LEARNING FROM CHEMICAL COPING BEHAVIORS OF WILDLIFE TO DISCOVER NEW APPROACHES FOR PEST MANAGEMENT

by

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DEDICATION

For all the mobility challenged ecologists out there pioneering. There are literally dozens of us.

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ABSTRACT

Pests, such as parasites and pathogens, persist throughout time and space as threats to public health and food security. The need for novel and sustainable approaches to managing these threats are in high demand. The current approach of discovering and developing chemical treatments to manage pests is tedious, not efficient, and often outpaced by traits of resistance in pests. Here, we propose a new approach to discovering new chemical pest management solutions by observing chemical coping behaviors in wildlife. We define a chemical coping behavior as the exploitation of naturally occurring chemicals within a host's environment to manage pests. Specifically, the use of greenery in nests by avian species may provide clues to plants that can deter ectoparasites. Plants use chemical defenses to cope with their own parasites, pathogens, and herbivores, which avian hosts can exploit to combat pests in nests. A local host-pest-plant interaction was investigated to discover the potential chemical diversity and bioactivity of greenery found in nests of golden eagles (*Aquila chrysaetos*). We found that each plant offered unique chemicals, but that the plant species underrepresented in nests compared to availability in the landscape provided greater diversity in volatile chemicals whereas overrepresented plant species provided greater diversity in water-soluble chemicals compared to other plants. Furthermore, we tested how concentration and diversity of volatile and water-soluble chemicals in plant species found in nests of golden eagles affected the behavior of a hematophagous parasite (*Cimex lectularius*, the common bed bug). We found that bed bugs spent less time resting and transitioned from grooming to exploration at an

increased frequency with high concentration and diversity of volatiles from plants found in nests of golden eagles. Observing the chemical coping behaviors in the wild could provide a sustainable framework for discovering diverse and robust sources of chemicals and modes of action that can used to manage pests of human concern.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACT	Artemisinin-based combination therapies
ANOVA	Analysis of variance
AT	*Artemisia tridentata*, big sagebrush
AUC	Area under the curve
BORIS	Behavioral observation research interactive software
CI	Confidence interval
cm	Centimeter
CV	*Chrysothamnus viscidiflorus*, green rabbitbrush
DDT	Dichlorodiphenyltrichloroethane
DW	Dry weight
EN	*Ericameria nauseosa*, gray rabbitbrush
ESI	Electrospray ionization
g	Gram
GABA	Gamma-aminobutyric acid
GC	Gas chromatography
GS	*Grayia spinosa*, spiny hopsage
HD	High definition
HPLC-MS	High pressure liquid chromatography-mass spectrometry
IPM	Integrated pest management
KPa	Kilopascals

m	Meter
MeOH	Methanol
mg	Milligram
min^{-1}	Per minute
mL	Milliliter
µL	Microliter
mm	Millimeter
m/z	Mass to charge ratio
QTOF	Quadrupole time of flight
SD	Standard deviation
UV	Ultraviolet
WW	Wet weight

CHAPTER ONE: INTRODUCTION TO ECOLOGICALLY BASED PEST MANAGEMENT DIRECTED BY FLORAL AND FAUNAL HOSTS

Pests such as pathogens, weeds, and herbivores threaten up to a third of the human food supply (Riegler, 2018). In addition, arthropod pests can be disease vectors that threaten to infect up to eighty percent of the world's population (World Health Organization 2017). Changing climate and growing human populations will require innovative strategies to combat pests of concern into the future (Deutsch et al., 2018; Marcos-Marcos et al., 2018). Chemical treatments remain an effective and powerful tool against pests but can easily be rendered useless through the onset of resistance traits developing in pests (Liang et al., 2018). Poor management of pest species through over-reliance on chemical control therapies threatens food security and global health (Hoy, 1998; Palumbi, 2001). For example, the synthetic pesticide, dichlorodiphenyltrichloroethane, more familiarly known as DDT, was credited with helping control malaria outbreaks and is proposed to have saved at least a billion people in the 1950s and 60s. However, DDT had widespread harmful effects on wildlife and the environment that can still be felt today (Turusov et al., 2002) and has resulted in DDT-resistant phenotypes of pests (Overgaard and Angstreich, 2007). Today, DDT is still considered an essential interim tool against malaria outbreaks, but its use has significantly declined over health and environmental concerns (Overgaard and Angstreich, 2007; Berry-Cabán, 2011). Other chemical treatments, such as organophosphates and carbamates have been used alternatively to manage pests, however

these pesticide classes share concerns about health and environmental safety. Additional tools are needed to fight resistant phenotypes and mitigate health and environmental concerns.

Midway through the 1990s, integrated pest management (IPM) became a widespread movement in developed agricultural communities to delay the development of pesticide-resistant phenotypes in many classes of pests, ranging from pathogens, such as fungi or viruses, weeds, and both vertebrate and invertebrate herbivores (Ehler, 2006; Barzman et al., 2015) and was later adopted for vector management (Chanda et al., 2017; Marcos-Marcos et al., 2018). For the purposes of this thesis, we have coopted the term pest to include any animal, plant or fungus that is detrimental to a host organism. IPM focuses on using a combination of tactics to delay resistance and sustainably manage pest populations while minimizing environmental impacts, including effects on non-target species (Barzman et al., 2015). Tactics of IPM can be broken up into four different types, which it is important to note are not mutually exclusive: cultural (which we have adapted to behavioral), physical, biological, and chemical (Figure 1.1). The behavioral or cultural tactics focus on broad-ranging, preventative defenses such as using crop rotations (Rusch et al., 2013) or intercropping (Tanyi et al., 2018). Physical tactics, including mechanical tactics, focus on limiting access to resources of a host via employment of structural materials, which are typically preventative but can also be therapeutic (e.g., traps or trenches, Vincent et al., 2003). Biological controls are best described as the recruitment or release of a pests' natural enemies, which includes parasitoids (Afiunizadeh and Karimzadeh, 2015) and predators (Cakmak, et al. 2009). Chemical tactics focus on target-specific, reactionary, therapeutic defenses against pests using synthetic and biorational

repellents and pesticides. IPM has evolved into a robust framework based on the ecology of pests and their environment. Gleaning IPM strategies in wild systems (e.g., plant and avian species, Figure 1.1) offers opportunities to discover innovative and novel solutions for pest management by humans.

The need to use a variety of tactics to manage pests and overcome resistant phenotypes is not unique to humans (Figure 1.1). As an example of a behavioral defense tactic, birds of prey have been thought to maintain alternative nests to avoid using nests that harbor pest infestations (Ontiveros et al., 2008; Lesko and Smallwood, 2012). Physical defense tactics employed by avian species include removing pests via grooming or molting plumage (Clayton et al., 2010). Biologically, innate and adaptive immune systems are particularly useful against pathogens. Innate immunity uses molecular patterns to monitor and identify pest species, such as those activated by viruses and microbes through a series of feedback mechanisms responding to the threat of the pest (Jones et al., 2016). Like humans, some wildlife species exploit chemical defenses of plants to use against their own pests. For example, wood ants (*Formica paralugubris*) harvest tree resin for its antimicrobial properties, and antifungal properties were increased after the ants combined the plant resin with their own metabolites (Chapuisat et al., 2007; Brütsch et al., 2017).

Plants are a reliable resource for humans to gain insight into managing traits of resistance in pests and therefore could be exploited by other hosts for a few reasons. First, plants have been locked in reciprocal evolution over millennia with pests, requiring the management of resistant traits in pests over long periods of time (Labandeira and Currano, 2013). Second, plants have limited means to avoid or escape pests while

sometimes persisting in extreme environments (Mithöfer and Maffei, 2017). Due to these long-lasting pest interactions and environmental limitations, plants as hosts have developed a diversity of broad-ranging to target-specific tactics (Figure 1.1) that manage resistance traits in pests (Kant et al., 2015). At the behavioral level of defense, plants have exhibited the ability to communicate pest attacks through volatile compounds that allow neighboring plants to 'prime' defenses against potential invasion (Karban et al., 2000; Heil and Karban, 2010). Plants also rely on physical barriers (e.g., thorns, Milewski et al., 1991) and biological tactics (e.g., recruitment of predators or parasitoids, De Moraes et al., 1998) to combat pests. Plants have also demonstrated innate immune responses, analogous to those found in animal taxa (Ausubel, 2005; Jones et al., 2016), that are triggered by pest damage that prompt feedback mechanisms such as inducible chemical defenses, which are relatively metabolically inexpensive when pests are not present (Shudo and Iwasa, 2001; Westra et al., 2015). Production of toxic chemicals, especially chemical mixtures, can be metabolically expensive to maintain and are targeted towards herbivores that have specifically adapted to detoxify, sequester, or excrete these toxins (Mithöfer and Maffei, 2017). Instances where plants have invested heavily in a diversity of therapeutic toxic chemicals might indicate an intense chemo-adaptive battle between host plant and plant pest. Plants naturally synthesize chemicals and possess a wide range of chemical tactics to deploy against pest species and delay development of resistance traits. As such, plants are ripe to be exploited by other taxa to defend against their own pests.

Here we use the term "chemical coping behavior" to describe a host taxon exploiting chemicals from a naturally occurring source to control their own pests.

Observations of chemical coping behavior in wild systems may provide much needed insight into sustainable sources of novel chemistry to manage resistance mechanisms through chemical interactions and diversifying target receptors of pests. For example, artemisinin-based combination therapies (ACTs), offer a historical and recent example of chemical coping behavior by humans. ACT was inspired by traditional Eastern medicinal use of the Chinese herb *Artemisia annua* (Elfawal et al., 2015; Antony and Parija, 2016) and currently provides the most effective treatment against difficult malaria cases (Wells, 2011; Antony and Parija, 2016). Recently, ACTs have become the most relied on treatment for drug resistant and susceptible malaria cases (Antony and Parija, 2016; Elfawal et al., 2015; Kavishe et al., 2017). Specifically, it was found that combining longer-lasting antimalarial drugs with short-acting artemisinin was effective against most drug-resistant malaria with low frequencies of resistance development (Kavishe et al., 2017). Combinatorial treatments of chemicals reduce exposure of any single chemical and thus delay resistance mechanisms (Chow and Yu, 1999; Gionchetti et al., 1999; Elfawal et al., 2015; Blasco et al., 2017; Kavishe et al., 2017).

Blood feeding arthropods are a particularly challenging pest species that can negatively influence the health of a range of hosts. Blood feeding is a life strategy found almost exclusively in the arthropods and creates opportunities for the vectoring of pathogens from the blood feeder to its food source. For example, the non-obligate blood feeding *Anopheles* mosquito has been wildly successful at adapting to a diversity of hosts and is responsible for transmitting malaria to hundreds of known taxa including insects, reptiles, birds, and mammals (Martinsen et al., 2008). Blood feeding pests present a unique challenge to the health of humans and livestock as these pests are extremely

intimate with their hosts' main system of transport, the circulatory system. Identification of chemical coping behaviors by of wildlife associated with blood feeding pests may provide researchers with a "divining rod" to discover unique chemicals and strategies to use those chemicals that could be used against pests.

Here we consider whether chemical coping behaviors observed in a local avian species, the golden eagle (*Aquila chrysaetos*), can lead us to the discovery of chemical defenses against pests. Golden eagles are ideal subjects to address chemical coping behaviors because they integrate greenery (fresh plant material) into their perennially reused nests, likely to reduce parasitic infestations. In general, birds that reuse their nests, such as golden eagles, are more susceptible to parasitic infestations and more likely to include greenery, introduced green plant material, in their nests than birds that build new nests every breeding season (Wimberger, 1984). Specifically, golden eagles meet many of the criteria for chemical coping against a blood feeding pest, the Mexican chicken bug (*Haematosiphon indorus*). In a recent study, Dudek (2017) established that golden eagles preferentially select certain plant taxa relative to abundance in the habitat to use as greenery in nests. Dudek (2017) found that the addition of greenery in the nests of golden eagles is correlated with a reduction in nest infestations by Mexican chicken bugs, *Haematosiphon inodorus*. Although previous studies have examined nest plant composition in relation to pest populations in avian nests (Gwinner and Berger, 2005; Scott-Baumann and Morgan, 2015), no published study to date has analyzed the chemical diversity of nest greenery or the direct effects of nest greenery on the behavior and activities of individual parasites. This knowledge may prove useful in the search of new bioactive chemicals and modes of action against pests.

In Chapter 2, we demonstrate that observations of nesting golden eagles can direct our attention to plants with diverse, unique, and potentially bioactive chemicals and chemical mixtures. We first targeted plants with potential chemical diversity based on observed frequency of plant taxa use in golden eagle nests relative to availability of these plants in nesting territories (see Dudek, 2017). We targeted big sagebrush species (*Artemisia tridentata*) that were detected in eagle nests in lower proportion than available in the territory as our "underrepresented" species. We targeted rabbitbrush species (*Ericameria* sp., previously classified as *Chrysothamnus*) and spiny hopsage (*Grayia spinosa*) that were detected in eagle nests in higher proportion than available in the territory as our "overrepresented" species. We performed gas chromatography and liquid chromatography coupled with mass spectrometry to determine diversity and uniqueness of chemicals in each targeted plant species. Both under- and overrepresented plant species contained a wide diversity of both volatile and water-soluble chemicals. The underrepresented plant, big sagebrush, contained the highest diversity of volatile chemicals compared to the other plants tested. The overrepresented plant, green rabbitbrush, contained the highest diversity of water-soluble chemicals compared to the other plants tested. We found evidence that the purported chemical coping behaviors of golden eagles led us to a wide diversity of unique, bioactive volatile and water-soluble chemicals that may have relevance to arthropod management.

In Chapter 3, we tested whether volatiles of plants used by golden eagles had bioactive potential against a blood feeding pest. We took a biorational repellent approach to see if plants under- and overrepresented by golden eagles influenced the behavior of the common bed bug, *Cimex lectularius* (Hemiptera: Cimicidae), an obligate blood

feeding, non-pathogen vectoring parasite that is closely related to Mexican chicken bugs. The common bed bug represented a model for the responses of the closely related Mexican chicken bugs to volatile chemicals associated with under- and overrepresented plants used by golden eagles in their nests. We observed exploration (host-searching), grooming, and akinesis (resting) behaviors of bed bugs under low and high concentrations of volatile chemicals. Both under- and overrepresented plants with high concentrations of volatiles significantly decreased akinesis behavior of bed bugs. This change in behavior of bed bugs was explained by two dimensions of the volatiles: concentration and diversity. We also observed a potential pattern in the sequential organization of behaviors by bed bugs that further explained the disruption of akinesis behavior under high concentrations of volatiles. Bed bugs generally allocated more time to grooming after exploration when exposed to higher concentrations of volatiles from plants found in nests of golden eagles.

Pests have been and will continue to be recognized for detrimental effects on hosts (Poulin, 1995; Windsor, 1998; Zhang and Wang, 2017; Pohl et al., 2018). Among a range of other tactics (behavioral, biological, and physical), chemical treatments are an especially powerful tool to manage pests of human concern. However, over-reliance on individual chemicals has led to the evolution of resistance in pests. Future pest management requires innovation to reduce the risk of resistant phenotypes developing and plants are uniquely adapted to direct us towards a sustainable approach. Examining and deciphering the diversity of chemicals used in plant taxa and when, where, and how these chemicals are then exploited by animals to control their pests has the potential to

reveal new mixtures of bioactive molecules with novel modes of action against pests of concern for both wildlife and humans.

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Figures

Figure 1.1 Comparison of defense tactics by human, plant, and avian wildlife hosts to manage pest species

Host tactics are often diverse with a focus on optimizing a defense against a specific pest. Hosts often share chemical, biological, physical, and behavioral tactics, which vary in their level of reactivity and specificity. It is important to note that these tactics are not mutually exclusive, for example grooming and preening (asterisk) can be categorized as both a physical/mechanical and behavioral/cultural tactic. Tactics are often deployed in tandem to maximize effectiveness against a pest.

CHAPTER TWO: DISCOVERING DIVERSE MIXTURES OF BIOACTIVE CHEMICALS IN PLANTS BY OBSERVING THE CHEMICAL COPING BEHAVIORS OF AN AVIAN HOST

Abstract

Pests represent a current and future threat to food security and human health. Managing crop damage and transmission of pathogens from pests requires innovative and sustainable approaches. Our current approach to discovering and developing chemical control treatments against pests is tedious, inefficient, and often outpaced by evolving chemical resistant phenotypes in pests. We propose that identifying observations of chemical coping behaviors in wildlife may provide an effective framework to facilitate discovery of bioactive chemical mixtures that can deter a wide range of pests. Chemical coping behavior is defined as the exploitation of naturally occurring chemicals within a host's environment as therapy against pests. For example, the use of greenery in nests by wild avian species may provide clues to plant species that can deter nest-dwelling parasites. Many plants use chemicals to deter pests, which wild avian hosts can exploit to combat pests in their nests. We used a local host-pest-plant interaction to discover the potential chemical diversity and bioactivity of greenery found in nests of golden eagles (*Aquila chrysaetos*). We hypothesized that plants species underrepresented in the nests of eagles would have greater chemical presence and diversity than those plants that are overrepresented in nests. Additionally, we hypothesized that overrepresented plant species would have unique chemicals that are not present in the underrepresented plant

species. We tested our hypotheses by comparing the diversity of volatile and water-soluble chemicals in four targeted plant species identified in nests of golden eagles that were under or overrepresented relative to their availability within nesting territories. Using a literature search, we demonstrated that the classes of chemicals in under- and overrepresented plant species are potentially bioactive against a range of pests. We also found that the highly available, underrepresented plant species had the highest diversity of chemicals and that the less available overrepresented plant species contained more unique chemicals than other targeted plants. Our results revealed that the underrepresented plant species (big sagebrush) provided the highest diversity in volatile chemicals and the overrepresented plant species (green rabbitbrush) provided the highest diversity in water-soluble chemicals. Additionally, we confirmed that inclusion of the overrepresented plant species offered unique chemicals not found in underrepresented plant species. This work provides a preliminary foundation to guide future studies that can further separate and identify structures of candidate chemicals for potential bioactivity against pests. As demonstrated herein, observing, and recognizing chemical coping behaviors in wildlife provides a potentially useful and sustainable strategy for discovering diverse sources of chemicals that can combat pests.

Introduction

Growth of human populations and climate change demands innovative strategies to manage the negative consequences that pest species have on food security and human health. It is predicted that in the next twenty years, food production will need to double to meet the needs of growing human populations (Lobell et al., 2008; Godfray et al., 2010; Campbell et al., 2016). It also is predicted that by 2050, the shifts in climate will decrease

global major crop yields due to losses caused by pests (Delcour et al., 2015; Campbell et al., 2016; Deutsch et al., 2018). In response to these effects, pesticide use is expected to increase in both frequency and application volume to protect our crops (Delcour et al., 2015). Beyond food security, changing climates are causing shifts in the ranges of arthropod vectors (Haines et al., 2006; Carvalho et al., 2017) and their pathogens (Rochlin et al., 2013; Samy et al., 2016; Wu et al., 2016; Armstrong et al., 2017; Sonenshine, 2018). For example, ticks are responsible for 95% of vector-borne disease in North America and some tick species are undergoing range expansion (Leo et al., 2017; Sonenshine, 2018). Human populations have experienced a spike in cases of tick-borne disease cases (Khatchikian et al., 2015; Kugeler et al., 2015; Oliver et al., 2017). Increased incidence of tick-borne disease due to an increase in pathogen transmission by arthropod vectors that is partly a consequence of greater growth and mobility of human populations (Alirol et al., 2011; Arthur et al., 2017), which is exacerbated by climate change (Lafferty, 2009; Pickett et al., 2010; Balogun et al., 2016).

Chemical treatments remain one of the most powerful tools to manage arthropod pests (Perring et al., 1999; Leal, 2014; Dambolena et al., 2017; Hill et al., 2018). While there are many examples of successful synthetic chemical treatments (Leal, 2014), some of the most successful chemical treatments, have been derived or synthesized from natural products (Veeresham, 2012; Pan et al., 2013; de la Parra and Quave, 2017; Garnatje et al., 2017; Kayser, 2018). For example, pyrethroids, synthetic derivatives of pyrethrum originally found in the dried flower heads of *Chrysanthemum* spp., are widely used in crop systems because of their effectiveness against targeted pests and minor toxicity to mammals (Dorman and Beasley, 1991; Soderlund, 2015; Field et al., 2017).

Ethnobotany, also known as traditional medicine, has been effective at directing discovery of bioactive chemicals from natural sources (Veeresham, 2012; Pan et al., 2013; de la Parra and Quave, 2017; Garnatje et al., 2017; Kayser, 2018). One example is the effective treatment of malaria with artemisinin-based combination therapies (ACTs) (Wells, 2011; Antony and Parija, 2016). ACTs were inspired from traditional Eastern medicinal use of Chinese herb *Artemisia annua* (Elfawal et al., 2015; Antony and Parija, 2016). The use of *A. annua* by humans represents an example of chemical coping behavior, which is defined as the exploitation of naturally occurring chemicals within a host's environment as a therapeutic. Unfortunately, phenotypes of resistance have developed to both pyrethroids and ACTs in targeted pests (Soderlund and Knipple, 2003; Antony and Parija, 2016; Hemingway et al., 2016; Naqqash et al., 2016; Tyagi et al., 2018). Until we better understand how to manage the underlying mechanisms of chemical resistance in pests, we will need to continue investing time and effort into discovering novel bioactive chemicals and modes of action (Sparks, 2013; Hardy, 2014; Sparks and Nauen, 2015).

To facilitate discovery of new chemical treatments for pests, we propose expanding our observations of chemical coping behaviors beyond those observed in traditional medicine from human systems. There is increasing evidence that wild and domestic animals display their own chemical coping behaviors where naturally occurring chemicals in their environment are exploited to provide therapy against pests (Clayton and Wolfe, 1993; Huffman, 2003; Rounak et al., 2011; de Roode et al., 2013). For example, ants harvest tree resin to protect their brood from harmful microbes (Brütsch and Chapuisat, 2014; Brütsch et al., 2017). Defensive anointing (Carroll et al., 2005;

Weldon, 2010, 2013), also known as ‘fur-rubbing’ (Carroll et al., 2005; Morrogh-Bernard, 2008; Bowler et al., 2015) and ‘anting’ (McAtee, 1938; Groskin, 1950; Ehrlich et al., 1986; Potter, 1970; Bush and Clayton, 2018), is common in the animal kingdom, which consists of a host topically applying a naturally occurring chemical source, such as plants or in some in cases ants, to deter ectoparasites. Endoparasites also can be deterred through exploitation of plant chemicals. For example, lambs infected with gastrointestinal parasites preferred to consume diets that contained tannins (a class of bitter phenols), which resulted in lower parasite loads (Lisonbee et al., 2009; Villalba et al., 2014).

Excessive dependence on chemical strategies to fight our pests has put us in the precarious situation of managing chemical resistant phenotypes in our pests. Current strategies typically rely on the discovery or synthesis of new ‘silver bullet’ chemicals that often ends with loss of efficacy over time as a result of imposed selection for resistance, which prompts the reiteration of selection for resistance traits (Theuretzbacher, 2011; Brown and Wright, 2016; Gould et al., 2018). This tedious cycle of resistance alongside the unintended environmental and health consequences of mismanaged pesticides led to the advent of integrated pest management (IPM, including integrated vector management), which has become essential to manage resistant phenotypes in pests (Pretty, 2018; Reznick et al., 2019). Integrated pest management frameworks focus on ecologically based combinatorial tactics that start with cultural/behavioral tactics that are preventative and untargeted and include reactive and targeted chemical interventions only when pest species pass a damage threshold (Figure 1.1). Wild organisms employ several tactics to lessen the burden of parasites that often mimic the methods of IPM. For example, alternative nest use is an example of behavioral host tactics used by raptor nest

re-users (Ontiveros et al., 2008; Lesko and Smallwood, 2012). Grooming and molting are an example of physical host tactics used by both vertebrate and invertebrate hosts (Boecking and Ritter, 1993; Mooring et al., 2004; Clayton et al., 2010). Many plant species use biological tactics such as recruitment of natural enemies of their pests (Turlings and Wäckers, 2004). Chemical defenses can be synthesized by hosts, such as the venom of *Crematogaster* ants (Marlier et al., 2004; Heredia et al., 2005) or alkaloids in the plant family Amaryllidaceae (López et al., 2002). Chemical defenses can also be sequestered from an external source such as the sequestration of unpalatable cardenolides from milkweeds (*Asclepias* spp.) by monarch butterflies (*Danaus plexippus*) to deter avian predators (Brower and Moffitt, 1974; Malcolm and Brower, 1989) or exploited such as wood ants (*Formica paralugubris*) harvesting tree resin to defend their nests (Chapuisat, 2007; Brütsch and Chapuisat, 2014; Brütsch et al., 2017).

