Investigation of elemental defense and trophic transfer of metals using beet armyworm, *Spodoptera exigua,* Hübner

by

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Abstract

Elemental defense is a phenomenon in which high concentrations of a metal in plant tissues can defend a plant from herbivores. Elemental defense is one of the hypotheses proposed to explain the evolution of metal hyperaccumulator plants. In this dissertation, I evaluated the toxicities of Co, Cu, Ni and Zn against a generalist herbivore, *Spodoptera exigua,* using artificial diet. First, I determined the minimum lethal (MLC) and minimum sublethal concentrations (MSC) for each metal against *S. exigua* larvae. I showed that MLC values (dry mass basis) for three metals (90 µg Co g^{-1}, 570 µg Ni g^{-1} and 280 µg Zn g^{-1}) were below published minimum hyperaccumulator levels, whereas that for Cu (530 µg Cu g^{-1}) was above. MSC levels (dry mass basis) were all recorded at concentrations lower than published minimum accumulator levels: 15 µg Co g^{-1}, 95 µg Ni g^{-1} and 200 µg Zn g^{-1}, with the exception, again of Cu (175 µg Cu g^{-1}). Furthermore, both MLC and MSC values for Zn were within normal tissue concentrations (20-400 µg Zn g^{-1}). Second, I evaluated the type of joint effect that occurs between Co, Cu, Ni and Zn when fed in combination to *S. exigua* larvae and between the four metals and three organic compounds (nicotine, mustard seed powder and tannic acid) when fed in combination to *S. exigua*. The Joint Effects Hypothesis suggests that combinations of metals, or a combination of a metal with an organic defense compound, may have increased defensive effects (these may be additive or synergistic). I tested for joint effects using both lethal and sublethal concentrations of the chemicals tested. I showed that all lethal and sublethal joint effects were either synergistic or additive (none antagonistic).

Third, I evaluated the survival, growth and development of a generalist predator (*Podisus maculiventris*) when fed herbivorous prey (*Spodoptera exigua*) reared on artificial diets amended with MSCs and MLCs of Co, Cu, Ni and Zn as an example of potential tritrophic interactions. Additionally, I determined if metals were biotransferred or bioaccumulated from diet to herbivore and to predator. Survival of *P. maculiventris* was not affected by feeding them larvae raised on diet amended with any of the four metals. Predators fed herbivores reared on Cu-amended and Zn-amended diets took a significantly longer time to mature to adults compared to control treatments ($P = 0.0001$ and $P < 0.0001$, respectively). Adult weights were significantly reduced only for predators that were raised on herbivores reared on diets amended with the MLC of Cu ($P = 0.018$). The presence of metals in artificial diets resulted in biotransfer of Ni, and bioaccumulation of Co, Cu and Zn, from the artificial diet to the herbivore and from the herbivore to the predator. Overall, my research showed the potential ecological importance of elemental defenses at relatively low metal levels, the role joint effects may play in lowering threshold levels for sublethal and lethal effects of the metals studied, and potential sublethal effects of metals on predators that feed on metal-containing herbivores.

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CHAPTER I: INTRODUCTION

Plants use different forms of defenses to increase their fitness in the presence of pathogens, herbivores and other natural enemies (Howe and Jander, 2008). Plant defenses can be physical, associational, behavioral or chemical defenses (Davis and Boyd, 2000). Chemical defenses can be grouped into either organic or elemental defenses (Davis and Boyd, 2000). Organic defenses are secondary metabolites that are byproducts of photosynthetic pathways in a plant, but do not have a major role in the growth and development of a plant (Bennett and Wallsgrove, 1994; Galeas et al., 2007). Organic defense compounds include alkaloids, phenolics, glucosinolates and terpenes (Bennett and Wallsgrove, 1994). Elemental defenses, unlike organic compounds, are not synthesized by plants but are sequestered by plants from their environment (Boyd, 2007).

Elemental defense is a newly recognized phenomenon in which plants use elements present in their tissue to reduce damage by natural enemies (Martens and Boyd, 1994; Boyd, 2004; Boyd, 2007;). Metal ions can defend a plant from herbivores by deterrence, acute toxicity or both (Boyd and Martens, 1998), depending on a herbivore's feeding mode. In acute toxicity, metals in plant tissue cause mortality when ingested by a herbivore. With deterrence, herbivores avoid high-metal tissue because they have taste receptors that enable them to directly or indirectly detect higher concentrations of metal in plant tissue (Poschenrieder et al., 2006).

Elemental defenses have two advantages over secondary defense compounds. First, elements are acquired from the soil: therefore, plants do not use any resources towards their synthesis (Freeman et al., 2006). By reducing the resources allocated to organic defenses, a plant is able to use those resources for growth and reproduction

(Thaler, 2002). Second, counterdefenses that are used by herbivores against organic defenses are not applicable because elements cannot be degraded metabolically (Martens and Boyd, 1994; Boyd and Martens, 1998).

Metal concentration in plant tissue can be subjectively categorized into three groups; normal, accumulator and hyperaccumulator (Reeves and Baker, 2000). Normal plants have low metal concentrations, while accumulators are plants with greater metal concentrations and in which the metal concentration quotient of the leaf/root is >1.0, whereas hyperaccumulators have at least 100-fold higher metal concentrations in their aboveground tissue than normal plants (Brooks et al., 1977; Reeves and Baker, 2000; van der Ent et al., 2012). The term hyperaccumulator was initially introduced by Brooks et al. (1977) to describe plants that had exceptionally high levels of Ni (>1,000 µg Ni g^{-1} dry mass) but it has been used since with other elements (Reeves and Baker, 2000; van der Ent et al., 2012).

Hyperaccumulators are found in most parts of the world, except Antarctica. However, most hyperaccumulators have been reported in Cuba (Proctor, 1999), New Caledonia, and Mediterranean Europe. Most hyperaccumulator plants are found on serpentine soils, which are derived from ultramafic rocks. These soils are characterized by high concentrations of chromium, cobalt, iron, and nickel, but have low levels of nitrogen, phosphorous, potassium and silicon (Brooks, 1987).

Organic and elemental defenses can have lethal or sublethal effects on herbivores (Trumble et al., 1998). In lethal effects, chemicals in plant tissue cause death when ingested by a herbivore, which can reduce or eliminate an entire population (Clancy and

Price, 1987). A sublethal effect can reduce the growth, development and reproductive rates of herbivores (Price et al., 1980; Clancy and Price, 1987; Jensen et al., 2007).

In the environment, plants are subjected to mixtures of chemicals. The presence of more than one defensive chemical in a plant may result in joint effects (those because of a combination of two chemicals), as either a combination of multiple metals or between metals and organic chemicals (Boyd, 2007). Joint effects can be additive, synergistic or antagonistic. An additive effect happens if the response to a combination of chemicals is equal to the response of two individual chemicals added together (Koppenhöfer and Kaya, 1997; Jhee et al., 2006). If the result of combined chemicals is significantly greater than what occurs for an additive effect, then a synergistic joint effect is present (Nelson and Kursar, 1999). An antagonistic joint effect is present if the result of combined chemicals is significantly less than that produced by an additive effect (Tabashnik, 1992).

Plant and animal interactions have major impacts on food webs. Therefore an interaction between herbivores and hyperacumulators may impact a community (Ping et al., 2009). Some herbivores are adapted to high concentrations of metals in the plants they consume and can sequester these metals in their own tissue (Jensen and Trumble, 2003). Boyd and Wall (2001) hypothesized that herbivores may concentrate metals in their tissues to defend themselves against parasitoids, predators and pathogens. However, the metal concentration in the herbivore tissues also may aid the transfer of metals from the soil or water to higher trophic levels in a food web (Boyd and Martens, 1998; Jensen and Trumble, 2003).

In the current study, I examined elemental defense and trophic transfer of Co, Cu, Ni and Zn. This study was divided into three major parts. First, I determined the minimum lethal and sublethal concentrations of Co, Cu, Ni, and Zn to Beet Armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), larvae in artificial diet. Second, I determined the type of joint effect for metal/metal combinations and for metals combined with one of three organic compounds, nicotine, mustard seed powder and tannic acid, using an artificial diet experimental system. I tested for both lethal and sublethal joint effects in these combination experiments. Third, I assessed the survival, growth, and development of the predator *Podisus maculiventris* fed *Spodoptera exigua* larvae raised on artificial diets amended with MSCs and MLCs of Co, Cu, Ni and Zn over a 30 day period. I also evaluated if the metals were biotransferred or biomagnified from the artificial diet to larvae of the generalist herbivore *Spodoptera exigua* and to the adults of the generalist predator *Podisus maculiventris*.

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CHAPTER II: EXPLORING LOWER LIMITS OF PLANT ELEMENTAL DEFENSE BY COBALT, COPPER, NICKEL, AND ZINC

Abstract-Elemental defense is a relatively newly recognized phenomenon in which plants use elements present in their tissue to reduce damage by herbivores or pathogens. In the present study, neonates of the generalist herbivore, *Spodoptera exigua,* were fed artificial diets amended with varying concentrations of four metals that are hyperaccumulated by plants (Co, Cu, Ni and Zn). After seven days, the number of larvae alive and their weights were recorded and analyzed to determine minimum lethal concentrations (MLC) and minimum sublethal concentrations (MSC) for each metal. MLC values (dry mass basis) for three metals (90 μg Co g^{-1}, 570 μg Ni g^{-1} and 280 μg Zn g^{-1}) were below published minimum hyperaccumulator levels, whereas that for Cu (530 μg Cu g^{-1}) was above. MSC levels (dry mass basis) were all recorded at concentrations lower than published minimum accumulator levels: 15 μg Co g^{-1}, 95 μg Ni g^{-1} and 200 μg Zn g^{-1}, with the exception, again of Cu (175 μg Cu g^{-1}). Furthermore, both MLC and MSC values for Zn were within normal tissue concentrations (20-400 μg Zn g^{-1}). These results indicate that elemental defense for Co, Ni and Zn may be effective at concentrations far lower than hyperaccumulator levels and so may be more widespread that previously believed. Results for those metals also provide support for the Defensive Enhancement Hypothesis, which suggests that hyperaccumulation evolved because increases in metal concentration from relatively low initial levels improved plant fitness due to increased defense against herbivores and pathogens. The defensive effectiveness of low concentrations of these metals (as demonstrated here) is an essential pre-condition for defensive enhancement to occur.

INTRODUCTION

Plants utilize chemical defenses to increase their fitness against natural enemies (Galeas et al., 2007; Howe and Jander, 2008). Plant chemical defense compounds can be classified into two groups: organic and inorganic compounds (Davis and Boyd, 2000). Organic compounds are also called secondary plant compounds because they are by-products of important metabolic pathways (Bennett and Wallsgrove, 1994) and typically do not have direct effects on plant growth, development, or reproduction. Major organic compounds that have been studied include terpenes, phenolics, alkaloids, glucosinolates, salicylic acid, and methyl jasmonate (Bennett and Wallsgrove, 1994). Inorganic compounds primarily are metals and have been termed elemental defenses (Martens and Boyd, 1994).

Elemental defense is a phenomenon in which plants use elements present in their tissue to reduce damage by natural enemies (Martens and Boyd, 1994; Boyd, 2004; Boyd, 2007). The elemental defense hypothesis was formally proposed by Martens and Boyd (1994) as an explanation for the function of hyperaccumulation in plants. Elemental defenses have two advantages over secondary defense compounds. First, elements are obtained from the soil; plants do not have to allocate resources towards their synthesis, thus potentially affecting plant growth (Thaler, 2002; Freeman et al., 2006). Second, elements cannot be degraded metabolically by herbivores; thus this mechanism of herbivore counterdefense would not be applicable (Martens and Boyd, 1994; Boyd and Martens, 1998).

Elemental defenses can protect a plant from herbivores by deterrence, acute toxicity, or both, depending on a herbivore’s feeding mode (Boyd and Martens, 1998). In deterrence, herbivores avoid ingesting high-metal tissue. Deterrence is initiated by chemoreceptors located on medial and lateral sensilla styloconica found on maxillary galea of larval mouthparts (Renwick, 2001). With acute toxicity (lethal effects), metals in plant tissue can cause death when ingested by a herbivore and either reduce or completely eliminate a herbivore population. Elemental defenses may also result in a sublethal effect on herbivores, such as reduced growth and increased development time, which may increase a herbivore's susceptibility to natural enemies (predators, parasitoids or pathogens) and result in a lower reproductive rate (Price et al., 1980).

Metal concentration in plant tissue can be subjectively classified into three categories; normal, accumulator and hyperaccumulator (Reeves and Baker, 2000). Generally, normal plants have relatively low metal concentrations, while accumulators are plants for which the metal concentration quotient of the leaf/root is >1.0, and hyperaccumulators have at least 100-fold more metals in their aboveground tissue than normal plants (Brooks et al., 1977; Reeves and Baker, 2000). The term hyperaccumulator was initially introduced by Brooks et al. (1977) to describe plants that had exceptionally high levels of Ni (>1,000 μg Ni g^{-1} dry mass) but it has been used since with other elements (Reeves and Baker, 2000; van der Ent et al., 2012). For Co, Cu and Zn the minimum concentrations for hyperaccumulator plants are 300 μg Co g^{-1} 300 μg Cu g^{-1}, and 3,000 μg Zn g^{-1}, respectively (van der Ent et al., 2012). Accumulator plants have reduced levels of metal but significantly more than that found in normal plants: Figure 1 illustrates the concentration ranges of normal, accumulator and hyperaccumulator plants according to the categories suggested by Reeves and Baker (2000), and recently modified for hyperaccumulation of Co, Cu and Zn by van der Ent et al. (2012), for the four metals (Co, Cu, Ni, Zn) studied here.

Hyperaccumulator plants have generated much scientific interest due to their ability to concentrate often potentially phytotoxic elements to relatively extreme levels (Reeves and Baker, 2000; Rascio and Navari-Izzo, 2011; van der Ent et al., 2012). Hyperaccumulators are distributed across most parts of the world and are found in all continents, excluding Antarctica. The largest numbers of species have been reported in Cuba (Proctor, 1999), New Caledonia (Brady et al., 2005), and Mediterranean Europe (Reeves et al., 1996; Reeves and Adigüzel, 2008). Most hyperaccumulator plants are found on serpentine soils and are endemic to these habitats (Brooks and Radford, 1978; Reeves and Baker, 2000). Serpentine soils originate from ultramafic rocks (Reeves et al., 1996) and are characterized by high concentrations of Cr, Co, Fe, and Ni, but have low concentrations of N, P, K and Si (Brooks, 1987).

Biogeographic and phylogenetic distributions of hyperaccumulators suggest that the hyperaccumulator trait probably evolved multiple times (Krämer, 2010), driven by ecological advantages (Pollard and Baker, 1997; Pollard, 2000). However, it is also speculated that more than one factor led to the evolution of hyperaccumulator species (Kazakou et al., 2008). Boyd and Martens (1992) summarized five hypotheses generated to explain the evolution of hyperaccumulators. For the tolerance and disposal hypothesis, plants concentrate metals in aboveground tissue and dispose of them when plant parts break off. For the drought resistance hypothesis, metals dissolved in plant cell cytoplasm can lower water potential enabling the plant to survive drought conditions. For the interference hypothesis, when a hyperaccumulator drops high-metal litter onto the surrounding substrate, metals leach out into the soil and increase surface soil metal concentration. With elevated metal levels in the surface soil, metal sensitive plants cannot survive but the metal tolerant hyperaccumulator plants thrive. For the inadvertent uptake hypothesis, a plant accumulates metal as a consequence of other mechanisms involved in acquiring nutrients from the soil. For the defense hypothesis, hyperaccumulators use metal ions in their tissues to reduce or eliminate damage by natural enemies.

The evolution of hyperaccumulation has been speculated to be an outcome of stepwise evolution driven by defensive benefits, termed the Defensive Enhancement Hypothesis (Boyd, 2007). Stepwise evolution of hyperaccumulation may occur if a plant with a low level of metal in its tissues is protected against natural enemies, providing it with a selective advantage compared to plants with lesser metal levels. This initial metal concentration, the Threshold Defensive Metal Concentration results in a defensive benefit that provides a fitness advantage on which natural selection can operate. If greater concentrations of metal lead to proportionally greater defensive benefits, further increases in tissue metal concentration will lead to still increased defensive benefits and higher fitness. Continual selection for greater metal concentration, driven by the effectiveness of greater levels of an elemental defense, eventually can reach or exceed the metal concentration defining hyperaccumulation.

An important question regarding the Defensive Enhancement Hypothesis is: What is the lowest concentration of a metal that can provide a defensive benefit to a plant? This Threshold Defensive Metal Concentration (TDMC) is important because it defines the lower limit of an elemental defense and therefore indicates whether elemental defenses operate at hyperaccumulator levels, accumulator levels, or even normal levels in plants.

Determination of TDMC levels for metals in Fig. 1 would allow inference to show which concentrations allow elemental defenses to become ecologically important.

