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Selenium in Ant Communities: Implications of Contamination on Ecosystem Ecology, Diversity, and Invasive Potential

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Selenium in Ant Communities: Implications of Contamination
on Ecosystem Ecology, Diversity and Invasive Potential

A Dissertation submitted in partial satisfaction
of the requirements of the degree of

Doctor of Philosophy

in

Entomology

by

Deborah Grace De La Riva

June 2016

Dissertation Committee:

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ABSTRACT OF THE DISSERTATION

Selenium in Ant Communities: Implications of Contamination on
Ecosystem Ecology, Diversity and Invasive Potential

by

Deborah Grace De La Riva

Doctor of Philosophy, Graduate Program in Entomology
University of California, Riverside, June 2016
Dr. John T. Trumble, Chairperson

Ants have often been described as keystone species for the critical roles they play that contribute to ecosystem functioning. Evidence from Europe and Australia has improved our understanding of the impacts of disturbance, including pollution, on native ant communities. However, there is a lack of information available for North American ant communities. The overall goal of this dissertation was to assess the effects of selenium, a globally widespread element and environmental contaminant in certain regions of the U.S., on the survival, behavior and reproduction of the invasive Argentine ant, *Linepithema humile*. The findings of this research highlight the unexpected role of pollution to act as a potential barrier to range expansion for invasive ant species.

Chronic ingestion of four environmentally common forms of selenium at environmentally reported concentrations, were found to cause mortality among Argentine ant workers. The extent of mortality was found to be dependent on the form of selenium. In addition, mortality and bioaccumulation of selenium increased with the duration of exposure and concentration. Furthermore, Argentine ants were not deterred by lethal concentrations of selenium in the diet, regardless of background sucrose concentrations.

The transfer of selenium via a tri-trophic system was assessed. Aphid honeydew, a major component of the diet for Argentine ants in their introduced range, was found to serve as a route of selenium transfer to ant colonies.

Sub-lethal concentrations of selenium were found to impact reproduction for Argentine ant colonies, where colonies exposed to selenium sucrose diets experienced reduced queen fecundity, queen mortality, and reduced viability for offspring. Selenium did not alter the competitive ability of Argentine ant workers during interactions with the native species, *Dorymyrmex bicolor*, but sub-lethal concentrations did contribute to a longer food discovery time for the native species.

Native ant communities residing in a selenium-contaminated habitat were also documented. Ant species composition differed across sites, but selenium did not appear to have an overall impact on diversity. Ant concentrations differed by species, but were comparable to levels found among other arthropod trophic groups. Results suggest that these ants residing have evolved a high tolerance to this particular pollutant.

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Introduction

Ants and Ecosystem Functioning

Ecological services provided by insects, in the United States alone, amount to a value of over $50 billion per year (Losey and Vaughan, 2006). Among the most ubiquitous animals on earth are ants, contributing greatly to this value via the direct and indirect beneficial services they provide to humans and ecosystems. Ants are considered to be keystone species and ecosystem engineers in many ecological communities (Power et al. 1996; Folgarait 1998; Lach et al. 2010) due to the critical roles they play in several ecological processes such as nutrient cycling, soil aeration, natural pest control, pollination and seed dispersal.

As agents in soil manipulation, ants can bring about changes in both physical and chemical characteristics of the soil (Folgarait 1998; MacMahon et al. 2000). Examples of such manipulation include improved soil aeration and drainage following construction of underground galleries (Folgarait 1998; MacMahon et al. 2000), an increase in organic matter and nutrients as result of food storage and accumulated insect feces, corpses and litter (Pętal et al. 1977; Folgarait 1998; MacMahon et al. 2000), as well as bringing subsoil to the surface during nest excavation (Folgarait 1998; MacMahon et al. 2000). Each of these activities can enhance the availability of resources to surrounding microorganisms, plants and animals in these communities (Lach et al. 2010).

Ants also have the potential to significantly alter arthropod communities in which they reside, as a result of their predatory, mutualistic and tending behaviors. A single colony of wood ants has been documented to have an intake of 459 g of solid food (insects) over a 14 h period; it is estimated that a large colony of wood ants is capable of

catching 100,000 insects per day (Holt, 1955). Taking advantage of their predatory

dominance, humans have used ants as agents of biological control in horticulture

industries around the world for centuries (Blüthgen and Feldhaar, 2010). For instance,

ants were shown to reduce the abundance of herbivores such as the fall armyworm

(Lepidoptera) and the corn leafhopper (Hemiptera) in maize fields in Nicaragua in the

absence of pesticides (Perfecto, 1991). The potential for ants as biological control agents

against lepidopteran pests was also demonstrated in coffee farms in Mexico (Vandermeer

et al. 2002). A meta-analysis conducted by Rosumek et al. (2009), comparing the

influence of ants on plant pests over a wide variety of herbaceous and woody plants

found that plants without ants suffered nearly twice as much feeding damage compared to

plants with ants. Plants in which ants were removed also suffered a reduction in biomass,

leaf production, and reproduction. However, consistently positive results might be

difficult to achieve in cases where plant pests offer ant predators a reward in the form of

honeydew, a sugar-rich liquid secreted by insects such as aphids and scale insects. In

these cases, the presence of ants can have a negative effect on plant fitness if ants choose

to protect herbivores against other natural enemies (Styrsky and Eubanks, 2007).

Nevertheless, there are still instances in which the presence of pest-tending ants has an

overall positive effect on plant fitness when predation on other, more damaging

herbivores also occurs (Rico-Gray and Oliveira, 2007; Styrsky and Eubanks, 2007).

Plants that have adapted structures providing their own rewards such as extrafloral

nectaries or other food bodies can avoid this problem altogether as is the case in the

mutualistic *Pseudomyrmex* (ant)-*Acacia* (plant) relationship. Ants guard plants from

herbivores as well as competing plants in return for food and shelter provided by plants (Rico-Gray and Oliveira, 2007).

In addition to the indirect effects on arthropods and plants discussed above via their engineering and predatory roles in an ecological community, ants can also have a direct effect on plant fitness and diversity. This is seen in instances when ants act as pollinators and seed dispersers. Reports on pollination by ants have mixed results ranging from instances in which visits to plants by ants for nectar ranges from nectar thievery to a positive effects on plant fitness. Despite their abundance and distribution, the ability for ants to play a role as pollinators has been controversial. Characteristics such as frequent grooming, bodies not suited for pollen adhesion, possible limits in foraging ranges among non-winged members and the presence of the antibacterial secretion from the metapleural gland of some species which inhibits pollen germination (Beattie et al., 1984), have all been stated as reasons for the inability of ants to pollinate. In addition, evidence for the presence of deterrent compounds in the floral nectar of several plant species (Guerrant and Fiedler, 1981), are thought to act as plant defenses against potential nectar robbers. However, ants have been reported to travel hundreds of meters in search of food (Lach et al. 2010) and have been demonstrated to carry pollen grains in which many cases of successful pollination have been observed to occur (Rico-Gray and Oliveira, 2007). Especially in the cases in which the relative abundance of other potential pollinators is low, ants have been shown to act as sufficient pollinators (Gómez et al., 1996).

In addition to impacts on plant fitness, ants can influence the diversity, composition and persistence of plants species in their communities via seed dispersal. Myrmecochory, seed dispersal by ants, is both geographically and ecologically widespread. Dispersal by ants has been found to occur in Australia, Africa, North America, Japan and the Meditterranean amounting to approximately 3,000 plant species (Rico-Gray and Oliveira, 2007). Selection for seeds by ants is largely a function of seed size (Samson et al. 1992; Christian 2001; Rowles and O'Dowd 2009) and the presence of a reward (MacMahon et al. 2000; Rico-Gray and Oliveira 2007) such as an eliasome. Eliasomes are lipid-rich structures attached to seeds of certain plant species. These structures are removed and consumed by ants without damaging the seed. Seeds are carried a distance and discarded along a foraging path after consuming the reward or brought back to the nest and discarded or buried. There are several benefits other than simple dispersion to these plants by ants. Seeds taken back to the nest or buried are safe from predation. Ant nests also provide a nutrient rich environment for seed growth. Competition between plants for microsites is also reduced (Rico-Gray and Oliveira, 2007). In contrast, plants producing seeds without eliasomes or seeds with eliasomes that have been removed by other non-dispersing insects or rodents are then at a disadvantage as their seeds are likely to be consumed or ignored (Ohara and Higashi, 1987). Secondary dispersal by ants, in which birds rather than ants are the primary disperser, has also been observed to occur in tropical forests and savannas (Rico-Gray and Oliveira, 2007). In these cases ants may be after the fleshy fruit and subsequently disperse seeds within. Predation by ants is a third possibility in many environments as the seeds

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themselves are high in lipids and protein (Janzen, 1971); however, seeds in such

environments may also contain physical and chemical defenses. Nevertheless, in each of

the three scenarios, ranging from direct dispersal to predation by ants, the effect on the

composition of the plant community is evident. Preference for one seed type or seed size

by ants can affect the abundance and diversity of plant species found in a given

community (Samson et al. 1992). This, in turn, has the potential to indirectly affect

competition between plant species as well as the abundance and diversity of arthropod

herbivores relying on those species.

Threats to Ecological Dynamics in Ant Communities

The fundamental role played by ants in many ecological processes in which they

occur cannot be ignored. However, natural and human caused disturbances can lead to a

loss in ecosystem services provided by ants, while at the same time act as a potential

barrier to movements of un-adapted or invasive ant species. One such contaminant that

may have the potential to impact both native and invasive arthropod communities is

selenium.

Selenium (Se) is a widespread naturally occurring trace element derived from the

weathering of Cretaceous marine sedimentary rock (Frankenberger and Benson, 1994).

The role of selenium (Se) as a potential environmental toxin for mammals, fish, birds and

plants has been established (Eisler, 1985; Frankenberger and Benson, 1994; Ohlendorf et

al., 1988, 1986; Santolo, 2009, 2007), however, comparatively few studies have

investigated the role of selenium on invertebrates (Hladun et al., 2013, 2012; Jensen et

al., 2005; Vickerman and Trumble, 2003). This element has captured the interest of many

scientists because there exists a fine line between deficiency and toxicity of this element to humans and wildlife (Council, 1983). In humans and animals, selenium acts in conjunction with the enzyme glutathione peroxidase as an important antioxidant (Council, 1983; Daniels, 1996), but intake beyond the daily requirement can lead to selenium toxicity. Symptoms of toxicity range from nausea and vomiting to nail and hair loss in humans following ingestion of se-rich food. Abnormal breathing, blind staggering and death in livestock have been reported following ingestion of se-accumulating plants (Council, 1983).

Selenium is not an essential element for the majority of plant growth and plants will most often not survive if selenium contamination is introduced into the soil and water. There are, however, many plants that have adapted the ability to successfully grow on Se-contaminated soils and have the potential to accumulate selenium at levels well beyond that present in the soil. These are referred to as selenium accumulators and they fall under three main categories: primary accumulators, secondary accumulators and non-accumulators (Eisler, 2007; Frankenberger and Benson, 1994). Primary accumulators also known as "hyperaccumulators" or "indicator species" are those that require 1-50 mg Se/kg for growth and can accumulate 100-10,000 mg Se/kg in their tissue. Common plants genera in this category include species *Astragalus, Stanleya, Machaeranthera,* and *Oonopsis*. Secondary accumulators do not require selenium for growth, but may contain 25-100 mg Se/kg when grown on Se-rich soil. Plant species accumulating found to accumulate these levels include species within the genera *Aster, Gutierrezia, Atriplex*, and *Comandra*. Lastly, plants containing 1-25mg Se/kg when

grown on seleniferous soils are considered non-accumulators (Eisler, 2007) and include

grasses, small grains and shallow-rooted plants (James et al., 1989). In the majority of

cases, primary and secondary selenium accumulating plants are the causes of selenium

poisoning to livestock (Eisler, 2007, 1985; Frankenberger and Benson, 1994; James et al.,

1989). This is thought to explain what Marco Polo described, during his travels in

thirteenth century western China, as a hoof disease among his horses after feeding on

certain plants (Frankenberger and Benson, 1994).

Although Se is naturally mobilized and leached out of sediment following rainfall,

certain anthropogenic activities such as mining or agricultural runoff can also contribute

to high levels of Se than would not be present otherwise (Eisler, 1985). This is especially

true in the western United States where large portions of land are thought to be

susceptible to irrigation induced selenium contamination due to underlying layers of

seleniferous bedrock (Seiler et al. 1999). Such was the case in the late 1960s to early

1980s at Kesterson Reservoir, located at San Joaquin Valley, California, where diverted

subsurface drainage water into the reservoir was found to contain high levels of selenium

resulting in the embryo deformities and mortality among the residing waterfowl (Garone,

1998; Ohlendorf and Santolo, 1994; Ohlendorf et al., 1986). The 12 ponds comprising the

reservoir have since been filled in and converted to a terrestrial habitat to prevent further

exposure and toxicity to wildlife. Several species of plants with the potential to

accumulate selenium are now growing in the habitat (Frankenberger and Benson 1994)

that include plant species within the plant families Brassicacae, Poaceae, Asteraceae, and

Fabaceae.

Contamination within this habitat and the presence of selenium accumulating plants is likely to have affected the subsequent diversity of arthropods. Among plants that have been identified as successful accumulators, Se has been found to be present at significant levels in the seeds, nectar, and pollen (Bañuelos et al., 2011; Hladun et al., 2011; Quinn et al., 2011), all of which ants feed upon. Unfortunately, there is a lack of available information on the effects of introduced selenium on arthropod communities where Se-rich soils have been reported to exist. There is currently no published information available from studies at this site on the impact or accumulation of this element for ants (CH2MHILL and Laboratory, 2000; CH2MHILL, 2015). Due to the fact that the majority of ant species are generalist omnivores, feeding on both plant resources and arthropods (Blüthgen and Feldhaar, 2010), several possible routes exist for exposure and ingestion of this potentially toxic element to ants. These include: exposure to soil or plant tissue during nest excavation, possible ingestion of pollen (Czechowski et al., 2008; Urbani and de Andrade, 1997), nectar and seeds from Se-accumulating plants, tri-trophic acquisition via ingestion of herbivores from these plants, and ingestion of honeydew secreted from these herbivores. Ants thus provide a novel and versatile system for studying acquisition of a pollutant, in this case selenium, and the consequences of ecosystem pollution on ant communities. One such consequence might include the impact on establishment of an invasive ant species, where pollution might serve as an obstacle in its success.

Biological Invasions

    Biological invasions have been described to resemble chemical disturbances in

that they can have similar detrimental effects on ecosystem dynamics (Heliovaara and

Vaisanen, 1993). For this reason, invasions present unique opportunities to study

ecological dynamics while also revealing traits in organisms that promote behavioral and

ecological dominance (Krushelnycky et al., 2010). It is estimated that 6500 species of

arthropods have been introduced in the US alone, with accidental introductions

accounting for 95% (Pimentel et al. 2005). The cost of damage in Texas alone by the red

imported fire ant, *Solenopsis invicta*, is estimated at $300 million/year with an additional

$200 million/year invested towards control of this ant (Pimentel et al. 2005). Introduction

of the Argentine ant, *Linepithema humile*, in southeastern Australia resulted in

elimination of a native seed dispersing ant species. Seed dispersal of a native plant

species was subsequently reduced and dispersal of an invasive plant species increased,

due to preference by Argentine ants (Rowles and O'Dowd, 2009). Invasion by the

Argentine ant in shrublands in South Africa also resulted in displacement of two native

seed dispersing ants preferring large seeds. Large-seeded plants then declined in density

due to an increase in vulnerability to predation by other animals. This evidence indicates

that plant species that rely on specialized partners for dispersal of their seeds are at

greater risk following a biological invasion by ants (Christian, 2001). Similarly, the

presence of the Argentine ant in Hawaiian high-elevation shrubland also caused a

reduction in abundance of several endemic arthropod species and an increase in

abundance of an invasive Isopod species (Cole et al., 1992).

Sufficient evidence exists for the consequences of a biological invasion on interactions, diversity and the persistence of native species in effected communities. There is also available evidence as to some of the underlying causes promoting or encourage successful invasion. Such factors include human and non-human caused disturbances such as flooding, soil perturbation and tree removal (Krushelnycky et al., 2010). Competitive behavior on the part of both the invading and native ant species is also an important factor in determining whether or not an invasive ant species can successfully invade (Holway and Suarez, 1999; Krushelnycky et al., 2010). Factors inhibiting the ability to compete also involve the health of the defending or invading colony. In one study, infection of red imported fire ant workers by a pathogen caused a reduction in their ability to prevent invasion from a native ant species (Keck, 2005). Sorvari et al. (2007) found that the immune responses, measured via encapsulation responses, of a wood ants were enhanced when exposed to moderate levels of heavy metals but suppressed when exposed to high levels. Kabashima et al. (2007) reported an effect of pesticide exposure on the competitive behavior of the red imported fire ant towards the Argentine ant. If chronic exposure to environmental toxins, such as selenium, were to similarly cause a reduction in the health of ant colonies in contaminated environments, they too might be less able to compete and thus be susceptible to displacement by invading species. On the other hand, contamination in a neighboring environment might also inhibit the spreading of an invasion species if native species have already developed resistance to the toxin. There is currently limited information on the effects of pollution on the invasive ecology of ants. This work

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provides a novel avenue and model system for investigating the potential for invasion in the presence of a pollutant. It will also give crucial insight into potential factors inhibiting or encouraging the successful establishment of an exotic invader.

Dissertation Objectives

In order to determine the potential for selenium to act as a barrier to range expansion for the invasive Argentine ant we proposed the following objectives/questions: 1) Determine if ecologically occurring levels of selenium are toxic to Argentine ants. 2) In addition, determine whether selenium in the diet would act as feeding deterrent. 3) If chronically ingested, at what levels does it bio-accumulate within worker ants? 4) Can selenium be acquired from sources other than floral nectar such as honeydew or insect prey? If so, is there a difference in the levels accumulated by ants depending on their source of ingested selenium? 5) Does chronic ingestion of sub-lethal levels of selenium have detrimental effects for queen fecundity and offspring viability? 6) Might sub-lethal levels also influence the competitive ability of a native and invasive ant species? 7) What influence does selenium contamination have on the diversity of native ant communities as measured by species richness, composition, and nest density?

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Chapter 2

Impact of Selenium on Mortality, Bioaccumulation and Feeding Deterrence in the

Invasive Argentine Ant, *Linepithema humile* (Hymenoptera:Formicidae)

Abstract

Ants are known for the important roles they play in processes contributing to ecosystem functioning in many habitats. However, pollutants can impact the ecosystem services provided by ants. The Argentine ant, an invasive species in North America, was investigated for the potential impact selenium may have on ants residing within a contaminated habitat. Mortality tests were conducted among worker ants feeding on a range of four environmentally common forms of selenium (seleno-L-methionine, methylselenocysteine, selenate and selenite) from an artificial nectar source. Accumulation of selenium in ant bodies at the end of two weeks was quantified with the use of hydride generation atomic absorption spectroscopy. Lastly, we conducted choice tests to determine whether ants might avoid a carbohydrate diet containing selenium by providing them a choice between control sucrose and sucrose containing selenium. The results of this study indicate that form and quantity of selenium, as well as time of exposure impact mortality in Argentine ant workers. Methylselenocysteine and selenate were found to be the most toxic among the four forms when presented in sucrose solutions, whereas seleno-L-methionine and selenite caused greater selenium body burdens. Furthermore, choice tests showed that ants did not prefer control sucrose solution to sucrose treated with selenium. These findings serve as first look into the possible detrimental impacts contaminants may pose for ants that frequent sugary nectar sources.