Observing and understanding the chemical interventions used by plants and wildlife through chemical coping strategies may offer much needed insight to sustainably manage pests. Plants, being of sessile nature, must manage the continual reciprocal evolution of resistance mechanisms within their pests (Rausher, 2001; Pavela and Benelli, 2016; Frickel et al., 2017; Mills, 2017). Many organisms rely on chemical diversity to disrupt the onset of resistance (Richards et al., 2015, 2016; Mitchell et al., 2016). One example is increased chemical diversity that can create bioactive synergies to reduce effective concentrations (Seeram et al., 2004; Bakkali et al., 2008; Richards et al., 2016) and delay resistance mechanisms (Roush, 1998; Rausher, 2001; Elfawal et al., 2015). Investigations into whether animals, other than humans, participate in chemical coping behaviors that exploit bioactive chemicals from plants are limited. Instead, studies

often focus on the behaviors of the host (Huffman, 2003) or the effects of the exploited sources on the pest species (Dubiec et al., 2013) rather than the chemical sources the host is exploiting. Perhaps the best example of all three of the former effects being investigated in one system is the use of the plant *Vernonia amygdalina* by chimpanzees (*Pan troglodytes schweinfurthii*). Chimpanzees of irregular health were observed consuming the bitter juice from the pith of *Vernonia amygdalina* (Huffman and Seifu, 1989). This plant is available year-round but was generally used in low frequency by chimpanzees, which suggested that the plant was used medicinally. Huffman et al. (1993) provided a case study where a chimpanzee inflicted by the nematode *Ternidens* species, which causes gastrointestinal distress, experienced a decline in parasite load after ingestion of *V. amygdalina*. The same study investigated the bioactive constituents of *V. amygdalina* and found the chemicals, vernodalin and vernonioside B_1, in the pith and leaves. There have been additional studies into the chemistry of *V. amygdalina* demonstrating anti-tumor and anti-microbial properties (Jisaka et al., 1993) and antifeedant properties (Ganjian et al., 1983). More studies are needed that couple investigations of chemical coping behaviors by a wider variety of hosts, the effects of exploited resources on parasites and pathogens, and the chemistry of the exploited resources.

Here, we used host-pest-plant interaction as a case study that demonstrated how observations of chemical coping behaviors in an avian host can direct the discovery of diverse bioactive chemical presences that could affect pests. We targeted observations in golden eagles (*Aquila chrysaetos*) for several reasons. First, many large birds of prey are habitual nest re-users (Wimberger, 1984), which creates an insulated microcosm where

decaying prey items maintain communities of potentially detrimental microbes, such as fungi, and arthropods. Second, pests within nests can negatively impact the success of avian young (Barclay, 1988). For example, 94% of golden eagle nests surveyed in southwestern Idaho contained a blood sucking pest, the Mexican chicken bug, *Haematosiphon inodorus* (Hemiptera: Cimicidae), an obligate blood-feeding ectoparasite (Dudek, 2017). These parasites have been attributed to nestling deaths in a variety of avian species (Usinger, 1947; Platt, 1975; Grubb et al., 1986; McFadzen and Marzluff, 1996). Third, many avian nests are known to contain aromatic greenery (Rodgers et al., 1988; Gwinner, 1997; Dubiec et al., 2013; Heinrich, 2013). Moreover, golden eagles were selective about the greenery included in their nests (i.e., under- or overrepresented in nests relative to availability in territories) and collected greenery was often replaced with plants of the same taxon (Dudek, 2017). Use of aromatic greenery has been hypothesized to reduce parasites found in nests of avian species (Scott-Baumann and Morgan, 2015). The use of this greenery by golden eagles in their nests had a positive impact on the hematocrit of golden eagle nestlings and suggests that nest greenery may have deterred the blood feeding parasites that compromise the health of nestlings (Dudek, 2017). Additionally, the plants observed in the nests of golden eagles belong to families with known chemical diversity that are biologically active against a variety of pest targets (Table 2.1, in order of prevalence found in golden eagle nests: Asteraceae, Amaranthaceae, Poaceae, Apiaceae, Brassicaceae, Saxifragales, Salicaceae, Betulaceae, Rosaceae, Polemoniaceae, and Amaryllidaceae) (Dudek, 2017)). For a more detailed description of golden eagle nesting territories see Dudek (2017).

Two classes of chemicals, terpenoids and phenolics, are of particular interest in chemical coping behaviors. Terpenoids (Gershenzon and Dudareva, 2007; Ashour et al., 2018) and phenolics (Haslam, 1988; Salminen and Karonen, 2011) have proven useful to human services in health, food, and environment and can be found in abundance in the well-studied family of Asteraceae, which includes the majority of plants used by golden eagles (Table 2.1). For example, both the genera *Artemisia* (including sagebrush found in eagle nests, Dudek, 2017) and *Chrysothamnus* (including rabbitbrush found in eagle nests, Dudek, 2017) are within the family Asteraceae and contain terpenoids (i.e., volatile chemicals) that repel herbivores (Giordano et al., 2017). These taxa also include water-soluble chemicals like phenolics that have insect antifeedant properties (Bohm and Stuessy, 2013) and sesquiterpenoids that have remarkable cytotoxic properties (Rodriguez et al., 1976; Ghantous et al., 2010). Notably, sesquiterpene lactones, found ubiquitously in the family Asteraceae (Compositae, Seaman, 1982), have significant human health implications by showing high bioactivity against malaria and cancer (Rodriguez et al., 1976; Chadwick et al., 2013). Given that sagebrush and rabbitbrush, both of which are found in the nests of golden eagles (Dudek, 2017), contain bioactive chemicals, these species are prime candidates for their potential to combat a variety of pests. However, none of the combinations of chemicals in the nest greenery used by golden eagles have been examined for potential bioactivity against pests to date.

We explored the potential bioactivity and chemical diversity of greenery found in nests of golden eagles through a literature search and by using analytical chromatography. We predicted that the chemical presence and diversity of volatile and water-soluble chemicals would differ between underrepresented and overrepresented

plants found in golden eagle nests. Specifically, we hypothesized that underrepresented plants (relative to high abundance within nesting territories) would have greater chemical presence and diversity than those plants that are overrepresented in nests (relative to low abundance within nesting territories). Additionally, we hypothesized that overrepresented plants would have unique chemicals that are not present in the underrepresented plant species. Our rationale was that geographically abundant (highly available in nesting territories) plants have greater overall exposure to a diversity of pests, which requires a greater diversity of chemical defenses to combat those pests. In contrast, plants that are less geographically available plants, which may indicate microhabitat specialization or poor competition with abundant plants, would have unique chemicals. Inclusion of a variety of plants from the landscape in nest greenery would create complex chemical diversity to combat a variety of pests than inclusion of any single plant. Golden eagles have a diversity of pests, which include the protozoa *Trichomonas gallinae* (Dudek, 2017) and various trematode species (Baker et al., 1996). No single chemical or plant is likely to be effective against all pests due to rapid evolution (Carrière et al., 2016). Additionally, reuse of nests by golden eagles (Kochert and Steenhof, 2012) increases interactions of pests with greenery and without chemical diversity, pests may be more likely to evolve mechanisms of resistance (Wimberger, 1984). We tested our hypotheses by comparing the diversity of volatile (which included terpenoids) and water-soluble (which included phenolics and sesquiterpenoids) chemicals in nest greenery that was underrepresented and overrepresented relative to its availability within territories of golden eagles.

Methods

Literature Review for Bioactivity in Nest Greenery of Golden Eagles

We relied on previous work that identified the plant species found in nests of golden eagles (Dudek, 2017). Briefly, use of plants by golden eagles was determined by taking photos of nests and using Program SamplePoint (Booth et al., 2006) to identify the dominant green plants in nests from photos. Additionally, nest greenery was collected and brought back to the laboratory where greenery was identified down to a reliable taxon using internet resources and local plant identification books (IDFG, 2011; Utz et al., 2013; Figure 2.1). We conducted a literature search for the potential bioactivity of plant taxa observed in nests of golden eagles by combining the plant taxa with the term "chemical defense" using Google Scholar. Plant family was always searched first, followed by genus and species, when known. This approach was by no means an exhaustive review of chemical defenses in these plant taxa. The goal of the literature search was to provide a general overview of the potential bioactivity of these plants that could be used to interpret under- and overrepresentation of plant taxa as greenery by golden eagles.

Selection of Targeted Plants for Chemical Analysis

We also relied on previous work by Dudek (2017) to target plants under- and overrepresented by golden eagles relative to availability within eagle territories for chemical analysis. Plant availability was determined using line-point-intercept within each of fifteen golden eagle territories where greenery of nests also was quantified. A preference foraging index (Chesson, 1983) was used to determine which of the plants identified in nests were under- or overrepresented (Figure 2.2). We analyzed Chesson's

index between plant species using a Kruskal-Wallis test (kruskal.test from stats package) using the base R (R version 3.5.2 (2018-12-20)) and RStudio (Version 1.3.959), followed by a post-hoc analysis using Dunn's multiple comparison test (dunnTest from FSA package). From this analysis, we chose to target chemical diversity of a single species of plant that was underrepresented in nests (big sagebrush, *Artemisia tridentata*) and three species of plants that were overrepresented (gray rabbitbrush, *Ericameria nauseosa*; green rabbitbrush, *Chrysothamnus viscidiflorus*; spiny hopsage, *Grayia spinosa*).

Plant Collection

We collected separate composites of the under- and overrepresented plant species from within four nesting territories of golden eagles (Dudek, 2017). Within each nesting territory, we identified two to three distinct patches (>50 m apart) and combined stems and leaves collected from two to five individual plants of each targeted plant species (approximately 2.0 g wet weight (WW) per plant) within each patch. Each of the patch composites were coarsely ground (<2 mm particle size) in liquid nitrogen with a mortar and pestle. Once ground, 2.0 g (WW) of each patch composite were combined to create a single nesting territory composite separately for each of the targeted species of plants. We then combined 8.0 g (WW) of each nesting territory composite to create a plant species composite for chemical analysis (see Figure 2.3 for complete process). We used gas chromatography to verify that the chemical diversity from plant species composites represented the patch composites (Appendix A).

Separation of Volatile Chemicals

Separation of volatile chemicals from a subset of each plant species composite (approx. 500 mg (WW)) was determined using headspace gas chromatography (GC).

Each composite was analyzed using an Agilent 6890 N GC (Santa Clara, CA) coupled with a Hewlett-Packard HP7694 headspace autosampler (Palo Alto, CA). The headspace program was as follows: 100 °C oven temperature, 110 °C loop temperature, and 120 °C transfer line temperature. The vial equilibrium and pressurization times were both 0.20 min, the loop fill time was 0.50 min, the loop equilibrium time was 0.20 min, and the injection time was 0.50 min. One mL of headspace gas from each sample was injected into an Agilent J&W DB-5 capillary column (30 m × 250 μm × 0.25 μm, Santa Clara, CA) with helium as the carrier gas at a constant flow of 1.0 mL min−1 and a splitless injector temperature of 250 °C. The temperature program for the GC was as follows: 40 °C for 2.0 min, then increased by 3 °C.min^{-1} to 60 °C, then by 5 °C.min^{-1} to 120 °C and finally by 20 °C.min−1 to 300 °C where the final temperature was held for 7 min. Inlet pressure was 80 KPa and the flame ionization detector was set at 300 °C. We used distinct peaks at individual retention times (min) to identify individual volatile chemicals from our plant species composite extracts (Figure 2.4A).

Separation of Water-Soluble Chemicals

We prepared two replicates of each plant species composite by serially extracting 100 mg of ground material three times using 90% high pressure liquid chromatography (HPLC) grade methanol (MeOH), 9.5% distilled water, and 0.5 % acetic acid (Sakakibara et al., 2003). Extraction consisted of sonicating sample solutions, placing them in a centrifuge, and then collecting the supernatant. Solvent extracts of each plant sample were then evaporated in a TurboVap LV Evaporator (Zymark, Hopkinton, MA, USA). Extracts were then reconstituted in 1.0 mL of HPLC grade MeOH and filtered through a 0.45 μm filter and stored in a 2.0 mL amber vial at −20 °C.

To separate the water-soluble chemicals from our plant species composite extracts, we performed HPLC coupled mass spectrometry (MS), using an ultra-high-resolution quadrupole time of flight (QTOF) mass spectrometer (Bruker maXis, Billerica, MA, USA). The electrospray ionization (ESI) source was operated under the following conditions: positive ion mode, 1.2 bar nebulizer pressure, 8 $L.min^{-1}$ flow of N_2 drying gas heated to 200 °C, 3000 V to −500 V applied between HV capillary and HV end-plate offset, mass range from 80 to 800 m/z, and the quadrupole ion energy at 4.0 eV. Sodium formate was used to calibrate the system in this mass range. HPLC separation was achieved using an XTerra MS C18 column, 3.5 µm, 2.1 × 150 mm (Waters, Milford, MA, USA) with a flow rate of 250 $\mu L.min^{-1}$ on an UltiMate 3000 HPLC (Thermo Scientific, Waltham, MA, USA). The mobile phase consisted of water with 5.0% acetonitrile and 0.1% formic acid (buffer A) and 5.0% acetonitrile and 0.1% formic acid (buffer B). A linear gradient method was used to separate analytes starting at 5% buffer B and increasing to 60% buffer B over 25 min. A 1.0 µL sample injection volume was used. We used specific charge (m/z) within distinct retention times (min) to quantify individual volatile chemicals from our plant species composite extracts (Figure 2.5A). We were able to tentatively identify water-soluble chemicals by m/z (Appendix B).

Chromatogram Alignment and Chemical Diversity Indices

Retention times were manually aligned for chemicals from GC and HPLC-MS (Figure 2.4B and 2.5B). Both datasets used parameters that minimized noise within the data. Those parameters included limitations on linear shifts of retention times among samples and only chemicals that appeared in all replicates of plant species composites were considered as present. Base R (R version 3.5.2 (2018-12-20)) and RStudio (Version

1.3.959) were used for analysis of chemical diversity using a wide data format of manually aligned retention times (area under the curve (AUC) for volatile chemicals) and m/z (intensity for water-soluble chemicals) in the vegan package using the diversity function denoting inverse Simpson index, hereafter diversity index (Dixon, 2003; Oksanen, 2015). The diversity index we used is weighted to account for both chemical richness and relative chemical concentration (AUC or intensity).

To create the upset chart demonstrating unique and shared chemicals (Figure 2.6), GC and HPLC-MS data were combined into a single data set. Each individual chemical (designated by either unique retention time for volatile chemicals or unique m/z within retention time for water-soluble chemicals) was assigned an identification number. Each identification number was assessed and determined to be either absent or present in the targeted plant species. In Rstudio, using the package UpsetR, this data was processed with the upset function. The statistical analysis was repeated for the GC data for volatile chemicals and the HPLC-MS data for water-soluble chemicals separately (Appendix C).

Results

Volatile Chemical Diversity

Of the four species of plants we investigated (one underrepresented and three overrepresented plant species), we found that each species varied in the total number of chemicals, number of unique chemicals, and chemical diversity of volatile chemicals (Table 2.2, Figure 2.4). A total of 81 chemicals were identified by retention times from the GC. We found that the underrepresented plant species, big sagebrush, contained the highest number of volatile chemicals (n = 64), highest unique volatile chemical contributions (n = 38, Figure 2.4) and highest diversity index (1/D = 9.32, Table 2.2).

Overrepresented plant species, green and gray rabbitbrush, contained comparable numbers of volatile chemicals (n = 28 and 23, respectively). Green rabbitbrush contributed 10 unique volatile chemicals and had a diversity index of 4.02 and gray rabbitbrush contributed two unique volatile chemicals and had a higher diversity index than green rabbitbrush of 7.36 (Table 2.2). Spiny hopsage had the lowest number of volatile chemicals (n = 8), and contributed no unique chemicals (Figure 2.4), and had the lowest diversity index for any plant species tested of 1.72 (Table 2.2).

Water-Soluble Chemical Diversity

Of the four plants we investigated, we found each species varied in the number, uniqueness, and diversity of water-soluble chemicals (Table 2.3 and 2.5). A total of 264 chemicals were identified by unique m/z at specific retention times using HPLC-MS. This number is likely an underestimation of the total chemical diversity present due to the method of separation. Specifically, it is likely that some chemicals may share similar features (such as the same molecular mass and retention) that can cause them to be interpreted as a single individual chemical when there is more than one chemical present (Nyiredy, 2004).

We found that the overrepresented plant species, green rabbitbrush contained the highest number of water-soluble chemicals (n = 125), highest unique water-soluble chemical contributions (n = 58, Figure 2.5), and had the highest diversity index (1/D = 31.38, Table 2.3) compared to other plant species. In contrast, big sagebrush and gray rabbitbrush, contained comparable amounts of water-soluble chemicals (n = 100 and 102, respectively, Figure 2.5). Big sagebrush contributed 53 unique water-soluble chemicals and had a diversity index of 15.50 whereas gray rabbitbrush contributed 39 unique water-

soluble chemicals and had a higher diversity index of 23.98 (Table 1.3) relative to big sagebrush. Spiny hopsage had the lowest amount of water-soluble chemicals (n = 78), the lowest contribution of unique chemicals (n = 38; comparable to unique contributions by gray rabbitbrush, Figure 2.5), and had lowest diversity index of 14.96 (Table 2.3) compared to other tested species.

Analysis of Shared and Unique Chemicals Occurring Across Targeted Plant Species

Unique chemicals (n = 237) far outnumbered shared chemicals (n = 108) across chemical classes in targeted plant species (Figure 2.6). Shared chemicals are those that align between one or more species for both volatile (GC) and water-soluble (HPLC-MS) chemicals. When considering all detected chemicals, big sagebrush had the highest number of total chemicals (n = 164) and highest number of unique chemicals (n = 90), followed by green rabbitbrush (n = 153 total chemicals, n = 68 unique chemicals). Shared chemicals across all four targeted plant species were higher than any other combination (n = 23). There were 15 shared chemicals among gray rabbitbrush, green rabbitbrush, and big sagebrush. Gray rabbitbrush and green rabbitbrush shared the most two-pair combinations (n = 18), followed by green rabbitbrush and big sagebrush (n = 16), and then gray rabbitbrush and big sagebrush (n = 11). There were less than 10 chemicals that were shared between spiny hopsage and any other species.

Discussion

Chemical coping behaviors in wild organisms such as golden eagles and their use of greenery in nests may provide an unbiased framework for discovering a diversity of potentially bioactive chemicals. Support for our hypotheses were variable. In support of our hypothesis that highly available greenery would contain the greatest chemical

diversity, we found that the underrepresented plant species, big sagebrush (*Artemisia tridentata*), which was highly available within golden eagle nest territories, contained the highest number and diversity of volatiles and total chemicals (volatiles and water-soluble combined) when compared with plants overrepresented in the nests of golden eagles (rabbitbrush, *Ericameria nauseosa, Chrysothamnus viscidiflorus*. and spiny hopsage, *Grayia spinosa*). Inconsistent with our hypothesis, the overrepresented plant species, green rabbitbrush (*Chrysothamnus viscidiflorus*), contained more water-soluble chemicals than the underrepresented and highly available sagebrush or any other species. Consistent with our hypothesis that overrepresented greenery would contain unique chemicals not found in underrepresented greenery, we found that unique chemicals within each species outnumbered combinations of shared chemicals with two of the overrepresented plant species contributing unique chemicals not found in the underrepresented plant species. However, contrary to our hypothesis, big sagebrush, contained the greatest number of unique chemicals. Each different combination of these four plant species has the potential to diversify the chemical profile found in the nest by lending access to chemicals that are species, genus, or family dependent. Below we examine the chemical diversity and potential bioactivity of greenery found within the nests of golden eagles as directed by their chemical coping behavior.

The chemical diversity observed in this system derives from, in part, plant taxa investing in different chemical classes dependent on the diverse abiotic and biotic interactions they experience in their environments. Volatile chemicals are primarily a distance cue processed by olfactory receptors, which can warn of toxicity (Camazine, 1985) or attract pollinators (Pichersky and Gershenzon, 2002) and natural enemies that

protect plants against pests (Pichersky and Gershenzon, 2002; Turlings and Wäckers, 2004). The high diversity of chemicals in big sagebrush likely reflects the wide availability of this plant in the sagebrush steppe ecosystem (Kelsey et al., 1983; Takahashi and Huntly, 2010; Kleinhesselink and Adler, 2018). As an abundant plant, big sagebrush encounters a wide variety of herbivores on the landscape (Wiens et al., 1991; Sanford and Huntly, 2010; Takahashi and Huntly, 2010) and likely requires investment in different chemical classes to protect against both specialist and generalist herbivores. Water-soluble chemicals (i.e., phenolics) often require contact to be processed by gustatory (Scott and Mark, 1987; Poudel and Lee, 2016) or pain receptors (Lynn, 1990) but some subclasses within the phenolics class of chemicals are capable of being detected visually if an organism has the ability to see into the ultra-violet (UV) spectrum (Krauss et al., 2002). Constituents of rabbitbrush species do fluoresce under UV conditions (McArthur et al., 1978) and many birds of prey can see into the UV spectrum (Rajchard, 2009; Lind et al., 2013) and detect odors at a distance (Potier, 2019). Given the greater diversity of water-soluble chemicals in rabbitbrush, these species may be used more than they are geographically available by eagles because the phenolics that they produce are more visually detectable by eagles than plants producing volatile chemicals.

The highest overall chemical diversity and number of unique chemicals across both chemical classes in big sagebrush may explain why it is present in nests (Figure 2.1), but not used in lower proportion than its abundance in the territory (Figure 2.2). The unique chemicals and high concentrations in big sagebrush could negatively impact nestling health. For example, big sagebrush has relatively higher concentrations of total (shared and unique) volatile chemicals (Appendix D) than any of the overrepresented

plant species. While water-soluble chemicals require extraction into another media, volatiles can be released from glands through disturbance of the leaves or heat (Loreto et al., 2006; Loreto and Schnitzler, 2010). Therefore, nestlings or adults moving on the nest, warming environmental temperatures, or emission of radiant heat from eagles could release the volatiles. Toxicity and irritation by big sagebrush chemicals could explain underrepresentation compared to overrepresentation of rabbitbrush in the nests of golden eagles. For example, even relatively small concentrations of sesquiterpene lactones, found abundantly in the Asteraceae family and more specifically in the *Artemisia* genus, can cause skin rashes (Mitchell and Dupuis, 1971; Hjorth et al., 1976; Ducombs et al., 1990; Christensson et al., 2009). However, some inclusion (47% of nests surveyed used big sagebrush at least once, Figure 2.1) at relatively low amounts (1% of all nest greenery observations, Figure 2.1) compared to rabbitbrush species (40% of all nest greenery observations, Figure 2.1) may offer protection from pests. The potential toxicity of big sagebrush might explain the variable inclusion of the plant in golden eagle nests, which may only be tolerated with high pest loads or infestations of specific pests.

Variable inclusion of specific plant species with varying chemical concentrations and uniqueness may provide a novel approach to combat pests. Chemicals often have dose-dependent consequences against pests (Siemens and Mitchell-Olds, 1996; Grandjean, 2016) and reliance on increasing dose may lead to resistance in pest species (Mallet, 1989). Incorporating chemical diversity as an additional dimension of treatments against pests, where unique chemicals vary over time, may combat the ability for chemical resistant phenotypes to form through different modes of action and inter-chemical synergies (Gardner et al., 1999; Rex Consortium, 2013). By coupling diverse

plant species, both the diversity of individual chemicals within and across chemical classes increases. The observed diversity and variation across taxa in this study likely underrepresents the true chemical diversity potential of this system. Based both on our separation and extraction techniques we could have overlooked certain chemical classes or even individual chemicals (Hegeman, 2010). Chemicals that are structurally similar, in molecular weight or polarity, can cause overlap in retention time peaks causing one peak to appear as one chemical rather than two individual chemicals. Slight structural differences in chemicals can change the functionality of that chemical. For example, isomers of azadiractins, which have the same molecular weight, have shown variation in antifeedant activity in invertebrate herbivores, where azadiractin A has been shown to induce higher levels of antifeedant behavior than isomers, B, D, or H (Koul, 2008). The type of solvent used to extract the chemicals from greenery has the possibility of excluding chemical classes based on structural properties (Kim and Verpoorte, 2010). We did not investigate chemicals that contain non-organic elements (e.g., alkaloids, organosulfates), which are found in big sagebrush in low quantities (Kinney and Sugihara, 1943), as they typically do not extract into methanol (Kim and Verpoorte, 2010). However, these compounds have notable bioactivity (Table 2.1) and should be investigated in future studies. In addition, we targeted a subset of the plant diversity used in nests by focusing on a single time point for detection of use. Tracking phenology of plant use and corresponding chemical diversity in nests relative to availability with phenology of pests could reveal behavioral adaption of eagles to combat temporal dynamics of pest occurrence and the rate of acquired resistance by pests to nest chemistry.

While we could not provide specific identification or functionality of the chemicals detected in our analyses, our results offer evidence that chemical coping behaviors of golden eagles can direct us to plants with diverse and unique bioactive chemicals (Table 2.1). Observed chemical coping behavior in humans has already brought us a cornucopia of new drug possibilities, such as artemisinin and pyrethrum mentioned earlier (de la Parra and Quave, 2017; Kayser, 2018). However, the potential to detect novel chemicals and predict their bioactivity against molecular targets is limited by the biases of the sensory drives in the model organisms we use in biomedicine, such as fruit flies, rats, mice, and humans (Fuller and Endler, 2018; Yohe and Brand, 2018; Renoult and Mendelson, 2019). In contrast to model systems, wild systems curated over evolutionary time provide novel and potentially highly targeted chemical solutions to combat a greater diversity of pests (Jones et al., 1991; Bednarek and Osbourn, 2009). Chemical coping behaviors by wild species provide unique sensory drives that have been established by tight associations with the chemical sources they exploit (Higham and Hebets, 2013). Chemicals can be detected and processed uniquely within each taxon because of different phylogenetic sensory investments (Fuller and Endler, 2018). For example, many avian species depend on both visual and olfactory cues from their environment for foraging and identifying natal grounds (DeBose and Nevitt, 2008; Corfield et al., 2015). As such, wild species that are actively selected upon for unique sensory systems are more likely than model organisms with limited sensory drives to detect and select for unique and diverse chemicals (Yohe and Brand, 2018).