A related question is whether the Evolutionarily Effective Defense Threshold is determined by lethal effects of a metal on herbivores or if sublethal effects can make important contributions to defensive effectiveness. It seems likely that lethal effects will accrue at higher metal concentrations than sublethal effects, and that their defensive value will be greater because they will severely impede herbivore populations, but even sublethal effects may be evolutionarily effective in some cases (Price et al., 1980; Boyd and Moar, 1999). Thus, determination of both lethal and sublethal levels of metals may provide information helpful to studies of elemental plant defense.

In this study, I determined the minimum lethal and sublethal concentrations of Co, Cu, Ni, and Zn to Beet Armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), larvae in artificial diet. According to Capinera (2006), *S. exigua* is a polyphagous herbivore with a broad plant host range including alfalfa, cabbage, peppers, soybean and cotton, along with some genera that contain metal accumulators. For example, it feeds on *Chenopodium* spp. (some species are Cu accumulators: Porębska and Ostrowska, 1999) and *Amaranthus* spp. (some species accumulate Zn: Prasad and Freitas, 2003). With a short life cycle that can be completed in 4-5 weeks depending on climate (Capinera, 2006), *S. exigua* is an ideal insect to raise and manipulate in a laboratory.

The four metals were selected because they are among those most commonly hyperaccumulated by plants (Reeves and Baker, 2000). Additionally, Ni and Co are found in high concentrations in serpentine soils (Brooks, 1987). About 75% of 579 reported hyperaccumulator plant species are Ni hyperaccumulators (van der Ent et al.

2012). Van der Ent et al. (2012) reported that the literature includes reports of 30 hyperaccumulators of Co, 32 of Cu and 12 of Zn. Additionally, Co was included in my study because it may be co-accumulated with Cu and because both it and Cu are metals with relatively low thresholds for the definition of hyperaccumulation: 300 µg g^{-1} (van der Ent et al., 2012).

METHODS

General Experimental Procedures. I maintained the *S. exigua* colony and conducted experiments in a rearing room with the temperature maintained at 27±2 degrees Celsius. I established the research colony using egg sheets from two different colonies: a colony in the laboratory of William Moar (Boyd and Moar, 1999) at Auburn University, Alabama (established from insects collected from cotton fields in Alabama) and a colony maintained by Benzon Research (Carlisle, PA, USA). I raised larvae in cups containing artificial diet, a combination of pinto beans, wheat germ, tortula yeast, casein, gelcarin, insect vitamin, methyl p-hydroxybenzoate, tetracycline and formaldehyde (Li et al., 2002). I kept adults in 4 liter glass jars (25-35 adults per jar) covered with single sheets of paper towel and fed them 10% sucrose solution. I collected egg sheets and sterilized them in 10% formalin for 30 seconds, rinsed them in distilled water, and air-dried them.

I used chloride salts of Co, Cu, Ni, and Zn (Fisher Scientific, Pittsburgh, PA, USA) to generate stock solutions and diluted the stock solutions into desired concentrations for bioassays. I placed neonates onto diet amended with the four metals (along with a control treatment containing no added metal) in 24 well trays within 8 hours of hatching as described by Thamthiankul et al. (2004). I monitored growth and development of larvae daily in all experiments for 7 days (Ali and Luttrell, 2009).

Use of chloride salts meant that there was the potential for the chloride, or the high level of added salts, to cause mortality or reduced weights rather than the metals: therefore I included a separate set of experiments using $CaCl_2$ (also obtained from Fisher Scientific, Pittsburgh, PA, USA). By showing that much higher concentrations of $CaCl_2$ were required to cause increased larval mortality and weight reduction compared to the four metals tested, I could determine that mortalities and weight reduction observed in experiments using metal chlorides likely were caused by the metals rather than the chloride or a salt effect.

I analyzed diet samples to determine actual concentrations of Co, Cu, Ni and Zn, expressed as $\mu g\ g^{-1}$ on a dry weight basis, using data for actual metal concentrations in diet analyzed at the Auburn University Soil Testing Laboratory. I prepared 30 ml diet samples with varying concentrations of Co, Cu, Ni, and Zn in the same manner as for the bioassays. I measured wet weight and volume for each sample, as well as the dry weight by drying each sample at 60 degrees Celsius for 5 days. I sent samples to the Auburn University Soil Testing Laboratory where samples were ground into powder, dry-ashed in a muffle furnace, oxidized with boiling nitric acid and dissolved in HCl (Gavlak et al., 1994). Samples were diluted with deionized water and analyzed for metal concentration using inductively coupled argon plasma spectroscopy (Jarrell-Ash ICAP 9000: Genesis Laboratory Systems, Grand Junction, CO, USA). I determined actual metal concentrations for two reasons: 1) as a check of the desired concentration in diet, and 2) to allow comparison with metal concentrations in plant tissue published in the literature (usually expressed on a dry weight basis) (Coleman et al., 2005). I did not analyze the actual concentration of $CaCl_2$ on a dry weight basis because I was not comparing $CaCl_2$ to what is found in plant tissue.

Minimum Lethal Concentration (MLC) Experiments. To determine MLC for each metal, I transferred neonates to artificial diets containing varying concentrations of Ca, Co, Cu, Ni and Zn (Table 1). For each concentration, I used a twenty-four well plate (Multiwell tissue culture plates, Becton Dickinson Labware, Lincoln Park, NJ, USA) with one neonate per well. Each trial included a range of experimental (metal-amended) treatments and a control (unamended) treatment. I incubated plates at 27±1 degrees Celsius and monitored larvae for 7 days. After 7 days, I recorded the total number of larvae alive in each plate as well as the instar of each larva.

I determined MLCs for each metal by comparing treatment mortalities to control mortality. I characterized mortality as the number of larvae that were dead as well as those that failed to molt to 2^{nd} instar by day 7 (Anilkumar et al., 2009). I defined MLC as the lowest metal concentration that resulted in insect mortality significantly greater than that of the control. I used three acceptable experimental trials for statistical analysis of each metal. An acceptable trial was a trial with control mortality less than 8% and a progressive increase in mortality as metal concentration in the diet increased. I analyzed data from trials of Ca, Co, Cu, Ni and Zn separately, using One-way Analysis of Variance (ANOVA) in StatView (SAS Institute, 2005). Survival values were arcsine square root transformed to satisfy the normality assumption (Zar, 1996) underlying ANOVA. Significant results from ANOVA ($P \leq 0.05$) were followed by Fisher's

Protected Least Significant Difference (PLSD) tests to determine the lowest concentration of each metal that resulted in significantly greater mortality than that of the control treatment ($P \leq 0.05$) (SAS Institute, 2005).

Minimum Sublethal Concentration (MSC) Experiments. To determine the MSC for each metal, I transferred neonates to artificial diets containing varying concentrations of Co, Cu, Ni and Zn (Table 2). For each concentration, I used one twenty-four well plate, with one larva per well. Each trial included a range of experimental (metal-amended) treatments and a control (unamended) treatment. I incubated plates at 27±1 degrees Celsius and monitored the larvae daily for 7 days. After 7 days, I recorded the total number of larvae alive in each plate as well as the instar of each larva. I weighed all living larvae from each plate as a group and divided the total weight by the total number of larvae alive per plate to obtain mean individual larval weight.

I determined MSC for each metal as the lowest metal concentration with mean larval weight significantly reduced compared to the control treatment weight. Percent weight reduction was calculated using the following equation:

$$\text{Percent weight reduction} = \left(\frac{(C_W - T_W)}{C_W}\right) \times 100\%$$

Where $\mathbf{C_w}$ is mean individual larval weight from the control and $\mathbf{T_w}$ is mean individual larval weight from the treatment (Abivardi and Benz, 1984).

I used three acceptable experimental trials for statistical analysis of each metal. I only considered trials with control mortality less than 8% to be acceptable. I analyzed data from trials using Co, Cu, Ni and Zn separately. I arcsine transformed the percent weight reductions and analyzed them using one-way ANOVA. After significant results from ANOVA ($P \leq 0.05$), I used Fisher's Protected Least Significant Difference (PLSD) tests to determine the lowest concentration of each metal that resulted in a significant weight reduction compared to the control treatment ($P \leq 0.05$) (SAS Institute, 2005).

RESULTS

Minimum Lethal Concentration Experiments. I recorded MLCs for all metals at relatively low concentrations (Fig. 2). ANOVA showed significant effects of all metals on survival (Table 3), and Fisher's PLSD tests showed the MLC for Co was 90 µg Co g^{-1}, the MLC for Cu was 530 µg Cu g^{-1}, and MLCs for Ni and Zn were 570 µg Ni g^{-1} and 280 µg Zn g^{-1}, respectively (Fig. 2). I used $CaCl_2$ to determine whether mortalities were caused by metals in the diet rather than a chloride or salt effect. ANOVA showed a significant effect of $CaCl_2$ on survival (Table 3) and Fisher's PLSD test showed that the MLC for $CaCl_2$ was relatively high: 133 µM (data not shown). MLC values for the four metals tested were at least 100-fold lower than that for $CaCl_2$ (Co = 0.34 µM, Cu = 1.35 µM, Ni = 1.85 µM and Zn = 1.24 µM). At the highest concentration of $CaCl_2$ in the artificial diet (400 µM), percent mortality was 19% while with the four metals tested a similar mortality was observed at exceptionally lower concentrations (Co = 0.34 µM, Cu 1.35 µM, Ni = 1.85 µM and Zn = 2.5 µM).

Minimum Sublethal Concentration Experiments. Significant effects of all metals on mean larval weight were observed (Table 3), and at concentrations less than the MLC values for each metal. Fisher's PLSD tests showed significant weight reduction (Fig. 3) at concentrations as low as 15 µg Co g^{-1} and 95 µg Ni g^{-1} while larvae raised on Cu–

amended diet showed a significant weight reduction at 175 µg Cu g^{-1} and those raised on Zn-amended diet showed a significant effect at 200 µg Zn g^{-1}.

DISCUSSION

My results show that elemental defenses could be effective at relatively low concentrations for all four metals tested. As illustrated in Figure 4, MLCs for three metals (Co, Ni, Zn) against *S. exigua* are below hyperaccumulator levels, while all MSCs are below or in accumulator ranges. Additionally, both the MLC and the MSC for Zn were below the minimum concentration used to define a normal concentration in plant tissues (Reeves and Baker, 2000). The MLC for Cu (530 µg Cu g^{-1}) was in the hyperaccumulator range, but it should be noted that van der Ent et al. (2012) recently reduced the hyperaccumulator threshold from 1,000 µg g^{-1} to 300 µg g^{-1}: by the former standard the MLC of Cu is below the hyperaccumulator threshold. It is important to note that the effects of added metal chloride salts were probably caused by metals in the diet and not the chloride or the overall addition of the salts. This conclusion is based on the evaluation of the MLC of $CaCl_2$ against *S. exigua* which, at 133 µM, was nearly 100-fold greater than the greatest MLC value (that for Cu at 1.35 µM) for any of the four metals used in the experiments.

Although the mode of action for each metal was not determined in my study, comparison of MLC and MSC values (expressed as molarities to allow comparisons on a per-atom basis) showed great variation between metals. *Spodoptera exigua* was particularly sensitive to Co compared to the other three metals. MLC (Fig. 2, Table 1) and MSC (Fig. 3, Table 2) values for Co were 0.17 µM and 0.053 µM, respectively, while MLC and MSC values for other metals were: 1.35 µM and 0.106 µM for Cu, 0.62

μM and 0.197 μM for Ni, and 1.24 μM and 0.787 μM for Zn. Compared to Co, MLC values for Cu and Zn were at least 7-fold greater and the MLC value for Ni was 3.7-fold greater. MSC values were 2-fold greater for Cu, 3.7-fold greater for Ni, and 15-fold greater for Zn when compared to the MSC value for Co.

All four metals evaluated are considered essential to living organisms as enzyme cofactors in regulated doses (Scheifler et al., 2002). However, differences in metal requirements could explain the variation in sensitivity of *S. exigua* to the metals. Regulation of Cu and Zn by metallothioneins might explain why Cu and Zn were the least toxic to *S. exigua* compared to Co and Ni (Giguère et al., 2003). Metallothioneins are proteins that bind to the group B metals (Cd, Cu and Zn) in both vertebrates and invertebrates (Jensen et al., 2007). When metallothioneins bind to group B metals in the midgut, they can either increase metal resistance or aid with detoxification of excess metals (Giguère et al., 2003). Wang et al. (2007) noted that excessive Co, which is required in very low concentrations in insects, induces the production of the enzyme caspase which regulates apoptosis. An increase in apoptosis may lead to thinning of the epithelium of the gut wall, allowing Co to penetrate the walls of the gut and causing toxicity. Unlike the other three metals, low concentrations of Ni in insects can induce an enhanced immune system but a higher concentration of Ni inhibits the immune system, causing the organism to become susceptible to pathogens (Sun et al., 2011).

Several studies have evaluated the effectiveness of metals against different herbivores using both plants and artificial diet (Pollard and Baker 1997; Boyd and Martens 1999; Boyd and Moar 1999; Coleman et al. 2005; Jhee et al. 2005). However, effective concentrations are variable between the metals and herbivores used. In comparison to other hyperaccumulated metals, Ni has received the most attention in the evaluation of elemental defenses, in part because over 75% of identified hyperaccumulators are Ni hyperacumulators (van der Ent et al., 2012). In my study, Ni caused significant mortality and weight reduction of *S. exigua* at concentrations as low as 140 and 230 µg Ni g^{-1} respectively, a 1.5-fold increase in the MLC from the MSC. Using artificial diet amended with Ni, Boyd and Moar (1999) reported 80% mortality of *S. exigua* at 640 µg Ni g^{-1} and a distinct decrease in larval weight at 535 µg Ni g^{-1}. Martens and Boyd (1994) observed Ni toxicity against the butterfly *Pieris rapae* (Hübner) (Lepidoptera: Pieridae) at 1000 µg Ni g^{-1}. Both studies reported effective concentrations higher than the MLC of Ni observed in the present study. However Coleman et al. (2005) reported reduced survival of *Plutella xylostella* (Hubner) (Lepidoptera: Plutellidae) caused by dietary Ni at 20 µg Ni g^{-1}, a concentration lower than what I observed. Differences in effective concentrations reported by Coleman et al. (2005) and Martens and Boyd (1994) could be due to different sensitivities to Ni by the herbivore species studied.

In my results, MLCs and MSCs for Zn were within normal tissue concentrations. Coleman et al. (2005) reported Zn toxicity against *P. xylostella* at 275 µg Zn g^{-1}. MLC results from my study and those of Coleman et al. (2005) show that the toxic concentration is significantly below the accumulator level (which is 2000 µg Zn g^{-1}: Fig. 1), falling within normal plant Zn concentrations (Reeves and Baker, 2000). Other studies of elemental defense by Zn have found defensive effects at greater concentrations. For example, Pollard and Baker (1997) observed preferential feeding by the locust *Schistocerca gregaria* (Forskal) (Orthoptera: Acrididae) and the slug *Deroceras* *caruanae* (Lessona and Pollonera) (Mollusca: Agrialimacidae) when offered low (528 µg Zn g^{-1}) and high (7432 µg Zn g^{-1}) Zn plants of *Noccaea caerulescens* (J. Presl & C. Presl) F.K. Mey (formerly *Thlaspi caerulescens*: Brassicaceae): both preferred the low Zn plants after sampling the high Zn plants. Behmer et al. (2005) reported that Zn in artificial diet was toxic to the locust *Schistocerca gregaria* at concentrations as low as 1500 µg Zn g^{-1}. When locusts were fed an artificial diet amended with intermediate (1500 µg Zn g^{-1}) and high (5500 µg Zn g^{-1}) Zn concentrations, the time it took to molt to the 5th stadium was prolonged, and the mass was significantly reduced, compared to both the control treatment and that amended with a low concentration (500 µg Zn g^{-1}). Both my study and that of Coleman et al. (2005) used herbivores from the same insect order (Lepidoptera), while the other two studies reported above used herbivores other than Lepidoptera: differences in sensitivity to Zn between these groups may explain the variations in the results.

Compared to Ni and Zn, only a few Co and Cu hyperaccumulator plant species have been identified (Faucon et al., 2007) and the recent review by van der Ent et al. (2012) suggested contamination of leaves with dust might account for at least some of the reports. Despite the small number of Co and Cu hyperaccumulators, Cu and Co can be toxic to invertebrate herbivores at relatively low levels. Coleman et al. (2005) evaluated Co and Cu toxicity against *P. xylostella* in artificial diets and showed that Cu significantly reduced survival at 175 µg g^{-1} and Co at 40 µg g^{-1}. These concentrations are both below the minimum levels used to define hyperaccumulators of Co and Cu, >1000 µg g^{-1} by Reeves and Baker (2000) and >300 µg g^{-1} by van der Ent et al. (2012). My results with *S. exigua* showed toxicity at greater levels (90 µg Co g^{-1} and 530 µg Cu g^{-1}), suggesting that hyperaccumulated Co or Cu have a defensive benefit from toxicity but with the defensive value of lower levels dependent on the importance of sublethal effects.

