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Selenium Accumulation in Brassicaceae Plant Species and its Biotransfer to Insect Pollinators

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Dedications

To my husband, Christopher Ross Moylan,

and

To the memory of my grandmother, Lourdes Dueñas Zerrudo

ABSTRACT OF THE DISSERTATION

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by

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Doctor of Philosophy, Graduate Program in Entomology
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Dr. John T. Trumble, Chairperson

Selenium (Se) has contaminated soils and plants in the western USA and other regions where pollination can be critical to the functioning of both agricultural and natural ecosystems, yet we know very little about how pollutants can impact insect pollinators. The overall goal of this dissertation was to investigate both the toxic effects of a plant-accumulated pollutant (Se) on a common pollinator, the honey bee (*Apis mellifera* L., Hymenoptera: Apidae).

I investigated the accumulation of Se in two phytoremediating species, *Brassica juncea* and *Stanleya pinnata*. The plants were irrigated in the greenhouse with selenate, and accumulated significantly quantities in the nectar (110 and 150 µg Se ml^{-1} ww) and

pollen (1700 and 12900 µg Se g^{-1} dw, respectively). These concentrations are much higher than the Se LC_{50}'s for certain insects.

I used proboscis extension reflex bioassays to determine if the Se affected the gustatory response of honey bee foragers. Antennal stimulation with selenomethionine reduced PER at higher concentrations. Foragers dosed with selenate had higher mortality and reduced responses to sucrose, which may lead to decreases in incoming floral resources needed to support coworkers and larvae in the colony.

In a semi-field study, the weedy plant *Raphanus sativus* (radish) was exposed to selenate watering treatments to evaluate the effects on pollination ecology. Honey bee pollinators were observed to readily forage on *R. sativus* for both pollen and nectar despite high floral Se concentrations. Se treatments increased seed abortion and decreased plant biomass, but herbivory by birds and aphids was also reduced, indicating a potential tradeoff of phytotoxicity and protection from Se.

I used artificial diet bioassays to determine the toxicity of four Se compounds that occur in accumulating plants. *Apis mellifera* larvae were chronically fed Se, and the inorganic forms were more toxic (LC_{50} selenate = 0.72 µg g^{-1}, LC_{50} selenite = 1.03 µg g^{-1}) than the organic forms (LC_{50} methylselenocysteine = 4.09 µg g^{-1}, LC_{50} selenomethionine = 6.04 µg g^{-1}). All four forms decreased the percentage of larvae that pupated, and selenate and methylselenocysteine significantly decreased larval growth rates. Taken together, the dissertation research from the past five years represents a crucial first step towards understanding the impact environmental stressors can have on pollinator-plant interactions and specifically, the honey bee, *Apis mellifera*.

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CHAPTER 1

Introduction

Selenium (Se) is a metalloid that occurs naturally in the alkaline soils from shale deposits of prehistoric inland seas (Emmons et al. 1896). Volcanic activity during the Cretaceous Period deposited ashes and magmas rich with Se into the sediments (Rosenfeld and Beath 1964). Geologic forces since then have caused an upheaval of the prehistoric seas, exposing the seleniferous shale sediments in areas now known as the California Coast Ranges (Trelease and Beath 1949). Cretaceous marine formations are also found in the western areas of the San Joaquin and Sacramento valleys as well as parts of the Rocky Mountain and Great Plains region (Trelease and Beath 1949; Presser 1994). Many regions of the central and western U.S. have Se soil and water levels up to 110 $\mu g\ g^{-1}$ Se (Seiler 1999). Anthropogenically seleniferous soils differ from the naturally seleniferous rocks and soils (such as in the California Coast Ranges) because they are caused by agricultural water moving Se to areas previously uncontaminated with Se. Agricultural water drainage solubilizes Se from naturally-occurring pyrite rock soils that contain significant amounts of Se (Presser 1994). The irrigation of these naturally seleniferous soils has caused the buildup of selenate (SeO_4^{2-}), the predominant and bioavailable form of Se in aerobic and alkaline environments. Selenate is the most common species of Se found in the root zone (Tokunaga et al. 1991) and can contaminate both water and soil (Cutter 1982; Dhillon and Dhillon 2001, Frankenberger and Benson 1994; Trumble and Sorensen 2008). Therefore Se can occur naturally in the soils from the California Coast Ranges, as well as anthropogenically, when it is transported via agricultural runoff as selenate to various areas of the San Joaquin Valley, where it would not naturally occur.

One of the worst cases of Se pollution reached its highest point in 1983 at the Kesterson Reservoir in the San Joaquin Valley (Merced County, California, USA), a major drainage site for many agricultural regions of California. The source of Se was determined to be from the California Coastal Ranges, which are adjacent to the Kesterson Reservoir (Presser 1994). Agricultural irrigation transported the Se via the San Luis Drain to the Kesterson National Wildlife Refuge, where there was a 64% rate of waterfowl death and offspring deformity due to the toxic buildup of Se (Presser 1994). Even with the current EPA Maximum Contaminant Level (MCL) for Se set at 0.05 mg L^{-1}, unregulated drainage waters may still continue to contaminate the grasslands near Kesterson Reservoir (Chilcott 2000). The San Joaquin River has exceeded the EPA MCL level 50% of the time from 1987 to 1997 (Presser and Luoma 2006). Selenium contamination in the San Joaquin Valley continues to be a problem due to the unlimited reservoir of selenium stored in the California Coast Ranges (Presser and Schwarzbach 2008).

The EPA MCL for Se was based on evidence from the well-documented case of Se poisoning in the Kesterson Reservoir as well as 96 hour acute and chronic toxicity testing of aquatic animals. However, the MCL does not consider bioaccumulation or biomagnification of Se in the food web. Studies have demonstrated the biomagnification of Se throughout the food web (Ohlendorf 2003). Se concentrations in 3 waterbird species were above the threshold to cause reproductive harm in 2005 (Paveglio and Kilbride 2007). From 2003 to 2006, bird eggs collected at the Panoche Drainage District contained Se concentrations 9 fold higher than the risk threshold for embryos (Presser

and Schwarzbach 2008). The potential for food-chain contamination presently at the Kesterson Reservoir has been determined to be less problematic due to a lack of negative impacts on wildlife (Wu 2004). Recent studies of Se contamination have focused on birds (Herbst 2006 in Nevada; Pavelgio and Kilbride 2007; Santolo 2007 in California; Vest et al. 2009 in Utah) and few studies have sampled plants or insects for current Se concentrations in Kesterson Reservoir or the surrounding areas. At the higher end of the dietary Se threshold for fish and birds (11 µg Se g^{-1} dry weight), several invertebrates including *Podisus*, *Daphnia* and *Myzus* showed reduced growth and increased mortality (Debruyn and Chapman 2007). Current toxicity thresholds for Se protect fish and birds, but may be toxic to the invertebrate prey they feed upon. Se can have a detrimental affect not only on vertebrate animals, but also the plants and insects in a polluted ecosystem.

Studies at Kesterson Reservoir conducted from 1990 to 2000 found the methylation rate of soil Se dissipation to be between 1 to 5% per year, or 46 to 230 years before soil Se will drop to normal levels of 4 mg kg^{-1} soil (Wu 2004). Current remediation efforts hope to accelerate Se removal from contaminated soils and water using plants (Bañuelos and Lin 2005; Pilon-Smits and LeDuc 2009), bacteria (Ghosh et al. 2008; Zhang et al. 2008), miroalgal-bacterial treatments as well as abiotic remediation technologies (Frankenberger et al. 2004). In addition, farm drainage systems such as constructed solar and evaporation ponds are also being implemented to capture and contain Se-contaminated agricultural runoff (Presser and Schwarzbach 2008). Although the selenium hazardous waste criterion is set at 1000 µg L^{-1}, it is not applied to integrated on-farm drainage management systems such as solar or accelerated evaporation ponds,

which do not need to meet the hazardous waste code for selenium (California Code of Regulations 2003). Large-scale treatment strategies such as evaporation ponds (at the Kesterson Reservoir and Tulare Basin in California) and artificially constructed wetlands (built by the Chevron Richmond Oil Refinery, California) created areas for Se to be volatilized or biotransformed by microorganisms, but they have caused serious ecological problems for migrating water birds nesting and feeding in these areas (Skorupa 1998). In addition, Se uptake in phytoremediating plants could biotransfer the Se from to the insects or animals that feed on them (Bañuelos et al. 2002; Pilon-Smits and Freeman 2006).

Selenium is also a micronutrient that is essential to many animals, including mammals, fish, and bacteria (Burau 1985). In particular, selenium is a critical component of glutathione peroxidase, a major cellular antioxidant enzyme. However, there is a narrow divide between the human dietary requirement for selenium (50 to 200 μg day^{-1}) and the level that causes toxicity (500 μg day^{-1}, National Research Council 1983). Low levels of selenium are beneficial for many organisms, but slightly higher levels can cause toxic effects. Selenium's toxicity is attributed to its similarity to sulfur (S). Se replaces S in amino acids and can change protein folding, disrupting cell metabolism and causing deformities in animals (Daniels 1996; Lemly 1997). Although Se is a micronutrient for many living organisms, a surplus of the element can cause its misincorporation into cysteine and methionine, thus leading to protein conformation changes and toxicity. Although Se is not considered to be essential for plants, low concentrations of Se can increase seed production in some species (Lyons et al. 2009).

Effects of high dietary levels of Se may cause toxicity through the misincorporation of Se into amino acids and proteins. The maintenance of the disulfide bridges is crucial to the folding and stability of the structure of proteins. At high dietary levels of Se, Se may replace sulfur (S) in amino acids such as cysteine. Substitution of Se for S in cysteine can cause an alteration in the proteins it occurs in due to differences between the sulfhydryl and selenol bridges causing the disruption of chemical bonds. Se has a larger atomic radius (Se = 0.5 Å) than S (S = 0.37 Å) which can cause protein conformation changes. Se-Se bridges are longer and weaker than S-S bonds (Brown and Shrift 1982). Diselenide bridges may not form at all, causing slightly different protein structure that can affect catalytic activity. For selenomethionine, protein structure may not be affected, but enzyme activity might be altered due to selenomethionine being more hydrophobic (Brown and Shrift 1982). Methoinine is used to transfer methyl groups to mRNA in eukaryotes (Perry 1976), and if a Se replaces S, it may reduce its ability to serve this function.

Plants employ several tactics for defending against herbivory, ranging from physical to chemical defenses that are produced within the plant. Plant-made defenses can be costly (Boyd 1998) and acquiring defenses from the environment may prove to be a less expensive tactic. Some plants growing in naturally metalliferous soils (such as the Se-containing Cretaceous age shales from the California Coast Ranges) can accumulate high levels of these elements within their tissues. Hyperaccumulator plant species absorb 100 to 10,000 mg/kg dw of elements such as cadmium (Cd), copper (Cu), nickel (Ni), lead (Pb), zinc (Zn), and selenium (Se) (Boyd 2007). These levels can be hundreds of times

greater than the normal range of elements found in plants. Plants that have evolved on naturally metalliferous soils may acquire toxic levels of the elements to defend against herbivores, as described by the elemental defense hypothesis (Boyd and Martens 1992). A growing number of studies support the elemental defense hypothesis by revealing the toxic and deterrent effects of metal and metalloid-containing plant tissues on herbivores (Boyd 2007).

Plants that rely on relationships with pollinators experience a tradeoff between defending against herbivores while attracting mutualists. Defensive compounds produced in the flower protect against florivores but may deter pollinators (Strauss 1997), especially if the compounds are found in nectar and or pollen. Several studies have found elevated levels of metals in the flowers and fruits in a number of hyperaccumulator species (Jaffre et al. 1976; Reeves et al. 1981; Freeman et al. 2006). Herbivores fed plant tissues containing high levels of metals, metalloids, or other accumulated elements have shown reduced development and survival (Boyd 2007), and several studies have shown some insect species cannot detect detrimental levels of Se (Trumble et al. 1998; Vickerman et al. 2002), but there are no studies to date examining the effects of Se-containing floral tissues on insect pollinator fitness. Hyperaccumulator plants may face opposing selection pressures to use metal accumulation to defend against herbivores while still attracting mutualists. Despite extensive research on the effects of accumulated metals or metalloids on several types of herbivores including leaf chewers, phloem feeders, and cell disruptors (Boyd and Martens 1998), few studies have examined the effects of accumulated elements on pollinators.

Se hyperaccumulators can absorb metal or metalloids to levels that can be hundreds of times greater than the normal range of elements found in non-accumulator plants (Brown and Shrift 1981). Non-accumulator plants normally accumulate 0.05 to 1 mg Se kg^{-1} dw (Brown and Shrift 1981). *Stanleya pinnata* is a perennial species native to the western USA and is a Se hyperaccumulator that can absorb more than 1000 mg Se kg^{-1} dw from soils containing as little as 10 mg Se kg^{-1} dw. In addition, high levels of Se were found in the flowers relative to its leaf tissues (Freeman et al. 2006; Galeas et al. 2007). However, these studies did not distinguish which specific parts of the flower (particularly the tissues collected by bees such as pollen and nectar) contained Se. In hyperaccumulating plants, selenate is converted to methylated seleno-amino acids which are not incorporated into proteins (Freeman et al. 2006; Parker et al. 2003; Terry et al. 2000). The non-protein seleno-amino acids can then be ingested by insect herbivores (Vickerman et al. 2002; Boyd 2007). In hyperaccumulators, Se can also be transformed into Se-Se-methylselenocysteine, and may then be released as the volatile dimethylselenide (DMSe) or dimethyldiselenide (DMDSe) (Terry et al. 2000).

Secondary accumulator plant species do not accumulate as high levels of Se as hyperaccumulators (Brown and Shrift 1982), and do not grow on naturally high-element soils like hyperaccumulators do. Secondary Se accumulators typically contain up to 350 mg Se kg^{-1} dw when grown in soils containing moderate levels of Se (Terry et al. 2000). Brassicaceae plants such as *Brassica juncea* will preferentially take up sulfur (S) over Se, and are thus categorized as secondary Se accumulators (Bañuelos et al. 1997; Feist and Parker 2001; Terry et al. 2000). *Brassica juncea* also accumulates Se mostly as selenate

(Parker et al. 2003; Terry et al. 2000), and experiences reduced growth when grown in soil containing 2 mg Se kg^{-1} (Bañuelos et al. 1997), suggesting there is a cost to accumulating Se in secondary accumulator plants. In secondary accumulator plants, selenate can be reduced to selenite (SeO_3^{2-}) and then incorporated into amino acids and proteins as selenomethionine or selenocysteine, which can also have toxic effects (Brown and Shrift 1981). Selenium can also be volatilized from *B. juncea* foliar tissues as dimethylselenide (DMSe) and other Se-containing volatiles (Kubachka et al. 2007; Meija et al. 2002).

The family Brassicaceae contains a high proportion of invasive species (Müeller 2009). Various species of Brassicaceae have been invading areas of the California Coast Ranges and the San Joaquin and Sacramento Valleys (Cal-IPC 2006) where natural as well as anthropogenic sources of Se occur. Certain species of the Brassicaceae typically accumulate sulfur (S), but also accumulate some amount of Se, categorizing them as secondary Se accumulators (Feist and Parker 2001; Terry et al. 2000). Se accumulation has been documented in several members of the Brassicaceae (White et al. 2007), and may also occur in other Brassicaceae species that are weedy or invasive. Invasive plants that can accumulate Se may act as a portal for entry of the contaminant into the local ecosystem via insects that feed upon them. Sorensen et al. (2009) found Se accumulation to levels as high as 1070 mg kg^{-1} in experimental manipulations of the invasive plant, saltcedar (*Tamarix ramosissima*). In addition, *Diohabda elongata* beetles fed the Se-containing plant tissues also accumulated 260 mg Se kg^{-1}, indicating the potential for biotransfer of Se from an invasive plant species to the insects feeding upon its tissues.

Weedy or invasive Brassicaceae plants may be able to accumulate Se in the field from natural or anthropogenic seleniferous soils and biotransfer it to insects such as pollinators that feed on and collect its tissues. If pollinators cannot detect and avoid Se compounds in the plant tissues they are foraging on and collecting for their progeny, they may suffer similar adverse effects as seen in other insect guilds.

Se may be a feeding repellent to insects (if it is detected at all) because insects may have evolved to avoid plants that have metal or metalloid based chemicals within them because of their toxicity. Selenium compounds found in floral tissues or volatilizing from plants in areas of normally low to nonexistent soil concentrations of Se may be picked up by an insect as a plant to avoid. However, insects have been found to visit Se-accumulating plants in areas of naturally seleniferous soils as herbivores (Galeas et al. 2008) and pollinators (Quinn et al. 2011), so insects in these areas may in fact use chemosensory cues of Se to choose plants that are the least damaged by other insect or mammalian herbivores. Se accumulation can reduce herbivory, making a plant more attractive to other herbivores as well as pollinators, relative to neighboring non-accumulators that are not able to fend off herbivore damage as well.

There have been mixed results in the literature regarding whether insects are attracted, deterred, or indifferent to Se in plant tissues. For an insect that may have evolved as a Se specialist, the diamondback moth *Plutella xylostella* Stanleyi (Freeman et al. 2006) may cue in on Se as a stimulant and preferentially feed on Se-hyperaccumulating plants, especially because it increases its fitness when feeding upon these plants by potentially protecting itself from predators by sequestering Se. Adult

parasitoids (*Cotesia marginiventris*) do not discriminate against beet armyworm hosts fed selenium-containing alfalfa (Vickerman et al. 2004), and females of the detritivore *Megaselia scalaris* did not discriminate against oviposition sites containing Se (Jensen et al. 2005).

Insects metabolize Se by first ingesting the selenium as selenate, selenite or selenoamino acids. If the Se is ingested as a selenoprotein or selenoamino acids, it is first broken down in the alimentary canal from proteins to amino acids. Methionine is an amino acid essential to insects (such as honey bees), whereas cysteine is non-essential (De Groot 1953). Metabolism of selenocompounds would involve incorporating selenoamino acids such as selenomethionine or selenocysteine into the insect's proteins where sulfur-containing amino acids would normally be present. In *D. melanogaster*, selenium is incorporated into proteins as indicated by selenoprotein mRNA expression of selenophosphate synthetase 2, and novel G-rich and BthD selenoproteins (Martin-Romero et al. 2001).

Insects may metabolize Se similar to microorganisms or other animals, and reduce ingested selenate to selenite, then selenide, which is then incorporated into selenoamino acids such as selenocysteine and selenomethionine. If insects ingest methylselenocysteine (the predominant chemical form of Se found in the hyperaccumulator *Stanleya pinnata*), they may demethylate the selenocysteine then incorporate it into proteins, which can cause toxicity. Insects that ingest high dietary amounts of Se may sequester the highest concentrations of Se in the Malpighian tubules, with a direct dose accumulation relationship found in *Tenebrio molitor* (Hogan and

Razniak 1991), suggesting Malpighian tubules play a role in the excretion of excess amounts of Se, possibly the anionic forms selenate and selenite. In addition, insects may excrete the ingested Se in the frass, in cocoons and pupal cases after eclosion (Vickerman et al. 2004), in exuvia shed at each molt, or in the eggs (which have high protein content and may sequester much of the selenoamino acids).

The level of toxicity to the insect may depend on what Se form the insect is ingesting from the plant. In the tolerant hyperaccumulator *S. pinnata*, selenate in the soil is converted into methylselenocysteine, the predominant form found in the leaves and flowers. The methylation of selenocysteine prevents its misincorporation into proteins in the plant. The diamondback moth, *Plutella xylostella Stanleyi*, is a specialist herbivore on *S. pinnata* (Freeman et al. 2006). In Se-tolerant *P. xylostella* caterpillars, the predominant form of Se was methylselenocysteine (90% of total Se), which was mostly localized to the hindgut. For Se-sensitive caterpillars, Se was mostly un-methylated selenocystine and selenocysteine, and was found throughout the body with no particular site of accumulation (Freeman et al. 2006). Digestive enzymes such as MeCys demethylase may remove the methyl group on the methylselenocysteine and releasing more toxic selenocysteine in the sensitive insect. The Se-tolerant diamondback moth, however, may re-methylate the selenocysteine after ingestion (Freeman et al. 2006). Based on these studies involving herbivore feeding on Se-accumulating plants, insects that have not evolved in naturally seleniferous areas such as *Plutella xylostella Stanleyi* will not have the suite of detoxification and tolerance mechanisms.

Pollinators such as *A. mellifera* and *Bombus sp.* collected from hyperaccumulator plants in naturally seleniferous areas of Colorado contained up to 270 µg Se g-1, although X-ray fluorescence (µXRF) distribution maps revealed methylselenocysteine throughout their bodies, it was not localized to any specific organs (Quinn et al. 2011). Methyselenocysteine did not appear to be demethylated, but the insects were collected in the field and may have not had time to digest and transform the compound. More studies need to be conducted to determine if methylselenocysteine can be somewhat toxic to honey bees, particularly if they manage to demethylate it. In addition, pollinators in polluted areas have not had a enough time to evolve a tolerance mechanism, and may be much more susceptible than the insects living in naturally Se-rich areas.

There are four objectives of this dissertation.

Objective 1. To test the hypothesis that ecologically relevant concentrations of Se will accumulate in the pollen and nectar of two phytoremediating Brassicaceae species. In addition, we will test the hypothesis that Se accumulation will negatively affect plant performance and reproduction in the secondary accumulator plant, *B. juncea*. An extensive body of research has examined the role of Se accumulation in plants, and their potential as phytoremediators of contaminated environments. Phytoremediation is a technology of using plants (and also microbes) to remove, transform and restore a contaminated site to a less toxic state. The two plant species examined in Objective 1, *Brassica juncea* (a Se secondary accumulator) and *Stanleya pinnata* (a Se hyperaccumulator), have recently been examined as potential phytoremediators of Se-

polluted soils due to their ability to accumulate and volatilize Se from the soil through their plant tissues (Bañuelos et al. 2002; Parker et al. 2003; Pilon-Smits and Freeman 2006; Pilon-Smits and LeDuc 2009). However, if the pollen or nectar of these phytoremediating plants contains high levels of Se, pollinators collecting these plant tissues could possibly be exposed to toxic levels of Se.

Objective 2. To determine whether feeding and gustatory behaviors in *Apis mellifera* L. are affected by the presence of Se. We will test the hypothesis that pollinators will respond to the presence of Se in artificial nectar through changes in feeding behavior of forager honey bees in the laboratory. We will test bee feeding behavior responses to the presence of Se using proboscis extension reflex (PER). Some insect herbivores avoid feeding on plants accumulating Se (Bañuelos et al. 2002; Vickerman and Trumble 1999) and may be deterred by Se. In addition, sublethal and lethal effects of Se on foragers will be tested by measuring the sucrose response threshold and dosing bees with various forms and concentrations of Se and measuring mortality.

Objective 3. To determine whether Se will accumulate in the floral tissues of *Raphanus sativus* L. (radish). *Raphanus sativus* is a Brassicaceae species that has naturalized throughout the world (Holm et al. 1997) and is a weedy plant that grows throughout in North America (Warwick and Francis 2005). In California, it is known to hybridize with *R. raphanistrum*, creating an invasive hybrid (Hegde et al. 2006; Panetsos and Baker 1967). The third objective determined whether Se accumulation in *R. sativus* impacts pollinator visitation, herbivore damage, and plant reproduction in the field using a common garden experiment. *Raphanus sativus* is a self-incompatible species

dependent on pollinators for seed set (Sampson 1964). Examination of this self-incompatible species will allow for additional insight into Se's impact on plant fitness that is attributable to insect pollination. If pollinator visitation rates are reduced due to Se, seed production and therefore plant fitness may be negatively affected.

Objective 4. To determine whether Se can affect honey bee (*A. mellifera*) larval development and survival. The hypothesis will test whether the concentrations of Se found in the previously tested plant species will have negative effects on larval fitness such as early mortality, as well as reductions in growth and development. Se accumulation in plant tissues reduces the fitness of several types of insects, but there are no studies to date examining the effects of floral tissues containing Se on honey bee fitness.

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CHAPTER 2

Selenium accumulation in the floral tissues of two Brassicaceae species and its impact on floral traits and plant performance

Abstract

Selenium (Se) is a metalloid that can occur naturally in soils from the Cretaceous shale deposits of a prehistoric inland sea in the western United States. Agricultural irrigation and runoff solubilizes Se from these shales, causing buildups of toxic levels of selenate (SeO_4^{2-}) in water and soil. Our main objective was to investigate the accumulation of Se in two Brassicaceae species chosen for their potential as phytoremediators of Se contaminated soils. We tested the hypothesis that Se will accumulate in the pollen and nectar of two plant species and negatively affect floral traits and plant reproduction. Certain species of Brassicaceae can accumulate high concentrations of Se in their leaf tissues. In this study Se accumulation in plant tissues was investigated under greenhouse conditions. Se accumulator (*Brassica juncea*) and Se hyperaccumulator (*Stanleya pinnata*) plants were irrigated in sand culture with 0 µM selenate (control), 8 µM selenate, and 13 µM selenate.

Nectar and pollen in *S. pinnata* contained up to 150 µg Se ml^{-1} wet weight and 12900 µg Se g^{-1} dry weight when irrigated with 8 µM selenate. Se levels in nectar (110 µg Se ml^{-1} wet weight) and pollen (1700 µg Se g^{-1} dry weight) were not as high in *B. juncea*. Floral display width, petal area and seed pod length were significantly reduced in the 13 µM selenate Se treatment in *B. juncea*. *Stanleya pinnata* floral traits and seeds were unaffected by the Se treatments.

This study provides crucial information about where some of the highest concentrations of Se are found in two phytoremediators, and may reveal the potential risks pollinators may face when foraging upon these accumulating plants. In the field,

duration of the plant's exposure, Se soil and water concentrations as well as other environmental factors may also play important roles in determining how much Se is accumulated into the leaf and floral tissues. Our greenhouse study shed light on two species' ability to accumulate Se, as well as determined the specific plant tissues where Se concentrations are highest.