In addition, wild species are hosts to a broad diversity of pests across space and time because of consistent, continual interactions (Poulin, 1995) and, therefore, hold the

potential to diversify the discovery of chemicals against a variety of pests. For example, migratory birds (Anseriformes and Accipitriformes*)* have greater diversity in their parasite communities than their conspecific resident counterparts (Leung and Koprivnikar, 2016). As such, wild and mobile species have a greater diversity of target pests that may need to be matched to specific chemical pesticides. Pest insects have adapted chemoreceptors (i.e., olfactory and gustatory) sensitive to their host's metabolites (Chaisson and Hallem, 2012; Clifford and Riffell, 2013; Syed, 2015). This makes each host observed performing chemical coping behaviors as a potential lead for using chemical bioactivity that is targeted to specific pest species. Pests often share nuisance traits, which can make them easily identified and targeted (Gandon et al., 2002). For pests with the potential for rapid evolution due to short life cycles (i.e., microbes and insects), chemicals can be rotated to limit exposure, which makes use of chemical diversity as a robust tactic for managing resistance and tolerance traits (Cloyd, 2010; Shonga et al., 2013).

We caution that greenery selection by golden eagles may not be based solely on chemistry or as a defense against pests. The only overrepresented plant not belonging to the Astereae tribe and notably non-aromatic, was spiny hopsage, which generated no chemical results from our literature search (Table 2.1). This species had both relatively low chemical overlap with the other species and did not substantially contribute unique chemicals (Figure 2.6). Other functional traits such as handling time, plant morphology or appearance, or mate preferences of the host may also provide explanations for selection of nest greenery. The most accepted and supported hypotheses for inclusion of greenery in the nest of a bird are: 1) the mate courtship hypothesis, which asserts that nest

greenery attracts females to nests (Veiga et al., 2006); 2) the drug hypothesis; and 3) the nest protection hypothesis. Both the drug and nest protection hypotheses assert that nestlings benefit from the chemicals of greenery, either directly (drug) or indirectly (nest protection hypothesis, Scott-Baumann and Morgan, 2015). Nest greenery is often aromatic and non-structural, which implies function, however more studies are needed that focus on how avian species are detecting and selecting green nest material alongside chemical diversity analysis. It is important to note that these alterative explanations for selection of chemical diversity are not mutually exclusive.

Even if the mechanism responsible for diversifying nest greenery is unknown, nests still provide a resource for discovering mixtures of diverse chemicals. We propose that observations of nest greenery do represent chemical coping behaviors by an avian host, and we demonstrate that those observations could direct us to diverse and novel mixtures of chemicals. Studies of chemical coping behaviors in wildlife has primarily been limited to discussing the possibility that animals are exploiting chemicals as therapy against pests and disease (Ehrlich et al., 1986; Huffman, 2001; Forbey et al., 2009; Rounak et al., 2011). We suggest that experimental tests that focus on assessing the bioactivity of diverse chemical mixtures (see Chapter 3), rather than single chemicals or even single plant species, will promote the discovery of novel mixtures bioactive chemicals and modes of action. Moreover, these discoveries can only occur if animals have access to diverse plant taxa and if collaborations between wildlife ecologists (who observe the temporal and spatial behaviors of wildlife) and chemists are fostered. Ultimately, preservation of biological and chemical diversity and the convergence of

experts in behavior, chemistry, and drug discovery are need for sustainable chemical pest management solutions.

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Tables

Table 2.1 Review of potential chemicals (classes, subclasses or individual) and potential mechanism of bioactivity against molecular or organismal targets for each plant taxa found in nests of golden eagles (*Aquila chrysaetos*) in Southwestern Idaho (Figure 2.1)

Taxa in Nest			**Chemical class (subclass within a class or individual chemical)**	**Potential bioactivity**	**Target of potential bioactivity**	**Reference**
Family	*Genus*	*Species*				
Amaryllidaceae			Alkaloids	Antifeedant	Acetylcholine inhibition	(Kilgore and Kutchan, 2016)
	Allium		Organosulphoids (thiosulfinates); Phenols (flavonoids); Alkaloids; Terpenoids (saponins)	Antifeedant, Deterrent	Microbes and invertebrate herbivores	(Block, 1992; Mann et al., 2011; Lanzotti et al., 2013)
Amaranthaceae			Phenols (phenolic acids, flavonoids, aurones); Alkaloids; Betalians (betacyanins, betaxanthins); Sterols (ecdysteroids); Terpenoids (triterpenes, saponins)	Antifeedant, Deterrent	Invertebrate herbivores	(Correa et al., 2016)
	Atriplex		Alkaloids; Terpenoids (saponins)	Antifeedant	Vertebrate herbivores	(Cibils et al., 1998; Rastogi and Shukla, 2013)
		confertifolia	Chemistry unknown; (no results during literature search)			
	Grayia		Chemistry unknown; (no results during literature search)			

Taxa in Nest			Chemical class (subclass within a class or individual chemical)	Potential bioactivity	Target of potential bioactivity	Reference
Family	*Genus*	*Species*				
		spinosa	Chemistry unknown; (no results during literature search)			
Apiaceae; Umbellifers			Polyacetylenes (phthalides, Z-ligustilide); Phenols (phenylpropanoids, coumarins, furanocoumarins, pyranocoumarins, myristicin); Terpenoids (monoterpenes, daucan-type sesquiterpenes; sesquiterpene lactones)	Antifeedant, Deterrent	Microbes, invertebrate and vertebrate herbivores	(Berenbaum, 1981, 1990, 2001; Christensen and Brandt, 2006; Schinkovitz et al., 2008; León et al., 2017)
	Perideridia		Chemistry unknown; (no results during literature search)			
	Lomatium		Chemistry unknown; (no results during literature search)			
Asteraceae; Compositae			Polyacetylenes; Phenolics (flavonoids)	Antifeedant	Microbes and invertebrate herbivores	(Konovalov, 2014; Bessada et al., 2015)
	Artemisia		Phenolics (flavonoids, coumarins, caffeoylquinic acids); Terpenoids; (monoterpenes, diterpenes, triterpenes, sequiterpenes, methyl jasmonate)	Antifeedant, Deterrent	Microbes and invertebrate herbivores	(Tan et al., 1998)

Taxa in Nest			Chemical class (subclass within a class or individual chemical)	Potential bioactivity	Target of potential bioactivity	Reference
Family	*Genus*	*Species*				
		tridentata	Terpenoids (monoterpenes, sesquiterpenes)	Antifeedant, Deterrent	Invertebrate and vertebrate herbivores	(Turi et al., 2014)
		cana	Terpenoids (monoterpenes, sesquiterpene lactones)	Antifeedant, Deterrent	Microbes	(Lee et al., 1969; Lopes-Lutz et al., 2008)
	Chrysothamnus		Polyacetylenes; Phenols (flavonoids, phenolic acids); Terpenoids (monoterpenes, diterpenes, sesquiterpenes)	Antifeedant, Deterrent	Microbes, invertebrate and vertebrate herbivores	(Hegazy et al., 2007)
		vicidiflorus	Polyacetylenes; Terpenoids (monoterpenes, diterpenes); Polymers (latex)	Antifeedant, Deterrent	Microbes, invertebrate and vertebrate herbivores	(Hegerhorst et al., 1987)
	Ericameria		Previously classified as *Chrysothamnus*; see above			
		nauseosa	Polyacetylenes; Terpenoids (monoterpenes, diterpenes); Polymers (latex)	Antifeedant, Deterrent	Microbes, invertebrate and vertebrate herbivores	(Hegerhorst et al., 1987)
	Tetradymia		Chemistry unknown; (no results during literature search)			

Taxa in Nest			**Chemical class (subclass within a class or individual chemical)**	**Potential bioactivity**	**Target of potential bioactivity**	**Reference**
Family	*Genus*	*Species*				
Betulaceae			Phenolics; Terpenoids	Antifeedant, Deterrent	Vertebrate herbivores	(Julkunen-Tiitto et al., 1996)
	Betula		Phenolics	Antifeedant	Vertebrate herbivores	(Palo, 1984, 1985; Sunnerheim et al., 1988)
Brassicaceae			Glucosinolate (myrosinase, nitriles, isothiocyanates, epithionitriles, thiocyanates)	Antifeedant	Microbes and invertebrate herbivores	(Rask et al., 2000)
	Brassica		Glucosinolates (myrosinase)	Antifeedant	Microbes and invertebrate herbivores	(Siemens and Mitchell-Olds, 1998)
	Lepidium		Glucosinolates	Antifeedant	Invertebrate herbivores	(Agrawal, 2000; Burow et al., 2007)
Poaceae			Chemistry known; None			
	Bromus		Chemistry known; None			
	Poa		Chemistry known; None			
	Triticum		Chemistry known; None			
Polemoniaceae			Chemistry known; None			

Taxa in Nest			Chemical class (subclass within a class or individual chemical)	Potential bioactivity	Target of potential bioactivity	Reference
Family	*Genus*	*Species*				
	Phlox		Phenolics (tannins)	Deterrent, Antifeedant	Microbes and invertebrate herbivores	(Levin 1971)
Rosaceae			Phenolics (tannins)	Antioxidant	Microbes	(Määttä-Riihinen et al., 2004; Oszmianski et al., 2007)
	Cercocarpus		Chemistry unknown; (no results during literature search)			
Salicaceae			Phenolics (phenolic glycosides); Salicylates	Antifeedant, Deterrent	Microbes, invertebrate and vertebrate herbivores	(Boeckler et al., 2011; Volf et al., 2015)
	Populus		Phenolics (condensed tannins, phenolic glycosides)	Antifeedant, Deterrent	Invertebrate and vertebrate herbivores	(Palo, 1984; Donaldson et al., 2006; Lindroth and Clair, 2013)
Grossulariaceae			Chemistry unknown; (no results during literature search)			
	Ribes		Phenolics (tannin, anthocyanins, proanthocyanidins. prodelphinidin)	Antifeedant, Deterrent	Microbes	(Bate-Smith, 1976; Määttä et al., 2001)
		aureum	Chemistry unknown; (no results during literature search)			

Table 2.2 Chemical summary for number of chemicals separated using gas chromatography of targeted plants found in nests of golden eagles (*Aquila chrysaetos*) in Southwestern Idaho (Figure 2.1). Chemicals were counted as individual peaks in chromatograms where each peak represents at least one chemical a.

Targeted plant species	Total # chemicals	Total # unique chemicals [a]	Diversity index [b]
Big sagebrush (*Artemisia tridentata*, underrepresented)	64	37	9.32
Green Rabbitbrush (*Chrysothamnus vicidiflorus*, overrepresented)	28	10	4.02
Gray Rabbitbrush (*Ericameria nauseosa*, overrepresented)	23	2	7.36
Spiny Hopsage (*Grayia spinosa*, overrepresented)	8	0	1.72

[a] A unique chemical was defined as a peak at a retention time that did not align with any other target plant species after analysis.

[b] Diversity index was calculated using the inverse Simpson index (Dixon, 2003; Oksanen, 2015) calculated by dividing the area under the curve (AUC) of a single retention time by the total AUC of the sample, then multiplying this number by 100 and then squaring it. Dividing that number by one gives the reciprocal of Simpson's index, which accounts for both chemical richness and relative chemical concentration.

Table 2.3 Chemical summary for number of chemicals separated using high pressure liquid chromatography mass spectrometry of targeted plants used by golden eagles (*Aquila chrysaetos*) in Southwestern Idaho as greenery in nests (Figure 2.1). Each mass-charge ratio (m/z) represents at least one chemical. Chemicals were counted based on unique retention time and charges where each charge was used to verify chemical uniqueness a.

Targeted plant species	Total # chemicals	Total # unique chemicals [a]	Diversity Index [b]
Big sagebrush (*Artemisia tridentata*, underrepresented)	100	53	15.50
Green Rabbitbrush (*Chrysothamnus vicidiflorus*, overrepresented)	125	58	31.38
Gray Rabbitbrush (*Ericameria nauseosa*, overrepresented)	102	39	23.98
Spiny Hopsage (*Grayia spinosa*, overrepresented)	78	38	14.96

[a] A unique chemical was defined as a mass-charge ratio found within a peak at a retention time that did not align with any other target plant species after analysis.

[b] Diversity index was calculated using the inverse Simpson index (Dixon, 2003; Oksanen, 2015) calculated by dividing the mass-to-charge (intensity) of a single retention time by the total intensity of the sample, then multiplying this number by 100 and then squaring it. Dividing that number by one gives the reciprocal of Simpson's index, which accounts for both chemical richness and relative chemical concentration.

Figures

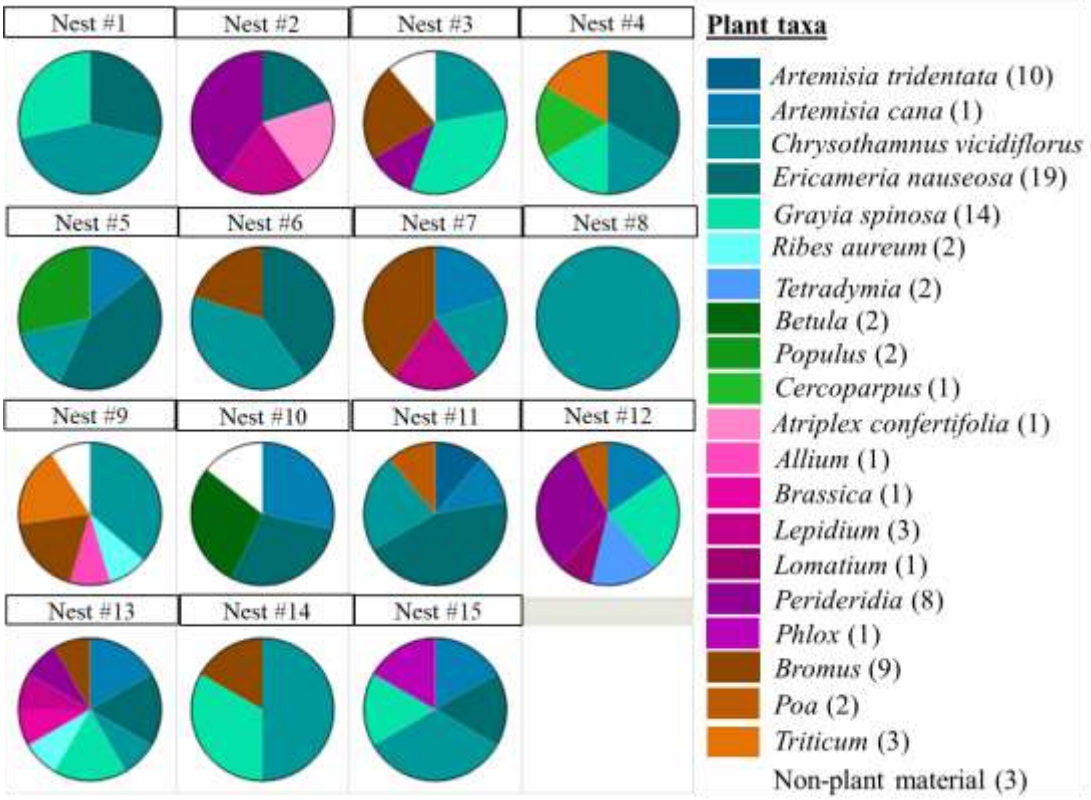

Figure 2.1 Pie charts showing diversity and proportion of plant taxa among nests of golden eagles (*Aquila chrysaetos*) surveyed in 2016 (adapted from Dudek, 2017)

The color-coded legend shows plant taxa identified within nests of golden eagles (*Aquila chrysaetos*). Blue shades represent shrubs, greens represent trees, pinks and purples represent forbs, and browns represent grasses. The number in parentheses is the total count of plant samples across all nests. Nest greenery was sampled up to three times per nest and all greenery was collected with a total of 111 plant samples analyzed.

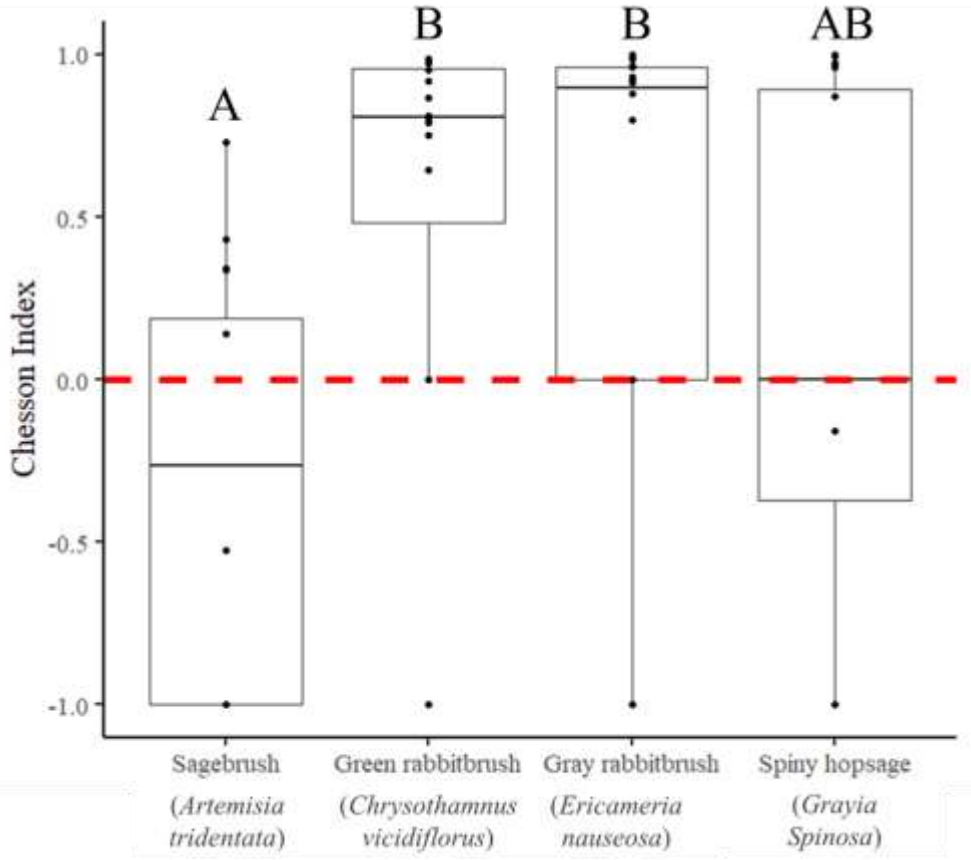

Figure 2.2 Chesson preference index of plant taxa used as nest greenery by golden eagles (*Aquila chrysaetos*).

Adapted from Dudek 2017. Preference of big sagebrush (*Artemisia tridentata*), green rabbitbrush (*Chrysothamnus vicidiflorus*), gray rabbitbrush (*Ericameria nauseosa*), and spiny hopsage (*Grayia spinosa*) in eagle nests. Box plots display Chesson preference index values. Boxes represent the median interquartile range and whiskers represent the 5-95% range. Bars not sharing a common letter (A or B) were significantly different from each other (Dunn's multiple comparison test: $p < 0.05$).

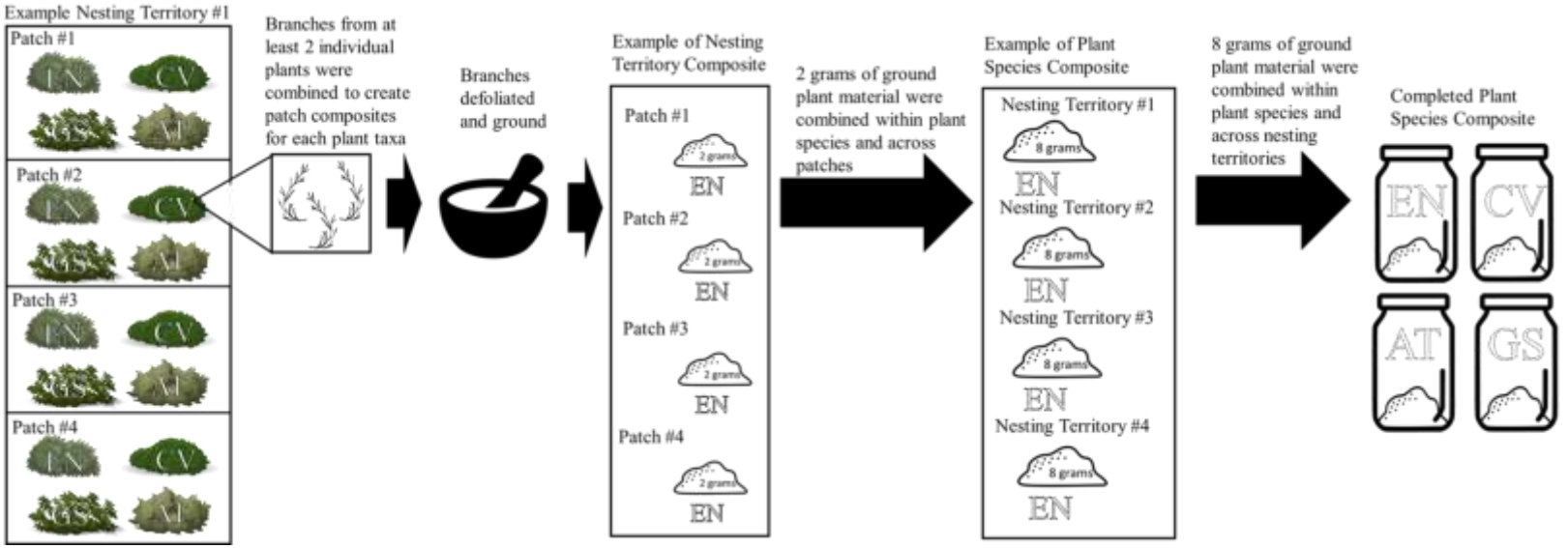

Figure 2.3 Diagram depicting the process of creating a species composite of each targeted plant taxa found in nests of golden eagles (*Aquila chrysaetos*).

This diagram follows the process used to create a plant species composite, which is used as a chemical representative across golden eagle territories. One underrepresented plant species, big sagebrush (AT) and three overrepresented plant species (gray rabbitbrush (EN); green rabbitbrush (CV), and spiny hopsage (GS)), were collected in patches across nesting territories. Patch composites were comprised of branches with leaves from at least two individual plants within a patch. Branches of patch composites were defoliated (leaf tissue removed), and leaves were finely ground and homogenized. Two grams (wet weight, WW) of each species-specific patch composite were then combined and homogenized (shaken) to create a nesting territory composite (8 grams). Next, eight grams (WW) of each nesting territory composite were combined and homogenized to create the final plant species composites (32 grams).

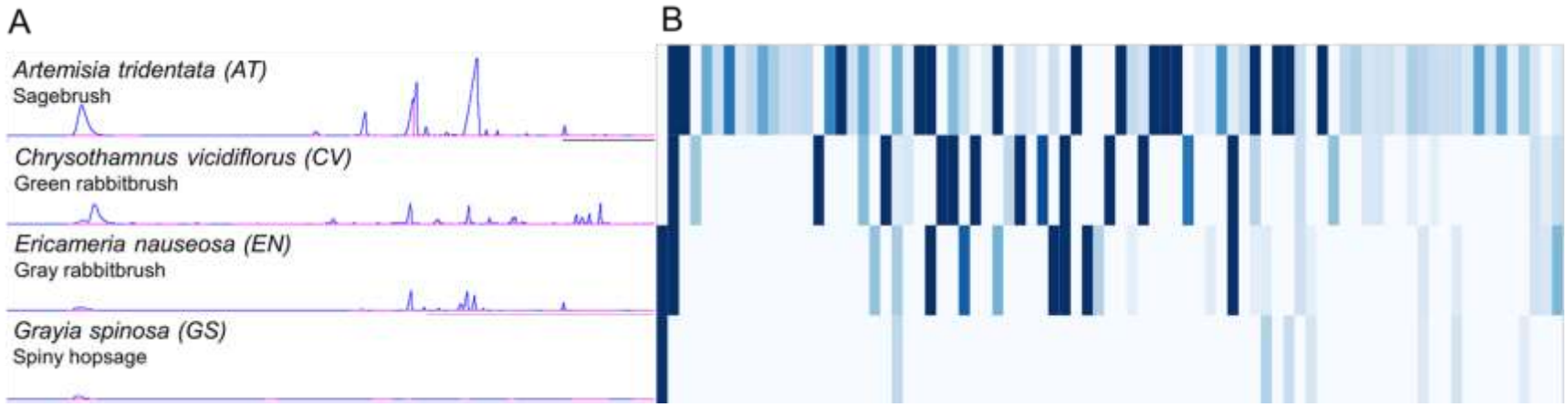

Figure 2.4 Gas chromatography of volatiles of four plant taxa found in greenery of golden eagle (*Aquila chrysaetos*) nests (see Table 2.2 for summary).

A. Representative gas chromatograms of volatile chemicals of *Artemisia tridentata* (AT, big sagebrush); *Chrysothamnus vicidiflorus* (CV, green rabbitbrush); *Ericameria nauseosa* (EN, gray rabbitbrush); and *Grayia spinosa* (spiny hopsage, GS) separated along the x-axis by retention time. B. Retention times were manually aligned and then analyzed in RStudio with vegan package. Blue color represents intensity on a scale of relative concentration (dark blue is >1200 area under the curve (AUC) and white is not detected) for each chemical separated along the x-axis by retention time.

