublethal selenium can have a significant effect on honey bee learning and memory within 24 hours. The small amount of selenium fed to the bees in this study is less than what bees could encounter in contaminated areas. The concentrations used are well within the ranges of selenium observed in the aerial tissues in several plant species grown in selenium contaminated soil in both greenhouse conditions and contaminated locations (Hladun et al., 2013a, 2012; Quinn et al., 2011). And the volume fed is much less than the crop load a honey bee could carry.

Our results are consistent with two different mechanisms potentially mediating the effects of toxic selenium exposure on honey bee behavior. In other species, the cellular level mode of action for selenium toxicity depends on the specific form of the selenium compound. In the case of inorganic selenium cellular damage is likely caused by oxidative damage, as has been shown in cultured cell lines and in mammals (Letavayova et al., 2006). With organic forms of selenium, the molecule may be

metabolized into selenocysteine that could then be misincorporated into proteins, causing misfolding and impaired cellular function (Hladun et al., 2013a). It is likely that selenium toxicity in honey bees is mediated in similar ways. Therefore, the specific mechanisms of the selenium-induced behavioral impairments we observed likely depend on the form of selenium to which the honey bees were exposed.

Selenium toxicity is likely acting in a non-selective manner. So, the specific impairments caused by excess selenium would be influenced by which tissues and organs are exposed to selenium containing compounds. Also, selenium would have a greater impact on organs that are more susceptible to and are less able to repair selenium-induced damage. It may be that the nervous system is simply one of the first organ systems to suffer irreparable damage and thus exhibit impaired functionality. Alternatively, non-neural peripheral toxic effects may also be sources of the selenium induced behavioral impairments through induction of malaise, or compromising function of organs playing a supportive role in brain function and health. Further studies that examine which organs and tissues in the bee are damaged following exposure to selenium and the correlation of this damage with the organ/tissue selenium content are needed to determine the exact mechanism mediating selenium-induced behavioral impairments in our experiments.

Selenium induced learning and memory impairments could impact honey bees' ability to function as pollinators and maintain healthy colonies. While foraging, honey bees must be able to quickly learn the locations and odor profiles of flower patches, from which they gather the nectar and pollen required for colony survival. Disruption or impairment of learning and/or memory could significantly impair the foragers'

efficiency in gathering these resources and their ability to pollinate the many crops depending on them for good productivity.

As our awareness of these environmental contaminants increases, it is becoming increasingly apparent that we must further our understanding of the harm caused by the plethora of toxins, diseases, pests, etc. that are challenging honey bee populations worldwide. Recent studies have detected the presence of multiple pesticides, heavy metals, and metalloids in honey bee colonies throughout the U.S. and Canada, some of which are already known to have negative impacts on honey bee behavior at the detected concentrations (Mullin et al., 2010; Pettis et al., 2013; Søvik et al., 2015). However, for most of the pesticides and other toxic chemicals present in the honey bees' environment, there is still little known about how sublethal levels of these chemicals affect the behavior of honey bees and other pollinators.

Identifying changes in behavior caused by a toxin will allow us to identify the sublethal concentrations at which honey bees first become impaired so we can work toward sufficiently cleaning highly contaminated areas and setting safe limits for toxin and pesticide presence in the environment. There may be interactions between these toxins and other challenges to honey bee health that could augment the influence the individual toxin or disease has on behavior and colony survival, so furthering our understanding of these potential synergistic relationships is of great import as well.

CHAPTER 4

LOCALIZING SELENIUM BIOACCUMULATION FOLLOWING CHRONIC EXPOSURE

INTRODUCTION

Honey bees (*Apis mellifera*) are important pollinators in the agricultural industry, contributing to the productivity of 70% of food crops (Gallai et al., 2009; Klein et al., 2007). Over the past seven decades there has been a significant decline of honey bee populations in multiple regions around the world through reduction in number of beekeepers and increased colony loss (Berenbaum, 2014; Ellis et al., 2010; Pettis and Delaplane, 2010; vanEngelsdorp and Meixner, 2010). Because of this population decline, there is heightened interest in factors influencing honey bee survival (Berenbaum, 2014; Dennis and Kemp, 2016; Kluser et al., 2010; Staveley et al., 2014). In addition to natural challenges – like predators, weather, and resource availability – honey bees face many human-generated factors that negatively influence their survival. One of these factors is the accumulation of toxic chemicals in the environment from agricultural, mining, industrial, and urban generated waste (Chen et al., 2013; Guo et al., 2012; Kabir et al., 2012; Z. Li et al., 2014; Rajaram and Das, 2008; Roberts and Johnson, 1978; Viglizzo et al., 2011).

Selenium is one toxin that has become a concern in multiple regions, including the western United States. Excess selenium is released into the environment during ore extraction and refining, fossil fuel extraction and burning, heavy agricultural irrigation,

fertilizer production, and other industrial processes (Lakin, 1972; Mehdi et al., 2013; Zhang et al., 2014). In areas contaminated with toxic levels of selenium, plants can accumulate high levels of this element in their floral tissues (Lakin, 1972; Mehdi et al., 2013; Quinn et al., 2011). Honey bees then collect the contaminated nectar and pollen and take it to the colony. As a result, the whole colony is exposed to potentially toxic levels of selenium.

If sufficiently high quantities are ingested, selenium is lethal to honey bees (Hladun et al., 2012). However, there is little known about how sublethal levels of selenium may impact honey bee behavior. It is important to understand the effects of sublethal toxicity since these potential selenium-induced behavioral changes could indirectly impact the health and survival of honey bee colonies in areas that are not currently regarded as sufficiently contaminated to be of concern. Additionally, selenium may interact synergistically with other toxins, augmenting the magnitude of the sublethal effects, as has been shown with some mixtures of toxic metals and combinations of various pesticides (Chu et al., 2002; Zhu et al., 2014).

Acute selenium exposure impairs learning and memory in worker honey bees. Burden, et al (2016) assessed the effect of acute sublethal selenium exposure on adult honey bee odor learning and memory in a laboratory setting. They found that a single sub-lethal dose (18 ng) of sodium selenate, an inorganic form of selenium found in plants, reduced performance on an odor learning task. A single dose (18 ng) of methylseleno-L-cysteine, an organic form of selenium also found in plants, did not impair odor learning. However, bees treated with either selenium compound, showed reduced long-term recall of the odor learning task 24-hour following conditioning.

Though behavioral impairments resulting from sublethal selenium toxicity have been documented in honey bees, there is little currently known about the underlying pathophysiology. In order to discover the pathophysiology of selenium toxicity, it is relevant to know where selenium accumulates in the honey bee. From localization of accumulated selenium, we can infer possible mechanisms mediating the behavioral impairments and form testable hypotheses for further investigation of these pathophysiological mechanisms. The distribution of selenium accumulated in honey bees has only been assessed in bees feeding on plants growing in selenium contaminated soil (Quinn et al., 2011). These studies confirm that honey bees bioaccumulate selenium from feeding on these flowers. However, multiple forms of selenium are typically present in floral tissues, and the potential for these studies to provide specific information on the metabolism and bioaccumulation of specific forms of selenium is limited. This prevents the identification of the pathophysiological mechanisms associated with ingestion of each of the selenium forms.

To identify potential mechanisms for the pathophysiology of selenium toxicity, we quantified and localized the accumulation of known molecular forms selenium in honey bees following exposure to selenium contaminated food. The selenium fed to the bees was of known molecular forms, so we could assess the potential differences in the degree of accumulation and the distribution of the accumulated selenium, We exposed the bees to two forms of selenium: Sodium selenate, which is a prevalent inorganic form of selenium found in plant tissues, and methylseleno-L-cysteine, a prevalent organic form of selenium found in plant tissues. Because we fed the animals known molecular forms of selenium, we could identify whether the honey bee tissues were

metabolizing the excess of each form of the ingested selenium and which molecular form of selenium was incorporated into tissues. We quantified the amount of selenium that accumulated in the honey bees exposed to each form of selenium to estimate the concentration of selenium in the bees used for localizing selenium bioaccumulation. We scanned whole bees using micro scanning x-ray fluorescence (μ-SXRF) mapping to determine which tissues and organs significantly accumulated selenium and used micro x-ray absorption near edge spectroscopy (μ-XANES) to determine the molecular speciation of the bioaccumulated selenium.

METHODS

Animals and chronic selenium exposure

Worker honey bees from colonies with open-mated New World Carniolan queens were used in these experiments (Cobey 1999). The queens were purchased from commercial honey bee breeders in northern California. Approximately 200 newly-emerged worker honey bees were collected and housed in plastic and wire mesh cages for 7 days. During this time each cage was in an incubator maintained at 34 °C and 60% relative humidity. The bees were provided with *ad lib* access to deionized water, 1 M sucrose, and a pollen-sucrose patty consisting of 20 parts ground pollen and 7.5 parts 1 M sucrose. For the selenium exposed groups, the sucrose solution and the pollen patty were both contaminated with a final concentration of 6 mg/kg sodium selenate or methylseleno-L-cysteine. This concentration of selenium is within the range of concentrations honey bees are exposed to when feeding on flowers grown in selenium contaminated soils, and it has been shown to be sublethal in honey bees (Burden et al.,

2016; Hladun et al., 2012). Overall survival for each treatment group was determined at the end of the 7-day exposure period and compared to control bees housed in the same conditions. The differences in the number of survivors in each group was analyzed using a Pearson's Chi-square test (IBM SPSS version 23). The bees that survived the selenium exposure were frozen at −80 °C until they were used in further analyses.

Quantifying accumulation of selenium following chronic exposure

To quantify the accumulation of selenium in honey bees chronically exposed to selenium-contaminated food, we used inductively coupled plasma – optical emissions spectroscopy (ICP-OES). Honey bees were dried in a drying oven at 80 °C for 5 days. They were weighed and replaced in the drying oven for an additional 2 days. Then, they were weighed a second time to confirm that they had reached a consistent dry weight. Up to 19 bees were pooled to generate each 0.5 g sample. The samples (n = 9) were digested in 20 ml Teflon-lined vessels containing 5 ml concentrated nitric acid at room temperature for approximately 24 h followed by microwave digest at 568 W for 30 min using a MARS microwave oven (CEM, Matthews, NC). The digested samples were diluted in nanopure water and analyzed using the iCAP6300 ICP Optical Emission Spectrometer (Thermo Scientific, Waltham, MA). Selenium recovery for this digestion protocol was verified with a National Institute of Standards and Technology tissue standard 1566B (oyster tissue). Recovery of the selenium from the oyster tissue was >99%.The differences in selenium content in the pooled samples was analyzed using a one way analysis of variance (ANOVA; IBM SPSS version 23).

Distribution of selenium accumulation following chronic exposure

To map the distribution of selenium accumulation in the honey bees following chronic exposure we analyzed the bees with micro scanning x-ray fluorescence (μ-SXRF) mapping at the Lawrence Berkeley National Laboratory Advanced Light Source (Beamline 10.3.2). Frozen samples were mounted on a Peltier stage cooled to −25 °C to prevent thawing and reduce beam damage. μ –SXRF maps were constructed using a scanning beam energy of 13 keV with a 200 μm × 50 μm or 50 μm × 20 μm beam and a 20 μm × 20 μm pixel size. The dwell time was 50 ms. Selenium K-edge micro x-ray absorption near edge spectroscopy (μ-XANES) was used to probe the molecular speciation of the selenium at specific points where the samples showed significant accumulation of the metalloid. Five bees from the sodium selenate treatment group and five bees from the methylseleno-L-cysteine treatment group were used for selenium mapping. A control bee was also scanned for comparison. Three out of the five bees were also used to probe the molecular speciation of the selenium at several points showing substantial accumulation.