Sublethal effects have been a rather controversial topic because the benefits of slowed growth and development of a herbivore have been argued as both beneficial and destructive to plants. With increased development time of a herbivore, there is an increase in feeding time; therefore, more plant material is consumed. In addition, reduced prey size due to slow growth and development might reduce predator growth and survival, leading to a reduction in predator population. This shortage of predators may lead to an increase in the herbivore population in subsequent generations, and increased herbivory (Clancy and Price, 1987). However, a sublethal effect would be considered beneficial to a plant if, because of reduced growth rate, and increased time for growth and development, a herbivore larva becomes more susceptible to predation or pathogens (Clancy and Price, 1987; Vickerman et al., 2004; Jensen et al., 2007). Additionally, prolonged development could lead to later maturity of herbivores so that they are still feeding when a plant has advanced in its development and the plant could have developed other defenses, such as trichomes, cellulose and lignin. These defenses may make it harder for herbivores to consume and digest plant material (Price, 1980; Clancy and Price 1987) and thus reduce herbivore growth and fitness.

If sublethal defenses are effective, then the MSC values in Fig. 4 extend the defensive benefit of the studied metals to lower concentrations. In the case of Cu and Ni, the MSC is well within the Accumulator Range, for Co is below the Accumulator Range, and for Zn is within the Normal Range. This suggests that elemental defenses may be more widespread than previously believed, benefitting plants that are in the accumulator range for Co, Cu and Ni and the highest part of the normal range for Zn (Fig. 4). These results also support the Defensive Enhancement Hypothesis as a potential explanation for the evolution of elemental hyperaccumulation (Boyd, 2007). Low concentrations of metals, through sublethal and/or lethal effects, can provide an initial defensive benefit to a plant. Natural selection may then drive evolution of even higher concentrations in their tissue to enhance defense against herbivory. Future research should explore whether sublethal doses contribute to the evolution of hyperaccumulators and hence support the Defensive Enhancement Hypothesis. Additional research should also determine if individual metals are more effective when combined with other metals or organic defensive compounds. This Joint Effects Hypothesis of Boyd (2007) suggests that combinations of defensive chemicals may lower the level at which a metal has a defensive effect, possibly to levels below those shown in my study. These joint effects may also contribute to the Defensive Enhancement Hypothesis as a potential explanation for the evolution of metal hyperaccumulation by plants.

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FIGURES

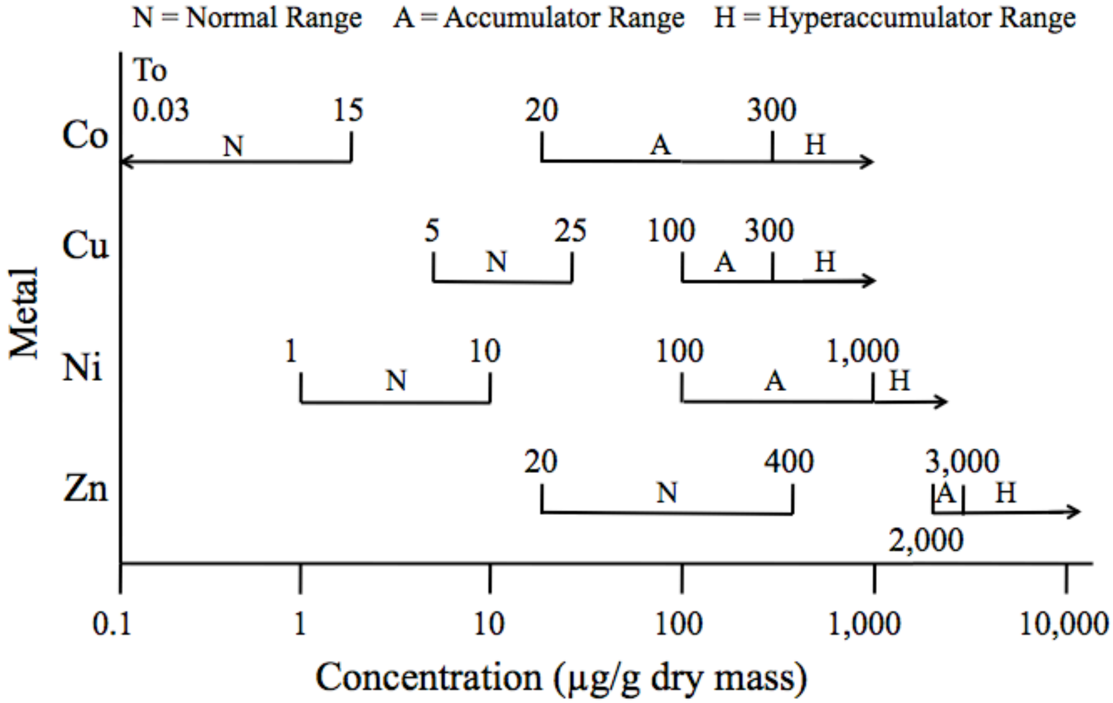

Figure 1. Ranges of elemental concentrations (log scale X-axis) in Normal (N), Accumulator (A) and Hyperaccumulator (H) plants for Co, Cu, Ni and Zn according to Reeves and Baker (2000), with hyperaccumulator levels for Co, Cu and Zn modified according to van der Ent et al. (2012). Gaps between categories for a metal are concentration ranges that are not named in the classification scheme of Reeves and Baker (2000).

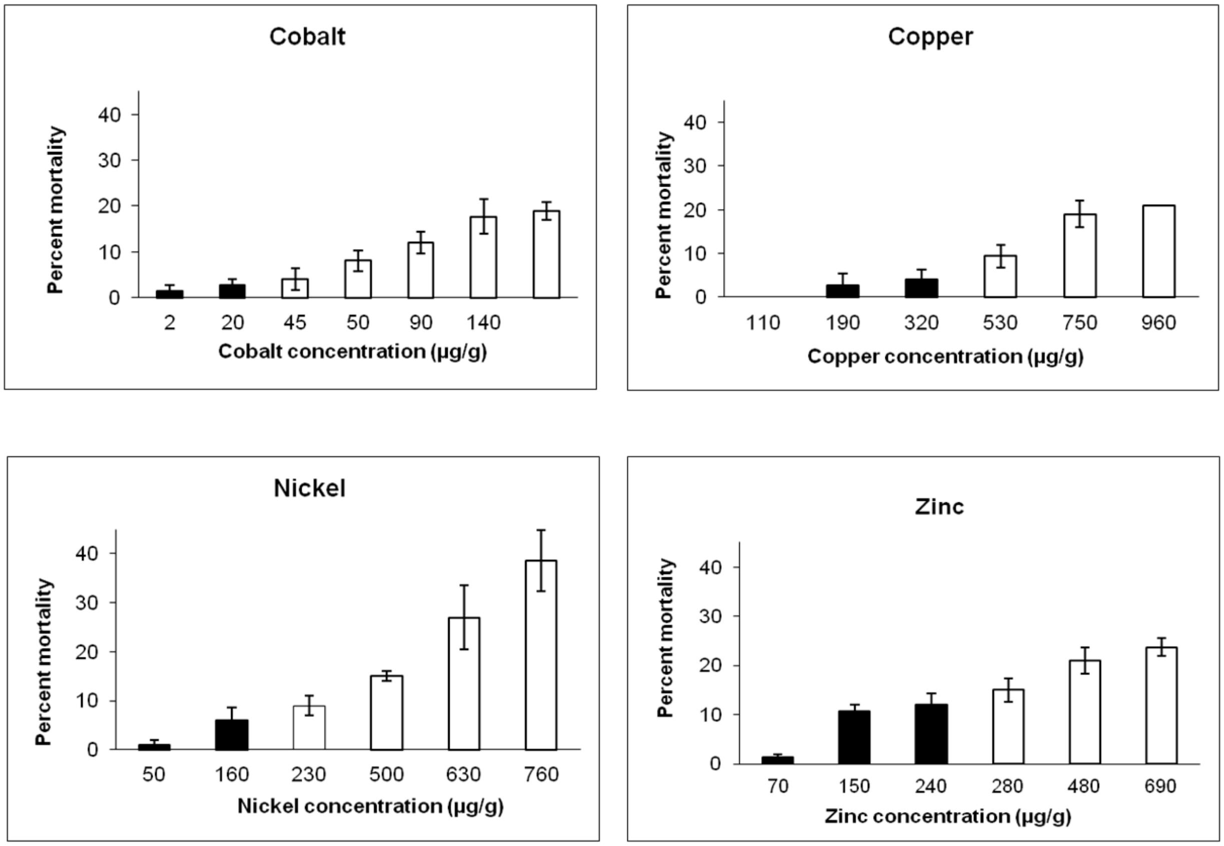

Figure 2. Results for Minimum Lethal Concentration (MLC) experiments (Means: error bars are SE). Error bar for Cu 960 µg/g is too small to show at the scale used. Metal concentrations in artificial diet (x-axis) are expressed on a dry mass basis. Open bars show concentrations (µg/g dry mass) of metals with significantly increased larval mortality compared to the control treatment (the far left bar in each graph).

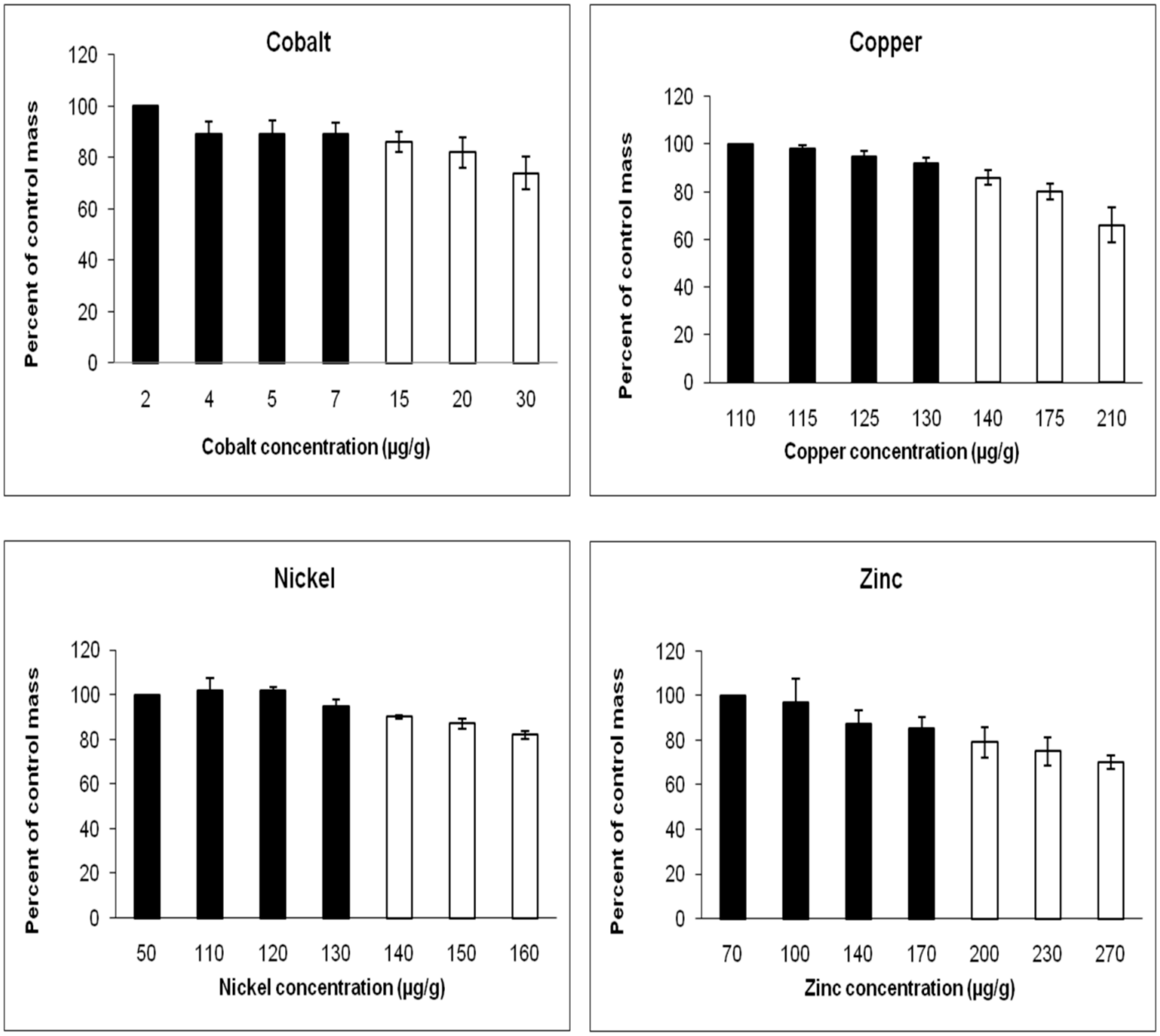

Figure 3. Results for Minimum Sublethal Concentration (MSC) experiments (means: error bars are SE). Metal concentrations in artificial diet (x-axis) are expressed on a dry mass basis. Open bars show concentrations (in µg/g dry mass) of metals with significantly reduced larval mass compared to the control treatment (the far left bar in each graph).

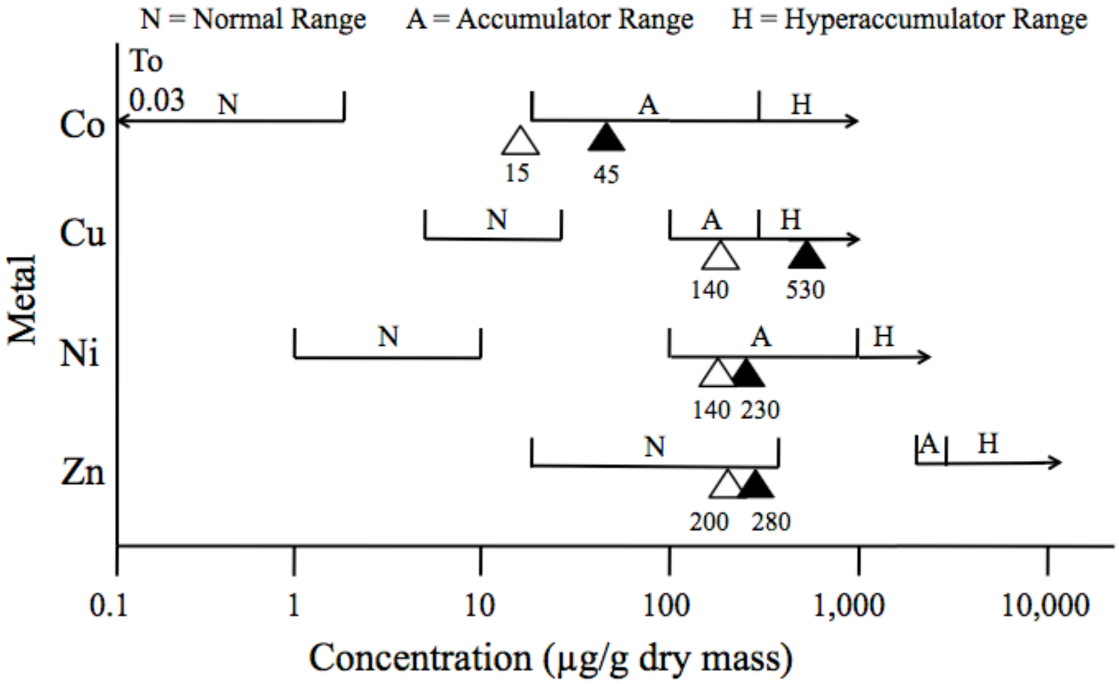

Figure 4. Sublethal (Minimum Sublethal Concentration: open triangle) and lethal (Minimum Lethal Concentration: filled triangle) concentrations of metals for Beet Armyworm larvae fed artificial diets in this study, relative to ranges of elemental concentrations (log scale X-axis) for Normal (N), Accumulator (A) and Hyperaccumulator (H) plants according to Reeves and Baker (2000), with hyperaccumulator levels for Co, Cu and Zn modified according to van der Ent et al. (2012).

TABLES

Table 1. Metal concentrations in artificial diet used for minimum lethal concentration experiments. Data in µM are reported as micromoles of metal chlorides per liter added to the diet: µg data were determined from analysis of samples of diet.

Metal	Units	Concentration							
Co	µM $CoCl_2$	0.00	0.07	0.13	0.17	0.34	0.51		
	µg Co/g	2	20	40	50	90	140		
Cu	µM $CuCl_2$	0.00	0.27	0.68	1.35	2.03	2.70		
	µg Cu/g	110	190	320	530	750	960		
Ni	µM $NiCl_2$	0.00	0.31	0.62	1.85	2.46	3.08		
	µg Ni/g	50	120	190	470	600	750		
Zn	µM $ZnCl_2$	0.00	0.50	1.00	1.24	2.50	3.73	4.97	7.46
	µg Zn/g	70	150	240	280	480	690	890	1300
Ca	µM $CaCl_2$	0.00	67	133	200	266	333	400	

Table 2. Metal concentrations in artificial diet for minimum sublethal concentration experiments. Data in µM are reported as micromoles of metal chlorides per liter added to the diet: µg data were determined from analysis of samples of diet.