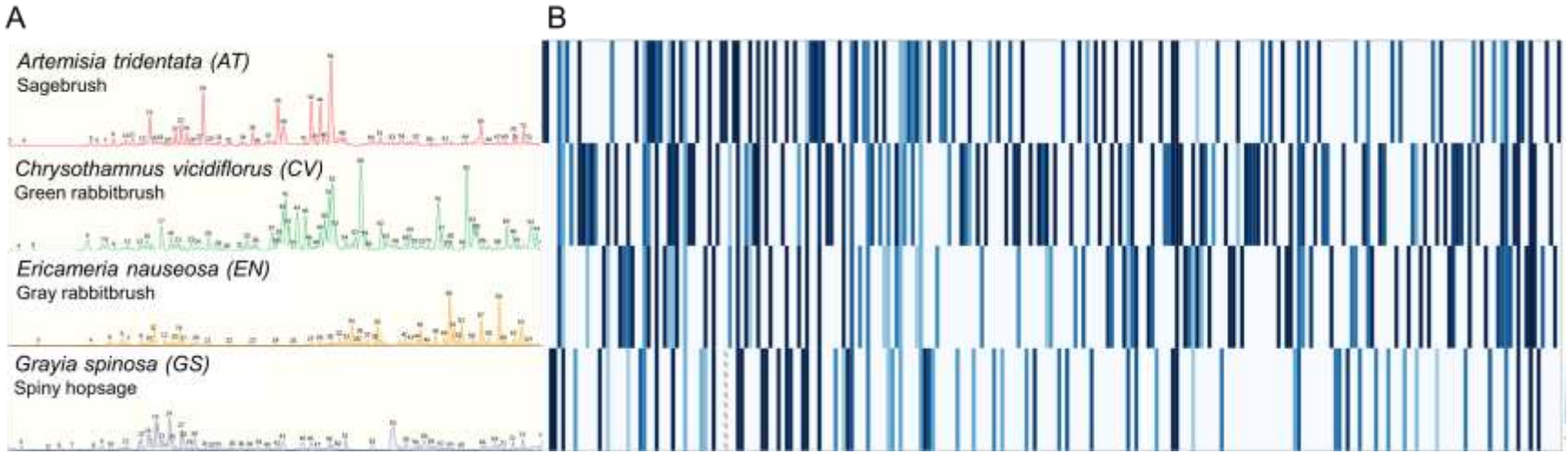

Figure 2.5 High pressure liquid chromatography and mass spectrometry of water-soluble chemicals for four plant taxa found in golden eagle (*Aquila chrysaetos*) nest greenery (see Table 2.3 for summary).

A. Representative liquid chromatograms of water-soluble chemicals in *Artemisia tridentata* (AT, big sagebrush); *Chrysothamnus vicidiflorus* (CV, green rabbitbrush); *Ericameria nauseosa* (EN, gray rabbitbrush); and *Grayia spinosa* (spiny hopsage, GS) (2 to 3 replicates per composite) separated along the x-axis by retention time. B. Retention times and m/z were manually aligned and then analyzed in RStudio with vegan package. Blue color represents intensity on a scale of relative concentration (dark blue is ≥300000 cnts and white is not detected (0 cnts) for each chemical separated along the x-axis by retention time.

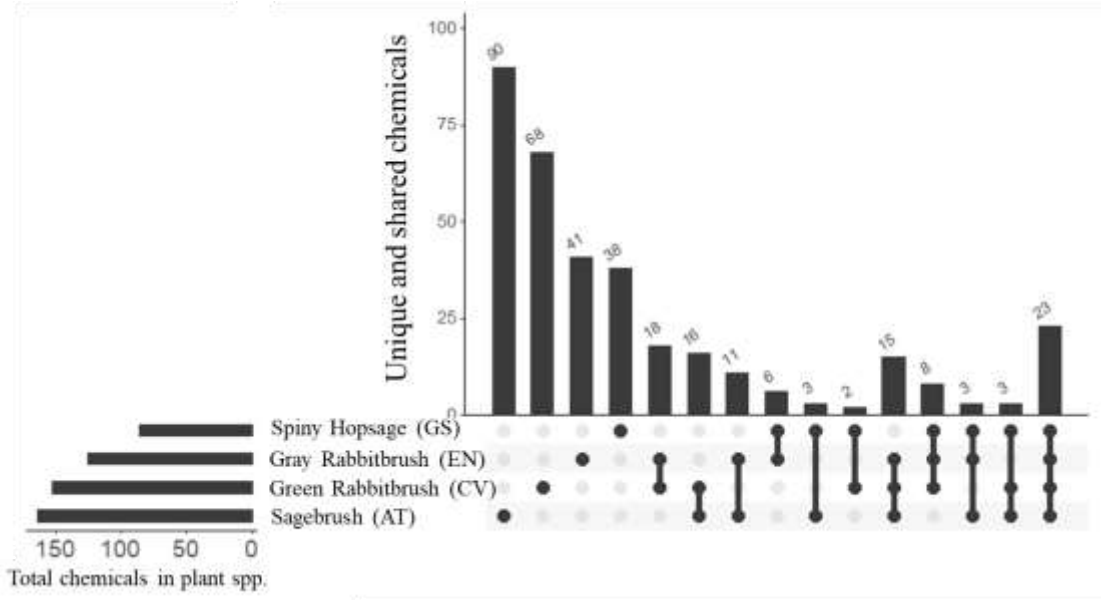

Figure 2.6 Shared volatile and water-soluble chemicals among targeted plant species used by golden eagles (*Aquila chrysaetos*) as nest material.

The horizontal bars to the left of the targeted plant species represent the total number of chemical contributions, both unique and shared, in each plant species. Top section of the chart (vertical bars) corresponds with the bottom section presented with dots. A single dot under a bar represents unique chemicals found only in *Artemisia tridentata* (AT, big sagebrush); *Chrysothamnus vicidiflorus* (CV, green rabbitbrush);

Ericameria nauseosa (EN, gray rabbitbrush); or *Grayia spinosa* (spiny hopsage, GS). Dots (two, three, or four) connected by a line represent chemicals shared by designated plant species. Numbers above the vertical bars represent the number of total unique (single dot) or shared (more than one dot) chemicals for each combination.

CHAPTER THREE: CHEMICAL CONCENTRATION AND DIVERSITY OF GREENERY IN AVIAN NESTS DISRUPT BEHAVIOR OF A COMMON BLOOD FEEDING PEST

Abstract

Climate change and chemical resistance in arthropods, specifically blood feeding arthropods, suggest that densities and distribution of these pathogen vectors are a concern to public health. Present and future management of blood feeding arthropods requires innovative and sustainable approaches. One approach is to exploit the chemical defenses of wild plants to combat pests. Using ecological knowledge from a native host-pest-plant interaction and a biorational repellent approach, we tested how concentration and diversity of volatiles in plant species found in nests of golden eagles (*Aquila chrysaetos*) affected the behavior of a hematophagous arthropod, the common bed bug (*Cimex lectularius*, hereafter bed bug). We hypothesized that bed bugs would vary in responses to volatiles from specific plant species and that exposure to a high concentration and diversity of volatile plant chemicals would cause greater disruption of behaviors than a low concentration and diversity. We found that behavior of bed bugs in response to volatiles was not influenced by plant species. However, within plant species, bed bugs spent less time in akinesis (resting behavior) when exposed to higher concentration of volatiles. Grooming bouts happened more frequently after exploration bouts than bouts of resting when bed bugs were exposed to higher concentrations of volatiles, regardless of plant species. The high concentration and diversity of volatiles found in nest greenery of

golden eagles resulted in bed bugs spending more time resting and more time grooming after exploration, which under natural conditions might result in less time searching for and feeding on hosts. Exploiting plants with higher concentrations and diversity of volatiles may help wildlife combat pests and direct drug discovery towards unique natural chemical mixtures to deter pests responsible for both wildlife and human diseases.

Introduction

Blood feeders, such as mosquitos, biting flies, fleas, lice, midges, ticks, and assassin bugs, have become well adapted to a variety of hosts over time (Waage, 1979; Balashov, 1984; Champagne, 2004). Blood feeding is a relatively uncommon foraging strategy that requires the development of highly specialized sensory structures to locate suitable hosts and specialized mouth parts to access a host's bodily fluids. Specialized mouthparts give hematophagous arthropods exclusive access to the nutrient transportation system (i.e., bloodstream) of hosts and make them particularly dangerous as vectors for a variety of pathogens (Balashov, 1984). Humans have resided alongside blood feeders since the times of the pharaohs (Panagiotakopulu and Buckland, 1999) and have suffered from the vector competence of these pests.

Notable historical cases include the epidemics of plague (McEvedy, 1988; Panagiotakopulu, 2004) and typhus (Cowan, 2000) from flea and lice vectors, respectively. Prevailing epidemics, such as those heavily affecting developing countries, like malaria (Cox, 2010) and dengue fever, are vectored by blood feeding mosquito species and have been responsible for millions of deaths (Brady et al., 2012). The arbovirus, dengue, which is vectored by *Aedes* mosquitoes and is difficult to treat, has experienced a rise in infection rate, with an estimated hundreds of million people at risk

of contracting the virus (Brady et al., 2012; Benelli and Mehlhorn, 2016). In addition to human hosts, blood feeders have great impact as vectors of disease in livestock and wildlife, which can lead to decreased populations and zoonotic spillover (Wharton and Norris, 1980; Hassell et al., 2017). Pathogens vectored by biting flies and ticks, such as Crimean-Congo hemorrhagic fever and West Nile virus, threaten our food security by infecting livestock with the potential to threaten our health (Gale et al., 2010; Dórea et al., 2016; Hassell et al., 2017). Blood feeding arthropods as well as the pathogens they carry have experienced increased occurrences of resistance to the active ingredients of our current chemical treatments (Hardy, 2014; Hemingway et al., 2016; Gomes et al., 2017; Gould et al., 2018). For example, knockdown resistance, resistance traits associated with DDT and pyrethroids, has been documented in many pest species like, the house fly (*Musca domestica*, Williamson et al., 1993) and the African malaria vector, *Anopheles gambiae* (Martinez-Torres et al., 1998). Combating growing and mobilized human populations and increasingly resistant phenotypes of arthropods requires innovative, effective, and sustainable pest management approaches.

One approach to manage blood feeders and overcome chemical resistance is to manipulate properties of the chemical receptors that mediate interactions between an organism and its environment (Wicher and Marion-Poll, 2018). Managing pests requires understanding the role of chemical receptors in relation to pest behavior. Locating a host is initiated by starvation and involves processing multi-modal cues (e.g., thermal, chemosensory, and visual) in addition to semiochemicals (i.e., chemicals that manipulate the behavior or physiology of an organism) manufactured by the host (Olson et al., 2009; Chaisson and Hallem, 2012). Detecting hosts, especially those that do not live in

concentrated populations and that are highly mobile, can be a significant challenge to blood feeding organisms. Highly specialized sensory organs within the olfactory and gustatory systems are needed by blood feeding arthropods to locate suitable hosts and mates (Luntz, 2001; Barrozo et al., 2017). Grooming of these organs in arthropods has been found to keep these organs functional, particularly when processing chemical stimuli (Daniel et al., 2001; Böröczky et al., 2013; Zhukovskaya, 2014). When not searching for hosts and mates or avoiding predators, it is advantageous for blood feeders to conserve energy through sheltering behavior. Sheltering is often mediated by aggregation cues in blood feeding arthropods (Wertheim et al., 2005; Olson et al., 2009). Volatile chemical blends are often responsible for providing these cues to arthropods at a distance (Bruce et al., 2005; Bruce and Pickett, 2011).

Given the reliance on olfaction by blood feeding pests to feed, breed, and hide, disruption of chemical receptors is a powerful tool to deter these pests. However, the current approach generally focuses on developing and delivering 'silver bullet' toxic chemicals to targeted pests. However, overuse of one chemical creates a selective pressure on the mode of action, which may result in chemical resistant phenotypes in the target species. For example, the over-reliance on pyrethroids has led to several pyrethroid-resistant phenotypes in pests of human importance (Soderlund and Knipple, 2003). In contrast, natural sources of chemicals in wild organisms rarely rely on a single chemical. Specifically, plants rely on unique and diverse odors (i.e., volatile chemicals) to attract pollinators (Pichersky and Gershenzon, 2002) or deter (Giordano et al., 2017) and defend against (Paré and Tumlinson, 1999) herbivores and pathogens. These chemical coping strategies of plants, also commonly known as plant secondary

metabolites, offer reliable sources of complex chemical mixtures that can disrupt pest behavior. Specifically, essential oils of plants are a compelling source to deter pests due to their high efficacy and nontoxic degradation, multiple modes of molecular action (e.g., inhibiting Cytochrome P450 enzymes, binding of gamma-aminobutyric acid (GABA) receptors, inhibition of the cholinergic system and modulating octopaminergic system), and relatively low toxicity (Pavela and Benelli, 2016; Benelli and Pavela, 2018). Plant derived chemical treatments, especially essential oils, have been investigated routinely for control of blood feeding pests as well as the pathogens that they vector (Pavela and Benelli, 2016; Benelli and Pavela, 2018; Pavela et al., 2019). If found effective against pests, essential oils are often fractionated to isolate active constituents. However, single chemicals often lose some efficacy possibly due to the cancellation of synergistic interactions with other constituents in the mixture (Shaalan et al., 2005; Tak et al., 2016). Mixtures of chemicals are often effective at delaying resistant traits within pests (Pavela and Benelli, 2016) and deserve greater attention for pest management due to broad spectrum of activity against pests.

We used a wild system to test how green nesting materials added to nests by golden eagles may lead us to help identify chemical mixtures that have the potential to disrupt behavior of a cosmopolitan blood feeding pest, the common bed bug (*Cimex lectularius*). We chose this system because, as described in Chapter 2, golden eagles in southwestern Idaho are thought to use aromatic greenery to reduce the negative health effects of Mexican chicken bugs, *Haematosiphon inodorus*, a blood feeding pest parasite of avian nestlings. In a recent survey, Dudek (2017) found *H. inodorus* in 94% of golden eagle nests. These hematophagous bugs have long been implicated in the deaths of

golden eagle nestlings (Usinger, 1947; Grubb et al., 1986; Platt, 1975; McFadzen and Marzluff, 1996). Specifically, Dudek (2017) showed that greenery in golden eagle nests correlated positively with hematocrit levels in nestlings, which is consistent with the hypothesis that the presence of the Mexican chicken bug was attributed to nestling deaths (Usinger, 1947; Platt, 1975; Grubb et al., 1986; McFadzen and Marzluff, 1996). Golden eagles add aromatic greenery to their nests, which has been hypothesized to reduce parasites found in nests (Clark, 1991; Petit et al., 2002; Gwinner and Berger, 2005; Pires et al., 2012; Scott-Baumann and Morgan, 2015). The greenery selected by golden eagles in their nests has chemical diversity (Chapter 2) and potential for bioactivity against pests (Table 2.1 in Chapter 2). These results suggest that pests of eagles may be deterred by both volatile and water-soluble chemicals in nest greenery. We investigated how volatiles of two plant species found in golden eagle nests influence the behavior of a pest related to the Mexican chicken bug, the common bed bug (*C. lectularius*).

We hypothesized that volatiles in gray rabbitbrush, a plant species overrepresented in the nests of golden eagles (Dudek, 2017), would disrupt the behavior of blood deprived bed bugs more effectively than volatiles of big sagebrush, an underrepresented plant species (Figure 2.2, Chapter 2). Specifically, we predicted that this disruption would result in a decrease in exploration and an increase in both grooming and akinesis (i.e., resting) behavior, in blood deprived cimicid subjects more effectively than a underrepresented plant species (big sagebrush, *Artemisia tridentata*) (Figure 2.2, Chapter 2). Additionally, we hypothesized that higher concentrations of volatiles would result in larger changes in bed bug behavior due to irritancy caused by volatile chemicals. Finally, we hypothesized that disrupted behaviors would be in response to both chemical

concentration and chemical diversity. To test these hypotheses, we analyzed the time budget of *C. lectularius* exposed to plant species (over- and underrepresented) found in the nests of golden eagles. Specifically, we assessed the time bed bugs allocated to exploration, grooming and akinesis and the sequence of these behaviors when bed bugs were exposed to high and low concentrations of volatiles from over- and underrepresented plant species.

Methods

Plant Collection and Preparation

Plant species were targeted based on inclusion in nests of golden eagles across four nesting territories (Dudek, 2017; Figure 2.1). Gray rabbitbrush was chosen to represent nest material that was overrepresented, and big sagebrush was chosen to represent nest material that was underrepresented (Figure 2.2). In the summer of 2016, samples of gray rabbitbrush and big sagebrush were collected in the field within nesting territories of golden eagles and brought back to the laboratory and stored at −20 °C before being processed. We made composites of each species from collections taken from at least two individual plants (approximately 2.0 g wet weight (WW) per plant) within at least two distinct patches (>50 m apart) for each species within four nesting territories of golden eagles (Dudek, 2017). The leaf material of each collection was coarsely ground (< 2 mm particle size) in liquid nitrogen with a mortar and pestle. Leaf material from each collection was composited first by patch, then by nesting territory, and then finally into a plant species composite (Figure 2.3). We created a subset of ground plant material for gray rabbitbrush and sagebrush that was serially extracted in methanol to remove both volatile and water-soluble chemicals from the plant biomass (9.0 g WW for each

composite, Figure 3.1). This extracted subset represented a low concentration of volatile chemicals compared to the original plant species composite (not extracted) (Figure 2.3. We weighed 30 mg of each plant treatment into containers with 12 mm circumference (Chromacol, PTFE lined phenolic caps, hereafter 'treatment containers') and then stored each sample covered in parafilm at −20 °C until we conducted behavioral assays on bed bugs.

Bed Bug Subjects

Cimex lectularius subjects of the laboratory strain Harlan, were donated by the Urban Pest Laboratory at the University of Florida, FL, USA. This colony consisted of bed bugs of mixed age and sex while being maintained at 21.1 °C and 30% relative humidity. The colony was fed twice a week on live chickens. Fifteen female and fifteen male bed bugs were removed from the colony and placed into containers isolated by sex. The containers were kept inside an incubator housed at 30 °C with a L14:D10 photoperiod. Bed bugs in the experiment had been deprived of blood for a minimum of five days and a maximum of nine days prior to the experiment to promote host-seeking behavior.

Experimental Design

Prior to testing, identification numbers of bed bugs were assigned to a sex (male or female), plant treatment (gray rabbitbrush or big sagebrush), and order of testing phases (high and low volatile concentration). For chemical analysis purposes, plant treatment containers were assigned identification numbers that were linked to subject identification numbers. Twenty-four bed bugs were randomly assigned identification numbers at the beginning of their respective first testing phase. Subjects were randomly

selected out of the appropriate sex container and then matched with the correlating plant treatment containers. Each subject experienced high and low volatile concentrations from only one plant treatment during both testing phases. Testing phases were separated by 24 hours to control for any residual behavioral response from volatile exposure.

Experimental Protocol and Arena

Experiments were performed in a controlled environment dark room. The room stayed at a consistent 22 °C ± 1 °C, and humidity of 52% ± 3% during the experiments. Lamps, with red light bulbs were placed on both sides of the designated recording area, containing the staging arena and acclimation arena, and maintained for the duration of the experiment. Red light has been shown to produce minimal photonegative effects on bed bug behavior (Weeks et al., 2013). An HD 1080P camera (ELP, Model CMOS 800TVL) was suspended above the recording area via a clamp stand and a ring support. A piece of white paper was secured below the recording area with tape to provide a neutral background for the image. Three arenas were created for experiments using clean glass Petri dishes (10 cm diameter x 1.5 cm height) (Fig. 3.2). A staging arena consisted of a clean Petri dish where plant treatments (specific species) were placed for each testing phase (concentration). An acclimation arena consisted of a clean upside-down Petri dish. A mobile experimental arena consisted of a clean glass Petri dish where nylon material (Max Collection Disposable Foot Sox, discontinued) was secured around the arena to serve as a surface for the bed bugs to walk on while preventing escape. Each bed bug was tested in their own respective experimental arena to prevent chemical cross-contamination. On the day of the experiment, bed bugs were removed from their sex-specific storage containers and placed in their individual experimental arenas. Arenas,

which were labeled with bed bug and plant treatment identification, were then placed in a dark chamber (approximately 76 cm wide by 30 cm tall and 76 cm deep) while not in the testing phase. Before beginning a testing phase, the subject was stacked on top of the acclimation arena within their respective experimental arena (Figure 3.3) to acclimate to the experimental conditions for a minimum of two minutes and a maximum of five minutes. During the acclimation phase, two treatment containers of plant material were placed directly across from each other in the bottom of the 100 mm diameter staging arena. The experimental arena was then transferred from the acclimation arena to the staging arena where the experimental arena was stacked on top of the staging arena. The nylon material (Max Collection Disposable Foot Sox, discontinued) separated the subject from the plant treatments in the staging arena. Behaviors of bed bugs exposed to plant treatments within the stacked experimental and staging arena was key logged in real time using the program BORIS (Friard and Gamba, 2016) for 10 consecutive minutes during the testing phase. After this first testing phase was complete, subjects were re-secured in their respective experimental arenas and placed back into the dark chamber until all subjects received testing. The experimental chambers of all subjects were then placed back into the incubator overnight (Figure 3.1). The process was then repeated for the second day of testing using the volatile concentration that was not used in the first day of testing. After each day of the testing phase, plant material in treatment containers from individual staging arenas were tightly sealed with parafilm and kept refrigerated or on ice (Figure 3.3 cooler) until they could be transferred to vials for chemical analysis using gas chromatography.

Description of Ethogram

We constructed an ethogram by separating behaviors into discrete, defined elements (Table 3.1, Appendix E). Four behaviors were observed and recorded over the subject's ten-minute testing phase. We recorded three state behaviors designed not to overlap temporally (exploration, grooming, and akinesis) and one point behavior (number of swipes of antennal appendages of grooming).

Exploration was defined as any locomotion by the subject that is not described as grooming, including attempted vertical movement. Climbing was not specifically quantified as a point behavior of exploration due to difficulty defining and capturing discrete climbing events. Because subjects were deprived of a blood meal at least five days prior to experiments, exploration represents a form of host-seeking behavior exacerbated by the radiation of heat of the observer recording behaviors (DeVries et al., 2016). Carbon dioxide from the observer likely did not factor in as a cue because the staging and experimental arena created an enclosed space.

Grooming is defined as the time spent grooming tarsi and in the 'grooming' position by the subject. The grooming position is defined as the front two tarsi of the subject coming together in front of the subject (i.e., tarsi in a praying position). To be discrete from akinesis or resting behavior, any resting that occurred while in the grooming position was recorded as grooming. While the grooming state was recorded, the number of individual grooming swipes of the antennal appendage were recorded for each subject. Antennal grooming swipes are defined as the subject's two tarsi coming together and moving downward along the antennal appendage. Grooming is typically

done to clean the appendage, particularly when sensory systems are agitated (e.g., novel environments and odors, Zhukovskaya, 2014).

Akinesis, also referred to as resting, is defined as a subject that is motionless with all tarsi planted on the nylon mesh. Akinesis generally represents a low risk behavior for predation and offers energy saving potential (Mellanby, 1938).

Analysis of Volatile Chemicals

Volatile chemicals from each treatment container (approx. 60 mg wet weight (WW)) were analyzed using headspace gas chromatography to verify high or low concentrations of volatiles for each plant treatment and separate individual chemicals for analysis of chemical diversity. All samples were analyzed using an Agilent 6890 N gas chromatograph (GC, Santa Clara, CA) coupled with a Hewlett-Packard HP7694 headspace autosampler (Palo Alto, CA). The headspace program was as follows: 100 °C oven temperature, 110 °C loop temperature, and 120 °C transfer line temperature. The vial equilibrium and pressurization times were both 0.20 min, the loop fill time was 0.50 min, the loop equilibrium time was 0.20 min, and the injection time was 0.50 min. One mL of headspace gas from each sample was injected into an Agilent J&W DB-5 capillary column (30 m × 250 μm × 0.25 μm, Santa Clara, CA) with helium as the carrier gas at a constant flow of 1.0 $mL.min^{-1}$ and splitless injector temperature of 250 °C. The temperature program for the GC was as follows: 40 °C for 2.0 min, then increased by 3 $°C.min^{-1}$ to 60 °C, then by 5 $°C.min^{-1}$ to 120 °C and finally by 20 $°C.min^{-1}$ to 300 °C where the final temperature was held for 7 min. Inlet pressure was 80 KPa and the flame ionization detector was set at 300 °C. Retention times of individual volatiles and individual areas under the curve (AUC) were quantified using Hewlett-Packard

ChemStation software version B.01.00 (Palo Alto, CA). AUC was used to represent the relative concentration of samples. We used distinct peaks at individual retention times to identify individual volatile chemicals. Analysis of chemical diversity was performed in Excel using wide data of manually aligned retention times with relative concentration (AUC) and then manually calculating the inverse Simpson index, hereafter diversity index (Dixon, 2003; Oksanen, 2015). The diversity index we used is weighted to account for both chemical richness and relative AUC. The diversity index was calculated by dividing the AUC of a single retention time by the total AUC of the sample, then multiplying this number by 100 and then squaring it. Dividing that number by one gives the reciprocal of Simpson's index.

Analysis of Ethogram

All analyses excluded Bugs 1 and 11 (subject identification). Bug 1 died soon after completing its first treatment. Bug 11 was excluded because it did not complete a full ten-minute testing phase (only 7.66 minutes was recorded) on the first day of the experiment due to an unintentional researcher error.

In JMP 14 Pro (SAS), an ANOVA was performed to test for an interaction between plant species (gray rabbitbrush and big sagebrush) and concentration (high and low) of volatiles. Because no significant interactions were found between the treatments (See Appendix F) each treatment comparison (species or concentration) was analyzed separately using the non-parametric Wilcoxon ranked sum test in RStudio (Version 1.3.959) using the software program R (version 4.0.2 (2020-06-22)). Specific plant species and concentration of volatile chemicals (both categorical and continuous) were each investigated as predictors of the proportion of times spent in four recorded behaviors

(Table 3.1). For those behavioral responses that differed significantly between high and low volatile concentrations, we used correlation analysis to test for relationships between individual responses and (1) relative volatile concentration (represented by AUC) and (2) chemical diversity (represented by Simpson's inverse diversity index). Cohort (i.e., subjects that experienced experiments on the same two days) and order (i.e., if subjects experienced high or low concentration of volatiles first or second) effects were tested as possible predictors of behavioral responses (See Appendix G).