Introduction

Ecosystem services provided by insects in the United States are estimated at a value of $50 million yr^{-1} (Losey and Vaughan, 2006). Ants are considered to be keystone species and 'ecosystem engineers' in many ecological communities (Power et al. 1996; Folgarait 1998; Lach et al. 2010) due to the critical roles and ecosystem services they provide. These fundamental processes include nutrient cycling (Pętal et al. 1977; MacMahon et al. 2000) soil aeration (Folgarait, 1998), pollination (Gómez et al., 1996), seed dispersal (Samson et al. 1992; MacMahon et al. 2000; Christian 2001) and natural pest control (Perfecto 1991; Vandermeer et al. 2002; Rosumek et al. 2009).

Unfortunately, natural and anthropogenic disturbances in the environment may have adverse implications for ecosystem functioning if they impact the organisms providing those services (Vanbergen and Initiative, 2013). Previous studies reported negative impacts of heavy metal pollution from Finnish smelters on wood ant physiology (Sorvari et al., 2007), changes in nest mound volumes and abundance (Eeva et al. 2004) as well as impacts of sulfur released from mining operations in Australia on abundance and richness in surrounding ant populations (Hoffmann et al. 2000). However, not all trends are negative (Grześ, 2009), because ant species have considerable variation in metal regulation physiology (Grześ, 2010). Interestingly, we found no reports on the impacts of other lesser-known but common contaminants on ants, such as the metalloid selenium.

Selenium (Se) is a widespread and naturally occurring element with the potential to act as a contaminant following anthropogenic activities. Due to its abundance in the

western United States (Brown et al. 1999), approximately 414,400 km^2 of land is susceptible to rainfall or irrigation-induced selenium contamination (Seiler et al., 1999). Particularly high concentrations of selenium accumulated at the Kesterson Reservoir in the Central Valley, CA during the 1980s wherein subsurface drainage water from the surrounding agricultural land was diverted into the reservoir. Embryo deformities and mortality among the breeding waterfowl and fish then resulted from the high levels of selenium in the water (Ohlendorf and Santolo, 1994).

Selenium is rarely present in its elemental form (Babula et al., 2008), but occurs in the environment in both inorganic and organic forms depending on soil conditions and biological activity in plants or animals (Daniels, 1996; Eisler, 2007; Mayland, 1994). Selenate is the form most common and readily available to plants, which can be reduced to selenite in acidic soils (Hoffmann, 2003). Both of these inorganic forms can be accumulated by plants and may be converted to organic forms such as selenomethionine (SeMet), methylselenocysteine (MeSeCys) and selenocysteine (Eisler, 2007; Hoffmann, 2003). This element has also captured the interest of many scientists because of the narrow range between deficiency and toxicity of this element to humans and wildlife (Council, 1983). Selenium is essential for growth in humans and animals and acts in conjunction with the enzyme glutathione peroxidase as an important antioxidant (Council, 1983; Daniels, 1996). A lack of selenium has been implicated with heart disorders such as Keshan Disease in certain regions of China, where soils are known to have very low selenium content (Daniels, 1996; Tan et al., 1994). However, because it is also chemically similar to sulfur, nonspecific incorporation into proteins and enzymes can

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lead to toxicity in many organisms (Daniels, 1996). In humans, initial symptoms of toxicity include vomiting, as well as nail and hair loss; in livestock, abnormal breathing, blind staggering and death have been reported (Council, 1983).

Plants may also be at risk of toxicity (Brown and Shrift, 1982; Mayland, 1994) following the mobilization of selenium by rain, irrigation, mining, and coal burning (Haygarth, 1994) unless adapted to survive on soils containing high levels of selenium. Interestingly, plants found growing in seleniferous soils are often capable of concentrating higher levels of selenium than what is present in the soil (Babula et al., 2008; Eisler, 2007). These plants are divided into three categories based on their accumulation capacity (Eisler, 2007): non-accumulators (1.0-25 mg Se kg^{-1}), secondary accumulators (25-100 mg Se kg^{-1}) and primary accumulators (up to 10,000 mg Se kg^{-1}). Structures frequently consumed by herbivorous/granivorous species of ants such as the seeds, pollen and nectar (Rico-Gray and Oliveira 2007; Lach et al. 2010) have been discovered to concentrate high levels of selenium within these plants. Nectar was reported to contain over 100 µg Se ml^{-1} (fresh weight) and pollen contained over 1000 µg Se g $^{-1}$ (dry weight) in both primary and secondary accumulating plants within the family Brassicaceae (Hladun et al. 2011; Quinn et al. 2011). Another study found that the prickly pear cactus, *Opuntia ficus-indica*, concentrated approximately 17 µg Se g^{-1} in the seeds, 47 µg Se g^{-1} in the fruit and over 100 µg Se g^{-1} in cladodes DW (Bañuelos et al., 2011). Due to their tolerance and ability to concentrate significant levels in their tissue, accumulating plants are often considered as potential phytoremediators (Parker and Page, 1994). High selenium levels accumulated by phytoremediators may pose potential risks

for wildlife ingesting these plants (Eisler, 1985), but may also serve as attractive oviposition sites for pest insects, despite the possibility of incomplete development (Vickerman et al., 2002).

Interestingly, the majority of studies investigating selenium's impact on wildlife have been conducted on mammals, fish, and birds, with comparatively few studies on invertebrates (Hladun et al., 2012; Jensen et al., 2005; Vickerman and Trumble, 2003). There is no study to date investigating the effects of environmental selenium on ant communities, despite their abundance and close association with plants (Folgarait, 1998; Rico-Gray and Oliveira, 2007). Ants provide a novel system for studying the toxic effects of selenium, because their feeding behavior allows for potential acquisition from nectar, extrafloral nectaries, seeds, pollen, as well as from direct feeding on herbivores and their honeydew.

The Argentine ant, *Linepithema humile*, an invasive species from South America first reported in U.S. the late 1800s (Newell and Barber, 1913), has established in several areas throughout the southeastern and southwestern United States, as well as on five other continents (Buczkowski and Silverman, 2006; Klotz et al., 2008). Argentine ants are well known for their sugar-seeking behavior. A study by Markin (1970a) in citrus groves reported 99% of food brought back to the nest by forgers consisted of honeydew, a sugary substance secreted by many phloem-feeding insects (Styrsky and Eubanks, 2007). Honeydew has also been reported as a source of heavy metal transfer to ants (Migula and Głowacka, 1996). Due to these characteristics, Argentine ants serve as a good model

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organism for investigating the impacts of various forms of selenium in nectar on ant survival, as well as its ability to accumulate within these ants.

Materials and Methods

Ant Collections and Rearing

Argentine ant colonies containing queens, brood and workers were collected in October 2011, June and July of 2012 and February 2013 from the Department of Agricultural Operations at the University of California, Riverside. Colonies were then transferred into plastic containers (31 x 26 x10cm) where inner walls had been coated with liquid teflon (DuPont^{TM} Teflon$^®$ PTFE TE-3859) to prevent escape. Nesting material was provided in the form of 9cm diameter plastic petri dish bottoms filled with plaster of paris and covered with cardboard to provide darkness. Nests were moistened with de-ionized water twice a week to maintain humidity. Water was provided in a cotton-plugged 50-ml falcon tube in addition to weekly replenishments of chopped cockroaches, *Peripalenta americana,* (as a protein source) and 25% sucrose water (3 times per week). Rearing was conducted under ambient laboratory conditions of 24 ± 1°C, 40 ± 10% RH and LD: 14:10. Colonies were allowed to acclimate to laboratory conditions for approximately 4-5 days before use in experiments. Only worker ants were used for all experiments.

Mortality Assays

Assays were carried out for two weeks in order to investigate the effects of chronic ingestion of four selenium forms purchased from Sigma Aldrich, St. Louis, MO; selenate as sodium selenate (Na_2SeO_4), selenite as sodium selenite (Na_2SeO_3), MeSeCys,

and SeMet. Chemicals were incorporated into solutions of 25% sucrose to achieve

concentrations of 0, 2, 4, 10, 20, 30, 40 and 50 μg Se ml^{-1} for all forms and 0, 0.5, 2.7,

5.4, 13.5, 27, and 54 μg Se ml^{-1} for selenate. Each replicate consisted of a plastic box (30

x 18 x 10.5 cm) containing 100 worker ants, water and shelter. Shelter was similar as that

provided for colony rearing, only 5cm diameter petri dishes were used this time. Ants

were allowed to acclimate over night and dead ants (injured from transport) were

removed the next day and replaced with live ants. Following the acclimation period, a 10

ml vial containing one of the test concentrations for a particular Se form was then added

to each box. At least three replicates were conducted for each form. Worker ants were

allowed to feed *ad libitum*. The numbers of dead ants were recorded and removed from

each box every 24 ± 2 hrs following initial feeding. Cotton for water and treatment vials

was checked daily to ensure that it remained moist. In addition, the treatment vials were

replaced with new ones at the end of one week to prevent interference from microbial

growth in the sucrose solutions.

Analysis of Selenium Accumulation

In order to quantify levels that may occur within ant populations foraging in

contaminated habitats, we chose to analyze the surviving ants from each mortality assay.

Because single ants did not provide enough material for selenium analysis, all ants from a

single box were combined and sacrificed by freezing. Ants were stored in a freezer (-

60°C) prior to freeze-drying (Labconco Corp., Kansas City, MO) at -40°C at -25 psi for

48 hrs. Dry weights were then measured using a microbalance before microwave

digestion.

Ant tissue was microwave digested in 110 ml Teflon-lined vessels containing 5ml HNO_3 at 200°C for 30min (CEM Corp., Matthews, NC). Digested filtrate was then gently heated on a hot plate for approximately 10 min to remove any excess NOx gases. The resulting filtrate was diluted in 6 M HCl and heated in a water bath at 90°C for 20 min prior to analysis for accumulated selenium using hydride generation atomic absorption spectroscopy (HG-AAS; Perkin-Elmer, Waltham, MA). Quality control was verified with NIST Standard Reference Material (oyster, NIST 1566B), with an average percent recovery > 90%.

Deterrence (Choice-Test) Assays

We investigated whether or not foraging ants would exhibit a preference between a diet containing selenium and a diet without selenium. Arenas (Fig. 2.1 A) consisted of four feeding stations, two untreated controls and two treatment stations containing 50 ug Se ml^{-1} of each form of selenium placed in an alternating fashion in the bottom center of a plastic box (30 cm x 18 cm x 10.5 cm (height)). As part of this study, control and treatment solutions were also prepared in 10% and 30% sucrose solutions to determine if differences in concentration of sugar in nectar are likely to influence feeding. Assays were carried out with the use of non-toxic red and blue food coloring dyes (McCormick & Co., Inc., Hunt Valley, MD), after Cassill and Tschinkel (1999), mixed in sucrose solutions so that food choice could easily been seen in the ant's gaster (Fig. 2.1 B). Dyes for control and treatment solutions were switched between each replicate to control any effect of color on ant preference. Tests containing only sucrose water (no selenium added) and dyes were also conducted as an added positive control to further test for color

preference. Each form was tested using at least six replicates of 50 worker ants each for both sucrose concentrations. Choice tests were conducted for two hours and observations were made at different time points (0,1, 2, 30, 60, 90, and 120 min) documenting the number of ants present at each treatment station. At the end of two hours, all 50 ants were removed and placed in a freezer overnight. The dead ants were removed from the freezer and crushed between two pieces of filter paper to allow absorption of dye onto the paper (Fig. 2.1 C). The number of ants containing red, blue, purple and non-colored gasters was scored blindly (observer was unaware of dye assignments for treatments) to remove any observer bias.
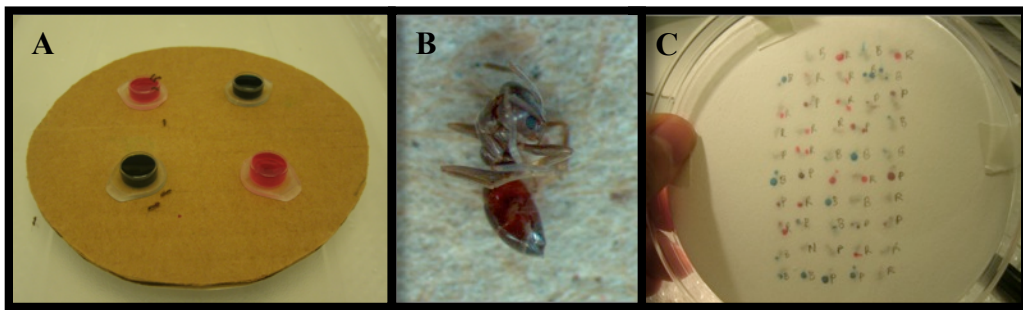
Fig. 2.1 (A) Choice test arenas of control and treatment stations, where same colored stations represent the same treatment (control or 50 ug ml^{-1} Se in sucrose solution). (B) Argentine ant worker with red gaster after ingesting sucrose containing red dye. (C) Filter paper showing spots of blue, red and purple colored dye from crushed ant gasters.

Statistical Analysis

Analysis was conducted using R version 2.14.1 (The R Foundation for Statistical Computing 2011). All response variables were examined for normality using the Jarque Bera Test, when samples sizes were greater than 50, and Shapiro Wilk's Test, when samples sizes were less than 50. Tests for homogeneity of variance were conducted using the Bartlett's Test or Levene's Test. For multiple comparison testing, a one-way

analysis of variance (ANOVA) was used when data when assumptions were valid. When

data had equal variance and could not be transformed for normality, a one-way Welch

Test was used. Post hoc comparisons were conducted using Tukey's HSD for ANOVA

and Games and Howell following Welch tests. The specific statistical tests used for

mortality, bioaccumulation, and choice experiments are described below.

Mortality

Cox Proportional Hazard Regression Models (Cox.PHR) were used to track

survival over time between each form using the R package "survival" (Therneau, 1999).

To follow up on differences in mortality seen between concentrations within forms,

multiple comparisons were conducted for time points of days 5, 7, 11 and 14 using

ANOVA. Data were corrected for control mortality using Abbott's formula (Abbott,

1925) prior to calculating LC_{50}s (lethal concentration that kills 50% of the population).

Lethal concentrations for day 7 and 14 were generated following the methods described

in Jeske et al. 2009 and using the R package "drc" (Ritz and Streibig, 2005).

Eq. (1):   % Mortality $= \dfrac{x - y}{x} \times 100$ x = % survival in untreated control

$$y = \% \text{ survival in treatment group}$$

Bioaccumulation

The initial model included the factors: treatment, form of selenium and the

interaction of treatment and form. The interaction between form and treatment was not

significant (ANOVA, F= 0.74, df = 21, P=0.78) and was removed from the model; the

two factors of form and treatment concentration were then analyzed separately. Because

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overall data could not be transformed to normality, they were analyzed using the non-parametric Kruskall Wallis test. Comparisons of accumulated selenium across treatments, within each form, were analyzed using Wilcoxin-Mann-Whitney for SeMet and Selenite (data were normal), a Welch test for MeSeCys and an ANOVA for Selenate. Comparisons across forms at each concentration treatment were conducted using ANOVA and post hoc Tukey's HSD. Accumulated levels reported here are from the combined weights (as described in the methods section) rather than from individual ants.

Choice Tests

Data from observations of ants feeding at stations during the assay were analyzed using a generalized linear model. Based on the AIC value, the negative binomial (NB) model was found to have a better fit compared to the poisson model. The final model included the number of ants present at each station as the response variable and the factors: form of selenium, color of dye, treatment, sucrose concentration, and time point during the assay. Multiple comparison tests using ANOVA or Welch tests were used to investigate ant choice by comparing the percentage of ants visiting the following options: control, treatment, both or no stations (gathered from scoring colored gasters at the end of the assay). Similar analyses were also conducted across dye colors alone to reveal a possible color preference among ant workers as a possible attraction factor to certain stations.

Results

Mortality

There was a statistically significant effect of form (CoxPHR; χ^2=1168.61, df=2,23, P<0.0001), and concentration (CoxPHR; χ^2=192.23, df = 7,23, P<0.0001) on mortality. There was also an interaction effect (CoxPHR; χ^2=236.28, df =14,23, P<0.0001) so form and concentration were not analyzed separately using one-way ANOVA. The data were then analyzed to compare time to significant mortality within each form. Figure 2 depicts the survival of ants over the course of two weeks, where cohorts fed MeSeCys and Selenate experienced greater mortality compared to Selenite and SeMet. Ants feeding on sucrose containing MeSeCys experienced significant mortality sooner, compared to all other forms. A comparison across treatments for MeSeCys revealed that by day 5, concentrations of 20-50 µg Se ml^{-1} produced significantly greater mortality (ANOVA; F=13.02, df=7,16, P<0.0001) than control and lower concentration treatments. In contrast, differences in mortality across treatments were not seen until after day 7 for all other forms of selenium. Comparisons on day 11 revealed greater mortality among the highest treatment of Selenate (54 µg Se ml^{-1}) than all other treatments (ANOVA; F=7.30, df=6,21, P<0.01). Differences in mortality among treatments did not occur for ants feeding on sucrose with SeMet until day 14 (ANOVA; F=2.75, df=7,24, P<0.05); no statistical difference in mortality across treatments was found for Selenite (Fig. 2.2).
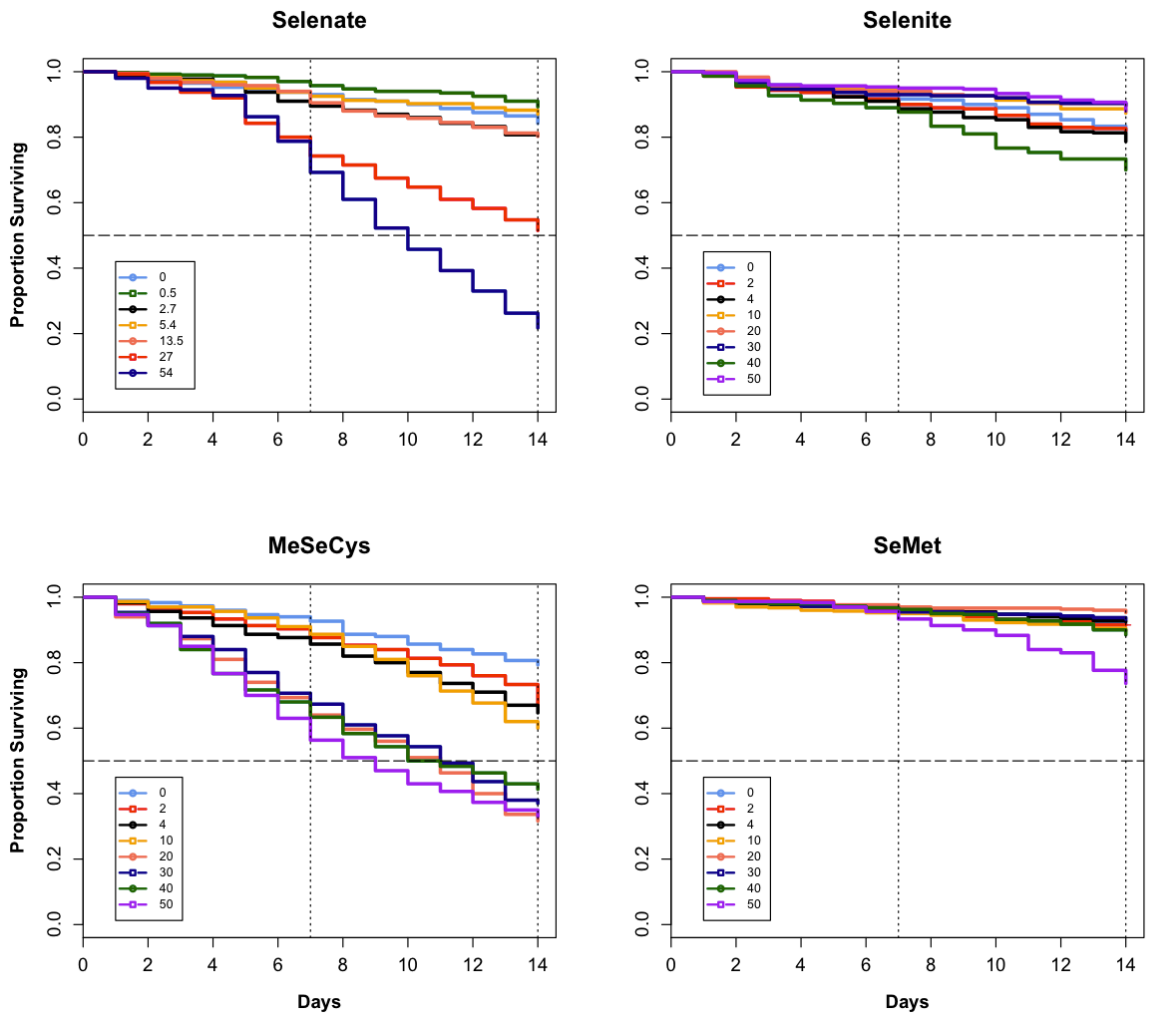
Fig. 2.2 Cox proportional hazard model plot displaying proportion survival/mortality of ants over 14 days following ingestion of selenate, selenite, methylselenocysteine (MeSeCys) and selenomethionine (SeMet). Legends within each graph correspond to concentrations fed in (µg Se ml^{-1}). Horizontal dashed lines denote 50% mortality. Vertical dotted lines separate mortality seen by day 7 and day 14.