Introduction

Plants employ several tactics for defending against herbivory, ranging from physical structures and escape in time or space to chemical defenses that are produced within the plant. Most plants employ innate defenses such as secondary compounds to guard against herbivore attack. However, plant-made defenses can be costly and acquiring defenses from the environment may prove to be a less expensive tactic. Certain species of plants have evolved on naturally metalliferous soils and may accumulate toxic levels of the elements to defend against herbivores, as described by the elemental defense hypothesis (Boyd and Martens 1992). A growing number of studies support the elemental defense hypothesis by revealing the toxic and deterrent effects of metal and metalloid-containing plant tissues on herbivores (for reviews see Boyd 2007; Trumble and Sorensen 2008).

Hyperaccumulator plants can sequester large amounts of metals or metalloids (such as As, Co, Cr, Cu, Mn, Ni, Pb, Zn, or in this case, selenium, Se) in their foliar tissues (Baker and Brooks 1989). They can absorb 1000 mg kg^{-1} Se dw or higher into shoot tissues (Brown and Shrift 1981; Reeves and Baker 2000), and may contain levels of elements several orders of magnitude higher than what is normally found in species at the same site. Se hyperaccumulators include plant species in the genera *Astragalus* (Fabaceae), *Stanleya* (Brassicaceae), *Oonopsis* and *Xylorhiza* (Asteraceae), and these species mainly occur on naturally seleniferous soils such as in the western USA. At least twenty Se hyperaccumulator plant species have been described (Reeves and Baker 2000). Secondary accumulators, on the other hand, can typically absorb up to 1000 mg kg^{-1} Se

when grown on contaminated soils containing moderate levels of the metalloid (Brown and Shrift 1981). Secondary accumulator plants do not accumulate extremely high concentrations of Se like hyperaccumulators. Certain Brassicaceae species growing in seleniferous soils can accumulate high levels of Se within their tissues (Brown and Shrift 1981). Non-accumulators such as forage or crop plants accumulate less than 100 mg kg^{-1} of Se and suffer toxic effects when growing in high-element soils. Plants normally accumulate 0.05 to 1 mg kg^{-1} Se dw, but hyperaccumulators can absorb concentrations hundreds of times greater than the normal range of elements found in non-accumulator plants.

An extensive body of research has examined the role of Se accumulation in plants. Two plant species, *Stanleya pinnata* and *Brassica juncea*, have recently been investigated as potential phytoremediators of polluted soils due to their ability to accumulate and volatilize Se from the soil through their plant tissues (Bañuelos et al. 2002; Parker et al. 2003; Pilon-Smits and Freeman 2006; Terry et al. 2000). *Stanleya pinnata* is a Se hyperaccumulator species that grows on naturally formed seleniferous soils in the Western USA (Rosenfeld and Beath 1964), and can absorb up to 10,000 mg kg^{-1} Se dry weight (dw) even when growing on soils containing only 2 to 10 mg kg^{-1} Se dw (Virupaksha and Shrift 1965). *Stanleya pinnata* will preferentially take up Se even when S is present as a competitive inhibitor (Bañuelos et al. 1997; Bell et al. 1992; Feist and Parker 2001; Terry et al. 2000; White et al. 2007). *Brassica juncea* is a Se secondary accumulator that typically contains up to 350 mg Se kg^{-1} dw when grown in soils contaminated with moderate levels of Se (Terry et al. 2000), and it preferentially

accumulates sulfur (S) over Se (Feist and Parker 2001; Parker et al. 2003). *Brassica juncea* accumulates Se mostly as selenate (SeO_4^{2-}, Parker et al. 2003; Terry et al. 2000), and experiences reduced growth when grown in soil containing 2 mg Se kg^{-1} (Bañuelos et al. 1997), suggesting there may be toxic effects of accumulating Se in secondary accumulator plants. In secondary accumulator plants, selenate can be reduced to selenite (SeO_3^{2-}) and then incorporated into amino acids and proteins as selenomethionine or selenocysteine, which can also have toxic effects (Brown and Shrift 1981).

Two recent studies by Freeman et al. (2006) and Galeas et al. (2007) found high levels of Se in the flowers of *S. pinnata* relative to its leaf tissues, suggesting the defense of fitness-linked reproductive organs (McKey 1979). However, these studies did not distinguish which specific parts of the flower (pollen, nectar, or petal) contained Se. Selenium concentrations in specific *B. juncea* and *S. pinnata* floral tissues such as pollen and nectar have not been examined to date.

The first objective of this study was to determine whether plants that accumulate Se in their leaves will also accumulate Se in their pollen, nectar, and other floral tissues. The second objective was to determine the toxic effects of Se uptake in terms of floral traits and plant performance in a hyperaccumulator and accumulator plant species.

Materials and Methods

Plant growth conditions. Seeds from the Se hyperaccumulator plant species *Stanleya pinnata* (Pursh) Britton (Desert Prince's Plume) were obtained from a commercial seed company (Western Native Seed, Coaldale, CO, USA). Seeds from the secondary Se

accumulator plant species *Brassica juncea* (L.) Czern (Indian mustard, cv. "Southern Giant Curled") were also obtained from a commercial seed company (Seedway Vegetable Seeds, Hall, NY).

Seeds of both species for Experiment 1 were germinated in the greenhouse (Environmental Sciences Greenhouses, University of California, Riverside, CA) in University of California Standard Soil Mix III and transplanted in 2007. Se treatments were then begun 20 days after transplanting. For Experiment 2, seedlings were transplanted to the greenhouse in 2008 and Se treatments were begun 24 days after transplanting. Seedlings were removed from germination flats and roots were rinsed with tap water to remove as much soil as possible, and were then transplanted to the irrigation sand culture after nutrients had already been added and passed through the sand so that carbonates in the sand would buffer the pH. Seedlings were transplanted to 7.5 l pots filled with silica sand (Weist Rentals and Sales, Riverside, CA). Five plants were transplanted per pot, and any plants that had died were replaced during the following week. Four pots were irrigated from a 120 l tank filled with water and nutrient solution. The basal nutrient solution and Se treatments were added according to Parker et al. (1991). The basal nutrient solution contained 1 mM NH_4NO_3, 1 mM $CaCl_2$, 0.25 mM KCl, 0.1 mM $MgSO_4$, 10 µM NaH_2PO_4, 1 µM $MnCl_2$, 1 µM $ZnCl_2$, 0.1µM $CuCl_2$, 3 µM H_3BO_3, 0.1 Na_2MoO_4, and 10 µM Fe-EDTA. Nutrient solution irrigation was activated on a daily timer, pumping solution into each pot five times a day for five minutes. Nutrient solution then drained out of the pots and back into the 120 l tanks. Water levels were maintained at 120 l in the tank by replacing evaporated water with deionized water.

Solution N and P levels were checked throughout the experiments and replenished as necessary. However, solution Se levels were not replenished, and were added only once at the start of the experiments (using protocols from Feist and Parker 2001). *Brassica juncea* showed reduced growth when irrigated with 2 mg Se kg^{-1} that was maintained at this concentration throughout the experiment (Bañuelos et al. 1997), thus only an initial exposure to the high Se concentration was used to minimize the toxic effects of Se and allow for greater flower production. In addition, a multi-year field study using Se-contaminated soils from the Kesterson Reservoir of California found *B. juncea* depleted the total soil Se inventory by almost 50% (Bañuelos et al. 1995), thus phytoremediators planted in Se-contaminated soils can deplete the Se in the soils around them from an initially higher concentration to a lower concentration over time. Tank pH was monitored in both experiments and averaged 7.78± 0.05 (Experiment 1) and 7.50 ± 0.08 (Experiment 2). Greenhouse temperatures were monitored throughout the experiments using a Hobo temperature sensor (Onset Computer Corp., Bourne, MA) and averaged 26.1ºC.

Experimental design and Se treatments. Selenium treatments were started after 20 to 24 days of seedling establishment in the sand culture. Selenium was added as sodium selenate (Na_2SeO_4, Sigma-Aldrich, St. Louis, MO) and is reported as concentrations of elemental Se. Treatment water concentrations were chosen based on Se treatment concentrations used in Feist and Parker (2001), as well as concentrations below 4 mg l^{-1}, the maximum Se concentrations contaminating the western San Joaquin Valley in CA (Burau 1985; Mikkelsen et al. 1986; Presser and Barnes 1985). The three treatment

levels of elemental Se added to the tanks were 0 µM selenate (0.0 mg Se l^{-1}) (control, nutrient solution only), 8 µM selenate (0.65 mg Se l^{-1}), and 13 µM selenate (1.0 mg Se l^{-1}). Pots from each experiment were arranged in a randomized block design in order to minimize the variation in temperature and light in the greenhouse. Each pot was used as a unit of replication for all responses measured except Se content in nectar for *B. juncea* because it produced such low quantities of nectar (< 0.02 µl per flower per pot). Nectar from the four pots irrigated by individual tanks were pooled together, thus irrigation tank became the unit of replication for this response.

In Experiment 1, *B. juncea* and *S. pinnata* plants were subjected to the three levels of treatments (0 µM selenate, 8 µM selenate, and 13 µM selenate). In Experiment 2, *B. juncea* plants were subjected to the 0 µM selenate and 8 µM selenate levels of Se treatments and *S. pinnata* was subjected to the 0 µM selenate, 8µM selenate, and 13 µM selenate treatments. Each treatment was replicated with up to 58 pots. The datasets of both Experiment 1 and Experiment 2 were compared for each response variable using a *t* test. Datasets of Se content in floral and leaf tissues were combined for both experiments due to no significant differences between the two (*t* test, $P > 0.23$). Experiment 1 and 2 datasets for display width ($P < 0.03$), anther length ($P < 0.004$) and petal area ($P < 0.0001$) were analyzed separately for *B. juncea*. Experiment 1 and 2 plant performance responses that showed no significant differences between the two experiments (total flower number, nectar per flower, seed pod length and total seed weight and proportion of developed seeds, $P > 0.05$ for all) were combined into one dataset. *Stanleya pinnata* plants did not flower in Experiment 1, thus Se content and plant performance data from

Experiment 2 only are reported. The photosynthetic photon fluence rate (PPFR, 400-700 nm) was 621 to 895 µmol m^{-2} s^{-1}. Additional high intensity lighting was provided in the greenhouse and programmed on a 16:8 day:night cycle.

Collection of plant tissues for Se uptake. We examined the effects of Se irrigation on plant tissue Se content by measuring the concentration of Se in floral and leaf tissues. Irrigation solution samples were collected 0, 41, 60, and 95 days after the selenate treatments were started. Irrigation solution was analyzed for S and Se.

Floral tissues were collected throughout the experiments, and included: pollen, nectar, anthers/stigmas, and petals. Petals and anther/stigmas were dissected away from other floral tissues and placed in microcentrifuge tubes. Eighty percent ethanol was added to tubes containing anthers/stigmas then sonicated for 3 min (Bransonic Ultrasonics Corp., Danbury, CT) to remove pollen. The anther/stigma portion of the flowers was then removed from the tubes with forceps and placed into separate microcentrifuge tubes. Tubes with ethanol and pollen were then centrifuged at 10,000 rpm for 3 minutes to pellet the pollen (Fisher Scientific accuSpin Micro 17R microcentrifuge, Fisher Scientific, Pittsburg, PA), and tubes were then placed in a fume hood to evaporate the ethanol. Leaf tissues were also collected at the end of the experiments to compare leaf Se concentrations to floral tissue concentrations. Two leaves of similar age were collected from each plant, rinsed with tap water, and then dried with clean paper towels. All floral and leaf tissues were frozen in a -60ºC freezer (Fisher Scientific, Pittsburg, PA) and then freeze-dried (Labconco Corp., Kansas City, MO) at -40ºC and -25 psi for at least 3 days. Nectar was not freeze-dried and is reported as wet weight in µg Se ml^{-1}. After freeze

drying, leaf tissues were ground to a fine powder using a mortar and pestle. Floral tissues and seeds were not ground due to their small weights. All freeze-dried plant tissues and nectar were stored in a -60ºC freezer until digestion.

Plant performance measurements. We examined the effects of Se irrigation on plant performance by measuring both floral traits and seed production in both *B. juncea* and *S. pinnata*. For floral traits, we measured two flowers per pot. Floral trait measurements included display width (distance across flower from the tip of one petal to the other), petal area (estimated as length x width), anther length (length of one anther from two flowers per pot), total flower number, and nectar produced per flower (collected from two flowers per pot). The total number of flowers produced per day were counted for each pot replicate throughout the experiment, and then summarized within pot to calculate total flower number. Nectar production was measured using microcapillary tubes (20 µL size for *B. juncea* and 50 µL size for *S. pinnata*) (Drummond "Microcaps", Drummond Scientific Co., Broomall, PA). Nectar volume was collected by first measuring the length of the microcapillary tube using digital calipers (Fisher Scientific, Pittsburg, PA). The microcapillary tube was positioned at the bottom of the nectary, collecting the entire nectar volume in the flower, and the length of the nectar in the tube was then measured using digital calipers. The total volume of nectar was calculated as the tube size (20 µL or 50 µL) divided by the length of nectar in the tube (mm) which was also divided by the length of entire tube (mm). The total sum of nectar collected during the entire experiment (for each pot replicate) was then summed and divided by the total number of flowers collected for nectar to calculate the nectar produced per flower.

Seed production was measured from up to two seed pods per pot as the seed pod length, proportion of developed seeds, and total seed weight. Seeds were categorized as developed or undeveloped; undeveloped seeds were small and wrinkled, indicating an undeveloped embryo. Seed viability was confirmed for developed and undeveloped seeds by germinating them on filter paper moistened with tap water in a growth chamber kept at a constant temperature of 21ºC and a 16:8 day:night cycle.

Atomic absorption and inductively coupled plasma optical emission spectroscopy measurements. Plant tissues were weighed using a microbalance (weighing to 0.00000 g, model 1712 MP8, Sartorius Corp., Goettingen, Germany) prior to microwave digestion. Plant material was microwave digested in 110 ml teflon-lined vessels containing a mixture of 1 ml H_2O, 2 ml 30% (v/v) H_2O_2, and 2 ml concentrated HNO_3 (Sah and Miller 1992). The vessels were heated for 20 min using a 570 W microwave oven (CEM Corp., Matthews, NC). Plant tissue filtrates and irrigation solution samples were then diluted with 6 M HCl, heated in a 90ºC water bath for 20 min and analyzed using hydride vapor-generated atomic absorption spectroscopy (HVG-AAS). Sulfur was analyzed using inductively coupled plasma optical emission spectroscopy (ICP-OES). Se and sulfur concentrations in irrigation water are reported in µM. Selenium concentrations in plant tissues are reported in ppm (µg g^{-1} for plant tissues or µg ml^{-1} for nectar). Samples were run in duplicate and Se spikes were added as internal standards to determine precision and recovery. Duplicate sample concentrations were within 10% of each other, and Se spike recovery was over 90%.

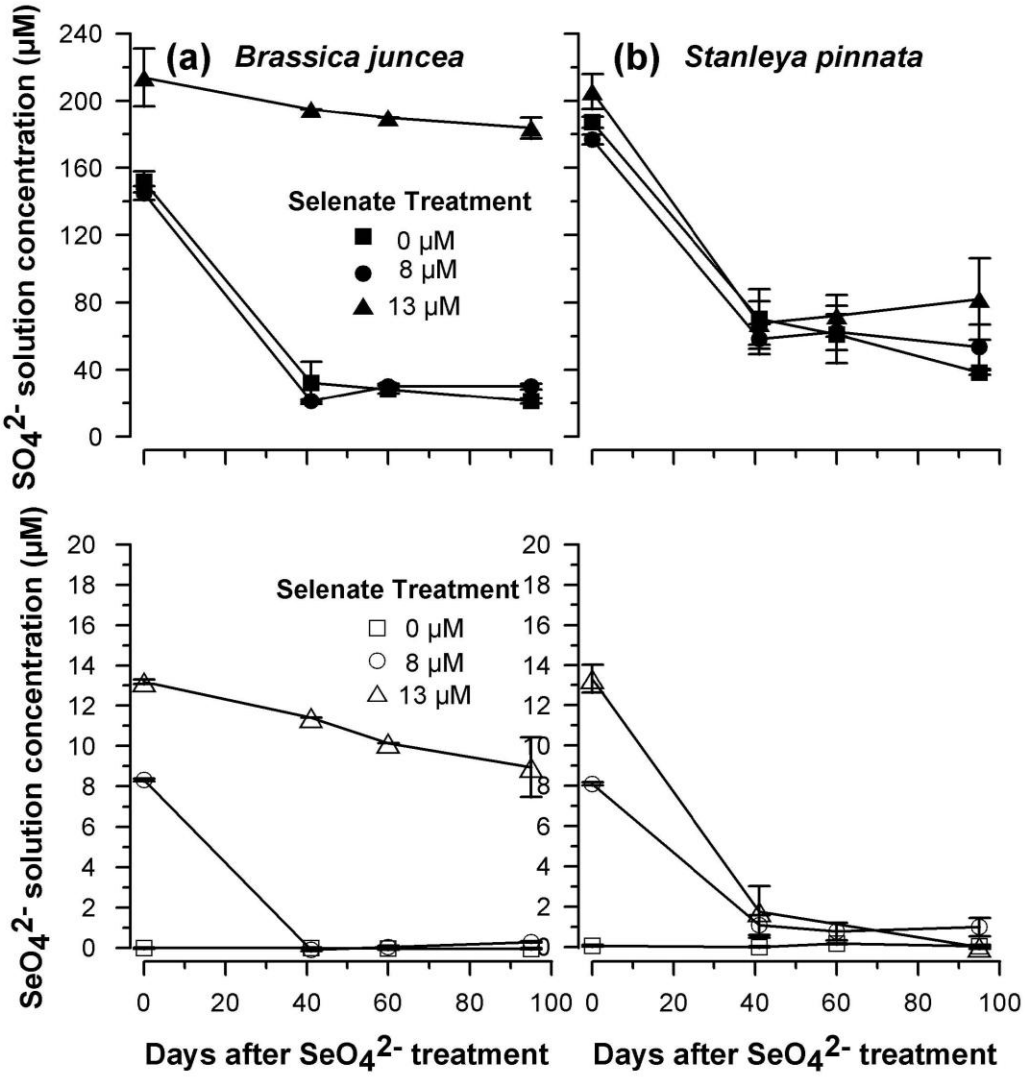
Statistical analyses. We examined the effects of Se irrigation on Se concentration in plant tissues and plant performance in *B. juncea* and *S. pinnata*. All data were averaged within pot using pot as the unit of replication for all responses except *B. juncea* nectar, which was averaged within tank due to the small volumes. Data were analyzed with SAS version 9.2 (SAS Institute, 2008, Cary, NC) using the General Linear Models (GLM) procedure with type III sums of squares. The basic model analyzed the effects of Se irrigation treatment and block (a fixed factor) on several response variables. The Se concentration response variables were analyzed in the following plant tissues: pollen, nectar, anther/stigmas, petals and leaves. Plant performance was analyzed as several responses, including: floral traits (display width, petal area, anther length, total flower number and nectar per flower) as well as seed traits (seed pod length, proportion of developed seeds and total seed weight). A standard Bonferroni correction was applied to the Se in plant tissue and plant performance analyses due to the large number of ANOVAs done conducted. Sulfur and Se concentrations in irrigation tank water were analyzed using regression in the REG procedure (SAS 2008). Assumptions of normality were examined using normal probability plots and the Shapiro-Wilks test in the UNIVARIATE procedure (SAS 2008). Se concentrations in plant tissues were log transformed to meet assumptions of normality for both *B. juncea* and *S. pinnata*. Floral traits and seed data were normally distributed without transformation for *S. pinnata*. *Brassica juncea* display width, petal area, nectar per flower, and total seed weights were log transformed to meet assumptions of normality.

Results

S and Se concentrations in irrigation tanks. Sulfur and Se concentrations were monitored at four timepoints during the experiments. For all irrigation tanks, the 0 µM selenate treatment contained less than 0.006 ± 0.02 µM Se during the entire duration of the experiment. *Brassica juncea* initial irrigation tank water concentrations averaged 8.32 µM Se (8 µM selenate treatment) and 13.01 µM Se (13 µM selenate treatment) (Fig. 1.1). *Stanleya pinnata* initial tank concentrations averaged 8.10 µM Se (8 µM selenate treatment) and 13.20 µM Se (13 µM selenate treatment) (Fig. 1.1). After the experiments concluded 95 days later, the final Se concentrations for *B. juncea* averaged 0.30 µM Se (8 µM selenate treatment) and 8.94 µM Se (13 µM selenate treatment). *Stanleya pinnata* final Se concentrations averaged 0.98 µM Se (8 µM selenate treatment) and 0.01 µM Se (13 µM selenate treatment). Sulfur and Se levels in irrigation tanks were correlated in both *B. juncea* ($r = 0.98$, $P < 0.0001$) and *S. pinnata* ($r = 0.96$, $P < 0.0001$) in the 8 µM selenate treatment. Both elements decreased in the irrigation solution over time. Sulfur and Se levels were not correlated in the 0 or 13 µM selenate treatments for *B. juncea* ($r < 0.02$, $P > 0.46$ for both) or *S. pinnata* ($r < 0.23$, $P > 0.08$ for both).

Leaf and floral tissue weights in B. juncea and S. pinnata. Pollen tissue weights averaged 0.008 ± 0.001 g for *B. juncea* ($n = 42$) and 0.01 ± 0.002 g for *S. pinnata* ($n = 32$). Anther/stigma tissue weights averaged 0.02 ± 0.002 g for *B. juncea* ($n = 20$) and 0.08 ± 0.008 g for *S. pinnata* ($n = 34$). Petal weights averaged 0.04 ± 0.008g for *B. juncea* ($n = 33$) and 0.06 ± 0.008 g for *S. pinnata* ($n = 22$). Leaf tissues averaged 0.10 ± 0.002 g for *B. juncea* ($n = 29$) and 0.10 ± 0.0003 g for *S. pinnata* ($n = 31$). Nectar

Figure 2.1. Mean concentrations of S (closed symbols, top panels) and Se (open symbols, bottom panels) in irrigation tank water over time in 0, 8, and 13 µM selenate treatments for (a) *B. juncea* (first flower = 35 days) and (b) *S. pinnata* (first flower = 45 days). Shown are means ± SE.

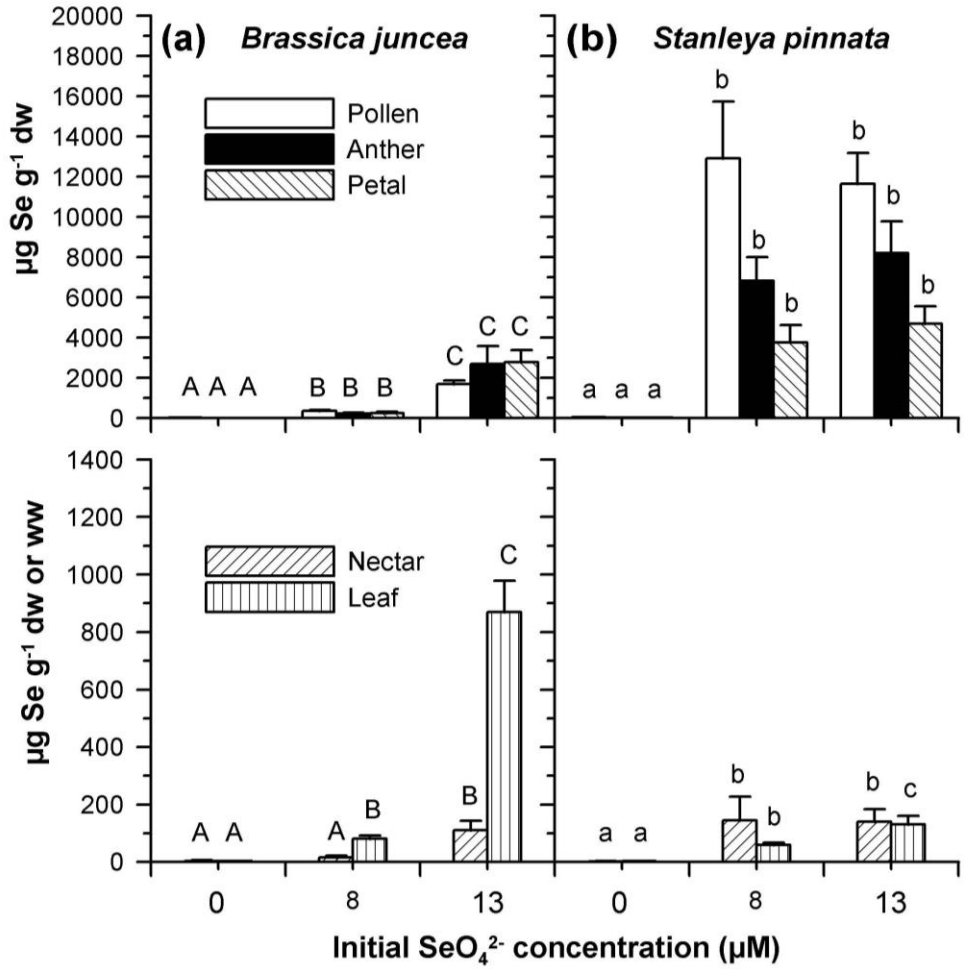


volumes analyzed ranged from 0.004 ± 0.0004 ml for *B. juncea* (n = 19) and 0.04 ± 0.006 ml for *S. pinnata* (n = 33).