Post-hoc qualitative analysis of sequential organization of behaviors was conducted due to observations of potential behavioral patterns under different volatile treatments. Frequencies of each behavior when subjects were exposed to high or low volatile concentrations were calculated separately. A transition matrix of the three state event behaviors observed (Table 3.1) were used to construct kinematic diagrams for each concentration treatment (combining data from over- and underrepresented plant species).

Results

Influence of Plants Species on Behavior

Plant species (gray rabbitbrush and big sagebrush) did not influence the proportion of time subjects allocated to any of the four behaviors recorded over a ten-minute period (Table 3.2, Figure 3.4). Exploration was the behavioral state subjects spent the greatest proportion of time in, but it did not differ between gray rabbitbrush and big sagebrush plant treatments. The proportion of time spent grooming was marginally higher when bed bugs were exposed to big sagebrush than gray rabbitbrush but was not statistically significant. We found no significant difference in the number of individual swipes of the antenna by subjects between gray rabbitbrush and big sagebrush. The

proportion of time allocated to akinesis did not differ between gray rabbitbrush and sagebrush. The exclusion of outliers did not influence results (Appendix H).

Influence of Concentration of Volatiles on Behavior

We compared the AUCs of high and low volatile concentrations to validate that plant treatments had been extracted of most volatile chemicals. We verified that low volatile concentration treatments (gray rabbitbrush: $\bar{x}$ = 164.39±323.93; big sagebrush: $\bar{x}$ = 126.60±288.76) were at least 95% lower than high volatile concentration treatments (gray rabbitbrush: $\bar{x}$ = 2234.94±4186.77; big sagebrush: $\bar{x}$ = 9057.94±3841.03). There was no significant effect of volatile concentration on three of the four behaviors recorded (Table 3.3, Figure 3.5). Bed bugs spent most of their time in exploring and this behavior did not differ between the high and low volatile concentration. Grooming and antennal swipes did not differ between the high and low volatile concentration. Bed bugs spent a significantly higher proportion of time in a state of akinesis when they were exposed to the low volatile concentration than when exposed to the high volatile concentration (Figure 3.5c). The exclusion of outliers did not influence results (Appendix H).

Influence of Concentration and Diversity of Volatiles on Behavior

Both chemical concentration (r^2 = 0.11, Figure 3.6b) and chemical diversity (r^2 = 0.09, Figure 3.6a) were negatively correlated with akinesis. Both parameters had relatively small effects on akinesis behavior (relative chemical concentration effect size = $-1.04e^{-5}$ and chemical diversity effect size = −0.01).

Sequential Organization of Behaviors Relative to Concentration of Volatiles

The predominant behavior observed in subjects was exploration. When bed bugs were exposed to the low volatile concentration, akinesis occurred after exploration 52%

of the time and was similar to the occurrence of grooming after exploration (48% of the time, Figure 3.7, left, arrows departing from blue box). In contrast, when bed bugs were exposed to the high volatile concentration, akinesis was less likely to follow exploration (32%) than grooming (68%, Figure 3.7, right, arrows departing from blue box). Akinesis was more likely to follow exploration when exposed to the low volatile concentration (52%, Figure 3. 6, left, arrows departing from blue box) than the high concentration.

Exploration frequently followed grooming behavior; 71% of the time under the low volatile concentration and 84% of the time under the high volatile concentration (Figure 3.7, arrows departing from red box). Akinesis was less likely than exploration to follow grooming when exposed to both the low volatile concentration (29%) and the high volatile concentrations (16%, Figure 3.7, left, arrows departing from red box).

Akinesis was more likely to be followed by exploration (71%) than grooming behavior (29%) when bed bugs were exposed to the low volatile concentration (Figure 3.7, left, arrows departing from green box). Similarly, akinesis was more likely to be followed by exploration (64%) than grooming (36%) when bed bugs were exposed to the high volatile concentration (Figure 3.7, right, arrows departing from green box).

Discussion

We used a biorational repellent approach to demonstrate that volatiles of plant species (over- and underrepresented) in nests of golden eagles can alter some behavioral responses of a blood feeding pest, the common bed bug. We predicted that the volatiles of an overrepresented plant species (gray rabbitbrush) would cause a larger effect of behavioral disruption than an underrepresented plant species (big sagebrush). In contrast to our hypothesis, there were no differences in behavioral response by bed bugs between

over- and underrepresented plant species found in golden eagle nests. However, there was a trend that the plant underrepresented by golden eagles, big sagebrush, may increase grooming behavior of bed bugs compared to the overrepresented plant. Furthermore, we predicted that higher concentration and diversity of volatiles would disrupt behavior more than lower concentrations and diversity. Only akinesis was disrupted by the high concentration of volatile chemicals. Specifically, higher concentration and diversity of volatiles decreased the time bed bugs spent resting. We also captured a trend that sequential organization of behaviors by bed bugs was influenced by higher concentrations of volatiles. Specifically, grooming followed exploration at a higher frequency than akinesis when bed bugs were exposed to the high volatile concentration, but not the low concentration. Results suggest that sequential organization might be an important dimension of behavior that is often overlooked in lieu of more easily quantifiable behavioral dimensions, such as frequency and proportion or duration of time. We explore how volatiles in plants used as nest greenery by golden eagles could offer a chemical resource for combatting pests.

Subjects spent the highest proportion of time in the state of exploration. These results are likely due to the bed bugs being blood deprived prior to experimentation. Exploring bed bugs were possibly looking for a meal and responding to the presence of host cues (e.g., body heat and carbon dioxide) coming from the observer. In obligate hematophagous arthropods, like bed bugs and kissing bugs, host-seeking is mediated by circadian cycles: akinesis to an awakened state, once awake they begin non-oriented exploration, then depending on host cues present (CO_2, heat, movement, and other host odors) exploration becomes oriented towards host cues (Guerenstein and Lazzari, 2009;

Lazzari, 2009; Romero et al., 2010). Once a host is located and the organism has fed, they retreat, typically using odors to find an aggregation of conspecifics. Long periods of time spent host searching can be detrimental to the survival of a blood feeder (Mellanby, 1938; Romero et al., 2010). Romero et al. (2010) found that short-term starvation (unfed for one week) elicited more activity (e.g., exploration) than that of well-fed bed bugs. It is likely that the more a bed bug is deprived of a blood meal, the more motivated they are to find a blood meal, especially in the presence of at least one host cue (Wigglesworth and Gillett, 1934). When searching for a host, especially when deprived of blood, bed bugs may minimize allocating energy to other behaviors like grooming. This is consistent with our findings that bed bugs allocated a high proportion of time spent to exploration regardless of treatment. Sequential behaviors are in response to certain stimuli inputs, especially in situations that impact the survival of an organism (Bell, 1990). Host-searching is likely governed by sequential behavior (Bell, 1990; Kanzaki, 1996), with each success of locating a host reinforcing that particular sequence. Coupling behavioral dimensions such as proportion of time spent in certain states with locomotive orientation and velocity (especially in relation to host cues) could parse out the effects of starvation between non-oriented and oriented exploration. Analysis of cohort effect (Appendix G) suggests that prolonged blood deprivation increases the proportion of time spent in the state of exploration, minimizing time allocated to grooming and akinesis to maximize energy allocated to finding a host (Mellanby, 1938; Scharf, 2016).

Grooming behavior is common in wildlife and is gaining recognition in behavioral research. For example, studies of insects show that grooming, can increase olfactory acuity (Zhukovskaya, 2012; Böröczky et al., 2013), be a response to a novel

environment (Zhukovskaya, 2014), or an immune defense against disease (Zhukovskaya et al., 2013). Grooming is a likely response to irritant volatiles but may also be a response to any novel stress, such as a laboratory assay. In the present study, subjects spent a moderate amount of time in the grooming state. Although not statistically significant, bed bugs tended to elicit a stronger grooming response in the presence of big sagebrush volatiles, which was found to contain a higher diversity of chemicals, than gray rabbitbrush. These results, considered alongside the potential pattern found in sequential organization where grooming is more likely to follow exploration under the high volatile concentration than akinesis, suggests that volatiles might be influencing bed bug grooming responses. However, neither plant species nor concentration significantly influenced the number of grooming events. It is possible that the volatile concentration of the plant material saturated olfactory receptors, which amplified or swamped behavioral responses regardless of plant species within the relatively small, stacked staging arena and experimental arena. However, our results do suggest that grooming is associated with exploratory behaviors, in both our time proportion (Appendix J) and sequential data, which would support the use of grooming to sharpen olfactory acuity while host-searching.

After locating a host and then feeding, bed bugs will set out to aggregate using odors. Aggregates of bed bugs are often found nearby hosts in warm, dark places with minimal air disturbance (Weeks et al., 2011) and protected from injury (Reis and Miller, 2011). Rejoining an aggregation, mating occurs after feeding, then rest or akinesis. Bed bugs are well-known for their ability to survive long periods of time without a meal. This ability is likely due, in part, to energy conservation and aggregation behaviors to mitigate

desiccation (Mellanby, 1938; Romero et al., 2010; Reis and Miller, 2011). We found evidence that volatiles do influence akinesis. Specifically, higher concentrations and diversity of volatiles reduced akinesis (Figure 3.5c). These chemical features are not mutually exclusive, where the chemicals present (diversity) and varying concentrations of those chemicals work in tandem to combat pests through synergistic effects or different modes of action (Richards et al., 2016). Lack of investment in akinesis may be a result of blood deprivation. Some studies have found that chemical repellants become less effective under the effects of starvation (Bomford and Isman, 1996; Lindgren et al., 1996). There are likely consequences of survival that are influenced by investing more time into exploration than akinesis. Chemically mediated actions like sheltering and mating can be reduced, while risky behaviors like predation avoidance can be increased (Scharf, 2016). Grooming behavior does not seem to be associated with akinesis behavior (neither proportionally nor sequentially), which suggests that while in states of akinesis, grooming is likely a neutral component to survival in this state.

Observing how chemicals specifically affect the behavior of an organism can be extremely challenging. Laboratory behavioral assays are essential to decipher how chemical stimuli are being processed and to identify basic behavioral responses, despite the behavioral contamination of handling and the synthetic nature of laboratory bioassays (Aak et al., 2014). It is crucial to assess appropriate concentrations of chemical treatments, either singular chemicals or mixtures, for experimental conditions to prevent overwhelming the chemosensory systems, which can make basic behavioral responses more difficult to decipher. Biologically appropriate chemical treatments can be identified by creating dose gradient treatments where dose-dependent responses can be evaluated.

In support, recent experiments have found that the number of antennal grooms by bed bugs increased with increasing concentrations of phenolics extracted from the underrepresented big sagebrush (Appendix I).

Moreover, behavioral responses to stimuli are often taxa specific (Bruce, 2015) and driven by distinct chemical interactions with plants such as avoidance by herbivores (Bruce and Pickett, 2011) or attraction by pollinator (Pichersky and Gershenzon, 2002). We were not able to quantify if volatiles had the ability to affect orientation towards a host, increasing the amount of energy and time needed to locate a host. However, our recent experiments have found that higher concentrations of phenolics extracted from big sagebrush caused bed bugs to avoid the source of phenolics (Appendix I), which may indicate that these plant-derived chemicals do influence orientation. To further understand chemically-mediated orientation, video tracking software such as Ethovision (Noldus et al., 2001) or freeware options like Tracktor (Sridhar et al., 2019) and DORIS (developed by the creator of BORIS (Friard and Gamba, 2016, http://www.boris.unito.it/)) can be coupled with current techniques in computing to assess additional behavioral dimensions such as locomotion orientation and sequential organization (Egnor and Branson, 2016). Future work also should consider important behaviors mediated by semiochemicals within diverse chemical classes, such as aggregation, mate-searching, or feeding behaviors. The push-pull strategy used in pest management uses semiochemicals that both push (repel) and attract (pull) pests to manipulate them into being easily removed or terminated (Nalyanya et al., 2000; Cook et al., 2007). Including chemical treatments that elicit opposing behavioral responses would

require a more complex design that would offer insight into chemical functionality in relation to behavioral responses of pests while expanding tools of pest management.

Finally, we acknowledge that our experiments were limited in several chemically relevant areas. While we focused on volatiles for this study, other chemical classes, such as phenolics and alkaloids, hold potential to disrupt important pest behaviors. Specifically, alkaloids have been shown to produce antifeedant behavior in lepidopterans and dipterans (Koul, 2008). Furthermore, it is possible that physical contact with the chemical mixture that includes both volatile and water-soluble chemicals (Chapter 2) rather than exposure to volatiles in the airspace may induce a greater grooming response. Water-soluble chemicals, specifically phenolics, are a large class of mostly bioactive chemicals (Table 2.1, Chapter 2, Appendix I) but they may require contact to disrupt gustatory receptors or neuroreceptors (i.e., inhibition of neurotransmitters or neuromodulators, Koul, 2008). Using survival and behavioral assays where contact is inescapable under different concentrations may provide insight into the potency of these chemical mixtures.

Although preliminary, our results suggest greenery could reduce the potential for pests to find hosts. Specifically, akinesis was reduced in the presence of the high volatile concentration, a behavior that is essential to survival in the face of starvation. The differences observed in the sequence of behaviors where grooming occurred more frequently after exploration under conditions of high volatile concentrations may push a subject to expend more energy than needed while in the state of exploration especially without increased akinesis. We provide one example showing that wildlife behavior can be a powerful tool to discover chemical mixtures used by plants and animals to

potentially deter or manipulate the behavior of their pests. Uniting observations of chemical coping behavior of wildlife across the landscape with emerging metabolomics techniques, that allow processing and quantifying large quantities of plant defense chemicals (Breitling et al., 2013), and controlled behavioral experiments that leverage behavioral computing tools could provide much needed insight to decipher functional roles of chemical mixtures and exploit them to better combat pests.

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Tables

Table 3.1 Ethogram (with behavior references) used by observer in BORIS to key-log behaviors of bed bugs (*Cimex lectularius*).

State Behavior	Point Behavior	Description of behavior	Reference
Exploration		All locomotion, including attempted vertical space climbing.	(Degen et al., 2015; Hesselberg, 2015)
Grooming		Time spent grooming and in the 'grooming position. The 'grooming' position consists of the front two tarsi of the subject coming together in front of the subject (i.e., in a praying position). To be discrete from resting behavior, resting in the 'grooming' position was recorded as grooming	(Walker and Archer, 1988; Böröczky et al., 2013; Zhukovskaya et al., 2013b; Yanagawa et al., 2014; Zhukovskaya, 2014)
	Antennal swipes	Individual counts of subject's two tarsi coming together in front of the subject (i.e., in a praying position) and moving downward along the antennal appendage	(Walker and Archer, 1988)
Akinesis		Motionless with all tarsi planted on nylon mesh. Low risk behavior for predation and has energy saving potential	(Olson et al., 2009)

Table 3.2 Behaviors performed by bed bugs (*Cimex lectularius*) during exposure to plant treatments. Mean, standard deviation (SD), 95% confidence interval (CI), W and p-value of Wilcoxon ranked sum tests are reported for proportion of time spent for exploration, grooming and akinesis and counts for antennal swipes.

Behavior	Plant Species	Mean	SD	95 % CI	W	p-value
Exploration (proportion of time)		0.70	0.19	0.76–0.64	303	0.16
	Gray rabbitbrush	0.74	0.19	0.66–0.83		
	Big sagebrush	0.66	0.20	0.58–0.75		
Grooming (proportion of time)		0.15	0.13	0.11–0.19	205	0.40
	Gray rabbitbrush	0.12	0.08	0.08–0.15		
	Big sagebrush	0.18	0.16	0.10–0.25		
Antennal swipes (counts)		24.3	17.6	18.9–29.6	244	0.97
	Gray rabbitbrush	22.1	11.5	17.1–27.2		
	Big sagebrush	26.4	22.2	16.6–36.2		

Behavioral State	Plant Species	Mean	SD	95 % CI	W	p-value
Akinesis (proportion of time)		0.15	0.14	0.10–0.19	238	0.92
	Gray rabbitbrush	0.14	0.13	0.08–0.20		
	Big sagebrush	0.15	0.16	0.08–0.22		

Table 3.3 Behaviors performed by bed bugs (*Cimex lectularius*) during exposure to two plant volatile concentrations. Mean, standard deviation (SD), 95% confidence interval (CI), W and p-value of Wilcoxon ranked sum tests are reported for proportion of time spent for exploration, grooming and akinesis and counts for antennal swipes. Significant results in bold.

Behavioral State	Volatile Concentration	Mean	SD	95 % CI	W	p-value
Exploration (proportion of time)		0.70	0.19	0.64–0.76	277	0.42
	High	0.72	0.21	0.63–0.82		
	Low	0.68	0.17	0.61–0.76		
Grooming (proportion of time)		0.15	0.13	0.11–0.19	268	0.55
	High	0.17	0.15	0.09–0.23		
	Low	0.13	0.10	0.08–0.17		
Antennal swipes (counts)		24.3	17.6	18.9–29.6	231	0.79
	High	24.8	20.4	15.7–33.9		
	Low	23.7	14.6	17.2–30.2		

Behavioral State	Volatile Concentration	Mean	SD	95 % CI	W	p-value
Akinesis (proportion of time)		0.15	0.14	0.10–0.19	143	**0.02**
	High	0.11	0.13	0.05–0.16		
	Low	0.19	0.15	0.12–0.25		

Figures

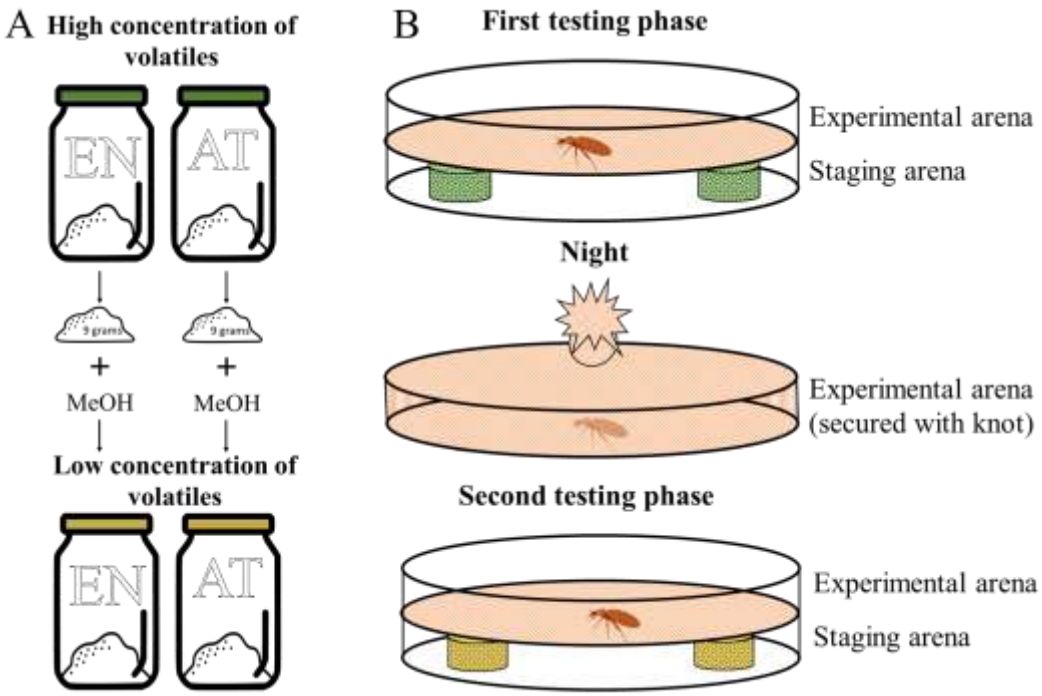

Figure 3.1 Preparation of treatments and set-up of behavioral assay to test response of bed bugs (*Cimex lectularius)* to plant volatiles.

A. One subset of the original plant species composites of *Ericameria nauseosa* (EN, gray rabbitbrush) and *Artemisia tridentata* (AT, big sagebrush) represent high volatile concentration (top, green treatment container) and a second subset was extracted with methanol to represent low volatile concentration (bottom, tan treatment container). See Figure 2.3 in Chapter 2 for explanation on how plant species composites were made. B. An individual bed bug was recorded for ten minutes in the presence of one treatment (in this case high volatile concentration in green) from a composite of one of the two plant species, then kept overnight within the experimental arena without a volatile treatment, then given the other volatile concentration (in this case low volatile concentration in tan) of the same plant species.

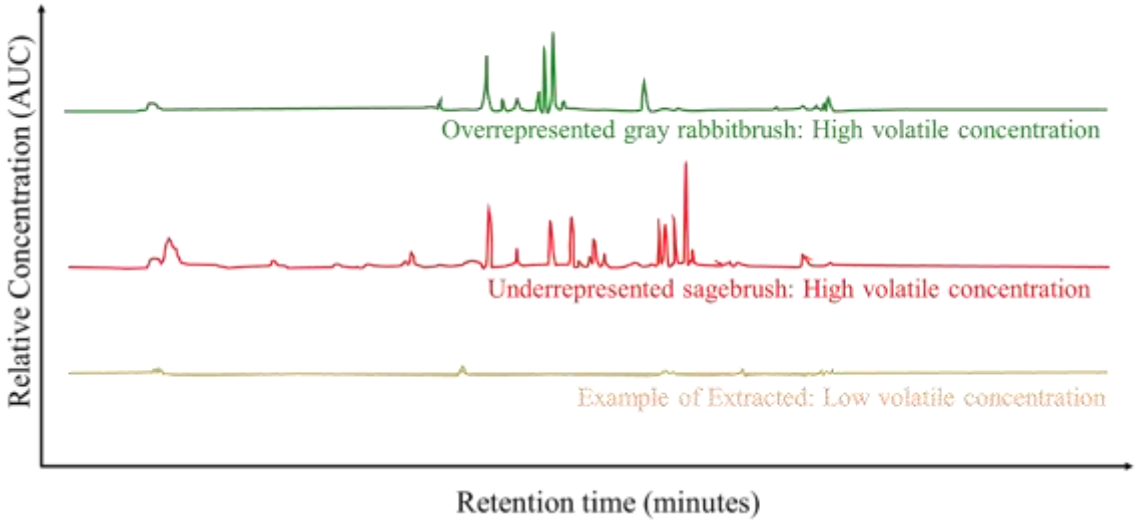

Figure 3.2 Overlaid reference chromatograms for plant treatments (see Figure 3.3).

Green chromatogram is the high volatile concentration (unextracted) of gray rabbitbrush (*Ericameria nauseosa*), which was overrepresented in nests of golden eagles (*Aquila chrysaetos*). Red chromatogram is the high volatile concentration (unextracted) of big sagebrush (*Artemisia tridentata*), which was underrepresented in nests of golden eagles. The tan chromatogram is an example of a low concentration (extracted with methanol) volatile profile (gray rabbitbrush).

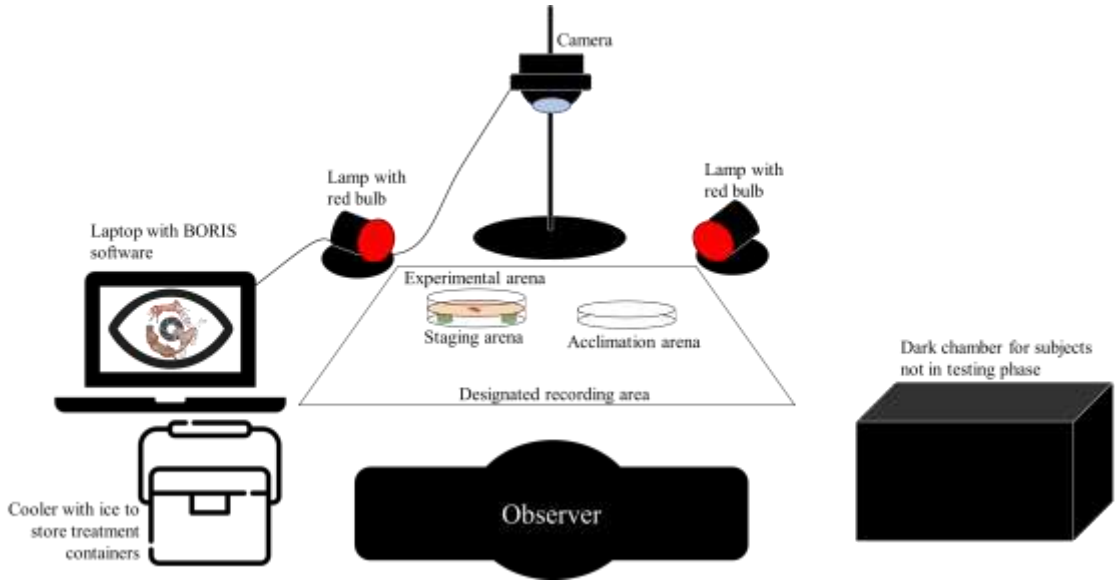

Figure 3.4 Schematic of experimental protocol and arenas used to test bed bug (*Cimex lectularius*) responses to plant volatiles.