Generation of LC_{50}s allowed for comparison of toxicity among forms and over time. Longer exposures generally decreased the LC_{50}s from day 7 to day 14 (Table 2.1). For example, approximately 88 µg ml^{-1} of MeSeCys was enough to cause 50% mortality after the first week, but the LC_{50} declined to 28 µg Se ml^{-1} after two weeks. The LC_{50}s for

SeMet and Selenite after the first week were not attainable at the given experimental concentrations (mortality was not high enough), however, after two weeks of chronic ingestion, LC_{50}s stabilized below 200 and 800 µg Se ml^{-1}, respectively.   Non-overlap of 95 % confidence intervals indicate that for Argentine ants, the order of highest to lowest toxicity among the four forms tested is as follows: MeSeCys > Selenate > SeMet > Selenite.

Table 2.1 Lethal concentrations that kill 50% of the population (LC_{50}) for four seleno-compounds after 7 and 14 days.

Selenium Form	Day	LC 50 (mg L^{-1})	95% Confidence Limits
Selenate	7	131.57	97.93-176.78
	14	34.8	32.48-37.29
Selenite	7	$44.02x^5$	$3.02\text{-}6.43x10^{12}$
	14	709.89	$134.37\text{-}3.75x10^3$
Methylselenocysteine (MeSeCys)	7	87.83	69.15-111.55
	14	27.68	24.00-31.92
Seleno-L-methionine (SeMet)	7	$29.4x10^3$	$40.73\text{-}21.24x10^6$
	14	176.17	97.3-318.95

Bioaccumulation

Ants from control treatments were found to contain small amounts of selenium (Table 2.2). This was attributed to their diet of cockroaches, which were fed Purina Dog Chow^{TM} that was subsequently found to contain low levels of selenium. Because all ants in the experiments were fed the same materials, and accumulations in the control ants

were quite low and not statistically different between controls, treatment accumulations

were not adjusted.

Table 2.2 Mean and median accumulated selenium in ants (μg Se g^{-1}) after two weeks of chronic ingestion of the four seleno-compounds.

Conc. Fed µg Se ml^{-1}	Selenate			Selenite			MeSeCys			SeMet		
	Mean	Median	P	Median	Median	P	Mean	Median	P	Mean	Median	P
0	1.81	0.38	-	1.15	0.19	-	4.15	3.63	-	1.38	0.85	-
2	5.88	3.75	0.98	15.66	13.755	*	7.91	8.45	0.567	29.86	11.51	**
4	7.33	6.39	0.94	14.09	14.26	*	13.63	15.89	0.502	85.54	22.92	**
10	21.52	28.68	0.3	28.53	27.61	*	24.72	17.54	0.487	57.3	60.04	**
20	46.31	43.44	*	43.48	36.47	*	48.94	36.82	0.283	98.32	96.32	**
30	62.26	65.75	**	67.96	59.79	*	55.76	53.97	0.563	160.76	150.56	**
40	74.18	86.82	**	171.47	94.98	*	61.43	74.98	0.26	193.61	150.67	**
50	77.24	78.12	**	108.68	114.65	*	82.51	82.86	*	150.13	152.29	*

Post hoc comparison p-value against control treatments within each form are given with significance codes: '**'0.01 '*'0.05

Between Form Comparisons

There was a significant difference in ant accumulation between fed selenium

forms (Kruskall-Wallis; χ^2=8.088, df=3, P<0.05). After two weeks, surviving ants were

found to contain selenium body burdens at levels greater than they had been provided in

treatments. Mean levels reached approximately twice as much for MeSeCys and

Selenate, but more than double for SeMet and Selenite at all concentrations (Table 2.2,

Fig. 2.3). Overall accumulation comparisons between forms revealed that ants that fed

on SeMet accumulated more selenium than ants feeding on MeSeCys (Wilcoxin-Mann-

Whitney, W=388, P<0.05) or Selenate (Wilcoxin-Mann-Whitney, W=301, P<0.05).

Comparisons across forms within concentrations (Fig. 2.3) documented an overall

statistically significant difference in accumulation between forms at 10 μg Se ml^{-1}

(ANOVA; F=4.77, df=3,12, P<0.05), 20 μg Se ml^{-1} (ANOVA; F=3.89, df=3,12, P<0.05), 30 μg Se ml^{-1} (ANOVA; F=8.44, df = 3,12, P<0.01) and 50 μg Se ml^{-1} (ANOVA; F=4.16, df=3,10, P<0.05). Pairwise comparisons confirmed that ants feeding on SeMet accumulated greater amounts of selenium than MeSeCys and Selenate at 10, 30, and 50 μg ml^{-1} (Tukey's HSD, p<0.05) and a greater amount than Selenite at 30 μg ml^{-1} (Tukey's HSD p<0.05). The highest mean accumulation (Table 2.2) was observed for ants fed 40 μg Se ml^{-1} as SeMet, with a mean body burden of 193 μg Se g^{-1}. Ants feeding on sucrose containing 40 μg Se ml^{-1} as Selenite had a mean accumulation of 171 μg Se g^{-1}. The lowest mean accumulations of selenium were seen for ants feeding on sucrose containing 2 μg Se ml^{-1} of MeSeCys or Selenate, where mean levels in ants reached ≈8 and 6 μg Se g^{-1}, respectively.
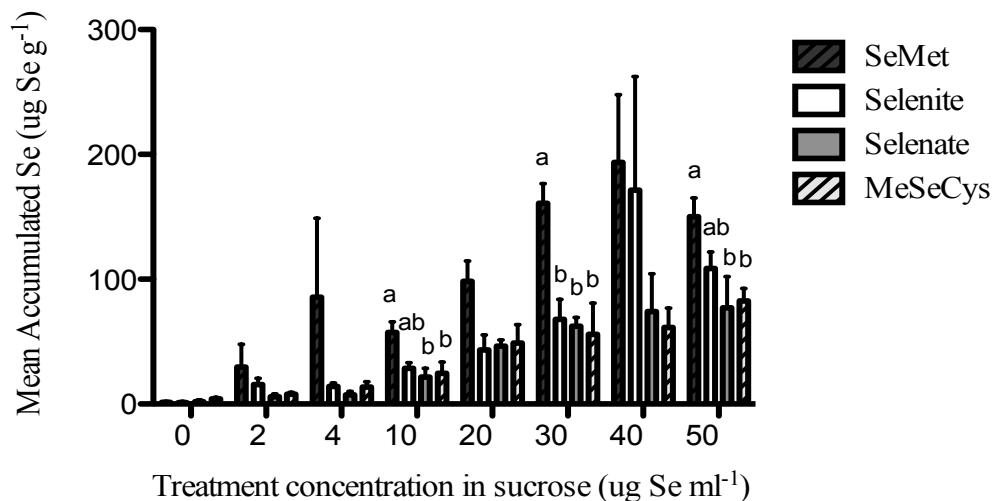
Fig. 2.3 Comparison of bioaccumulation between forms for accumulated selenium in ants following two-week ingestion at various concentrations. Letters represent means that are significantly different from each other within a given concentration. Differences were confirmed using ANOVA and post hoc Tukey's pairwise comparisons.

Within Form Comparisons

There were also differences in the level at which treatment concentrations were significantly different from each other (Kruskall-Wallis; χ^2=82.637, df=7, P<0.0001). Table 2.2 gives post hoc comparison p- values of treatments against controls for each form. Bioaccumulations at all treatment concentrations were significantly greater than control accumulation for SeMet (Wilcoxin; W=0, P<0.01) and Selenite (Wilcoxin; W=0, P<0.05). In contrast, accumulation in ants feeding on sucrose with MeSeCys only reached mean levels statistically greater than controls (Welch test; F=9.1, df=7,8.94, P<0.01), when fed the highest treatment concentration of 50 μg Se ml^{-1} (Games and Howell, P<0.05). A difference in accumulation between concentrations was also seen for ants fed sucrose containing Selenate (ANOVA; F=8.94, df=7,16, P<0.01), but this was only reached at treatment concentrations ≥ 20 μg Se ml^{-1} (Tukey's HSD, P<0.01).

Choice Tests

Analysis of ant visits to stations during the assays only revealed significance against the factors of "time" (GLM NB; χ^2=80.174, df=6, P<0.0001) and "form" (χ^2=87.081, df=3, P<0.0001). Significance of time was not unexpected, as ant activity at all stations began to taper after the first hour. Because there was no statistical difference in ant visits between treatments (χ^2=0.495, df=1, P=0.482), nor was there a significant interaction between treatments and form (χ^2=4.304, df=3, P<0.230), this suggests that the significance difference in ant visits between assays of selenium forms appears to have occurred by chance.

When using data taken from scoring ant gasters at the end of each assay, the analysis showed that for all forms of Se, there was an overall statistically significant difference detected (ANOVA/ Welch test; df=3, P<0.001) in the percentage of ants choosing to feed at 1) the sucrose-only control feeding stations, 2) the stations containing some form of Se plus sucrose, 3) those that fed on both the treated and control solutions and, 4) those not feeding at all. However, post hoc comparisons (Table 2.3) revealed this significant difference existed only for the option of no feeding (Games and Howell or Tukey HSD; df=3, P<0.05). Furthermore, in all cases, there was no statistically significant difference in the percentage of ants feeding at control versus selenium-treatment stations when provided choices in 10% sucrose (MeSeCys P=0.999; Selenate P=0.636; SeMet P=0.997; Selenite P=0.992) or 30% sucrose (MeSeCys P=0.067; Selenate P=0.192; SeMet P=0.326; Selenite P=0.843). Thus, there was no evidence that the ants responded to the presence of any form of Se that was tested, regardless of the toxicity or background sucrose concentration (Table 2.3 A, B).

An analysis of color was also conducted to determine if the color of the dye (red or blue) might have affected choice of the feeding station (even though the color was changed between replicates). For all assays, an analysis of feeding as indicated by the color of the gasters indicated an overall statistical difference (ANOVA or Welch test; df=3, P<0.001,), but this difference was only evident between those that had not fed (no color in the gaster) versus those that had fed (red, blue, or purple gasters). In all trials, there was no difference in visits to red versus blue stations (Games and Howell or Tukey HSD; all trials P ≥0.06). In addition, as a positive control, ants were offered choices of

sucrose containing no selenium. Again there was an overall statistical difference (Welch Test; F=19.98, df=3,31, P<0.001), but no statistically significant difference between numbers of ants that fed on red versus blue stations (Games and Howell; P=0.3). Thus, there was no evidence that the color of the dyes used in the study had any measurable impact on food choice by the workers.

Table 2.3 Choice test post hoc comparison p-values based on data from percentage of colored gasters at the end of each assay.

A	Selenate				Selenite				MeSeCys				SeMet			
	Games and Howell				Tukey's HSD				Games and Howell				Games and Howell			
Choice	C	T	B	N	C	T	B	N	C	T	B	N	C	T	B	N
C	-	0.636	0.975	0.137	-	0.992	*	***	-	0.999	0.178	**	-	0.997	0.567	*
T	-	-	0.718	*	-	-	0.065	***	-	-	0.264	**	-	-	0.476	*
B	-	-	-	**	-	-	-	**	-	-	-	***	-	-	-	**
N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

B	Selenate				Selenite				MeSeCys				SeMet			
	Games and Howell				Tukey's HSD				Games and Howell				Tukey's HSD			
Choice	C	T	B	N	C	T	B	N	C	T	B	N	C	T	B	N
C	-	0.192	0.799	*	-	0.843	***	***	-	0.067	**	***	-	0.326	0.962	***
T	-	-	0.476	**	-	-	***	***	-	-	0.093	**	-	-	0.593	***
B	-	-	-	*	-	-	-	1.0	-	-	-	***	-	-	-	***
N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

The post hoc test used is listed below each form. Letters represent possible ant choices where C= control, T=treatment, B=both and N=none. Results from comparisons are also separated for treatments mixed in A) 10% sucrose and B) 30% sucrose. Significance codes: '***'0.001 '**'0.01 '*'0.05

Discussion

Argentine ant workers experienced significant mortality within two weeks of chronically ingesting sucrose containing selenium. The selenium forms methylselenocysteine and selenate exhibited greater toxicity than selenomethionine and selenite. In addition, methylselenocysteine appears to have greater acute toxicity as evidenced by significant mortality as early as day five, whereas all other forms had more of a delayed effect. This is similar to a study by (Jensen et al. 2005) that found

selenocysteine was the most toxic form of Se tested against a phorid fly (*Megaselia scalaris*: Diptera: Phoridae). However, these results are in contrast to other reports which found methylselenocysteine to be among the least toxic to larvae and adult honeybees (*Apis mellifera*: Hymenoptera: Apidae; Hladun et al. 2013) and of intermediate toxicity to beet armyworms (*Spodoptera exigua*, Lepidoptera: Noctuidae; Vickerman and Trumble 1999). Even so, the LC_{50}s reported here suggest all forms have the potential to cause substantial mortality at the ecologically-relevant ranges that have been reported in plant structures (Hladun et al. 2011; Quinn et al. 2011), especially in instances where plants convert inorganic forms to their less toxic form methylselenocysteine.

Surviving workers were also found to bioaccumulate selenium for all forms of selenium fed at nearly twice the concentrations provided in sucrose across all treatments. Interestingly, ants feeding on sucrose containing the two forms that were found to cause lower toxicity accumulated the highest body burdens. Selenomethionine and selenite were discovered to have a delayed toxic effect, however ants feeding on sucrose containing these forms accumulated over 150 μg Se g^{-1}, whereas those feeding on the more toxic methylselenocysteine and selenate averaged of 85 μg Se g^{-1} or less. The choice tests determined that this pattern did not occur due to avoidance of any particular form of Se, so we predict that selective feeding will not play a critical role in natural settings for Argentine ants. Differences in toxicity and accumulation caused by different selenium forms are likely due to the ability/difficulty of each form to be metabolized or absorbed within the ant. It is also probable that the form present upon ingestion is different than that which is causing toxicity, if forms are undergoing conversion within

the worker ants. This might also help to explain the discrepancies between reports on

toxicity of certain selenium species between invertebrates. As of now, the behavior of

selenium within the ant body is unclear, but identification of the particular selenium

species within ant bodies should help to reveal the mechanisms of toxicity.

Ants were not deterred by selenium in sucrose for any form, regardless of the

sugar concentration. The observation that Argentine ants were not deterred by Se from

feeding is not unexpected, as the literature includes reports of some insect species

demonstrating no avoidance of Se and relatively few that do not have an adverse reaction

(Vickerman and Trumble 1999; Jensen and Trumble 2003; Hanson et al. 2004; Galeas et

al. 2008; Hladun et al. 2013). Furthermore, when nectar extracts from plant species

containing possible defensive compounds were tested against several species of ants,

results indicated predominantly non-avoidance of ants to nectar, whereas ants showed

mixed results towards floral tissue extracts (Guerrant and Fiedler, 1981). Therefore,

predicting the responses of invertebrate species to new and potentially toxic materials

will require much more information than is currently available. For example, low

selenium levels were found to contribute to larval resistance against a baculovirus when

added to the diet of two Lepidopteran species (Noctuidae) – the cabbage looper,

Tricoplusia ni (Popham et al., 2005) and the tobacco budworm, *Heliothis virescens*

(Shelby and Popham 2007). However, in most reports in the literature, selenium's impact

on growth and development of insects has documented primarily negative results (Lemly

1997; Jensen et al. 2007; Popham and Shelby 2007; Shelby and Popham 2007; Hladun et

al. 2013).

The results of this study point to several potentially detrimental implications for ant communities residing in habitats containing selenium-rich soils where plant resources available to ants have been reported to contain significant levels of this element. If ants are undeterred by selenium in their diet, workers will continue to gather and provide toxic food to other members of their colony. Previous studies on heavy metal accumulation in ants have shown that body burdens are often highest in workers due to different feeding strategies among castes or because of dilution, due to trophallaxis, before reaching the brood and reproductives (Grześ, 2010). Nonetheless, sub-lethal concentrations may still impact larval development, as was seen for larval honeybees fed selenium (Hladun et al. 2013). Furthermore, accumulation of selenium by ants, even at the lowest levels reported in this study, may be sufficient to pose a problem for other organisms that depend on ants as food. At least in the case of the invasive Argentine ant, the rate of mortality shown here has the potential to impact colony size and development. This may in turn affect their ability to exploit new territory and compete against other ant species, as large numbers are often a key factor in their success (Holway and Suarez, 1999).

This is the first in depth study to investigate the toxic effect of selenium in ants. Additional studies are needed to investigate the impact of selenium on other ant species, particularly a native ant species that occurs in selenium-contaminated areas for comparison with these findings. Given the relatively high body burdens of selenium in ants in our experiments, studies on the potential of selenium to be transferred from invertebrates to predatory organisms also appears warranted. Future work should also investigate the sub-lethal impact of selenium on other members of the ant colony, such as

the reproductives and brood, as fecundity and brood development are also likely to influence colony persistence and survival in contaminated habitats. Lastly, information on the tissue location(s) of selenium as well as the selenium species occurring within an ant after ingestion will give us a better understanding as to selenium's toxic and/or possible beneficial role in invertebrates.