RESULTS

Survival following chronic selenium exposure

Newly emerged bees were chronically exposed to selenium contaminated food for 7 days. Chronic exposure to 6 mg/kg sodium selenate or methylseleno-L-cysteine

significantly increased mortality during the 7 days of treatment compared to control bees (Pearson Chi-square: $\chi^2 = 41.254$, df = 2, $p < 0.001$; Figure 4.1A).

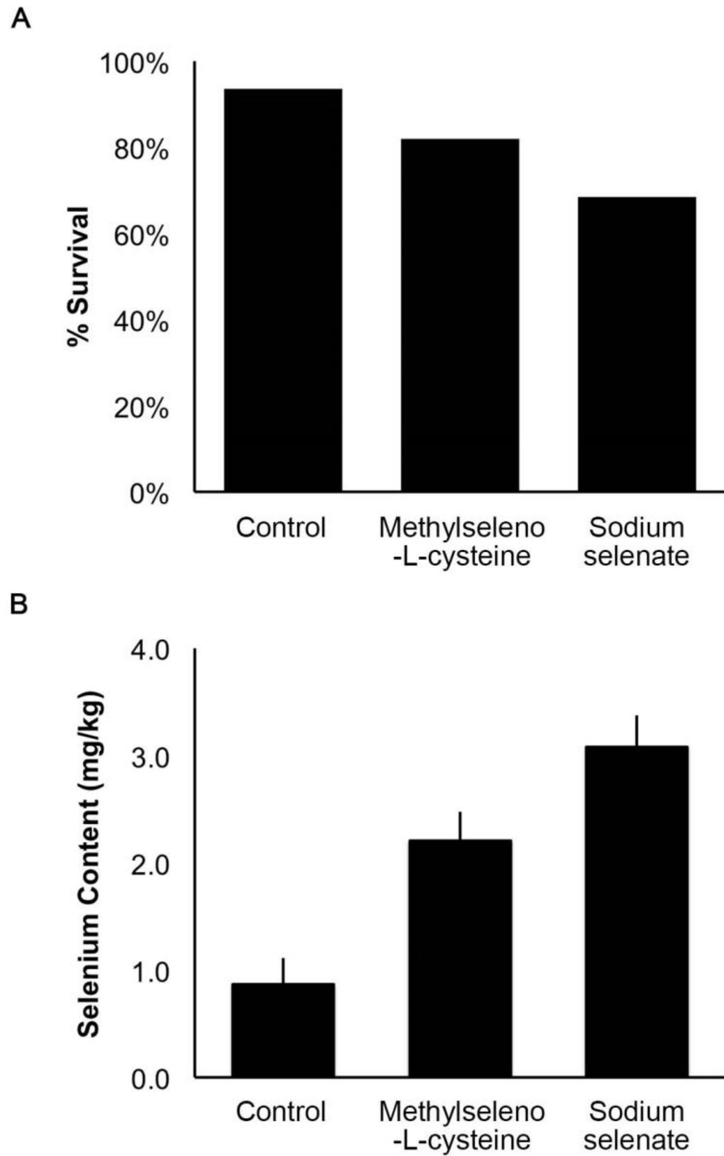


Figure 4.1. The survival of bees chronically exposed to selenium-contaminated food and the quantification of selenium accumulation following exposure. A. The % survival of newly-emerged bees exposed to sucrose and pollen contaminated with 6 mg/kg

methylseleno-L-cysteine or 6 mg/kg sodium selenate for 7 days (n = 200 bees / treatment group). B. The quantification of selenium accumulation to newly emerged bees chronically exposed to food contaminated with 6 mg/kg selenium for 7 days (n = 9). Error bars represent ± 1 Standard deviation from the mean.

Quantifying accumulation of selenium following chronic exposure

We used ICP-OES to quantify the accumulation of selenium in bees chronically exposed to contaminated food. Both groups of bees chronically exposed to selenium accumulated significantly more selenium in their bodies than controls bees (ANOVA, $F = 51.386$, $df = 2$, $p < 0.001$; Figure 4.1B). Controls bees had an average of 0.87 mg selenium per kg dry tissue weight. In bees chronically exposed to methylseleno-L-cysteine, selenium accumulated to an average concentration of 2.21 mg per kg dry tissue weight. Bees exposed to sodium selenate accumulated more selenium in their bodies than bees exposed to methylseleno-L-cysteine, with an average concentration of 3.08 mg per kg dry tissue weight.

Mapping the distribution of selenium accumulation following chronic exposure

μ-SXRF mapping revealed differential accumulation of selenium in bees chronically treated with sodium selenate and methylseleno-L-cysteine (Figure 4.2). Three of the five bees analyzed from each of the selenium-exposed groups are shown in the figure. Specific points showing selenium accumulation were probed using μ-XANES for the speciation of selenium in those tissues.

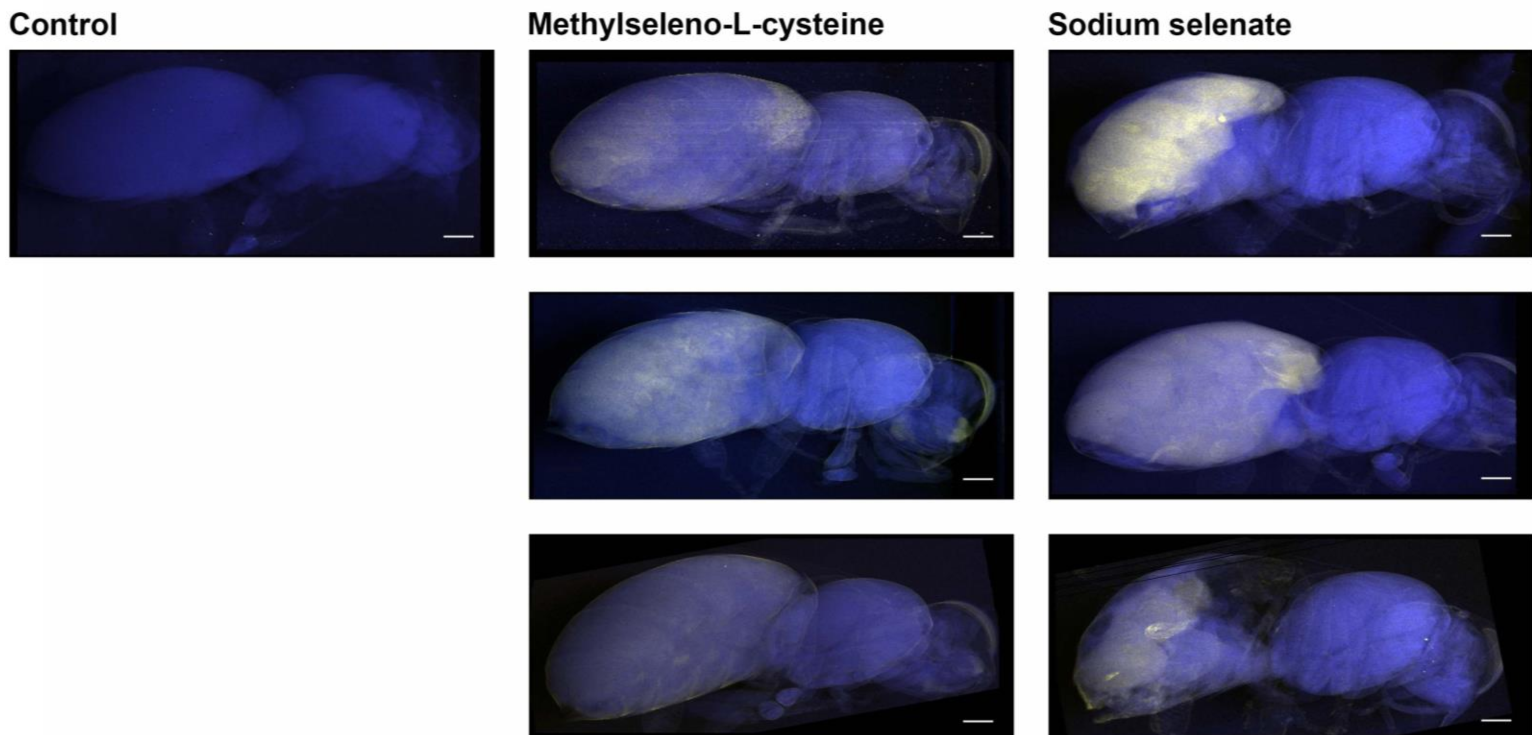


Figure 4.2. μ-SXRF maps of selenium accumulation in newly emerged bees chronically exposed to 6 mg/kg selenium for 7 days. The blue color represents the elastic scatter of the x-ray beam and shows a general outline of the bees' bodies. The areas in yellow and white represent areas with selenium accumulation above the detection limit. Scale bars represent 1 mm.

In bees chronically exposed to sodium selenate, the detectable selenium accumulation was restricted to the abdomen. Most of the detectable selenium was located in structures that correspond to the rectum of the bees' intestinal tract. Some bees also had patterns of selenium accumulation in structures that resemble malpighian tubules or trachea. μ-XANES analysis revealed that in all the locations probed for selenium speciation approximately 50% (range = 44-60%) was in the ingested form, selenate. The remaining selenium detected was in an organic molecule that is most likely selenomethionine (range = 40-56%). One of the bees analyzed was an exception to this pattern. A form of selenium most likely to be selenomethionine accounted for 55% of the total selenium detected in this bee, and only 24% of the selenium was in the form of selenate. The remaining 21% of the total selenium was in a molecular form referred to as grey selenium.

In bees chronically exposed to methylseleno-L-cysteine, a large portion of the accumulated selenium was located in the abdomen. In contrast to the bees exposed to sodium selenate, the distribution of accumulated selenium in methylseleno-L-cysteine treated bees was more diffuse and evenly dispersed in the abdomen. This accumulation was likely either in the hemolymph or in the fat body, which lines much of the inner surface of the abdomen. In some of these bees, there were areas within the abdomen that had higher selenium content and a globular appearance, which is consistent with accumulation in the fat body. In addition to accumulation of selenium in the abdomen, bees chronically exposed to methylseleno-L-cysteine also showed accumulation in the outer rim of the eye and in triangular globular structures in the head that are likely the salivary glands. For all of the bees analyzed and all of the specific points in the abdomen,

head, and eye that were probed for the molecular speciation of selenium, 100% of the selenium was in the organic form selenomethionine.

DISCUSSION

Determining the pathophysiology of behavioral modifications caused by sublethal selenium exposure in honey bees is reliant on knowing the distribution of bioaccumulated selenium in the animals' tissues and the molecular form of the accumulated selenium. We show that the quantity and localization of selenium bioaccumulation differs with the molecular form of selenium ingested following chronic exposure to contaminated food. The bees fed sodium selenate accumulated a significantly higher quantity of selenium than those fed methylseleno-L-cysteine. It may be that methylseleno-L-cysteine contaminated food may be distasteful to the bees after several days of exposure, causing them to consume less of the selenium than bees fed sodium selenate contaminated food. However, the bees fed the organic methylseleno-L-cysteine showed a wider distribution of the selenium in the honey bees' bodies than those fed inorganic sodium selenate. Once ingested and absorbed, methylseleno-L-cysteine apparently is more readily incorporated into the tissue.