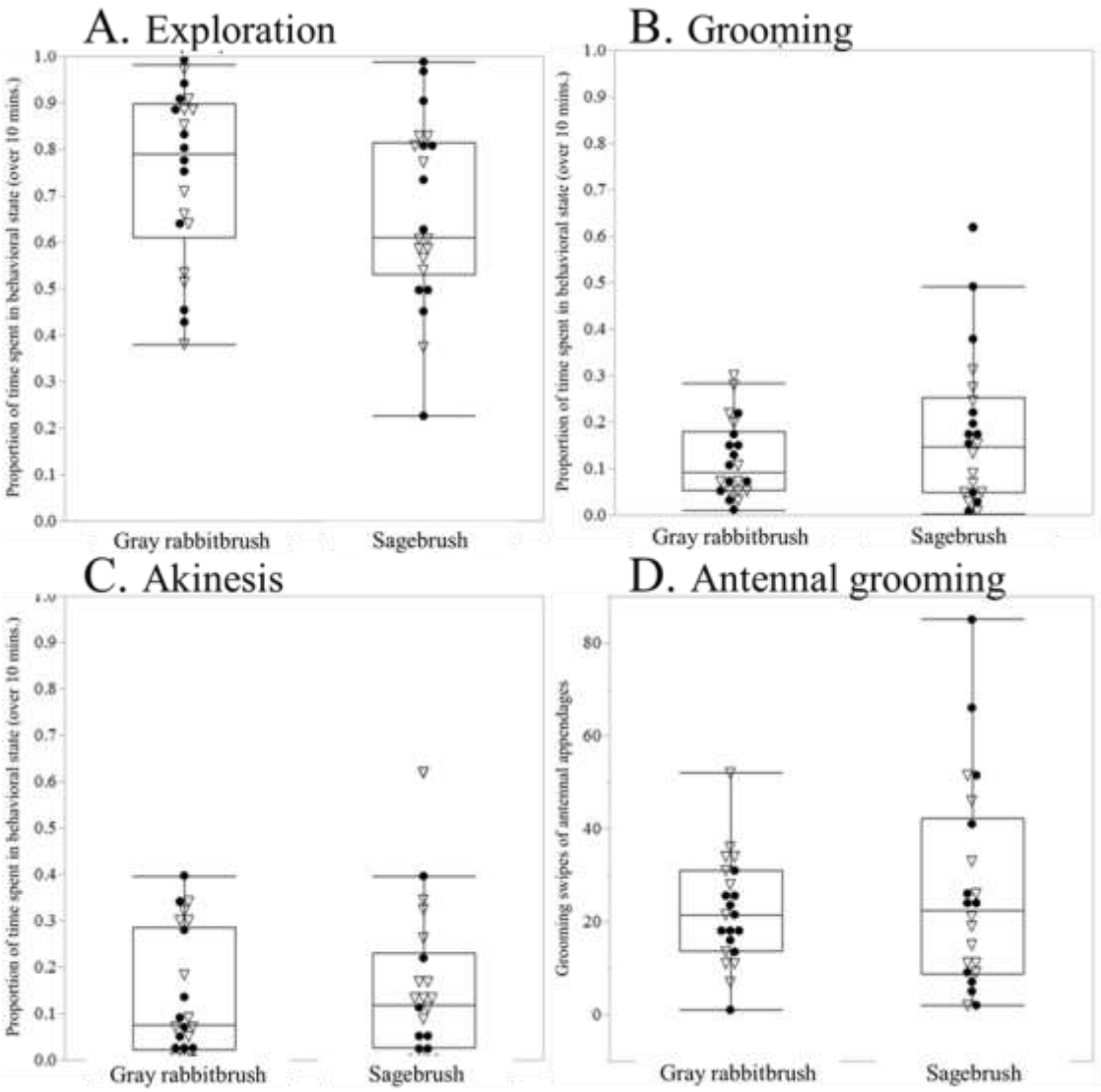

Figure 3.5 Bed bug (*Cimex lectularius*) behavior allocated to three state behaviors: (A) exploration, (B) grooming, and (C) akinesis and one point behavior: (D) antennal grooming when exposed to volatiles of overrepresented (gray rabbitbrush, *Ericameria nauseosa*) or underrepresented (big sagebrush, *Artemisia tridentata*) plants.

Box plots comparing plant treatments (gray rabbitbrush and big sagebrush) where boxes represent the median interquartile range and whiskers represent the 5 to 95% range. Solid circles represent the high volatile concentration and open triangles represent the low volatile concentration.

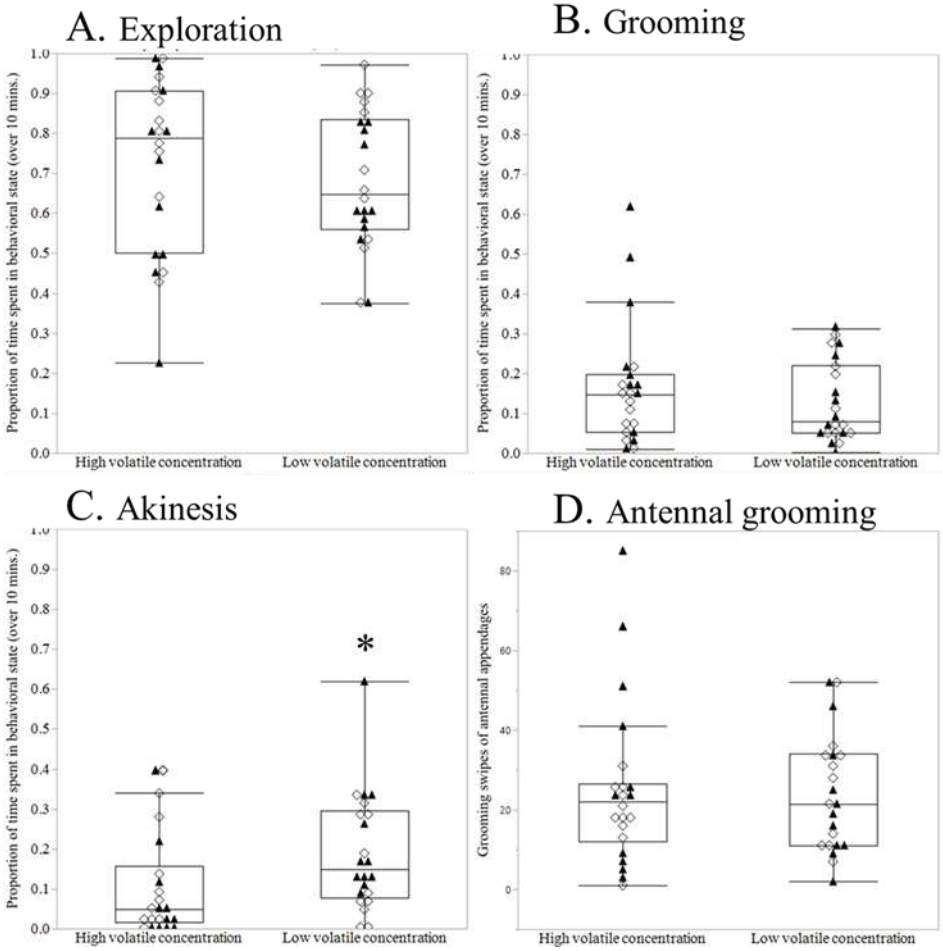

Figure 3.6 Bed bug (*Cimex lectularius*) behaviors allocated to three state behaviors: (A) exploration, (B) grooming, and (C) akinesis and one point behavior: (D) antennal grooming when exposed to high or low plant volatile concentrations.

Box plots comparing volatile concentration (high and low) where boxes represent the median interquartile range and whiskers represent the 5 to 95% range. Asterisk above box plot denotes significant difference ($p < 0.05$). Open diamonds signify overrepresented plant species (gray rabbitbrush, *Ericameria nauseosa*) and solid triangles signify underrepresented plant species (big sagebrush, *Artemisia tridentata*).

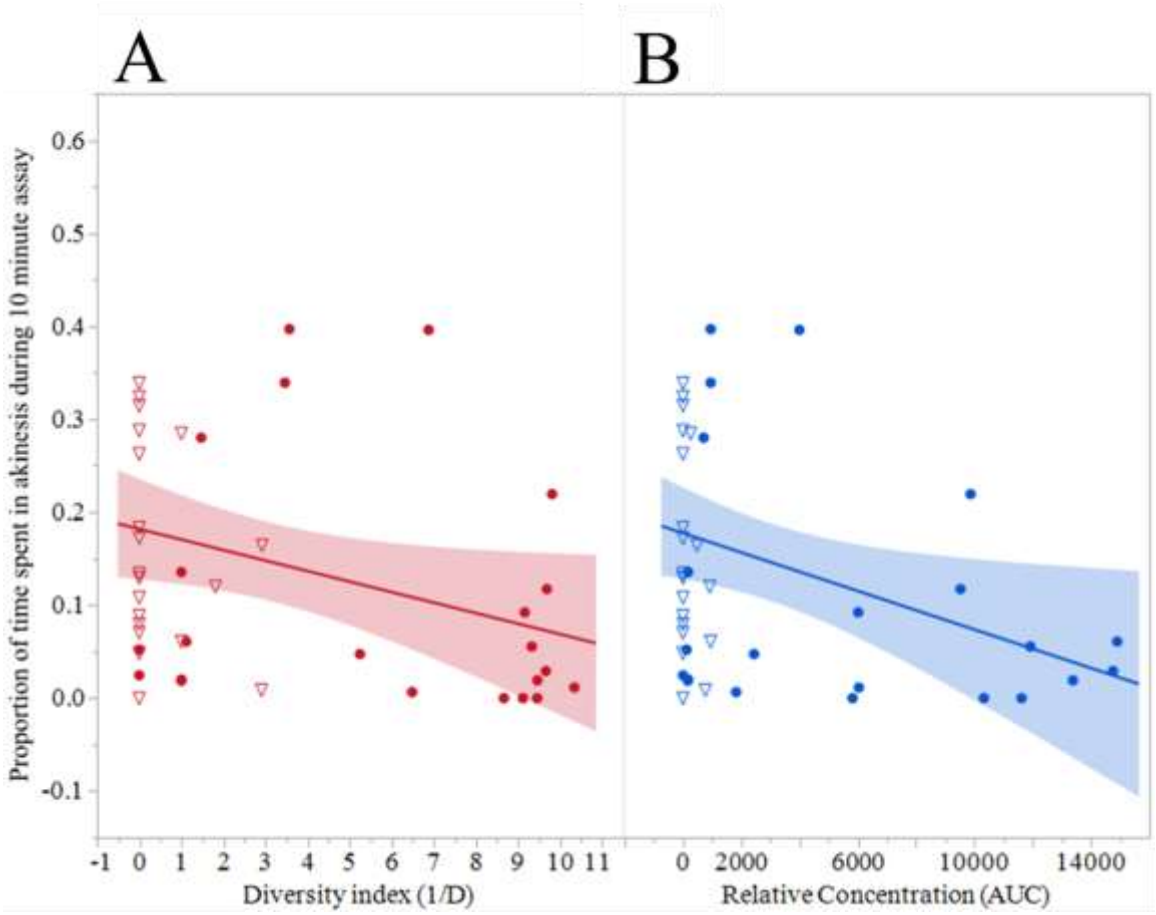

Figure 3.7 Akinesis in bed bugs (*Cimex lectularius*) exposed to plant volatiles and its relationship to (A) volatile diversity index and (B) relative concentration of volatiles. .

The relationship between proportion of time allocated to akinesis during a ten-minute assay and (A) diversity index (represented by the inverse Simpson's diversity index, $r^2 = 0.06$ $p < 0.04$) and (B) relative concentration of volatiles (represented by the total area under the curve from chromatographs, $r^2 = 0.07$, $p < 0.02$). Solid circles represent the high volatile concentration and open triangles represent the low volatile concentration.

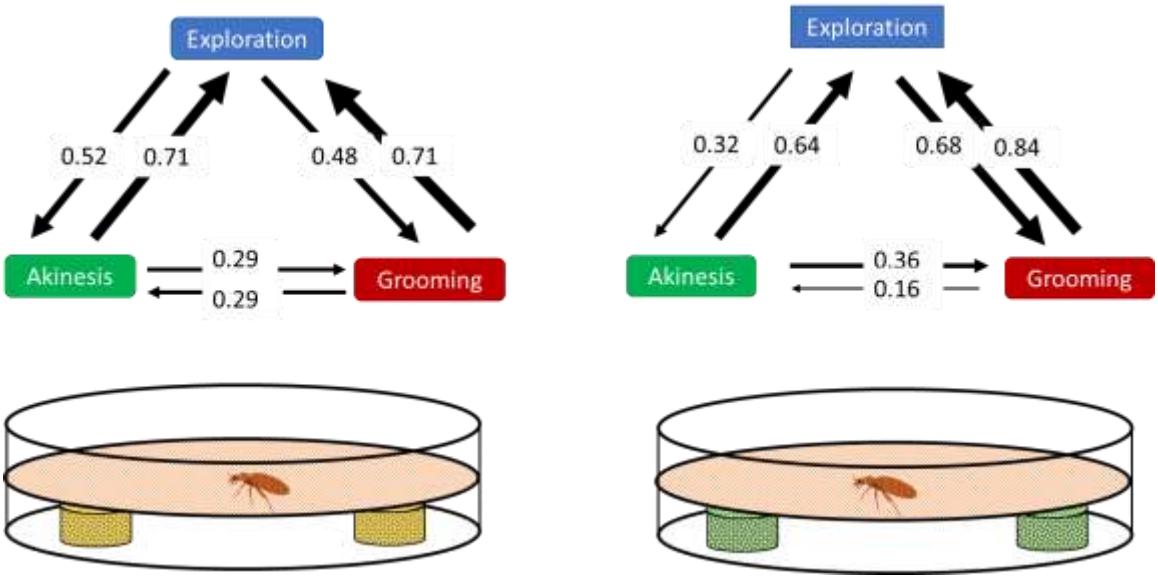

Figure 3.8 Sequential behavior organization of bed bugs (*Cimex lectularius*) under low plant volatile presence (left, tan) and high plant volatile presence (right, green).

The average frequency of occurrences of pooled data for underrepresented and overrepresented plant treatments were used to calculate transitions between two behaviors for high and low concentrations of volatile chemicals. The higher numbers within arrows and increased thickness in arrow lines represent higher probabilities of sequential transitions occurring between two sequential behaviors.

CHAPTER FOUR: CONCLUSION

As pests continue to threaten global health (World Health Organization, 2017) and food security (Riegler, 2018), while developing phenotypes of resistance (Gould et al., 2018), the need to develop and sharpen pest management strategies will persist. Among a range of other tactics (behavioral, biological, and physical), chemical treatments are an especially powerful tool to manage pests of human concern. However, over reliance has led to inevitable phenotypes of resistance. Future pest management requires innovation to reduce resistant phenotypes and wild systems are uniquely adapted to direct us in a sustainable approach. We have shown that observations of wildlife using chemical coping behaviors can lead us to sources of chemical diversity with bioactive potential to disrupt pest behavior. Examining and deciphering the diversity of chemicals in plant taxa, which are then exploited by animals, is only the first step to discover new pest management strategies. The next step should be to conduct these observations in a spatio-temporally explicit context such that changes in chemical diversity is observed relative to chemical availability and pest presence. Specifically, a more comprehensive survey of the types of plants being brought back to the nest by golden eagles and at what time and frequency would provide land managers with insight into how golden eagles are using their surrounding landscape. Additionally, the ecology of the Mexican chicken bug, which has the potential to be a greater threat to birds of prey in the future, needs more thorough investigation. Information pertaining to the annual cycle, feeding routine, and habitation within the nest would be particularly useful in the management of these

species. Lastly and perhaps the most challenging would be a deeper investigation into if golden eagles are being selective of their nesting material and if so, how they are selecting nesting material, whether it be chemically or otherwise. This investigation would be useful to land managers to make decisions about conserving and restoring functional plants on the landscape and also to the field of chemical ecology at large to help to parse out the function of aromatic nest material associated with avian species.

Wildlife, both faunal and floral, can provide useful insight to combatting pests by using chemical defense strategies, while balancing phenotypes of resistance that may occur. Observing dynamics in chemical coping behaviors in tandem with ecological dynamics in specific pests that are linked to metabolomic techniques can create a targeted framework to locate chemicals and chemical mixtures to be used in pest management. We suggest that experimental tests that focus on assessing the bioactivity of diverse chemical mixtures (see Chapter 2), rather than single chemicals, will promote the discovery of novel bioactive chemicals and modes of action. In addition, we recommend that researchers evaluate functional dimensions such as feeding, mating, and finding shelter as well as sequential behavioral dimensions in addition to chemical dimensions, like concentration and diversity.

Uniting observations of chemical coping behavior of wildlife across the landscape with emerging metabolomics techniques, that allow processing and quantifying large quantities of plant secondary metabolites (Breitling et al., 2013), and controlled behavioral experiments that leverage behavioral computing tools could provide much needed insight to decipher functional roles of chemical mixtures and exploit them to better combat pests. Finally, these discoveries can only occur if two things are fostered.

The first is conserving the diversity of animals we can observe and ensuring they have access to diverse plant taxa. The second is facilitating collaborations among wildlife ecologists who can observe the temporal and spatial behaviors of wildlife, chemists who can employ metabolomic techniques with expertise in chemical class extraction and separation, behavioral ecologists who can assess important pest behaviors and design assays to specifically address drivers of these behaviors, and practitioners who are willing and knowledgeable to enact applications of these tactics. Ultimately, preservation of biological and chemical diversity and the convergence of experts in behavior, chemistry, pest management, and drug discovery are needed for sustainable chemical pest management solutions.

References

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APPENDIX A

Diversity Index of Plant Species-Level Composites Represent Patch-Level Composites of Plant Species

In Chapter 2, we summarize how species-level composites were compiled through combining patch composites (each collection consisting of 2-3 individual plants) and then combining patches within nesting territory and then combining nesting territories for a species (Figure 2.3). However, the amount of variation that can be found in the chemical composition of individual plants and patches within a given plant species can be immense due to differences in abiotic and biotic stressors an organism may encounter in the environment. Here we demonstrate that despite inter-species chemical variation, represented by a diversity index, species-level composites (blue, Figure A.1) generally represent the volatile composition of patch-level composites of plants (red, Figure A.1). Spiny hopsage was not included because patch-level composites had not been catalogued or processed. Diversity indices of big sagebrush ($\mu_{\text{species-level composite}} = 7.26$, $\mu_{\text{patch-level composite}} = 8.00$) and green rabbitbrush ($\mu_{\text{species-level composite}} = 3.89$, $\mu_{\text{patch-level composite}} = 4.43$) did not differ greatly between species-level and patch-level composites (differences are below one, $\mu_{\text{sagebrush}} = -0.74$, $\mu_{\text{green rabbitbrush}} = -0.54$), whereas the species-level composite diversity of gray rabbitbrush ($\mu_{\text{species-level composite}} = 6.41$, $\mu_{\text{patch-level composite}} = 5.04$) exceeded the patch-level composite diversity (a difference greater than one, $\mu_{\text{gray rabbitbrush}} = 1.37$). Results suggest that chemical diversity in the species-level composites of big sagebrush and green rabbitbrush generally reflects the diversity found across geographically distinct patches of these species. In contrast, the chemical diversity in the species-level composite of gray rabbitbrush may be overestimated compared to the variation that exists at patches. Results also indicated that golden eagles have an opportunity to select for higher diversity of chemicals among patches within a species. Future studies should compare the diversity

of chemicals across patches within territories, not just within a plant taxon, to chemical diversity selected by eagles in their nests.

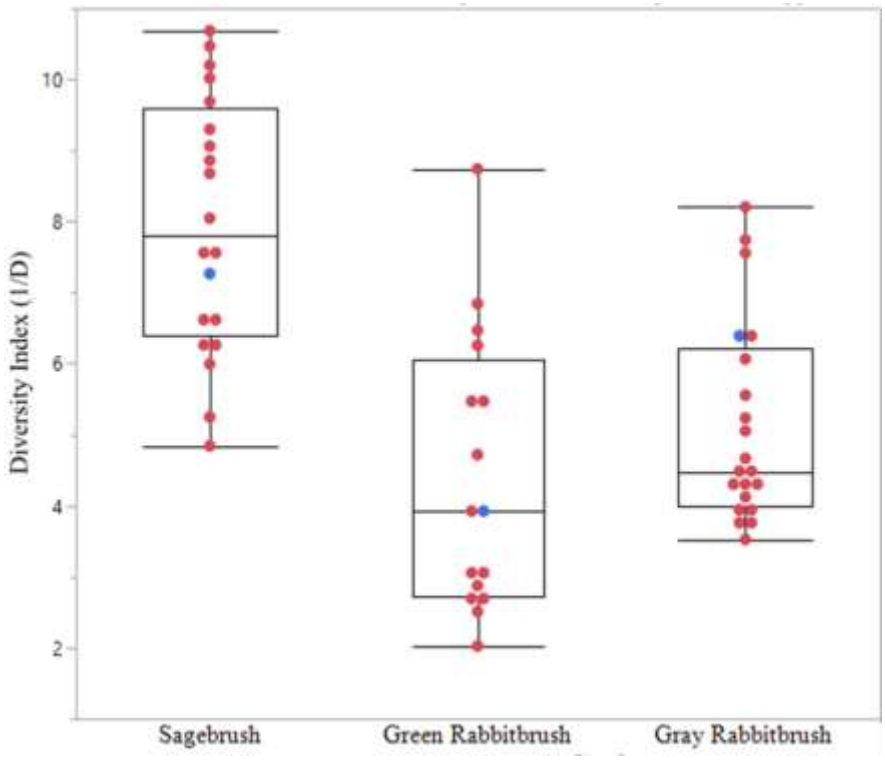

Figure A.1 Diversity index variation within patch-level composites within territories (red) compared to plant species composites (blue) for each plant species.

Box plots comparing inverse Simpson's diversity index of volatile chemicals between three patch composites (underrepresented: big sagebrush (*Artemisia tridentata*) and overrepresented: green rabbitbrush (*Chrysothamnus vicidiflorus*) and gray rabbitbrush (*Ericameria nauseosa*)), where boxes represent the median interquartile range and whiskers represent the 5 to 95% range. Spiny hopsage was not analyzed at this time because patch composites had not been catalogued or processed.

APPENDIX B

Tentative Chemical Identification of Water-Soluble Chemicals Detected in HPLC-MS

As described in Chapter 2, water-soluble chemicals were detected using high pressure liquid chromatography–mass spectrometry (HPLC-MS). Liquid chromatography coupled with mass spectrometry analysis provides both retention times and mass to charge ratios (m/z) that can be used in identification of chemicals within a sample. We cross referenced the observed m/z with molecular weights of chemicals isolated from plants the same genus (Table B.1) using the keyword search of the Core KNApSAck database (http://www.knapsackfamily.com/knapsack_core/top.php). The genus *Artemisia* was searched for the observed m/z in *Artemisia tridentata*. The genera *Chrysothamnus* and *Ericameria* were searched for the observed m/z in *Chrysothamnus vicidiflorus* and *Ericameria nauseosa*, due to the recent reclassification of *Ericameria nauseosa* from the genus *Chrysothamnus* into the recently new genus of *Ericameria*. *Grayia spinosa* was excluded from this analysis because the KNApSAck database contained no metabolite data for the genus *Grayia*.

These tentative chemical assignments lay the foundation for chemical identification within these plant species. An updated comprehensive chemical composition of these species is needed to better understand the bioactive potential of these plants and to decipher why they are under- or overrepresented in the nests of golden eagles.

Table B.1 Tentative assignments of water-soluble chemicals (HPLC-MS) using observed mass-charge ratio (m/z) comparisons to chemical molecular weight (MW) and cross-referencing to KNApSAck metabolite database.