Metal	Units	Concentration								
Co	µM $CoCl_2$	0.00	0.007	0.013	0.02	0.027	0.053	0.08	0.107	0.133
	µg Co/g	2	4	5	7	9	15	20	30	40
Cu	µM $CuCl_2$	0.00	0.026	0.053	0.08	0.106	0.021	0.032	0.043	0.053
	µg Cu/g	110	115	125	130	140	175	210	240	280
Ni	µM $NiCl_2$	0.00	0.049	0.098	0.148	0.197	0.246	0.03		
	µg Ni/g	50	60	75	85	100	110	120		
Zn	µM $ZnCl_2$	0.00	0.19	0.393	0.59	0.787	0.983	1.18		
	µg Zn/g	70	100	140	170	200	230	270		
Ca	µM $CaCl_2$	0.00	67	133	200	266	333	400		

Table 3. ANOVA results for Minimum Lethal Concentration (MLC) and Minimum Sublethal Concentration (MSC) experiments.

Experiment	Metal	*F*	*df*	*P*-value
Minimum Lethal Concentrations	Co	5.1	5,12	0.0094
	Cu	11.1	5,12	0.0004
	Ni	15.4	5,18	<0.0001
	Zn	8.1	6,17	0.0003
	Ca	10.7	6,14	0.0001
Minimum Sublethal Concentrations	Co	9.5	6,35	<0.0001
	Cu	24.7	8,27	<0.0001
	Ni	8.1	6,14	0.0007
	Zn	2.9	6,14	0.0498

CHAPTER III: ELEMENTAL DEFENSE AND THE JOINT EFFECTS HYPOTHESIS: AN INITIAL TEST USING *SPODOPTERA EXIGUA* (LEPIDOPTERA: NOCTUIDAE)

Abstract-Metal hyperaccumulation may be an elemental defense, in which high concentrations of a metal in plant tissues decrease herbivore survival or growth rate. The Joint Effects Hypothesis suggests that a combination of metals, or a combination of a metal with an organic defense compound, may have enhanced defensive effects. This enhancement may be additive or it may be synergistic (defined as enhancement exceeding additive expectations): in either case the concentration of metal for which a defensive benefit accrues to a plant will be lowered. I tested the Joint Effects Hypothesis using neonates of *Spodoptera exigua* fed artificial diet. Metal-metal experiments amended diet with pairs of metals, using four metals hyperaccumulated by some plants (Co, Cu, Ni and Zn). I also conducted metal-organic compound experiments, pairing each metal (Co, Cu, Ni and Zn) with nicotine, mustard seed powder and tannic acid. I tested for joint effects using both lethal and sublethal concentrations of the chemicals tested: lethal concentration tests used LC_{20} levels and sublethal concentration tests used concentrations that reduced larval weight by 10-25%. Of six metal-metal pairs, three (Co + Cu, Cu + Zn and Ni + Zn) had synergistic joint effects in lethal concentration tests and the other three pairs (Co + Ni, Co + Zn and Cu + Ni) had additive joint effects. For sublethal tests, all metal-metal pairs had additive joint effects except for Co + Cu (which was synergistic). For lethal tests of metal-organic combinations, synergistic joint effects were present for all metals + tannic acid, for Co, Cu, or Zn + mustard seed powder, and for Cu + nicotine. Additive joint effects were detected for Co, Ni or Zn + nicotine and for Ni + mustard seed powder. For sublethal tests, Co or Zn + mustard seed powder, Co + tannic acid, Cu + nicotine, and Zn + tannic acid had synergistic joint effects whereas Co + nicotine, Cu + mustard seed powder, Cu + tannic acid, and Ni with all three organic compounds had additive joint effects. The synergistic and additive joint effects documented here show that combinations of metals, or metals and organic compounds, enhance the defensive effect of a metal by allowing it to harm a herbivore at lower concentrations than if that metal were present singly. These results support the Joint Effects Hypothesis and suggest that defensive effects of metals may have contributed to the evolution of hyperaccumulation by some plants.

INTRODUCTION

Plants use different forms of defenses to increase their fitness in the presence of pathogens, herbivores and other natural enemies (Howe and Jander, 2008). Plant defenses can be categorized as physical, associational, behavioral or chemical defenses (Davis and Boyd, 2000). Chemical defenses can be grouped into either organic or elemental defenses (Davis and Boyd, 2000). Organic defenses are secondary metabolites including alkaloids, phenolics, glucosinolates and terpenes (Bennett and Wallsgrove, 1994). These chemicals are byproducts of photosynthetic pathways in a plant that do not play a significant role in plant growth and development (Bennett and Wallsgrove, 1994; Galeas et al., 2007). Elemental defenses, unlike organic compounds, are not synthesized by plants but are sequestered from the environment by plants (Boyd, 2007).

Elemental defense is one of the hypotheses reviewed by Boyd and Martens (1992) as an explanation for the evolution of hyperaccumulator plants. Hyperaccumulators are plants that have exceptionally high concentrations of metal in their tissues (Brooks et al., 1977). The term hyperaccumulator was first used by Brooks et al. (1977) to describe plants with an unusually high concentration of Ni, but it is now used to describe exceptionally high concentrations of other elements in plants (Reeves and Baker, 2000; van der Ent et al., 2012). About 580 plant species have been identified as hyperaccumulators and over 75% are Ni hyperaccumulators (van der Ent et al., 2012). Other metals/metalloids hyperaccumulated by plants may include As, Cd, Co, Cr, Cu, Mn, Se, Tl and Zn (van der Ent et al., 2012), although confirmation of hyperaccumulation of some metals (Co, Cu, Cr, Pb and Tl) is still needed (van der Ent et al., 2012). Some plants can accumulate or hyperaccumulate more than one metal concurrently (Reeves and Baker, 1984), termed co-accumulation. Although it is not well understood, co-accumulation is believed to be due to the presence of a metal detoxification system that is not metal selective but might be specific to certain groups of metals (Reeves and Baker, 1984). Metals that are commonly co-accumulated are Zn and Ni, and Zn and Pb, as well as Co and Cu (Reeves and Baker, 2000).

In the environment, plants are exposed to mixtures of chemicals. The presence of more than one defensive chemical in a plant may result in joint effects (those stemming from a combination of two chemicals), either between multiple metals or between metals and organic chemicals (Boyd, 2007). Joint effects can be additive, synergistic or antagonistic. An additive effect occurs when the response to a combination of chemicals is equal to the response of two individual chemicals added together (Koppenhöfer and

Kaya, 1997; Jhee et al., 2006). In a synergistic joint effect, the result of combined chemicals is significantly greater than what occurs for an additive effect (Nelson and Kursar, 1999). An antagonistic joint effect occurs when the result of combined chemicals is significantly less than that produced by an additive effect (Tabashnik, 1992). Joint effects between pairs of metals, and between metals and organic compounds, were demonstrated by Jhee et al. (2006) who reported additive joint effects between metal/metal and metal/organic compounds at low concentrations against larvae of the diamondback moth, *Plutella xylostella* (Linnaeus) (Lepidoptera: Plutellidae).

Chemical defenses (both organic and elemental) can have lethal or sublethal effects on herbivores (Trumble et al., 1998). With lethality, chemicals in plant tissue cause death when ingested by a herbivore, reducing or eliminating an entire population (Clancy and Price, 1987). A sublethal effect causes reduced growth rate and increased development time of a herbivore, which may make a herbivore more susceptible to parasitoids, pathogens or predators (Clancy and Price, 1987; Jensen et al., 2007). Additionally, sublethal effects may lower the reproductive rate of a herbivore resulting in lower population size (Price et al., 1980).

In the current study, I evaluated the type of joint effect that exists between four representative metals and three organic compounds using an artificial diet experimental system. First, I evaluated the type of joint effect between Co, Cu, Ni and Zn when fed in combination to larvae of a test herbivore, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae). I analyzed both lethal and sublethal effects of all pairwise metal combinations (6 metal/metal combinations). Next, I assessed the type of joint effect between each metal when combined with nicotine, mustard seed powder or tannic acid.

Again, I evaluated both lethal and sublethal joint effects of each of the four metals in pairwise combinations with each organic compound (12 metal/organic combinations).

Spodoptera exigua is a polyphagous insect (Capinera, 2006) that can feed on more than 90 plant species in 18 plant families, including cabbage, pigweed, cotton, peppers, sunflowers (Greenberg et al., 2001), alfalfa, soybeans, and *Chenopodium* sp. (Capinera, 2006). The life cycle of *S. exigua* is greatly influenced by climate and can be as short as 4-5 weeks (Capinera, 2006), which makes it an ideal candidate to rear and manipulate in laboratory experiments. *Spodoptera exigua* also has been the subject of elemental defense studies in recent years. Boyd and Moar (1999) reported that tissue of the Ni hyperaccumulator *Streptanthus polygaloides* A. Gray (Brassicaceae) was toxic to *S. exigua* when plants hyperaccumulated Ni. Vickerman and Trumble (1999) studied the feeding response of *S. exigua* to different forms and concentrations of Se, an element hyperaccumulated by some plants (Reeves and Baker, 2000), concluding that forms and concentrations of Se in artificial diet affected the feeding habits of 3rd instar larvae but not younger larvae. Using artificial diet, Trumble et al. (1998) reported that the growth and development of *S. exigua* are affected by the presence of Se in plants. They concluded that selenomethionine causes direct toxicity, while growth-inhibiting forms of Se (such as sodium selenate, sodium selenite and selenocystine) produce sublethal effects by slowing larval development.

I chose Co, Cu, Ni and Zn because they are the metals often hyperaccumulated by plants (Reeves and Baker, 2000). A recent review (van der Ent et al., 2012) reported that 450 of nearly 580 reported hyperaccumulators hyperaccumulate Ni, while 30 species hyperaccumulate Co, 32 hyperaccumulate Cu, and 12 hyperaccumulate Zn. Additionally, Ni and Co are present in high concentrations in serpentine soils (Brooks, 1987). Furthermore, Co and Cu, along with Ni and Zn, are pairs of metals reported in some cases to be co-accumulated by plants, in addition to Zn and Pb (Reeves and Baker, 2000).

I selected organic compounds to represent some of the major groups of plant chemical defenses. I chose nicotine to represent alkaloids, mustard seed powder because it contains glucosinolates, and tannic acid to represent quantitative phenolic organic compounds (Bennett and Wallsgrove, 1994). Nicotine is a qualitative plant defense alkaloid found in the plant families Solanaceae, Liliaceae and Amaryllidaceae (Bennett and Wallsgrove, 1994). Nicotine is one of the secondary metabolites commonly found in the Cd hyperaccumulator *Solanum nigrum* L. (Solanaceae) (Dawson et al., 1960). When present in plant tissue, alkaloids such as nicotine may protect a plant from herbivores and pathogens (Bennett and Wallsgrove, 1994). Nicotine poisons acetylcholine receptors, therefore it is toxic to organisms with neuromuscular junctions (Baldwin, 2001). Mustard seed powder (White mustard: *Brassica hirta* L. (Brassicaceae) was used because members of the genus contain glucosinolates as defensive compounds (e.g., Moyes et al., 2000). Glucosinolates are qualitative plant defenses common in plant species belonging to the family Brassicaceae (Bennett and Wallsgrove, 1994; Moyes et al., 2000), which includes many hyperaccumulators (Reeves and Baker, 2000). Their potency deters polyphagous insect herbivores but also they serve as feeding cues by crucifer-feeding specialists (Bartlet et al., 1999). Tannic acid is a quantitative plant defense that is constitutive in many plants, including the Ni hyperaccumulator *Psychotria douarrei* (G. Beauvisage) Däniker (Rubiaceae) (Davis et al., 2000) as well as conifers and oaks (Nomura and Itioka, 2002). Tannic acid is a hydrolysable tannin that belongs to the polyphenol group of organic compounds (Becker and Makkar, 1999). Unlike nicotine, tannin concentrations generally increase as a plant ages and tannins affect herbivores by reducing the digestibility of plant materials (Guan et al., 2009). Tannins, like most organic compounds in the phenolic family, act by complexing with dietary protein as well as by reacting with digestive enzymes produced in the gut of a herbivore (Bennett and Wallsgrove, 1994; Makkar, 2003).

METHODS

I used a laboratory-raised colony of *S. exigua* that was started with insects from two different origins: egg sheets purchased in 2008 from Benzon Research Inc. (Carlisle, PA, USA) and a colony in the laboratory of William Moar (Boyd and Moar, 1999) at Auburn University, Alabama established from insects collected from cotton fields in Alabama. I raised insects on artificial diet and maintained them in a rearing chamber at 27±2 degrees Celsius. The artificial diet is a mixture of pinto beans, wheat germ, tortula yeast, casein, gelcarin, insect vitamin, methyl p-hydroxybenzoate, tetracycline and formaldehyde (Li et al., 2002). I maintained adults on a diet of 10% sucrose solution soaked in cotton balls and placed in a petri dish inside 4 liter glass jars (25-35 adults per jar) and covered with single sheets of white paper towel. I replaced the cotton balls with freshly soaked cotton balls and removed paper towels that had eggs on them every day and replaced them with fresh paper towels. I sterilized the egg-covered towels in 10% formalin, rinsed them in distilled water for 1 minute, and air-dried them before placing them in plastic Ziploc bags. For experiments described below, I transferred neonates onto artificial diets within 16 hours of hatching as described by Thamthiankul et al. (2004).

Metal/Metal Combination Experiments: Lethal Concentrations. I used chloride salts of Co, Cu, Ni and Zn (Fisher Scientific, Pittsburgh, PA, USA) to generate stock solutions of the metals and diluted the stocks to desired concentrations before use in experiments. I determined metal concentrations to use in these experiments by performing single metal experiments, amending diet with varying concentrations of Co (20 to 140 µg Co g $^{-1}$), Cu (190 to 960 µg Cu g $^{-1}$), Ni (120 to 750 µg Ni g $^{-1}$), and Zn (150 to 1300 µg Zn g $^{-1}$). In each experiment, I used twenty-four well plates with one larva and about 1.25 ml of diet per well. Each replication included a plate for each concentration of each metal as well as a control treatment (unamended diet). I incubated experiments at 27±2 degrees Celsius and monitored them for 7 days (Ali and Luttrell, 2009). On day 7, I recorded the number of larvae alive in each plate and the instar of each larva. I characterized mortality as the number of larvae that were dead plus those that failed to molt to 2nd instar (Anilkumar et al., 2009). I used probit analysis (Robertson et al., 2007) to determine the LC_{20} for each metal using the results of the single metal experiments from Chapter 2. The LC_{20} is the concentration predicted to cause 20% larval mortality for each metal individually.

I used LC_{20} values for each metal (concentrations used are shown in Table 1) to perform experiments for all pairwise metal/metal combinations (a total of 6). I used this relatively low LC value as a practical consideration: positive joint effects could result in relatively high mortalities and I was trying to avoid combination values nearing or exceeding 100%. In each experiment, I used twenty-four well plates with one larva per well. Each replication included a combination treatment, a single metal treatment for each metal, and a control treatment (unamended diet). I incubated experiments at 27±2 degrees

Celsius and monitored them for 7 days. On day 7, I recorded the number of larvae alive in each plate and the instar of each larva. I characterized mortality as the number of dead larvae plus those that failed to molt to 2nd instar (Anilkumar et al., 2009). I used three satisfactory trials to generate the data for each metal-metal pair: a satisfactory trial was a trial with control mortality ≤ 8%.

*Metal/Metal Combination Experiments: Sublethal Concentration*s. To determine sublethal concentrations to use in experiments, I conducted preliminary experiments for each metal. I amended diet with varying concentrations of Co (4 to 40 μg Co g^{-1}), Cu (115 to 280 μg Cu g^{-1}), Ni (60 to 120 μg Ni g^{-1}), and Zn (100 to 270 μg Zn g^{-1}) and determined the concentration that caused larval weight reduction in the range of 10-25% (compared to an unamended control). I then designed experiments using sublethal concentrations (Table 1) for each of the 6 possible metal/metal combinations. In each experiment, I used twenty-four well plates with one larva per well. For each replication I included a combination treatment, a single sublethal metal treatment for each metal and a control treatment (unamended diet). I incubated experiments at 27±2 degrees Celsius and monitored them until day 7, when I recorded the number of larvae alive in each plate, the total weight of larvae from each plate, and divided total weight by the number of larvae alive to calculate mean larval weight. I calculated percent larval weight reduction using the formula:

$$\text{Percent weight reduction} = \left(\frac{(C_W - T_W)}{C_W}\right) \times 100\%$$

Where $\mathbf{C_w}$ is mean individual larval weight from the control and $\mathbf{T_w}$ is mean individual larval weight from the treatment (Abivardi and Benz, 1984). I used three satisfactory experimental trials for each pair of metals, defining a satisfactory trial as one with control mortality ≤ 8%.