Se accumulation in B. juncea and S. pinnata plant tissues. *Brassica juncea* plants irrigated with 8 and 13 µM selenate treatments significantly accumulated Se into pollen, anthers/stigmas, petals, and leaves (ANOVA, $P < 0.0001$ for all) (Figs 1.2). Petal and anther/stigma tissue contained the highest Se concentrations (2800 µg Se g^{-1} dry weight (dw) and 2700 µg Se g^{-1} dw in the 13 µM selenate treatment). Pollen concentrations were also high (1700 µg Se g^{-1} dw in the 13 µM selenate treatment). *B. juncea* nectar irrigated with 8 and 13 µM selenate treatments significantly accumulated Se into nectar (up to 110 µg Se ml^{-1} wet weight (ww), $P < 0.01$) (Fig.1.2). Leaf and nectar concentrations were low relative to the other plant tissues. Block had no significant effect on Se accumulation in any *B. juncea* plant tissues ($P > 0.02$ for all, insignificant with a Bonferroni correction). Seeds from *B. juncea* treated with 8 and 13 µM selenate contained 220 and 940 µg Se g^{-1} dw respectively.

Stanleya pinnata plants irrigated with 8 and 13 µM selenate treatments also significantly accumulated Se into pollen, nectar, anthers/stigmas, petals and leaves ($P < 0.0001$ for all) (Fig. 1.2). Pollen contained the highest concentrations of Se compared to all other tissues (12900 µg Se g^{-1} dry weight in the 8 µM selenate treatment), followed by anther/stigma tissues (8200 µg Se g^{-1} dw in the 8 µM selenate treatment) and petal tissues (4700 µg Se g^{-1} dw in the 13 µM selenate treatment). Nectar contained up to 150 µg Se ml^{-1} wet weight in the 8 µM selenate treatment. Leaf tissues had the lowest Se concentrations (130 µg Se g^{-1} dw in the 13 µM selenate treatment). Seeds from *S.*

Figure 2.2. Se levels in (a) *B. juncea* and (b) *S. pinnata* after selenate treatment in floral tissues (top panels) pollen, anther/stigmas, petals, leaves and nectar (bottom panels). Shown are means ± SE, with letters above means indicate statistically significant differences between groups (α = 0.05) using Tukey's HSD test.



pinnata plants irrigated with 8 and 13 µM selenate contained 3300 and 6000 µg Se g^{-1} dw respectively. Block had no significant effect on Se accumulation in *S. pinnata* plant tissues ($P > 0.44$ for all).

Effects of Se on plant performance in Brassica juncea and Stanleya pinnata. For experiment 1, the 13 µM selenate treatment reduced *B. juncea* floral display width by 31% (ANOVA, $P < 0.0001$) and petal area by 44% ($P < 0.0001$, Table 1.1). However, in both experiments 1 and 2, the 8 µM selenate treatments had no effect on these floral traits ($P > 0.08$ for all), only the highest Se treatment reduced display width and petal area. In Experiment 1, block had a significant effect on display width ($P < 0.006$). Se treatment had no significant effect on anther length ($P > 0.05$).

For experiments 1 and 2 combined, both the 8 and 13 µM selenate treatments reduced total flower number, but it was not significant with a Bonferroni correction. For experiments 1 and 2 combined, the 13 µM selenate treatment reduced seed pod length by almost 50% ($P < 0.0001$, Table 1.1), but the 8 µM selenate treatment actually produced slightly larger seed pods. Se treatments had no effect on nectar per flower, proportion of developed seeds, or total seed weight ($P > 0.20$ for all). For both experiments combined, block had no significant effect on *B. juncea* flower number, nectar per flower, seed pod length, proportion of developed seeds, or total seed weight ($P > 0.10$ for all).

Se treatments had no significant impact on any aspect of *S. pinnata* floral or seed traits ($P > 0.20$ for all, Table 1.1). *Stanleya pinnata* could tolerate these treatment levels and maintain its floral traits and seed production. Block had no significant effect on all *S. pinnata* plant performance responses ($P > 0.10$).

Table 2.1. Effects of 0, 8, and 13 µM initial selenate treatments on *S. pinnata* and *B. juncea* floral traits (display width, anther length and petal area) and plant performance (flower number and seed pod length). Shown are means ± SE. Letters next to the means indicate statistically significant differences between groups ($\alpha = 0.05$) using Tukey's HSD test.

	Floral display width (mm)			Anther length (mm)			Petal area (mm^2)			Flower number			Seed pod length (mm)		
	N	Mean ± SE		N	Mean ± SE		N	Mean ± SE		N	Mean ± SE		N	Mean ± SE	
Experiment 2															
Stanleya pinnata															
0 µM SeO_4^{2-} treatment	6	25.13 ± 3.76	a	6	3.80 ± 0.62	a	6	45.91 ± 10.59	a	7	26 ± 8	a	9	30.57 ± 4.47	a
9 µM SeO_4^{2-} treatment	8	22.92 ± 1.45	a	8	3.64 ± 0.40	a	8	38.76 ± 3.17	a	8	25 ± 6	a	4	27.87 ± 7.40	a
18 µM SeO_4^{2-} treatment	3	23.25 ± 1.75	a	3	3.22 ± 0.47	a	3	37.34 ± 4.57	a	3	60 ± 3	a	3	30.33 ± 7.59	a
Experiment 1															
Brassica juncea															
0 µM SeO_4^{2-} treatment	34	12.84 ± 0.34	a	34	1.86 ± 0.03	a	34	58.66 ± 2.39	a	58	109 ± 27	a	36	22.23 ± 1.15	b
9 µM SeO_4^{2-} treatment	10	12.76 ± 0.83	a	11	2.01 ± 0.14	a	10	57.66 ± 5.70	a	39	53 ± 11	a	22	27.67 ± 1.03	a
18 µM SeO_4^{2-} treatment	11	8.92 ± 0.59	b	11	1.62 ± 0.06	a	11	12.84 ± 0.34	b	11	22 ± 6	b	4	11.41 ± 3.82	c
Experiment 2															
0 µM SeO_4^{2-} treatment	24	11.35 ± 0.31	a	24	1.67 ± 0.06	a	24	43.14 ± 2.05	a						
9 µM SeO_4^{2-} treatment	25	10.75 ± 0.32	a	25	1.68 ± 0.05	a	25	37.13 ± 2.20	a						

Discussion

The objectives of this study were to investigate 1) whether plants that accumulate Se in their leaves will also accumulate Se in their pollen, nectar, and other floral tissues, and 2) to determine the toxic effects of Se uptake in terms of floral and seed traits in a secondary accumulator and hyperaccumulator plant species. Our predictions were that Se would minimally accumulate in the pollen and nectar of both species and that Se would have a stronger negative effect on plant performance and floral traits in the secondary accumulator *B. juncea* compared to the Se hyperaccumulator *S. pinnata.*

Effects of Se treatments on uptake into leaves and floral tissues. *Brassica juncea* accumulated up to 22 and 85% less Se in the nectar and pollen, respectively, compared to the hyperaccumulator plant, *S. pinnata*. *Brassica juncea* plants showed no significant difference in nectar Se concentration between the 0 and 8 µM selenate treatments. However, *S. pinnata* accumulated similar concentrations of Se in nectar at both treatment levels (about 140 µg Se ml^{-1}, Fig. 2B). Se accumulation in *Stanleya pinnata* may have peaked at the 8 µM selenate treatment level, since there were no significant differences between Se concentrations at the 8 and 13 µM treatment levels in any of the floral tissues. Se follows the same sulfate assimilation pathway in both plant types, but *B. juncea* preferentially accumulates S instead of Se (Feist and Parker 2001; Terry et al. 2000). $MgSO_4$ was added to the irrigation tanks once at the beginning of the experiments at a concentration of 0.1 mM, and this was the only significant source of S available to the plants. Sulfur was not completely depleted from the *B. juncea* irrigation tanks at the end of the experiments. However, *B. juncea* contained almost seven times as much Se in its

leaf tissues compared with *S. pinnata*. At the end of the experiments, tanks irrigating *S. pinnata* contained less Se than *B. juncea* tanks in the 13 µM selenate treatment (0.01 µM vs. 8.94 µM), suggesting *S. pinnata* removed more Se from the solution. The hyperaccumulator *S. pinnata* may have mobilized much of its leaf Se into the reproductive tissues or volatilized the Se out of its leaves into the atmosphere. Field studies using *S. pinnata* have demonstrated an increase in Se in reproductive tissues (flowers and seeds) corresponding with a reduction in leaf Se (Galeas et al. 2007). Selenium may be utilized as an elemental defense by protecting fitness linked organs such as flowers and sequestering high concentrations of Se in the floral parts instead of the leaves at later developmental stages.

Secondary accumulators vs. hyperaccumulators: Effects of Se uptake on plant performance. We hypothesized that Se would have a stronger negative effect on plant performance and floral traits in the accumulator *B. juncea* compared to the hyperaccumulator *S. pinnata*. Hyperaccumulators can take up over 4000 mg Se kg^{-1} without showing reduced growth (Shrift 1969), whereas in our study, *B. juncea* suffered toxic effects on plant performance in terms of reduced flower size, flower number, and seed pod length. In addition, plants appeared smaller at the highest Se treatment (personal observation). Several *Brassica* land races showed signs of Se toxicity in terms of reduced dry matter yield and leaf surface area (Bañuelos et al. 1997). Selenium's toxicity is attributed to its similarity to sulfur (S). Se replaces S in amino acids and can change protein folding, causing reduced growth and deformities (Daniels 1996; Lemly 1997). However, Se hyperaccumulators can circumvent these toxic effects by

methylating the selenocysteine for storage or volatilization (Terry et al. 2000). Se accumulators such as the crop plant *B. juncea* take up low to moderate levels of Se into their plant tissues when growing on soils with moderate levels of Se, whereas Se hyperaccumulators such as *S. pinnata* can take up high levels of Se into their plant tissues even when growing on soils with low levels of Se (Terry et al. 2000). Hyperaccumulators such as *S. pinnata* are thought to have evolved on seleniferous soils, and can metabolize and biotransform selenate into non-protein selenoamino acids (such as Se-methyl-selenocysteine), which secondary accumulators cannot (Brown and Shrift 1981; Brown and Shrift 1982; Terry et al. 2000). Methylation of the selenoamino acids may protect the hyperaccumulators such as *S. pinnata*, but not secondary accumulators such as *B. juncea*, from the toxic effects of these compounds.

A large portion of the Se was depleted from the tanks at the beginning of the flowering period for both species (Fig. 1A and B). In particular, *Stanleya pinnata* began flowering 45 days after the Se treatments were started. Within forty-one days after treatments were added, Se concentrations in the irrigation tank water dropped to 1.08 µM Se (8 µM initial selenate treatment), and 1.74 µM Se (13 µM initial selenate treatment). A recent study by Galeas et al. (2007) found that Se mobilizes to different plant tissues in Se hyperaccumulator plants. In the early part of the growing season, hyperaccumulators transport Se to the leaf tissues, whereas later in the season, Se is moved from leaf tissues into reproductive tissues such as flowers and seeds. In our study, Se may have been mobilized within the plant from leaf tissues into the flowers, although leaves were collected for Se testing only at the end of the experiment. In hyperaccumulators, Se

mobilization to the fitness-linked floral tissues such as flowers and seeds may provide support for optimal defense theory (McKey 1979) and the elemental defense hypothesis (Boyd 1998; Boyd 2007). However, in order to link the adaptive significance of Se accumulation in terms of increased fitness and as a defense of reproductive tissues, additional studies will be required. Also, leaf and floral tissues would have to be collected at several timepoints throughout the experiment to determine whether Se was being mobilized within the plant.

Although Se levels were high in the floral tissues of our greenhouse study, Se concentrations in the leaves of *B. juncea* and *S. pinnata* have varied across field studies. Galeas et al. (2007) found *S. pinnata* leaf concentrations of 500 to 2000 mg Se kg^{-1} dw and flower concentrations of 1800 mg kg^{-1} dw in the field throughout a 7 month growing season. In addition, a study by Bañuelos et al. (2007) found the leaves of transgenic *B. juncea* grown for phytoremediation of soil contaminated with 4 mg Se kg^{-1} contained only about 30 to 50 µg Se g^{-1} dw in the field. In our greenhouse study, *B. juncea* accumulated Se concentrations in the pollen and nectar that could be potentially toxic to pollinators, but Se concentrations of leaves in field studies (such as Bañuelos et al. 2007) suggest flower concentrations may be lower. The duration and soil concentration of Se exposure as well as other environmental factors may play important roles in determining how much Se is accumulated into the leaf and floral tissues. Although the leaves in our study had higher *B. juncea* concentrations and lower *S. pinnata* concentrations compared to the studies mentioned above, our experiments are relevant because they focused on a 3 month period which captured the peak flowering period of both species when irrigated

with ecologically relevant Se concentrations (up to 1.4 mg Se l^{-1}). Our study provides a snapshot of the Se concentrations during the flowering period that could be available to pollinators visiting flowers on Se-accumulating plants.

Several studies have found evidence for plant-produced (secondary chemical) defenses in floral tissues such as petals, nectar (Adler 2000; Detzel and Wink 1993; Gegear et al. 2007; Kessler and Baldwin 2007; McCall and Karban 2006) and even pollen (Praz et al. 2008). Some hyperaccumulator plant species also accumulate elevated levels of metals and metalloids in their flowers and fruits (Freeman et al. 2006; Jaffre et al. 1976; Reeves et al. 1981), possibly as an elemental defense. Certain insect species cannot detect and avoid Se (Trumble et al. 1998; Vickerman et al. 2002), but there are no studies to date examining the effects of Se-containing plant tissues on insect pollinator visitation in terms of deterrence. If insect pollinators cannot detect and avoid toxic compounds in the floral tissues they are foraging upon and collecting for their progeny, they may suffer similar adverse effects such as mortality and reduced development as has been seen in other insect guilds (Trumble et al. 1998; Vickerman et al. 2002; Hanson et al. 2003; Hanson et al. 2004; Freeman et al. 2007; Sorensen et al. 2009). Alternatively, Se is a micronutrient that is essential to many animals when ingested in low quantities (Burau 1985) and may be a beneficial antioxidant to pollinators that feed upon Se-containing floral tissues.

Brassica juncea and *S. pinnata* have gained interest as phytoremediators of Se-contaminated soils (Parker et al. 2003; Pilon-Smits and Freeman 2006). In particular, *B. juncea* has been genetically modified to increase its ability to accumulate and volatilize

Se (Bañuelos et al. 2007; Pilon-Smits and LeDuc 2009). In our study, *S. pinnata* had low concentrations of Se in the leaves, suggesting this species may volatilize Se as well. Phytoremediation using these species may expose pollinators to Se-containing tissues, unless plants are harvested before flowering. Transgenic plants are harvested when 25% of the plants flower (as mandated by the USDA-Animal and Plant Health Inspection Service) and a similar approach to managing non-transgenic phytoremediators may protect beneficial pollinators from exposure to potentially toxic floral tissues. This study provides crucial information about where some of the highest concentrations of Se are found in two phytoremediators, and may shed light on the potential risks pollinators may face when foraging upon these accumulating plants.

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CHAPTER 3

Selenium toxicity to honey bee (*Apis mellifera* L.) pollinators: Effects on behaviors and survival

Abstract

We know very little about how soil-borne pollutants such as selenium (Se) can impact pollinators, even though Se has contaminated soils and plants in areas where insect pollination can be critical to the functioning of both agricultural and natural ecosystems. Se can be biotransferred throughout the food web, but few studies have examined its effects on the insects that feed on Se-accumulating plants, particularly pollinators. In laboratory bioassays, we used proboscis extension reflex (PER) and taste perception to determine if the presence of Se affected the gustatory response of honey bee (*Apis mellifera* L., Hymenoptera: Apidae) foragers. Antennae and proboscises were stimulated with both organic (selenomethionine) and inorganic (selenate) forms of Se that commonly occur in Se-accumulating plants. Methionine was also tested. Each compound was dissolved in 1 M sucrose at 5 concentrations, with sucrose alone as a control. Antennal stimulation with selenomethionine and methionine reduced PER at higher concentrations. Selenate did not reduce gustatory behaviors. Two hours after being fed the treatments bees were tested for sucrose response threshold. Bees fed selenate responded less to sucrose stimulation. Mortality was higher in bees chronically dosed with selenate compared with a single dose. Selenomethionine did not increase mortality except at the highest concentration. Methionine did not significantly impact survival. Our study has shown that bees fed selenate were less responsive to sucrose, which may lead to a reduction in incoming floral resources needed to support coworkers and larvae in the field. If honey bees forage on nectar containing Se (particularly selenate), reductions in population numbers may occur due to direct toxicity. Given that

honey bees are willing to consume food resources containing Se and may not avoid Se compounds in the plant tissues on which they are foraging, they may suffer similar adverse effects as seen in other insect guilds.

Introduction

Over 60% of the world's crop species are animal pollinated, with honey bees constituting a large component (Kearns et al. 1998; Klein et al. 2007). The value of the honey bee (*Apis mellifera* L., Hymenoptera: Apidae) as managed pollination services in the United States is estimated to be up to 14 billion dollars per year (Delaplane and Mayer 2000; Morse and Calderone 2002; Southwick and Southwick 1992). Declines in honey bee populations due to pesticide poisoning have been a focus of recent research (Desneux et al. 2007), but the role of soil-borne pollutants on honey bee survival has not been examined. Few studies have focused on the toxicological effects of metal or metalloid pollutants on bee behaviors and survival.

Honey bees forage over very large areas and bring plant materials (nectar, pollen and propolis) back to their hives, and thus may collect significant amounts of toxic contaminants. Plant pollinators such as honey bees and their honey products have been investigated as potential bioindicators of metal and metalloid pollutants (Celli and Maccagnani 2003; Kevan 1999). Varying amounts of contaminants that are toxic to insects have been found in honey, propolis, and pollen from honey bee hives located in close proximity to polluted sites around the world (Achudume and Nwafor 2010; Bibi et al. 2008; Bogdanov 2006; Bromenshenk et al. 1985; Jones 1987; Leita et al. 1996;

Yarsan et al. 2007). With regards to the soil-borne pollutant, selenium (Se), pollen collected by bees from plants growing in fly ash from coal-burning electrical power plants contained 14 mg Se kg^{-1} (De Jong et al. 1977). In an urban, uncontaminated area of Poland, honey bee foragers collected from stationary hives contained 7.03 mg Se kg^{-1} (Roman 2010). Honey collected from different regions of Turkey contained 38 to 113 µg kg^{-1} (Tuzen et al. 2007). Honey collected from hives located in seleniferous areas of Colorado contained up to 0.73 mg Se kg^{-1} (Quinn et al. 2011). These findings raise the following issues: 1) Does nectar and pollen from plants growing in high metal or metalloid soils contain levels of these elements that, when collected, are toxic to brood or workers? 2) What is the potential for adverse effects on pollinator health of widespread contamination of selenium? Although there has been some interest in using honey bees and their products as bioindicators of pollution, few studies have examined the effects of foraged plant tissues containing soil-borne pollutants such as Se on pollinator health.

Selenium (Se) is a metalloid that occurs naturally in certain alkaline soils from shale deposits of prehistoric inland seas (Emmons et al. 1896). Agricultural water drainage dissolves Se from these naturally seleniferous soils and has caused the buildup of selenate (SeO_4^{2-}), the predominant and bioavailable form of Se. One of the worst cases of Se pollution occurred at the Kesterson Reservoir in the San Joaquin Valley (Merced County, California, USA), a major drainage site for many agricultural regions of California (Wu 2004). The EPA maximum contaminant level (MCL) of 0.05 mg L^{-1} for Se in drinking water was based on evidence from this well-documented case of Se poisoning as well as 96 hour acute and chronic toxicity testing of aquatic animals. However, the MCL does

not consider bioaccumulation or biomagnification of Se. Studies have demonstrated the biomagnification of Se throughout the food web (Ohlendorf 2003), but few studies have examined the effects of plants and the insects that feed on them in Se-contaminated sites. However, in several studies examining Se levels in arthropods collected from accumulating plants, various floral visitors contained up to 75 µg Se g^{-1} dry weight (dw) (Glaleas et al. 2008), honey bees contained 14.8 µg Se g^{-1} dw and bumble bees contained 251 µg Se g^{-1} dw (Quinn et al. 2011). Thus, there is the possibility for biotransfer of Se from plant to pollinator.

Despite its toxic properties, selenium is also a micronutrient that is essential to many organisms, including mammals, fish, and bacteria (Burau 1985), but slightly higher levels can cause toxic effects. Selenium's toxicity is attributed to its similarity to sulfur. Se replaces sulfur in amino acids such as cysteine and methionine and can change protein folding, disrupt cell metabolism (Daniels 1996; Lemly 1997), and alter the activity of enzymes if the Se replaces S near the active site (Schrauzer 2000). Inorganic forms of Se can also cause oxidative stress (Spallholz 1997) and DNA damage (Coombs and Gray 1998). Although Se is a micronutrient for many living organisms, a surplus of the element can cause developmental deformities and toxicity.

There is good evidence that Se accumulation can have negative effects on plant growth, insect herbivores, their predators and parasites, and the detritivores that feed on decaying plant and animal tissues (Vickerman and Trumble 1999; Jensen and Trumble 2003), yet we know very little about how pollutants such as Se impact pollinators. Herbivores fed plant tissues containing high levels of metals, metalloids (such as Se), or

other accumulated elements have shown reduced development and survival (Boyd 2007), and several studies have shown some insect species can not detect detrimental levels of Se (Trumble et al. 1998; Vickerman et al. 2002), but there are no studies to date examining the effects of Se-containing floral tissues on insect pollinator behaviors and survival.

Our overall objective was to determine whether the two main forms of Se commonly found in accumulating plants, selenate and selenomethionine (de Souza et al. 1998; Kahakachchi et al. 2004; Pedrero et al. 2006) can have sublethal or lethal effects on the honey bee (*Apis mellifera* L., Hymenoptera: Apidae). Our first objective examined whether the presence of Se affected honey bee gustatory behavior via two different chemosensory organs (antenna or proboscis). Our second objective was to examine whether Se has sublethal effects on the honey bee's feeding behaviors, particularly if it can alter the bee's responsiveness to sucrose. Our third objective tested whether increasing concentrations of Se can cause mortality when administered as a single or chronic dose to honey bee foragers. If pollinators cannot detect and avoid Se compounds in the pollen and nectar on which they are foraging and collecting for their progeny, they may suffer similar adverse effects as seen in other insect guilds.

Results

Antennal response assays. The proboscis extension reflex (PER) involves stimulating a honey bee's antennae with a sucrose solution. The bee will then reflexively

extend its proboscis in response to the stimulation. We examined whether honey bees exhibited a reduced PER response to sucrose solutions that contained selenate, selenomethionine or methionine over a range of concentrations spanning five orders of magnitude, from 0.6 to 6000 µg ml^{-1}. Honey bee foragers' PER responses to antennal stimulation by selenate were not significantly different than the responses to 1 M sucrose at any of the 5 concentrations (logistic regression, $X_6^2 < 3.43$, $p > 0.06$ for all; Figure 3.1). Responses to selenomethionine plus sucrose were significantly lower than the 1 M sucrose control at 60 µg ml^{-1} ($X_1^2 = 11.80$, $p < 0.001$), 600 µg ml^{-1} ($X_1^2 = 22.40$, $p < 0.0001$) and 6000 µg ml^{-1} ($X_1^2 = 46.51$, $p < 0.0001$; Figure 3.2). For methionine, responses were only significantly lower than the 1 M sucrose control at the 60 µg ml^{-1} ($X_1^2 = 4.19$, $p < 0.05$) and 6000 µg ml^{-1} treatments ($X_1^2 = 8.15$, $p < 0.001$; Figure 3.3). PER responses to antennal stimulation with solutions containing 1 M sucrose plus selenate (Figure 1) or methionine (Figure 3.3) were significantly higher than responses to water at all 5 concentrations ($X_6^2 > 6.75$, $p < 0.01$ for all). Responses to antennal stimulation by sucrose plus selenomethionine were significantly higher than responses to water at the 4 lowest concentrations ($X_5^2 > 11.42$, $p < 0.001$ for all; Figure 3.2). However, at the 6000 µg ml^{-1} concentration, the response (22%) was not significantly different from that for water (17%, $X_1^2 = 0.02$, $p = 0.88$).

Figure 3.1. Honey bee behavioral responses to antennal stimulation with selenate. Honey bees were stimulated with 1 M sucrose, water, and selenate in 1 M sucrose (N = 83). Asterisks indicate significance of *$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$ (Logistic regression with multiple comparisons) between 1 M sucrose and treatment lines.

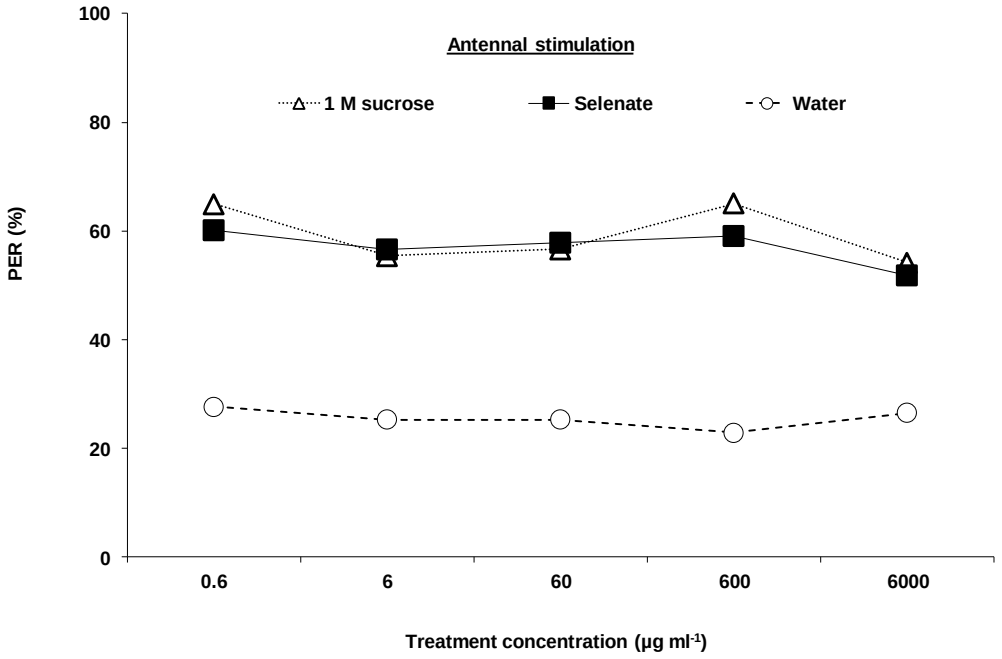


Figure 3.2. Honey bee behavioral responses to antennal stimulation with selenomethionine. Honey bees were stimulated with 1 M sucrose, water, and selenomethionine in 1 M sucrose (N = 94). Asterisks indicate significance of *P < 0.05, **P < 0.001, ***P < 0.0001 (Logistic regression with multiple comparisons) between 1 M sucrose and treatment lines.

