Effects of rare earth elements on the generalist herbivore, *Melanoplus sanguinipes* (Fabricius) (Orthoptera: Acrididae)

Jane Allison

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Department of Biology
Faculty of Science
University of Ottawa

Abstract

The rare earth elements (REEs) are contaminants of emerging concern with environmental levels rising with increasing demand for these elements. REE pollution may arise from multiple sources. Increased extraction to meet the growing demand for emerging technologies will likely result in elevated environmental levels, due in part to mining waste, in part to industrial activity and in part to landfills holding discarded REE containing technologies. Monazite rocks, rich in REEs are commonly mined to produce phosphate-based fertilizers. Direct mining and fertilizer application are another substantial source of REE pollution. Despite growing environmental levels of REEs, the impact on organisms in terrestrial systems, where pollution originates, are poorly studied. Plants are known to accumulate REEs in root and shoot tissue through contaminated soils. Herbivorous species feeding directly on plant tissues with elevated levels of REEs may likewise accrue these metals in their body with unknown consequences for growth, development, reproduction, health, and survival. The goal of my thesis was to explore the risk posed by REE contamination to terrestrial invertebrates using *Melanoplus sanguinipes* (the lesser migratory grasshopper) as a model system. Two REEs of economic importance were selected for study, cerium (Ce) and neodymium (Nd). First, in chapter 2, I assessed if *Melanoplus sanguinipes* readily consumes REEs using high levels of contamination over a short exposure period to look for acute effects of consumption. Adults consumed both Ce and Nd indiscriminately with consequences for motor function when exposed to Ce and growth when exposed to Nd. REEs are persistent pollutants that may be underestimated by short exposure periods. In chapter 3, I set out to understand if living in a polluted environment (chronic exposure) would have negative impacts on survival and sublethal effects on growth, development, and reproduction. My results indicate that females carry high concentrations of Ce in their bodies while males carry higher levels of Nd.

Overall survival was affected by exposure to Ce and a range of sublethal effects including delayed development, reduced adult mass and shortened adult lifespan (males exposed to Ce) were seen. In chapter 4, I investigated the possibility that accumulation of REEs in the body could impact energy stores (lipids, glycogen, free carbohydrates and protein), energy production (citrate synthase), affect redox balance, cause cellular damage (lipid peroxidase) and induce antioxidant (catalase) and biotransformation (glutathione-s-transferase) defenses. Energy production was increased at the lowest dose tested and slight declines in lipid stores were identified. However, these changes were not associated with an increase in detoxification processes, nor was there evidence of a loss of redox balance or cellular damage. Taken together, my thesis points to the need for both lethal and sublethal endpoints when characterising the risk of persistent pollutants.

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Chapter 1 An introduction to the rare earth elements

The lanthanide series comprises elements with atomic number 57 (lanthanum) to 71 (lutetium). Also included are yttrium (atomic number 36) and scandium (atomic number 21) due to their similar chemical and physical properties (Hu et al., 2006). Although termed the “rare” earth elements (hereafter REEs), soils throughout the world commonly contain lanthanide metals. The term “rare” refers instead to the lack of large deposits characteristic of elements such as silver and gold (Hu et al., 2006). Instead, the REEs often occur in mixture with each other. This rarity, along with the reactivity and chemical similarity of REEs has traditionally made the process of extracting and refining them from surrounding rock complex (Castor & Hedrick, 2006; Long et al., 2012).

Nonetheless, REEs were once widely mined prior to China’s development of a low cost means of production that significantly reduced worldwide activity (Stone, 2009). Once mining activity at Mountain Pass Mine in California was halted, China became the world’s main supplier of REEs. China currently possesses an estimated 36% of the world’s global reserves of REEs and produces 95% or more of the world’s supply (Paul & Campbell, 2011; Rüttinger et al., 2010). As a result, China has a monopoly on these valuable resources, raising concerns over supply security.

More recently, reduced exports from China have renewed interest in REE mining, particularly in North America. Canada and the United States have begun to develop and reopen REE mining facilities like those at Thor Lake and Strange Lake in Canada and the pre-existing REE mine at Mountain Pass in California (Long et al., 2012).