Metal/Organic Compound Combination Experiments: Lethal Concentrations. For metal/organic compound combinations I used Co, Cu, Ni and Zn in combination with nicotine, tannic acid and mustard seed powder. As with the metal-metal combination experiments, I used preliminary experiments to determine LC_{20} values of nicotine (Fisher Scientific, Pittsburgh, PA, USA), tannic acid (Sigma, Saint Louis, MO, USA) and mustard seed powder (Science Lab, Houston, TX, USA). Again, I used a relatively low LC for practical reasons: positive joint effects could result in relatively high mortalities and I was trying to avoid combination values nearing or exceeding 100%. I then designed experiments using LC_{20} concentrations (Table 1) of all pairwise metal/organic compound combinations (12 combinations total), using twenty-four well plates with one larva in each well. Each replication included a combination treatment, a single metal treatment (LC_{20}) for each metal, a single organic compound treatment (LC_{20}) and a control treatment (unamended diet). I incubated experiments at 27±2 degrees Celsius and monitored them for 7 days. I counted the number of larvae alive in each plate on day 7, recorded each larva's instar and determined mortality as the number of dead larvae plus those that failed to reach the 2nd instar. I used three satisfactory experimental trials (defined as a trial with control mortality ≤ 8%) to generate data for each metal/organic defense compound pairing.

Metal/Organic Compound Combination Experiments: Sublethal Concentrations. From preliminary single organic compound experiments, I determined sublethal concentrations (10-25% larval weight reduction) for each organic compound (Table 1). I paired each metal with all three organic compounds individually (12 total combinations), using twenty-four well plates (one larva per well). Each replication included a combination treatment, a single metal treatment, a single organic compound treatment and a control treatment (unamended diet), incubated at 27±2 degrees Celsius for 7 days. I recorded the number of larvae alive on day 7 in each plate, the total weight of larvae in each plate, and calculated mean larval weight. I calculated percent weight reduction using the formula:

$$\text{Percent weight reduction} = \left(\frac{(C_W - T_W)}{C_W}\right) \times 100\%$$

Where $\mathbf{C_w}$ is mean individual larval weight from the control and $\mathbf{T_w}$ is mean individual larval weight from the treatment (Abivardi and Benz, 1984). The dataset comprised three satisfactory trials, defining a satisfactory trial as one with control mortality ≤ 8%.

Statistical analyses. I conducted a preliminary statistical analysis for each combination experiment to demonstrate that a joint effect had occurred. Using Mann-Whitney U tests in StatView (SAS Institute, 2005), I compared mortality or larval weight inhibition for each single chemical treatment to that of the combination treatment. If the combination treatment result was significantly greater ($P = 0.05$) than that for each individual chemical treatment, this step confirmed that a joint effect had occurred.

I then conducted a second analysis to determine if the joint effect was additive, synergistic, or antagonistic. I calculated an expected result (additive percent mortality or weight reduction) as described by Salama et al. (1984), using single chemical treatment data, and compared the expected value to the experimental value from the combination treatment. I used a χ^2analysis to determine whether the combination mortality or larval weight reduction was significantly different from the expected value ($P = 0.05$). A synergistic effect had occurred if the value from the combination treatment was greater than that for the expected value, an additive effect was demonstrated if the combination result did not differ from the expected value, and if the combination result was less than the expected value then an antagonistic joint effect had occurred.

RESULTS

Metal/Metal Combination Experiments. Based on Mann-Whitney U tests, combination results for all treatments were significantly different from individual chemical treatments, for both lethal (Fig. 1A) and sublethal (Fig. 1B) experiments. Analysis of mortality data revealed that three of the six metal-metal combinations showed additive joint effects while the other three (Co + Cu, Cu + Zn and Ni + Zn) showed synergistic joint effects (Table 2). Two of the synergistic combinations (Co + Cu and Cu + Zn) had the highest percent mortalities (74 and 66%, respectively: Fig. 1A) while the Co + Zn combination had the lowest mortality recorded (59%) in the metal/metal experiments. Joint effects in the sublethal experiments were mainly additive (Table 3): five of the six combinations showed additive joint effects, but the Co + Cu combination again was synergistic with a 77% larval weight reduction observed (Fig. 1B).

Metal/Organic Compound Combination Experiments. Mann-Whitney U tests showed that lethal combination results for all treatments were significantly different from individual chemical treatments (Fig. 2). All metal/organic combinations were either synergistic or additive for the lethal experiments (Table 4): eight of the twelve combinations had synergistic joint effects while the other four (Co + nicotine, Ni + nicotine, Ni + mustard seed powder and Zn + nicotine) had additive joint effects, with 51, 36, 44 and 51% mortalities, respectively (Fig. 2). The synergistic combinations had percent mortalities (Fig. 2) ranging from 53% (Co + tannic acid and Ni + tannic acid) to as high as 71% (Cu + mustard seed powder). Results for sublethal combinations showed half synergistic and half additive joint effects (Table 5). All the Ni + organic combinations were additive, as well as Co + nicotine, Cu + mustard seed powder and Cu + tannic acid. Synergistic joint effects were observed for Co + mustard seed powder, Co + tannic acid, Cu + nicotine and all the Zn + organic combinations: Zn/organic compound combinations had the highest weight reductions recorded (ranging from 84-90%: Fig. 3).

DISCUSSION

In my combination experiments, concentrations of metals that caused both sublethal and lethal joint effects were below the minimum concentrations used to define hyperaccumulators (Reeves and Baker, 2000). Additionally, all joint effects for both larval mortality and weight reduction were either additive or synergistic (none were antagonistic) for both metal/metal and metal/organic combinations, and in both lethal and sublethal experiments. Synergistic interactions were statistically significant but were not as pronounced as has been reported in some other cases. For example, Jensen et al.

(2007) reported that a mixture of selenate (Se) and methyl mercury chloride (MeHg) reduced larval growth of the southern house mosquito (*Culex quinquefasciatus*) significantly (*P* = 0.003) compared to each chemical individually. The fact that no interactions between metals resulted in antagonism supports that idea that plants may have evolved metal hyperaccumulation for insect protection.

My results showed that elemental defenses, in combination with each other or with organic compounds, lower the metal concentrations needed to affect *S. exigua*. Table 6 compares the concentration of an individual metal against the concentration of that same metal in combination that was able to generate similar mortality. In general, a much greater concentration of a single metal in the artificial diet was needed to cause mortality similar to that produced in combination experiments. For example, 74% mortality resulted when 180 μg Co g^{-1} and 800 μg Cu g^{-1} were added in combination to artificial diet, whereas a similar percent mortality required 2.5-fold more Co (450 μg Co g^{-1}) or 3.1-fold more (Cu 2,450 μg Cu g^{-1}) in single metal experiments. Another example is the Ni + Zn combination, for which 59% mortality was achieved with 900 μg Ni g^{-1} and 1,600 μg Zn g^{-1} in combination but with individual metals similar percent mortality was achieved with 1.2-fold more Ni (1,100 μg Ni g^{-1}) and 1.6-fold more (2,500 μg Zn g^{-1}) added to artificial diet. Furthermore, a similar pattern was observed when metals were combined in sublethal experiments (Table 6). The levels of metals required for the sublethal combinations were reduced by 1.2-fold for Ni to as high as 2.3-fold in Co.

With these results, I have demonstrated that elemental defense could be effective against herbivores at lower concentrations when metals are combined with other metals or with organic defense compounds. To my knowledge, only Jhee et al. (2006) has demonstrated the existence of joint effects between metal/metal and metal/organic compound combinations. In artificial diet, they demonstrated additive joint effects between pairwise combinations of Ni, Cd, Pb and Zn, as well as for Ni combined with tannic acid, atropine, and nicotine against the diamondback moth *Plutella xylostella* (Jhee et al., 2006).

The Defensive Enhancement Hypothesis suggests that evolution of hyperaccumulation may have been driven by elemental defense (Boyd, 2007). The joint effects that I have demonstrated in metal/metal or metal/organic compound combinations show that hyperaccumulation may evolve more readily because the level of metal needed for a defensive effect is lower when a metal occurs in combination with another metal or an organic compound. This Joint Effects Hypothesis, offered by Boyd (2007) as one way by which defensive enhancement can occur, is supported by the data in Table 6: in general, a low concentration of a single chemical, when combined with a second chemical, may cause increased mortality or decreased growth of herbivores. This joint effect would enhance plant fitness and allow natural selection to favor further increases in plant metal concentration, potentially leading to hyperaccumulation levels of a metal.

Although metal/metal and metal/organic compound joint effects are relatively unexplored, joint effects have been demonstrated between insecticides and plant chemical defenses, and between different plant defense mechanisms. For example, Lichtenstein et al. (1974) showed that green extracts (containing d-Carvone, Apiol, Dill-apiol and Myristicin) from dill plants *Anethum graveolens* L. (Apiaceae) had a synergistic joint effect with carbamates (carbaryl and carbofuran) and an organophosphate (parathion) insecticide against two dipterans: a fruit fly, *Drosophila melanogaster* Meigen (Diptera:

Drosphilidae), and a mosquito, *Aedes aegypti* L. (Diptera: Culicidae). A recent study by Anilkumar et al. (2009) demonstrated synergistic interactions between *Bacillus thuringiensis* (Bt) Cry1Ac and gossypol or cotton powder when fed in combination to larvae of Cry1Ac-resistant bollworm *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae). Guan et al. (2009) concluded that an additive joint effect exists between tannic acid and Cry1Ac against the cotton bollworm *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). Dyer et al. (2003) showed that the three amides found in the shrub *Piper cenocladum* C. DC. (Piperaceae) had synergistic joint effects against the herbivores *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) and two herbivorous ants; *Atta cephalotes* (Linnaeus) (Hymenoptera: Formicidae) and *Paraponera clavata* (Fabricius) (Hymenoptera: Formicidae).

The joint effects demonstrated here also have implications for the concept of tradeoffs in plant defenses. Agrawal (2011) hypothesized that plants in nutrient-poor habitats may rely on defenses that do not require allocation of already limited resources to synthesize them, implying that plants alternate defenses based on resource limitation. For example, they suggested that plants in low-nitrogen areas may trade off nitrogen-based alkaloids, which are costly to a plant, for less expensive phenolics. Boyd (2007) suggested that the existence of joint effects would allow a tradeoff to occur in hyperaccumulators, in which metals in plant tissue may allow reduced levels of defensive organic compounds. Such a tradeoff has been shown in some cases: Tolrà et al. (2001) reported a decrease in glucosinolate concentrations when *Thlaspi caerulescens* J and C. Presl. (Brassicaceae) hyperaccumulated Zn and Davis and Boyd (2000) demonstrated that the presence of Ni in the hyperaccumulator *Streptanthus polygaloides* reduced concentrations of some glucosinolates compared to the non-hyperaccumulator *Streptanthus insignis* Jeps. (Brassicaceae).

The Tradeoff Hypothesis is not limited to chemical defenses alone. Tradeoffs may be present between chemical and structural defenses, or between chemical and associational defenses. Hanley et al. (2007) suggested that there might be a tradeoff between structural defenses (thorns, prickles, spines) and chemical defenses, with some plants using a structural defense rather than a costly chemical defense. Resource limitations might drive a plant growing in a low-nutrient habitat to utilize physical defenses, rather than resource-demanding secondary metabolites (Hanley et al., 2007; Agrawal, 2011). Other plants may trade off chemical defenses for associational defenses: those involving other organisms (Agrawal, 2006). For example, evaluation of Central American *Acacia* plant species showed that non-ant *Acacia* plants contained hydrogen cyanides, while those with ant associations lacked those defensive chemicals (Rehr et al., 1973).

While my results demonstrated that joint effects occurred in both lethal and sublethal experiments, sublethal effects may not be great enough to affect natural selection (compared to lethal effects). The ecological value of sublethal effects to a plant is debatable because the benefits of slowed growth and development of a herbivore have been explored as both advantageous and harmful to plants. Sublethal effects may be advantageous to a plant if an increase in larval development period means that the plant will have developed past a vulnerable stage due to an increase in defenses such as cellulose and lignin (Price, 1980; Clancy and Price 1987). Additionally, reduced growth and development of herbivores may increase their susceptibility to predators and pathogens (Clancy and Price, 1987; Vickerman et al., 2004; Wise et al. 2006; Jensen et al., 2007). However, sublethal effects may be harmful to a plant if the extended development time of a herbivore would increase feeding time and lead to an increase in plant destruction (Clancy and Price, 1987). In my experiments, the concentrations of metals required to cause a sublethal effect in both metal/metal and metal/organic combinations were 4- to 11-fold lower than the effective concentrations for lethal combinations (Table 6).

It is important to note that the mustard seed powder used in my experiments did not only contain glucosinolates, therefore, the synergy observed between mustard seed powder and metals could be an interaction between the metals used and other chemicals in the mustard seed powder. According to Bell et al. (1971), mustard seed powder contains less than 2% glucosinolates and there are more than 14 different groups of glucosinolates. Further experimentation using purified isolates of glucosinolates found in hyperaccumulators will be needed to test whether specific glucosinolates and metals have joint effects similar to those found in my experiments.

The presence of positive metal/metal and metal/organic combined effects in my experiments supports the Joint Effects Hypothesis (Boyd, 2007). Although this is an initial test using only one herbivore species, my results suggest that joint effects may be ecologically important and may have contributed to the evolution of metal hyperaccumulation. Future research should evaluate other potentially important ecological factors, including specialist versus generalist herbivores, herbivore feeding mode, arthropod herbivores versus non-arthropod herbivores and whether joint effects impact evolution of herbivore resistance to plant defenses and pesticides. Other avenues worth pursuing may include exploration of the influence of dosage (e.g the lethal concentration level used) on combination effects (instead of just the LC_{20} used in my experiments) and investigating more complex mixtures of chemicals (for example, metal/metal/organic and metal/organic/organic combinations).

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FIGURES

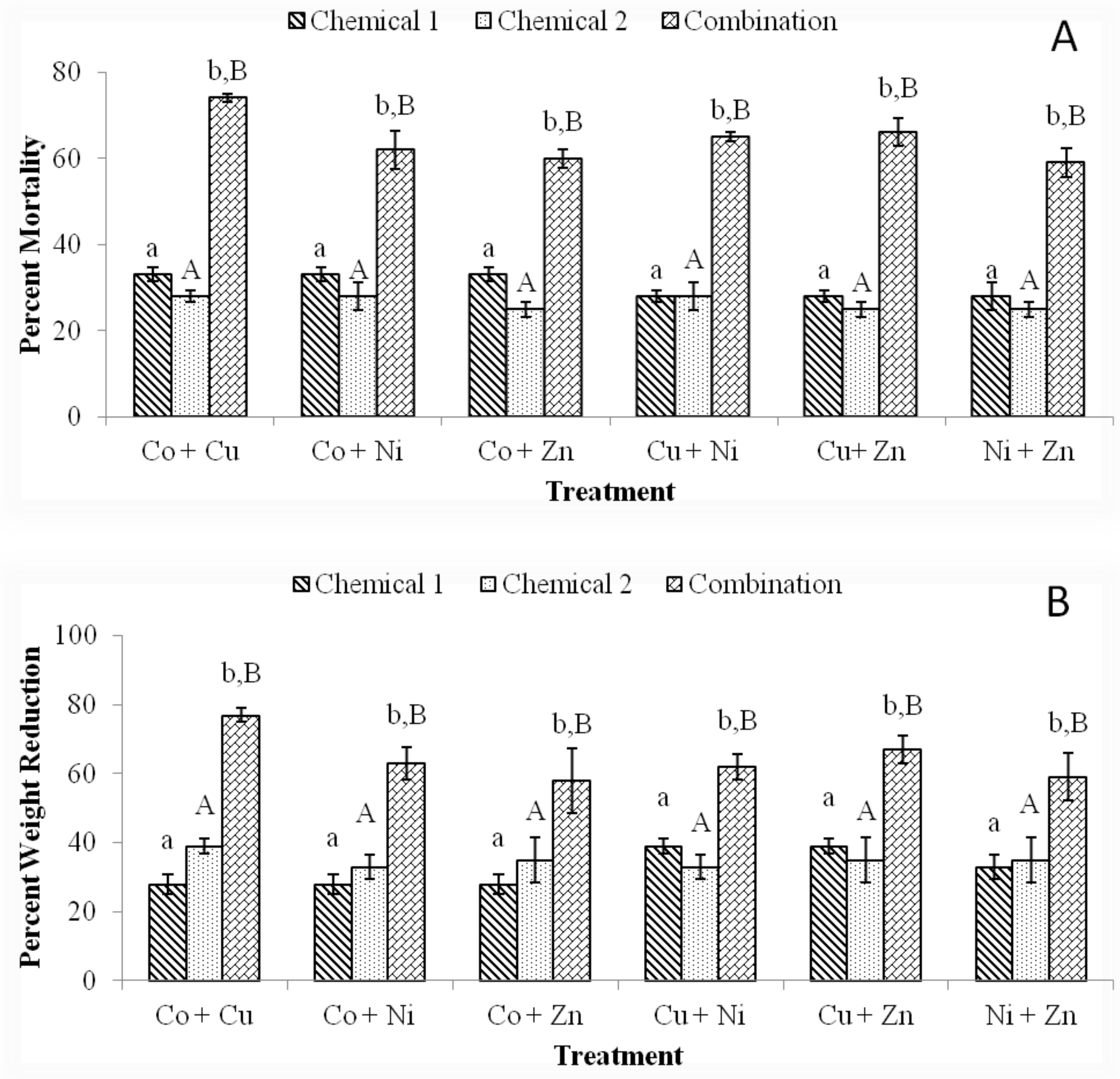

Figure 1. Metal/metal joint effects in artificial diet against *Spodoptera exigua* larvae. A) Lethal effects expressed as percent mortalities. B) Sublethal effects expressed as percent weight reduction compared to control larval weight. Error bars indicate the SE of the means (N=3 in all cases). For each pair of metals, letters denote results of statistical comparison of each individual chemical treatment against the combination treatment: lower case for comparison of Chemical 1 against the combination and upper case for comparison of Chemical 2 against the combination (Mann-Whitney U test, $P = 0.05$)