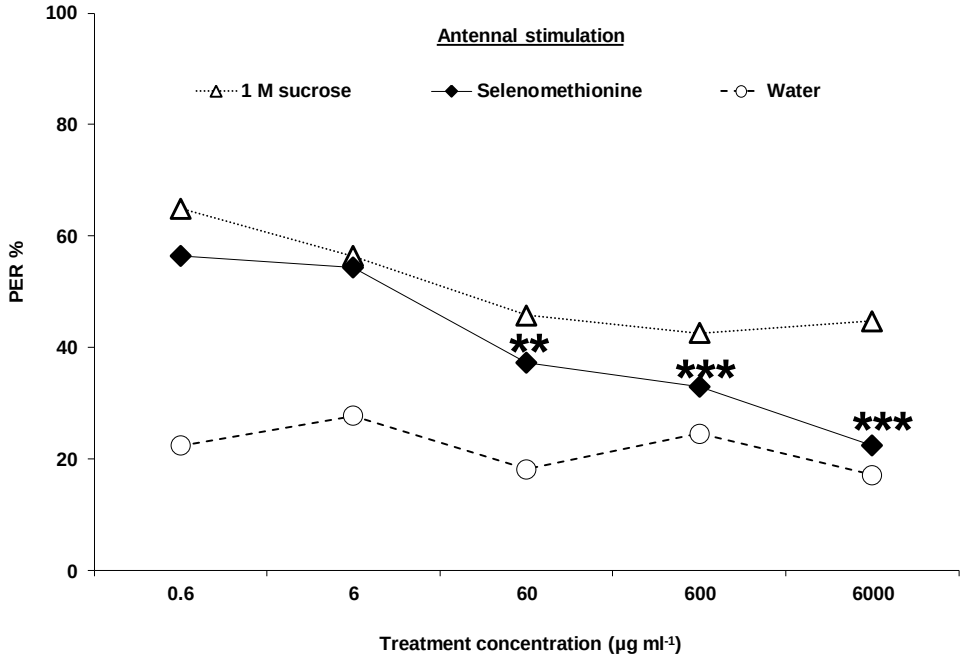
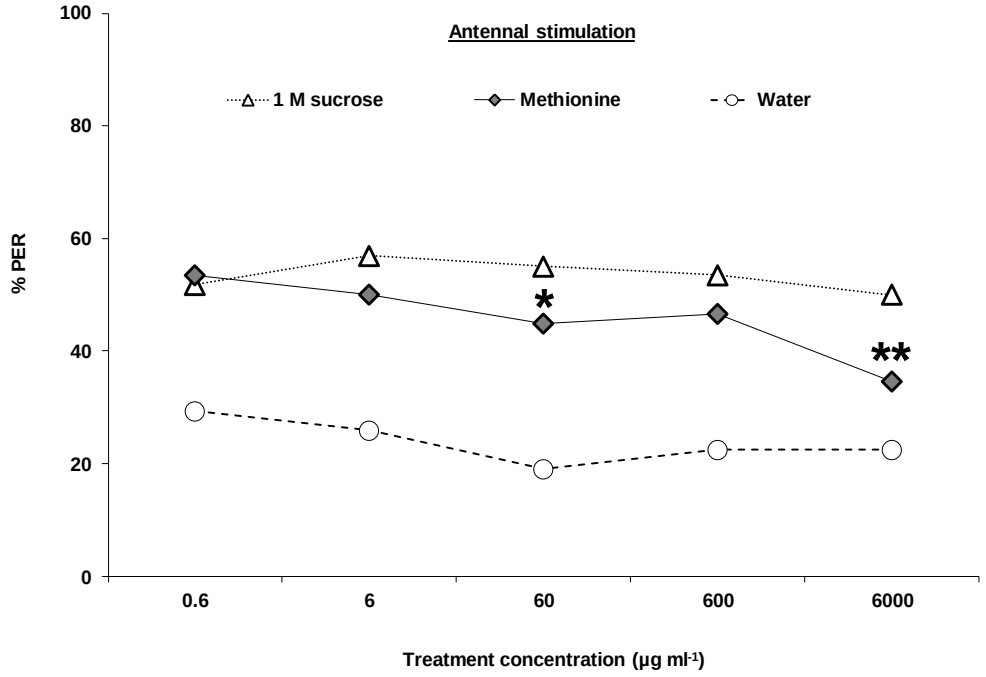


Figure 3.3. Honey bee behavioral responses to antennal stimulation with methionine. Honey bees were stimulated with 1 M sucrose, water, and methionine in 1 M sucrose (N = 58). Asterisks indicate significance of *$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$ (Logistic regression with multiple comparisons) between 1 M sucrose and treatment lines.



Proboscis response assays. As well as receptors on the antennae, honeybees also have gustatory receptors on the proboscis. We examined if the presence of selenate, selenomethionine or methionine, at the same 5 concentrations, affected the willingness of bees to actually consume 1 M sucrose solutions. Bees that were given sucrose solutions containing selenate showed no significant differences in consumption of the droplet between 1 M sucrose and any of the 5 selenate concentrations (logistic regression, $X_5^2 < 1.45$, $p > 0.23$ for all; Figure 3.4). Proboscis stimulation with the water treatment elicited a significantly lower response than 1 M sucrose or any of the 5 selenate concentrations ($X_6^2 > 13.99$, $p < 0.002$ for all; Figure 3.4). There were no significance differences in the percent of bees consuming the droplet between 1 M sucrose and any of the selenomethionine ($X_5^2 < 0.79$, $p > 0.37$ for all; Figure 3.5) or methionine ($X_5^2 < 0.76$, $p > 0.38$ for all concentrations; Figure 3.6) treatments. Consumption responses to proboscis stimulation with water were significantly lower than responses to 1 M sucrose and selenomethionine ($X_6^2 > 13.99$, $p < 0.002$ for all concentrations) or methionine ($X_6^2 > 14.46$, $p < 0.0001$ for all concentrations).

Figure 3.4. Honey bee behavioral responses to proboscis stimulation with selenate. Honey bees' proboscises were stimulated with 1 M sucrose, water, and selenate in 1 M sucrose (N = 23-30).

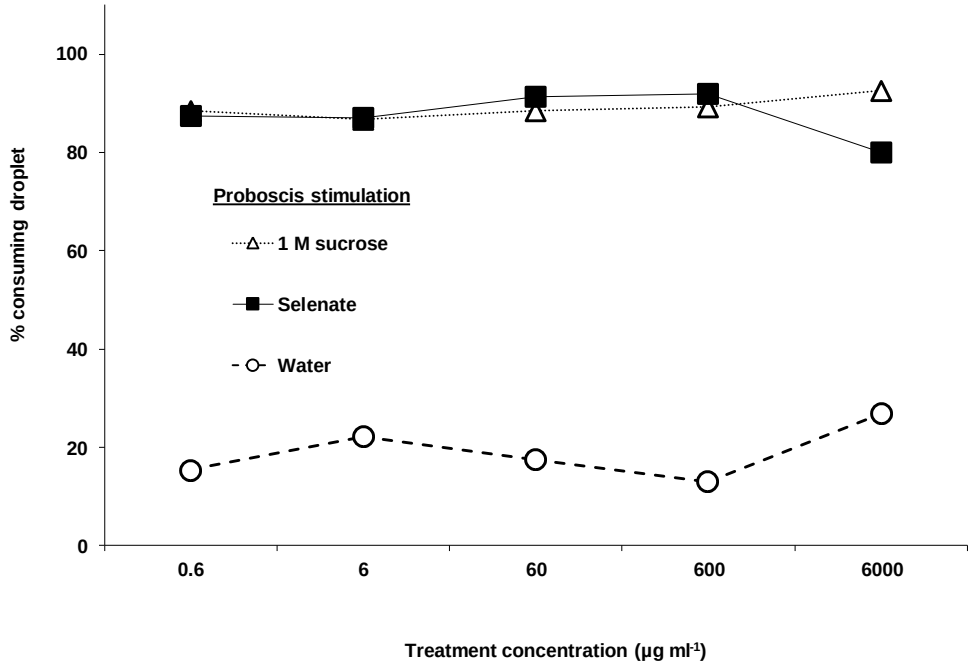


Figure 3.5. Honey bee behavioral responses to proboscis stimulation with selenomethionine. Honey bees' proboscises were stimulated with 1 M sucrose, water, and selenomethionine in 1 M sucrose (N = 22-31).

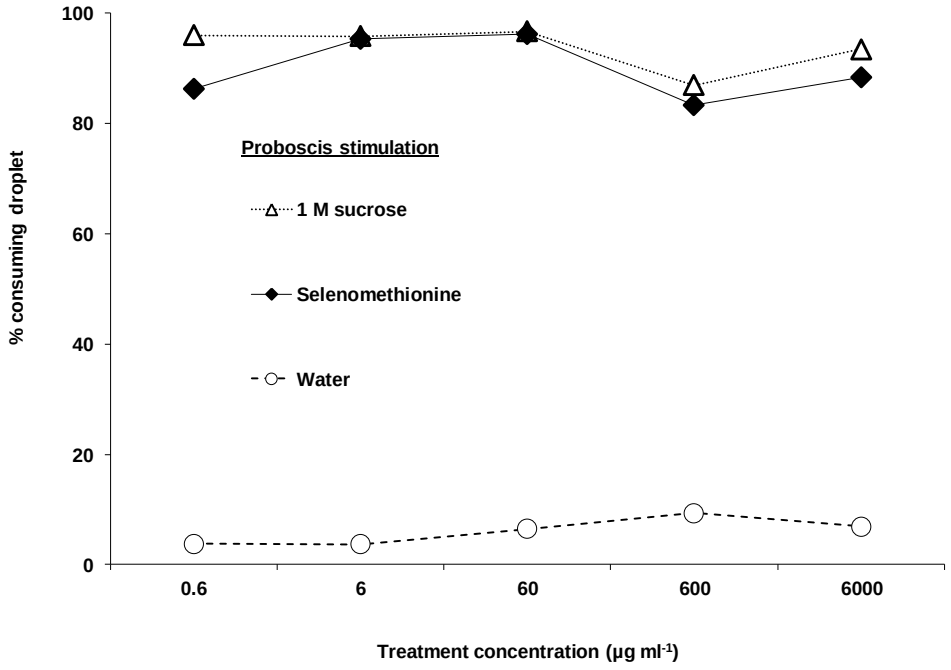
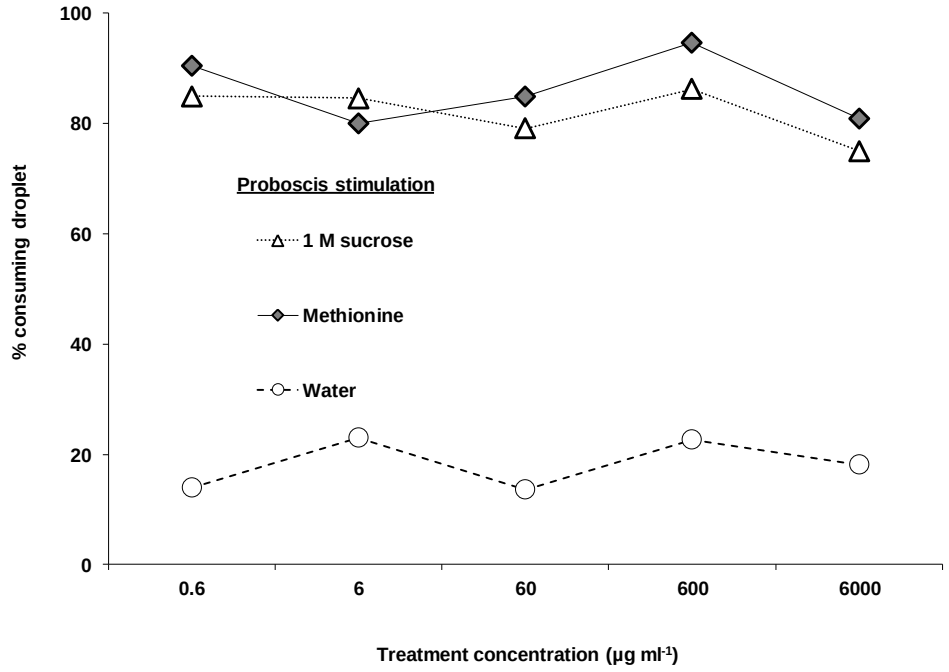


Figure 3.6. Honey bee behavioral responses to proboscis stimulation with methionine. Honey bees' proboscises were stimulated with 1 M sucrose, water, and methionine in 1 M sucrose (N = 19-26).



Sucrose response threshold assays. The effects of selenate, selenomethionine, and methionine consumption on the responsiveness of honey bee foragers to sugars were determined using sucrose response thresholds (SRT), or the lowest sucrose concentration that elicits a PER response. Bees from all selenate treatment groups showed a dose-dependent change in PER to increasing concentrations of sucrose (logistic regression, $X_6^2 = 58.09$, $p < 0.0001$, Table 3.1). The sucrose response threshold occurred between 3 and 10%, except for the group of bees fed 60 µg selenate ml^{-1}, whose response to sucrose never significantly differed from that of water. Selenate feeding treatment had a significant effect on proboscis extension response ($X_5^2 = 13.34$, $p < 0.02$), resulting in a decrease in overall average PER for all selenate feeding treatments (Table 3.1). The percentage of bees responding with proboscis extension dropped from 48% in bees fed the control (1 M sucrose) to as low as 17% in the 6000 µg ml^{-1} selenate-fed bees. However, there was no significant interaction between the sucrose antennal treatment and the selenate feeding treatment ($X_{30}^2 = 37.30$, $p = 0.17$), indicating that selenate feeding did not alter the sucrose response threshold of 3 to 10% (Table 3.1).

All selenomethionine treatment groups showed a dose-dependent change in PER to increasing concentrations of sucrose ($X_6^2 = 40.08$, $p < 0.0001$). The sucrose response threshold occurred between 3 and 10%, except for the 0.6 and 6 µg ml^{-1} treatment groups whose sucrose response thresholds were as high as 30% (Table 3.1). Selenomethionine feeding treatment did not have a significant effect on sucrose response threshold ($X_5^2 = 4.41$, $p = 0.49$). In addition, the interaction of selenomethionine feeding treatment and the sucrose antennal treatment was not significant ($X_{30}^2 = 41.15$, $p = 0.09$).

All methionine treatment groups showed a dose-dependent change in PER to increasing concentrations of sucrose ($X_6^2 = 57.93$, $p < 0.0001$). The sucrose response threshold occurred between 1 and 10% (Table 3.1). Methionine feeding treatment did not have a significant effect on sucrose response threshold ($X_5^2 = 7.98$, $p = 0.16$). The interaction of methionine feeding treatment and sucrose antennal treatment was also not significant ($X_{30}^2 = 24.94$, $p = 0.73$).

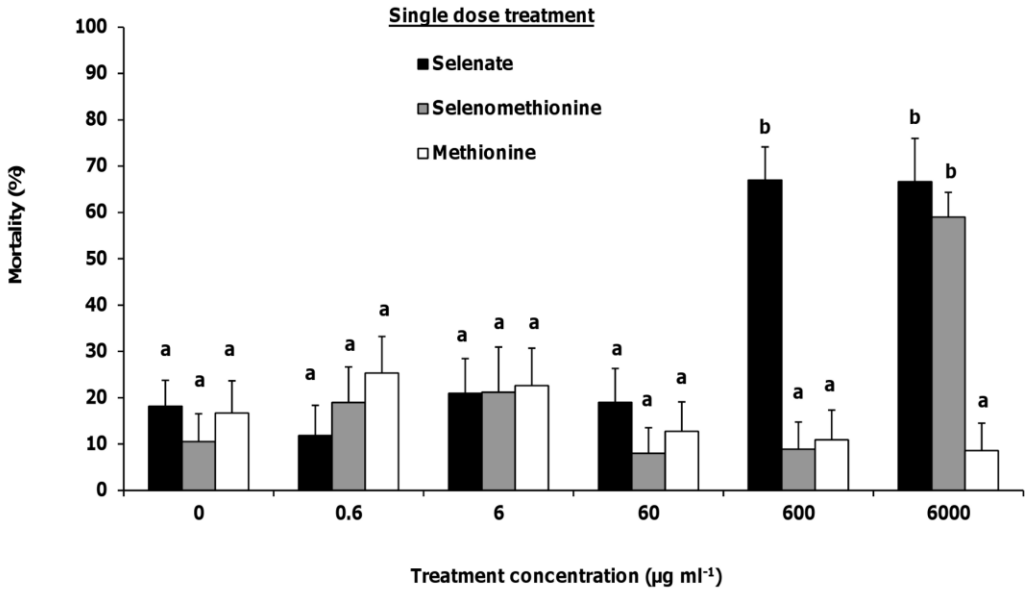
Table 3.1. Honey bee sucrose response thresholds after selenium feeding treatments. Percentages of bees responding to antennal stimulation with six concentrations of sucrose after treatment with selenate, selenomethionine, or methionine. Asterisks indicate significance of *$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$ (Logistic regression with multiple comparisons) between antennal stimulation with water and increasing sucrose concentrations.

			% Sucrose concentration (antennal treatment)							
	Feeding treatment concentration (µg ml^{-1})	N	0.1	0.3	1	3	10	30	Average % of non-responsive bees	Average % response to water
Selenate	0	36	22 ± 7	28 ± 8	31 ± 8	47 ± 8	78 ± 7 ***	75 ± 7 ***	52	26 ± 3
	0.6	34	18 ± 7	21 ± 7	24 ± 7	29 ± 8 *	35 ± 8 **	47 ± 9 **	71	14 ± 2
	6	31	23 ± 8	23 ± 8	26 ± 8	29 ± 8 *	35 ± 9 **	48 ± 9 **	69	15 ± 3
	60	28	29 ± 9	32 ± 9	32 ± 9	39 ± 9	46 ± 10	46 ± 10	63	35 ± 4
	600	31	13 ± 6	13 ± 6	16 ± 7	16 ± 7	35 ± 9 **	39 ± 9 **	78	10 ± 2
	6000	32	13 ± 6	9 ± 5	9 ± 5	16 ± 7	25 ± 8 *	31 ± 8 **	83	8 ± 2
Selenomethionine	0	19	16 ± 9	16 ± 9	16 ± 9	47 ± 12 *	42 ± 12 *	79 ± 10 ***	64	11 ± 3
	0.6	20	5 ± 5	10 ± 7	10 ± 7	15 ± 8	25 ± 10	40 ± 11 *	82	9 ± 3
	6	18	22 ± 10	33 ± 11	33 ± 11	28 ± 11	44 ± 12	50 ± 12 *	65	23 ± 4
	60	19	16 ± 9	11 ± 7	11 ± 7	32 ± 11 *	26 ± 10	58 ± 12 **	75	17 ± 4
	600	20	30 ± 11	35 ± 11	20 ± 9	30 ± 11	50 ± 11 **	40 ± 11	66	19 ± 4
	6000	20	30 ± 11	20 ± 9	15 ± 8	20 ± 9	35 ± 11 *	35 ± 11 *	74	15 ± 3
Methionine	0	20	20 ± 9	15 ± 8	10 ± 7	50 ± 11 *	50 ± 11 **	75 ± 10 ***	63	13 ± 3
	0.6	20	5 ± 5	5 ± 5	5 ± 5	25 ± 10	30 ± 11 *	55 ± 11 **	79	8 ± 3
	6	20	10 ± 7	15 ± 8	20 ± 9	30 ± 11	45 ± 11 **	50 ± 11 **	72	8 ± 3
	60	20	5 ± 5	5 ± 5	5 ± 5	15 ± 8	25 ± 10 *	55 ± 11 **	82	7 ± 2
	600	20	30 ± 11	25 ± 11	30 ± 11 *	40 ± 11 *	45 ± 11 **	60 ± 11 **	62	16 ± 3
	6000	19	5 ± 5	16 ± 9	21 ± 10	37 ± 11 *	37 ± 11 *	53 ± 12 **	72	11 ± 3

Total consumption and single dose mortality. Honey bee foragers were fed a single dose of Se or sulfur as selenate, selenomethionine, or methionine plus sucrose at 5 concentrations, and then mortality was scored for 5 days. Treatments were compared to bees fed 1 M sucrose as the control. There was no significant difference in total consumption of selenate (ANOVA, $F_{5,232} = 0.79$, $p = 0.56$), selenomethionine ($F_{5,108} = 1.26$, $p = 0.29$) or methionine ($F_{5,129} = 2.19$, $p = 0.06$) at the 5 concentrations. Bees ingested an overall average of 21.94 ± 0.47 µl of selenate in 1 M sucrose (N = 18-21), 21.83 ± 0.97 µl of selenomethionine in 1 M sucrose (N = 18-20), and 20.51 ± 0.63 µl of methionine in 1 M sucrose (N = 21-24) across all concentrations.

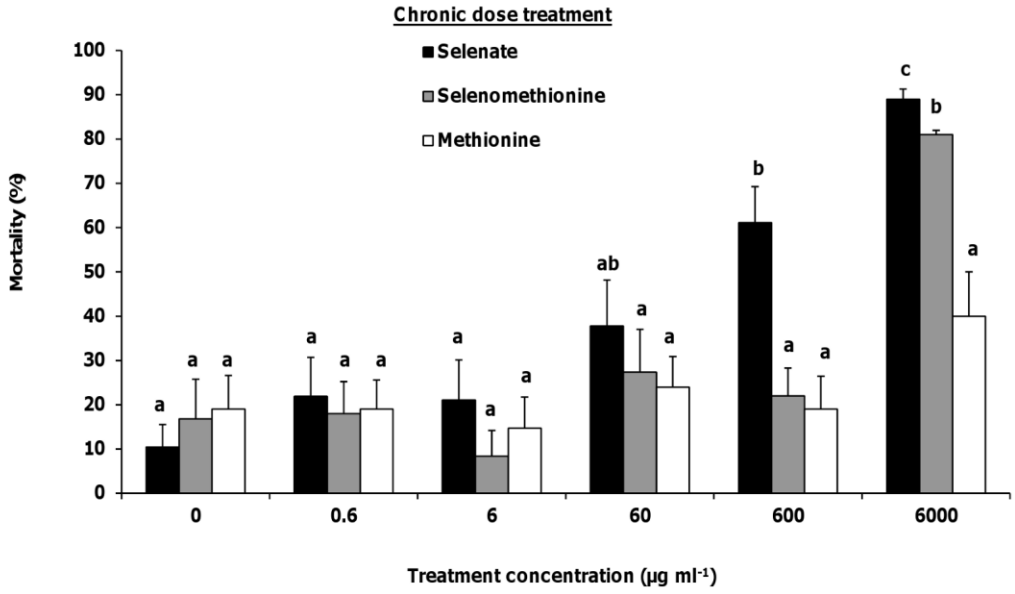
Single dosage with selenate significantly increased final percent mortality in honey bee foragers at the 600 µg ml^{-1} (Kruskal-Wallis, $X_1^2 = 29.83$, $p < 0.0001$) and 6000 µg ml^{-1} ($X_1^2 = 37.31$, $p < 0.0001$) treatment levels compared to 1 M sucrose (Figure 3.7). Mortality reached as high as 67% at the 6000 µg ml^{-1} selenate concentration. Selenomethionine consumption also had a significant effect on mortality (Figure 3.7), and increased mortality to 59% at the highest concentration ($X_1^2 = 24.22$, $p < 0.0001$). Methionine consumption had no significant effect on mortality at all concentrations (Figure 3.7). Overall mortality across all methionine concentrations ranged from 9 to 23%.

Figure 3.7. Honey bee forager mortality from a single dose of selenium. Percentages of honey bee mortality after a single dosage of selenate (N = 20-22), selenomethionine (N = 17-20) or methionine (N = 21-24) in 1 M sucrose at 6 concentrations. Control bees received 0 µg ml^{-1}, or 1 M sucrose only. Mortality was recorded for 5 subsequent days. Final percent mortality is shown. Letters above the means indicate statistically significant differences between groups (α = 0.05) using the Mann-Whitney U test. Values are means ± standard error (SE).



Chronic dose mortality. Honey bee foragers were fed Se or sulfur as selenate, selenomethionine, or methionine plus sucrose at 5 concentrations for 5 days, and then mortality was scored on each day. Treatments were compared to bees fed 1 M sucrose as the control. Chronic dosing with selenate significantly increased mortality (Figure 3.8) at the 60 µg ml^{-1} ($X_1^2 = 5.40$, $p < 0.02$), 600 µg ml^{-1} ($X_1^2 = 17.81$, $p < 0.0001$) and 6000 µg ml^{-1} ($X_1^2 = 32.84$, $p < 0.0001$) concentrations compared with bees fed 1 M sucrose. Selenate consumption for 5 days increased mortality to as high as 89% in the 6000 µg ml^{-1} concentration. Similar to single dose mortality, chronic doses of selenomethionine increased mortality only at the highest concentration ($X_1^2 = 24.70$, $p < 0.0001$; Figure 8), although more bees died with a chronic dose (81%) compared to the single dose (59%). Chronic dosing with methionine at all concentrations did not have a significant effect on mortality ($X_1^2 < 3.19$, $p > 0.07$), although mortality was higher for chronic dosing compared to single dosing in the 6000 µg ml^{-1} treatment group (13% vs. 40%).