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Chapter 3

Evidence for the Transfer of a Contaminant via a Tri-Trophic Plant-

Herbivore-Ant System

Abstract

Uptake of environmental contaminants by lower trophic groups can have negative effects on higher trophic groups. We tested the ability of selenium, an environmental contaminant found in high concentrations in the nectar and pollen of certain accumulating plants, to be transferred to ants via aphid tissue and aphid honeydew. Plants of the selenium accumulator, *Raphanus sativus* (wild radish)*,* were watered with three different selenium treatments (0, 0.25 and 0.5 μg Se mL^{-1}). Aphids, *Myzus persicae,* and Argentine ant colonies, *Linepithema humile,* were added to each caged plant and allowed to interact freely. Ant colonies were supplemented with one of three different food options to encourage the consumption of aphids, aphid honeydew, or aphids and honeydew. To determine the potential for selenium to biomagnify between each trophic step, the accumulation of selenium by each trophic group and a trophic transfer factor (TTF) was calculated. We compared the amount of selenium acquired by ants as a factor of diet and caste. Plants, aphids, and worker ants accumulated selenium in a dose dependent manner. Ant queens did not contain detectable amounts of selenium. The TTF for plants to aphids was >1, indicating biomagnification, whereas the TTF for aphids to worker ants was <1, indicating only biotransfer. Accumulated levels by worker ants did not statistically differ as a result of diet. Honeydew contained comparable amounts of selenium to plant selenium levels. Access to toxic compounds via honeydew and insect protein may have negative effects on the range expansion of invasive ant species, such as the Argentine ant.

Introduction

Ants have been regarded as keystone species for their abundance and their involvement in critical ecological processes (Folgarait, 1998; MacMahon et al., 2000; Pętal et al., 1977; Power et al., 1996) that can impact both the surrounding flora and fauna. They have also been investigated for their potential use as bioindicators of change following habitat disturbances such as flooding (Ellis et al., 2001), fire (Andersen et al., 2006; Hoffmann and Andersen, 2003), deforestation (Dunn, 2004; Floren and Linsenmair, 2001), urbanization (Burbidge et al., 1992; Lessard and Buddle, 2005) and pollution (Bengtsson and Rundgren, 1984; Gramigni et al., 2011; Hoffmann et al., 2000; Nummelin et al., 2007). Furthermore, environmental pollution has the potential to detrimentally impact ant communities via changes in species composition, abundance, richness (Eeva et al., 2004; Grześ, 2009; Hoffmann et al., 2000), behavior (Barbieri et al., 2013; De La Riva and Trumble, 2016; Sorvari and Eeva, 2010) and health (Sorvari et al., 2007) .

The modes of transfer of environmental contaminants to ants have not been experimentally determined. Previous research has documented the accumulation of heavy metals in plants and insects collected near ant colonies (Bengtsson and Rundgren, 1984; Del Toro and Floyd, 2010; Heikens et al., 2001). These organisms are likely sources of contamination for ants, whose diets are often comprised of both plant resources and insects. For example, Głowacka et al. (1997) reported heavy metal levels present in psyllid honeydew as a potential source of contamination, but did not measure its transfer to ants. Starý and Kubizňáková (1987) measured pollutant levels in wood ants tending

aphid colonies and determined that honeydew was the most likely route of metal acquisition, but they were unable to rule out other possible sources of exposure such as floral nectar, ingestion of seeds, or other insect prey. In addition, there has been no work conducted comparing the amount of the contaminant transferred when ingested via a protein versus a carbohydrate source.

We chose the invasive Argentine ant, *Linepithema humile*, because it is omnivorous, feeding on both carbohydrate and protein sources (Tillberg et al., 2007) It has also become a problem in agricultural settings in its introduced ranges for tending honeydew-producing plant pests (Bartlett, 1961; Daane et al., 2007; Markin, 1970). This diet flexibility provides the Argentine ant with substantial ecological advantages over obligate carnivory during range expansion (Tillberg et al. 2007). Selenium (Se), an abundant soil-borne metalloid in the western U.S. (Brown et al., 1999), was used as the target contaminant. Although naturally derived from Cretaceous shales, selenium can also be mobilized following human activities such as coal burning, mining, or irrigation (Eisler, 2000). Toxicity can occur in animals ingesting selenium-accumulating plants (Eisler, 1985). Previous studies investigating the concentration of selenium in accumulating plants found high concentrations in resource consumed by ants, such as the nectar, pollen and seeds (Hladun et al., 2011; Prins et al., 2011; Quinn et al., 2011). When given an artificial nectar source contaminated with selenium, De La Riva et al. (2014) found that Argentine ant workers were not deterred even by lethal concentrations in artificial nectar.

Therefore, a study was initiated with the following objectives: 1) Determine whether selenium can be transferred to ants from plants via an aphid intermediate; 2) Determine the concentration of selenium within each trophic group (plant, aphid, ant) to determine the trophic transfer factor; 3) Compare the difference in accumulation of selenium by different castes within the colony, and; 4) Determine whether ants might acquire different levels of selenium when ingesting protein vs. carbohydrates (honeydew).

Materials and Methods

Plant and Insect Material

Seedlings of *Raphanus sativus* (cultivar "White Globe", Livingston Seed Co., Columbus, OH) were transplanted to small plastic pots containing UC soil mix III (Matkin and Chandler, 1957), watered three times per week and maintained in a greenhouse environment. Miracle Gro nutrient solution (Scotts Co., Marysville, OH) was added to the watering regime once every other week. When plants were 6 weeks old, 36 healthy plants were transferred to 2.5 L pots to begin treatments with selenium.

Green peach aphids, *Myzus persicae*, were colonized from infested pepper plants in greenhouses located at the UCR Agricultural Operations Field Station. They were placed onto caged radish plants and colonized for 2-3 generations before further use. A large colony of Argentine ants, *Linepithema humile*, containing queens, brood and several thousand workers was collected in a wooded grassy area on the UCR campus (33°58'34" N, 117°19'57"W, 312 m). Thus, the genetic variability of the ants used in these tests was as standardized as possible. Ants were then separated into 36 sub-colonies, each

containing ~300 workers, 6 queens and brood. Sub-colonies were placed individually in small plastic boxes (20 L x 15 W x 10 H cm) lined with liquid Teflon® (PTFE TE-3859, DuPont Fluoroproducts, Wilmington, DE) to prevent escape. Each box had a lid and a 1 in. breathing hole covered with mesh. A 9 mm Petri dish containing a moistened bottom layer of Plaster of Paris was provided as nest material and a cotton-plugged vial of 25% sucrose, a vial of water and approximately 2-3 chopped cockroaches (*Gromphadorhina portentosa)* were provided as food prior to the beginning of the experiment. Ants remained in these boxes until they were introduced in to arenas described below.

Experimental Design

Each of the 36 potted radish plants were placed in separate plastic containers (31 L x 26 W x 10 H cm, Supplemental Fig. 3.1) and arranged randomly in space in a greenhouse at the UCR Agricultural Operations Field Station. A 1.27 cm layer of Plaster of Paris was poured over the soil at the base of each plant (this was necessary to prevent nesting of ants in the soil following their later introduction). Plants were then randomly assigned one of three selenium treatments (0, 0.25 and 0.5 µg Se mL^{-1}) for a total of 12 plants per treatment. Selenium treatments were chosen to be ecologically relevant and less than 1 µg Se mL^{-1} to prevent any potential repellency of plants to aphids (Hladun et al. 2013).

Treatment solutions were prepared by dissolving sodium selenate powder (Na$_2$Se0$_4$, 98% purity, Sigma-Aldrich, St. Louis, MO) in double distilled water to yield the target concentration. The plants were treated three times per week for the remainder

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of the experiment by pouring 500 mL of the solution into the plastic containers and allowing the soil to draw up the liquid from the bottom of each pot.

Two days following the initial selenium treatments and addition of plaster, any developing flowers or buds were removed by snipping at the peduncle to ensure that nectar would not later be available to ants. Green peach aphids were then added by cutting leaf sections from the untreated radish plants used to rear the aphid colonies and laying them on the foliage of each of the 36 plants. Each leaf section added contained ~100 aphids. The potted radish plants were then bagged (Supplemental Fig. 3.2) in order to cage the aphids on each plant.

After one week of allowing aphids to acclimate and feed on the radish plants, the 36 sub-colonies of Argentine ants were brought to the greenhouse and paired with a plant. Ants from individual sub-colonies were allowed to enter a caged arena to access the aphids via a plastic tube (30.5 cm length and 1.9 cm diameter) that ran from one of the plastic nest boxes to one potted plant. Glue was added around the holes of the box and the pot at the tube connection sites to ensure ants would not escape. All nest boxes were also placed in a slightly larger plastic container and moist soil was added around the smaller box. This was done to maintain a cool humid climate for the ants within the greenhouse. Each of the 36 ant sub-colonies was then assigned one of three supplemental food options (Table 3.1), in order to manipulate their preference for harvesting honeydew versus eating the aphids as a protein source. This resulted in a total of 4 replicate systems (plant-aphid-ant interactions) per treatment-food option combination. Colonies given a 15 mL vial of *water only* were predicted to gather honeydew as well as aphids for protein in

order to feed their brood; colonies provided with *protein* (fresh dry ice-killed, aphids that

fed on control plants without Se) were expected to preferentially gather only honeydew

from the living aphids in the test arena. Because there was no way to prevent Argentine

ants from feeding on honeydew in the presence of live aphids, we did not allow the

remaining colonies access to living aphids on the plant. Instead, they were provided with

carbohydrates (25% sucrose) and leaves containing ~25 freshly killed aphids from their

paired plant. Because removing large numbers of aphids from those plants would have

resulted in the population crashing and no aphids to later analyze, we waited one week

before providing ants with contaminated aphids. However, this delay did allow the ants to

acclimate to the feeding system by first providing them uncontaminated aphids.

Table 3.1 Ant food options.

Supplement	Expectation	Total Diet
water only	contaminated aphid tissue + contaminated honeydew	Water + **contaminated aphids + contaminated honeydew**
protein (<u>un</u>contaminated aphids)	contaminated honeydew	<u>un</u>contaminated aphids + **contaminated honeydew**
carbohydrates (25 % sucrose)	contaminated aphids	sucrose + **contaminated aphids**

Items listed under "Supplement" are those that were provided to ant colonies in their nest
boxes, in order to manipulate their preference for feeding on the items listed under "
Expectation" in the caged arena. Items listed in bold font under " Total Diet" were the
sources of selenium acquisition by foraging ants.

Honeydew Collection

Collecting honeydew from aphids in the arena would have added another complication to the set-up, so we prepared a group of separate plants and aphids for the task. Twelve radish plants were grown in plastic pots (10.16 W x 10.16 L x 8.9 cm H) using soil, water and fertilizer methods described above. At approximately 6 weeks of age, these plants were randomly separated in space, placed in small plastic boxes (20 L x 15 W x 10 cm H) and given one of three selenium treatments as before (0, 0.25 and 0.5 µg Se mL^{-1}). Treatments were administered by pouring 150 mL of the target treatment solution in the plastic box and allowing the soil to draw up liquid from the bottom of the potted plant. Treatments were administered three times per week.

Aphids were introduced a few days later by placing leaf sections containing ~100 uncontaminated aphids on each of the 12 fresh plants. A square piece of foil paper (~12.5 x 12.5 cm) was placed at the base of each plant to collect droplets of honeydew. The foil was collected two weeks later, brought back to the lab and placed in a -60 °C freezer overnight. Then, each piece of foil was inspected for molted aphid exuviae. Frozen honeydew allowed for easier removal of the aphid exuviae through the use of a fine paintbrush. Once the foil was cleared of debris, the total wet weight of the honeydew from each piece of foil was obtained using a microbalance. The honeydew was then removed from the foil by taking smaller sections of each foil sample and rinsing them in warm double distilled water in a glass funnel. A 1.5 mL microcentrifuge tube was placed at the end of the funnel to collect the rinse. A glass rod also proved helpful in rubbing the honeydew free of the foil during each rinsate. Each foil sample was rinsed using a total of

~1.5 mL of water. The microcentrifuge tubes containing the rinsate honeydew samples were placed in a -40 °C freezer until further use.

Selenium Analysis

At two weeks following the addition of ants, the experiment was terminated and all plant, aphid, and ant material was then sacrificed. Ant nest boxes were placed in a -40°C freezer. All live aphids were removed from each bagged plant and placed in collection vials. A portion of the plant foliage was taken from each plant and was rinsed off in double-distilled water to remove any remaining debris or aphids. Foliage samples were standardized by utilizing fully expanded leaves from the center of plant, and then placed in separate collection vials. Both aphid and plant samples were then stored in a -40 °C freezer. Ants were removed from the nest boxes and the workers and queens were placed in separate collection vials, and then placed back in the freezer. We were unable to obtain enough ant brood mass for analysis. All frozen plant and insect tissues were freeze-dried (Labconco Corp.) at -40 °C and -25 psi for 72 hr and 48 hr, respectively. Dried samples were weighed on a microbalance prior to microwave digestion. All plant, insect and honeydew samples were digested with 5 mL concentrated HNO_3 for 20 min, at 1200 W in a microwave oven (CEM Corp., Matthews, NC). Quantification of selenium concentration was carried out by diluting a portion of the digestate (0.25-1 ml) in a 6 M-HCl matrix, heating for 20 min in a 90 °C water bath, and analyzing each sample with hydride generation atomic absorption spectroscopy. Standard reference material for insects (oyster tissue, NIST 1566B) and plants (wheat durum, NIST 8436), Se spikes, and

blanks (H_2O) were used to verify recovery. Selenium recoveries in reference material were over 90%.

Data Analysis

Statistical analysis was carried out using R v.3.2.2 (2015, The R Foundation for Statistical Computing). Concentration data for plants, aphids and ants could not be normalized with transformation. Therefore, a non-parametric Kruskal-Wallis test with a post hoc Dunn Test was used to compare concentrations accumulated by each group across selenium treatments. To determine which fixed factor (selenium treatment vs. food option) resulted in the greatest amount of selenium accumulated by ants, we conducted a two-way analysis of variance. Concentration data contained zeros, which could not be normalized with log transformation, and so the zeros were changed to 0.00001 to achieve normalization. Multiple comparisons of honeydew concentrations across selenium treatments were conducted using a Welch Test for normal data with unequal variance. Separation of means was conducted with the post hoc Games and Howell test for normal data with unequal variance.

We determined the trophic transfer factor (TFF) for each trophic exchange by calculating the ratio of selenium concentration in the organism in comparison to its food item (after DeForest et al. 2007). For example, the selenium concentration in the aphids was compared to the concentration in the plants, and the selenium concentration in the ants was compared to the concentration in the aphids.

Results

Worker ants were observed to tend aphids for their honeydew and collecting

fresh-killed aphids. Brood was included to encourage the gathering of insect tissue by

workers for protein. In ant colonies provided untreated sucrose and freeze-killed aphids

on leaf sections directly from their paired plant, we observed the removal of those aphids

from the leaf sections by workers. Those workers were also found to contain selenium in

their bodies (Fig. 3.1 A), which was presumably obtained following the ingestion of body

fluids from the aphids and/or feeding on larval regurgitant of those aphids. Similarly, in

colonies provided untreated aphids or water only, we observed both aphid-tending in

arenas and gathering of killed aphids. Workers from both of those groups were also found

to contain detectable levels of selenium indicating that ingestion of contaminated

honeydew or both contaminated honeydew and selenium-laden aphids had occurred,

respectively.

The concentration of selenium present in plant and insects followed a dose

dependent trend (Fig. 3.2), where a greater concentration of selenium added to the soil

resulted in higher accumulated selenium for radish foliage (Kruskal-Wallis: X^2=23.3,

df=2, P<0.0001), aphid tissue (Kruskal-Wallis: X^2=24.2, df=2, P<0.0001) and worker

ants (Kruskal-Wallis: X^2=19.5, df=2, P<0.0001). However, the post hoc Dunn Tests

revealed that plants and aphids only contained significantly higher concentrations of

selenium when treatment groups were compared to control groups (0 vs. 0.25: all

P<0.0001; 0 vs. 0.5: all P<0.001; 0.25 vs. 0.5: P= 0.12-0.24). In contrast, worker ants

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A

B
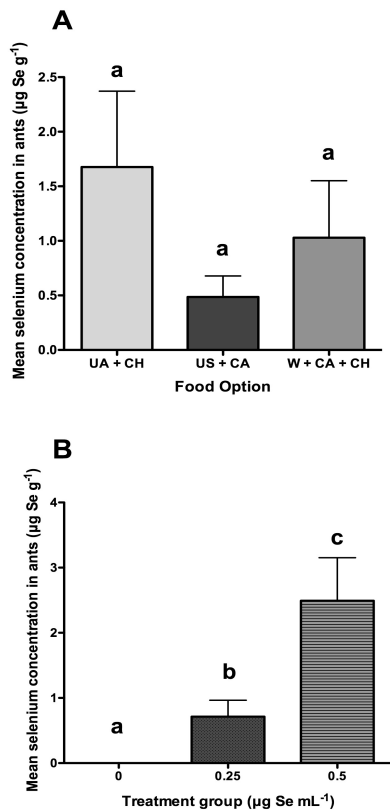
Fig. 3.1 A) Mean selenium concentration in worker ants as a result of different food options: Uncontaminated aphids + contaminated honeydew (UA+CH) refers to colonies fed on contaminated honeydew when provided killed, uncontaminated aphids ; uncontaminated sucrose+contaminated aphids (US+CA) refers to colonies that consumed uncontaminated sucrose and freeze-killed, contaminated aphids (the only source of Se was aphids from contaminated plants); and water+contaminated aphids+contaminated honeydew (WC+CW) are results from colonies that consumed both aphids from contaminated plants and their contaminated honeydew when provided water only (the source of Se was provided in both aphids and honeydew). **B)** Mean selenium concentration in worker ants as a result of selenium transfered from plants grown in soil with three concentrations of Se. Statistically different means are represented with different letters (Kruskal-Wallis test with post hoc Dunn Test, α=0.05).

collected from arenas treated with 0.5 µg Se mL^{-1} contained greater selenium body burdens compared to those from the 0.25 µg Se mL^{-1} group (post hoc Dunn Test, P=0.02). Worker ant concentrations did not differ as a result of different food options (Two Way ANOVA: F=2.18, df=2,28, P=0.13, Fig. 3.1 A) versus the amount of selenium accumulated as an effect of treatment (Two Way ANOVA: F=12.49, df=2,28, P<0.001, Fig. 3.1 B). Queen ants did not contain detectable amounts of selenium in any of the three treatment groups.
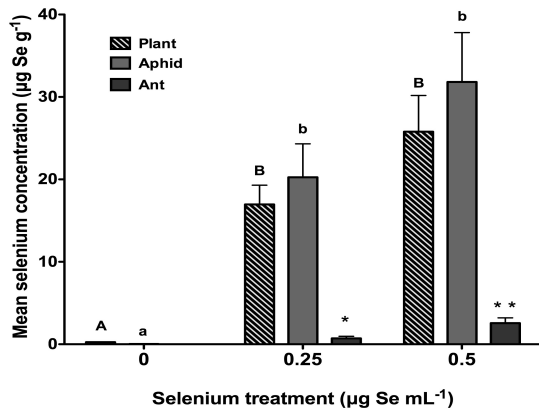
Fig. 3.2 Average selenium concentrations (μg Se g^{-1}) accumulated by plants, aphids, and ants following three different soil selenium treatments. Different letters of the same case or those with different numbers of asterisks represent statistically significant differences (Kruskal-Wallis test with post hoc Dunn Test. α=0.05). Mean concentrations in plants are compared using capital letters, mean concentrations in aphids are compared using lowercase letters, and mean concentrations in ants are compared using numbers of asterisks.