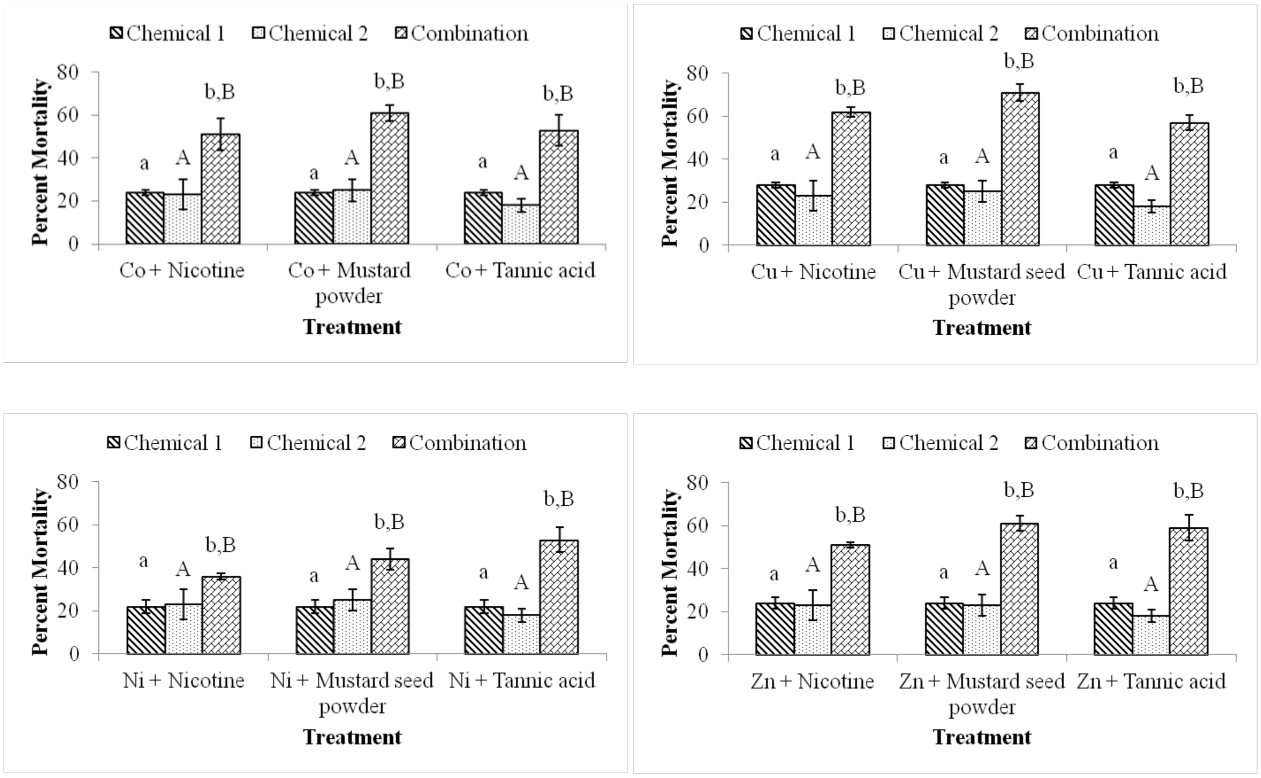

Figure 2. Metal/organic compound lethal joint effects (expressed as percent mortalities) in artificial diet against *Spodoptera exigua* larvae. For each pair of chemicals, letters denote results of statistical comparison of each individual chemical treatment against the combination treatment: lower case letters compare Chemical 1 against the combination and upper case letters compare Chemical 2 against the combination (Mann-Whitney U test, *P* = 0.05).

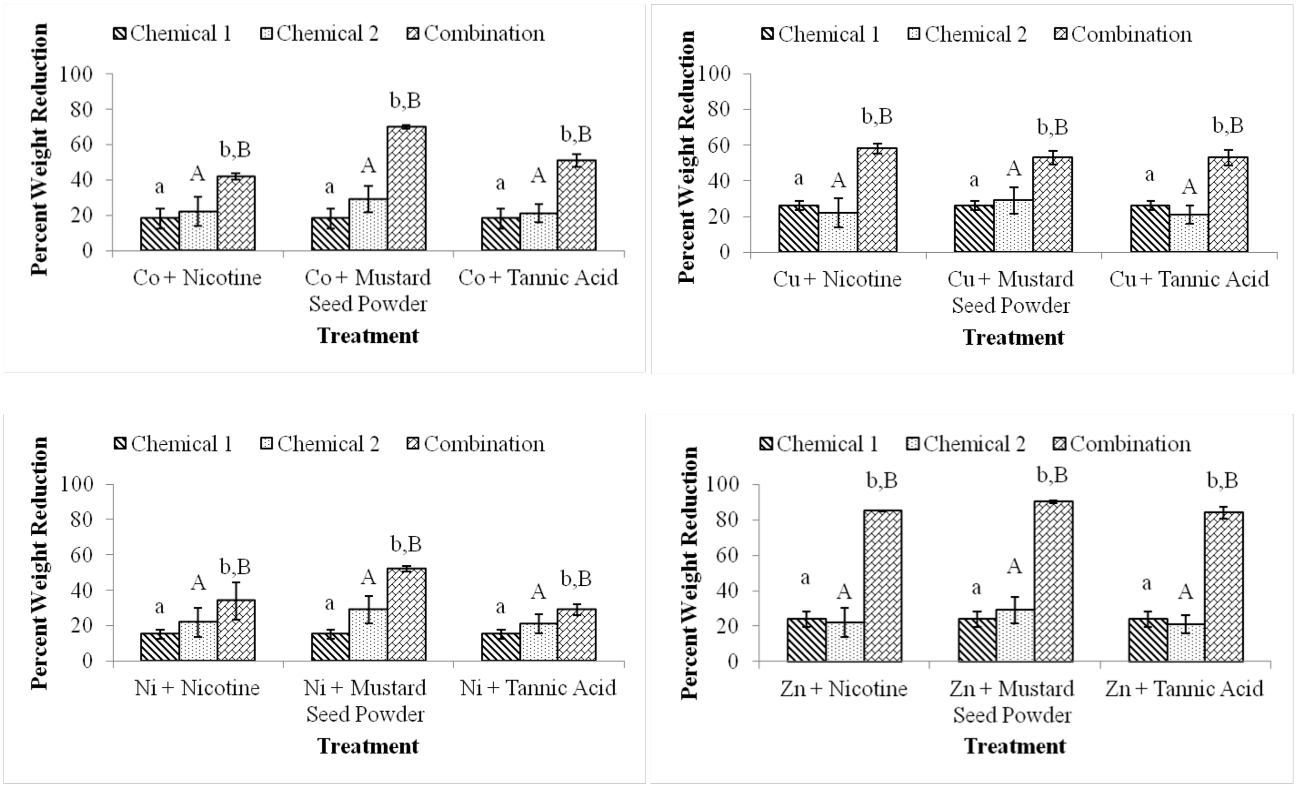

Figure 3. Metal/organic compound sublethal joint effects (expressed as percent weight reduction compared to control larval weight) in artificial diet against *Spodoptera exigua* larvae. Error bars indicate the SE of the means (N = 3 in all cases). For each pair chemicals, letters denote results of statistical comparison of each individual chemical treatment against the combination treatment: lower case letters compare Chemical 1 against the combination and upper case letters compare Chemical 2 against the combination (Mann-Whitney U test, *P* = 0.05).

TABLES

Table 1. Metal and organic compound concentrations used in artificial diet for metal/metal and metal/organic combination experiments. Data expressed in µM are micromoles of metal chlorides per liter of diet. Data expressed as µg/g dry weight were obtained from analysis of diet samples. Organic compounds were not analyzed in diet samples: therefore they are expressed only in micromoles/l (nicotine, tannic acid) or g/l (mustard seed powder) of diet.

Combination	Chemical	Experiment	
		Lethal	Sublethal
Metal/Metal	µM $CoCl_2$	0.69	0.08
	µg Co/g	180	20
	µM $CuCl_2$	3.94	0.106
	µg Cu/g	800	180
	µM $NiCl_2$	2.9	0.246
	µg Ni/g	900	150
	µM $ZnCl_2$	6.92	3.93
	µg Zn/g	1600	140
Metal/Organic Compound	µM $CoCl_2$	0.69	0.08
	µg Co/g	180	20
	µM $CuCl_2$	3.94	0.106
	µg Cu/g	800	180
	µM $NiCl_2$	2.9	0.246
	µg Ni/g	900	150
	µM $ZnCl_2$	6.92	3.93
	µg Zn/g	1600	140
	µM Nicotine	1.38	0.90
	Mustard Seed Powder (g/l)	4.15	1.04
	µM Tannic Acid	2.97	0.40

Table 2. Lethal joint effects between Co, Cu, Ni and Zn against *Spodoptera exigua* larvae in artificial diet. Expected mortalities were calculated using the mean mortality of individual chemical treatments as described by Salama et al. (1984). The critical χ^2value (used to determine if effects were additive) is 3.84 (*df* = 1).

Treatment	**Percent Larval Mortality**		χ^2	**Joint Effect**
	Observed	**Expected**		
Co + Cu	74	51	10.4	Synergistic
Co + Ni	62	52	1.92	Additive
Co + Zn	60	50	2.00	Additive
Cu + Ni	65	52	3.25	Additive
Cu + Zn	66	46	8.70	Synergistic
Ni + Zn	59	45	4.43	Synergistic

Table 3. Sublethal joint effects between Co, Cu, Ni and Zn against *Spodoptera exigua* larvae in artificial diet. Expected larval weight was calculated using the mean percent larval weight reduction of individual chemical treatments using the approach for mortality of Salama et al. (1984). The critical χ^2 value (used to determine if effects were additive) is 3.84 (*df* = 1).

Treatment	**Percent Larval Weight Reduction**		χ^2	**Joint Effect**
	Observed	**Expected**		
Co + Cu	77	56	7.74	Synergistic
Co + Ni	63	51	2.71	Additive
Co + Zn	58	53	0.57	Additive
Cu + Ni	62	59	0.14	Additive
Cu + Zn	67	61	0.61	Additive
Ni + Zn	59	56	0.20	Additive

Table 4. Lethal joint effects between Co, Cu, Ni and Zn combined with nicotine, mustard seed powder and tannic acid and fed to *Spodoptera exigua* larvae in artificial diet. Expected mortalities were calculated using the mean mortality of individual chemical treatments as described by Salama et al. (1984). The critical χ^2 value (used to determine if effects were additive) is 3.84 (*df* = 1).

Treatment	**Percent Larval Mortality**		χ^2	**Joint Effect**
	Observed	**Expected**		
Co + Nicotine	51	41	2.52	Additive
Co + Mustard Seed Powder	61	42	7.86	Synergistic
Co + Tannic Acid	53	37	6.28	Synergistic
Cu + Nicotine	62	44	7.41	Synergistic
Cu + Mustard Seed Powder	71	45	14.4	Synergistic
Cu +Tannic Acid	57	41	6.33	Synergistic
Ni + Nicotine	36	40	0.46	Additive
Ni + Mustard Seed Powder	44	41	0.23	Additive
Ni + Tannic Acid	53	36	7.67	Synergistic
Zn + Nicotine	51	41	2.52	Additive
Zn + Mustard Seed Powder	61	42	7.86	Synergistic
Zn + Tannic Acid	59	37	12.9	Synergistic

Table 5. Sublethal joint effects between Co, Cu, Ni and Zn combined with nicotine, mustard seed powder and tannic acid and fed to *Spodoptera exigua* larvae in artificial diet. Expected larval weight was calculated using the mean percent larval weight reduction of individual chemical treatments using the approach for mortality of Salama et al. (1984). The critical χ^2 value (used to determine if effects were additive) is 3.84 (*df* = 1).

Treatment	**Percent Larval Weight Reduction**		χ^2	**Joint Effect**
	Observed	**Expected**		
Co + Nicotine	42	35	1.17	Additive
Co + Mustard Seed Powder	70	42	19.6	Synergistic
Co + Tannic Acid	51	35	7.43	Synergistic
Cu + Nicotine	58	42	5.66	Synergistic
Cu + Mustard Seed Powder	53	48	0.59	Additive
Cu +Tannic Acid	53	42	2.68	Additive
Ni + Nicotine	34	33	0.02	Additive
Ni + Mustard Seed Powder	52	40	3.69	Additive
Ni + Tannic Acid	29	33	0.47	Additive
Zn + Nicotine	85	40	49.7	Synergistic
Zn + Mustard Seed Powder	90	44	48.2	Synergistic
Zn + Tannic Acid	84	46	33.1	Synergistic

Table 6. Comparison of metal concentrations added to artificial diet individually and in metal/metal or metal/organic combinations to achieve similar mean values of larval mortality/larval weight reduction. The percent mortalities and percent weight reductions for combinations are shown as a range of all combinations for each metal (3 metal/metal and 3 metal/organic combinations for each metal).

Experiments	Metal	Individually		Combination	
		Concentration	Percent Larval Mortality/Weight Reduction	Concentration	Percent Larval Mortality/Weight Reduction
Lethal (Mortality)	Co	450 µg Co/g	68	180 µg Co/g	51-74
	Cu	2450 µg Cu/g	73	800 µg Cu/g	57-74
	Ni	1100 µg Ni/g	66	900 µg Ni/g	36-65
	Zn	2500 µg Zn/g	62	1600 µg Zn/g	51-66
Sublethal (Weight Reduction)	Co	45 µg Co/g	60	20 µg Co/g	42-77
	Cu	280 µg Cu/g	66	180 µg Cu/g	53-77
	Ni	210 µg Ni/g	53	150 µg Ni/g	29-63
	Zn	270 µg Zn/g	70	140 µg Zn/g	58-92

CHAPTER IV. BIOTRANSFER, BIOACCUMULATION AND EFFECTS OF HERBIVORE DIETARY CO, CU, NI AND ZN ON GROWTH AND DEVELOPMENT OF THE INSECT PREDATOR *PODISUS MACULIVENTRIS* (SAY) (HEMIPTERA: PENTATOMIDAE).

Abstract-Increased availability of metals in the environment can have detrimental impacts on the growth and development of organisms at all trophic levels in a food web. Metals can be biotransferred or bioaccumulated from lower to higher trophic levels. In the current study I evaluated the survival, growth, and development of a generalist Hemipteran predator (*Podisus maculiventris*) when fed herbivorous prey (*Spodoptera exigua*) reared on artificial diet amended with two different concentrations of Co, Cu, Ni and Zn and determined if metals were biotransferred or bioaccumulated from the diet to herbivore and predator. *P. maculiventris* were fed *S. exigua* larvae raised on artificial diet amended with sublethal (Minimum Sublethal Concentration or MSC) or lethal (Minimum Lethal Concentration or MLC) concentrations of each metal. Survival of *P. maculiventris* fed metal-raised herbivore larvae was not significantly different from the untreated control. Predators that were fed herbivores raised on MLC levels of both Cu-amended and Zn-amended diets matured to adult after a significantly longer time than on the untreated control ($P = 0.0001$ and $P < 0.0001$, respectively). Adult weights were significantly reduced for predators that were raised on herbivores reared on diets amended with MLC levels of Cu ($P = 0.018$). The presence of metals in artificial diets resulted in biotransfer for Ni, whereas Cu and Zn were bioaccumulated from the artificial diet to the herbivore and from the herbivore to the predator. These results show that

metals in a food web can affect the growth and development of a hemipteran predator. Furthermore, metals can be transferred from a lower trophic level to another higher trophic level, but whether it is bioaccumulation or biotransfer is metal-dependent.

INTRODUCTION

Elevated levels of metals in the environment can have detrimental impacts on food webs. Increased metal concentrations may occur as a result of natural (Jensen and Trumble, 2003) or anthropogenic processes (Shah and Nongkynrih, 2007) that deposit metals in soil, water, or onto plant surfaces (Hsu et al., 2005). Bioavailable metals can then be introduced into a terrestrial food web by consumption or inhalation, and then mobilized into higher trophic levels (Vickerman and Trumble, 2003; Mogren and Trumble, 2010).