Figure 3.8. Honey bee forager mortality from chronic doses of selenium. Percentages of honey bee mortality after chronic dosage of selenate (N = 18-21), selenomethionine (N = 19-20) or methionine (N = 19-20) in 1 M sucrose at 6 concentrations. Control bees received 0 µg ml^{-1}, or 1 M sucrose only. Bees were fed 20 µl of each treatment for 6 days. Mortality was recorded for 5 subsequent days after the first dosage. Final percent mortality is shown. Letters above the means indicate statistically significant differences between groups ($\alpha = 0.05$) using the Mann-Whitney U test. Values are means ± standard error (SE).



Discussion

Our first objective examined whether the presence of Se affected the gustatory behaviors of honey bees via two different chemosensory organs (antenna or proboscis). Honey bee sensillae used to taste sugars and salts have been found on mouthparts associated with the proboscis (Whitehead 1978; Whitehead and Larsen 1976; Wright et al. 2010). Taste sensilla on the antennae respond to sugars (Haupt 2004) and salt solutions (de Brito Sanchez et al. 2005). Se deters feeding in certain insect (Hanson et al. 2003; Hanson et al. 2004; Vickerman and Trumble 1999), and mammalian (Franke and Potter 1936; Quinn et al. 2008) herbivores, and may reduce feeding behaviors such as PER in honey bees. However, some insects cannot detect Se and will ingest it in laboratory feeding studies (Vickerman et al. 2002; Jensen et al. 2006). In our study, the presence of selenate in sucrose did not reduce the responses of honey bees to stimulation of the antennae or proboscis. However, antennal stimulation with selenomethionine significantly reduced PER at 600 µg ml^{-1} and higher, indicating that there was some decrease in response. Antennal stimulation with methionine also reduced PER at higher concentrations, suggesting that deterrence may be due to the methionine portion of the selenomethionine molecule. Methionine causes behavioral deterrence in the leaf-chewing herbivores *Spodoptera litura* F. (Lepidoptera: Noctuidae) (Hirao and Arai 1991), *Grammia geneura* Strecker (Lepidoptera: Arctiidae) (Bernays and Chapman 2001) and *Mamestra brassicae* L. (Lepidoptera: Noctuidae) (Wieczorek 1976) under experimental conditions. Selenomethionine and methionine may interfere with the honey bee's perception of the sucrose reward when antennae are stimulated, thus reducing PER.

In a study by de Brito Sanchez et al. (2005), antennal stimulation with solutions containing sucrose and the bitter substance quinine inhibited PER and reduced electrophysiological responses to sucrose in the honey bee. Alternatively, honey bees may respond less to the amino acid depending on the amount of amino acid already in their hemolymph prior to capture (Kim and Smith 2000). Honey bees that have recently fed on a protein rich plant source may be less responsive to it in subsequent feeding stimulations.

In the proboscis response assays, the bees could choose to drink a small droplet of Se or sulfur containing sucrose solution. There was no significant difference between consumption of the 1 M sucrose alone (control) and selenomethionine or methionine in 1 M sucrose treatment droplets, indicating that the decrease in response was mediated by the antennae and not the proboscis. Honey bee foragers prefer to feed upon sugar solutions containing certain amino acids (Alm and Simpson 1990; Kim and Smith 2000; Inouye and Waller 1984). Methionine is an essential amino acid for honey bee development (de Groot 1953), although higher concentrations in nectar may act as a deterrent. In our study, deterrence was specific to antennal stimulation, suggesting that receptors detecting either methionine or selenomethionine may not be present on the proboscis.

Our second objective examined the effects of Se ingestion on the sucrose responsiveness of honey bees. Foraging honey bees evaluate floral resources based on the sugar concentrations in nectar, and adjust their foraging and recruitment behaviors accordingly (Page et al. 1998). The sucrose response threshold is an important

benchmark for bees to recruit to a floral resource. In our study, the sucrose response threshold, or the point when the probability of responding to sucrose was significantly greater than water, was not significantly altered by feeding honey bees with Se compounds or methionine prior to testing for sucrose responsiveness. However, selenate did significantly reduce the overall responsiveness of the foragers to sucrose as fewer bees fed selenate responded to any sucrose concentration compared to bees fed 1 M sucrose alone. Selenate may lower the honey bee's overall level of responsiveness and arousal, reducing its ability to evaluate relevant stimuli such as a rich floral resource. Honey bees fed toxins such as ethanol (Mustard et al. 2008), the pesticides fipronil (El Hassani et al. 2005), or thiamethoxam (Aliquane et al. 2009) showed reduced responsiveness to sucrose. If honey bee foragers ingest nectar containing selenate, foraging behaviors may be altered and bees may be less responsive to floral resources.

Our third objective examined the lethal effects of Se ingestion in honey bee foragers when applied at single or chronic dosages. Se as a micronutrient is essential for survival, but higher concentrations can be toxic to insects (Jensen and Trumble 2003). Se ingestion increased mortality and development time in *Cotesia marginiventris* Cresson (Hymenoptera: Braconidae) (Vickerman et al. 2004), *Heliothis virescens* F. (Lepidoptera: Noctuidae) (Popham and Shelby 2007), *P. maculiventris* (Vickerman and Trumble 2003) and *S. exigua* (Trumble et al. 1998; Vickerman and Trumble 1999; Vickerman et al. 2002). In our study, selenate was more toxic than selenomethionine or methionine when fed to honey bee foragers as either a single or chronic dose. Selenomethionine was toxic only at the highest dosage. In other insect plant-feeders, selenomethionine was as toxic

as selenate in *S. exigua* (Trumble et al. 1998), but more toxic than selenate in *H. virescens* (Popham and Shelby 2007). In the detritivore *Megaselia scalaris* Loew (Diptera: Phoridae), selenomethionine was more toxic than selenate (Jensen et al. 2006). In insects fed various forms of Se, selenocompounds concentrated in the hindgut of the Se-tolerant *Plutella xylostella* L. (Lepidoptera: Plutellidae) (Freeman et al. 2006), whereas Se concentrated in the Malpighian tubules of the Se-intolerant *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) (Hogan and Razniak 1991), suggesting these are the sites of sequestration and detoxification. Se detoxification in tolerant insects has been attributed to their ability to sequester Se as methylated forms of selenocompounds (Freeman et al. 2006), which can prevent their misincorporation into proteins. In addition, trimethylselenonium-like species were found in the parasitoid *C. marginiventris*, suggesting they may detoxify the selenium accumulated from contaminated hosts by using methylation and volatilization (Vickerman et al. 2004). Honey bees may employ similar mechanisms of detoxification by methylating or even volatilizing the Se.

Bees chronically fed 60 µg ml^{-1} selenate and higher experienced a significant decrease in survival. Greenhouse studies irrigating *Brassica juncea* (Indian mustard) (Hladun et al. 2011) and *Raphanus sativus* (radish, unpublished data) with selenate treatment levels comparable to contaminated water in the western San Joaquin Valley of California revealed flowers accumulated up to 60 µg Se ml^{-1} Se in the nectar of both plant species. In the field, plants growing in soils containing 5 to 10 mg Se kg^{-1} accumulated approximately 1800 mg Se kg^{-1} dw in their flowers (Galeas et al. 2007), and

insect floral visitors to hyperaccumulator and non-accumulator plants contained an average of 44 and 10 µg Se g^{-1} dw respectively (Galeas et al. 2008). For the hyperaccumulator plant *Stanleya pinnata*, flowers accumulated 2323 mg Se kg^{-1} dw, with nectar containing 244 µl Se ml^{-1} fw (Quinn et al. 2011). Pollen collected by bees from New England aster growing in fly ash from coal-burning electrical power plants contained 14 mg Se kg^{-1} (De Jong et al. 1977). Floral visitors on Se-accumulating plants contained up to 75 µg Se g^{-1} dw (Galeas et al. 2008), honey bees contained 14.8 µg Se g^{-1} dw and bumble bees contained 251 µg Se g^{-1} dw (Quinn et al. 2011) when collected from seleniferous field sites in Colorado. Several greenhouse and field studies suggest there is the potential for honey bee foragers to acquire toxic levels of Se from certain species of plants growing in Se-contaminated areas.

Se in plant tissue or artificial diet has been shown to have negative effects on several insect species, yet we know very little about how soil-borne pollutants can impact pollinators. Insect herbivores fed plant tissues containing high levels of metals, metalloids (such as Se), or other accumulated elements have shown reduced development and survival (Boyd 2007), and several studies have shown some insect species cannot detect detrimental levels of Se (Trumble et al. 1998; Vickerman et al. 2002). If nectar contains Se in the form of selenate, honey bees may not avoid these plants. If the foraging honey bees feed on nectar containing Se (particularly selenate), reductions in population numbers may occur due to direct toxicity. The older, foraging population of workers may be reduced, and younger workers may need to precociously forage to maintain the constant flow of resources into the colony. On the other hand, if the nectar

contains selenomethionine, bees may detect and avoid these flowers. Additionally, our study has shown that fewer bees respond to sucrose when fed selenate. If a forager bee does survive the ingestion of selenate, she may be less responsive, forage and recruit less, and not properly evaluate valuable floral resources. Fewer responsive foragers may reduce the incoming floral resources needed to support coworkers and larvae. Taken together, effects on survival and foraging behaviors may significantly reduce the productivity and longevity of the colony. Our study is the first to examine the sublethal and lethal effects of a plant-accumulated pollutant on honey bee feeding preference, sucrose response threshold and mortality.

Materials and methods

Compounds tested. Sodium selenate (henceforth, selenate, Na_2SeO_4, 98% purity), seleno-DL-methionine (henceforth, selenomethionine, $C_5H_{11}NO_2Se$, 99% purity) and DL-methionine (henceforth, methionine, $C_5H_{11}NO_2S$, 99% purity) were all purchased from Sigma-Aldrich (St. Louis, MO). These forms of Se were chosen for comparison to toxicity assays using *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) (Trumble et al. 1998; Vickerman and Trumble 1999; Vikerman et al. 2002). Compounds were prepared at 5 treatment levels so that each treatment contained the following concentrations of Se or sulfur: 0.6 µg ml^{-1}, 6 µg ml^{-1}, 60 µg ml^{-1}, 600 µg ml^{-1}, and 6000 µg ml^{-1}. A control containing 0 µg ml^{-1} (1 M sucrose alone) was also included. In previous experiments, two non-hyperaccumulator plant species, *Brassica juncea* L. (Indian mustard) (Hladun et

al. 2011) and *Raphanus sativus* L. (radish) (Hladun, *unpublished data*), accumulated up to 60 µg ml^{-1} total Se in the nectar when irrigated with selenate in the greenhouse. Therefore treatments included this concentration and two orders of magnitude higher and two lower. Stock solutions were prepared in 1 M sucrose solution (99.9% purity, Fisher Scientific, Pittsburg, PA). Sucrose solution alone and deionized water alone were used for control treatments.

Animals. Tests were performed from June 2010 until January 2011 at the University of California – Riverside (UCR, Riverside, CA) using honey bee (*A. mellifera*) foragers collected at the entrance of a hive maintained at Agricultural Operations at UCR. The queen was not changed during the course of these experiments to minimize genetic variation. Bees were captured in small glass scintillation vials and chilled briefly at 4ºC until immobile. Each individual was restrained in a harness comprised of a 3.8 cm long piece of drinking straw with a diameter of 7 mm. A thin strip of duct tape secured between the head and thorax permitted movement of the antennae and proboscis. Each bee was fed *ad libitum* with 1 M sucrose solution after harnessing. Bees were then left for 24 hours in a humid box at room temperature within the laboratory before use in experiments.

Antennal response assays. Honey bee taste sensillae have been found on mouthparts associated with the proboscis (Whitehead 1978; Whitehead and Larsen 1976; Wright et al. 2010), as well as on the antennae (Haupt 2004), therefore we tested the bee's response to stimulation of both. Honey bee foragers were tested with a range of Se concentrations to determine whether they would respond with PER to antennal

stimulation with Se. Assays were based on methodology from de Brito Sanchez et al. (de Brito Sanchez 2005), and delivered the test compound dissolved in 1 M sucrose to the antennae, eliciting PER. PER responses were scored as (+), proboscis extended upon antennae stimulation, or (-), proboscis retained after antennae stimulation. Bees that did not extend their proboscis even when their antennae were stimulated with sucrose were recorded as non-responsive. We determined the response thresholds for 2 Se compounds (selenate and selenomethionine) and 1 sulfur compound (methionine) dissolved in 1 M sucrose at 5 concentrations (0.6 µg ml^{-1}, 6 µg ml^{-1}, 60 µg ml^{-1}, 600 µg ml^{-1}, and 6000 µg ml^{-1}). In addition, 1 M sucrose only and water only touched to the antennae served as controls. Bees were stimulated with solution contained within a Gilmont micrometer glass syringe (Gilmont Instruments, Barrington, IL). Immediately before the assay, each honey bee was tested for their motivation to feed by touching the antennae with a droplet of 1 M sucrose solution and observing the proboscis extension. Only bees extending their proboscis were chosen for subsequent trials. Antennal stimulation with water in between each treatment stimulation served as a control for sensitization, with an intertrial time of about 3 minutes.

Proboscis response assays. Proboscis response assays were based on methods used in Wright et al. (2010). Each bee's antenna was stimulated with a 1 M sucrose droplet to elicit the PER (Kuwabara 1957), then each bee was fed 0.6 µl of the treatment solution. The proboscis stimulation treatment involved exposing the proboscis to selenate, selenomethionine, or methionine dissolved in 1 M sucrose solution in a 0.6 µl droplet administered with a Gilmont syringe. The small volume used to stimulate proboscises

ensured that bees would not feed enough to reach satiation and become less responsive. Groups of bees were tested with either selenate, selenomethionine or methionine dissolved in 1 M sucrose at 5 treatment concentrations (0.6 µg ml^{-1}, 6 µg ml^{-1}, 60 µg ml^{-1}, 600 µg ml^{-1}, and 6000 µg ml^{-1} as Se or sulfur). Proboscis exposure to a droplet of 1 M sucrose or water acted as positive and negative controls, respectively. Bees were scored as (+), bee consumed entire 0.6 µl droplet, or (-), bee did not consume droplet.

Sucrose response threshold assays. To examine the effects of the consumption of selenium on the responsiveness to sugars, bees were fed an acute dose of selenate, selenomethionine or methionine and then their sucrose response thresholds were determined. The sucrose response threshold assays were based on methods from Mustard et al. (2008) and Page et al. (1998). Honey bees were captured and harnessed as described above. Twenty four hours later, bees were fed 20 µl solutions of 1 M sucrose containing 0 (control), 0.6 µg ml^{-1}, 6 µg ml^{-1}, 60 µg ml^{-1}, 600 µg ml^{-1}, and 6000 µg ml^{-1} of Se or sulfur in the forms of selenate, selenomethionine or methionine. Two hours after the bees had consumed the treatment, they were assayed for sucrose response threshold. Each bee's antennae were stimulated with sucrose solutions at increasing concentrations of 0.1%, 0.3%, 1%, 3%, 10% and 30%, interspersed with antennal stimulation with water. Water stimulations were interspersed between sucrose stimulations to serve as a control for increased sensitization or habituation on subsequent responses from repeated sucrose stimulation. After antennae were stimulated, proboscis extension (+) or retention (-) was recorded. Intertrial times were 3 minutes.

Total consumption and single dose mortality assays. Bees were captured and harnessed as described above and fed 1 M sucrose only *ad libitum*. Twenty four hours later, bees were fed treatments of Se or sulfur as selenate, selenomethionine, or methionine dissolved in 1 M sucrose at 6 concentrations (0, 0.6, 6, 60, 600, and 6000 µg ml^{-1}) for a total of 18 treatment groups. Bees were fed using a Gilmont syringe. The total volume consumed from each treatment was calculated. Bees remained harnessed for 5 days after the single dosage and mortality per day was scored in control and treated groups and has been presented as final mortality after 5 days. Surviving bees were fed 1 M sucrose *ad libitum* on each of the 5 subsequent days.

Chronic dose mortality assays. Based on the average volume of treatment solution consumed in each treatment in the single dose assay, bees were fed 20 µl for each control and treated group on day 0, and were fed an additional 20 µl of treatment solution on each of the 5 subsequent days. Treatments consisted of selenate, selenomethionine, or methionine dissolved in 1 M sucrose at 6 concentrations (0, 0.6, 6, 60, 600, and 6000 µg ml^{-1} as Se or S) for a total of 18 treatment groups. Throughout the assay, bees were evaluated in control and treated groups for mortality per day.

Statistical analysis. Antennal response, proboscis response, and sucrose threshold response probabilities were analyzed as a binary variable using repeated-measures logistic regression with each bee as a unit of replication. Data were analyzed using the GENMOD procedure in SAS (version 9.2, SAS Institute, Cary, NC) with *post hoc* multiple comparisons. Antennal and proboscis response compared PER probabilities in the 1 M sucrose control group to the treated groups unless otherwise noted. Sucrose

response threshold assays compared response probabilities between the water trials and each sucrose concentration. Total consumption was analyzed for each treatment group using ANOVA (GLM procedure) and *post hoc* Tukey's HSD test. For mortality assays, as recommended in the EPA Ecological Effects Test Guidelines (OPPTS 850.3020), mortality was 20% or less in all control groups. Based on preliminary studies feeding harnessed foragers with 1 M sucrose, mortality increased above 20% by day 6, therefore we concluded the toxicity bioassays at day 5. Each honey bee represented a unit of replication. Pairwise comparisons were made of mortality in the 1 M sucrose (control) group to each treatment level and within each Se form. Se forms were not compared to each other. Mortality data was not normally distributed; therefore comparisons were made using the nonparametric Kruskal-Wallis test with *post hoc* separations using the Mann-Whitney *U* test (NPAIR1WAY procedure).

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CHAPTER 4

Effects of selenium accumulation on phytotoxicity, herbivory, and pollination ecology in radish (*Raphanus sativus* L.)

Abstract

Selenium (Se) has contaminated areas in the western USA where pollination is critical to the functioning of both agricultural and natural ecosystems, yet we know little about how Se can impact pollinators. In a two-year semi-field study, the weedy plant *Raphanus sativus* (radish) was exposed to three selenate treatments and two pollination treatments to evaluate the effects on pollinator-plant interactions. Honey bee (*Apis mellifera* L.) pollinators were observed to readily forage on *R. sativus* for both pollen and nectar despite high floral Se concentrations. Se treatment increased both seed abortion (14%) and decreased plant biomass (8-9%). Herbivory by birds and aphids was reduced on Se-treated plants, indicating a potential reproductive advantage for the plant. Our study sheds light on how pollutants such as Se can impact the pollination ecology of a plant that accumulates even moderate amounts of Se.

Introduction

Up to 80% of the world's 250,000 flowering plant species (Kremen et al. 2007) and 60% of crop species (Roubik 1995) are animal pollinated, with insect pollinators such as honey bees being critical components to the crop species in particular. Pollinators such as honey bees and their honey products have been investigated as potential bioindicators pollutants, and varying amounts of elements that are toxic to insects have been found in honey, propolis, and pollen of honey bee hives located in proximity to polluted sites (Bogdanov 2006). However, few studies have focused on pollutants effects on plant-pollinator interactions or the fitness consequences on bee populations.

Selenium (Se) is a metalloid that can occur naturally in soils from the Cretaceous shale deposits of a prehistoric inland sea in the western United States. Agricultural irrigation and runoff dissolves Se from these shales, causing accumulation of toxic levels of selenate (SeO_4^{2-}) in water and soil (Brown et al. 1999). Selenate is the most common species of Se found in the root zone (Tokunaga et al. 1991) and can contaminate both water and soil (Cutter 1982; Dhillon and Dhillon 2001; Frankenberger and Benson 1994; Trumble and Sorensen 2008).

Several studies have reported elevated levels of metals in the flowers and fruits of specialized plant species known as hyperaccumulators that have evolved to use certain elements as a defense against herbivores (Boyd 2007; Freeman et al. 2006; Jaffre et al. 1976; Reeves et al. 1981). High levels of Se have been found in flowers relative to leaf tissues (up to 9000 mg Se kg^{-1} for *Astragalus bisulcatus* ((Hook.) A. Gray, Galeas et al. 2007), but this study did not distinguish which specific parts of the flower (pollen, nectar,

or petal) contained Se. These hyperaccumulators tend to be found in rather limited areas where elevated concentrations of specific elements naturally occur (Boyd 2007; Feist and Parker 2001). However, certain species of Brassicaceae that have not evolved elemental defense can also have moderately high Se levels (Brown and Shrift 1981) when growing on Se-polluted soils. Foliar herbivores fed plant tissues containing high levels of metals, metalloids, or other accumulated elements have shown reduced developmental rates and survival (Boyd 2007; Butler and Trumble 2008). Several reports have indicated some insect species cannot detect detrimental levels of Se (Trumble et al. 1998; Vickerman et al. 2002), but there is no published study to date examining the effects of the pollutant Se on the pollination ecology of a non-hyperaccumulator plant.

Certain crop species can accumulate Se when grown in soils with elevated Se (Carvalho et al. 2003). Members of the Brassicaceae such as *B. juncea* experience reduced growth when grown in soil containing 2 mg Se kg^{-1} (Bañuelos et al. 1997), suggesting there is a cost to accumulating Se. Se may have similar phytotoxic effects on *Raphanus sativus* L. (radish) which is known to accumulate Se mostly as selenate (Pedrero et al. 2006). Selenate can be reduced to selenite (SeO_3^{2-}) and then incorporated into the amino acids as selenomethionine or selenocysteine, and then into proteins, which can also have toxic effects (Brown and Shrift 1981). Se volatilizes from foliar tissues as dimethylselenide (DMSe) and other Se-containing volatiles (Meija et al. 2002; Kubachka et al. 2007), and may cause changes in feeding site preferences and deterrence for herbivores as well as pollinators. The potential effects on pollination and subsequent plant reproductive success is largely unknown for non-specialist plants.

Raphanus sativus has been examined as a model for studying plant responses to pollutants (Kostka-Rick and Manning 1993). This species is a common weed throughout California and is cultivated throughout the world (Snow and Campbell 2005). It is an annual, self-incompatible plant (thus ideal for pollination studies) that has been examined extensively in herbivore and pollinator studies (Stanton 1987; Strauss et al. 2004) as well as for its hybridization with *Raphanus raphanistrum* L. (Hedge et al. 2006). Our previous greenhouse studies confirm that radish can accumulate Se into its leaves and roots, as well as into its pollen and nectar (Hladun et al. *unpublished data*) at concentrations well above the LC_{50} for an insect herbivore (*Spodoptera exigua* Hübner, Lepidoptera: Noctuidae, Trumble et al. 1998).

We conducted a manipulative semi-field study to examine how the soil-borne pollutant Se can affect plant performance and reproduction, herbivory, and pollinator visitation. Our main objectives were to test the hypotheses; 1) the pollutant Se will cause a reduction in plant reproduction due to pollinator deterrence or phytotoxicity to the plant, and 2) Se will have a beneficial effect by reducing herbivore damage without a plant losing attractiveness to pollinators and therefore maintaining plant reproductive output.

Materials and methods

Experimental treatments. For year 1, on 27-Jan-2010, *R. sativus* (crop radish, cv. "White Globe", Livingston Seed Co., Columbus, OH USA) was planted in steam sterilized potting mix (50% sand, 25% bark, 25% peat moss) within 18.93 l pots. Pots

were placed approximately 0.5 m apart, placed within bins to capture runoff, in a plot of land measuring 35 m x 22 m. Experiments were conducted at the Department of Agricultural Operations at the University of California (Riverside, CA). Two Se treatments (0 and 0.51 mg Se l^{-1}) and 2 pollination treatments (natural and hand) were manipulated in a factorial design for a total of 4 treatment combinations. Plants were assigned to treatments in a randomized block design, with 3 plants per treatment combination and 12 plants per block, for a total of 6 blocks and 72 plants. Block was included as a fixed factor to account for differences in proximity to the honey bee hive.

For year 2, on 2-Feb-2011, crop radish seeds were planted as described above. Two Se treatments (0 and 0.51 mg Se l^{-1}) were applied along with an additional high Se concentration (1.53 mg Se l^{-1}) for a total of three Se treatments. Two pollination treatments (natural and hand) were again included in a factorial design for 6 total treatment combinations. Plants were assigned to treatments in a randomized block design, with 1 replicate plant per treatment combination per block and 6 plants per block, for a total of 12 blocks and 72 plants. During both years, we watered plants with Se-treated tap water three times a week with 500 ml of treatment water. Se treatments were added as sodium selenate (Na_2SeO_4, Sigma-Aldrich, St. Louis, MO), the form commonly found in contaminated waters and soils (Tokunaga et al. 1991) and concentrations are reported in elemental Se. Se treatment levels were ecologically relevant because concentrations were within the range of the high end of reported concentrations for contaminated sites (2 mg l^{-1}) (Seiler et al. 1999) and the highest mean Se concentrations from stream sediments and soils in CA (0.58 mg kg^{-1}) (Grossman et al. 2007), but were

below 4 mg l^{-1}, the maximum Se concentrations contaminating the western San Joaquin Valley in CA (Burau 1985; Presser and Barnes 1985).