Aphid honeydew contained selenium (Fig. 3.3) at concentrations slightly less than those found in aphid tissue, but comparable to the levels seen in plant tissue. Honeydew collected from plants grown in soil treated with 0.25 and 0.5 μg Se mL^{-1} contained statistically greater levels than honeydew collected from plants watered with no selenium (Welch Test, F=21.3, df=2, 4, P<0.01, post hoc Games and Howell P<0.05); however, concentrations of Se in honeydew from aphids feeding on plants grown in two treatments with Se-amended soil were not different from each other (Games and Howell P=0.66).

Fig. 3.3: Mean honeydew concentration excreted by aphids as result of selenium transferring from plants grown in soil with three concentrations of selenium. Different letters indicate means that are statistically different from each other (One Way test with post hoc Games and Howell, α=0.05).
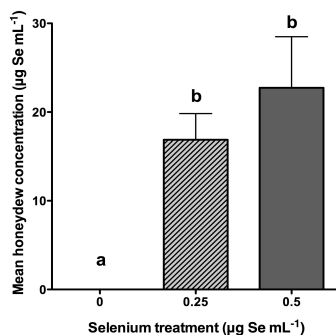
The trophic transfer factor of selenium from plants to aphids was <1 in the control group, but >1 in both selenium treated groups, suggesting the ability of selenium to

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biomagnify during this first trophic step (Table 3.2). In contrast, the trophic transfer

factor from aphid tissue to ants and aphid honeydew to ants was <1, indicating that

biotransfer of selenium had occurred without biomagnification.

Table 3.2 Selenium concentrations in each trophic group

Soil Treatment (µg Se/ g)	Plant	Aphid	Honeydew	Ant	Trophic Transfer Factor (TTF)		
					Aphid/Plant	Ant/Aphid	Ant/Honeydew
0	0.25	0.03	0.00	0.00	0.12	0.00	0.00
0.25	16.96	20.26	16.88	0.71	1.19	0.04	0.04
0.5	25.78	31.82	22.74	2.56	1.23	0.08	0.11

Table 3.2 Mean selenium concentrations (µg Se g^{-1}) for each trophic group, honeydew
(µg Se mL^{-1}) and the trophic transfer factors (TTF) at each trophic step. Ratios of metal
concentrations upper trophic groups to lower trophic groups that are >1=biomagnification; those
<1=biotransfer.

Discussion

Ants acquired selenium from the aphids feeding on treated plants. We are certain

that selenium was not transferred to the ants from the plants because there were no floral

nectar, pollen, or extrafloral nectar available. No detectable amounts of selenium were

measured in queens, but this is not entirely unexpected, as previous studies have also

found differences in metal levels across castes. For instance, Hladun et al. (2013a) found

that honey bee adult foragers fed selenium contained significantly greater body burdens

compared to larvae. Similar trends have been reported for red wood ant (*Formica* sp.)

colonies residing in metal-contaminated sites where differences were found 1) among

workers vs. pupae (Migula and Głowacka, 1996), 2) workers vs. pupae and newly

emerged sexual progeny (Starý and Kubizňáková, 1987) and, 3) outside workers vs.

inside workers, pupae and queens (Maavara et al., 1994). Unfortunately, we were unable

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to gather enough brood mass from the nests in order to obtain accurate analysis for selenium accumulated by larvae and the pupae. Final counts of brood were not taken after the tests ended, but it is possible that some of the brood may have died during the experiment if selenium was ingested (De La Riva and Trumble, 2016). Future research is necessary to determine the reason behind this pattern of "negative bioaccumulation" (Maavara et al., 1994) from workers to brood and reproductives. It is expected that individuals that are directly exposed to the source of the pollutant, such as foragers, will contain relatively greater levels, but whether the reduction in body concentration observed among members inside the colony is due to a dilution affect after many incidents of trophallaxis or to workers purposefully protecting other members via an evolved "socio-biological tolerance system," as hypothesized by Maavara et al. (1994), is yet to be elucidated. It is possible that lower levels previously reported in pupae were due to the elimination of toxins between the larval and pupal stage by molting of the exocuticle and/or excretion via the meconium (Dallinger, 1993; Newman and Unger, 2003). Nevertheless, this does not explain the lower levels seen in other adult members within the colony, as ants do not molt after emerging to the adult stage.

Previous studies appear to agree on the ability of selenium to biotransfer across trophic groups (Mathews and Fisher, 2008; Vickerman and Trumble, 2003), but opposing results have been reported for the ability of selenium to biomagnify (Barwick and Maher, 2003; Liu et al., 1987). When comparing selenium levels across trophic groups in our study, our findings suggest selenium was biomagnified from the soil to the plants and again between plants and herbivores. Although selenium was found in statistically greater

concentrations among the aphids compared to levels found in the radish foliage, lower levels of selenium were found in worker ants tending/ingesting those aphids. These findings support previous studies reporting differences in metal excretion abilities among various arthropods (Dallinger, 1993; Grześ, 2010), but are in contrast to higher metal levels seen in ants compared to aphids by Starý and Kubizňáková (1987). It appears that green peach aphids are eliminating selenium in their honeydew, but also retaining substantial amounts in their tissue, presumably from the action of metal binding proteins such as metallothioneins found in other invertebrates (Amiard et al., 2006; Roesijadi, 1992). The presence of much lower selenium levels occurring in the ants suggests that the ants may be excreting selenium. This was a slightly unexpected result due to the fact that workers of the same species contained body concentrations nearly twice as high as the concentration of selenium present in the sucrose diet provided in a previous experiment (De La Riva et al., 2014), whereas ants exposed to aphids with body burdens greater than 30 μg Se g^{-1} and/or honeydew greater than 30 μg Se mL^{-1} were found to contain less than 10 μg Se g^{-1} in their own tissues (Fig. 3.2). However, it is important to point out that workers in that previous experiment were not exposed in the presence of brood or reproductives and it is possible that selenium in their bodies was passing through at a much lower rate than might occur when other caste members are present.

Our findings indicate that there is no statistical difference in the amount of selenium acquired by ants from honeydew versus aphids used as protein sources versus both (Fig. 3.1 A), but that the main factor involved was the concentration of selenium added to the soil (Fig. 3.1 B). Adult ants are unable to ingest solid material, so acquisition

of selenium from freeze killed aphids could have occurred by ingesting fluids such as

hemolymph or regurgitated material from larvae. This could explain the slightly lower

concentration observed in worker ants provided aphids that fed on Se-contaminated

plants and untreated sucrose compared to those that ingested contaminated honeydew or

both.

Body concentrations in aphids in both selenium treatment groups averaged above

20 μg Se g^{-1}. Concentrations as low as 5 μg Se mL^{-1} have been shown to negatively

impact both queen fecundity and viability of developing offspring in Argentine ant

colonies (De La Riva and Trumble, 2016). Because their diet includes sugary resources

such as honeydew in their introduced range (Tillberg et al., 2007), this could be expected

to cause detrimental effects for populations Argentine ants expanding into contaminated

habitats. Toxic honeydew might also bring about negative effects for native species

residing in that same habitat that also seek out honeydew in addition to plant nectar (De

La Riva and Trumble, 2016). It is likely that exposure levels will differ across species as

a result of diet preferences and seasonally within a particular population, depending on

the presence or absence of developing brood. Mogren et al. (2013) found that levels of

arsenic transferred to predators differed based on their feeding strategy, where mantids

consuming entire arsenic-laden mosquitoes accumulated higher levels than spiders that

consumed only internal mosquito body fluids. Species-specific accumulation patterns of

heavy metals have also been described for ants residing in the same habitat (Rabitsch,

1997), suggesting possible differences in both metal regulation and diet preferences.

Future research is necessary to determine the effect of pollution on arthropod

communities composition and whether differences exist among ant-hemipteran

relationships in non-polluted versus polluted habitats.

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Chapter 4

Selenium Exposure Results in Reduced Reproduction in an Invasive Ant Species and

Altered Competitive Behavior for a Native Ant Species

Abstract

Competitive ability and numerical dominance are important factors contributing to the ability of invasive ant species to establish and expand their ranges in new habitats. However, few studies have investigated the impact of environmental contamination on competitive behavior in ants as a potential factor influencing dynamics between invasive and native ant species. Here we investigated the widespread contaminant selenium to investigate its potential influence on invasion by the exotic Argentine ant, *Linepithema humile*, through effects on reproduction and competitive behavior. For the fecundity experiment, treatments were provided to Argentine ant colonies via to sugar water solutions containing one of three concentrations of selenium (0, 5 and 10 μg Se mL^{-1}) that fall within the range found in soil and plants growing in contaminated areas. Competition experiments included both the Argentine ant and the native *Dorymyrmex bicolor* to determine the impact of selenium exposure (0 or 15 μg Se mL^{-1}) on exploitation- and interference-competition between ant species. The results of the fecundity experiment revealed that selenium negatively impacted queen survival and brood production of Argentine ants. Viability of the developing brood was also affected in that offspring reached adulthood only in colonies that were not given selenium, whereas those in treated colonies died in their larval stages. Selenium exposure did not alter direct competitive behaviors for either species, but selenium exposure contributed to an increased bait discovery time for *D. bicolor*. Our results suggest that environmental toxins may not only pose problems for native ant species, but may also serve as a potential obstacle for establishment among exotic species.

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Introduction

Competition between neighboring ants has been implicated as a driving factor in shaping ant assemblages within a given community (Cole, 1983; Rosengren, 1986; Valone and Kaspari, 2005). The coexistence of competing ant species is known to depend on key behavioral characteristics of the species, such as their ability to dominate in exploitative (indirect) or interference (direct) competitive interactions for food and territory (Fellers, 1987; Lebrun and Feener, 2007). These behaviors are considered to be an important factor in explaining successful establishment and expansion of certain invasive species in their introduced ranges, including the red imported fire ant, *Solenopsis invicta* (Porter and Savignano 1990) and the Argentine ant, *Linepithema humile* (Holway and Suarez 1999; Holway et al. 2002). Unlike most native species, the Argentine ant is reportedly dominant in both exploitation and interference competition (Human & Gordon, 1996, 1999). The generation of exceptionally large population densities following introduction into new ranges have also contributed to their competitive success (Holway, 1999). The successful growth in populations of the red imported fire ant has been attributed to their greater ability to engage in mutualistic partnerships for carbohydrates, in their less competitive introduced ranges (Wilder et al., 2011). However, competition alone has been shown to play a relatively minor role in shaping ant assemblages in comparison to other environmental factors, such as resource type and availability (Sanders and Gordon, 2003), habitat complexity (Sarty et al., 2006), or disturbance (King and Tschinkel 2006).

Anthropogenic disturbances such as fire, tree removal, flooding, and urbanization can alter ant species composition and richness (Andersen et al., 2006; Dunn, 2004; Graham et al., 2009; Hoffmann and Andersen, 2003; Nakamura et al., 2007; Pacheco and Vasconcelos, 2007), and thus have the potential to change competitive interactions. In some cases, such disturbances may actually encourage the colonization of invasive ant species (King and Tschinkel, 2008; Suarez et al., 1998), which may further alter dynamics within those communities. Previous studies have also investigated the effects of metal pollution on colony size, density, and species richness, where impacts ranged from neutral to detrimental (Eeva et al., 2004; Grześ, 2009; Hoffmann et al., 2000; Petal, 1978), but few studies have investigated the effects of pollution on competitive behavior and fecundity. There is some evidence for altered behavior among ants following exposure to pesticides. Research into competitive interactions between the red imported fire ant and the Argentine ant, revealed that the aggressive behavior by the red imported fire ant was reduced following ingestion of a pesticide treated bait (Kabashima et al. 2007). Similarly, exposure to a neonicotinoid pesticide resulted in altered aggressive behavior for both a native and invasive species (Barbieri et al., 2013). In an effort to reduce population growth, pesticides have been investigated for their potential to inhibit colony reproduction by specifically targeting the queens of pest species (Lofgren and Williams, 1982). Environmental contaminants may have similar effects on competitive behaviors and reproduction; therefore, more research is necessary to elucidate the mechanisms whereby changes in interspecific interactions among neighboring ant species may occur within a disturbed habitat.

Selenium (Se) is a soil-borne pollutant with particular abundance in the western United States (Brown et al., 1999). Although naturally occurring, soil and plant contamination with Se can increase via human activities (e.g. irrigation, mining and coal burning) that cause Se to become mobilized in the environment (Haygarth, 1994; Ohlendorf and Santolo, 1994). For instance, soil contamination with Se resulting from agricultural irrigation has become a serious problem affecting over 600,000 ha of farmland in eight western states (Brown et al., 1999). Selenium is also an essential dietary component for animals that helps maintain proper growth and development (Council, 1983), but toxicity can also occur following ingestion of Se that accumulates in plants that tolerate seleniferous soils (Eisler, 1985). Such plant species have been documented to contain moderate to high levels of Se in the nectar and pollen (Hladun et al., 2011; Quinn et al., 2011), which are resources consumed by ants (Blügthen and Fiedler, 2004; Czechowski et al., 2008; Markin, 1970). De La Riva et al. (2014) determined that Argentine ants experienced toxicity to concentrations of Se substantially below those reported in the nectar and pollen. In addition, these ants displayed no aversion to even highly toxic concentrations presented in artificial nectar. Therefore, the objectives of this study were to determine effects of Se on competitive behavior and reproduction in order to document the potential influence of Se pollution on ant community dynamics and establishment/range expansion of the invasive Argentine ant.

Materials and Methods

Collection and Study Organisms

Argentine ant colonies used in both the fecundity experiment and the competition experiment were collected from the same location at the University of California, Riverside, Agricultural Operations (30°57'47"N, 117°20'20"W, 308 m). One excavation was made for each for each experiment, where each excavation is enough to gather several thousand workers and approximately 10-25 queens. Workers of the pyramid ant, *Dorymyrmex bicolor,* were collected a short distance from the UCR campus (33°59'31"N, 117°19'58"W, 314 m). We were unable to use pyramid ants in the fecundity experiment due to the inability in excavating whole colonies. This species of pyramid ant, also belonging to the subfamily Dolichoderinae, was chosen to act as an opponent for the Argentine ant in the competition experiment, for the following reasons: 1) similar size, 2) the bicolored pattern would allow it to be easily distinguished during observations, 3) similar diet of insects, honeydew and nectar, and 4) overlapping range in southwestern California. *Dorymyrmex bicolor* was also chosen because these native ants are common in Se-contaminated sites (De La Riva and Trumble, unpublished), and have presumably evolved at least some tolerance to Selenium.

Fecundity Assay

The initial excavated Argentine ant colony was separated into 18 smaller sized colonies. This was done to reduce variability, both genetic and physiological across the 18 sub-colonies as all ants were presumably exposed to the same environment. Each sub-colony contained 1 queen: 100 workers that were placed in small plastic food storage

boxes (20 L x 15 W x 10 cm H) lined with liquid Teflon® (PTFE TE-3859, DuPont Fluoroproducts, Wilmington, DE) to prevent escape. The lids of each box had a hole in the center that was covered with mesh for ventilation. Each box contained a nest, a cotton-plugged vial of 25% sucrose, a vial of water, and approximately 3 g of chopped cockroaches, *Gromphadorhina portentosa*. Nests were composed of a 14.6 cm glass pipet that contained a small piece of cotton inside blocking the tapered end and a piece of foil paper around the outside of the pipet to create darkness (Supplemental Fig. 4.1). Distilled water was added to the cotton weekly at the tapered end of the pipet to maintain humidity within the nest. Colony boxes were placed in an environmental chamber at 28 °C (the optimal oviposition temperature for this species, Abril et al. 2008), 12:12 LD and ~60% RH. Ants were given a week to acclimate to their new surroundings before tests were conducted.

After one week of acclimation, boxes were inspected for worker deaths that may have occurred from accidental injury during the colony preparation. Dead workers were removed and replaced with live workers from the original colony. In order to simulate a toxic nectar source, we introduced Se to the colonies by removing the original sucrose solutions and replacing them with one of three different sucrose treatments (0, 5 or 10 µg Se mL^{-1}). We were confident that the ants would ingest the provided diets, because a previous choice experiment revealed that Argentine ants did not avoid sucrose containing 50 µg Se mL^{-1}, regardless of background sucrose concentrations (De La Riva et al., 2014). Selenate was the form of Se tested because it is a predominant form of selenium found in soils and taken up by plants (Pilon-Smits et al., 2013). Treatment solutions were

prepared by dissolving sodium selenate powder (Na$_2$SeO$_4$, 98% purity, Sigma-Aldrich, St. Louis, MO) in 25% sucrose solutions to yield the target concentration. Cotton-plugged vials were inspected weekly for moisture or microbial growth and replaced, if necessary. Fresh treatments were provided every 4 weeks. There were a total of six replicate colonies per treatment.

In order to track oviposition and development within each box, nests were removed weekly to record the number of eggs, larvae, pupae and newly emerged adults present. Newly emerged adults were easily identified by the typical pale coloration of ant exoskeletons, which may not darken fully until several hours after eclosion. A small piece of plumbers putty was placed on the open end of the pipet to prevent escape during counting. The colony box was also inspected periodically to replace dead workers in order to keep the number of workers constant.

Competition Assay

The experimental design consisted of the addition of 40 worker ants of *L.humile* or *D. bicolor* added to small plastic colony boxes (species kept separate). These boxes were in all ways similar to those provided for fecundity assays, except for the nests. Nests were made from 9-cm-diameter plastic Petri dishes containing a layer of Plaster of Paris on the bottom half (Supplemental Fig. 4.2). This layer was kept moist to maintain humidity within the nest by drawing in water from an attached cotton wick that was periodically soaked with ~1-2 mL of DDI H$_2$O. Cardboard was placed over the top to provide darkness. In order to investigate the influence of Se ingestion on behaviors related to competitive ability, worker ants were exposed to Se treatments of 0 µg Se mL^{-1}

or a sub-lethal level of 15 μg Se mL^{-1} (De La Riva et al., 2014) for one week before interacting with the opposing species. Treatment sugar solutions were also prepared as in fecundity assays using sodium selenate.

After one week of exposure to treatments, treatment solutions were removed from the colony box and the ants were starved for 24 hours. Following this, a pair of colony boxes (one *L.humile* and one *D. bicolor*) was arbitrarily chosen and placed on either side of a central test arena. The central test arena consisted of a larger plastic box (32.5 L x 26.5 W x 9.5 cm H), with approximately 2.5 cm layer of sand (Zoo Med Desert White Repti Sand, Petco). A small weigh boat (41 L x 41 W x 8 mm H) containing a cotton ball soaked in 25% sucrose was placed at the center of the arena so that its edges were flush with the top of the sand. Ants were then allowed to enter the arena with the use of simultaneously placed cardboard bridges (Supplemental Fig. 4.3). A video camera recorded all interactions and behaviors that occurred within the arena for 30 min from the time the bridges were added. Assays included combinations of treated and non-treated interspecific and intraspecific pairings, with a minimum of three replicates per combination. The following behaviors (Table 4.1) were recorded from the videos: 1) latency to bait discovery, the time it took the first ant of each group to find the bait after entering the arena, 2) duration and frequency of wrestling bouts, 3) frequency of neutral behaviors (antennation), 4) frequency of retreats, and 5) frequency of aggressive behaviors (lunges/chase, spasm, bite, gaster tilt). The initial 10 interactions for each opponent group were those that were scored. In this way, the observer could ensure that behaviors exhibited were not influenced by a previous interaction that ant had with the

opposing species. The number of ants on the bridge was also recorded at the time

intervals of 1, 5, 10, 15, 20, 25, and 30 min to determine possible sub-lethal effects on

climbing behavior. In addition, the number of casualties (ants that were dead or maimed

to the point of immobility) for each species in the arena at the end of each assay was

counted. All video reviews were conducted blindly (the reviewer was not aware of the

treatments involved).