From the μ-XANES analysis, the three forms of selenium we detected in the honey bees were grey selenium, selenate, and selenomethionine. Grey selenium was found in one spot scanned in one of the bees chronically exposed to sodium selenate. The presence of grey selenium in this bee is likely due to beam damage during the μ-XANES scan or misidentification of the selenium species during analysis of the scan data. Selenate was found only in the bees fed sodium selenate. Since detectable selenate

accumulation was primarily restricted to an area of the abdomen that corresponds to the rectum, it may be that this represents a portion of the selenate that was not absorbed from the gut and metabolized. Selenomethionine was one of the predominant molecular forms of selenium found in bees fed sodium selenate and the only form found in those fed methylseleno-L-cysteine. It has been shown in other animals that both forms of selenium are metabolized into selenomethionine (Letavayova et al., 2006). It appears that honey bees may more readily metabolize methylseleno-L-cysteine into selenomethionine than they do selenate, perhaps because there are fewer intermediate forms between methylseleno-L-cysteine and selenomethionine than between selenate and selenomethionine.

In other animals selenomethionine is randomly incorporated into proteins in place of methionine (Letavayova et al., 2006). This may cause protein misfolding, alter enzyme function, and may interfere with the interactions between the proteins and other biomolecules (Jackson and Combs, 2008). It is likely that a similar process is occurring in honey bees. In bees fed toxic levels of methylseleno-L-cysteine, selenomethionine is incorporated into proteins in the eye and salivary glands as well as what appears to be the fat body in the abdomen. These structures may have a greater concentration of proteins with high methionine content and/or a more rapid turnover of those proteins, allowing more selenomethionine to be incorporated into the tissue. In vertebrates some of the excess selenium is stored in the liver, after being processed and bound to the protein albumin (Mehdi et al., 2013). Similarly, the fat body in honey bees may also be sequestering the excess selenium to reduce the damage from selenium toxicity as occurs. The bees fed sodium selenate may also have some accumulation of selenomethionine in

these tissues but the reduced metabolism of the selenate into selenomethionine may result in less accumulation in these tissues.

Interestingly, there was no detectable selenium accumulation in the brain or ventral nerve cord of the honey bees exposed to sodium selenate or methylseleno-L-cysteine. An acute sublethal dose of selenium caused significant impairments in learning and memory impairments within 3 h (Burden et al., 2016). The absence of selenium accumulation in the brain makes it less probable that direct neurotoxicity in the central nervous system is a sufficient explanation for the learning and memory impairments observed following an acute sublethal dose of selenium. Rather the results are more likely attributable to a peripheral mechanism of selenium toxicity.

Several peripheral mechanisms are compatible with the behavioral impairments and selenium distributions observed. Selenate, the ingestion of which caused the greatest and most rapid decrease in learning and memory performance (Burden et al., 2016), appeared to primarily remain in or near the rectum, indicating that it may not have been absorbed from the gut. One way that selenium located in the gut may be influencing learning and memory performance is by altering the gut microbiome. In honey bees, the health of the gut microbiome in individual bees has been linked to the health and survival of the whole colony and its resistance to disease or parasite infestation (Hamdi et al., 2011). Also, in mammals, antibiotic-mediated alterations of the gut microbiome caused a depression in learning performance (Cryan and O'Mahony, 2011; Li et al., 2009). Administration of probiotics, to repopulate the gut microbiome was sufficient to alleviate learning and memory impairments induced by a bacterial infection in the gut (Gareau et al., 2011). It is possible that the health of the gut microbiome has an effect on learning

and memory in honey bees as well, and that selenium toxicity is able to induce that microbiota alteration.

Alternatively, enteroendocrine cells in the lining of the gut may be directly detecting the presence of selenium in the gut lumen or detecting the alterations of the gut microbiome and/or other gut contents caused by selenium ingestion (Bohórquez et al., 2015). Since at least in mammals, these cells appear to communicate electrochemically with neurons, information on gut contents or microbiome status may be relayed to the central nervous system and be altering neural circuit function (Bohórquez et al., 2015). It is possible a similar mechanism is mediating the physiological responses to toxin ingestion in honey bees too (Hurst et al., 2014).

Also, the toxin may be causing oxidative damage to peripheral tissues both in the gut and following absorption from the gut lumen. This is more likely in the bees fed sodium selenate, since ingestion of inorganic forms of selenium is more likely to increase oxidative damage in animals than are organic forms (Drake, 2006; Mézes and Balogh, 2009; Spallholz, 1994; Stewart et al., 1999). The significant transformation of both ingested forms of selenium into selenomethionine may be a method for reducing the toxic potential of the selenium (Mézes and Balogh, 2009; Stewart et al., 1999). And, this suggests that if oxidative damage is involved in selenium toxicity pathophysiology in honey bees, it plays a lesser role than other potential mechanisms.

Chronic effects of sublethal selenium toxicity may also be partially attributable to the significant incorporation of selenomethionine into proteins, as discussed above (Jackson and Combs, 2008; Letavayova et al., 2006). The consequential alterations in protein function may cause a reduction in the functionality of other peripheral organs.

This diminished peripheral functionality could trigger a multitude of pathologies, depending on the organ(s) affected and the degree of communication from these peripheral tissues to the central nervous system.

Any of these peripheral mechanisms could be altering the motivation or ability to learn, recall the learned task, or express the associated behavioral response. One convincing mechanism for linking these peripheral physiological effects of selenium ingestion to alterations in central nervous system function is the induction of a state of post-ingestional malaise. Following ingestion of substances known to induce malaise in other species (lithium chloride, quinine, or amygdaline) honey bees exhibit a reduction in locomotion and in performance on long-term recall of an olfactory learning task (Ayesteran et al., 2010; Wright et al., 2010). Malaise may be reducing overall motivation to feed, resulting in lower responsiveness towards a food cue. Or, malaise may be mediating a conditioned aversion towards the odor used during conditioning and a consequential devaluation of the expected food reward during recall test trials.

These mechanisms may involve dopaminergic and/or serotonergic signaling in the brain (Ayesteran et al., 2010; Wright et al., 2010). In honey bees, conditioned aversion toward the conditioned odor or the malaise inducing substance could reduce dopaminergic or increase serotonergic signaling, which has been shown to be involved in the valuation of positive rewards, like food (Ayesteran et al., 2010; Wright et al., 2010). The response profiles during the recall phase of the learning experiment from work by Ayesteran, et al (2010) is similar to the types of impairments observed following selenium ingestion (Burden, et al 2016). Therefore, it is probable that selenium ingestion

is also inducing a form of post-ingestional malaise and subsequently conditioned taste aversion to the selenium compounds.

Our work has raised several hypotheses for how sublethal selenium toxicity is causing learning and memory impairments in honey bees and in general. It is especially interesting to note that these mechanisms are most likely caused by mechanisms in peripheral organs and tissues rather than direct neurotoxicity to the central nervous system. Testing these hypotheses to further understand the pathophysiology of selenium toxicity is of value. In addition to better understanding selenium toxicity, specifically, these future directions of research would further our knowledge of metalloid and metal toxicity in general since the mechanisms of selenium toxicity likely are similar in many respects to the toxic effects of other metalloids and metals. This further research will also highlight the neural mechanisms mediating the effects of the status of peripheral organs and tissues on the function of higher order processing in the central nervous system and help us better understand how an organism can successfully interact with the challenges its environment.

CHAPTER 5

EFFECTS OF DNA METHYLTRANSFERASE INHIBITION ON LONG-TERM BEHAVIOR CHANGES

INTRODUCTION

DNA methylation is a mechanism that can underlie experience-dependent behavioral plasticity in adult animals. For example, in mammals, DNA methylation is shown to play an important role in the long-term behavior changes associated with memory consolidation, cocaine addiction, and chronic defeat stress (Elliott et al., 2010; LaPlant et al., 2010; Miller and Sweatt, 2007; Miller et al., 2010, 2008). Genes associated with these behaviors showed alterations in DNA methylation patterns following the behavioral change. And, in each of these cases, behavioral changes were attenuated when DNA methyltransferases (DNMTs), one class of enzymes that modulate DNA methylation, were inhibited or knocked down. In honey bee (*Apis mellifera*) workers, DNA methylation is known to be involved in two forms of long-term behavioral plasticity: (1) Memory formation and (2) the transition from the in-nest nurse duties performed by young workers to the foraging duties typically performed by older bees (Biergans et al., 2015, 2012; Herb et al., 2012; Lockett et al., 2010; Robinson, 1987).

Honey bees' memory can be tested using associative olfactory conditioning and extinction training. Associative olfactory conditioning consists of repeated pairing of an odor with sucrose reinforcement. The animal learns to associate the odor with the sucrose reinforcement and begins responding to the odor by extending its proboscis to feed prior

to presentation of the sucrose (Bitterman et al., 1983; Giurfa and Sandoz, 2012; Smith and Burden, 2014). If following olfactory conditioning the conditioned odor is presented multiple times without reinforcement, the response to the conditioned odor will diminish through a process of extinction learning (Bitterman et al., 1983). Over time the conditioned response normally recovers, which is one argument that extinction is a form of learning that also involves memory consolidation (Gil et al., 2007).

When DNMT inhibitor treatment is coupled with such testing, memory recall in honey bees is significantly altered. Topical treatment with the DNMT inhibitors zebularine or RG108 prior to conditioning causes an elevation in the responses to novel odors when tested 24 h following olfactory conditioning (Biergans et al., 2015, 2012). This increase in generalization of the odor memory to novel odors indicates a reduction in the specificity of the memory to the conditioned odor. In honey bees topically treated with zebularine, extinction learning is either enhanced or impaired depending on when the treatment occurs (Gong et al., 2016; Lockett et al., 2010). Recovery of the conditioned response following extinction is elevated with treatment occurring either before or after initial conditioning, indicating that inhibition of DNMTs results in an attenuation of the memory of extinction training. Furthermore, RG108 treatment resulted in a significant decrease in global DNA methylation and increased the relative expression of genes known to be involved in learning and memory (Biergans et al., 2015).

These studies show that inhibition of DNA methylation affects mechanisms of olfactory memory formation and/or recall in honey bee workers. However, only a single dose of DNMT inhibitor was used in the experiments. The specific dose administered of any drug, including DNMT inhibitors, can have a significant effect on the behavioral

outcome of learning and memory experiments, even to the extent of reversing the behavioral effect of the drug for some doses (Davis and Svendsgaard, 1990). For example, u-shaped dose response curves, in which the drug has a different effect on an animal's behavior at moderate doses than at low or high doses, are commonly observed in learning and memory studies (Davis and Svendsgaard, 1990). Therefore, the range of behavioral responses to different drugs and different levels of treatment with the same drug can reveal important information about the role the targeted mechanism plays in memory formation and the behavioral expression of those memories. To date studies of memory consolidation in honey bees have used only single dosages, so it is not known how varying the dose of DNMT inhibitor may affect memory consolidation.