Pollination was also manipulated to determine if Se accumulation in the plant altered pollen limitation. Pollination treatments were applied twice during the peak flowering period (Year 1: April 30 2010 and May 21 2010, Year 2: April 29 2011 and May 20 2011). Two unopened flowers of similar age per plant were arbitrarily chosen and covered with mesh bags the day before pollination treatment to prevent any visitation. The next day, pollen was collected from 5 different greenhouse-grown radish plants used for the sole purpose of pollen donation for the application of hand pollination treatments on the field plants. Pollen viability was evaluated for each paternal line using Alexander's stain (Alexander, 1980) and averaged 92.9 ± 1.4 % (n = 20). Bags were then removed and saturating amounts of pollen were applied evenly to the stigmas as the hand pollination treatment. Plants assigned the natural pollination treatment were also bagged to control for any bag effects, and were removed to allow pollen deposition from bee visitation. A honey bee hive maintained adjacent to the plots, was the main source of natural pollination at this site.

Plant performance and reproduction. For floral traits, two flowers per pot were measured during peak flowering period using morphological measurements based on Conner and Via (1993). Floral trait measurements included display width (distance across flower from the tip of one petal to the other), petal area (estimated as length x width), corolla tube length, pistil and stamen length. The total number of flowers produced per

day was counted for each plant throughout the experiment, and then summarized within plant to calculate total flower number.

Aboveground biomass and root biomass were harvested at the end of the experiment, dried in an oven at 70°C and weighed. Fruit on the whole plant were examined at the end of each experiment year and scored as intact, frugivory, or aborted (only the pedicel present). Seed production was measured for 5 randomly chosen fruit per plant. Fruit were broken open and total seed number and weight were quantified using a microbalance (weighing to 0.00001 g, model 1712 MP8, Sartorius Corp., Goettingen, Germany).

Herbivory. Herbivory was scored once a week for 11 weeks beginning on Feb 12 2010 and on Feb 23 2011. Each week, the total number of leaves were counted and damage to three randomly chosen leaves were estimated and averaged as the percent of leaf tissue removed. Herbivore damage by the imported cabbageworm (*Pieris rapae* L., Lepidoptera: Pieridae) was rare. The predominant herbivore found both years was cabbage aphid (*Brevicoryne brassicae* L., Hemiptera: Aphididae), and their total numbers on leaves and flower buds were quantified. Aphid mummies were also counted during each weekly herbivory census in order to collect data on Se's effects on higher trophic levels. A previous study found Se can impair the development and weight of a parasite in a host that had been feeding on Se-treated plants (Vickerman et al. 2004). The observation of aphid mummies was based on the characteristic swollen, papery brown stature an aphid turns into when parasitized by a wasp. Frugivory was observed to be from house finches, *Carpodacus mexicanus*. Fruits were ripped open by the birds, the

seeds inside eaten, and therefore the fruit was scored as "frugivory" only if a torn, empty husk remained.

Pollen limitation and pollinator visitation. Seed production and viability from flowers used in the pollination treatments was quantified as described above. Visitation by the predominant pollinator, the honey bee, was observed during peak flowering period from May 15 2010 to May 20 2010 (Year 1) and from May 13 2011 to May 26 2011 (Year 2) for 5 minute observation periods per day at the same time of day (between 1400 and 1600 hours). The total number and duration of honey bee visits were recorded for each plant. Seed viability was confirmed in two randomly chosen fruit per plant by germinating them on filter paper moistened with tap water in a growth chamber kept at a constant temperature of 21ºC and a 16:8 day:night cycle. Final germination percentage (FGP) was calculated as the total number of seeds that germinated after 7 days divided by the total number of seeds produced in each cross.

Se analyses in plant and insect tissues. Se treatment effects on plant tissue Se content was examined by measuring the concentration of Se in floral and leaf tissues. Two leaves and five flowers of similar age were collected from each plant during the peak flowering period. Honey bees were collected as they foraged during peak flowering period. Pollen loads were removed from corbiculae and analyzed separately. Cabbage aphids and ladybird beetle (Coleoptera: Coccinellidae) predators were also collected from plants treated with Se. All floral, leaf and insect tissues were frozen in a -60ºC freezer (Fisher Scientific, Pittsburg, PA) and then freeze-dried (Labconco Corp., Kansas City, MO) at -40ºC and -25 psi for at least 3 days. After freeze drying, leaf and flower tissues

were ground to a fine powder using a mortar and pestle to homogenize tissues. All freeze-dried plant tissues were stored in a -60ºC freezer until digestion. All Se concentrations in plant tissues are reported in mg kg^{-1} dry weight.

All plant tissues were weighed using a microbalance prior to digestion. Plant material was microwaved in 110 ml teflon-lined vessels containing a mixture of 1 ml H_2O, 2 ml 30% (v/v) H_2O_2, and 2 ml concentrated HNO_3 (Sah and Miller 1992). The vessels were heated for 20 min using a 570 W microwave oven (CEM Corp., Matthews, NC). Insect tissues were weighed using a microbalance prior to microwave digestion. Insect material was microwave digested in vessels containing 10 ml concentrated HNO_3, then were heated for 30 min in the microwave. Plant and insect tissue samples were analyzed using inductively coupled plasma optical emission spectroscopy (ICP-OES) (PerkinElmer Inc., Shelton CT). Se concentrations in plant and insect tissues are reported in mg kg^{-1}. Samples were run in duplicate and Se spikes were added as internal standards to determine precision and recovery. The NIST Standard Reference Material 8436 (durum wheat flour) was used as a standard for plant tissues, and NIST 1566B (oyster) was used for insect tissues. Duplicate sample concentrations were within 10% of each other, and Se spike recovery and NIST Se recovery were over 90%.

Statistical analyses. Results were analyzed with general linear models (PROC GLM, SAS 9.2; SAS Institute, Cary NC, USA) with type III sum of squares; independent variable included Se treatment, pollination treatment, year, and their interactions. Block was included as a fixed factor, and the experiment was blocked in space to minimize variation. MANOVAs were conducted on plant performance, herbivory, pollination, and

Se in insect and plant tissues. When MANOVAs were significant, subsequent ANOVAs were conducted. Mean separations were conducted between groups ($\alpha = 0.05$) using *post hoc* Tukey's HSD test. Assumptions of normality were examined using the Shapiro-Wilks test. The response variables aboveground biomass, root biomass, leaf damage, total bee visits and bee visit duration per bout were log-transformed to meet assumptions of normality and homogeneity of variance.

Results

Plant performance and reproduction. Se treatment or its interaction with year had no significant effect on floral traits or flower number (MANOVA, Wilks' $\lambda < 1.16$, $P > 0.34$). Block (Wilks' $\lambda = 1.63$, $P < 0.005$) and year (Wilks' $\lambda = 9.19$, $P < 0.001$) significantly affected floral morphology. Year significantly affected display width (ANOVA, $F_{1,32} = 7.52$, $P < 0.01$), corolla tube length ($F_{1,32} = 6.60$, $P < 0.02$), short stamen length ($F_{1,32} = 13.36$, $P < 0.001$), and long stamen length ($F_{1,32} = 4.96$, $P < 0.04$). Block had a significant effect on total flower number ($F_{1,32} = 5.77$, $P < 0.001$).

Se treatment (MANOVA, Wilks' $\lambda = 4.01$, $P < 0.005$) and year (MANOVA, Wilks' $\lambda = 72.68$, $P < 0.001$) had a significant effect on plant performance and reproduction. The interaction of Se treatment x year and block were not significant (MANOVA, Wilks' $\lambda < 2.14$, $P > 0.09$). The 1.53 mg l^{-1} Se treatment reduced the biomass by 20% compared to controls (Table 4.1; Fig. 4.1.a). In year 2, Se treatments significantly increased the proportion of aborted fruit up to 15% (Table 4.1; Fig. 4.1.b), whereas the proportion of frugivory on fruit was reduced by 14% (Fig. 4.1.c). Se treatments reduced the number of

seeds per fruit by up to 21% (Table 4.1; Fig. 4.1.d). Aboveground biomass weighed more in year 1 (mean ± SE: Year 1 187.58 ± 11.27 g; Year 2 43.96 ± 2.63 g), and the proportion aborted (Year 1 0.33 ± 0.02; Year 2 0.29 ± 0.02) and frugivory (Year 1 0.21 ± 0.04; Year 2 0.30 ± 0.02) fruit was higher in year 1. Plants also produced more seeds in year 1 (Year 1 5.7 ± 0.22; Year 2 3.99 ± 0.15). Se did not have a significant effect on dry belowground biomass (mean ± SE: 0 mg l^{-1} Se = 27.46 g (N = 18); 0.51 mg l^{-1} Se = 27.57 g (N = 18), 1.53 mg l^{-1} Se = 22.68 g (N = 12)).

Fig. 4.1 Se treatment and year effects on aboveground biomass (a), proportion of aborted fruit (b), proportion of seeds with bird (house finch, *Carpodacus mexicanus*) frugivory (c), and the average number of seeds per fruit (d) in *Raphanus sativus* (radish). Se treatment levels: 0.0 mg l^{-1} (control), 0.51 mg l^{-1}, and 1.53 mg l^{-1}. Values are means ± standard error (SE). Letters above the means indicate statistically significant differences between groups ($\alpha = 0.05$).

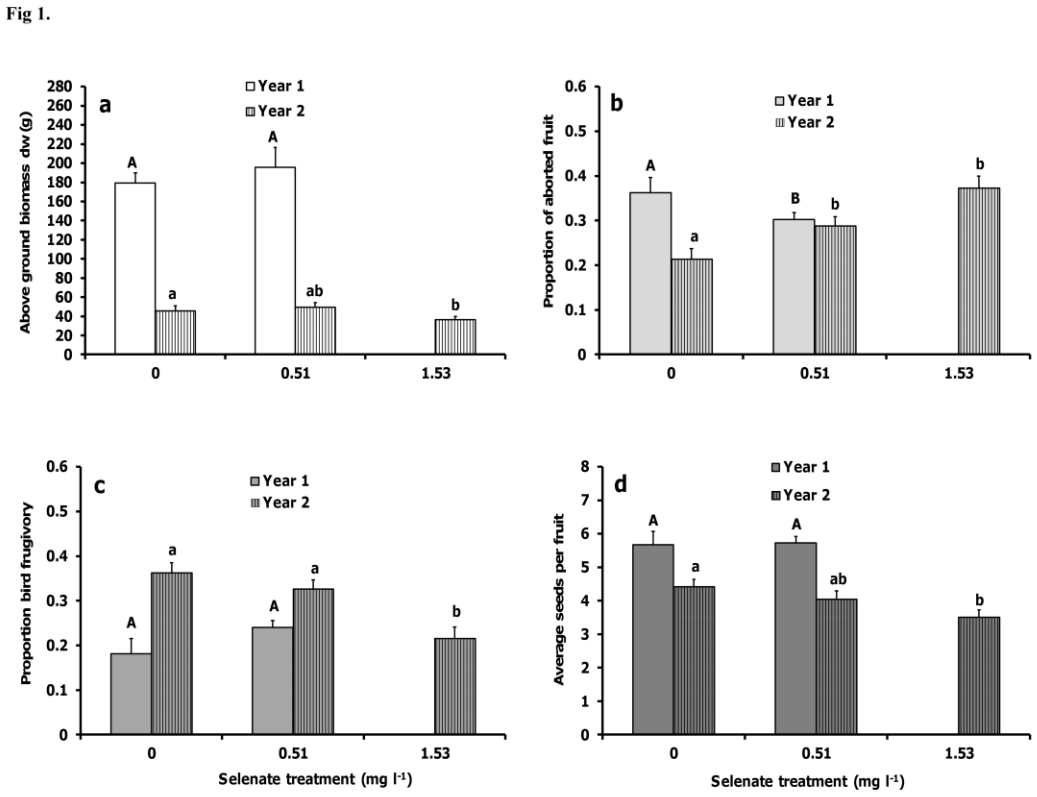


Table 4.1 ANOVA showing the effects of selenium treatment, year, their interaction and block on aboveground biomass, root biomass, proportion of aborted fruit, proportion of frugivory, number of seeds per fruit and weight per seed. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

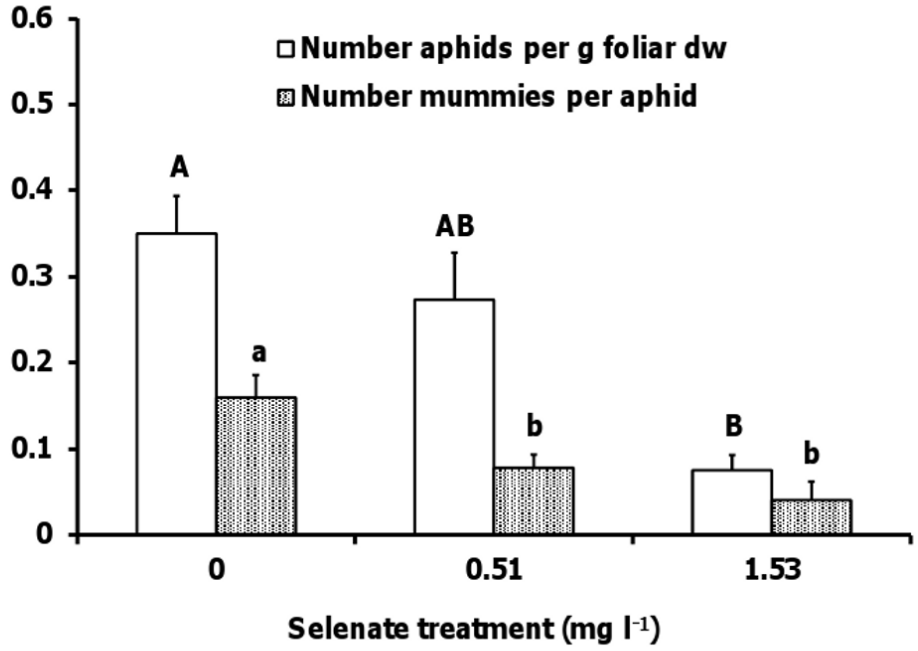
Source	*df*	Aboveground biomass		Root biomass		Proportion of aborted fruit	
		F	*P*	*F*	*P*	*F*	*P*
Selenium treatment	2	5.28	0.01*	2.11	0.14	10.20	0.0004***
Year	1	291.28	0.0001***	2.91	0.10	8.00	0.008***
Selenium treatment x Year	1	0.02	0.88	0.33	0.57	6.15	0.02*
Block	11	4.54	0.0004***	0.73	0.70	1.03	0.45
Error	32						

Source	*df*	Proportion of fruivory fruits		Number of seeds per fruit		Weight per seed (g)	
		F	*P*	*F*	*P*	*F*	*P*
Selenium treatment	2	5.11	0.01*	3.98	0.03*	1.10	0.34
Year	1	8.18	0.008**	28.44	0.0001***	0.03	0.87
Selenium treatment x Year	1	1.42	0.24	0.73	0.40	0.06	0.8
Block	11	0.85	0.60	1.71	0.12	0.74	0.70
Error	32						

Herbivory. Se treatment (MANOVA, Wilks' $\lambda = 10.02$, $P < 0.001$) had a significant effect on herbivory. Year and block did not significantly affect the number of aphids per g dry foliar biomass, or the number of mummies per aphid (MANOVA, Wilks' $\lambda < 2.74$, $P > 0.08$). Therefore the herbivory data for both years were pooled. The interaction of Se treatment and year was also not significant (MANOVA, Wilks' $\lambda = 0.29$, $P = 0.19$). Low and high Se treatments significantly reduced aphid numbers compared to control plants (Fig. 2; ANOVA, $F = 14.75$, $P < 0.001$). The number of mummies were also significantly reduced by both Se concentrations (Fig. 4.2; $F = 12.91$, $P < 0.001$). There was no effect of Se treatment on leaf number or average leaf damage (ANOVA, $F < 0.91$, $P > 0.41$).

Fig. 4.2. Se treatment effects on the number of aphids (*Brevicoryne brassicae*) per gram dry weight of foliar biomass and the number of mummies per aphid. Se treatment levels: 0.0 mg l^{-1} (control), 0.51 mg l^{-1}, and 1.53 mg l^{-1}. Values are means ± standard error (SE). Letters above the means indicate statistically significant differences between groups (α = 0.05).

Fig 2.



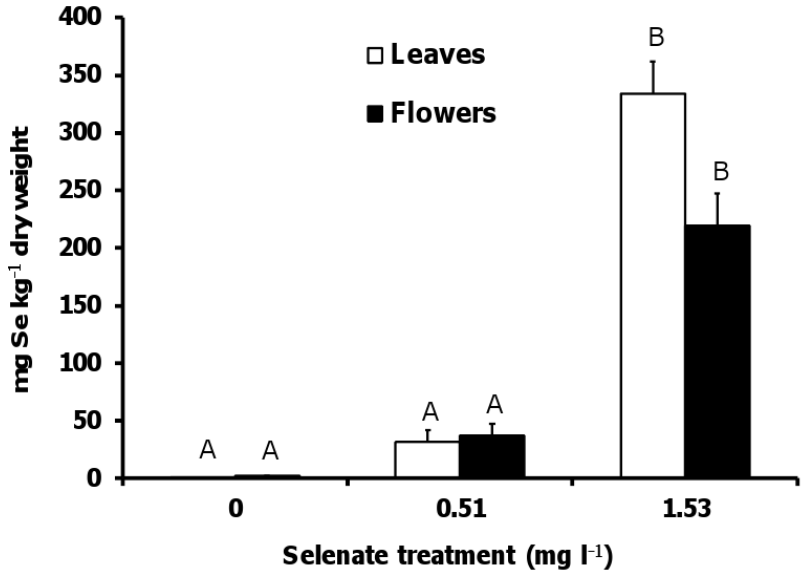
Pollen limitation and pollination. Pollination treatment (Wilks' $\lambda = 0.95$, $P = 0.43$) and the interaction of Se treatment x pollination treatment were not significant (Wilks' $\lambda = 1.01$, $P = 0.43$), indicating plants were not pollen limited due to Se treatment. Overall, plants that received the natural pollination treatment (pollen deposited only by naturally occurring pollinators, mostly honey bees) produced 25% more seed than plants given the hand pollination treatment (mean ± SE: Natural pollination: 2.30 ± 0.52 seeds, hand pollination: 1.71 ± 0.25 seeds), although the difference was not significant (Wilks' $\lambda = 0.95$, $P = 0.43$). There was no significant difference in final germination percentage for plants treated with natural pollination compared to hand pollination (Natural pollination: 50.4%, hand pollination: 54.5%). Block, Se treatment, year, pollination and their interactions also had no significant effect on pollen limitation (Wilks' $\lambda < 2.08$, $P > 0.13$).

Overall, the primary pollinator, the honey bee, visited flowers frequently and was an efficient pollinator, as indicated by the lack of pollen limitation in the pollination treatments listed above. Se treatment, Se treatment x year, and block had no significant effect on pollinator visitation (MANOVA, Wilks' $\lambda < 0.96$, $P > 0.53$). Year had a significant effect on pollinator visitation (Wilks' $\lambda = 33.75$, $P < 0.001$). Year had a significant effect on visit duration per flower ($F = 9.42$, $P < 0.005$) and total honey bee visits ($F = 104.40$, $P < 0.001$). Honey bee visit durations were 51% shorter in year 2 (mean ± SE: Year 1 21.25 ± 1.61; Year 2 10.48 ± 1.39). There were also far fewer total honey bee visits to plants in year 2 compared to year 1 (Year 1 15.85 ± 2.30; Year 2 1.26 ± 0.19).

Se concentrations in plant and insect tissues. Selenate-treated plants significantly accumulated Se into the flowers and leaves (Fig. 4.3; ANOVA, $F > 16.98$, $P < 0.001$). Pollen loads collected from the corbicula of honey bees observed to visit both control and Se-treated plants contained 6 to 2830 mg Se kg^{-1} (n = 7). Honey bee forager bodies contained 3 to 27 mg Se kg^{-1} (n = 11). Cabbage aphids collected from plants treated with Se contained 20 to 60 mg Se kg^{-1} (n = 5). Ladybird beetles collected near the cabbage aphids contained 141 to 217 mg Se kg^{-1} (n = 4).

Fig. 4.3. Se treatment effects on Se accumulation levels in leaves and flowers. Se treatment levels: 0.0 mg l^{-1} (control), 0.51 mg l^{-1}, and 1.53 mg l^{-1}. Values are means ± standard error (SE). Letters above the means indicate statistically significant differences between groups (α = 0.05).

Fig 3



Discussion

This study reveals the effects of a plant-accumulated pollutant on pollinators, frugivores and insect herbivores. Herbivory by birds and aphids was reduced at the highest Se treatment level, whereas pollinator visitation by honey bees was maintained at rates similar to control plants. Field studies have demonstrated reduced insect and mammalian herbivory (Galeas et al. 2008; Quinn et al. 2008) and fewer flower visitors present on Se-hyperaccumulating plants (Galeas et al. 2008). Our manipulative semi-field study suggest that while *R. sativus* plants experience some phytotoxicity from Se, these effects are minimized by the preservation of attractive floral traits as well as the reduction in herbivory, thus maintaining pollination and reproductive output in Se-accumulating plants.

The phytotoxic effects of Se in radish included reduced biomass and increased fruit abortion. Greenhouse-grown radish plants irrigated with similar levels of Se showed reduced biomass and seed set in the absence of herbivores (Hladun *unpublished data*), suggesting non-hyperaccumulator plants will suffer reductions in plant performance when exposed to Se concentrations of 1.53 mg Se l^{-1}in the field. *Brassica juncea* showed phytotoxic effects of reduced dry matter yield and leaf surface area when grown in soils containing 2 mg Se kg^{-1} (Bañuelos et al. 1997). In greenhouse studies, *B. juncea* suffered toxic effects from Se irrigation in terms of reduced flower size and number (Hladun et al. 2011). However, in the presence of herbivores, Se may protect plants, allowing them to outcompete non-accumulators that may also be present in the polluted landscape.

In several laboratory and field studies, herbivores fed plant tissues containing high levels of metals, metalloids, or other accumulated elements have exhibited toxic effects (Boyd 2007; Butler and Trumble 2008). In our study, aphid numbers were significantly reduced on plants watered with both Se treatment levels. Even the low Se treatment acted as a deterrent. Leaf concentrations as low as 10 mg Se kg^{-1} sufficed in deterring green peach aphids (*Myzus persicae* (Sulzer), Hemiptera: Aphididae, Hanson et al., 2004). In other insect species, Se ingestion increases mortality and development time, including the leaf-chewing herbivores *Heliothis virescens* F. (Lepidoptera: Noctuidae, Popham and Shelby 2007) and *S. exigua* (Trumble et al. 1998; Vickerman and Trumble 1999; Vickerman et al. 2002) as well as the predator *Podisus maculiventris* Say (Hemiptera: Pentatomidae, Vickerman and Trumble 2003). Several insect herbivore species and their predators are susceptible to Se toxicity, and the primary herbivore in our study was not tolerant of even low levels of Se.

Pollutants can alter tritrophic interactions (Heliövaara and Väisänen 1993), particularly if it is a soil-borne contaminant accumulated by a plant, passed onto the herbivore, and then biotransferred to the natural enemy. Parasitoids can be more susceptible to certain pollutants than their herbivore hosts (Fuhrer 1985), although the pollutant may have a direct toxic effect on the insect, or an indirect effect by reducing the number of prey available to the natural enemy. One study examining the effects of Se on a tritrophic system found detrimental effects on the parasitoid *Cotesia marginiventris* (Vickerman et al. 2004). The braconid wasp weighed less and took longer to develop when parasitizing a herbivore host that was fed Se-containing plant material. In our

study, there were fewer aphid mummies (most likely caused by a parasitoid wasp) on Se treated plants. In addition, a common generalist predator, the ladybird beetle (Coleoptera: Coccinellidae), was collected and analyzed for Se. High Se concentrations in the predators from Se-treated plants indicates biotransfer of the contaminant across several trophic levels. The predator accumulated about three times more Se than the aphid host. At Se-contaminated sites such as Kesterson Reservoir in CA, predatory invertebrates generally had higher Se concentrations than the herbivores (Vickerman and Trumble 2003). Additional studies are required to determine whether Se can biomagnify from the second to third trophic levels, and whether this can alter natural enemy populations.

A recent study using both hyperaccumulator (*Stanleya pinnata* (Pursh) Britton, Desert Prince's Plume) and non-hyperaccumulator (*B. juncea*) plants found honey bee and bumble bee pollinators visited control and Se-containing plants equally, further confirming that certain pollinators will not discriminate against hyperaccumulating plants despite concentrations as high as 3200 mg Se kg^{-1} in the flowers (Quinn et al. 2011). Our study revealed that honey bee pollinators will visit *R. sativus* that have accumulated selenium into flowers at concentrations well above the LC_{50} for a common insect herbivore, the beet armyworm (*S. exigua*, Trumble et al. 1998). Despite the high levels of Se (up to 219 ± 28 mg Se kg^{-1} dw), pollinators foraged on radish flowers and were observed to collect both pollen and nectar. At naturally seleniferous field sites, hyperaccumulator plants absorb up to 9000 mg Se kg^{-1} dw into the flowers (Galeas et al. 2007). Although there were fewer floral visitors to hyperaccumulators, the insects that

did visit flowers contained up to 75 mg Se kg^{-1} dw (Galeas et al. 2008). Pollen collected by honey bees from aster plants growing in fly ash from coal-burning electrical power plants contained 14 mg Se kg^{-1} Se (DeJong 1977), and nectar from radish plants grown in the greenhouse contained up to 100 µg Se ml^{-1} (Hladun *unpublished data*). Based on these concentrations, honey bees have the potential to bring food resources back to the hive that are contaminated with Se at levels shown to be toxic to other insect species.