At relatively high concentrations, metals have negative effects on the biotic densities and diversity across a food web (Hsu et al., 2005). Concentrations of metals found in contaminated habitats have been reported to cause either immunostimulation or immunosupression in terrestrial organisms (Sun et al., 2011): the exact effect is dependent on the form and the nature of the metal and response from the organism (Bañuelos et al., 2002). For example, lower concentrations of Ni can stimulate the immune system but higher concentrations can cause death by inhibiting the immune system (Sun et al., 2011). Both Cu and Zn are essential elements that are regulated by most organisms by binding to metallothioneins in the cytosol, but higher concentrations are toxic (Jensen and Trumble, 2003). Co can become toxic at high concentrations, inducing increased apoptosis that reduces cell differentiation and development (Wang et al., 2007).

Although metals can become bioavailable due to both natural and anthropogenic processes, most research has focused on anthropogenic causes; naturally occurring metals have received less focus (Boyd et al., 2007). Naturally occurring areas of elevated metal concentration can be found in metalliferous soils, including serpentine soils (Brooks et al., 1977). Serpentine soils are formed from weathering of ultramafic rocks and have high concentrations of Co, Cr, Fe and Ni, but low levels of K, N, P and Si (Brooks, 1987; Reeves et al., 1996). Because of the unique ecological conditions created by low nutrient levels and elevated metal concentrations, some organisms are adapted and endemic to serpentine soils, including many metal hyperaccumulator plants (Brooks and Radford, 1978; Reeves and Baker, 2000; van der Ent et al., 2012). Hyperaccumulators are plants that have exceptionally high concentrations of metals in their tissue (Brooks et al., 1977). Hyperaccumulators have been reported for As, Cd, Co, Cr, Cu, Mn, Ni, Pb, Se, Tl and Zn (van der Ent et al., 2012), although confirmation of actual hyperaccumulation, instead of surface contamination, is needed for a number of these metals (Co, Cr, Cu, Pb and Tl: van der Ent et al., 2012).

Plant–animal interactions have major impacts on a food web, and interactions between herbivores and hyperaccumulators may affect herbivores (Ping et al., 2009). Some herbivores are adapted to high concentrations of metals in the plants they consume and can sequester these metals in their tissue without any apparent negative physiological impact (Jensen and Trumble, 2003; Boyd, 2009). Boyd and Wall (2001) hypothesized that some herbivores may sequester metals in their tissues to defend themselves against predation and attack by pathogens. These herbivores may facilitate the trophic transfer of metals, with plants playing a major role in creating the pathway from the soil into the higher trophic levels (Boyd and Martens, 1998; Jensen and Trumble, 2003). Additionally, the movement of metals from plants into herbivores can lead to transfer of metal pollutants from contaminated to uncontaminated sites because, unlike most whole plants, herbivores are mobile (Boyd, 1998).

Because some herbivores can develop resistance to elemental defenses by sequestering metals from plants, plants can increase the bioavailability of metals in terrestrial environments (Bruno 1984; Jensen and Trumble, 2003). Bioavailable metals may be either biotransferred or bioaccumulated from one trophic level to the next (Vickerman et al., 2004). In biotransfer, metals are passed from one level to a higher trophic level at the same concentration or lower, whereas during bioaccumulation (biomagnification) metal concentrations in the higher trophic level are increased in comparison to the lower trophic level (Gray, 2002).

In my research I evaluated the survival, growth, and development of *Podisus maculiventris* (Say) (Hemiptera: Pentatomidae) fed the polyphagous herbivore, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), larvae raised on artificial diets amended with Co, Cu, Ni and Zn over a 30 day period. I also determined if the metals were biotransferred or biomagnified from the artificial diet to *S. exigua* larvae and to adults of the generalist Hemipteran predator *P. maculiventris*. I selected Co, Cu, Ni and Zn because they are commonly reported as hyperaccumulated by plants. Out of nearly 450 reported hyperaccumulators, more than 320 are Ni hyperaccumulators, 30 species hyperaccumulate Co, 24 hyperaccumulate Cu and 11 species hyperaccumulate Zn (Reeves, 2003).

Spodoptera exigua, commonly known as beet armyworm, is a polyphagous herbivore with a broad host plant range (Capinera, 2006). Host plants for *S. exigua* include both cultivars and non-cultivars from over 18 different families (Greenberg et al., 2001). Plant families attacked by *S. exigua* include Amaranthaceae, Apiaceae, Asteraceae, Fabaceae, Liliaceae, Malvaceae and Solanaceae (Vickerman and Trumble, 1999). According to Capinera (2006), *S. exigua* can complete its life cycle in 4-5 weeks depending on the climate. Females lay eggs in clusters of 50-150 per day over a period of 3-7 days. Eggs hatch within 3-4 days and larvae spread to different parts of a plant. Larvae molt through five instars and the duration of each instar is affected by temperature. Pupation occurs either on the surface or under the surface of soil or artificial diet: pupae are brownish in color and measure up to 20 mm long. The pupation period of *S. exigua* may be as short as 6 days in warm weather or as long as 6 months in cold weather. Adults have a silvery-gray to brownish body and wings and the entire body is about 12 mm long.

Podisus maculiventris is a generalist predator commonly known as the spined soldier bug (De Clercq, 1998). It is native to North America and is widely distributed from Mexico to Canada (De Clercq, 1998). First instars usually ingest only fluids (mostly plant juices) and begin predation during the 2nd instar (Mahdian et al., 2006). *P. maculiventris* has piercing, sucking mouthparts and feeds mostly on eggs and larvae of Coleoptera and Lepidoptera, including those in the genus *Spodoptera* (Mahdian et al., 2006). *P. maculiventris* is commonly found on alfalfa, cotton, cabbage, beans and other plants that are associated with Coleopterans and Lepidopterans (Mahdian et al., 2006). I used *P. maculiventris* because, besides it being a widespread polyphagous predator, its response to manipulations in diet trace element concentrations has been recently investigated (Coudron et al., 2012) and it has been used in a prior study involving biotransfer of Se from *S. exigua* (Vickerman and Trumble, 2003).

METHODS

I used a colony of *S. exigua* raised in the laboratory since 2008 from a combination of insects from two different origins: egg sheets purchased in 2008 from Benzon Research Inc. (Carlisle, PA, USA) and a colony from the laboratory of William Moar (Boyd and Moar, 1999) at Auburn University (Alabama, USA) which was established originally from insects collected from cotton fields in Alabama. The colony was raised on artificial diet and maintained in an insect rearing chamber at 27±2 degrees Celsius. The artificial diet is a blend of pinto beans, wheat germ, tortula yeast, casein, gelcarin, insect vitamin, methyl *p*-hydroxybenzoate, tetracycline and formaldehyde (Li et al., 2002). I reared adults on a diet of 10% sucrose solution soaked in cotton balls and placed in a petri dish inside 4 liter glass jars (25-35 adults per jar), covered with single sheets of white paper towel and secured with a rubber band. Every day, I replenished the food with freshly soaked cotton balls, removed paper towels and replaced them with fresh ones. I sterilized the egg-covered paper towels in 10% formalin for 5-10 min., rinsed them in distilled water for 1 minute, and air-dried them for at least 30 min. before placing them in sealed plastic Ziploc bags. For all the experiments, I used newly hatched larvae within 16 hours of hatching as described by Thamthiankul et al. (2004).

I used 1st instar *P. maculiventris* nymphs obtained from the laboratory of Thomas Coudron, Biological Control of Insects Research Laboratory, USDA-Agricultural Research Service, (Columbia, Missouri). The colony was established from adults collected from alfalfa plants in Boone County, Missouri during the summer of 2002 (Coudron et al., 2012).

Survival, Growth and Development of P. maculiventris. I raised *S. exigua* larvae on two different concentrations of each of four metals (Co, Cu, Ni and Zn) as well as a control treatment (unamended diet). The two concentrations used for each metal were determined in previous experiments as the Minimum Lethal Concentration (MLC: the lowest concentration that caused significant mortality of *S. exigua* larvae) and Minimum Sublethal Concentration (MSC: the lowest concentration that caused a significant weight reduction of *S. exigua* larvae compared to unamended diet) (Table 1). I transferred *S. exigua* neonates to artificial diet amended with the MSC or MLC of each metal, or to control diet. I used twenty-four well plates (Multiwell tissue culture plates, Becton Dickinson Labware, Lincoln Park, NJ, USA) with one larva per well and incubated plates at 27±1 degrees Celsius until larvae were needed for feeding to *P. maculiventris*.

For all treatments, I used five *P. maculiventris* nymphs placed into paper cups during the 1st through early 3rd instars, moving nymphs that progressed to late 3rd through 5th instars into individual cups to avoid the risk of cannibalism. I used 30 nymphs per treatment (90 per metal). I raised 1st instar *P. maculiventris* nymphs on distilled water until they molted to 2nd instar before starting metal and control treatments. Thereafter, I fed nymphs metal-raised *S. exigua* larvae or control larvae. Every other day, I fed each *P.*

maculiventris nymph a fresh host larva. Younger nymphs were offered 3rd instar larvae and the older nymphs were offered 4th – 5th instar larvae. To ensure that all nymphs received an equal amount of food, I fed all nymphs similar age and size of larvae for all the treatments, regardless of size and developmental stage of predator, for 30 days. When nymphs matured into adults, I removed them from cups, recorded the total number of days to adulthood and weighed each individually before freezing them for metal analysis. After30 days, I recorded the total weight and the developmental stage of each *P. maculiventris* that had not matured into an adult.

Metal Trophic Transfer. I analyzed adult predators, herbivore larvae reared on treated diet, and untreated diet samples to determine the concentrations of metals in all treatments. I collected all surviving adult *P. maculiventris*, representative samples of *S. exigua* larvae from each diet treatment, and untreated the diet. I measured wet weights of diet, *S. exigua* larvae*,* and *P. maculiventris* individuals and dried all samples (diet, herbivores and predators) using a Heatblock 3 hot plate (Henry Troemner LLC, Thorofare, NJ, USA) at 100 degrees Celsius and recorded dry weights. I analyzed dried samples for metal concentrations using Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) (Optima 7300DV, PerkinElmer, Walburn, MA, USA). I digested artificial diet, *S. exigua* and *P. maculiventris* samples separately for 24 hours in 150 µl metal free concentrated nitric acid (Fisher Scientific, Pittsburgh, PA, USA) for 1 hr in partially sealed tubes at 95 degrees Celsius. I centrifuged the digested samples, collected supernatants and diluted them to 1 ml with distilled water. As controls, I included nitric acid blanks and processed them along with samples. I determined metal concentrations by comparing sample intensities with standard curves created from four certified metal standards: Standards A, B, C and D (all with varying concentrations of Ag, Ca, Co, Cu, Fe, K, Na, Mg, Mn, Mo, Na, Ni, P and S) (PerkinElmer, Walburn, MA, USA). The ICP-OES can detect Co, Cu, Ni and Zn at concentrations as low as 0.005 ppm in the liquid sample.

Bioaccumulation factors were determined using the following equations (Scheifler et al., 2001). The Bioaccumulation Factor (BAF) from diet to herbivores was calculated as:

$$\mathrm{BAF} = C_{(\mathrm{MSC\ or\ MLC})h}/C_{(\mathrm{MSC\ or\ MLC})d}$$

Where $C_{(\mathrm{MSC\ or\ MLC})d}$ is the mean MSC or MLC of each metal in the diet and $C_{(\mathrm{MSC\ or\ MLC})h}$ is the mean concentration of each metal measured in the herbivore.

The BAF from herbivore to predator was calculated as:

$$\mathrm{BAF} = C_{(\mathrm{MSC\ or\ MLC})p} /C_{(\mathrm{MSC\ or\ MLC})h}$$

Where $C_{(\mathrm{MSC\ or\ MLC})p}$ is the mean MSC and MLC of each metal measured in the predator and $C_{(\mathrm{MSC\ or\ MLC})h}$ is the mean concentration of each metal measured in the herbivore.

Statistical Analyses. I compared survival of predators fed herbivores raised on MSCs and MLCs of Co, Cu, Ni and Zn as well as the control (unamended diet). I used survival analysis in the program StatView (SAS Institute, 2005), using the Kaplan-Meier estimate and with treatment significance determined by the Peto-Peto-Wilcoxon rank test at $P \leq 0.05$. Data for each metal were analyzed separately to determine if metal level in diet fed to larvae (control, MSC, MLC) significantly affected predator survival.

I used one-way analysis of variance (ANOVA, $P \leq 0.05$) to determine the effect of metal concentration on time to adulthood and final weight of predators, using a separate analysis for data from each metal. I arcsine-transformed data for time to adulthood and predator weights for each metal to meet the underlying ANOVA assumptions of normality (Zar, 1996). Significant results from ANOVA ($P \leq 0.05$) were followed by the Fisher's Protected Least Significant Difference (PLSD) Test at $P \leq 0.05$ to determine if there were significant differences between treatments (control, MSC, MLC) for each metal (SAS Institute, 2005).

I analyzed metal concentration data from ICP analysis to determine the effect of trophic level and dietary metal concentration on metal levels. Data for each metal were analyzed separately. I used a two-way ANOVA ($P \leq 0.05$) with trophic level (diet, herbivore, predator) and dietary metal concentration (control, MSC, MLC) as the main factors and including the interaction term (SAS Institute, 2005).

RESULTS

Survival, Growth and Development of P. maculiventris. Survival of *P. maculiventris* was not significantly affected by feeding on *S. exigua* larvae raised on diet containing MSC and MLCs of each metal (Peto-Peto-Wilcoxon rank tests; $P > 0.05$; Table 2; Fig. 1). Based on one-way ANOVAs, both time to maturity and adult weights of *P. maculiventris* were not significantly affected by feeding upon *S. exigua* raised on diets amended with Co or Ni (Table 3). However, one-way ANOVA showed a significant treatment effect on both time to maturity and weight for *P. maculiventris* that were fed *S. exigua* raised on diets amended with Cu. When results for different diet Cu concentrations were compared, predators fed herbivores raised on the MLC of Cu took significantly longer to mature, and weighed significantly less, than predators fed herbivores raised on either the control or the MSC diets (Table 3). Similarly, one-way ANOVA showed a significant treatment effect on both time to maturity and weight for *P. maculiventris* that were fed herbivores raised on diets amended with Zn. *P. maculiventris* that were fed *S. exigua* raised on Zn took significantly longer to mature to adults compared to the control. Predators fed herbivores raised on diets amended with the MLC of Zn took significantly longer to mature, and weighed less, than predators that were fed control herbivores or herbivores raised on diets amended with the MSC of Zn (Table 3).

Metal Trophic Transfer. Two-way ANOVA showed that both trophic level and initial dietary metal concentration had significant effects on metal transfer (Table 5). For Co treatments, metal concentrations increased from diet to herbivore to predator for the MSC treatment, but for the MLC treatment the metal concentration only increased from the diet to the herbivore and decreased slightly from the herbivore to the predator (Fig. 2). When metal concentrations of each trophic level were compared, Co concentrations increased within each trophic level as the metal level increased, with higher levels of Co being measured for the MLC treatment for all the trophic levels. Cu and Zn concentrations showed a positive correlation with trophic level, with the highest concentrations of each metal measured in the predator and the lowest concentration of each metal measured in the diet (Fig. 2). Within each trophic level, concentrations of Cu increased as the concentration in the diet increased. For example, the highest concentrations of Cu were measured in predators that were fed herbivores raised on MLCs of Cu in the diet. For Zn, concentrations were similar for herbivores fed MSC or MLC levels, and were again similar for predators fed MSC or MLC level herbivores (Fig. 2). A very different pattern was observed for Ni: Ni concentrations showed a negative correspondence with trophic level and Ni concentrations decreased from the diet to the herbivore and to the predator for both MSC and MLC treatments (Fig. 2).

For all four metals, there was an interaction between the metal concentration in the diet and the concentration measured in the trophic level (Table 5). Both Cu and Zn showed a positive interaction, shown most strongly with Cu, where metal concentration increased in the trophic level as the concentration in the diet increased (Fig. 2). Cobalt showed a different pattern: for the MSC treatment concentrations were similar for all trophic levels but, when the MLC was added to the diet, the concentration increased from the diet to the herbivore but decreased from herbivore to predator (Fig. 2). Nickel concentrations decreased from the diet to the herbivore and to the predator for both MSC and MLC treatments. However, differences between concentrations in each trophic level were relatively small for the MSC treatment and relatively large for the MLC treatment (Fig. 2).

DISCUSSION

My results demonstrate that the presence of metals in herbivores may impact the growth and development of a predator. Although survival of the predator was not significantly affected by the concentration of metals in the herbivore, growth and development of *P. maculiventris* was significantly decreased by the presence of relatively high (MLC) levels of Cu and Zn. Furthermore, in all cases, metals were transferred from the artificial diet into the herbivores and subsequently into the predators.

Metals can have diverse modes of action, resulting in varying degrees of toxicity, yet they can have similarities such as their general toxicity and long residual persistence in the environment (Jensen and Trumble, 2003). In my experiments, response to metals by *P. maculiventris* varied between the four metals resulting in biotransfer of Ni (Table 6) and bioaccumulation of Co, Cu, and Zn (Table 6). This result is in agreement with results reported by Ping et al. (2009), who reported that Cu and Zn were transferred at greater levels from soil to plants to insect larvae, whereas Cd and Pb concentrations decreased from soil to plants to insect larvae.