DNA methylation is also involved in the worker bees' nurse-to-forager transition. During its lifetime, each worker bee shifts from performing nurse duties – such as queen and brood care, honey processing, and nest maintenance – and begins flying out to forage for nectar, pollen, and other resources required by the colony. Widespread physiological changes occur during this transition, which are correlated with changes in expression of many genes involved in neural plasticity, metabolism, and hormone regulation (Hernández et al., 2012; Huang et al., 1994; Maleszka et al., 2009; Robinson, 1987; Whitfield et al., 2006, 2003). Analysis of DNA methylation patterns in nurses and foragers revealed that there are also widespread differences in DNA methylation between these two groups, including in many genes known to be differentially expressed in nurse and forager bees (Herb et al., 2012). Though worker bees complete this behavioral transition as they age, these differences in gene expression and in DNA methylation

patterns are more strongly correlated with the behavioral role than the chronological age of the bee (Lockett et al., 2012).

However, a causal relationship between the different DNA methylation patterns associated with the nurse or forager state and the occurrence of the nurse-to-forager transition has yet to be established. But, it has not yet been determined if the differential DNA methylation patterns in nurses and foragers are active components of the mechanisms that drive and/or stabilize the transition or if the nurse-to-forager transition drives the alterations to the methylome. It is also not known whether the methylation of loci showing differential methylation between nurses and foragers is altered during the transition or if it is static within an individual. Showing that inhibiting DNMT activity causes acceleration or delay in the timing of this behavioral transition would provide evidence that the DNA methylation status is dynamically regulated as it participates in driving and/or stabilizing this behavioral transition. If the difference in methylation of a CpG between nurses and foragers is static, there is the question of the origin of these differences and how they function in the nurse-to-forager transition.

Overall, we need to better understand the relationship between DNA methylation and experience-dependent behavioral plasticity. This is important when using pharmacological means and when discussing cause-effect relationships, as exemplified by the work in the honey bee. Thus, we first investigated how the behavioral outcomes of an olfactory conditioning and memory recall paradigm are affected by the DNMT inhibitor RG108 administered over a dose response curve. Thereafter, we studied whether the timing of the nurse-to-forager transition is susceptible to alteration via DNA methylation inhibition by zebularine and RG108 treatment.

METHODS

Experiment 1. The effect of DNMT inhibitor treatment on learning and long-term memory

Animals

For this experiment we used worker bees from colonies with open-mated New World Carniolan queens (Cobey 1999). The queens from these colonies were purchased from commercial honey bee breeders in northern California. All colonies were housed and regularly maintained in a sheltered area on the Tempe campus of Arizona State University.

Olfactory conditioning and memory tests

Forager honey bees returning from a foraging flight were captured at the hive entrance, marked with a small spot of enamel paint (Testors, Vernon Hills, IL), and released back into the colony. Three days later, we collected the marked foragers, which by then had at least 3 days of foraging experience. These bees were briefly anesthetized on ice, restrained in simple harnesses (Smith and Burden, 2014), fed 3-5 µl 1 M sucrose, and placed in a humidified plastic box to acclimatize to the harnesses for approximately 1 h. The bees were then divided into 5 treatment groups, and 0.5 µl RG108 (Concentrations: 10 µM, 100 µM, or 1000 µM; Sigma-Aldrich, St. Louis, MO) in insect saline (0.13 M NaCl, 0.007 M $CaCl_2$, 0.006 M KCl, 0.002 M $MgCl_2$, 0.16 M Sucrose, 0.025 M Glucose, 0.01 M Hepes, 0.02 M Ascorbic Acid, pH 6.7, 500 ± 5 mOsm/L) or saline alone was injected into the head capsule through the median ocellus 60-90 min prior to beginning conditioning. The order in which the treatment groups were treated with RG108 was randomized to minimize bias in responses arising from the amount of

time between capture and conditioning and between injection and conditioning. Just prior to beginning conditioning, the bees' sucrose responsiveness was tested by stimulating their antennae with a small drop of 1 M sucrose. Only bees exhibiting the proboscis extension response (PER) in response to this stimulation were used in the experiment, as they were likely sufficiently motivated to learn the task.

Olfactory PER conditioning consisted of 6 trials of either 0.2 M 1-hexanol or 2-octanone (Sigma-Aldrich, St. Louis, MO). All odors were diluted in heavy mineral oil (Sigma-Aldrich). The choice of which odorant was used as the conditioned odor was alternated across repetition of the experiment. Odor cartridges consisted of a 1 cc glass syringe barrel (BD Medical, Franklin Lakes, NJ) with a small strip of filter paper (Sigma-Aldrich, St. Louis, MO) inside and a small piece of silicone tubing (Cole-Parmer, Vernon Hills, IL) placed in the wide end of the barrel to constrict the opening. 10 μl of an odor was placed on the filter paper in each odor cartridge. The odors were puffed onto the bees' antennae by directing airflow through the odor cartridge. During odor stimulation, an airstream (~400 ml/min) was directed through the odor cartridge for 4 s. A DirectSoft 05 PLC (Automation Direct, Cumming, GA) triggered a 3-way solenoid valve (The Lee Co., Westbrook, CT) to control airflow through the odor cartridge. An exhaust port directly behind the bees' location in the conditioning arena removed odor-laden air from the conditioning arena to allow for discrete odor stimuli.

During a conditioning trial, the bee was placed in the conditioning arena and exposed to single odor stimulus forward-paired with a 0.4 μl 1 M sucrose reward offered 3 s after odor onset. The sucrose reward was presented using a Gilmont syringe (Cole-Parmer, Vernon Hills, IL). The inter-stimulus interval was 6 min. The presence or

absence of PER exhibited by each bee during a conditioning trial was recorded using a binary scoring system. A positive score was recorded if the bee extended its proboscis beyond an imaginary line drawn between the tips of its opened mandibles during odor stimulation and before presentation of the reward. A negative score was recorded when the failed to bee showed PER or only showed PER outside of the timeframe just described. Following conditioning each bee was fed to satiation with 1 M sucrose and placed in a humidified plastic box overnight.

Approximately 24 h following conditioning, each bee was exposed to 3 unreinforced odor trials to test their recall of the conditioned odor and the degree of generalization exhibited to two novel odors. One novel odor was either 0.2 M 1-hexanol or 2-octanone, the odor not used as the conditioned odor. Bees exhibit significant generalization between these odors (Guerrieri et al., 2005). The second novel odor was 0.2 M 2-nonanol, which is more perceptually distinct from the other two odors, and therefore fewer bees generalize their response to this odor than to the perceptually similar novel odor. Test trials were identical to the conditioning trial except for the absence of the reward. Also the bees were given a 10 s time window in which to respond to the odor stimulus beginning with odor onset. Immediately after the recall test, the bees were once again tested for their sucrose response by stimulating their antennae with 1 M sucrose to assess whether their motivation level alone could explain their response profile during test trials.

Statistical analysis

All statistical analyses were performed in SPSS Statistics v. 23. The bees' responses to conditioning trials were analyzed using generalized estimating equations with a logistic link function (Logistic GEE). The predictors in the logistic GEE equations were the concentration of RG108 (DOSE), the trial number (TRIAL), and the square of the trial number ($TRIAL^2$). The variation resulting from the repeated testing of individuals during conditioning was accounted for by the within subject variable BEEID. In the logistic GEE model for the long-term test recall data, we added one further variable, LEARNSCORE, accounted for differences in overall responsiveness during the test trials that could be attributed to the number of conditioning trials to which each bee responded. We included all main effects terms and all 2-way interaction terms in the models. Those interaction terms that were not significantly related to the probability of bees responding to the conditioning and test trials were subsequently removed from the model. The reduced models are shown below. The logistic GEE analysis was repeated with a reduced data set in which the bees that did not respond to any conditioning trial were removed. We analyzed the responses to the long-term test trials of bees that exhibited at least one response during conditioning in order to better assess the effect of RG108 treatment on long-term recall in bees that showed evidence of learning the task.

Experiment 2. The effect of DNMT inhibitor treatment on the nurse to forager transition

Animals

For this experiment, we used worker bees from colonies of a low pollen collecting strain of honey bees (*Apis mellifera*) that was developed by Page and Fondrk, (1995).

This strain has been used to study the genetic bases of division of labor, foraging preferences, and other aspects of honey bee behavior (Ihle et al., 2010; Page et al., 1995; Schulz et al., 2004). We used this strain of bees so our results would be comparable to an earlier study of DNA methylation in nurse and forager honey bees (Herb et al., 2012).

DNMT inhibition and behavioral observations

Three colonies were examined for workers placing their heads inside a cell containing brood for brood feeding and cleaning, which is characteristic of nursing (Winston, 1987). Because the differences in DNA methylation patterns seen in nurses and foragers is highly correlated with their behavioral role irrespective of chronological age (Lockett et al., 2012), we did not control for age in this experiment. Identified nurse bees were collected, briefly cold-anesthetized, and divided into 3 treatment groups (n = 218-265 / treatment). The members of each treatment group were marked with the same color of enamel paint on the dorsal abdomen. The bees were treated topically with 0.5 μl 10 mM RG108 (Sigma-Aldrich, St. Louis, MO), 10 mM zebularine (Tocris Biosciences, Bristol, UK), or the vehicle DMSO (Sigma-Aldrich, St. Louis, MO) on their dorsal thorax. After recovering from the anesthesia, the bees were placed in a single host colony.

The foragers were then removed from the host colony by rotating the hive entrance 180° and placing it on top of a second hive box, which was oriented exactly as the host colony's original orientation. Foragers originally from the host colony entered the second hive box instead of the host colony upon returning to the hive location after a foraging flight. This left only the nurse bees and the queen in the original colony. At the

end of the day the hive box containing the foragers was removed. This removal of foragers from a colony causes a portion of the nurse bees to begin foraging precociously (Huang and Robinson, 1996).

Two days later we began observing the colony entrance daily, from 0830 h to 1230 h, to monitor the initiation of foraging behavior in the treated bees. The number of bees from each treatment group returning to the colony was recorded. These returning bees were captured, checked for a nectar or water or pollen load, and placed on ice. A number of nurse bees equal to the number of collected foragers from each treatment group were also collected. The brains were dissected and stored in 80% ethanol until they were used for DNA methylation analysis.

DNA methylation analysis

Genomic DNA was extracted from 10 individual brains in each treatment group, bisulfite converted, and sequenced as in Herb, et al (2012). Briefly, DNA extraction was performed using the Masterpure kit (Epicentre). Bisulfite conversion was accomplished using the Zymo DNA-Methylation Gold kit. Regions of the genome containing four CpGs, which were previously shown to be differentially methylated in nurses and foragers (Herb et al., 2012) , were then amplified using nested PCR. Methylation levels were quantified using the Biotage PSQ HS96 pyrosequencer. The percent methylation for the four CpGs was calculated with Q-CpG methylation software (Biotage).

Statistical analysis

All statistical analyses were completed in SPSS Statistics v. 23. The relative methylation levels of each CpG in individuals from the treatment groups were analyzed using a Kruskal-Wallis test. Pairwise post hoc comparisons were completed using the Mann-Whitney U test when appropriate.