Pollutants found at toxic levels in the plant tissues honey bees forage upon and feed to their progeny may cause fitness effects for the colony that are not currently recognized. If a weedy plant such as *R. sativus* grows in a Se-contaminated area, and can maintain its attractiveness to pollinators as our study has demonstrated, there is the potential for biotransfer of Se from the accumulating plant to the colony. Several weedy Brassicaceae species have the ability to accumulate Se(White et al. 2004; 2007), and may concentrate the element in the flowers, allowing Se to biotransfer to pollinators through the portal of an accumulating plant. In addition, certain species of plants are used to accumulate and disperse Se in contaminated soils through phytoremediation, which has developed into an important strategy for land reclamation (Pilon-Smits and Freeman 2006; Vickerman et al. 2004). Such large-scale Se accumulation by phytoremediating plant species has the potential to alter local ecosystems. This may adversely affect plant mutualists such as pollinators and efforts should be made to minimize pollinator exposure to Se-rich flowers.

Our study confirms that Se can accumulate in the flowers of *R. sativus*, and will be foraged upon by pollinators. If pollinators do visit Se-accumulating plants in polluted

areas, depending on the widespread nature of the contamination, they may not have many alternate resources and will receive significant doses of the element. However, selenium is also a micronutrient that is essential to many organisms, including mammals, fish, and bacteria (Burau 1985). Pollinators may dilute the amount of Se they receive by foraging on both non-accumulator and accumulator plants, and low levels of Se may have beneficial impacts on colony health such as reduced disease or predation (Barillas et al. 2011). Studies are currently underway to elucidate the fitness consequences of Se on honey bee adult and larval development and survival. Further studies are needed to determine the impact of soil-borne pollutants such as Se and their impact on plant-pollinator interactions.

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CHAPTER 5

Impacts of selenium on the development and survival of larval honey bees (*Apis mellifera* L.)

Abstract

Apis mellifera L. (Hymenoptera: Apidae) is an important agricultural pollinator in the United States and throughout the world. In areas of selenium (Se) contamination, honey bees may be at risk due to the biotransfer of Se from foraged plant products such as nectar and pollen. Several forms of Se can occur in accumulating plants, and the toxicity of four compounds (selenate, selenite, selenomethionine and methylselenocysteine) was assessed using artificial diet bioassays. Honey bee larvae were chronically exposed to the toxicants over a 12 day period. The inorganic forms were more toxic (LC_{50} selenate = 0.72 µg g^{-1}, LC_{50} selenite = 1.03 µg g^{-1}) than the organic forms (LC_{50} methylselenocysteine = 4.09 µg g^{-1}, LC_{50} selenomethionine = 6.04 µg g^{-1}). All four forms of Se decreased the percentage of larvae that pupated. In addition, selenate and methylselenocysteine significantly decreased larval growth rates. Overall, inorganic forms of Se caused more rapid mortality, but the organic forms had substantial sublethal effects on development. Previous research showed that foragers from honey bee hives within or adjacent to Se-contaminated areas will collect contaminated pollen and nectar, and the larval LC_{50} values are very low, even modest transfer to brood will likely cause increased development times and potentially increased mortality. In addition, the toxicities of the various forms of Se to honey bee larvae are discussed in comparison to other insect herbivores and detritivores.

Introduction

Selenium contamination is a global problem originating from a multitude of sources that include mine tailings, production of glass, pigments, inks, and lubricants; and leaching and concentration of Se in drainage water through agricultural irrigation or rainfall on naturally seleniferous soils (Vickerman et al. 2004). In California's San Joaquin Valley, extensive agricultural irrigation has resulted in significant selenium contamination of over 525,000 acres (Frankenberger and Benson 1994). Imported irrigation water containing low concentrations of salts is applied to farmland and leaches the natural occurring soil elements, such as Se, which contaminates the water at concentrations approaching 10 μg Se/L (Mayland 1994). The well-established toxicity of Se to wildlife and humans has caused this element to be regulated by the Toxic Substances Control Act and the Clean Water Act. Once an endpoint for the drainage of Se-contaminated waters, the Kesterson National Wildlife Refuge serves as an example of the toxicological effects of selenium on wildlife, with a 64% rate of deformity and death of embryos and hatchlings of wild birds. Similar situations exist farther south in the Tulare Lake Bed area, the Salton Sea Area and nine other areas in the western United States (Presser 1994). Throughout the central and eastern United States, power plant coal-fly ash receiving ponds create similar environments with Se toxicosis evident in wildlife (Fan et al. 2002).

As a result of the extensive terrestrial contamination, the use of plants to accumulate and disperse Se through phytoremediation has developed into a potential strategy for land reclamation (Pilon-Smits and Freeman 2006; Vickerman et al. 2004). Such large-scale Se

accumulation by phytoremediating plant species has the potential to alter local ecosystems and may adversely affect plant mutualists such as pollinators. However, at least one common weed species found in California (radish, *Raphanus sativus* L.) that is not considered a phytoremediating species is capable of accumulating very high levels of Se in the pollen and nectar. In a semi-field study, honey bee pollinators did not discriminate against foraging on Se-accumulating radish (Hladun et al. *in review*), and in a naturally seleniferous landscape, bees were observed to visit Se hyperaccumulators (Quinn et al. 2011). Certain species of larval herbivores do not avoid Se in plant tissues (Vickerman et al. 2002) and may not detect certain forms of Se (Trumble et al. 1998), and therefore are willing to ingest toxic levels of the element. If honey bee larvae display a similar pattern in feeding, they may consume detrimental quantities of Se in their diet.

Selenium has several different oxidation states including selenate (Se^{+6}), selenate (Se^{+4}), elemental Se (Se^{0}), and selenides or organic forms of Se (Se^{+2}). In most cases, sodium selenate is transported via agricultural irrigation water, then transformed within plants to the organic forms, selenomethionine and selenocysteine (Frankenberger and Benson 1994). Selenomethionine is of particular interest, as it simulates Se toxicosis of wildlife in laboratory feeding studies and is produced by euryhaline microphytes prevalent in agricultural drainage systems (Fan et al. 2002). Interestingly, selenomethionine is a form that is not detected by some insects, allowing rapid ingestion of toxic doses (Trumble et al. 1998). The toxic effects of these selenoaminoacids are likely due to replacement of sulfur with selenium in amino acids, resulting in the incorrect folding of proteins and consequently nonfunctional proteins and enzymes

(Daniels 1996; Lemly 1997). Selenium bioaccumulation has been documented in corixids (Thomas et al. 1999), chironomids (Malchow et al. 1994; Fan et al. 2002), muscids (Simmons et al. 1988), edaphids (Fan et al. 2002) and noctuids (Vickerman et al. 2002). In all cases this occurred through oral exposure, with the bioconcentration factor ranging from 1 to16886. No studies to date have examined Se toxicity in the larvae of Apidae.

Raphanus sativus (radish) is known to accumulate Se in the foliar tissues mostly as selenate (Pedrero et al. 2006). In non-hyperaccumulator plants, selenate can be reduced to selenite (SeO_3^{2-}), assimilated into amino acids as selenomethionine or selenocysteine (Brown and Shrift 1981), then incorporated into proteins. The hyperaccumulator *Stanleya pinnata* (Pursh) Britton (Desert Prince's Plume) and non-hyperaccumulator *Brassica juncea* Czern (Indian mustard) were observed to be actively foraged upon by both honey bee and bumble bee pollinators, further confirming that certain pollinators will not discriminate against Se-accumulating plants despite concentrations as high as 3200 mg Se l^{-1} in the flowers (Quinn et al. 2011). Honey bee pollinators foraged on *R. sativus* flowers that contained concentrations up to 219 mg Se l^{-1} dw (Hladun et al. *in review*). Nectar from *R. sativus* plants grown in the greenhouse contained up to 100 mg Se l^{-1} (Hladun et al. *unpublished data*), and *B. juncea* contained 110 mg Se l^{-1} (Hladun et al. 2011). Based on these concentrations, honey bees have the potential to bring food resources back to the hive that are contaminated with Se at levels shown to be toxic to other insect species. Honey collected from different regions of Turkey contained 0.04 to 0.11 mg l^{-1} (Tuzen et al. 2007). Honey collected from hives located in seleniferous areas of Colorado contained up to 0.73 mg Se l^{-1} (Quinn et al. 2011). In particular, Se in nectar

and honey may be biotransferred from nurse bees to the developing brood as a contaminated food source. The primary objective of this study therefore was to determine whether four forms of Se found in floral tissues of accumulating plants can have a detrimental effect on the larval survival and development in a common pollinator, the honey bee (*Apis mellifera* L., Hymenoptera:Apidae).

Materials and methods

Compounds tested. Sodium selenate (henceforth, selenate, Na_2SeO_4, 98% purity), sodium selenite (henceforth, selenite, Na_2SeO_3, 99% purity), seleno-L-methionine (henceforth, selenomethionine, $C_5H_{11}NO_2Se$, 98% purity), and Se-(methyl) selenocysteine hydrochloride (henceforth, methylselenocysteine, 95% purity) were all purchased from Sigma-Aldrich (St. Louis, MO). These forms of Se were chosen for comparison to toxicity assays using *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae, Trumble et al. 1998; Vickerman and Trumble 1999; Vickerman et al. 2002) and *Megaselia scalaris* (Loew) (Diptera: Phoridae) (Jensen et al. 2005). In previous experiments, two non-hyperaccumulator plant species, *Brassica juncea* L. and *R. sativus* (radish), and accumulated up to 60 mg Se l^{-1} in the nectar when irrigated with selenate in the greenhouse. Pollen contained 1700 mg Se l^{-1} (Hladun et al. 2011) and 710 mg Se l^{-1} (Hladun, *unpublished data*) in *B. juncea* and *R. sativus*, respectively. Therefore we chose ecologically relevant concentrations well below what is found in greenhouse-grown plants in order to account for the possible dilution of Se from the foragers

collecting from both non-accumulating and accumulating plants and to calculate LC_{50} concentrations.

Animals. Tests were performed from March through June 2012 at the University of California – Riverside (UCR, Riverside, CA) using honey bee (*A. mellifera ligustica*) foragers collected from a hive maintained at Agricultural Operations at UCR. The queen was not changed during the course of these experiments to minimize genetic variation. Using methods based on Peng et al. (1992) and Aupinel et al. (2005) the queen was confined to a frame containing empty cells using an excluder cage for 24 hours. The cage allowed workers to move freely from the confined frame and the surrounding colony, while preventing the queen from leaving the cage. This method ensured eggs of a similar age. The queen was then removed and the frame replaced in the cage to prevent any further oviposition. Four days later, the frame was removed and the resulting 1-day old larvae were grafted onto artificial diet.

We reared honey bees *in vitro* using a chronic feeding assay that provisioned the larvae once (Kaftanoglu et al. 2010) with Se-laced artificial diet. Although LC_{50} concentrations can still be used to compare the susceptibility among species, bioassay parameters that consider more than just survivorship can reveal the more subtle, sublethal effects of a toxin. Exposing the species at different life stages may reveal different susceptibilities (Stark and Banks 2003). In addition, chronic exposure represents a more realistic scenario that the bees may experience when foraging in a contaminated area. In our current study, we exposed honey bees to Se during all larval stages, and collected both mortality and development data daily.

Several recent studies have standardized the methods for rearing *A. mellifera* larvae on artificial diet for the purposes of assessing contaminant toxicity. Laboratory *in vitro* feeding assays are preferred over *in vivo* rearing within the colony in order to accurately administer doses in a known quantity of food (Aupinel et al. 2007). Mortality can be reduced in the bioassay through minimal handling (Hendriksma et al. 2011) as well as a single, mass provisioning of food to sustain the individual throughout larval development (Kaftanoglu et al. 2010). Adding other components to the royal jelly such as sugars and yeast extract can also provide sustenance similar to nectar for brood growth and energy (Aupinel et al. 2005; Kaftanoglu et al. 2010; Rembold and Lackner 1981). The improvements in technique for rearing *A. mellifera* larvae *in vitro* has made laboratory toxicity tests more reliable, and by adding Se to the water component of the diet, can mimic contaminated nectar.

Development and survival studies. Selenium-spiked diet was prepared in six concentrations of 0, 0.2, 0.4, 0.6, 1 and 2 mg Se as selenate or selenite l^{-1} diet. For selenomethionine, the six concentrations tested were 0, 2, 4, 6, 7, and 9 mg Se as selenomethionine l^{-1} diet. For methylselenocysteine, the concentrations tested were 0, 2, 4, 6, 7, 9, and 10 mg Se as methylselenocysteine l^{-1} diet. These concentrations were chosen based on preliminary range-finding tests that determined dosage levels as described in EPA Ecological Effects Test Guidelines (OPPTS 850.3020). Artificial diet was prepared as described in Kaftanoglu et al. (2010). The diet consisted of 53% (w/w) commercial frozen royal jelly, 6% glucose, 6% fructose, 1% yeast extract, and 34% distilled water. Se compounds were dissolved into the water portion to yield final target

concentrations in the diet. All grafting tools, petri plates and cell cups, and well plates were UV sterilized before use to minimize contamination (Air Clean 600 PCR Workstation, ISC Bioexpress, Kaysville, UT). Larvae were provisioned once with 200 mg of artificial diet placed inside of queen cell cups (Glory Bee Foods, Inc., Eugene, OR). After grafting, petri dishes were kept in an incubator at 34.1 ± 0.14°C and 92.7 ± 0.35% RH under a 0:24 (L:D) photoperiod. At the prepupal stage on day 11, larvae were weighed and moved to 24 well plates (Costar 3526 cell culture plates, Corning Inc., Corning, NY) to allow more space for pupation. The confounding effect of damage due to grafting was eliminated by removing individuals that had died within 2 days after grafting.

Prepupae were weighed using a microbalance (weighing to 0.00001 g, model 1712 MP8, Sartorius Corp., Goettingen, Germany) on day 11 (Hendriksma et al. 2011). The date of pupation was recorded daily along with mortality for up to 12 days. The days to pupation and the number of individuals that survived to pupal stage were used to calculate the percent pupation.

To determine the potential changes in growth rates, we calculated a growth index (GI) and relative growth index (RGI) (Zhang et al. 1993). Selenium delays development and growth in both *Spodoptera exigua* (Trumble et al. 1998) and *Culex quinquefasciatus* (Jensen et al. 2007), therefore we used similar calculations. Larvae were scored as being in one of three developmental stages: larva, prepupa, or pupa. The numbers of alive and dead individuals in these three stages were scored on a daily basis for up to 12 days. All stages were identified based on descriptions in Snodgrass and Erickson et al. (1992).

Growth index was calculated for days 4 through 12 using equations described by Zhang et al. (1993). The maximum GI in each control replicate was used to calculate RGI's for controls and all treatments by dividing the treatment GI / control GI.

Relative toxicity of selenium forms. The mean lethal concentration that kills 50% of the *A. mellifera* larval population was calculated for each Se form to determine which forms were most toxic, and to compare the LC_{50}'s to other insects in different feeding guilds. Three replicates per treatment level (4 forms x 6 concentrations (7 for methylselenocysteine) containing up to 29 larvae were placed on diet containing 0, 0.2, 0.4, 0.6, 1, or 2 mg l^{-1} selenate or selenite, 0, 2, 4, 6, 7, or 9 mg selenomethionine l^{-1} or 0, 2, 4, 6, 7, 9, or 10 mg^{-1} methylselenocysteine. We calculated the LC_{50} concentrations after chronically feeding the larvae beginning when they were one day old, and continued for nine days. Day 9 was chosen for the relative toxicity values for all 4 Se forms in order to mimic chronic exposure to Se that a terrestrial insect may encounter (Jensen et al. 2007).

Statistical analyses. Four to five replicate petri dishes containing up to 29 larvae were tested at each concentration for the four different Se forms. Results were analyzed with general linear models (PROC GLM, SAS 9.2; SAS Institute, Cary NC, USA) with type III sum of squares; the independent variable was Se treatment concentration. Each Se form was analyzed separately, and prepupal weight, days to pupation, percent pupation, and average percent mortality (after 12 days) were the responses analyzed with ANOVA using replicate as the unit of replication. Mean separations were conducted between groups ($\alpha = 0.05$) using *post hoc* Tukey's HSD test. Assumptions of normality

were examined using the Shapiro-Wilks test. For methylselenocysteine, the number of days to pupation was log-transformed, and percent pupation was arcsine transformed to meet assumptions of normality and homogeneity of variance.

Growth indices were analyzed using MANOVA (PROC GLM) with repeated measures. The independent variable was Se treatment concentration, GI was the dependent variable, and day (over 9 days, or days 4 through 12) was the repeated variable. Mean separations were conducted between groups ($\alpha = 0.05$) using *post hoc* Tukey's HSD test. Each Se treatment concentration was replicated with two to five replicates containing up to 29 larvae each. Each Se form was analyzed separately, and growth indices were summarized within day using replicate as the unit of replication.

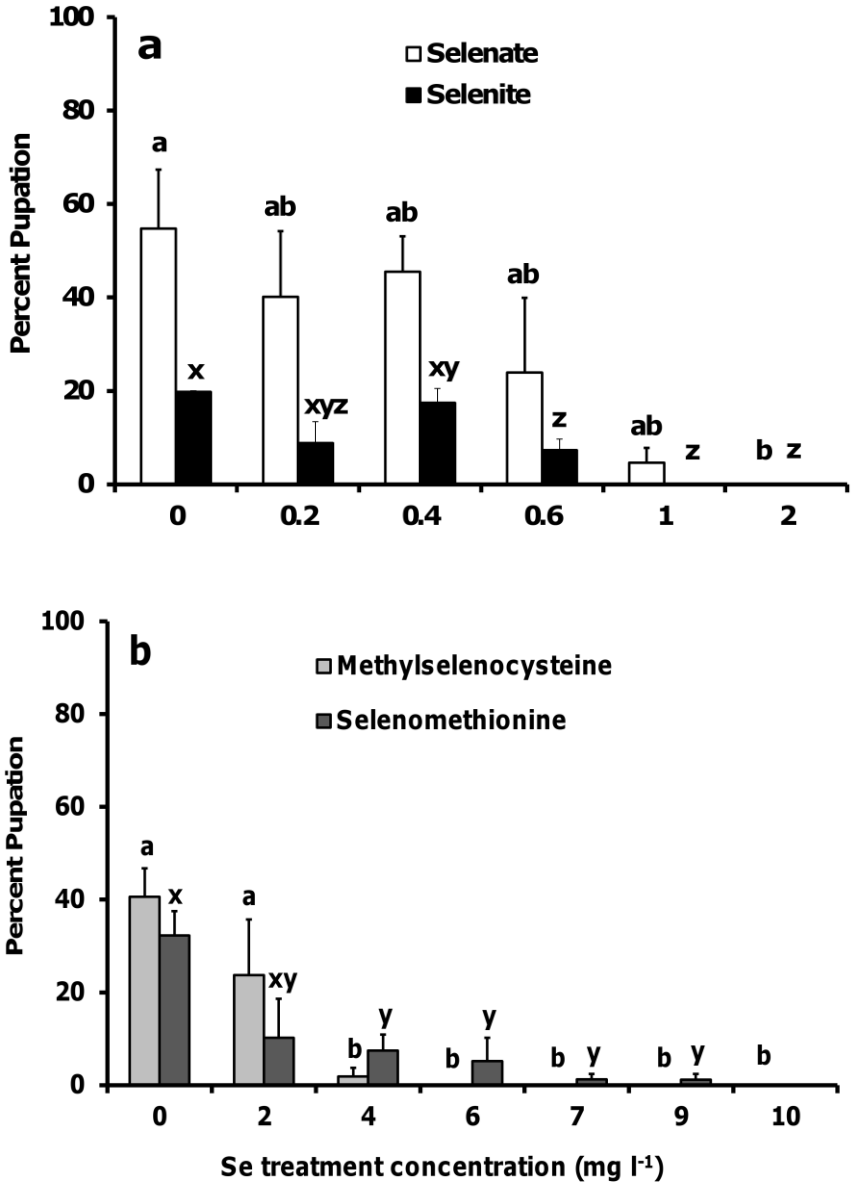
For mortality assays, as recommended in the EPA Ecological Effects Test Guidelines (OPPTS 850.3020), mortality was 20% or less in all control groups. Abbott's formula was used to correct for control mortality (Abbott 1925). For LC_{50} assays, two replicates were used for each treatment. LC_{50} concentrations were calculated using probit analysis (PROC PROBIT, SAS 9.2; Institute, Cary NC, USA). LC_{50}'s were modeled with Gompertz distributions for selenate, and normal distributions for selenite, methylselenocysteine, and selenomethionine.

Results

Pupation and mean mortality. All four forms of Se significantly decreased the percent of larvae that pupated (ANOVA, $F > 4.44$, $P < 0.02$). There was a dose dependent decrease in the number of individuals that pupated by day 12. Selenate reduced pupation by 27 to 100% (Fig. 5.1.a). None of the larvae pupated in the 1 or 2 mg $^{-1}$ selenite treatment groups (Fig. 5.1.a). Methylselenocysteine and selenomethionine reduced the percent pupation by 42 and 68%, respectively in the lowest Se treatment (Fig. 5.1.b). None of the individuals in the 6 mg l^{-1} and higher methylselenocysteine treatment groups pupated. However, there were no significant differences in the number of days to pupation (ANOVA, $F < 3.91$, $P > 0.06$) or prepupal weight (ANOVA, $F < 0.97$, $P > 0.47$) for all four Se forms.

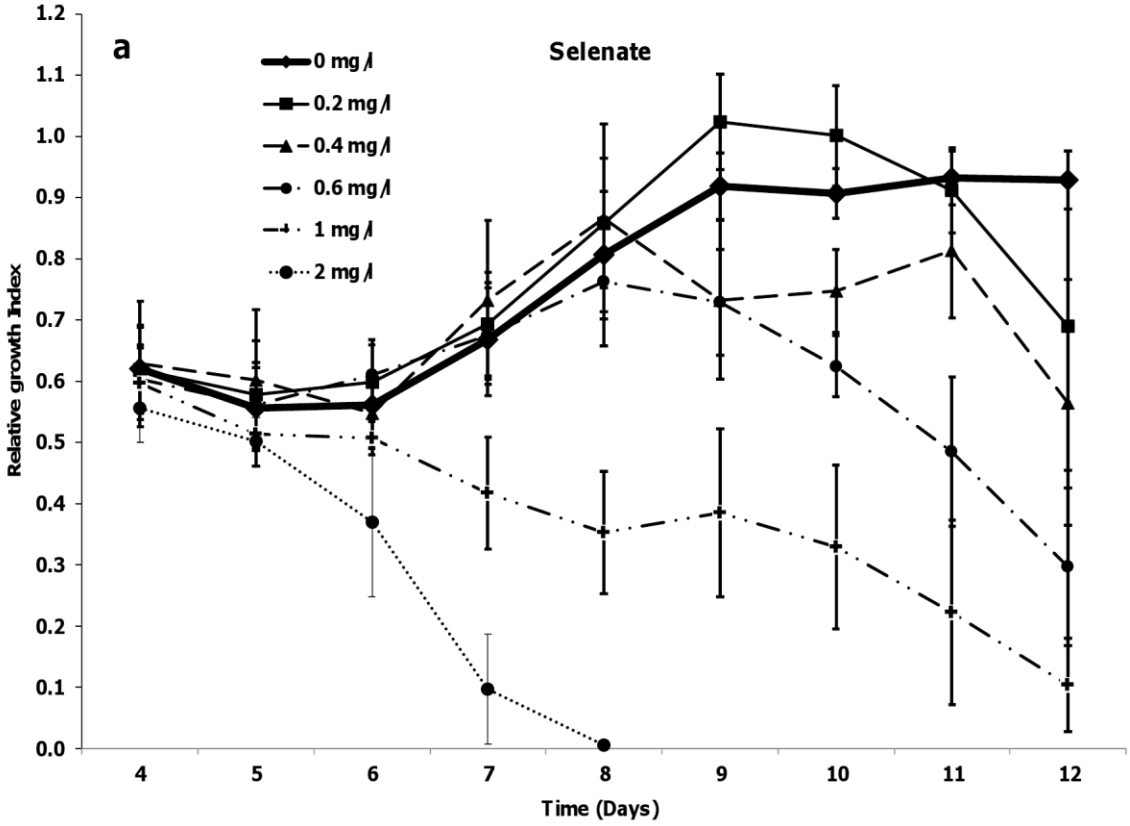
Chronic exposure to selenate and methylselenocysteine has a significant effect on larval survival. Selenate (ANOVA, $F_{5,17} = 9.84$, $P < 0.001$) and methylselenocysteine (ANOVA, $F_{6,17} = 3.60$, $P < 0.05$) significantly increased the average percent mortality. Larvae chronically fed 2 mg l^{-1} selenate in the diet experienced over three times as much mortality as larvae fed control. Larvae in the 9 mg l^{-1} methylselenocysteine group had twice as much mortality as the control.

Fig. 5.1. Effects of Se forms added to artificial diet on (a) percent pupation of selenate (N = 3) and selenite (N = 3, selenate, a-b, selenite, x-z), (b) selenomethionine and methylselenocysteine (N = 3, selenomethionine a-b, methylselenocysteine x-z) for *A. mellifera*. Bars (mean ± SE) with the same letters are not significantly different within SE species at the $P < 0.05$ level (ANOVA, Tukey's HSD).