Plant Species					
Compound ID number	**Ret. Time (min)**	**Likely Formula**	**MW ($g \cdot mol^{-1}$)**	**Observed m/z**	**Compound candidates**
Artemisia tridentata					
5	9.4	C15H26O2	238.1932	239.0942	alpha - bisabolol oxide
8	11.2	C15H26O	222.1984	223.0992	beta-Eudesmol/Cedrol/Elemol/Kongol
15	14.2	C26H28O14	564.1479	565.1618	Isoschaftoside
21	16.1	C21H20O10	432.1056	433.1169	Apigenin-7-O-glucopyranoside
22	16.2	C15H24O4	268.1674	269.178	Arbusculin E
23	16.6	C15H20O2	232.1463	233.1565	6,7-Dehydroartemisinic acid
28	18.4	C15H18O4	262.1205	263.1315	(-)-Artemisin
31	19.8	C15H22O4	266.1518	267.1621	(-)-Arbusculin D
32	20.5	C15H22O3	250.1568	251.1672	(+)-Arbusculin A
35	22.5	C15H22O2	234.1619	235.1722	Artemisinic acid
36	22.9	C20H30O	286.4500	287.0580	Ferruginol
37	23.7	C10H8O4	192.0422	193.1247	Scopoletin
39	24.5	C17H14O8	346.0688	347.0808	Axillarin/Eupatolitin
40	24.9	C15H22O2	234.1619	235.1726	Artemisinic acid
41	26.7	C16H12O6	300.0633	301.0740	Rhamnocitrin
41	26.7	C15H24O2	236.1776	237.1876	Davanone
42	27.2	C15H18O3	246.1256	247.1367	Achillin/Santonin

Plant Species					
Compound ID number	**Ret. Time (min)**	**Likely Formula**	**MW ($g \cdot mol^{-1}$)**	**Observed m/z**	**Compound candidates**
42	27.2	C15H22O2	234.1619	235.1728	Artemisinic acid
44	27.9	C17H20O5	304.1310	305.1427	Matricarin
45	28.3	C18H16O8	360.0845	361.0957	MethylAxillarin
46	28.8	C18H16O8	360.0845	361.0967	MethylAxillarin
47	29.5	C15H22O2	234.1619	235.1725	Artemisinic acid
48	29.6	C15H24O2	236.1776	237.1881	Davanone
50	32.1	C15H22O2	234.1619	235.1723	Artemisinic acid
51	32.8	C19H18O8	374.1001	375.1114	Chrysosplentin/Castican
61	38.1	C15H22O2	234.1619	235.1720	Artemisinic acid
65	41	C15H22O2	234.1619	235.1726	Artemisinic acid
Chrysothamnus viscidiflorus					
8	10.9	C11H12O2	176.0837	177.0582	Lachnophyllum ester
13	12.5	C17H14O6	314.0790	313.1450	Bucegin/Luteolin 3',4'-dimethyl ether/Kaempferol 3,5-dimethyl ether/Ermanin
39	23.9	C20H32O3	320.2351	319.0873	3alpha-Hydroxy-7,13E-labdadien-15-oic acid/Viscidic acid A
40	24.2	C16H12O7	316.0583	317.0713	Quercetin 3-O-methyl ether/Rhamnetin/Isorhamnetin
46	25.8	C16H14O6	302.0790	303.0926	Homoeriodictyol
47	26.5	C16H14O6	302.0790	303.0928	Homoeriodictyol
48	26.8	C20H30O3	318.2195	317.2171	3-Oxo-7,13E-labdadien-15-oic acid

Plant Species

Compound ID number	Ret. Time (min)	Likely Formula	MW ($g \cdot mol^{-1}$)	Observed m/z	Compound candidates
49	27	C16H12O7	316.0583	317.0715	Quercetin 3-O-methyl ether/Rhamnetin/Isorhamnetin
50	27.3	C17H14O7	330.0740	331.0874	3,3'-Dimethylquercetin
53	28.3	C17H14O7	330.0740	331.0886	Quercetin 3,4'-dimethyl ether
54	28.6	C19H18O9	390.0951	391.1102	5,7,4'-Trihydroxy-3,6,8,3'-tetramethoxyflavone
53	28.9	C18H16O8	360.0845	361.0989	Padmatin 3-acetate
57	29.9	C16H12O7	316.0583	317.108	Quercetin 3-O-methyl ether/Rhamnetin//Isorhamnetin
61	31.9	C22H34O4	362.2457	361.2075	Viscidic acid B
62	32.5	C17H14O8	346.0689	345.1038	Taxifolin 3-acetate
68	33.6	C16H14O6	302.0790	303.2375	Homoeriodictyol
72	35.7	C19H18O8	374.1002	375.1141	Quercetagetin 3,5,6,3'-tetramethyl ether
73	35.8	C18H16O7	344.0896	345.1029	Pachypodol/Quercetin 7,3',4'-trimethyl ether
76	37.2	C17H14O6	314.0790	315.2020	Bucegin/Luteolin 3',4'-dimethyl ether/Kaempferol 3,5-dimethyl ether/Ermanin
82	39.2	C16H14O6	302.0790	303.2389	Homoeriodictyol
85	40	C17H14O6	314.0790	315.2019	Bucegin/Luteolin 3',4'-dimethyl ether/Kaempferol 3,5-dimethyl ether/Ermanin
86	40.5	C15H24	204.1878	205.1993	beta-Germacrene C/Italicene
88	41.6	C15H24	204.1878	205.1994	beta-Germacrene C/Italicene

Compound ID number	Ret. Time (min)	Likely Formula	MW ($g \cdot mol^{-1}$)	Observed m/z	Compound candidates
Plant Species					
90	42.9	C15H24	204.1878	205.1996	beta-Germacrene C/Italicene
95	44.7	C15H24	204.1878	205.1998	beta-Germacrene C/Italicene
Ericameria nauseosa					
60	39.9	C2030O	286.4590	287.2404	Ferruginol
57	38.75	C21H22O8	402.1314	403.2521	Nobiletin
48	34.8	C15H22O5	282.1467	283.2094	artemisinin
36	28.8	C9H6O3	162.0316	163.0775	7-Hydroxycoumarin
43	31.8	C9H6O3	162.0316	163.0773	7-Hydroxycoumarin
59	39.4	C15H10O5	270.0528	271.2458	Apigenin
59	39.4	C19H18O8	374.1001	375.2578	Casticin
60	39.9	C15H10O6	286.0477	287.2404	Luteolin
17	15.7	C27H30O16	610.1533	611.1674	Rutin
18	15.9	C27H30O16	610.1533	611.1670	Rutin
56	38.4	C16H14O6	302.079	303.2356	Homoeriodictyol
8	11	C11H12O2	176.0837	177.0571	Lachnophyllum ester
9	12	C11H12O2	176.0837	177.0572	Lachnophyllum ester
15	14.5	C11H12O2	176.0837	177.0573	Lachnophyllum ester
51	36	C17H24O9	372.142	373.2414	Syringin
51	36.2	C16H12O6	300.0633	301.2199	Kaempferide
56	38.4	C16H12O5	284.0684	285.2257	Acacetin
58	39.1	C17H14O6	314.079	315.2355	Kaempferol 3,5-dimethyl ether

Plant Species

Compound ID number	Ret. Time (min)	Likely Formula	MW ($g \cdot mol^{-1}$)	Observed m/z	Compound candidates
65	42.4	C16H12O7	316.0583	317.2514	Rhamnetin

APPENDIX C

Analysis of Shared and Unique Chemicals Occurring Across Targeted Plant Species by Chemical Class (Volatile and Water-soluble)

In Chapter 2 we combined gas chromatography and liquid chromatography–mass spectrometry to evaluate shared and unique chemicals in four targeted plant species. The functionality of chemicals can vary widely within a class and more so between classes, so assessing chemical diversity at different scales (i.e., chemical classes) can provide valuable information into chemical investment across plant taxa. Here, we used an upset chart to show how the four targeted plant species (one underrepresented and three overrepresented plant species), differ in shared and unique chemicals within each of the two chemical classes investigated (volatile and water-soluble).

Using an upset chart of volatile chemicals detected in gas chromatography, we found that each species varied in the total number, uniqueness, and diversity of volatile chemicals (Figure C.1), which was consistent with chemical summaries presented in Chapter 2 (Table 2.2). Within volatiles, unique chemicals (n = 49) outnumbered shared chemicals (n = 32) which is consistent with our combined chemical data. Big sagebrush had the highest number of total chemicals (n = 64) and more than threefold greater number of unique volatiles (n = 37) than the other species. Only one chemical was shared across all four plant species. The members of Asteraceae family shared six volatile chemicals, with big sagebrush and green rabbitbrush sharing the most volatiles across all shared pairs (n = 9).

Using an upset chart of the water-soluble chemicals detected using liquid chromatography–mass spectrometry, we found that each species varied in the total number of chemicals, chemical uniqueness, and chemical diversity (Figure C.2), which was consistent with chemical summaries presented in Chapter 2 (Table 2.3). Within water-soluble chemicals, unique chemicals (n = 188) also outnumbered shared chemicals

(n = 76), which is consistent with our combined chemical data. All four plant species shared many more water-soluble chemicals (n = 22) than volatile chemicals (n = 1, Figure C.1). The members of Asteraceae family shared nine chemicals, with big sagebrush and green rabbitbrush sharing the most volatiles across all shared pairs (n = 16). Interesting, water soluble chemicals in big sagebrush and green rabbitbrush are more chemically similar than those in green rabbitbrush and gray rabbitbrush.

Consistent with Chapter 2 findings, the inclusion of each plant species increases the chemical diversity present in the nest. Results also suggested that volatile diversity (hence olfaction), which was highest in the underrepresented plant species may not be what golden eagles are using to select plants for their nests, but instead they select for water-soluble chemical diversity, which was highest in the overrepresented plant species.

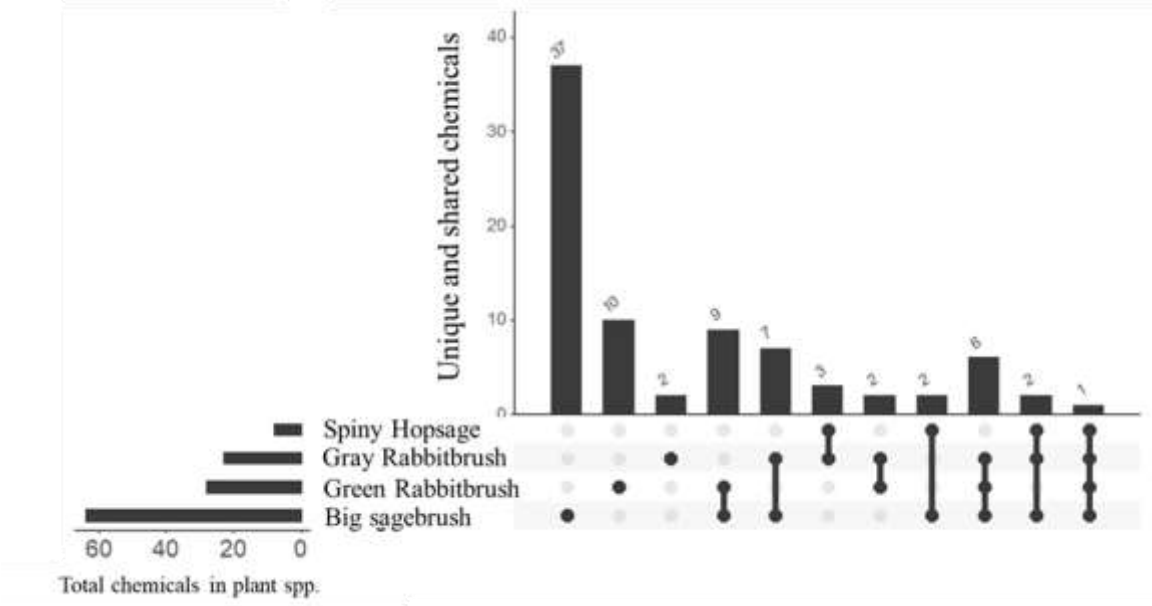

Figure C.1 Unique and shared volatile chemicals among targeted plant species used by golden eagles (*Aquila chrysaetos*) as nest material.

The horizontal bars to the left of the targeted plant species represent the total number of chemical contributions, both unique and shared, in each plant species. Top section of the chart (vertical bars) corresponds with the bottom section presented with dots. A single dot under a bar represents unique chemicals found only in *Artemisia tridentata* (big sagebrush); *Chrysothamnus vicidiflorus* (green rabbitbrush); *Ericameria nauseosa* (gray rabbitbrush); or *Grayia spinosa* (spiny hopsage). Dots (two, three, or four) connected by a line represent chemicals shared by designated plant species. Numbers above the vertical bars represent the number of total unique (single dot) or shared (more than one dot) chemicals for each combination.

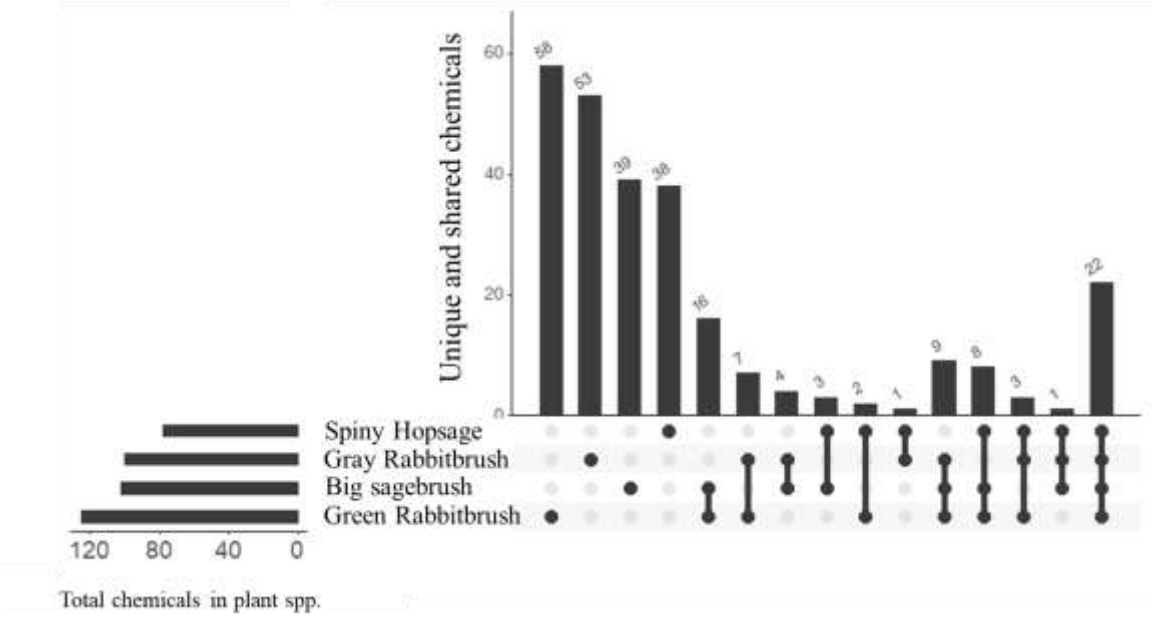

Figure C.2 Unique and shared water-soluble chemicals among targeted plant species used by golden eagles (*Aquila chrysaetos*) as nest material.

The horizontal bars to the left of the targeted plant species represent the total number of chemical contributions, both unique and shared, in each plant species. Top section of the chart (vertical bars) corresponds with the bottom section presented with dots. A single dot under a bar represents unique chemicals found only in *Artemisia tridentata* (big sagebrush); *Chrysothamnus vicidiflorus* (green rabbitbrush); *Ericameria nauseosa* (gray rabbitbrush); or *Grayia spinosa* (spiny hopsage). Dots (two, three, or four) connected by a line represent chemicals shared by designated plant species. Numbers above the vertical bars represent the number of total unique (single dot) or shared (more than one dot) chemicals for each combination.

APPENDIX D

Analysis of Volatile Concentration of Three Plant Species using Gas Chromatography

In Chapter 2, we discuss that the underrepresentation of big sagebrush by golden eagles maybe due to higher concentrations and potentially the presence of specific chemicals that could be harmful to nestling health. Based on principles of toxicology, higher concentrations would be potentially harmful to eagles. We used headspace gas chromatography to analyze volatile concentrations in patch-level composites from seven nesting territories. To adequately capture volatile concentration of patch-level composites, we had intended to use wet weights of 400 mg for rabbitbrush composites and 200 mg for sagebrush composites. However, due to a conversion error, we used a wet weight of 40 mg for gray and green rabbitbrush and a wet weight of 20 mg for big sagebrush. To determine concentration of volatile chemicals (area under the curve/mg dry weight (AUC/mg DW)), we divided the total AUC of a patch-level composite sample by the DW of that sample. A one-way ANOVA analysis found that mass specific volatile concentration of patch-level composites was significantly different among plant species ($F_{(2,52)} = 43.94$, $p < 0.0001$, Figure D.1). A Tukey's honestly significant difference post-hoc test revealed that the volatile concentration of big sagebrush was significantly higher than both rabbitbrush species, with no statistical differences in volatile concentration between the two rabbitbrushes. Big sagebrush had 4.3-fold higher concentration of mass specific volatiles on average (μ = 1637.25 AUC/mg DW) than green rabbitbrush (μ = 380.07 AUC/mg DW) and 7.6-fold higher concentrations then gray rabbitbrush (μ = 215.77 AUC/mg DW). In addition, big sagebrush had higher variation in mass specific concentration of volatiles (SD = 834.35) than either green (SD = 145.74) or gray (SD = 80.97) rabbitbrush. The difference in wet weights analyzed could provide an explanation for the variance of volatile concentration seen in patch-level samples of big sagebrush

that was absent in rabbitbrushes (Figure D.1). Spiny hopsage was not analyzed because sample collections had not been catalogued or processed. Our findings support that big sagebrush could be underrepresented compared to both rabbitbrushes due to higher concentrations of volatiles.

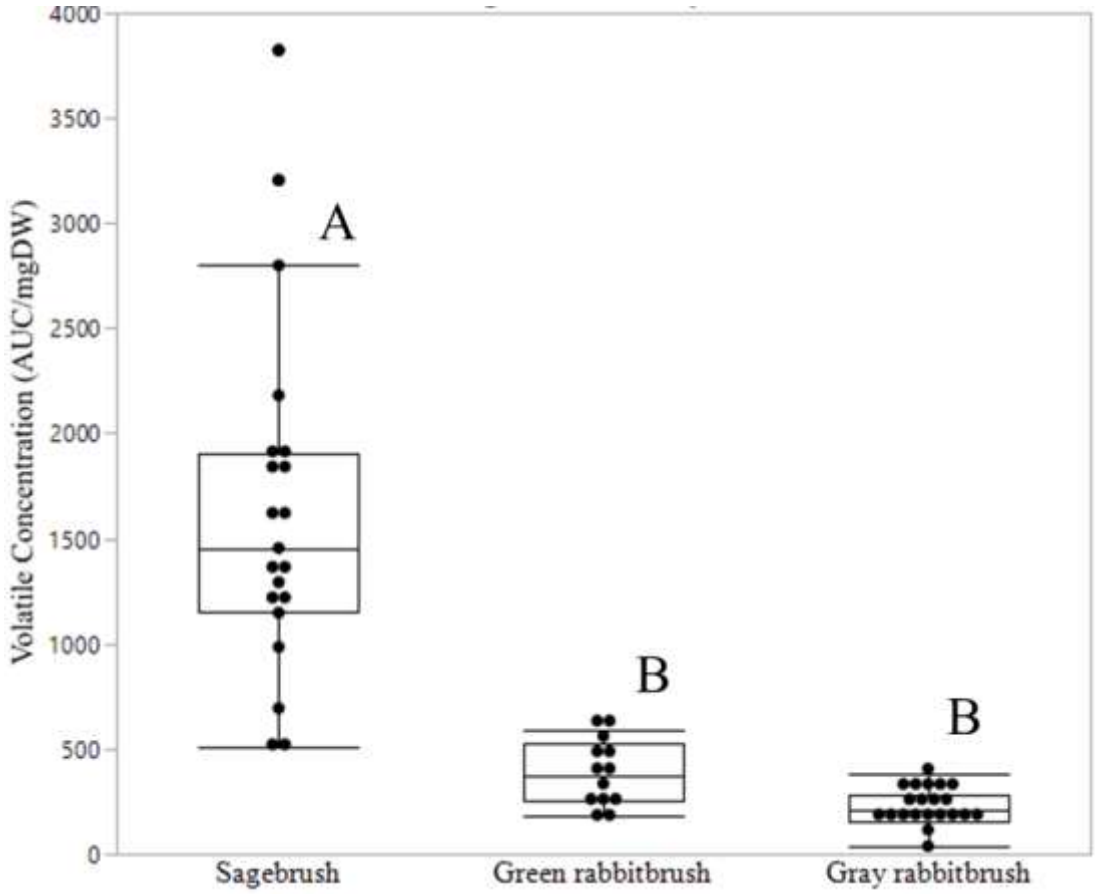

Figure D.1 Plant volatile concentrations (area under the curve/mg dry weight (AUC/mg DW)) in patch-level composites across three targeted species. Boxes represent the median interquartile range and whiskers represent the 5 to 95% range. Bars not sharing a common letter (A or B) were significantly different from each other (Tukey HSD test: $p < 0.05$)

APPENDIX E

Example Time Budget of an Ethogram for an Individual Bed Bug

In Chapter 3, we described how the ethogram was developed so that behavioral states were discreet and would not overlap. In Figure E.1, we provide an example where Bug 6 (subject) was assigned gray rabbitbrush for the plant species treatment, where it experienced the low volatile concentration during testing phase one (top) and the high volatile concentration during testing phase two (bottom). Antennal swipes only occurred in the grooming behavioral state. The grooming state was differentiated from akinesis by the grooming position where tarsi come together in front of the subject (i.e., in a praying position).

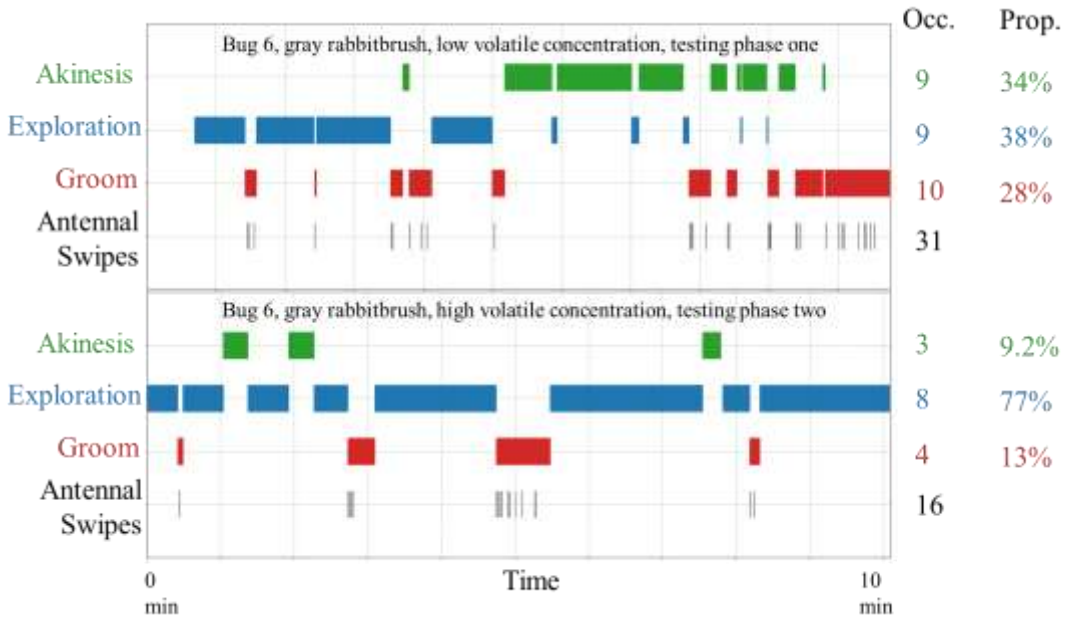

Figure E.1 Example of a time budget of an individual bed bug (*Cimex lectularius*) exposed to plant volatiles generated in BORIS.

Occurrences (Occ., number) of behavioral state during the testing phase and proportion of time spent (Prop., percent) in behavioral state during the testing phase. The top panel is the time budget of Bug 6, which was assigned gray rabbitbrush plant species treatment at the low concentration during the first testing phase. The bottom panel is the time budget of Bug 6 during the second testing phase when it received a high

concentration of volatiles of gray rabbitbrush plant species treatment. The three behavioral states: exploration (blue), grooming (red) and akinesis (green) were recorded with no overlap and the behavioral point event (antennal swipes in black) was nested within the grooming behavioral state.

APPENDIX F

Interaction Analysis between Plant Species Treatment and Volatile Concentration on the Proportion of Time Spent in Behavioral States

In Chapter 3, we analyzed plant species treatment and volatile concentration separately as effects on the proportion of time spent in a behavioral state. This was because there was no significant interaction between these two parameters. A least squares regression was performed with volatile concentration nested within plant species treatment due to experimental design (subject experienced only one plant species treatment but both levels of volatile concentrations). Results for an interaction between plant species treatment and volatile concentration are as follows: exploration ($F_{(3,40)}$ = 0.77, p = 0.52), grooming ($F_{(3,40)}$ = 2.03, p = 0.12), akinesis ($F_{(3,40)}$ = 2.01, p = 0.13, Figure F.1). These results could indicate that there is no interaction between plant species treatment or volatile concentration or indicate that the experimental design of this study (i.e., sample size, concentration used) was inadequate at deconstructing the interacting effects of these parameters.

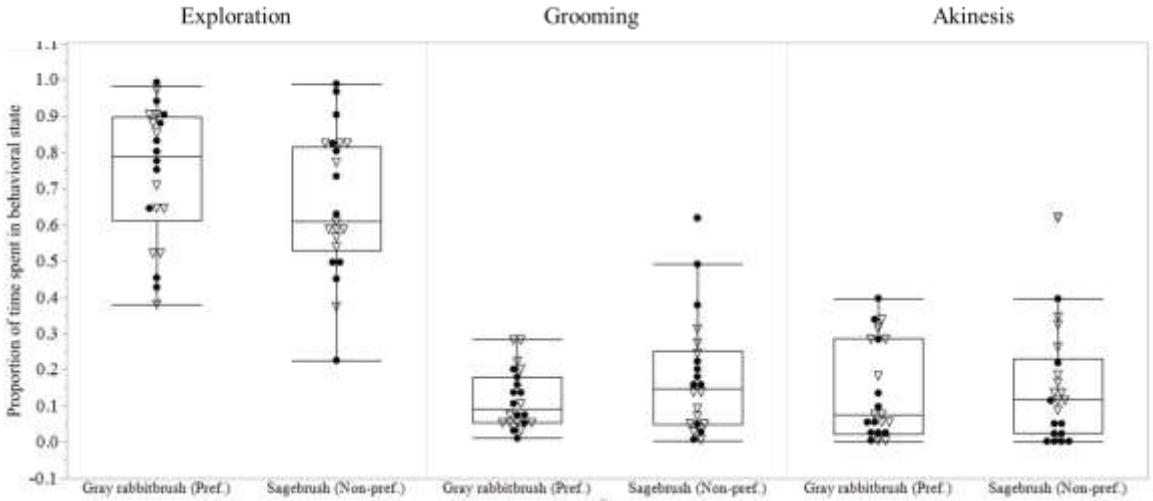

Figure F.1 Interaction between plant treatments and volatile concentrations on the proportion of time bed bug (*Cimex lectularius*) spend in each behavioral states (exploration, grooming, and akinesis). Solid circles represent high volatile concentrations and open triangles represent low volatile concentrations. Boxes represent the median interquartile range and whiskers represent the 5-95% range.

APPENDIX G

Analysis of Cohort and Order Effects

Controlling for sources of variation in an experiment, including those variables that are not of interest, is crucial to properly interpret statistical results. Here we investigated two possible sources of variation as experimental blocks: cohort and order effects. Cohorts in this experiment can be described as a block of subjects that shared testing phase days over an interval of two days. There were three cohorts (A, B, C) over a period of six days. Subjects that were in Cohort A were deprived of blood for five days, Cohort B were deprived of blood for seven days, and Cohort C were deprived of blood for nine days. Prolonged blood deprivation may increase the proportion of time spent in the state of exploration, minimizing time allocated to grooming and akinesis and maximizing energy expenditure, which has consequences on survival (Mellanby, 1938; Scharf, 2016). As such, we might expect that Cohort C, which experienced the longest time of blood deprivation, would spend more time exploring than other cohorts. We analyzed the cohort effect using an ANOVA followed by a Tukey Honestly Significant Difference test to compare each cohort for each behavior (Figure G.1). We found that cohorts were different in time allocated to exploration ($F_{(2,41)} = 6.39$, $p = 0.004$) and grooming ($F_{(2,41)} = 4.64$, $p = 0.02$), but not for akinesis ($F_{(2,41)} = 1.55$, $p = 0.23$). Cohort C explored significantly more than Cohort B ($p = 0.003$) and there was a trend that Cohort C explored longer than Cohort A ($p = 0.07$). This result could suggest that length of blood deprivation could influence behavioral outcomes and controlling for this factor may increase the ability to detect behavioral responses to chemical treatments in future studies. Cohort B groomed significantly more than Cohort C ($p = 0.01$). No difference was found of grooming behavior in Cohort A with Cohort B ($p = 0.28$) or Cohort A with Cohort C ($p = 0.32$). These results suggest that as blood deprivation is prolonged non-

essential behaviors that require energy are minimized. Cohorts did not differ in akinesis behavior ($p = 0.22$).