Table 4.1 Definitions for direct interactions observed during competition assays. These
behaviors are adapted from those defined in Human & Gordon, 1999.

Antennate:	antennating another individual upon face to face contact
Retreat:	running quickly in the opposite direction of opponent after an encounter or chase
Spasm:	twitching or spasm-like movement of body after contact
Lunge/Chase:	jumping towards and moving quickly towards opponent
Bite:	biting any area of the opponent's body
Wrestle:	initiating a full body grapple with the opponent
Gaster Tilt:	bending of the gaster in an effort to direct it towards the opponent

After reviewing the video recordings for assays in which two different species

were interacting, there were several cases in which one species was unable to make it to

the bait due to interception by the opposing species. Additional assays (n=23) were then

conducted in which each species was allowed to enter the arena alone. This enabled us to

determine if selenium exposure had any effect on the latency of each species to the bait in the absence of interspecific competition.

Statistical Analysis

All data analyses were performed using R version 2.14.1 (The R Foundation for Statistical Computing 2011). A multiple comparison of the time to death of queens across treatments was conducted using a non-parametric Kruskal-Wallis test and post hoc pairwise t-test with Bonferonni adjustment ($p<0.05$). The number of eggs produced by queens across treatments was analyzed using a generalized linear model (GLM) with negative binomial distribution (NB). Model selection was based on the Akaike Information Criterion (AIC), and the final model included the number of eggs as the response variable, treatment as a fixed factor, and the time of death by queens as a co-factor. For exploitation competition, data on latency to the bait was analyzed using a GLM with Gaussian distribution. The model included seconds as the response variable and species, treatment, and competition as the fixed factors. A two-way analysis was carried out for each species separately, when we found no interaction or effect of species. For interference competition, the overall effect of species and treatment on competitive behavior was analyzed with a MANOVA. Subsequent analyses of the frequency of each separate behavior, and the number of casualties for each opponent at the end of the assay, were conducted using GLMs with Poisson distribution. Final models included the behavior or number of casualties as the response variable with species and treatment as the fixed factors.

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Results

Fecundity

Chronic ingestion of selenium had a negative impact on queen survival and reproduction. Queens in colonies fed 10 µg Se mL^{-1} died significantly sooner than queens in control colonies (Kruskal-Wallis: χ^2=8.6,df=2, P=0.01; Post hoc: pairwise t-test with Bonferonni adjustment, Fig. 4.1). No queens remained alive after 8 weeks in colonies given 10 µg Se mL^{-1} or after 11 weeks for colonies given 5 µg Se mL^{-1}. One queen from the 0 µg Se mL^{-1} died during week 2 for unknown reasons; however, two control colonies still contained healthy reproducing queens when the experiment ended after 14 weeks.
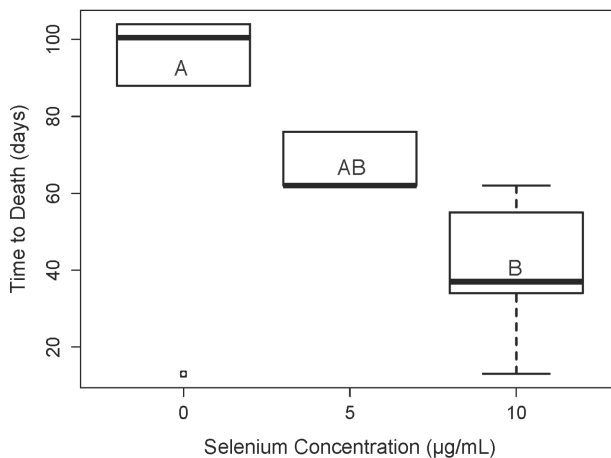
Fig. 4.1 Time to death (days) for Argentine ant queens across selenium treatments. Different letters represent means that are significantly different from each other (post hoc pairwise t-test).

The amount of brood produced by queens was also negatively impacted by selenium ingestion (Fig. 4.2). Colonies provided 5 and 10 µg Se mL^{-1} produced significantly fewer eggs than colonies fed no selenium (GLM.NB, χ^2=8.5,df=2, P=0.01). In addition, viability and development of their offspring were also affected (Table 4.2).

Offspring from control colonies reached adulthood, with one queen producing as many as six offspring that survived from egg to adult by week 14. In contrast, offspring from selenium treated colonies did not develop beyond the larval stage.

Fig.4.2 Comparison of the average number of eggs ± SEM produced by queens over time (weeks) for each selenium treatment category.
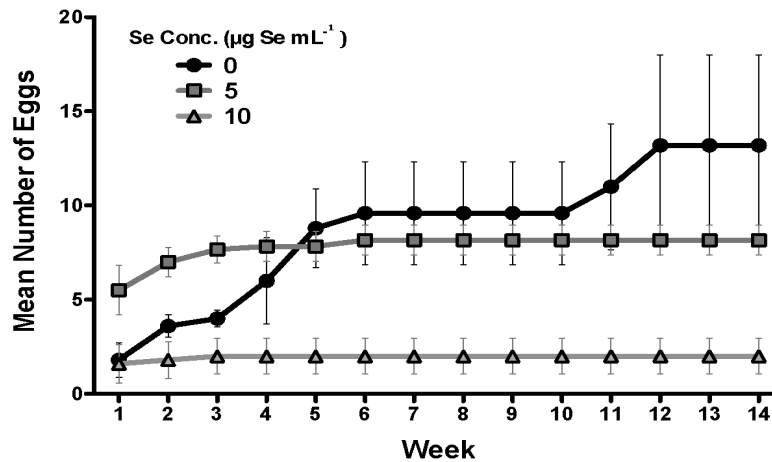
Table 4.2 Mean number of offspring that reached each developmental stage for each treatment category. The average number is in bold and the ranges are in parenthesis.

Developmental Stage	Control (0 μg Se mL^{-1})	Low (5 μg Se mL^{-1})	High (10 μg Se mL^{-1})
Egg	**13.2** (4-28)	**8.83** (5-10)	**3** (0-5)
Larva	**6.8** (1-14)	**4.67** (2-12)	**1.6** (0-8)
Pupa	**2.2** (0-10)	**0**	**0**
Adult	**1.4** (0-6)	**0**	**0**

Exploitation Competition

In the absence of selenium and competition, the average time it took Argentine ants to find the bait (171.38 ± 43.27 sec) was not statistically different than that for

pyramid ants (250.71 ± 58.08 sec, species effect: GLM, $\chi^2=0.59$, df=1,51, P=0.44). The time it took Argentine ants to find the bait was not affected by the presence of a competitor ($\chi^2=0.001$, df=1,20, P=0.44) or treatment ($\chi^2=1.02$, df=1,21, P=0.31), nor was there a significant interaction between the two factors ($\chi^2=2.05$, df=1,19, P=0.15, Fig. 4.3 A). In contrast, pyramid ants located the bait faster in the presence of a competitor ($\chi^2=6.44$, df=1,27, P=0.01; Fig. 4.3 B). Selenium treatment alone had no effect on latency to bait for pyramid ants ($\chi^2=2.82$, df=1,28, P=0.09), but the combination of selenium exposure and a competitor resulted in a slower location of the bait (treatment*competition: $\chi^2=4.71$, df=1,26, P=0.03).
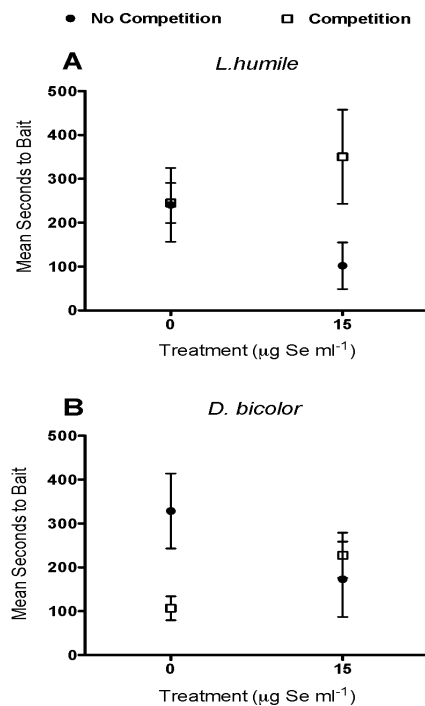
Fig. 4.3 Interaction plots show the effect of competition and selenium treatment on the average latency to bait (seconds) for *L. humile* (A) and *D. bicolor* (B).

Interference Competition

Selenium treatment had no overall effect on any of the behaviors exhibited by either species in direct interactions (MANOVA: F=0.47, df=7,230,P=0.86) nor was there an interaction between selenium treatment and species (MANOVA: F=0.26, df=7,230, P = 0.96). There was, however, a difference in the frequency of behaviors exhibited by each species (MANOVA: F=10.6, df=7,230, P < 0.0001). Table 4.3 shows the mean frequencies of the responses for each species upon encountering the opponent, where pyramid ants engaged in significantly more chasing and spasms and Argentine ants displayed significantly more retreats and gaster tilting. Both species had similar counts of antennation, biting, and initiating wrestling bouts. Assays in which the same species was used as the opponent resulted in no wrestling, spasms, biting, chasing or gaster tilting, but only antennation for both species and a single instance of retreating by an Argentine ant. Selenium treatment did not have an effect on the number of deaths/immobile ants remaining at the end of each assay (Z=-1.338, df=1,25, P=0.18); however, there was a significant difference in the number of casualties for each species (Z=2.09, df=1,26, P=0.04), where Argentine ants experienced nearly twice as many deaths as pyramid ants.

Table 4.3 Comparison of behaviors exhibited by the Argentine ant (*L.humile*) and pyramid ant (*D. bicolor*). Values shown below each species are means ± standard error of the means. n = number of encounters.

Response	*L.humile*	*D.bicolor*	Z	df	n	P
Antennate	0.66 ± 0.07	0.70 ± 0.05	-0.3	1	240	0.76
Retreat	0.80 ± 0.05	0.36 ± 0.04	-3.1	1	240	< 0.01
Spasm	0.23 ± 0.03	0.70 ± 0.05	3.2	1	228	< 0.01
Lunge/Chase	0.40 ± 0.05	0.87 ± 0.06	2.7	1	240	< 0.01
Bite	0.23 ± 0.03	0.40 ± 0.04	0.8	1	228	0.41
Wrestle	0.22 ± 0.03	0.36 ± 0.05	1.5	1	228	0.13
Gaster Tilt	0.76 ± 0.07	0.43 ± 0.04	-2.2	1	274	< 0.05
Casualties	8.3 ± 0.74	4.8 ± 0.64	2.09	1	28	< 0.05

Discussion

Two important intrinsic factors contributing to the success of invasive ant species, such as the Argentine ant are its competitive ability and its numerical abundance (Holway, 1999). Unlike many native species that are subject to a trade-off between discovering and dominating resources such as food and territory, the large numbers and aggressive nature of Argentine ants has allowed them to excel at both (Human and Gordon 1996,1999; Holway 1999; Bertelsmeier et al. 2015). However, other extrinsic components, such as habitat disturbance, should not be overlooked as an important factors influencing invasion and establishment of Argentine ants, because resulting changes in species composition, richness, and abundance (Floren and Linsenmair, 2001; Philpott et al., 2000) within a habitat are likely to leave communities susceptible to invasions (Gibb and Hochuli, 2003; King and Tschinkel, 2006). However, if native species have adapted a tolerance or avoidance to environmental toxins, expansion and establishment by the invasive species will likely be reduced.

Here we found a negative impact for reproduction among Argentine ant colonies fed selenium. Concentrations chosen for this study (5 and 10 μg Se mL^{-1}) are environmentally relevant and are actually among the lower range of concentrations that can occur in tissues of plants considered non-accumulating (up to ~20 μg Se g^{-1}); concentrations in selenium 'accumulating' plants may have up to 1000 μg Se g^{-1} while 'hyperaccumulating' plants may exceed 5,000 μg Se g^{-1} (Brown and Shrift 1982; Terry et al. 2000). Chronic ingestion of selenium was also found to impact queen mortality since queens from all colonies given 10 μg Se mL^{-1} were dead by week 8. The age of the queens at the beginning of the study was unknown and does not serve as a factor in fecundity (Keller and Passera, 1990); however the life expectancy of queens in Argentine ant colonies can extend over a year (Newell and Barber, 1913). Two of the queens from the control group were still alive and seemingly healthy at the end of the assay as they were still producing offspring. It is possible that they may have continued to do so for several more weeks had we not terminated the experiment at 14 weeks. The viability of the brood was also negatively impacted in colonies fed selenium, where offspring did not develop beyond the larval stage. In contrast, queens in control colonies produced brood that developed into adulthood. This developmental time falls within the range expected for Argentine ants (33-114 days from egg to adult; Newell and Barber 1913).

Previous studies have reported negative effects of metal exposure on ingestion, taxis/locomotion, and oviposition for various arthropods (Mogren and Trumble, 2010), yet few studies have investigated pollution effects on competitive behaviors. For both

species in this study, selenium did not alter the behaviors involved in direct competitive interactions. However, the combination of selenium exposure and the presence of a competitor resulted in a greater latency to the bait for the native pyramid ants that was not seen for Argentine ants. An increase in the time required to find resources in the environment could affect acquisition, especially when those resources are ephemeral, and further influence the competitive dynamics between species within a given habitat. The finding that these two species responded differently to the combination of a selenium and competitive pressure is not surprising, because ant species differ in both their competitive abilities (Parr and Gibb, 2010) and their tolerance to pollutants (Grześ, 2010). Insects have been reported to employ various strategies of metal regulation (Dallinger, 1993). Although the presence of such metal binding proteins, detoxifying enzymes and mineral concretions reported for other arthropods (Amiard et al., 2006; Egli et al., 2006; Roesijadi, 1992; Wilczek et al., 2004) have not been specifically reported in ants, the accumulation of metals in the same target tissues of the midgut and Malpighian tubules (Rabitsch, 1997) also seen in other insects (Brown, 1982; Humbert, 1978; Suzuki et al., 1984), suggests that they possess similar strategies. Furthermore, previous studies have reported differences in the accumulation of metals across ant species collected from the same habitat (Rabitsch, 1997, 1995; Starý and Kubizňáková, 1987). This suggests the possibility for differences in metal regulation physiology across ant species, and may help to explain differences in behavior seen in this study. The selenium levels in this study were enough to inhibit reproduction in Argentine ant colonies, but they were not enough to result in obvious changes in competitive behavior. This is in contrast to other

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studies that found changes in aggressive behaviors among invasive and native ants following exposure to pesticides (Barbieri et al., 2013; Kabashima et al., 2007) and, in one case, to copper pollution (Sorvari and Eeva, 2010). It is also possible that more significant changes in behavior may have become evident had the experiment been carried out longer than a week. However, longer exposure may have resulted in greater mortality as well.

For many ants, there are several possible routes of acquisition of environmental pollutants. For instance, within a contaminated habitat ants may not only acquire toxins from the soil, nectar and pollen, but also seeds, prey insects or insect cadavers, and honeydew. Each species may also display differences in their feeding preferences, which may result in different degrees of exposure depending on the food sources available. The pyramid ant was not deterred by the concentrations of Se in our tests, and this species has been found nesting in severely contaminated sites where soil concentrations ranged from 7-200 μg Se g^{-1} (De La Riva and Trumble, unpublished). This suggests a tolerance for Se. However, even though Argentine ants feed readily on food containing highly toxic concentrations of Se in their diet (De La Riva et al. 2014), our data show they lack a tolerance for even relatively low concentrations in their food source. Thus, the ability of Argentine ants to establish at sites within even low Se contamination would likely be inhibited by a physiological toxicity causing a reduction in queen survival and high larval mortality. This likely would lead to either population elimination or, at a minimum, loss of numerical dominance. The level of reduction in Argentine ant populations that would impact their ability to successfully compete for resources during range expansion is

unknown. Regardless, the differential toxicity of Se for these two species appears to play a much more substantial role in mediating interactions than the observed changes in competitive behaviors. Future research is necessary to understand this and other impacts of pollution, as a disturbance, on different stages of an invasion.

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Chapter 5

Arthropod Communities in a Selenium-Contaminated

Habitat with a Focus on Ant Species

Abstract

The selenium contamination event that occurred at Kesterson Reservoir (Merced Co., CA) during the 1970s is a frequently cited example for the negative effects of contamination on wildlife. Despite the importance of arthropods for ecosystem services and functioning, relatively little information is available as to the impacts of pollution on arthropod community dynamics. We conducted surveys of the arthropod community present at Kesterson Reservoir in order to assess the impacts of selenium contamination on arthropod diversity, with a focus on ant species richness, composition and density. Trophic groups were compared to determine which arthropods were potentially receiving the greatest selenium exposure. Plant samples were analyzed to determine the selenium content by site and by location within plant. Soil concentrations varied across the study sites, but not across habitat types. Topsoil contained higher levels of selenium compared to core samples. Plants contained similar concentrations of selenium in their leaves, stems and flowers, but flowers contained the greatest range of concentrations. Individuals within the detritivores, decomposers and predators accumulated the greatest concentrations of selenium, whereas nectarivores contained the lowest concentrations. Species composition differed across the sites: *Dorymyrmex bicolor* was only located at the site containing the greatest soil selenium concentration, but *Solenopsis xyloni* was found at most sites and was predominant at 6 of the sites. Selenium concentrations in ants varied by species and collection site. Nest density was also found to differ across sites, but was not related to soil selenium or any of the habitat variables measured in our study.

Selenium was not found to impact species richness, but was a significant variable for the occurrence of two out of the eight native species identified.

Introduction

An understanding of the responses exhibited by arthropods to habitat variation or stress can be important for predicting changes that may occur within arthropod communities following disturbance. In particular, changes that occur among ant populations may reveal potentially negative consequences for ecosystem functioning, due to the critical roles ants play in various ecological processes (Folgarait, 1998). In Australia, ants serve as useful biological indicators of ecosystem changes following such disturbances as fires, mining, deforestation, urbanization and pollution (Anderson and Majer, 2004; Hoffmann and Andersen, 2003). The impacts of heavy metal pollution on ant populations have also been extensively investigated in Europe (Grześ, 2010). Such investigations have reported pollution-induced effects on abundance (Bengtsson and Rundgren, 1984; Eeva et al., 2004), colony size (Eeva et al., 2004), species diversity (Bengtsson and Rundgren, 1984; Grześ, 2009), behavior (Sorvari and Eeva, 2010), and health (Sorvari et al., 2007). However, there is a lack of similar studies available for ant populations in North America, despite the existence of both natural and anthropogenic sources of environmental contamination.

Selenium is a naturally occurring element that enters the environment through the weathering of Cretaceous sedimentary rock, but can be concentrated and mobilized following human activities such as mining, smelting, coal burning and irrigation (Haygarth, 1994). Selenium is globally widespread, but present in varying concentrations

across regions within a given country (Oldfield 2002). In the United States, selenium is particularly abundant in soils of several western states (Brown et al., 1999), where approximately 414,400 km^2 of land is considered susceptible to rain or irrigation-induced selenium contamination (Seiler et al., 1999). Selenium is an essential dietary requirement for animals (including insects), and several reports have linked regions with high incidences of dystrophy, cardiovascular disease, and certain cancers to selenium deficiencies (Council, 1983; Oldfield, 2002). Previous studies suggest selenium may also play a similar important role for some insects (Martin-Romero et al., 2001; Popham et al., 2005). However, exposure to excess amounts of Se can also cause negative impacts, such as vomiting, hair loss, yellowing of nails in humans and blind staggers and hoof deformations in animals (Council 1983). In insects, selenium exposure can increase mortality, decrease reproduction, and modify behaviors (De La Riva and Trumble 2016; Burden et al. 2016; Hladun et al. 2016).