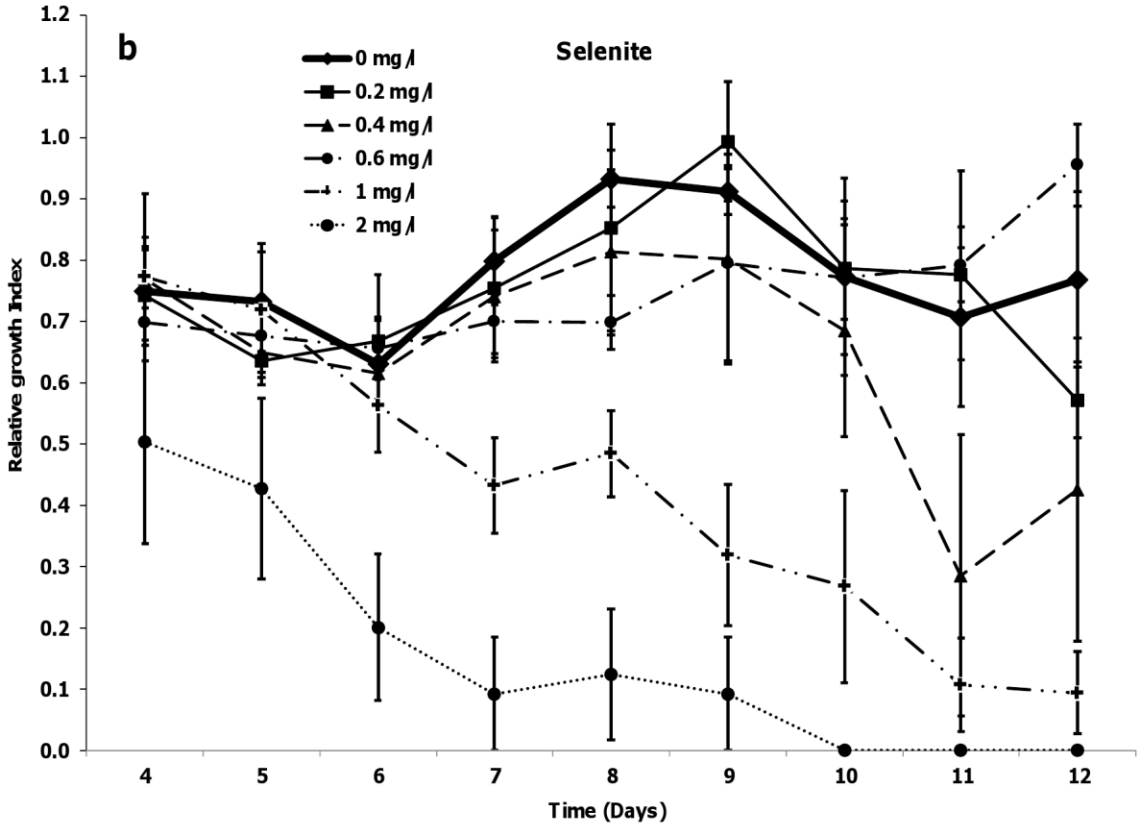
Relative growth indices. The RGIs were calculated for *A. mellifera* larvae exposed to selenate, selenite, methylselenocysteine and selenomethionine and are shown for days 4 through 12 (Fig. 5.2). For selenate, day (MANOVA, Wilks' $\lambda_{8,9}$ =11.32, $P < 0.001$) as well as the interaction of day and Se treatment (MANOVA, Wilks' $\lambda_{40,42}$ =1.87, $P < 0.03$) had an overall significant effect on RGI. There was no significant difference between treatments until day 7. The 2 mg selenate l^{-1} treatment had a significantly lower RGI compared to all other treatments (Tukey HSD test, $P < 0.05$, Fig. 5.2.a). By day 8, both the 1 and 2 mg selenate l^{-1} treatments had significantly lower RGI's compared to treatments 0 through 0.6 mg selenate l^{-1}.

Fig. 5.2.a. Relative growth indices of *A. mellifera* exposed to a range of concentrations of selenate over a 9 day period. Bars represent standard errors for each treatment on the day of observation (summarized by replicate).



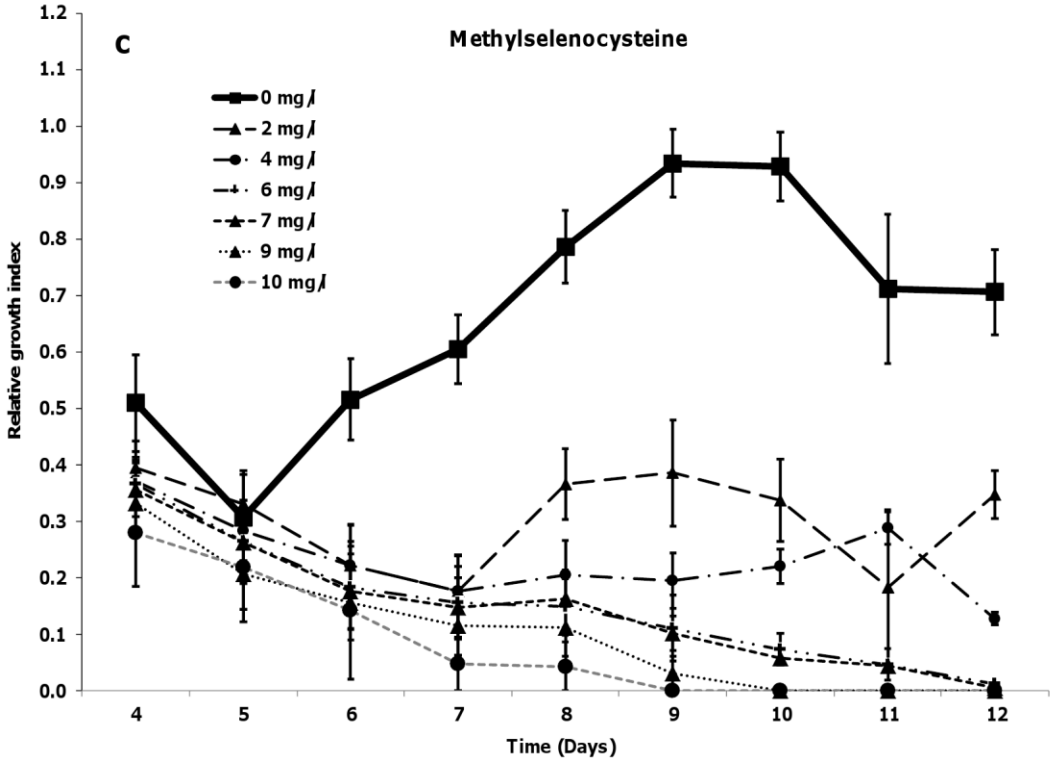
For selenite, day (MANOVA, Wilks' $\lambda_{8,7}$ =8.66, $P < 0.01$) had a significant effect on growth index. However, the interaction of day and Se treatment (MANOVA, Wilks' $\lambda_{40,33}$ = 1.1, $P = 0.39$) did not affect RGI (Fig. 5.2.b), indicating there was no significant difference between treatments across all days.

Fig. 5.2.b. Relative growth indices of *A. mellifera* exposed to a range of concentrations of selenite over a 9 day period. Bars represent standard errors for each treatment on the day of observation (summarized by replicate).



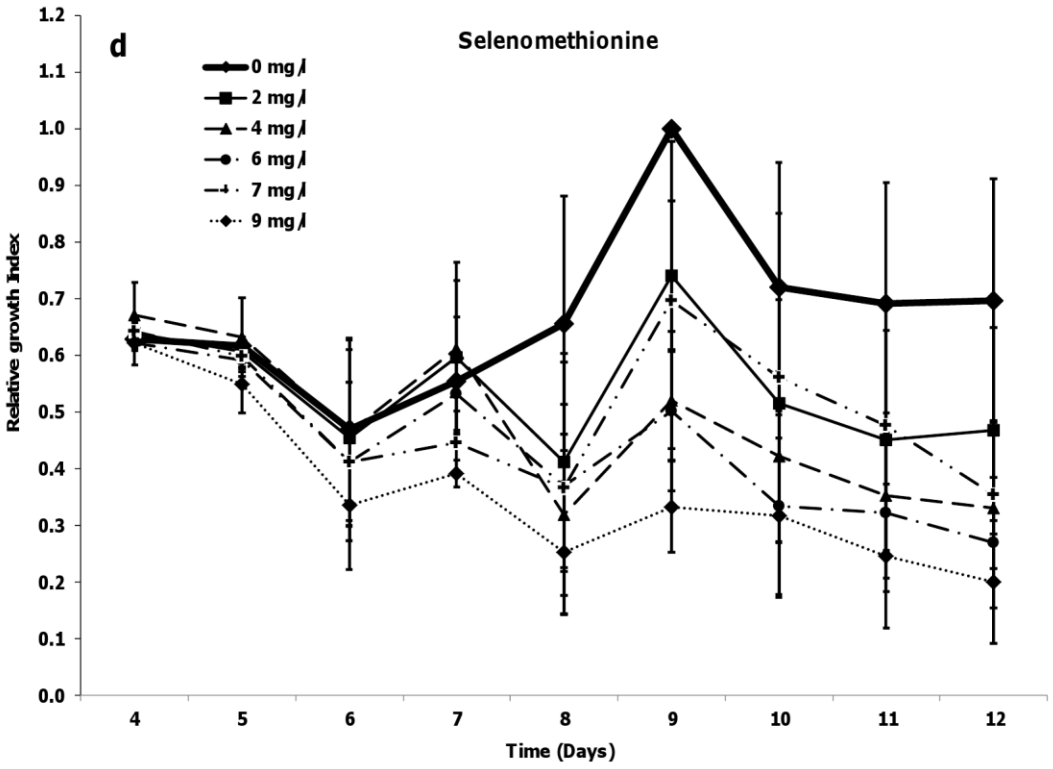
For methylselenocysteine, day (MANOVA, Wilks' $\lambda_{8,16}$ =10.02, $P < 0.001$) as well as the interaction of day and Se treatment (MANOVA, Wilks' $\lambda_{48,83}$ =2.04, $P < 0.01$) had an overall significant effect on RGI. Larvae fed the control (0 mg l^{-1}) had significantly higher RGI's compared to the 4, 6, 7, 9, and 10 mg methylselenocysteine l^{-1} treatments starting on day 7 (Tukey HSD test, $P < 0.05$, Fig. 5.2.c). By day 8, the lowest treatment concentration, 2 mg methylselenocysteine l^{-1} also had significantly lower RGI's compared to control, and the trend continued until day 12.

Fig. 5.2.c. Relative growth indices of *A. mellifera* exposed to a range of concentrations of methylselenocysteine over a 9 day period. Bars represent standard errors for each treatment on the day of observation (summarized by replicate).



For selenomethionine, day (MANOVA, Wilks' $\lambda_{8,11}$ =8.66, $P < 0.001$) had a significant effect on growth index. However, the interaction of day and Se treatment (MANOVA, Wilks' $\lambda_{40,51} = 0.95$, $P = 0.56$) did not affect growth index (Fig. 5.2.d), indicating there was no significant difference between treatments across all days.

Fig. 5.2.d. Relative growth indices of *A. mellifera* exposed to a range of concentrations of selenomethionine over a 9 day period. Bars represent standard errors for each treatment on the day of observation (summarized by replicate).



Relative toxicity of selenium forms. The log-dose probit analysis calculated the LC_{50}'s, or the concentrations that can kill 50% of the population. LC_{50}'s were calculated after the larvae were chronically fed the Se compounds for 9 days. As indicated by the LC_{50} concentrations, selenate, selenite and methylselenocysteine were the most toxic to honey bee larvae, followed by selenomethionine (Table 5.1). All four Se forms had lower lethal concentrations for *A. mellifera* larvae compared to three insect species (Table 5.2) with different feeding regimes.

Table 5.1. Mean lethal concentrations (LC_{50}) from *A. mellifera* bioassays exposing larvae chronically to two inorganic and two organic Se compounds in artificial diet. LC_{50}'s and 95% confidence limits were calculated using log-dose probit analysis (SAS Version 4.2, 2008).

Selenium species	Number of insects tested	LC_{50} concentration ($\mu g\ g^{-1}$ wet weight)	95% Confidence limits
Selenate	373	0.7	0.6-0.8
Selenite	268	1.0	0.9-1.3
Methylselenocysteine	281	4.1	0.7-5.4
Seleno-L-methionine	207	6.0	4.5-7.6

Table 5.2. Mean lethal concentrations (LC_{50}) for three insect species fed Se compounds in artificial diet. LC_{50}'s were calculated using log-dose probit analysis in the studies cited.

Insect species	Selenium forms	LC_{50} ($\mu g\ g^{-1}$, $mg\ l^{-1}$)	References
Megaselia scalaris (Diptera: Phoridae)	Selenate	258	Jensen et al. 2005
	Selenite	392	
	Seleno-L-methionine	130	
	Selenocysteine	83	
Culex quinquefasciatus (Diptera: Culicidae)	Selenate	11.8	Jensen et al. 2007
Spodoptera exigua (Lepidoptera: Noctuidae)	Selenate	21.41	Trumble et al. 1998
	Selenite	9.14	
	Seleno-DL-cystine	15.21	
	Seleno-DL-methionine	21.18	

Discussion

Apis mellifera is an important agricultural pollinator in the United States and throughout the world. In areas of selenium (Se) contamination, honey bees may be at risk due to the biotransfer of Se from foraged plant products including nectar and pollen. In particular, honey bee larvae are more susceptible to ingestion of Se-containing food than adults. The average percent mortality for adult *A. mellifera* foragers was much lower for selenate and selenomethionine when chronically fed for 5 days compared to larvae. Foragers were able to tolerate concentrations as high as 6 mg selenate l^{-1} and 60 mg selenomethionine l^{-1} (Hladun et al. 2012). The forager's ability to tolerate higher concentrations of Se may act against the colony as a whole. In pesticide toxicity studies, foragers that succumb to pesticides quickly prevent exposure to the brood, queen, and coworkers (Atkins and Kellum 1986). However, honey bee foragers are not deterred by Se in sucrose solution (Hladun et al. 2012) or in accumulating plants (Hladun et al. *in review*), suggesting they will actively collect contaminated pollen and nectar, and survive the intake of elevated concentrations of contaminated nectar and pollen. When the contaminated floral resources are then distributed to the hive coworkers, the Se may be passed on to brood and have toxic effects on the more susceptible larvae.

In our study, selenate, selenite and methylselenocysteine were more toxic than selenomethionine. Several forms of Se can occur in the flowers of accumulating plants, particularly selenate, selenite, selenomethionine and methylselenocysteine in different proportions. In the weedy plant *B. juncea*, methylselenocysteine is the predominant form (67% of total Se), followed by selenocysteine (16%) and selenate (11%) (Quinn et al.

2011). Methylselenocysteine is thought to be relatively less toxic compared with selenocysteine (de Souza et al. 1998; Freeman et al. 2006).

All four forms of Se decreased the percentage of larvae that pupated. *Megaselia scalaris* and *Cotesia marginiventris* (Cresson) experienced similar sublethal effects when fed with selenate, including increased number of days to pupariation (Jensen et al. 2005) and reduced pupal weight (Vickerman et al. 2004). In our study, selenate and methylselenocysteine significantly decreased growth indices for *A. mellifera* over time. Selenate ingestion reduced the relative growth rate of the herbivorous caterpillar, *S. exigua* (Trumble et al. 1998), although all concentrations tested were higher than those used in this study. While Se replaces S in amino acids such as cysteine and can change protein folding, disrupting cell metabolism and causing deformities in animals (Daniels 1996; Lemly 1997), the methylation of selenocysteine may prevent its misincorporation into proteins (Brown and Shrift 1981). Thus, the mechanisms for sublethal effects of methylselenocysteine on development are unknown, and further studies are warranted.

In a recent review *A. mellifera* LD_{50}'s were compared to those from other insect species for several insecticides, and they were not found to be more susceptible (Hardstone and Scott 2010). However, in our study the LC_{50}'s for larval *A. mellifera* to the metalloid Se are substantially lower than for other insect species (Jensen et al. 2005, 2007; Trumble et al. 1998). *Apis mellifera* has fewer detoxification genes used for pesticides compared to other insects (Claudianos et al. 2006), and they may also be lacking metal or metalloid detoxification genes as well, which may contribute to the honey bee's sensitivity to the toxicant. Additional experiments examining whole colonies

(especially brood responses) at Se-contaminated sites will be required to document potential effects on population dynamics of *A. mellifera*.

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CHAPTER 6

Conclusions

Summary of Se research and its implications for honey bee health. While most recently published studies have focused on declines in honey bee populations due to pesticide poisoning and colony collapse disorder, the role of pollutants on honey bee behavior and survival has not been examined. This is despite the fact that anthropogenic pollutant concentrations are increasing dramatically in the USA and throughout the world. Our studies reveal that pollutants can have critical sublethal and lethal effects on an important pollinator. The soil-borne pollutant selenium (Se) can biotransfer from the plant to the pollinating bee, and will have both lethal and sublethal consequences upon ingestion of contaminated floral tissues. Se accumulates in non-hyperaccumulating plants such as *Brassica juncea* (Hladun et al. 2011) and *Raphanus sativus* (Hladun et al., *in review*). Se-contaminated areas invaded by these mustards and radishes may provide a portal for Se to enter the ecosystem if other weedy, invasive Brassicaceae behave in a similar manner.

Apis mellifera foragers do not discriminate against Se in sucrose in a laboratory setting, and willingly ingest toxic concentrations of Se. Beyond acute toxicity, honey bees experience sublethal effects in the form of reduced response to sucrose and general malaise (Hladun et al. 2012). Fewer responsive foragers may reduce the incoming floral resources needed to support coworkers and larvae by foraging and recruiting less, but more experiments in the field examining worker activity after Se exposure is needed.

Plants may suffer phytotoxic effects from the Se directly, but this cost may be somewhat offset by the benefits of reduced herbivory (Hladun et al., *in review*). Pollinators do not discriminate against Se-accumulating plants. If bees are willing to visit

the flowers and bring contaminated pollen and nectar back to the hive, it may reduce the worker population, thus reducing other incoming food resources, and leading to the weakening of the colony as a whole. Se in various forms has lethal and sublethal effects on both honey bee adults and larvae. Inorganic forms of Se kills *A. mellifera* larvae earlier and the organic forms have sublethal effects on development (Hladun et al., *in prep*). Brood may experience increased mortality when fed contaminated food resources, thus reducing the future worker population, and increased development times may lengthen exposure to pests and diseases. On the other hand, honey bees may dilute the amount of Se they receive by foraging on both non-accumulator and accumulator plants, and low levels of Se may have beneficial impacts on colony health such as reduced disease or predation (Barillas et al., 2011). Foragers, pollen, honey and wax from within the hives in contaminated areas of the San Joaquin Valley of California can be sampled to determine the actual concentrations of Se being brought back to and stored in the hive. This work represents a crucial first step towards understanding the impact environmental stressors can have on honey bee populations.

Pollinators in the toxic landscape as bioaccumulators and bioindicators of pollutants. Honey bees forage over very large areas and bring plant materials (nectar, pollen and propolis) back to their hives, and thus may collect significant amounts of toxic contaminants, thus making them ideal bioindicators of pollutants (Celli and Maccagnani 2003; Kevan 1999). Varying amounts of contaminants that are toxic to insects have been found in honey bee hives and their products, particularly when located in close proximity to polluted sites, and most research regarding pollutants and bees focus on their use as

bioindicators. Moving beyond using bees as bioindicators, there is a complete lack of toxicological data on environmental contaminants' effects on pollinators beyond pesticides. Synergistic effects may occur between pollutants and other stressors such as pests, invasive species, pathogens and habitat loss (Potts et al. 2010).

Soil pollutants can biotransfer to pollinators through dermal exposure to ground-nesting bees or transport into floral tissues by an accumulating plant, which can then be collected by bees. The increasing abundance of soil due to extensive farming and industrialization may put pollinators at risk. Agroecosystems are contaminated with fertilizers such as manures and biosolids that contain higher concentrations of elements (such as Cu, Zn, B, Fe, and Mn) than agricultural soils. Soil contamination by fertilizers is a problem in China, but not so much in other countries where metal concentrations in organic fertilizers are regulated, such as in the United States (He et al. 2005). Fungicides are repeatedly applied to fruit crops in the US and infuse the soil with increasing concentrations of Cu. Point sources of soil pollution from mining activities can create mine spoils (disposal of metal-rich excavation wastes) or mine tailings from acid ores, thus releasing high concentrations of metals into the environment. Industrial activities such as fly ash from power generators as well as automotive emissions dispense Pb, Cd, Cr and other metal or metalloid pollutants into the atmosphere, and these elements can be found in honey bee hives adjacent to urban areas (Conti and Botrè 2000). Airborne pollutants such as Pb can expose the entire hive, or may be deposited onto flowers and collected by bees.

The current expansion of research on pollutants' effects on pollinators is beginning to advance from the individual to the community level. Pollutants can impact the functional ecology of an ecosystem, and a recent study focused on the species richness and abundance of a wild bee community adjacent to a smelter plant in Europe (Moroń et al. 2012). The population numbers of solitary wild bees such as *Megachile centuncularis* and *Hoplitis adunca* were greatly reduced along an increasing gradient of heavy metal pollution. Special conservation strategies for polluted areas may need to be implemented to supply the area with artificial nests and alternative food resources with wild flower seed mixes that do not accumulate metals (Moroń et al. 2012) in order to minimize exposure.

Not only honey bees are at risk, and there is a need for toxicology studies using other bee species besides *Apis mellifera*. Native pollinating bees contribute almost $3.07 billion of fruits and vegetable production in the United States (Losey and Vaughan 06). In addition, toxicological studies need to focus on more than just generalists (such as the honey bee), but specialist pollinators as well. Traits that can make bees more susceptible to environmental stressors (such as pollutants) include feeding habit and foraging range (see Williams et al. 10). Polylectic species (generalist feeders) may not collect pollen from plants that accumulate high levels of heavy metals. Oligolectic species (specializing on feeding on only a few plant species, even only one genus or species, such as *Hoplitis adunca* specializing on *Echium* in Moroń et al. 2012) are limited to certain plants that may be contaminated. From the plant's perspective, if specialist pollinators are indeed more susceptible and eliminated in the contaminated environment, certain

plant species that depend solely on these specialists may lose their ability to reproduce. The mean flight distance for most solitary bee species is <200 m (Gathmann and Tscharntke 2002), and they are more likely to be forced to forage on plants in localized, contaminated areas. Social bees such as honey bees (up to 7 km^2) and bumble bees (1-2 km^2) cover large distances during foraging and may be more likely to encounter uncontaminated food resources to dilute any toxins. In addition, foragers can act as a first line of defense against environmental stressors, succumbing in the field before bringing the toxin back to her coworkers, brood, and queen. Thus, there is a great need for additional research on the toxicological and ecological impacts on non-*Apis* bees, the community effects on native bee populations as a whole, and the fitness consequences for plants due to the alteration of pollination ecology in the polluted landscape.

Future directions. The research described in this dissertation was limited in that it only examined a single element, selenium, and its impact on insect-plant interactions. Future directions for research will involve examining other pollutants (both soil and airborne) and their effects on *Apis mellifera*. Experiments involving the sublethal effects of toxicants on honey bee learning, colony health, and foraging behaviors in the field are planned for the years ahead.

Olfactory conditioning assays using proboscis extension reflex (PER) can reveal the sublethal effects of contaminants on learning and memory in the honey bee (Devillers and Pham-Delegue 2002). Aversive learning assays involve training bees to an odor and conditioning them with a contaminated reward. By adding toxins to artificial nectar (administered in a sucrose solution), the taste or smell of the chemical associated with the

food may decrease the value of the reward, causing reductions in learning (Wright et al. 2010). By adding toxins to artificial nectar (administered in a sucrose solution), the potential deterrent taste of the chemical associated with the food may decrease the value of the reward. In addition, Se as a volatile may have a repellent effect. Simple olfactory learning assays can be used to detect the behavioral effects of toxicity. Honey bees dosed with the toxicant may lose their ability to learn or recall odors, either due to their reduced sucrose response (as seen in Hladun et al. 2012) or changes in olfactory perception and memory. Honey bee behaviors may not be affected physiologically, and foragers may learn and recall a trained odor just as rapidly despite being dosed with the pollutant. However, the sublethal behavioral effects will most likely be dependent on the dose concentration. Honey bees may be able to tolerate low levels of a toxicant (as was the case with selenium), but this may vary from element to element.

Choice tests can determine if honey bee foragers show a preference for an uncontaminated food source. Using a modified methodology from Detzel and Wink 1993, bees can be placed in a cage with small Petri dishes containing a control (sucrose only) and a contaminant treatment in sucrose solution. The dishes will be weighed before and after the assay to determine whether the bees prefer to feed on one food source or the other. The number of visits to each dish can be quantified as well as total consumption.

Adults and larvae within the colony can be exposed to environmental contaminants when pollen and nectar collected by foraging bees are brought back to the colony. If foraging bees are killed in the field due to toxin exposure, then there may be reduced food resources brought back to the colony. Using nucleus colonies, bees will be fed

contaminated artificial pollen and nectar as a sole food source. Colony health metrics can be measured such as development (mean area of new storage cells filled), brood production (change in mean area of brood cells) and survival (total numbers of workers). Nest size from comb construction, food stores and population size (measured as the entire weight of the colony), and population growth in terms of amount of capped and uncapped brood can be used as measures of whole-colony fitness (Seeley 1985). To determine the sublethal effects of contaminants on foraging behaviors, the mean number of pollen and nectar foragers returning to the hive entrance per minute can be quantified. Foraging traffic after contaminant feeding treatments have begun may reveal a reduction in response to food resources as has been demonstrated in foragers fed the contaminant selenium in laboratory sucrose response threshold assays (Hladun et al. 2012).

The quantity of pollutants are increasing with our rapidly expanding human population worldwide, and the primary pollinators that are needed to produce the food to sustain this population may be negatively impacted by anthropogenic pollutants beyond selenium. Scientific studies are need to determine which contaminants will be the most important to regulate and minimize pollinator exposure. By informing beekeepers and growers of the types of plants that can accumulate pollutants in contaminated areas, steps can be taken by either moving hives to uncontaminated areas or removing critical weedy plants that concentrate pollutants in nectar and pollen. However, without the basic information about which pollutants in particular are most detrimental to bees, we cannot preserve ecosystem services such as pollination. Further research is needed examining the impact of pollutants on foraging behaviors, insect detection, learning, development,

and survival. In addition, the interaction of pollinator and plants in the polluted environment must be closely evaluated in order to reveal the sometimes subtle impacts on plant fitness in both agricultural and natural ecosystems.

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