RESULTS

Experiment 1. The effect of RG108 treatment on learning and memory consolidation

All Bees

During conditioning, the % PER exhibited by all treatment groups increased over the trials for the data set including all bees used in the experiment (Figure 5.1A, Table 5.1). There was a significant effect of DOSE on the percentage of bees responding to the conditioning trials. The bees treated with 1000 μM RG108 exhibited significantly higher % PER on all conditioning trials relative to the bees treated with saline. Bees treated with 10 μM RG108 showed a significantly slower rate of learning than saline treated bees.

There was a significantly lower response to the perceptually similar and perceptually different odors relative to the conditioned odor on the recall tests 24 h after conditioning (Figure 5.1A, Table 5.2). This is typical for this combination of conditioned and test odors (Guerrieri et al., 2005). There was, however, no significant effect of RG108 treatment on the responses on any of the long-term recall test trials (Figure 5.1A, Table 5.2).

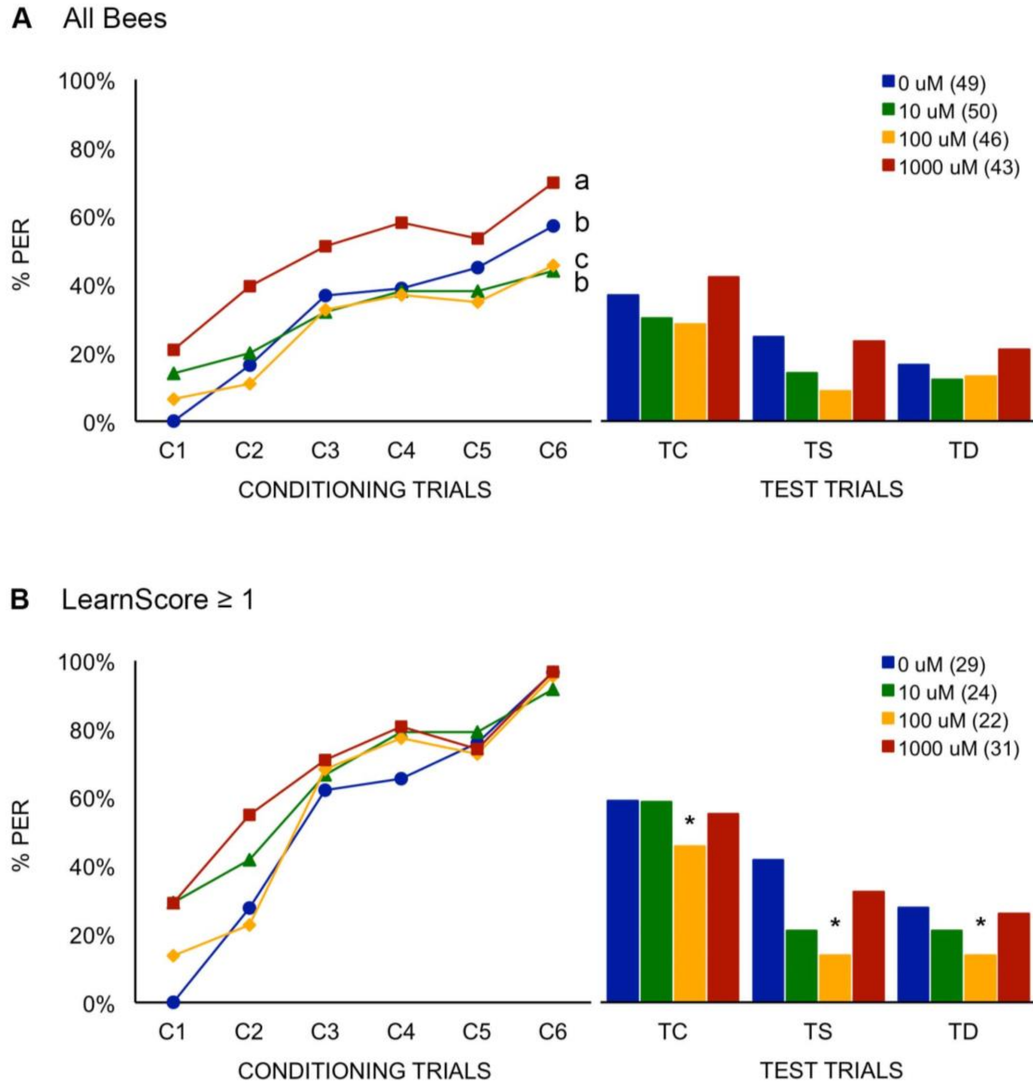


Figure 5.1. The percentage of bees exhibiting PER (% PER) to each trial during conditioning (C1-C8) and to the long-term recall test given approximately 24 h following conditioning (TC, TS, TD). Bees were either treated with 10 µM, 100 µM, or 1000 µM RG108 or saline injected into the head capsule. The graphs include either (A) all bees that survived the entire experiment or (B) only bees that responded to at least 1 conditioning trial (LearnScore ≥ 1) and survived the entire experiment. The sample size of each

treatment group is indicated by the number in parentheses located next to its designation in the legend.

Table 5.1. Logistic GEE analysis of the bees' responses during conditioning, 3h after treatment with the DNMT inhibitor RG108.

		All Bees		LearnScore ≥ 1	
Predictor	Contrast	β[1] Wald χ^2	p-value	β[1] Wald χ^2	p-value
DOSE	0 μM vs. 10 μM	0.951 3.977	0.046*	1.707 7.285	0.007*
DOSE	0 μM vs. 100 μM	0.303 0.373	0.541	0.677 1.022	0.312
DOSE	0 μM vs. 1000 μM	1.533 12.301	<0.001*	1.947 13.580	<0.001*
TRIAL	- - -	0.550 84.312	<0.001*	0.993 68.366	<0.001*
DOSE × TRIAL	0 μM vs. 10 μM	-0.259 8.493	0.004*	-0.355 4.275	0.039*
DOSE × TRIAL	0 μM vs. 100 μM	-0.135 2.036	0.154	-0.137 0.532	0.466
DOSE × TRIAL	0 μM vs. 1000 μM	-0.198 5.090	0.024*	-0.377 5.826	0.016*

1. The parameter estimate indicating the relationship of the predictor to the percentage of bees responding during conditioning and recall.

* p-value ≤ 0.05

Table 5.2. Logistic GEE analysis of responses during long-term recall test trials by bees treated with RG108.

		ALL BEES		LEARNSCORE ≥ 1	
Predictor	Contrast	β[1] Wald χ^2	p-value	β[1] Wald χ^2	p-value
DOSE	0 μM vs. 10 μM	-0.555 1.565	0.221	-0.715 2.017	0.155
DOSE	0 μM vs. 100 μM	-0.568 1.806	0.179	-1.073 4.724	0.030*
DOSE	0 μM vs. 1000 μM	-0.417 1.088	0.297	-0.584 1.792	0.181
ODOR	Conditioned vs. Novel Similar	-1.118 23.909	<0.001*	-1.251 25.444	<0.001*
ODOR	Conditioned vs. Novel Different	-1.302 29.464	<0.001*	-1.577 38.382	<0.001*
LEARNSCORE	- - -	0.502 45.857	<0.001*	0.403 13.489	<0.001*

1. The parameter estimate indicating the relationship of the predictor to the percentage of bees responding during recall.

* p-value ≤ 0.05

Bees with LearnScore ≥ 1

We suspected that the lack of treatment effect during the long-term recall test trials may have been due to the significant proportion of bees that did not respond to any trials during the conditioning phase of the experiment. So, we analyzed a reduced data set, comprised of only bees that responded to at least one conditioning trial, to examine the long-term memory recall of bees that showed evidence of learning the conditioned

association (Figure 5.1B, Table 5.1). The bees treated with 10 μM and 1000 μM RG108 exhibited a significantly greater percentage of bees responding to the initial conditioning trials than the bees treated with saline, indicating a higher spontaneous response to the odor stimulus. By the last conditioning trial, all groups exhibited a similar percentage of bees responding, which in part is due to the removal of the bees that did not respond to any of the conditioning trials.

During the long-term recall trials there were significantly lower percentages of the bees responding to the perceptually similar and perceptually different novel odors compared to the conditioned odor, as was expected with these odors (Guerrieri et al., 2005). There was also a significant effect of RG108 treatment with this dataset (Figure 5.1B, Table 5.2). The group treated with 100 μM RG108 exhibited a lower percentage PER to the conditioned odor and both of the novel test odors compared to the saline-treated bees. There was no significant effect of the 10 μM and 1000 μM RG108 treatment groups on the percentage of bees responding to the test trials as compared to the saline-treated bees. Though the bees treated with 10 μM and 100 μM RG108 exhibited a trend of decreasing responsiveness during the long-term recall trials, the group treated with 1000 μM was elevated to response levels similar to the control group. This generated a u-shaped curve over the treatment levels.

Though the group treated with 100 μM was the only group that reached significance, all of the groups treated with RG108 showed a greater decrease between the percentage of bees exhibiting PER to the conditioned odor and the percentage of bees responding to the perceptually similar novel odor (10 μM: 38%, 100 μM: 32%, 1000 μM: 23%) than the decrease seen in the control group (17%).

Experiment 2. The effect of RG108 & zebularine treatment on the nurse to forager transition

We investigated whether treatment with the DNMT inhibitors zebularine and RG108 was sufficient to alter the timing of the nurse to forager transition. Following treatment with the DNMT inhibitors and removal of the preexisting foragers from the host colonies, we observed the number of treated bees that began foraging within 5-7 days of the treatment. There was no effect of either zebularine or RG108 treatment on the number of bees that became precocious foragers (Table 5.3; Pearson's Chi-square: $\chi^2 = 0.776$, $p = 0.679$).

Table 5.3. The number of bees treated with DMSO, RG108, and zebularine returning to the colony from foraging flights during hive observations.

Treatment	Foragers Returned
DMSO	14
RG108	16
Zebularine	18

The methylation analysis was performed on four CpGs at two loci that were previously shown to have differential methylation in nurses and foragers (Herb et al., 2012). Two of the CpGs were located in the locus LOC551297, which is a putative gene similar to *sorting nexin 14*. The differentially methylated region, contained within an

exon of the gene, is associated with alternative splice variants of the gene product (Herb et al., 2012). The other two CpGs were located in the locus LOC412742, a putative gene similar to similar to *Imitation SWI CG8625-PA, isoform A*. The differentially methylated region of LOC412741 overlaps with the 3' end of the gene (Herb et al., 2012).

We analyzed the methylation for each CpG in 10 nurses and 10 foragers from each treatment group. In our analysis, the relative methylation of each CpG in an individual typically fell near 0%, 50%, or 100% methylation (Figure 5.2). For LOC551297 CpG 1, all individuals had relative methylation near 100%, indicating methylation of this CpG on both chromosomes containing this locus. About half of the bees in each treatment group showed approximately 50% methylation at LOC551297 CpG 2, which likely means that only one of the two chromosomes containing this locus was methylated (hemi-methylated). The rest of the bees had approximately 100% relative methylation at this locus. For LOC412742 CpGs 1 and 2, there were individuals with 0%, 50%, and 100% relative methylation at the two loci.