The potential for selenium toxicity in wildlife became evident in the 1980s when high concentrations of selenium at Kesterson Reservoir were found responsible for the deaths and deformities exhibited by birds breeding in the reservoir's evaporation ponds (Ohlendorf et al., 1986). The holding ponds of Kesterson Reservoir, located in Merced Co., CA, were originally meant to act as both a wetland habitat for migrating birds and a location to divert excess agricultural drainage water from the San Joaquin Valley's irrigated fields. However, damage to avian and fish populations occurred when subsurface drainage waters carrying selenium from the valley's selenium-abundant soils concentrated at the reservoir (Garone, 1998). The use of the reservoir as a repository for

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drainage water was then terminated and the ponds were filled in and the vegetation plowed in an effort to prevent further exposure to wildlife. Scientists have continued to monitoring wildlife in the area, with the majority of studies focusing on birds and small mammals (Ohlendorf et al., 1988; Santolo, 2009, 2007). A few publications have reported selenium levels occurring invertebrates at Kesterson (Ohlendorf, 2002; Ohlendorf et al., 1988; Santolo and Yamamoto, 1999; Santolo, 2007), but no published information is available as to the impact on ants, pollinators, or terrestrial insects at the population or community level.

The objectives of this survey were as follows: 1) Document the ant populations present; 2) determine whether selenium concentrations present in the environment are impacting ant species composition and abundance; 3) compare bioaccumulation levels of selenium across ant species and ant functional groups; 4) identify other insect taxa residing at Kesterson Reservoir; 5) compare selenium levels across different insect trophic groups.

Materials and Methods

Study Sites

Surveys were conducted during the spring of 2013 and 2014 at Kesterson National Wildlife Refuge, at the site of the former Kesterson Reservoir (37° 13' 53" N, 120° 53' 26" W, ~8 km east of Gustine, CA). The original 12 holding ponds no longer remain after the drying, filling and disking of the habitat; monitoring in the area has since been conducted based on three trisections of the 2100 ha land instead of the former 12 pond areas (Ohlendorf and Santolo, 1994). Our surveys were conducted on the southern

end of the reservoir (Fig. 5.1) in sites at Trisection 1 (previously ponds 1-4) and the

southern end of Trisection 2 (previously ponds 5-7, 9) in the three main habitat types

(filled, open and grassland). Filled habitats were previously lower elevation areas that

were filled in with soil, open habitats were the lands that formerly contained cattails that

were disked, and grassland habitats were the upland areas that existed before the reservoir

ponds were filled (Ohlendorf and Santolo, 1994). Collections were conducted in habitats

at the southern end of Kesterson because we expected soil selenium concentrations to be

greatest in the south (Trisection 1) and decrease northward, due to the fact that the

reservoir previously received drainage water that flowed from the southern to the

northern ponds (Wahl et al., 1994).
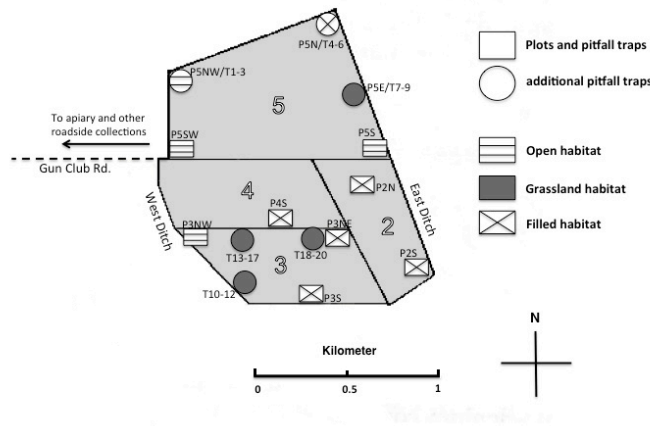
Fig. 5.1 Map of labeled collection site locations within Kesterson Reservoir that were previously ponds 2, 3, 4 and 5. Squares represent sites that contained six replicate 4x4 m plots containing pitfall traps. Circles only are those sites that only contained additional pitfall traps. Habitat types were determined using previously established maps of the reservoir (Ohlendorf and Santolo, 1994).

Data Collection

At each of the main collection sites (represented by squares in Fig. 5.1), six

collection locations were arbitrarily chosen encompassing a sampling area of ~1000 m^2

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site. A 4 m x 4 m plot was established at each of the six collection locations within each site. A sugar/carbohydrate ant bait for foragers was distributed within each plot (crumbled Pecan Sandies, Keebler[TM]) (Agosti et al., 2000). Baiting allowed for easier location of individual foraging ants, as well as helping to determine nest locations when foragers were followed back to their nest. Two people conducted collections within each plot for a total timed duration of 15 min. Hand collections (blunt featherweight forceps, BioQuip Products, Inc., Rancho Dominguez, CA) and aspirator collections were made of foraging ants, individuals exiting each ant nest, and other wandering ground insects. Samples were stored in coolers containing ice in 49 x 85 mm plastic vials. Due to the potentially high selenium levels at the southernmost collection site, we used a small shovel to scoop the top 13 cm of each nest (ants and soil), rather than aspirating directly from the nest. All samples were bagged in 1 L Ziploc bags and placed on ice. In order to determine the occurrence of species that may have had foraging activity periods at times other than when hand collections were made, we used pitfall traps (Agosti et al., 2000). Two pitfall traps (118 mL cups, 7 cm dia.) were placed (one per plot corner) diagonally from each other. These were filled ¾ of the way with water and mixed with a small amount of dish soap. Blue and yellow pan traps (355 mL, 18 cm dia.) were placed (one per corner) on the unoccupied corners of each plot, also diagonal from each other. Pan traps were also filled with a mixture of water and dish soap. A small rock was placed at the center of each pan trap to prevent tipping from wind. Pitfall traps and pan traps were left out for ~72 hr before collecting and emptying the contents into plastic collection bags (118 mL). These were also stored in ice coolers. Pitfall traps and pan traps were also

individually placed in other sites around Kesterson (represented by circles in Fig. 5.1) in

order to sample insects at sites from which we were unable to conduct hand collections.

Sweep netting for insects on flowering vegetation was conducted within the reservoir and

at several sites just outside the reservoir (roadside, near a local honey bee apiary, ditches

on the east and west edges of the reservoir). Upon returning to the laboratory, insect

samples were removed from the ice then stored in vials containing 80% ethanol prior to

species identification. Once identified, insects were placed either singly or with other nest

members into 1.5 mL centrifuge tubes. Bagged samples that contained topsoil and ants

from the southernmost site were emptied into separate trays. All ants belonging to a

single nest were removed from the soil, rinsed with double distilled water to remove soil

debris, and placed into a 1.5 mL centrifuge tube. A portion of the remaining soil was

taken for selenium analysis.

 Soil samples were also taken at each plot using a metal soil core sampler (~1.5 cm

dia.). Three core samples were taken at a depth of ~40 cm within each plot and were

mixed in a bucket. A composite sample from the mixture was bagged and stored on ice.

Soil moisture, soil pH, soil type, soil description (bare with small rocks/gravel, bare with

detritus/organic material, salt crusted), % ground cover, and a description of the %

vegetative type (grass, small shrubs, large shrubs, weeds) were recorded for each plot.

Data for temperature, % RH, and precipitation was taken from the online California

Irrigation Management Information System (CIMIS) using recordings from the Kesterson

weather station (#92). Flowering plants were sampled in order to assess selenium

exposure levels to pollen and nectar visiting insects. The majority of flowering plants

were located along ditches near the eastern and western edges of the reservoir. Plant samples from these locations as well as a few from within the habitat and near a local apiary and roadside, were collected by taking cuttings along the stalk of flowering sections and by taking leaf clippings. These samples were placed in separate Ziploc bags and stored on ice until further separations could be made in the laboratory.

Selenium Analysis

Insect and plant samples were freeze dried (Labconco Corp., Kansas City, MO) at -40 °C and -25 psi for 48 hr and 72 hr, respectively. Dried samples were then weighed on a microbalance prior to microwave digestion. Insect and plant samples were digested in 110 mL Teflon-lined vessels in 5 mL of HNO_3 for 20 min at 200 °C, 300 psi and 1200 W in a microwave oven (CEM Corp., Matthews, NC). Quality control was conducted using Se spikes, blanks, NIST Standard Reference Material 8436 (durum wheat flour) for plant samples and NIST 1566b (oyster tissue) for insect samples. Selenium recoveries for spikes and reference materials were on average > 90%. Solid soil samples were submitted on ice to Test America (Irvine, CA), where analysis of Se was conducted using method 6020-ICP/MS (Reporting Limit = 1.00 mg/kg; Minimum Detection Limit = 0.5 mg/kg). Selenium spike recoveries for soil were on average, over 90%.

Data Analysis

All statistical analyses were performed using R version 3.2.2 (The R Foundation for Statistical Computing, 2015). Normality and checking for equal variance were conducted using Shapiro Wilk's Test and Bartlett's Test, respectively. A non-parametric

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Kruskal-Wallis test or Welch Test was used to perform multiple comparisons when data could not be normalized by transformation.

In order to determine whether selenium concentrations in soil differed across habitats (filled, open, grassland) or by collection sites we performed multiple comparisons using Kruskal-Wallis tests. For analysis of plant concentrations across family, habitat, and plant part (flowers, stems, leaves, and whole samples for those that were too small to separate) we first compared two linear models containing interactions and no interactions of the three factors. When the results indicated no statistical significance for interactions of those factors (F=1.25, df=35, P=0.30), we analyzed each separately with a Welch Test. All arthropod samples were categorized into five trophic groups based on their general feeding patterns (Predators (P), Herbivores (H), Nectarivores (N), Omnivores (O), Detritivores/Decomposers (D) and Ant). Although ants fall under the category of omnivores, we were interested in comparing their selenium accumulation to those of the other arthropod categories. A Kruskal-Wallis test with post hoc Dunn Test and Bonferroni adjustment was used to compare selenium concentrations across trophic groups, and habitats. Extra habitat categories were included in the analysis for the insects that were collected at locations in the surrounding vicinity of the reservoir (road, apiary, west and east ditch).

A comparison of Se body burdens in ants across ant species and collection sites was conducted using a Kruskal-Wallis Test and post hoc Dunn Test with Bonferroni adjustment. In order to determine which habitat variables had an impact on ant species richness and nest density we first conducted a principle component analysis (PCA,

package *vegan* and *FactoMineR*) of all measured habitat variables (soil selenium concentration, % vegetative cover, soil-type, % large shrub, % small shrub, % weeds, % grass, moisture, pH, elevation, and habitat type). The first principal component explained ~28% of the variation and the second principal component explained ~17% of the variation. The four habitat variables with the highest contributions to the first dimension were soil-type (18%), grass (14%), moisture (15%), and pH (17%). A Spearman analysis of correlations between factors indicated that those variables were also highly correlated. In addition "cover" was highly correlated with "grass" at 81%. This suggests that the first principal component is distinguishes between two broad habitat types: 1) higher % grass and cover, lower soil moisture, higher pH, and bare or organic soil type vs. 2) lower % grass and cover, higher moisture, lower pH, and salty soil. We then used the individual principal component coordinates within the first dimension as a proxy in analysis against species richness, nest density, and the occurrence of each individual ant species. Generalized linear models with Poisson distribution were used to analyze species richness and nest density, whereas binomial distribution was used in the analysis for the presence or absence of each ant species. Soil selenium concentration loaded highest on the fourth principal component (28%), which only explained 10% of the total variation. A Spearman correlation of selenium against the other factors indicated that it was not highly correlated to any other variable. We, therefore, analyzed it as a separate factor. To determine whether ant species composition might have also been influenced by the presence or absence of other ant species across the different collection sites, we conducted a Co-Occurrence Analysis (EcoSimR package 0.1.0, *SIM9* algorithm) with a

C-score metric to indicate aggregation vs. avoidance (Gotelli, 2000; Parr and Gibb, 2010).

Results

Soil selenium concentrations for soil core samples did not differ across the established filled, open, and grassland habitat types (Kruskal-Wallis: X^2=5.6, df=2, P=0.06), but they did differ across collection sites (Kruskal-Wallis: X^2=39.96, df=8, P<0.0001, Fig. 5.2). Core samples from the southernmost site (P2S) had the highest concentrations ranging from 16-35 mg Se kg^{-1} (post hoc Dunn test with Bonferroni, Ps < 0.001). Topsoil concentrations from ant nests collected at this location were much higher than core samples and ranged from 7-200 mg Se kg^{-1} (Table 5.1). In contrast, soil cores from collection sites P3S and P4S contained the lowest concentrations at ranges of 0-2.8 and 0 mg Se kg^{-1}, respectively (post hoc Dunn Test with Bonferroni, Ps: 0.04-0.0001).
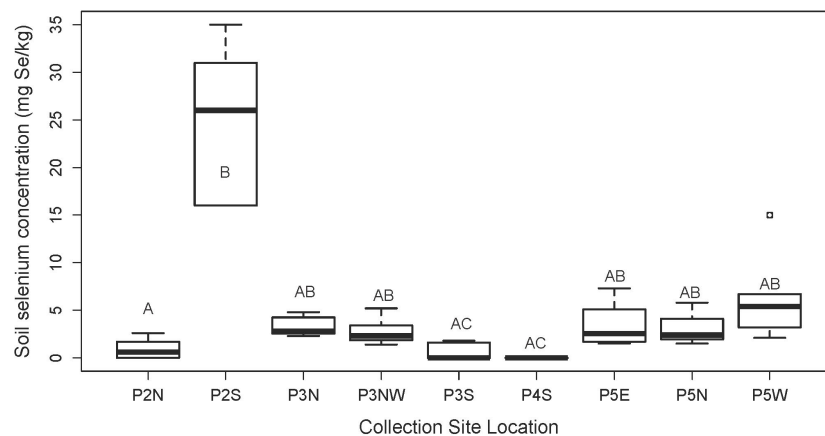
Fig. 5.2 Soil selenium concentrations across collection sites. Site "P5W" also refers to "P5SW." There was no soil sample for "P5NW". Different letters represent samples that contained statistically different concentrations (Kruskal-Wallis, post hoc Dunn test with Bonferroni p. adj., α=0.05)

Nest Sample ID	Soil Conc. (mg Se/ kg)	Ant Species	Ant Conc. (µg Se/ g)
P2-1-1N1	7	*S.xyloni*	20.67
		D.bicolor	10.33
P2-1-1 N2	99	*S.xyloni*	29.37
		D.bicolor	14.59
P2-1-1 N3	87	*S.xyloni*	29.06
		D.bicolor	25.37
P2-1-1 N4	200	*S.xyloni*	34.59
P2-1-6 N1	16	*D.bicolor*	10.12
P2-1-6 N2	52	*S.xyloni*	17.57

Table 5.1 List of Se concentrations for ant nest soil with a comparison of accumulated selenium between two closely nesting ant species.

During both years, the majority of flowering plants were found at the surrounding edges of each pond as well as in the surrounding vicinity of the reservoir, rather than inside the habitats. We analyzed plants belonging to five plant families: Brassicaceae, Azoaciae, Amaranthaceae, Apiaceae, and Asteraceae (Supplemental Table 5.1) for analysis of selenium as a factor of habitat/collection location, family, and plant part. An initial comparison of models indicated that there were no significant interactions between the three factors (ANOVA, F= 1.26, P=0.3) and so we analyzed each factor separately. Interestingly, the selenium contained in plants did not vary as a result of family (Welch Test: F=3.5, df=4,4.2, P=0.12), collection site (Welch Test: F=3.9, df=3,8.8, P=0.051) or plant part (Welch Test: F=4.09, df=3.3,4, P=0.09). However, flowers contained the greatest range of concentrations from 0-27 µg Se g^{-1}, whereas the stems, leaves, and whole samples contained concentrations below 5 µg Se g^{-1}.

Arthropods across 13 different orders and 29 families were analyzed for selenium accumulation (Supplemental Table 5.2). Arthropod concentrations were also found to differ across habitat types and additional collection locations (Kruskal-Wallis: X^2=13.18,

df=6, P=0.04). Except when compared to arthropods collected from plants along the ditch on the east end of the reservoir (post hoc Dunn test: P= 0.12), arthropods collected from the grassland habitat accumulated significantly greater concentrations compared to other habitat sites (open, filled) and additional habitat locations, such as plants along the roadside, apiary, and west ditch (post hoc Dunn test: all Ps = 0.04-0.005). Insects collected from the flowering plants near a local apiary and the ditch on the western site of the reservoir contained the lowest average concentrations of 0 and 0.3 μg Se g^{-1}, respectively. Selenium concentrations accumulated by arthropods were also found to differ across trophic groups (Kruskal-Wallis: X^2=32.7, df=5, P<0.0001, Fig. 5.3), where nectarivores contained the lowest body concentrations (0-14 μg Se g^{-1}) of selenium compared to all other trophic groups (post hoc Dunn test with Bonferroni: all Ps<0.01). Detritivores/decomposers had the greatest range of concentrations from 0-178 μg Se g^{-1} followed by predators with 0-123 μg Se g^{-1}. A few herbivorous insects also contained high Se levels such as 73 μg Se g^{-1} in a stink bug and 35 μg Se g^{-1} within a caterpillar. Ants fall under the category of omnivores, feeding on both plant resources and insect, and did not differ in accumulated selenium from other insects in that category (post hoc Dunn test: P=0.49).
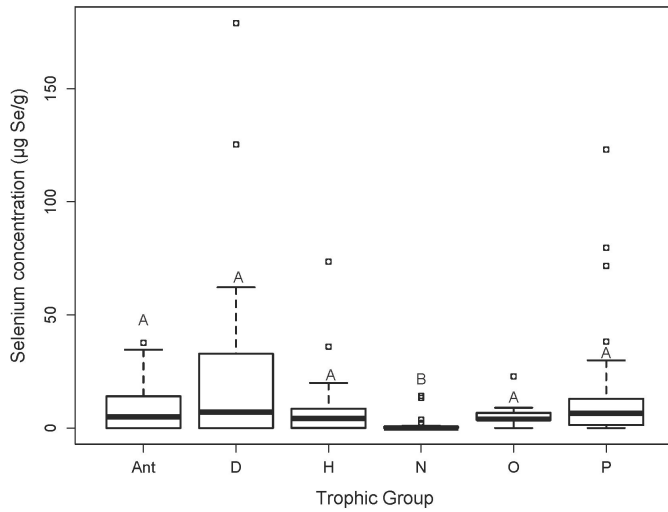
Fig. 5.3 Selenium concentrations compared across trophic groups, where D=detritivores/decomposers, H=herbivores, N=nectarivores, O=omnivores, P=predators and Ant=ants). Ants are often considered omnivores, but were separated in order to compare their accumulation to all other groups. (Kruskal-Wallis, post hoc Dunn test with Bonferroni p. adj., α=0.05)

Eight different native ant species were found residing at Kesterson: *Solenopsis xyloni*, *Dorymyrmex bicolor*, *Dorymyrmex insanus*, *Pheidole hyatti*, *Forelius mccooki*, *and two species of Formica* (Voucher specimens of collected ants were submitted to the UC Riverside Entomology Museum collection: UCRC ENT 461246-461263). Species composition differed across collection sites (Fig. 5.4). For instance, the ant species *D. bicolor* was only found nesting in the southernmost habitat containing the highest selenium soil concentrations. In contrast, the fire ant species *S. xyloni* was present at 10/14 sites, followed by *P. hyatti* at 50% of the sites. Species richness did not differ across collections sites (GLM with Poisson: Z=-0.2-1.0, df=12, 67, all Ps >0.33), nor did it vary as a factor of selenium concentration (GLM with Poisson: Z=-0.4, df=7,47, P=0.7) or habitat factors (GLM with Poisson: Z=-0.7, df=7,47, P=0.5). Nest density did vary across collections sites with the greatest total number of nests located at P5S (15 nests, GLM with Poisson: Z=2.7, df=12,67, P<0.01), P2S (12 nests, GLM with Poisson: Z=2.1, df=12,67, P=0.03) and P4S (11 nests, GLM with Poisson: Z=2.7, df=12,67, P=0.04).