The order in which chemical treatments were used within a cohort also was evaluated as a source of variation. We predicted that experiencing the low volatile concentration first would habituate subjects to the arena, therefore, reducing any stress responses during the second testing phase. In contrast, experiencing the high volatile concentrations first had the potential to intensify stress responses due to both the presence of odor and novel stresses. We analyzed the effect of chemical treatment order using an ANOVA for each behavior (Figure G.2). We found that there were no statistically significant order effect: exploration ($F_{(3,40)} = 1.02$, $p = 0.39$), grooming ($F_{(3,40)} = 2.00$, $p = 0.13$), and akinesis ($F_{(3,40)} = 1.65$, $p = 0.19$). These findings suggest that whether a subject experienced the high or low volatile concentration during the first testing phase had no influence on behavioral outcomes.

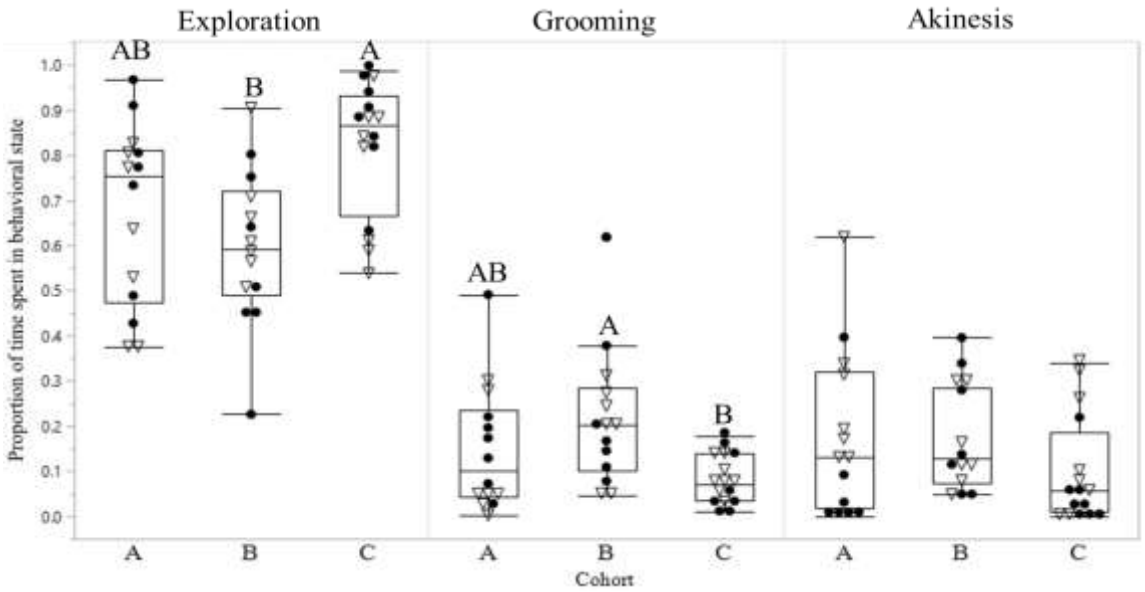

Figure G.1 Proportion of time spent in state behaviors across three cohorts (A, B, C) of bed bugs (*Cimex lectularius*) exposed to plant volatiles. Subjects in Cohort A were deprived blood for five days, Cohort B were deprived blood for seven days, and Cohort C were deprived blood for nine days. Solid circles represent the high volatile concentration and open triangles represent the low volatile concentration. Boxes represent the median interquartile range and whiskers represent the 5-95% range. Bars not sharing a common letter (A or B) were significantly different from each other ($p < 0.05$), while lack of letter indicates no significant difference between plant taxa.

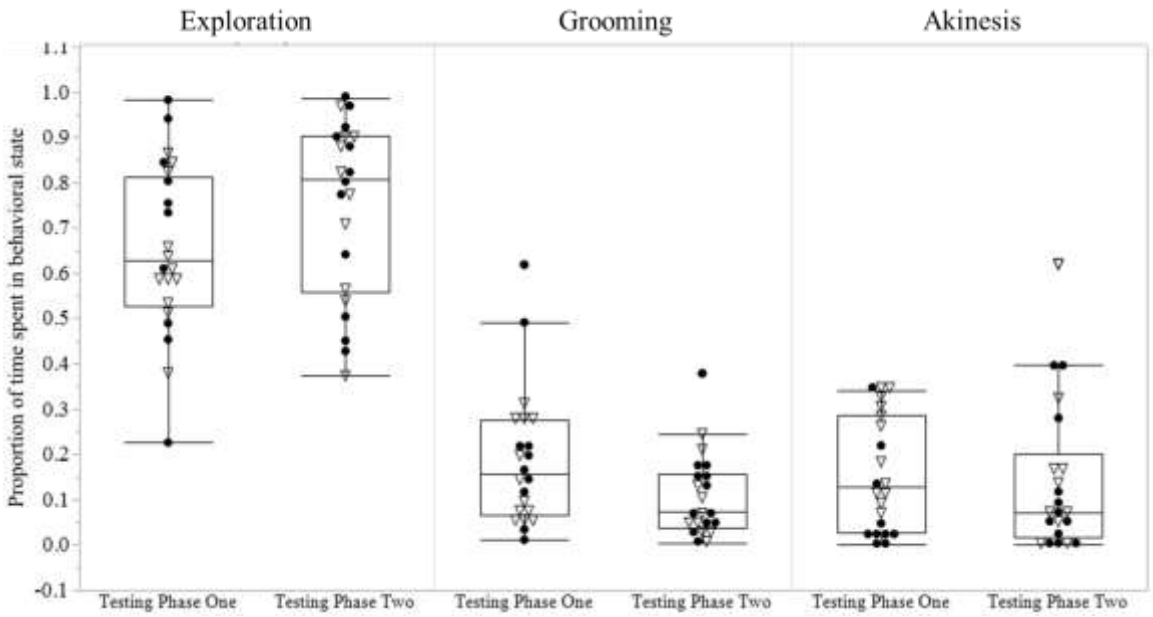

Figure G.2 Proportion of time spent in state behaviors by bed bugs (*Cimex lectularius*) when the plant volatile treatment was tested in phase one (first) or phase two (second) for an individual bed bug. Solid circles represent the high volatile concentration and open triangles represent the low volatile concentration. Boxes represent the median interquartile range and whiskers represent the 5-95% range.

APPENDIX H

Outlier Removal in Plant Treatments and Volatile Concentrations Analysis

Outliers were removed within behaviors and results compared with the full data set (Table G.1). Outliers were determined in RStudio (Version 1.3.959) using the software program R (version 4.0.2 (2020-06-22)) by using the boxplot.stats function, which uses Tukey's method to identify the outliers ranged above and below the 1.5*Interquartile range. This function was performed for each behavioral state response variable (proportion of time spent in behavioral state). Within the grooming proportion of time and antennal swipe count, two outliers were identified: Bug 10, big sagebrush lant species treatment, high volatile concentration, testing phase one; and Bug 13,big sagebrush treatment, high volatile concentration, testing phase one.

A Wilcoxon ranked sum test was performed in RStudio (Version 1.3.959) using the software program R (version 4.0.2 (2020-06-22)), with the exclusion of the outliers. There were no differences in significant results in plant species treatments with outliers removed (Table H.1). In Chapter 3, there were no significant effect of volatile concentration on grooming behavior and removal of outliers did not change statistical outcomes for grooming or exploration (Table H.2). However, with the removal of grooming outliers, the effect of volatile concentration on akinesis lost significance (p-value $_{\text{with outliers}} = 0.02$, p-value $_{\text{without outliers}} = 0.07$). It is worth noting both outliers were a combination of big sagebrush plant species treatment and high volatile concentration further demonstrating high variation in behavioral responses to high concentrations of volatiles from big sagebrush.

Table H.1 Summary statistics for behavioral state of bed bugs (*Cimex lectularius*) during exposure to plant treatments (gray rabbitbrush or big sagebrush) with inclusion of outliers and when outliers were removed. Mean, standard deviation (SD), 95% confidence interval (CI), W, and p-value of Wilcoxon ranked sum tests are reported for proportion of time spent in exploration, grooming, and akinesis and counts for antennal swipes.

Behavioral State	Plant Species	Mean	Mean (outliers removed)	SD	SD (outliers removed)	95 % CI	95% CI (outliers removed)	W	W (outliers removed)	p-value	p-value (outliers removed)
Exploration		0.70	0.73	0.19	0.17	0.64–0.76	0.68–0.79	303	223	0.17	0.51
	Gray rabbitbrush	0.74	0.74	0.19	0.19	0.66–0.83	0.66–0.83				
	Big sagebrush	0.66	0.72	0.20	0.16	0.58–0.75	0.64–0.80				
Grooming		0.15	0.13	0.13	0.09	0.11–0.19	0.10–0.16	205	196	0.40	0.95
	Gray rabbitbrush	0.12	0.12	0.08	0.08	0.08–0.15	0.08–0.15				
	Big sagebrush	0.18	0.14	0.16	0.11	0.10–0.25	0.09–0.19				

Behavioral State	Plant Species	Mean	Mean (outliers removed)	SD	SD (outliers removed)	95 % CI	95% CI (outliers removed)	W	W (outliers removed)	p-value	p-value (outliers removed)
Antennal swipes		24.3	21.7	17.6	13.0	18.3–29.6	17.6–25.9	244	222	0.97	0.53
	Gray rabbitbrush	22.1	22.1	11.5	11.5	17.1–27.2	17.1–27.2				
	Big sagebrush	26.4	21.2	22.2	15.0	16.6–36.2	13.7–28.7				
Akinesis		0.15	0.14	0.14	0.13	0.10–0.19	0.10–0.18	238	196	0.95	0.92
	Gray rabbitbrush	0.14	0.14	0.13	0.13	0.08–0.20	0.08–0.20				
	Big sagebrush	0.15	0.14	0.16	0.12	0.08–0.22	0.08–0.20				

Table H.2 Summary statistics for behavioral state of bed bugs (*Cimex lectularius*) during exposure to volatile concentrations (high and low) with inclusion of outliers and when outliers were removed. Mean, standard deviation (SD), 95% confidence interval (CI), W, and p-value of Wilcoxon ranked sum tests are reported for proportion of time spent in exploration, grooming, and akinesis and counts for antennal swipes.

Behavioral State	Volatile Conc.	Mean	Mean (outliers removed)	SD	SD (outliers removed)	95 % CI	95 % CI (outliers removed)	W	W (outliers removed)	p-value	p-value (outliers removed)
Exploration		0.70	0.73	0.19	0.17	0.64–0.76	0.68–0.79	277	239	0.42	0.30
	High	0.72	0.76	0.21	0.18	0.63–0.82	0.68–0.85				
	Low	0.68	0.70	0.17	0.16	0.61–0.76	0.63–0.78				
Grooming		0.15	0.13	0.13	0.09	0.11–0.19	0.10–0.16	268	203	0.55	0.95
	High	0.17	0.13	0.15	0.09	0.09–0.23	0.08–0.17				
	Low	0.13	0.13	0.10	0.10	0.08–0.17	0.08–0.17				

Behavioral State	Volatile Conc.	Mean	Mean (outliers removed)	SD	SD (outliers removed)	95 % CI	95 % CI (outliers removed)	W	W (outliers removed)	p-value	p-value (outliers removed)
Antennal swipes		24.3	21.7	17.6	13.0	18.3–29.6	17.6–25.9	231	167	0.79	0.37
	High	24.8	19.8	20.4	12.4	15.7–33.9	14.0–25.6				
	Low	23.7	23.7	14.6	13.6	17.2–30.2	17.3–30.0				
Akinesis		0.15	0.14	0.14	0.13	0.10–0.19	0.10–0.18	143	134	**0.02**	0.07
	High	0.11	0.11	0.13	0.14	0.05–0.16	0.05–0.18				
	Low	0.19	0.17	0.15	0.12	0.12–0.25	0.11–0.22				

APPENDIX I

Preliminary Analysis of Phenolic Concentrations on Bed bug Behavior

As part of a Murdock Partners in Science award, we mentored Jenna Raino from Skyview High School, Nampa, Idaho to discover and broaden the public and student perception of the functional role that chemical diversity plays in the health of wildlife and humans. We used this project to develop the antennal grooming bioassays used in this thesis. In general, we found concentration dependent changes in antennal grooming (Fig 1 in Figure I.1) and orientation of movement (Fig 2 in Figure I.1) by bed bugs for both extracts and whole leaves of big sagebrush. This work was presented by Jenna Raino at the Murdock Partners in Science 2018 National Conference, San Diego, CA January 2018 and represents some of the broader impacts of this thesis on K-12 teachers.

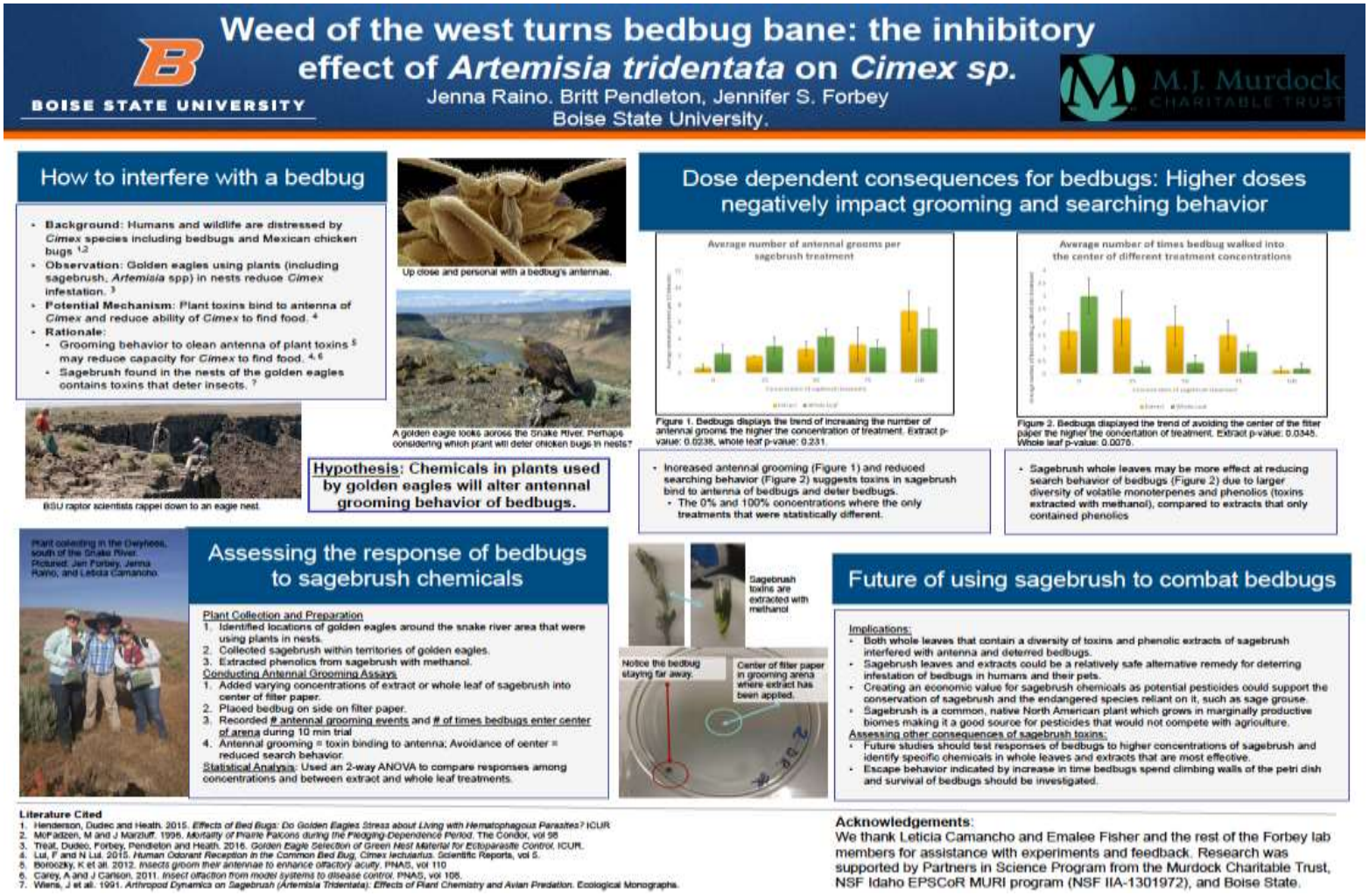

Figure H.1 Poster presented by Jenna Raino, Murdock Partners in Science recipient, at the Murdock Partners in Science 2018 National Conference. San Diego, CA January 2018.

APPENDIX J

Correlations Between Behavioral States: Exploration, Grooming and Akinesis

In Chapter 3, we investigated the proportion of time spent in three behavioral states: exploration, grooming, and akinesis. Because these behavioral states were proportions of a 10-minute testing phase they were correlated, this also causes the data to fail the test of normality when looking at behavioral states separately. Using RStudio (Version 1.3.959) and the software program R (version 4.0.2 (2020-06-22)), we ran Spearman correlation analyses between each behavioral state and found strong correlations between the grooming and exploration states ($r = -0.67$, $t(42) = -5.91$, $p < 0.0001$, Figure J.1) as well as akinesis and exploration states ($r = -0.70$, $t(42) = -6.27$, $p < 0.0001$, Figure J.2). Akinesis and grooming showed no correlation ($r = -0.05$, $t(42) = -0.37$, $p = 0.71$, Figure J.3).

Our results suggest that grooming is associated with exploratory behaviors, in both our time proportion and sequential data, which would support the use of grooming to sharpen olfactory acuity while host-searching.

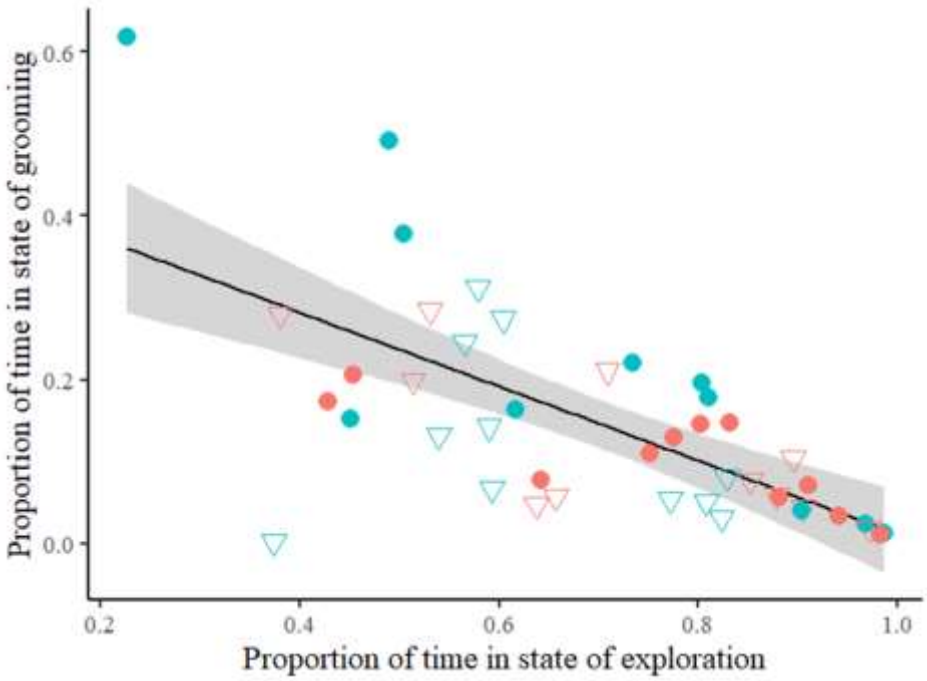

Figure J.1 Correlation between the behavioral states of grooming and exploration by bed bugs (*Cimex lectularius*) exposed to plant volatiles. Data points from bed bugs exposed to gray rabbitbrush are orange and big sagebrush are blue. The high volatile concentration data are depicted by solid circles and the low volatile concentration data are depicted by open triangles.

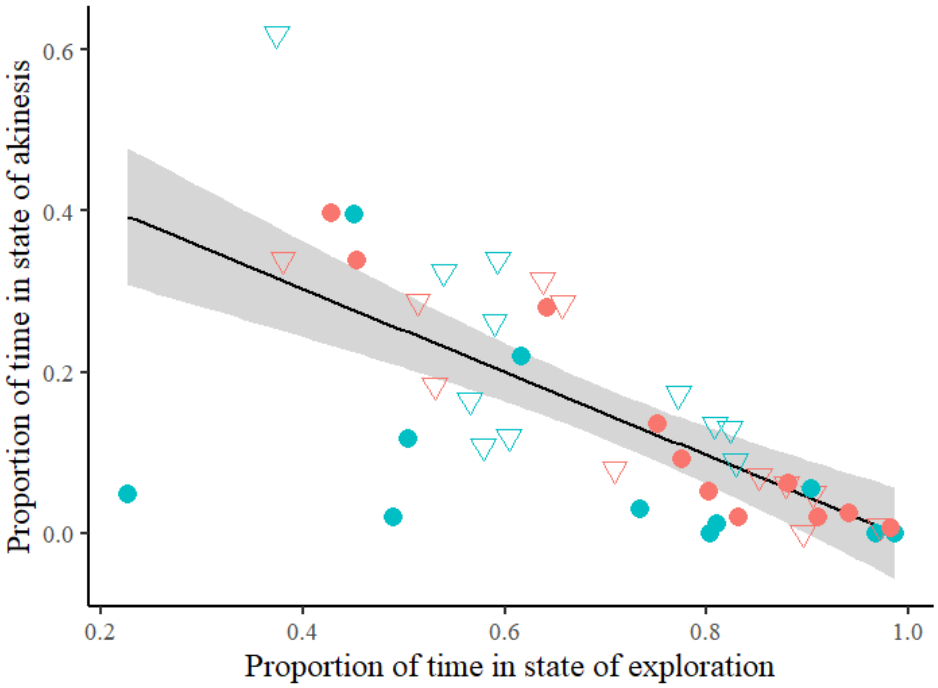

Figure J.2 Correlation between the behavioral states of akinesis and exploration by bed bugs (*Cimex lectularius*) exposed to plant volatiles. Data points from bed bugs exposed to gray rabbitbrush are orange and big sagebrush are blue. The high volatile concentration data are depicted by solid circles and the low volatile concentration data are depicted by open triangles.

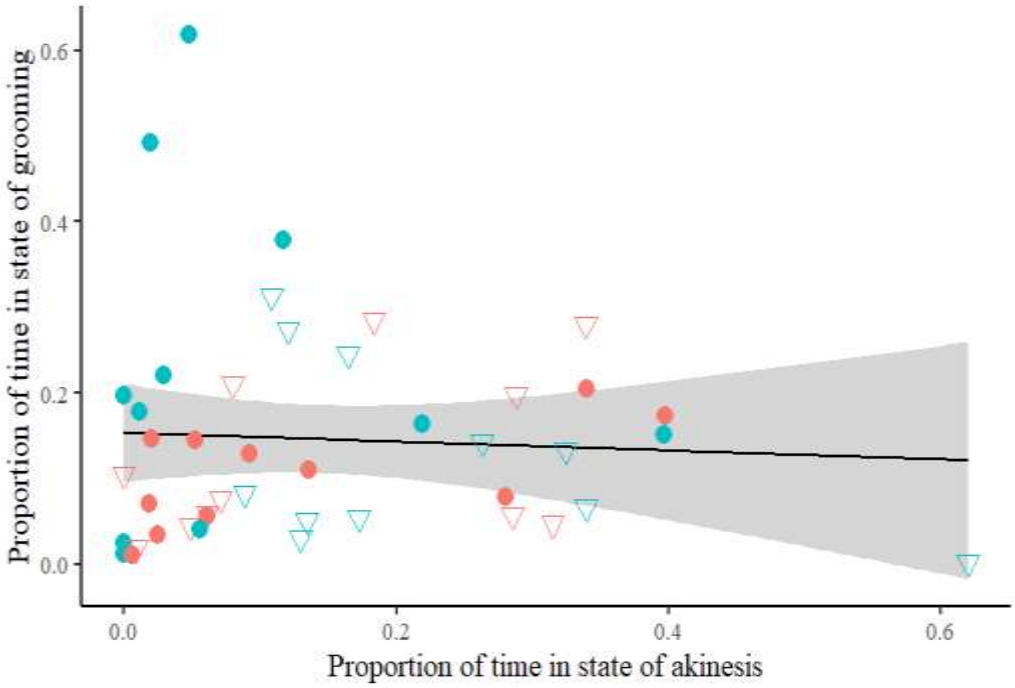

Figure J.3 Correlation between the behavioral states of akinesis and grooming by bed bugs (*Cimex lectularius*) exposed to plant volatiles. Data points from bed bugs exposed to gray rabbitbrush are orange and big sagebrush are blue. The high volatile concentration data are depicted by solid circles and the low volatile concentration data are depicted by open triangles.