The relative methylation of CpG 1 was significantly lower in the untreated nurses and foragers than in the groups treated with zebularine and RG108 (Kruskal-Wallis, LOC551297 CpG 1: $\chi^2 = 21.730$, $p = 0.001$). Pairwise post hoc comparisons revealed that the vehicle-treated groups had lower relative methylation at CpG1 than did the groups treated with the DNMT inhibitors. This is the opposite of what was expected if DNMT inhibitor treatment was influencing the methylation status of this CpG and well within the degree of error expected from the methylation analysis techniques employed (Brian Herb, personal communication). We did not see any other significant effect of treatment on the relative methylation of these four CpGs in nurses or foragers or between

the nurses and foragers (Kruskal-Wallis, LOC551297 CpG 2: $\chi^2 = 7.756$, $p = 0.170$; LOC412742 CpG 1: $\chi^2 = 9.683$, $p = 0.085$; LOC412742 CpG 2: $\chi^2 = 8.185$, $p = 0.146$).

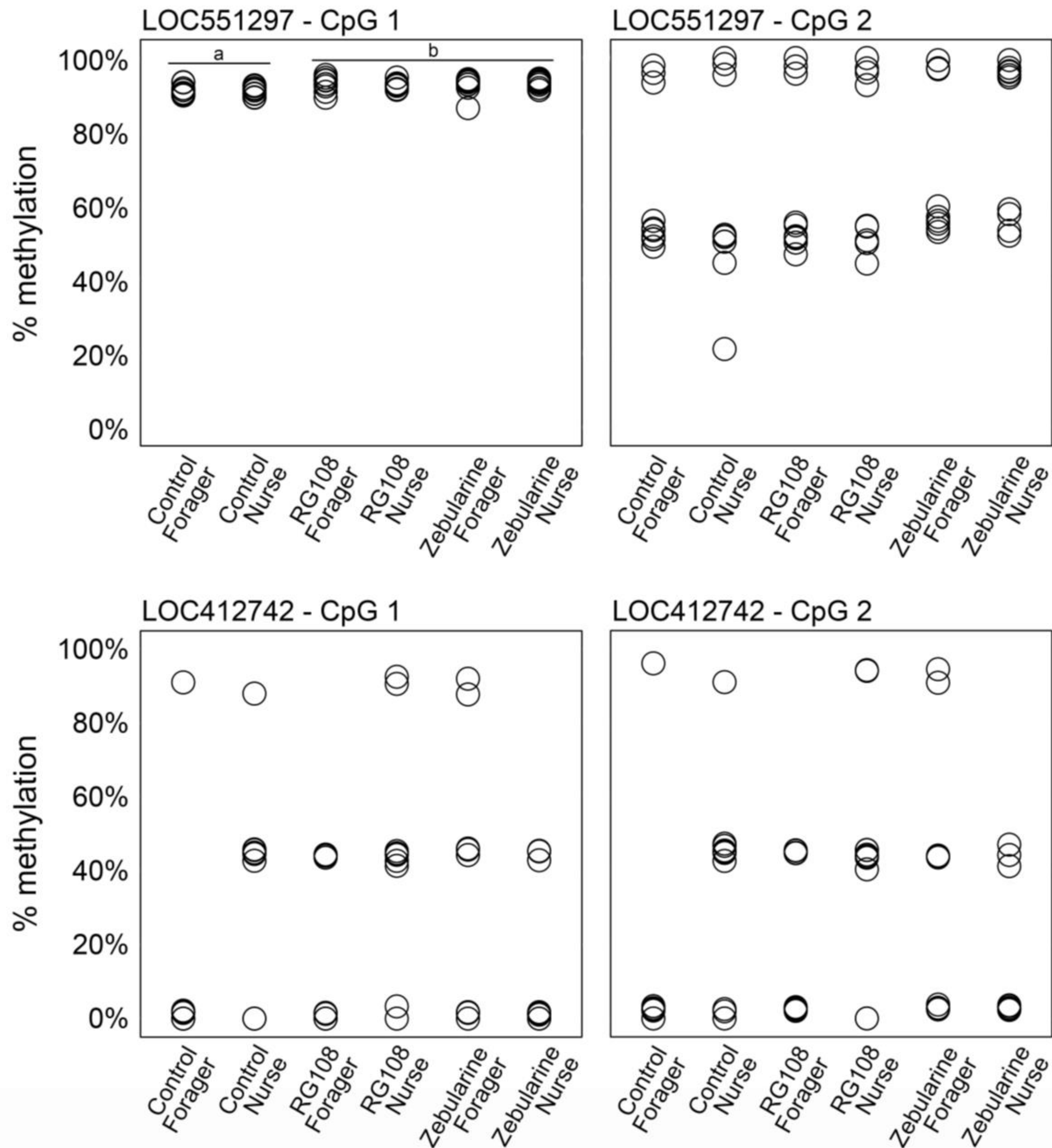


Figure 5.2. The relative methylation levels of 4 CpGs at 2 loci in 10 nurses and 10 foragers from each treatment group. The bees were treated topically with DMSO, RG108,

or Zebularine 2-4 days prior to collection. The loci examined were previously identified as differentially methylated regions in nurses and foragers.

DISCUSSION

Our aim for this study was to better understand how treatment with DNMT inhibitors influenced two well-studied types of behavioral plasticity in honey bees, an olfactory learning and memory task and during the nurse-to-forager transition. DNA methylation has been reported to be involved in both forms of long-term behavioral plasticity. However, less is know how inhibition of DNA methylation affects behavioral plasticity. We demonstrate that the dose of DNMT inhibitor administered can dramatically alter the expression of long-term olfactory memory and generalization of the conditioned response to novel odors in worker honey bees. We also show that though DNA methylation appears to be involved in the behavioral transition from nurse duties to a forager role, this transition is not sensitive to disruption through DNMT inhibition.

DNMT inhibition and memory consolidation

In this study we show that DNMT inhibition via RG108 treatment affected performance during long-term olfactory memory and olfactory generalization tests. Our results revealed a u-shaped dose response curve with the largest effect of RG108 treatment. Treatment with 100 μM RG108 (~17 ng) resulted in a decrease in the percentage of bees exhibiting PER to the conditioned odor to the perceptually similar novel odor. This effect was significant when the bees responded to at least one of the

conditioning trials. The lowest dose of RG108 also reduced generalization in bees that responded to at least one conditioning trial, but this did not reach significance.

The dose response curve for the percentage of bees responding to the long-term recall test trials is roughly u-shaped for the doses we tested. This is commonly observed with drug dose response curves, and can often be attributed to non-specific action of the drug at very high doses in addition to the expected mechanism of action (Davis and Svendsgaard, 1990). Alternatively, this type of non-linear relationship between treatment and behavioral outcome may reveal additional ways that pharmacologically impaired mechanism is influencing behavioral plasticity. Other studies reporting an effect of DNA methylation on memory formation used topical treatment with larger doses of RG108 (~680 ng & ~1360 ng). This treatment caused an increase in generalization of the conditioned response to the novel odor, abnormal extinction learning, and an increase in the recovery of the conditioned response following extinction training (Biergans et al., 2015, 2012; Gong et al., 2016; Lockett et al., 2010). The increased degree of generalization associated with the RG108 treatment used in these other studies may be representative of the responses expected at the high end of this u-shaped dose response curve, beyond the doses we tested.

All of the RG108 treated groups exhibited a greater decrease between the percentage of bees responding to the conditioned odor and the percentage of bees responding to the perceptually similar novel odor (23-38%) than was shown in control bees (17%). Though this decrease in generalization was significant only for the group treated with 100 μM RG108, the treatment had consistent effects on all treatment groups.

Further testing is necessary to determine at what concentration the drug ceases to reduce generalization and begins increasing the bees' response to novel odors.

The group treated with 1000 µM RG108 had significantly higher percentage of bees responding to the conditioning trials than the control group. This increased learning performance may be caused by a generalized stress response independent of the expected action of the drug, which would alter the bees' sensitivity to olfactory stimuli and the sucrose reward. High doses of a drug can induce a non-specific physiological stress response, which can have a significant impact on the outcome of behavioral tasks, including learning and memory (Fischer and Vail, 1980).

DNMT inhibition and the nurse to forager transition

We showed that treatment with DNMT inhibitors is not sufficient to alter the timing of the transition from nurse duties inside the nest to the out-of-nest forager duties. This major behavioral transition is influenced by many stimuli, so inhibition of a single molecular mechanism does not appear to be able to delay or accelerate the many physiological changes associated with the behavioral transition. This lack of a drug effect also could reflect the type of role DNA methylation is playing during this transition. A dynamic alteration of DNA methylation patterns may not be driving the transition or actively maintaining the new behavioral state; rather, it may be more subtly influencing the bees' ability to make the behavioral switch.

We assessed the differences in relative methylation of four CpGs in 2 loci in nurses and foragers treated with the DNMT inhibitors, zebularine or RG108, or with the vehicle, DMSO. These CpGs were previously shown to have higher relative methylation

in the brains of foragers than in the brains of nurses or reverted nurses (Herb et al., 2012). In our study, there were no significant differences in the relative methylation of three of these CpGs between the treated and control bees that can be attributed to DNMT inhibition or behavioral status. One CpG (LOC LOC551297 CpG 1) did have significantly higher DNA methylation in bees treated with the DNMT inhibitors. However, there are several sources of these differences that can be attributed to mechanisms besides the drug effect. First, all of the individuals showed approximately 100% methylation at this CpG. This compressed the variance for all of the groups and making the differences between the groups easier to detect statistically than with the other CpGs, which had individuals with approximately 0%, 50%, and 100% relative methylation. Also, this difference is within the range of error typically seen in the methylation analysis and sequencing technique we employed (Brian Herb, personal communication).

For all of the CpGs we examined, individual bees had levels of relative methylation tightly clustered around 0%, 50%, and 100%. This pattern in the methylation levels of these CpGs seems to indicate that the methylation status of each CpG was consistent in the majority of cells in the brain. Herb, et al (2012) used pooled brains from 7 individuals in each sample to obtain a sufficient volume of DNA for some of the methylation assays employed in the study. The differences in relative methylation between samples of nurses, foragers, and reverted nurses are reflecting the methylation statuses of multiple individuals and therefore do not exclude the possibility of static methylation at these CpGs. The relative methylation of a CpG in a pooled sample would then be partially determined by the proportions of bees with no methylation, hemi-

methylation, and full methylation at each CpG as well as any differences in methylation within a single individual's brain.

The patterns of DNA methylation at the loci we tested seem to indicate that these loci are not dynamically methylated during the nurse-to-forager transition. The source of the methylation patterns at these CpGs and others showing similar methylation patterns in whole brain samples might be genomic imprinting (Delaval and Feil, 2004; Elango et al., 2009; Howell et al., 2001). Alternatively, these CpGs may be obligatory epialleles. Throughout the honey bee genome there are approximately 220,000 SNPs that may be able to alter the number of CpGs in the allele or influence the methylation status of nearby CpGs through *cis*- or *trans*-acting sequences (Wedd et al., 2016). The inter-individual variation we saw in the methylation of these CpGs may be due to underlying sequence differences in the DNA. If these CpGs reflect obligatory epialleles, they may be cooperating with genetic polymorphisms in altering overall gene expression or the abundance of certain splice variants rather than acting more independently.

The methylation statuses of these loci may be static rather than undergoing an alteration in methylation status triggered by the nurse-to-forager transition or by stimuli influencing the timing of the transition. Therefore, the function of a specific methylation pattern at these loci may be to help confer a propensity for a particular physiological state (e.g. more metabolically active) via the expression level or alternative splicing of the gene with which it is associated. The propensity for a certain physiological state (e.g. more metabolically active) may allow a bee to make the switch from the nurse state to the forager state more easily and thus be more likely to become a forager earlier in life than others of its cohort.