However, neither selenium soil concentrations (GLM with Poisson: Z=-0.5, df=7,47, P=0.6) nor the habitat factors measured in our survey (GLM with Poisson: Z=-0.8, df=7,47, P=0.4) were found to explain this variation.
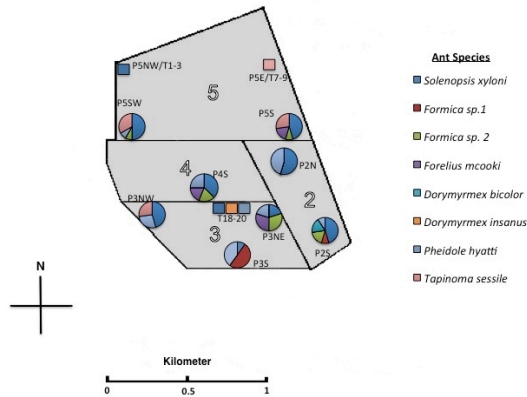
Fig. 5.4 Ant species composition at each collection site. The size of each slice in the pie graphs represents the relative proportion of each species at that location. Small squares represent the occurrence of a species in pitfall traps.

We determined whether soil selenium concentrations and/or our measured habitat variables might explain the presence or absence of each ant species across the different sites (Table 5.2). Soil Se was a significant factor for *D. bicolor* and *Formica sp*.2 and habitat factors (Dim.1 from PCA) were significant for *S. xyloni* and *Formica sp*.2.

Ant Species	Factors	Z	P value (α=0.05)	
Solenopsis xyloni	Se	0.23	0.82	
	Dim1	-2.48	0.01	★
Dorymyrmex bicolor	Se	1.98	0.05	★
	Dim1	-	-	
Dorymyrmex insanus	Se	-	-	
	Dim1	-	-	
Forelius mccooki	Se	-1.45	0.15	
	Dim1	0.92	0.36	
Formica sp.1	Se	-1.97	0.06	
	Dim1	2.85	0.01	
Formica sp.2	Se	2.28	0.02	★
	Dim1	-2.31	0.02	★
Pheidole hyatti	Se	-1.80	0.07	
	Dim1	0.36	0.72	
Tapinoma sessile	Se	0.20	0.84	
	Dim1	-1.95	0.06	

Table 5.2 Results from individual ant species occurrence as a factor of soil selenium concentration (Se) or habitat factors (Dim1) that explained the majority of the variation for the first prinicipal component of the PCA analysis of all measured habitat variables. Test method: GLM with binomial distribution, df=47, α=0.05. Stars next to p-values highlight factors of significance. Dashed lines represent analysis that could not accurately be performed due to low occurrence across different sites.

To determine whether competition might have played a role for ant species where habitat factors and selenium were not predictors, we conducted a co-occurrence analysis. The observed distribution of co-occurrence between all ant pairs was not different from the simulated distribution (Co-Occurrence Null Model, Algorithm *SIM9*, P=0.4, C-score=3.14), indicating that their co-occurrence with each other was neither a result of aggregation nor avoidance between pairs. Finally, we found that selenium concentration differed across ant species (Kruskal-Wallis: X^2=20.8, df=7, P<0.0001, Fig. 5.5) with the highest median accumulated selenium occurring in *D. bicolor* and the lowest in *F. mccooki* and *Formica spp.* The pyramid ant species, *D. insanus* was omitted from the test due to the low sample size of one nest. The variation in concentration among *P. hyatti* and *S. xyloni* might be explained by the location at which those samples were collected, as ant concentrations also differed as a factor of collection site (Kruskal-Wallis: X^2=30.9, df=7, P< 0.0001). Ants collected at collection site P2S contained greater selenium body burdens compared to sites P3NE, P3NW, and P4S (post hoc Dunn test with Bonferroni: Ps=0.0004-0.03). However, species nesting closely together in the same habitat (*D. bicolor* and *S. xyloni* were found together in the several of same soil nest samples taken from P2S) were still found to accumulate different levels of selenium (Table 5.1).
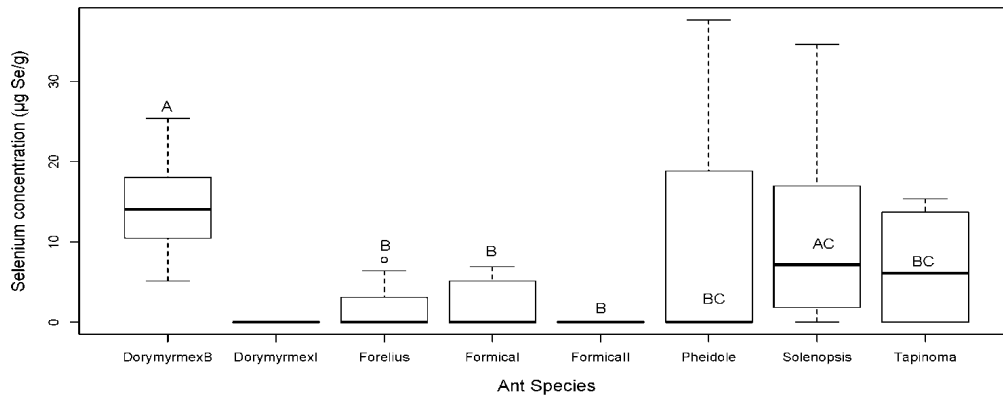
Fig. 5.5 Accumulated selenium across ant species. The ant species, *Dorymyrmex insanus* "DorymyrmexI" was graphically represented, but was not included in the analysis due to the small sample size. (Kruskal-Wallis, post hoc Dunn test with Bonferroni p. adj., α=0.05)

Discussion

Soil selenium concentrations differed across collection sites and ranged from 0 mg Se kg^{-1} in plots at sites P4S and P3S to 35 mg Se kg^{-1} in site P2S. Topsoil samples (~13 cm depth) directly from ant nests mounds contained greater levels of Se than core samples (~40 cm depth), with a maximum concentration of 200 mg Se kg^{-1} at one nest site. This supports previous reports that stated the highest selenium concentrations in soil at the reservoir are contained in the top 15 cm and decline with increasing depths (Wahl et al. 1994 [and references therein]). The morphology and depth of ant nests is species specific. For instance, nests of harvester ants, genus *Pogonomyrmex,* have depths ranging from 2-3.5 m (Tschinkel, 2003), whereas ant species within the genus *Dorymyrmex* commonly nest in depths no deeper than 10-15 cm (Cuezzo and Guerrero, 2011). This suggests that the native ant *D. bicolor,* identified in our study possesses a high tolerance to Se, due to the fact that it was only located in the topsoil at the site containing the

highest selenium concentrations. Depending on the depth and structure of the ant nests

for other species residing at Kesterson, its possible that some of the other species may be

escaping Se contact by modifying the depth of the nests. However, initial excavation and

periodic maintenance of those nests is likely a source of Se transfer to workers. It is also

possible that nest depth and thus exposure to concentrated soils may change with season,

as some ant species have been reported to adjust their distance from the surface in

response to ambient temperature (Bollazzi et al., 2008). In contrast to previous studies

which found the highest selenium soil concentrations in open habitats, compared to filled

and grassland (Wahl et al. 1994; CH2M HILL 2000; CH2M HILL 2015), soil

concentrations in our study did not vary. However, this is not surprising because our

study was conducted predominantly at the heavily contaminated southern end of the

reservoir, rather than across the entire reservoir.

Plant samples collected during our study were analyzed to assess Se levels

available to herbivores as well as insect pollinators/nectarivores. Our findings indicated

that Se levels accumulated by plants did not statistically differ across plant family or

collection site. However, the majority of flowering plants during our spring surveys

(predominantly Brassicaceae) were located at the surrounding edges rather than among

plants within the habitat. Total Se concentrations also did not differ by plant structure, but

flowers contained the greatest range of concentrations, exceeding 20 μg Se g^{-1} in some

Brassica sp. samples, whereas other structures did not contain levels above 5 μg Se g^{-1}.

Previous experiments have described the ability for selenium accumulating species within

Brassicaceae to concentrate levels exceeding 100 μL Se mL^{-1} FW in the nectar and 1000

µg Se mL^{-1} in the pollen (Hladun et al., 2011; Quinn et al., 2011). It was therefore surprising that nectarivores (native bees, syrphid flies, and honeybees) in our study contained the lowest selenium body concentrations compared to other trophic groups. This suggests the possibility that arthropods seeking nectar sources may be experiencing different levels of exposure throughout the year as the location and timing of flowering at the reservoir vary with season.

A comparison of accumulated levels across trophic groups revealed that trophic groups were not statistically different except when compared to nectarivores, as described above. This suggests that biomagnification does not appear to be occurring between trophic groups within this habitat. Nevertheless, there were several notably high selenium containing samples among detritivores/decomposers, herbivores and predators. The wide range in concentrations observed for detritivores was mostly due to the concentrations accumulated by Isopods, which ranged from 29-179 µg Se g^{-1}. Although detritus was not sampled in our study, these findings support recent conclusions for dead/organic plant material serving as an important entry pathway for selenium into trophic food webs in this habitat (CH2M HILL, 2015). Despite previous reports for the greatest concentrations in soil, plant, and detritus samples occurring in the open habitat compared to filled and grassland habitats, our study indicated that arthropods collected from the grassland habitat contained the greatest body burdens. However, grassland habitat in our sampling area was sparse compared to the northern end of the reservoir, and was largely surrounded by both filled and open habitat types. Except for nesting or burrowing arthropods, such as ants and some spiders, which remain relatively sedentary over longer

periods, it is very likely that several insects analyzed may have been travelling between habitats in search of food. Overall concentrations for arthropods reported in our study, are similar to those found in previously sampled invertebrates from this habitat (Santolo and Yamamoto, 1999; Santolo, 2007).

Several studies have investigated the impacts of disturbance on ant diversity (Hoffmann and Andersen 2003 [and references therein]). The majority of the research exploring the effects of pollution on ants has come from Europe, where findings include impacts on abundance (Bengtsson and Rundgren, 1984; Eeva et al., 2004), colony size (Eeva et al., 2004), species diversity (Bengtsson and Rundgren, 1984; Grześ, 2009), behavior (Sorvari and Eeva, 2010) and health (Sorvari et al., 2007). Currently, there is a lack of comparable information available for North American ant species. In our study, species composition differed across collection sites, but selenium contamination as a possible factor was only significant for the occurrence of two species, *D. bicolor* and *Formica sp.*2. In addition, selenium appears to have little impact on ant species richness and density. Taken together, this suggests that these native species have a high tolerance to selenium. This is in contrast to experiments that have shown detrimental impacts of selenium on the survival (De La Riva et al., 2014) and reproduction (De La Riva and Trumble, 2016) of the invasive Argentine Ant, *Linepithema humile*. According to an updated document mapping the global distribution of Se (Oldfield, 2002), there is a high incidence of selenium deficient soils in the native range of Argentine ants. This might help to explain the previously reported susceptibility of Argentine ants to selenium as well as lead to important predictions for the response of other invasive species from the

same area, such as *Solenopsis invicta*, the red imported fire ant. Sub-lethal impacts of selenium on native species, which were not captured by our measurements, are also possible. For instance, (Eeva et al., 2004) found that populations of wood ants *Formica* sensu stricto were able to nest and reproduce in habitats containing high levels of heavy metals, but exhibited smaller colony sizes compared to those nesting in non-polluted sites. Additional research is necessary to elucidate the reasons behind these differences in tolerance.

Selenium concentrations accumulated by ants were influenced by species and nesting site. For example, *D. bicolor* was among the highest accumulating species and was found nesting in soil with the highest level of Se contamination. However, diet preferences and possible differences in metal regulation between species should not be ruled out, as was evidenced by the different concentrations accumulated by *D. bicolor* and *S. xyloni,* despite their identical locations.

This study highlights the importance of arthropods as a potential pathway for transfer of contaminants to higher trophic groups. More work is necessary to explore the physiological mechanisms and/or evolutionary reasons behind tolerance of pollutants among different arthropods. Knowledge of existing differences in the metal regulation ability between native and invasive arthropod species can lead to important predictions and management decisions for areas susceptible to invasion. Future research on arthropods at Kesterson Reservoir should consider comparing differences in trophic groups with vegetation and flowering changes throughout the year. A greater sampling

effort is necessary to compare arthropod communities across the entire reservoir area to

elucidate the existence of additional species or changes in community dynamics.

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Chapter 6

Conclusions

Insects are vital members of ecological communities for their influence in ecosystem processes. Ants are especially unique in that they can belong to more than one trophic group and participate in various environmental activities, which include seed dispersal, soil aeration, predation, pollination, nectar robbing, and developing mutualistic associations with plants or herbivores. However, this wide variety of involvement can also increase their chances of exposure to environmental contaminants. Previous studies from Europe and Australia have reported on the detrimental impacts of anthropogenic disturbances, including pollution, on native ant communities. Here we investigated the impacts of selenium, a globally widespread element and an environmental contaminant in certain regions of the United States, on the survival, behavior and reproduction of an invasive ant species. An exploration of the influence of soil selenium levels on species composition, richness, and density of a native ant community was also conducted.

We determined that selenium does pose a potential threat to range expansion for the Argentine ant, especially in areas of the environment with abundant or contaminated levels. Our findings demonstrated that selenium concentrations documented to occur in the floral nectar, pollen and seeds of accumulating plants are lethal to Argentine ants. The extent of the impact to the colony will depend on the chemical form ingested as well as the duration of exposure. Furthermore, foraging ants were undeterred by lethal concentrations of selenium in the diet, regardless of background sucrose concentrations, indicating the potential for ants to continue recruiting to toxic food sources. In their introduced ranges, Argentine ants are not only pests in the home, but are also active tenders of honeydew producing insects. Results from our tri-trophic study using a

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secondary-accumulator of selenium, *Raphanus sativus* (wild radish) and the herbivore *Myzus persicae* (green peach aphid) indicated that selenium could be transferred to Argentine ant workers via honeydew and insect prey. This was determined by analyzing plant, aphid and ant tissue for total selenium concentrations. According to the average levels accumulated by each trophic group, selenium has the ability to biomagnify from plants to herbivores in this system. However, the trophic transfer factor from aphid tissue and honeydew to ants was < 1, indicating only biotransfer took place at this trophic step. Nevertheless, these findings suggest the possibility for contaminants to be transferred to both primary and secondary consumer arthropods, where levels of transfer and accumulation will likely depend on the metal regulation physiology of the subject. Interestingly, Argentine ant queens did not contain detectable levels of selenium. This was not unsurprising, as previous studies have reported similar results between castes. However, it is unclear as to whether this was simply due to dilution of the food between workers before reaching the queens or if workers were purposefully not exposing queens to the toxin. Based on our results from the feeding choice assays, where ants did not display a preference between a non-toxic diet and one containing lethal concentrations of selenium, we suspect that for this system, it is most likely the result of the former situation.

An understanding of the behavioral traits responsible for the successful establishment of invasive species is key in making steps to prevent or predicting future introduction consequences to ecosystems. For this reason, Argentine ants have been studied to elucidate the reasons behind their ability to displace native ant species in their

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introduced ranges. The impact of selenium on two factors contributing to the success of Argentine ants, their numerical dominance and competitive ability, was also explored. To understand the effect of a contaminant on population size, we were interested in determining whether sub-lethal levels of selenium might impact queen fecundity. Chronic ingestion of sub-lethal levels of selenium was found to significantly reduce the number of eggs laid by queens and disrupt development, where offspring from exposed colonies did not survive past the larval stage. Furthermore, long-term exposure of selenium also resulted in queen mortality. As far as impacts on competitive ability, we investigated the influence of sub-lethal levels of selenium on direct (interference competition) and indirect behaviors (exploitative competition) for a native ant species, *Dorymyrmex bicolor* and the Argentine ant. Selenium did not alter direct competitive behaviors such as lunging, grappling chasing, biting, etc. for either species; however, in the presence of a competitor, selenium exposed individuals of the native species took longer to find the bait compared to unexposed individuals. In the field, a greater duration would translate into longer time for recruits to arrive. Especially in the cases where the food source is ephemeral, a greater duration of discovery could mean the difference between obtaining that source of nutrition or not for the colony. The overall findings of these two experiments suggest that the influence of the contaminant on the ability for the invasive species to establish will depend both on its ability to regulate and detoxify the pollutant and the ability for the native species already residing in the habitat to do so as well. At least in the case for Argentine ants, a reduction in the number of healthy queens

would almost certainly impact colony sizes and their ability to compete for food and territory with native ant species.

Previous research from Europe and Australia have reported on the impacts of heavy metal pollution on species diversity for native ant communities, but there is a lack of comparable studies for North American ant communities. In order to address this issue, we conducted a survey of arthropods inhabiting Kesterson Reservoir (Merced Co., CA), an area that experienced high levels of selenium contamination between the 1970s and 1980s. Monitoring of wildlife within the site has continued since remediation efforts from 1987, but the majority of studies have focused on birds and small mammals. Our survey was conducted during the spring of two years in the southern end of the site, which received the majority of the contamination. Soil selenium concentrations differed across our collection sites, with the highest levels occurring at the southernmost site. Selenium concentrations were also highest among surface soils. Ant species composition differed across the collection sites, where *Dorymyrmex bicolor* was found residing in habitats with the greatest levels of selenium. In contrast, *Solenopsis xyloni* was present at nearly every location. Species richness and nest densities did not differ as a result of soil concentrations. Ants were found to contain comparable concentrations of selenium as other omnivores, herbivores, predators and detritivores. Suprisingly, nectarivores/pollinators contained the lowest concentrations compared to all other trophic groups. A comparison of selenium concentrations across ant species revealed that the degree of accumulation is likely influenced by nest location and diet preferences. Overall, we determined that native ant species residing at Kesterson possess a high tolerance to

selenium. It is possible that selenium may still be impacting these native ant species to a degree that was not elucidated by our measurements.

The native range for Argentine ants in South America has been documented to contain many cases of selenium deficiency among livestock, which suggests that possibility that North American ant species have perhaps evolved detoxification strategies specific to chemicals occurring in those habitats. Future research is necessary to determine the mechanisms behind their differences in susceptibility. A better understanding of differences in metal regulation physiology may help to predict the effects of selenium and other potentially novel compounds to additional invasive ant species, such as the red imported fire ant, *Solenopsis xyloni*. The findings of this research highlight the importance for additional exploration into the influence of natural and anthropogenically introduced environmental contaminants on invasive ecology.