The bioaccumulation of Cu, Zn, and Co may be due to similarities in pathways of storage and regulation of these metals within *S. exigua* (Scheifler et al., 2002). Both Cu and Zn are in the group B metals that bind to metallothioneins in the larval gut of most insects (Ping et al., 2009), reduce ion availability and release them back into the system when needed to replenish low ion levels (Mariňo et al., 1998). In mollusks, both Cu and Zn are controlled by a homeostatic response by increasing the excretion rate and storage in the extra-cytosolic compartments (Giguère et al., 2003). Because of the way Cu and Zn are controlled physiologically in insects, it is possible that, in my experiments, Cu and Zn were bound to metallothioneins in *S. exigua* larvae and, when the contents of the larvae were consumed by the predator, the bound metal ions were bioaccumulated in the predator. Although Co was also bioaccumulated, Co is regulated differently from Cu and Zn in insects. When there is excess Co in the epithelial layer of the gut, Co ions induce increased production of caspases. Caspases regulate cell death (apoptosis) and can slow cell differentiation and development (Wang et al., 2007).

Although Cu, Zn, and Co were bioaccumulated, Ni was the only metal transferred from the diet to *P. maculiventris* in decreasing concentrations (Table 6). Nickel biotransfer rather than bioaccumulation was reported by Migula et al. (2007) who evaluated transfer of Ni in two different cases involving the Ni hyperaccumulator *Berkheya coddii* Roessl. (Asteraceae) to the beetle *Chrysolina pardalina* F. (Coleoptera: Chrysomelidae) and its predator *Rhinocoris neavei* (Bergroth) (Hemiptera: Reduviidae), as well as from *Berkheya coddii* to the aphid *Protaphis pseudocardui* (Hemiptera: Aphididae) to the predacious ant *Polyrhachis sp.* (Hymenoptera: Formicidae). Boyd et al. (2006) reported high levels of Ni in the grasshopper *Stenoscepa sp.* (Orthoptera: Pyrgomorphidae) collected from the Ni hyperaccumulator *Berkheya coddii*, also concluding that Ni was biotransferred and not bioaccumulated. Phipps et al. (2002) concluded that Ni levels are generally reduced in higher trophic levels, and are reduced in adult insects compared to juveniles.

Lack of a significant impact by the MSCs of all the four metals on *P. maculiventris* survival (Fig. 1) and growth (Table 3) in my experiments does not necessarily mean lower metal levels do not affect a food web. Sublethal effects of metals and other pollutants are most likely to result in chronic effects rather than mortality (Karaouna-Renier and Zehr, 2003). A newly described category of sublethal effects is info-disruption, wherein a pollutant can disrupt an organism's chemical senses that would inhibit them to communicate with their biotic and abiotic surroundings (Boyd, 2010). While many terrestrial insects are unable to detect metals in their diets, alterations in insect behaviors such as ingestion, locomotion and reproduction have been reported and these in turn may affect food webs (Mogren and Trumble, 2010).

My research shows that dietary Co, Cu, Ni and Zn can impact diet-herbivore-predator interactions. While I did not observe that sequestered metals defended herbivores against predators as hypothesized by Boyd and Wall (2001), I showed that some dietary metals transferred from the diet to the prey, and from the prey to the predator, affect growth and development of the predator, *P. maculiventris*. Furthermore, dietary metals can be biotransferred or bioaccumulated from a lower to a higher trophic level in a food web, depending on the metal. Future research should evaluate the effectiveness of higher concentrations of Co, Cu, Ni and Zn against *P. maculiventris* because the concentrations used in my study were the lowest concentrations that could cause either lethal or sublethal effects. Another factor that could be explored is the feeding mode of the predator. A chewing predator, or one that swallows its prey whole, will likely receive a different dose of metal compared to a piercing sucking predator such as *P. maculiventris* because they may not be able to exclude metals stored in the exoskeleton or other parts of the prey. Additionally, the Joint Effects Hypothesis (Boyd, 2007) suggests that metals in plants can act positively (with each other or with organic defense compounds) to increase the defensive benefits to the plant. Does this Joint Effects Hypothesis extend to other trophic levels? Metals are often found in the environment as mixtures (Jensen et al., 2006): these chemical combinations could potentially act in a reinforcing manner to provide an increased defensive benefit to herbivores against predators. This could negatively impact the effectiveness of the accumulated metal as a plant defense, further complicating the balance of costs and benefits that likely underlie the evolution of elemental plant defenses.

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FIGURES

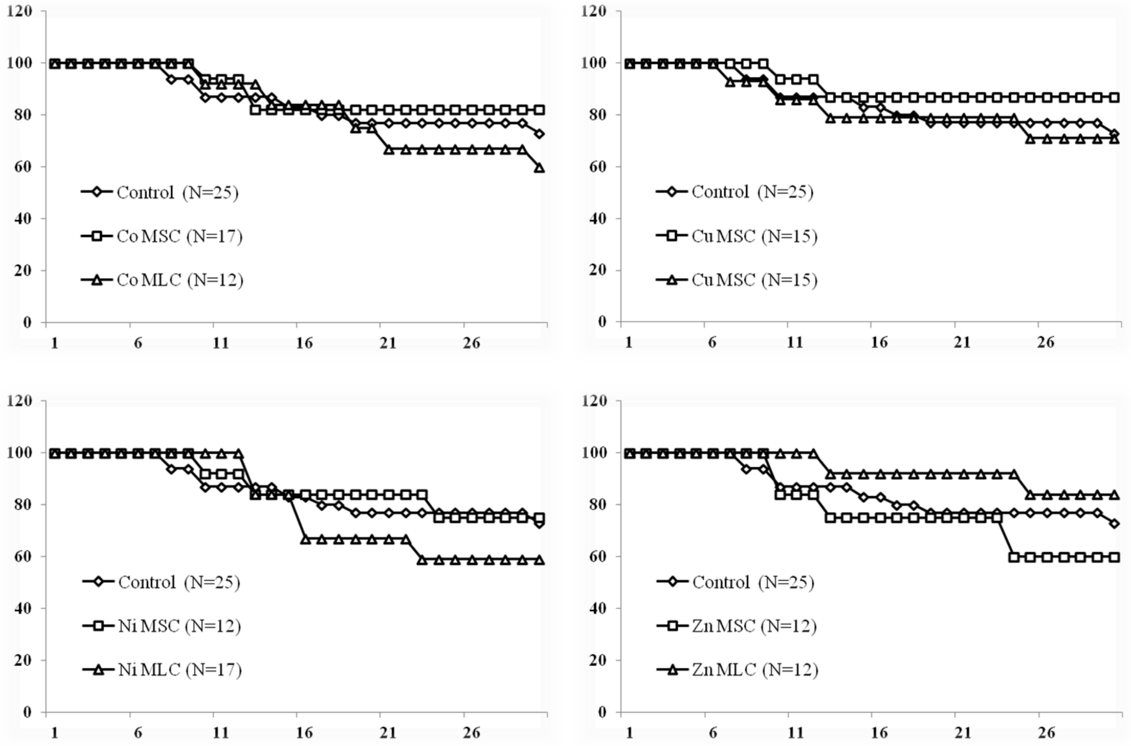

Figure 1. Percent survival of *Podisus maculiventris* raised on *Spodoptera exigua* reared on artificial diet amended with Minimum Sublethal Concentrations (MSC) and Minimum Lethal Concentrations (MLC) of Co, Cu, Ni and Zn as well as a control treatment over 30 days.

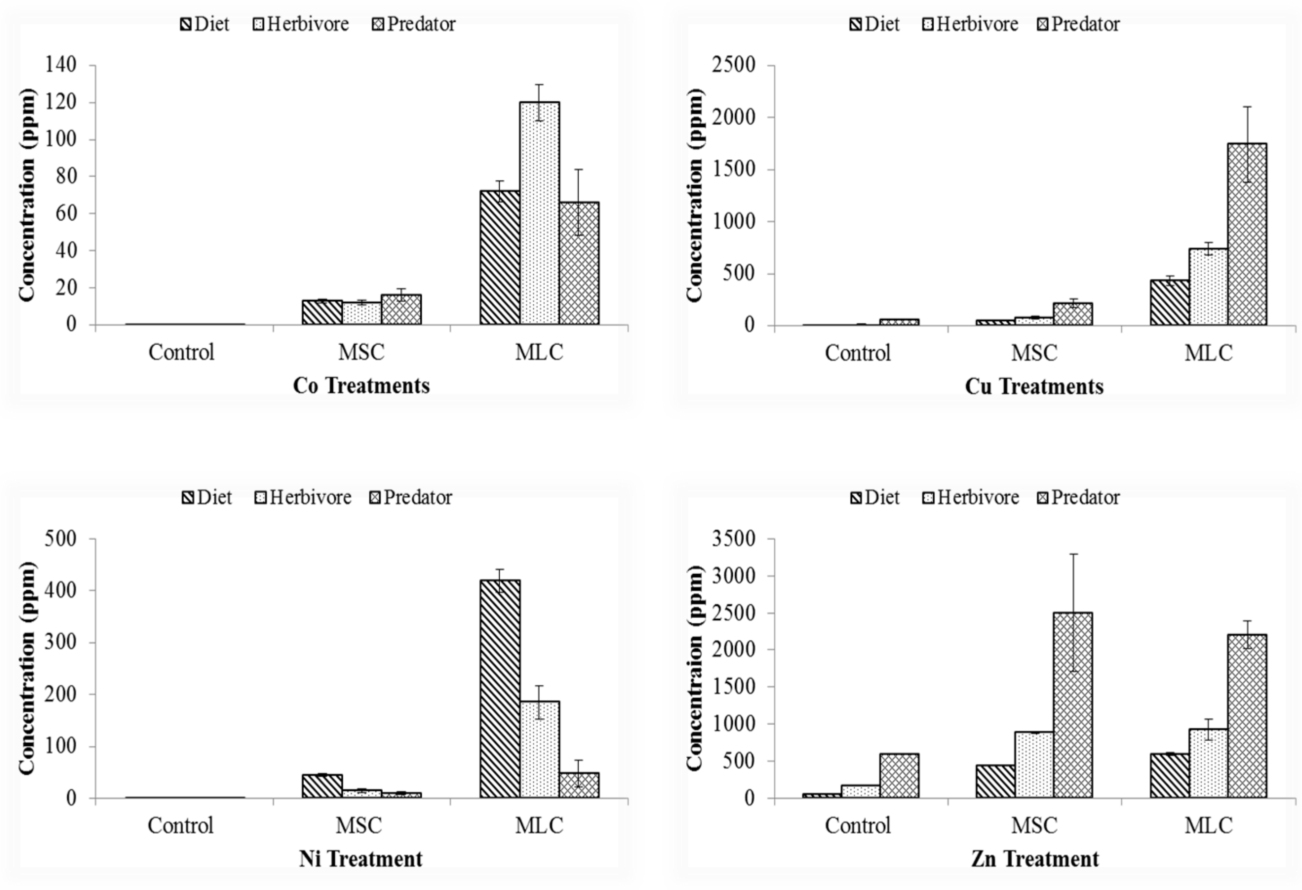

Figure 2. Mean metal concentrations (µg/g, dry mass basis) in artificial herbivore diet, herbivores *Spodoptera exigua.*and predators, *Podisus maculiventris*. Error bars indicate SE of the means (some bars are too small to be shown at the scale used).

TABLES

Table 1. Metal concentrations (in micromoles of metal chloride added per liter of artificial diet) of artificial diet fed to *Spodoptera exigua* larvae to generate prey for feeding experiments. Values in µg/g are expressed on a dry weight basis using data for actual metal concentrations in artificial diet analyzed at the Auburn University Soil Testing Laboratory.

		Concentration	
Metal	Units	Sublethal (MSC)	Lethal (MLC)
Co	µM $CoCl_2$	0.053	0.34
	µg Co/g	15	90
Cu	µM $CuCl_2$	0.106	1.35
	µg Cu/g	140	530
Ni	µM $NiCl_2$	0.197	1.85
	µg Ni/g	140	470
Zn	µM $ZnCl_2$	0.787	1.24
	µg Zn/g	200	280

Table 2. Survival analysis (Peto-Peto-Wilcoxon test) results showing *P*-values and χ^2 values for the effects of metal in the herbivore, *Spodoptera exigua* on the survival of *Podisus maculiventris.*

Metal	χ^2	*df*	*P*-value
Co	1.34	2	0.5116
Cu	1.04	2	0.5930
Ni	5.87	2	0.0533
Zn	1.66	2	0.3766

Table 3. Mean number of days to adulthood and weights of adults for *Podisus maculiventris* raised on *Spodoptera exigua* fed artificial diets amended with MSCs and MLCs of Co, Cu, Ni and Zn as well as control (unamended) diet. Mean values with the same letters are not significantly different from each other (Fisher's PLSD test; $P > 0.05$). SE is the standard error of means.

Metal	Experiment	Treatment					
		Control	SE	MSC	SE	MLC	SE
Co	Time to adult (days)	24.0^{a}	0.3	24.3^{a}	0.5	24.6^{a}	0.5
	Weight (mg)	59.7^{a}	3	61.0^{a}	3	61.9^{a}	4
Cu	Time to adult (days)	24.0^{a}	0.3	24.6^{a}	0.5	27.5^{b}	0.9
	Weight (mg)	60.0^{b}	3	64.0^{b}	3.5	49.6^{a}	3
Ni	Time to adult (days)	24.0^{ab}	0.3	23.6^{a}	0.3	25.1^{b}	0.7
	Weight (mg)	60.0^{a}	3	59.7^{a}	5	66.4^{a}	6
Zn	Time to adult (days)	24.0^{a}	0.3	23.8^{a}	0.5	28.0^{b}	0.7
	Weight (mg)	60.0^{b}	3	54.6^{ab}	3.5	49.4^{a}	4.5

Table 4. One-way ANOVA results for effects of each metal in the herbivore, *Spodoptera exigua* on the growth and development of *Podisus maculiventris*. Each analysis involved three treatments: diet amended with a metal at MSC and MLC as well as unamended diet.

Effect	Metal	*F*	*df*	*P*-value
Time to Adulthood (Days)	Co	0.84	2,40	0.44
	Cu	11.6	2,42	0.0001
	Ni	2.41	2,34	0.10
	Zn	22.4	2,35	<0.0001
Adult Weights	Co	0.18	2,40	0.83
	Cu	4.46	2,42	0.018
	Ni	0.47	2,34	0.63
	Zn	2.8	2,35	0.074

Table 5. Two-way ANOVA results showing *P*-values for concentrations of metal in diet, herbivore and predator.

Metal	Effect	*F*	*df*	*P*-value
Co	Trophic Level	19.6	2,72	<0.0001
	Metal Concentration	73.9	2,72	<0.0001
	Trophic Level*Metal Concentration	20.2	4,72	<0.0001
Cu	Trophic Level	9.96	2,72	0.0002
	Metal Concentration	27.4	2,72	<0.0001
	Trophic Level*Metal Concentration	5.92	4,72	0.0004
Ni	Trophic Level	28.0	2,60	<0.0001
	Metal Concentration	100	2,60	<0.0001
	Trophic Level*Metal Concentration	19.4	4,60	<0.0001
Zn	Trophic Level	22.8	2,53	<0.0001
	Metal Concentration	12.2	2,53	<0.0001
	Trophic Level*Metal Concentration	2.62	4,53	0.045

Table 6. Mean metal concentrations (µg/g) and Bioaccumulation Factors (BAF) in larvae of the herbivore *Spodoptera exigua* (raised on metal amended artificial diet) and adults of the predator *Podisus maculiventris* (fed metal-raised larvae). Artificial diet of the herbivore was amended with Minimum Sublethal Concentration (MSC) and Minimum Lethal Concentration (MLC) levels of Co, Cu, Ni and Zn.

Experiment	Diet	Herbivore (*Spodoptera exigua*)		Predator (*Podisus maculiventris*)	
	Concentration	Concentration	BAF	Concentration	BAF
Control	0.09	0.07	0.77	0.15	2.14
Co $_{\mathrm{MSC}}$	13.0	12.0	0.92	16.0	1.33
Co $_{\mathrm{MLC}}$	72.0	120	1.67	66.0	0.55
Control	6.00	16	2.67	54.0	3.38
Cu $_{\mathrm{MSC}}$	47.0	77.0	1.64	216	2.81
Cu $_{\mathrm{MLC}}$	433	738	1.70	1742	2.36
Control	0.82	0.53	0.65	0.46	0.87
Ni $_{\mathrm{MSC}}$	45.0	15.0	0.33	10.0	0.67
Ni $_{\mathrm{MLC}}$	419	185	0.44	48.0	0.26
Control	0.01	0.07	3.48	0.03	3.42
Zn $_{\mathrm{MSC}}$	7.00	10.0	2.01	790	2.82
Zn $_{\mathrm{MLC}}$	16.0	143	1.55	186	2.73