Conclusions

The relationship between DNA methylation and experience-dependent behavioral plasticity is complex. And, modulating the relationship through pharmacologically inhibiting DNA methylation supplies additional layers of complexity to the behavioral outcome of such experiments, especially when the behavioral responses to the drugs have not be fully characterized. Our results highlight this complexity as well as provide additional characterization of the effect of DNMT inhibitors on the plasticity of behaviors in which DNA methylation plays a role.

We also show that loci with static DNA methylation may also play a role in behavioral plasticity, perhaps acting as a way of predisposing an individual for making the nurse-to-forager transition. This priming of an individual for a particular physiological state may also be occurring during other forms behavioral plasticity. Much of the research on the role of DNA methylation in behavioral plasticity has focused on the dynamically methylated loci in the brain. However, the concept of a role for statically methylated loci in enabling animals to exhibit behavioral plasticity is also a distinct possibility and warrants further investigation.

CHAPTER 6

DISCUSSION

In this thesis, I investigate the sublethal effects of heavy metal and metalloid exposure on honey bee behavior. Through my research I highlight the idea that the physiological impact of a toxic substance on non-neural organs likely have a significant effect on neural function and behavior. I also explore the idea that a toxin's ecological impact may depend on its sublethal effects on animal behavior in addition to its lethal effects. In this chapter, I highlight those results, discuss the implications and limitations of my research, and propose directions for future investigation of these ideas.

MAJOR FINDINGS

When presented with heavy metal contaminated sucrose, honey bees responded with a unique response profile to each metal. These diverse response profiles likely reflect variations in the mechanisms for detecting the metal, the taste perception of the toxin, and the ability of the metals to alter the bees' sensitivity to sucrose or motivation to feed. While bees failed to reject sucrose contaminated with cadmium after antennal or proboscis stimulation, they showed a strong aversion to copper contaminated sucrose at even the lowest concentrations tested. It appears that at the concentrations I tested, bees are either unable to detect cadmium or do not find it unpalatable. The presence of copper may be detected independently or may be interfering with the detection of sucrose. Ingestion of either cadmium or copper did not alter the bees' sucrose sensitivity

indicating no adverse affects on motivation or sensory sensitivity following acute exposure. Lead, on the other hand, elicited a complex response profile from the bees. It may be interfering with normal sucrose sensory transduction in exposed receptors or altering the perceived sweetness of sucrose and normal sucrose sensitivity. Previous studies in other organisms showed that lead interferes with calcium signaling, which is important in sensory transduction and neural signaling (Jadhav et al., 2000; Suszkiw, 2004; Xiao et al., 2006) and is one likely explanation of my results.

Acute metalloid exposure also modifies honey bee behavior, as evidenced by impairments in performance on a learning and memory task following selenium ingestion. A single dose of as little as 18 ng of selenium was sufficient to impair performance. Honey bees exposed to the inorganic sodium selenate exhibited decreased learning and long-term recall of the task, while individuals exposed to the organic methylseleno-L-cysteine exhibited impaired long-term recall, though they appeared to learn the task normally. These results may be due to conditioned taste aversion, induced by malaise following ingestion of selenium and reducing valuation of the expected reward (Ayesteran et al., 2010; Mark et al., 1991; Wright et al., 2010). Or, there may be additional interference with neural processes involved in sensory perception, learning, memory formation, or memory recall.

To identify possible mechanisms mediating the learning and memory impairments caused by sublethal selenium exposure, I localized the accumulation of selenium in honey bees chronically exposed to selenium. I used bees chronically exposed to selenium to be able to detect the broader sites of selenium accumulation since an acute dose would not likely be detected beyond the unabsorbed selenium located in the gut lumen. I found

no evidence of selenium accumulation in the brain of honey bees chronically exposed to inorganic or organic forms of selenium. Instead, accumulation was localized to the abdominal structures of the fat body and the rectum, the outer layer of the eye, and the salivary glands in the form of selenomethionine or selenate. It is likely that the selenomethionine is being misincorporated into proteins and altering their functionality (Letavayova et al., 2006). Since we were not able to observe selenium accumulation in the honey bee brain, it is likely that the effect of selenium toxicity on honey bee learning and memory involves peripheral mechanisms that indirectly alter neural function. However, further testing for the presence of tissue damage in the brain is required to confirm the absence of direct neurotoxicity. A similar depression in performance during long-term memory recall in bees exposed to lithium was attributed to post-ingestional malaise reducing reward valuation (Ayesteran et al., 2010; Hurst et al., 2014). It is probable that selenium toxicity elicits a similar state, making conditioned taste aversion a likely mechanism for selenium induced learning and memory impairments.

Heavy metal and metalloids have been shown to alter DNA methylation (Davis et al., 2000; Ray et al., 2014; Sanders et al., 2014; Senut et al., 2014). However, it is not known if these toxins are directly altering the function of enzymes that alter or maintain DNA methylation or affecting epigenetics indirectly through broader mechanisms. If they are directly affecting DNA methylation it is possible that they could be impairing the DNA methylation dependent mechanisms underlying memory formation and consolidation. To assess the potential for metals to affect behavior through DNA methylation, it is necessary to first understand how DNA methylation may be involved in specific behaviors. I show that inhibiting DNMTs before a learning and memory task

impairs memory recall in bees that learned the task. This protocol can be used as a basis of and positive control for studies investigating the effects of metal and metalloid toxicity on DNA methylation. I also test whether inhibiting DNMTs is sufficient to alter the timing or stability of the nurse-to-forager transition. Though this protocol is less likely to be useful as a positive control for the effects of metalloid and metal toxicity on DNA methylation, it did highlight the question of whether the differences in DNA methylation patterns seen in nurses and workers are driven by the behavioral change or if they are driving the transition.

IMPLICATIONS

My results highlight the potential for the physiological status of non-neural organs to affect cognitive function. Though communication between the nervous system and non-neural organs has long been recognized, recent work has revealed that the physiological status of non-neural organs has more of an effect on cognitive function than previously considered (Critchley and Harrison, 2013; Gianaros and Wager, 2015). Of special interest is the health of the gut microbiome, which has been shown to affect cognitive processes and thus indicates some sort of communication between these symbiotic inhabitants of the gut and the host's brain (Foster and Mcvey Neufeld, 2013; Li et al., 2009). Recent identification of enteroendocrine cells, located in the lining of the gut lumen, that are capable of direct communication with the peripheral nervous system may be detecting alterations in the gut microbiome or the presence of the metal or metalloid in the gut contents directly and provide a link between the gut lumen and the nervous system (Bohórquez et al., 2015). Activation of the immune system also has been

linked to deficits in learning and memory, and may be impairing normal physiology throughout the body (Donzis and Tronson, 2014; Mallon et al., 2003; Yirmiya and Goshen, 2011). As highlighted by these examples, the physiological status of other non-neural organs may be more involved the functioning of the nervous system than previously considered. As our understanding of the interconnectedness of the various organ systems in the body with the nervous system increases, our definition of neurotoxicity may also need to grow in order to consider the effect broad physiological changes may have on neural function and behavior.

The presence of behavioral modifications following heavy metal and metalloid exposure highlights the possibility that sublethal toxicity has a significant ecological impact and should be included in the risk assessments of contaminated areas on the organisms living in and surrounding the location. If an animal's neural function is impaired through sublethal toxin exposure, its survival and/or fitness may be indirectly impaired through a reduced ability to escape predators, navigate properly, obtain food, or find mates. In addition to the effects of individual toxins, the evidence of synergistic interactions in the actions of multiple toxins and environmental challenges is indicative of the need to identify sublethal effects of toxin exposure, since in combination with other toxins, sublethal doses may have a significant effect on the animal. Therefore, there is a need to understand sublethal effects of toxin exposure to more fully understand the potential toxins or mixtures of toxins have of altering the normal function of an organism and how that may affect the population, community, or ecosystem.

Honey bees have been proposed as useful bioindicators of environmental health. This is predominantly through the ability to monitor toxin content in the environment

through measuring the buildup of toxins in the wax, honey, pollen, and propolis in the nest and accumulation in the honey bees' bodies (Conti and Botrè, 2001; Mullin et al., 2010; Pettis et al., 2012; Zhelyazkova, 2012). Honey bees are sensitive to sublethal toxin exposure and show behavioral modifications during and following that exposure. Therefore, their use as bioindicators can expand from monitoring environmental toxin content to identifying contamination levels that are capable of impairing neural function prior to increasing mortality. This information can then be used to inform policies on acceptable limits for toxin content in an ecosystem.

LIMITATIONS

PER-based assays have been successfully employed to study sensory and cognitive function for decades (Giurfa and Sandoz, 2012). Though they are useful in assessing the effects of a treatment on cognitive function, interpreting the results and inferring potential mechanisms can be challenging. The results are the outcome of a complex mixture of the animals' genotypes, motivation to feed, previous experience, and sensory and motor systems integrity (Frost et al., 2012; Latshaw and Smith, 2005; Urlacher et al., 2010). As my results highlight, the physiological status of non-neural organs also plays an important role in determining the behavioral outcome of the assays and is a variable that cannot be controlled for. Additionally, elements of the protocol may be altering the behavior of the bees, and affecting the outcome of the experiment. For example, restraining the bees for PER assays affects the bees' willingness to consume food contaminated with toxic substances. Bees that are restrained are more likely to consume quinine contaminated water or strong sodium chloride solutions than are

unrestrained bees (de Brito Sanchez et al., 2015). This could have an effect on the bees' willingness to consume heavy metals and metalloids as well, making them less likely to consume the toxins and consequently reducing the risk of exposure to the elements than my results indicate.

The detection limits of μ-SXRF mapping and the identification of selenium speciation by μ-XANES allow for some ambiguity as to the extent and localization of selenium accumulation in honey bees. Though control bees did not show any detectable selenium accumulation with μ-SXRF, quantification of selenium with ICP-OES in similarly treated bees revealed an average concentration of ~1 mg/kg in these controls. Presumably the selenium in control bees is distributed throughout their bodies, as there are not detectable deposits. Even bees fed 0.6 mg/L selenium over 7 days showed no detectable selenium accumulation, despite their chronic exposure to the metalloid. Though μ-SXRF mapping and μ-XANES are very sensitive methods for detecting elements in tissue, the sensitivity of the scans can vary considerably with the strength of the x-ray beam, positioning of the specimen, and sensitivity of the detector, making it difficult to determine an actual detection limit for the technique. In addition, the energy of selenium-scattered x-rays is very close to the energy generated from elastic collisions with the tissue in general, making it more difficult to determine if low amplitude signals are from selenium in the tissue or should be attributed to elastic collision. This poses the question of what other locations in the bee are accumulating low levels of selenium and whether there is sufficient selenium in the brain to alter neural function directly, even though it was not detectable. Though some ambiguity exists when determining early accumulation sites or the locations of accumulation following exposure to low

concentrations of selenium, this technique is still able to provide detailed information about selenium accumulation and metabolism within limits.

Retrospectively, the methods I used to treat bees with DNMT inhibitors are not ideal. To deliver a known quantity of the DNMT inhibitor, RG108, to the brain, I injected the drug solution directly into the head capsule of the bee. The invasive nature of the injection could be the cause for the high percentage of bees not responding to any trial during the olfactory task. And, this may alter the responses of those bees that learned the task, perhaps reducing the behavioral effect of the drug treatment. For the experiment investigating the role of DNA methylation in the nurse-to-forager transition, only a topical application of the DNMT inhibitors resulted in sufficient survival of the treated bee for the experiment to proceed. However, topical application of drugs reduces the ability to control the exact drug concentration delivered to the brain of the bee and could increase the between subjects variability of the results. Clearly, further optimization of DNMT inhibitor delivery for both the learning and memory task and the nurse-to-forager transition experiment would be of value.

In the experiment assessing the role of DNA methylation in the nurse-to-forager transition, we are limited in the number of CpGs we survey to assess the effect of DNMT inhibitor treatment on the differences in methylation patterns between nurses and foragers. We determine the methylation status of four CpGs at two loci previously identified as reliably having different methylation states in nurses and foragers (Herb et al., 2012). The expense and technical expertise required for the methylation assays prohibit a wider survey of CpGs. Though our results from these four CpGs are intriguing, this is a small sample of the many CpGs in the honey bee genome. Other CpGs may be

dynamically methylated during the nurse-to-forager transition, and may show a drug effect. However, the CpGs we test appear to be statically methylated, with a consistent methylation status throughout the many cell types in the brain. It would be fascinating to broaden our survey of the effect of DNMT inhibition on methylation statuses of many more CpGs at diverse loci to gain a more general understanding of the proportions of CpGs statically and dynamically regulated during the nurse-to-forager transition.

FUTURE DIRECTIONS

In this work, I investigated the effects of acute exposure to four heavy metal and metalloid toxins on honey bee behavior and neural function. In a metal or metalloid contaminated environment honey bees would be chronically exposed to these toxins. Therefore, identifying the modifications in neural function and behavior following chronic exposure to these toxins is ecologically relevant. Chronic exposure may attenuate the behavioral modifications I observe as the bees become accustomed to feeding on toxic levels of the metal or metalloid. Or, accumulation of the metal or metalloid may augment the behavioral modifications I report.

I have not addressed how sublethal exposure during critical developmental states affects the brain and behavior. In heavy metal or metalloid contaminated environments, honey bees would also normally be exposed to these toxins throughout larval and pupal development. Sublethal toxin exposure during larval and pupal stages could adversely affect the development of many organs, including the brain, which could affect neural function in the adult bee. In vertebrates, fetal exposure to lead, cadmium, iron and mercury can significantly alter cognitive function in the child (Senut et al., 2012; Wright

and Baccarelli, 2007). How development in the presence of a heavy metal or metalloid toxin influences adult bee cognitive function and behavior is an area that has not yet been explored, but is an intriguing avenue of future research.

Because contaminated environments typically contain multiple toxic metals and metalloids, it is important to broaden this work to include metals and metalloids that I did not investigate in this thesis. Some of these are arsenic, zinc, nickel, manganese, and mercury (Bai et al., 2012; Crossgrove and Zheng, 2004; Holmgren et al., 1993; Mebane et al., 2012; Wurtsbaugh et al., 2011). Of these additional metals, manganese and nickel have already been shown to affect insect pollinator foraging behavior (Meindl and Ashman, 2014, 2013; Søvik et al., 2015). In addition to studying the sublethal effects of these additional metal and metalloid toxins, studying the synergistic interactions between these toxins, or between metals and other common toxins such as pesticides is ecologically relevant. And, this is important to understanding the true impact that toxins have on honey bee neural function and health. Studies have already examined the synergistic interactions of various pesticide combinations on honey bee health and susceptibility to pathogens and parasites (Aufauvre et al., 2012; Johnson et al., 2009; Zhu et al., 2014), but there are currently no studies on the interactions of multiple heavy metals and metalloids on honey bee health or behavior. In addition to interactions between multiple metals and metalloids, these toxins may exhibit synergistic interactions with pesticides or environmental challenges such as climate change. Alternatively, a metal or metalloid may diminish the harmful effects of another toxin or a pesticide. Elements involved in antioxidant processes are the most likely to exhibit this type of

interaction. Investigating these interactions may lead to potential methods for reducing the impact of other toxic substances on honey bees.

Since restraint in harnesses can influence bees willingness to consume unpalatable substances (de Brito Sanchez et al., 2015), it would be interesting to develop experiments to test the willingness of unrestrained honey bees to consume heavy metal and metalloid food sources when given a choice between contaminated and uncontaminated food. Experiments testing free-flying forager willingness to forage on flowers with metal contamination have been conducted using bumblebees and wild pollinators in the field (Meindl and Ashman, 2014, 2013). And, de Brito Sanchez, et al (2015) employed a y-maze style choice test for testing honey bees freely walking between water and two unpalatable solutions: quinine and strong sodium chloride. Color choice assays with free-flying bees have been used to test multiple aspects of honey bee visual learning (Andrew et al., 2014; Jones et al., 2015; Kunze and Gumbert, 2000). Experiments to those mentioned above coupled with the metal and metalloid treatment could reveal additional aspects of honey bees' risk of exposure to these toxins.

The bees' varying response profiles to the heavy metals I tested indicate likely differences in their ability to detect each of the metals. In the case of lead, there is the additional question of whether this metal is altering the bees' ability to detect or their perception of the value of sucrose solutions offered during the experiments. Electroantennograms and electrophysiological recordings from sensory cells in the proboscis coupled with metal or metalloid stimulation could reveal whether honey bees are able to detect the metals in the offered solutions (Claudianos et al., 2014; de Brito Sanchez et al., 2005; Wright et al., 2010). Additionally, these types of recordings could

be used to determine if the sensory transduction of sucrose receptors is altered following lead exposure through stimulation with sucrose solutions of varying concentration before and after exposure to lead.

In bees chronically exposed to methylseleno-L-cysteine, there is a significant accumulation of selenium in the outer layer of the eye in the form of selenomethionine. Selenomethionine is incorporated into proteins instead of methionine and can alter protein function. Therefore, it is possible that this selenomethionine accumulation may be altering the function of the visual system. Honey bees can learn to associate visual stimuli with a reward, in experiments similar to the olfactory learning tasks I use (Hori et al., 2006). Coupling these visual learning paradigms with chronic selenium treatment can reveal whether the bees' vision is altered by selenomethionine accumulation in the eye.

Mechanisms potentially mediating the learning and memory impairments induced by selenium toxicity include alterations in gut microbiome health, oxidative damage to non-neural organs, immune system activation, impaired protein function, and abnormal DNA methylation dynamics or patterns. Testing these hypotheses is a logical next step in understanding the effects of metalloid toxicity on honey bee neural function and behavior. Additionally, further testing to confirm the absence of a direct neurotoxic effect is necessary. Current DNA sequencing technology makes it possible to sequence the collective genomes of the microbiome and thus identify differences in the species composition following heavy metal or metalloid exposure (Engel et al., 2012; Moran et al., 2012). Oxidative damage may cause lipid peroxidation and catalyze the formation of protein carbonyls and DNA adducts, which can be tested for with well-established biochemical assays (Dalle-Donne et al., 2003; Oliver et al., 1990). This is the most likely

form of tissue damage that might be present in the brain, even though I was not able to detect selenium in that organ. The absence of tissue damage in the brain would support the hypothesis that the behavioral modifications are due to non-neural toxic effects.

The tissue damage potentially caused by metal or metalloid toxicity may also activate the immune system. Assays might include detecting differences in the transcription of immune related genes or profiling hemocyte and other hemolymph particulates following metal or metalloid exposure (Cornman et al., 2013; Holt et al., 2013; Marringa et al., 2014). To assess the effect of metal or metalloid toxicity on protein function, the protein that incorporates the metal or metalloid must first be identified and its function characterized in the absence of the toxin. Though intensive, this work would provide valuable insights into normal physiology of the honey bee as well as a better understanding of metal or metalloid toxicity.

To establish that metal and metalloid toxicity affects DNA methylation in honey bees and how those epigenetic alterations affect behavior is not trivial. Though important advances have been made in understanding how DNA methylation is dynamically regulated during learning and memory, the surveys of CpGs have necessarily been limited to a few loci in the genome and our understanding of the link between the methylation patterns at these loci and the animals' behavior is still rudimentary (Biergans et al., 2015). However, assessing the potential of heavy metal or metalloid exposure on the DNA methylation patterns at these loci may not only reveal the effects of metal or metalloid toxicity it will further the understanding of how dynamic DNA methylation regulates the behavioral output of the animal.

Since these mechanisms discussed so far are predominantly located in non-neural organs, there is a need to identify the neural pathways relaying this peripheral information to the central nervous system and the neural networks mediating the behavioral modifications. Behavioral impairments caused by toxin exposure can be mediated by a conditioned aversion to the stimuli associated with the exposure or malaise reducing energy expenditure (Hurst et al., 2014; Wright, 2011; Wright et al., 2010). However, the peripheral neural networks detecting the physiological insults to the affected organs in the honey bee have not been identified. In the brain dopaminergic and serotonergic signaling are involved in altering the perceived value of a typically rewarding stimulus, like food (Ayesteran et al., 2010; Wright, 2011); however the link between the circuits affected by these neuromodulators and the peripheral signals has not been elucidated. It is likely that these peripheral neural pathways are diffuse, making it difficult to identify them. But, with the increasingly important connection between the physiological status of the whole body and cognitive function, this is an increasingly important avenue of future research.

Though honey bees are the most economically valuable insect pollinator, other insect pollinator species are of great ecological value (Biesmeijer et al., 2006; Steffan-Dewenter et al., 2005). Understanding the effects of heavy metal and metalloid toxicity on multiple insect pollinator species is ecologically relevant. Some studies have tested the effect of heavy metal or metalloid content in nectar rewards on foraging behavior (Meindl and Ashman, 2014, 2013). And, another examined the proximity to a source of metal contamination on survival of a wild bee species (Moroń et al., 2014). But much is still unknown about the effects of these toxins on wild insect pollinator behavior. It is not

known whether wild insect pollinator species are more or less sensitive to heavy metal and metalloid toxicity, but identifying the toxin concentrations that begin to impact these species neural function and survival is important. It is more difficult to assess the effects of toxin exposure on many of these insects since they are not so easily reared and manipulated as honey bees and because we know much less about their biology and behavior than we do about the honey bee. Still, as these species face continually increasing environmental pressures (Kosior et al., 2007; Potts et al., 2010a), the need for understanding the effects of these challenges on their behavior and survival also increases.

CONCLUSIONS

As the influence of non-neural tissues and even endosymbionts on the functionality of the nervous system becomes more apparent, our focus in neurotoxicology must shift to include the effects of toxins on non-neural organs as causative factors in toxin-induced behavioral modifications. My results highlight the need for this shift and point to further avenues of exploring the link between nervous system function and the physiological status of the rest of the body. The presence of sublethal effects of metal and metalloid toxicity also highlights the importance of understanding how low level toxic insult is able to alter an animal's neural function in order to fully comprehend the potential ecological impact of the toxin.

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APPENDIX

STATEMENT BY AUTHOR

I certify that the work contained in this dissertation includes a previously published paper of which I am co-author. All co-authors have granted their permissions for including that article in this dissertation.

SIGNED: Christina Marie Burden