1.1 Neodymium and cerium

In tandem with decreased exports from China, demand for REEs is increasing at an estimated 8% annually (Long et al., 2012). Fuelling much of this demand are emerging technologies, particularly those that provide alternatives to fossil fuel consumption (Long et al., 2012). Of significant economic importance are neodymium (Nd) and the most abundant of the REEs, cerium (Ce). Neodymium (atomic number 60, atomic mass 144.24 g mol^{-1}) and cerium (atomic number 58, atomic mass 140.12 g mol^{-1}) form part of the subgroup termed the light rare earth elements (LREEs), which also includes lanthanum (La), praseodymium (Pr), samarium (Sm), europium (Eu) and gadolinium (Gd). Bastnäsite and monazite rocks commonly contain LREEs (Chu, 2010). Natural crustal levels vary greatly by location and soil type but average estimates of levels of Nd are approximately 40 mg kg^{-1} and approximately 66 mg kg^{-1} of Ce (Greenwood & Earnshaw, 1997; Tyler, 2004). Data for Canadian soils is unavailable. These levels are comparable to other metals such as copper (60 mg kg^{-1}) and zinc (70 mg kg^{-1}), although the REEs have received far less attention in environmental studies (Tyler, 2004). In fact, Ce comprises more of the earth's crust than does copper or lead (Castor & Hedrick, 2006; Long et al., 2012).

Increasing demand for neodymium oxide has more than doubled the price from $108 USD in 2010 to $245 USD in 2011 (Paul & Campbell, 2011). In 2008, increasing demand for Nd outstripped supply (Rüttinger et al., 2010). Among the primary uses of Nd is as a component of neodymium-iron-boron permanent magnets used in MRI machines and for sound amplification in electronics (Stone, 2009). Hybrid-electric vehicles (approximately 1 kg per 5 vehicles) and wind turbines (approximately several hundred kg per turbine) also contain Nd (Chu, 2010). Neodymium-based magnets have also been investigated for their use in magnetic refrigeration (Stone, 2009). As per 2009, 80% of the global use of neodymium oxide was for magnet production (Chu, 2010). Producers of glass and ceramics employ Nd for color, in addition to its use in glass lasers (Chu, 2010). Cerium (III) oxide is commonly used to make catalytic converters and as a fuel additive to reduce carbon monoxide emissions (Chu, 2010; Sabiha-Javied et al., 2010). Like Nd, alloys of Ce contribute to the manufacture of magnets and electrodes used primarily by the welding industry and in the manufacture of glass. Cerium and Nd also serve as fluid cracking catalysts during oil refining (Chu, 2010). Increased mining activity to extract Nd and Ce to meet the demand for emerging technologies may result in elevated environmental levels, due in part to mining waste and in part to landfills holding discarded REE containing technologies.

1.2 Sources of pollution

Elevated levels of REEs from direct mining and processing have been discovered adjacent to facilities in China. Soil concentrations in excess of 1000 mg kg^{-1} (for Nd) have been documented up to one kilometer from the source (Li et al., 2010; Liang et al., 2014). This pollution may not be limited to areas immediately surrounding processing plants. Elevated soil concentrations of REEs due to mine tailings have been found up to approximately 6 km from a processing plant in China, with concentration strongly correlated to distance from the source (Li et al., 2010). Detected Nd levels ranged from 5726 mg kg^{-1} at 0.4 km from the source to 88 mg kg^{-1} at 6 km. Cerium levels were even higher at 12,170 mg kg^{-1} at 0.4 km from the source and only dropped to 205 mg kg^{-1} at 6 km (Li et al., 2010). Concentrations in polluted soils from industrial locations in The Netherlands run as high as 400 mg kg^{-1} for Nd and 900 mg kg^{-1} for Ce, about ten times higher than the background soil levels of 40 and 80 mg kg^{-1} respectively (Slooff et al., 1993). Oil-refining activity in the U.S. also results in the release of REEs to the environment, reported at 60-80 tonnes per day (approximately 66-88 US tons) (Slooff et al., 1993). As REE containing technologies are discarded, levels of contamination are expected to rise around landfill sites. The full impact of this source of pollution for local environments remains unknown.

In addition to direct mining and industrial pollution, phosphate fertilizer production and application is increasing levels of all REEs in the environment. These fertilizers are commonly applied on the Canadian prairies, with 1990 levels at approximately 775 000 tons (IPNI, 1999). Release of REEs, including Nd and Ce, following fertilizer application has been reported. In 1994 in the Netherlands, roughly 85 tonnes of Nd (93.7 US tons) were released into the environment from phosphate fertilizer production (Sneller et al., 2000). The application of phosphate fertilizers may already be causing substantial increases in REEs, including Nd and Ce, especially in agricultural areas (Todorovsky et al., 1997; Turra et al., 2011; Volokh et al., 1990). This expected increase in REE deposition in agricultural settings necessitates a more thorough assessment of potential environmental impacts.

1.3 Impact on terrestrial species

Little attention has been paid to REEs, likely because they are considered neither essential to life nor acutely toxic (Tyler, 2004). As a result, threshold limits and maximum permissible concentrations are poorly established in the literature. However, this expected increase in REE deposition necessitates a more thorough assessment of potential environmental impacts.

Plants are known to accumulate REEs, including Nd and Ce, in their root and shoot tissue through contaminated soils (Thomas et al., 2014). Accumulation is thought to be linked to the similarity in ionic radii between REEs and calcium (Hu et al., 2004; Pickard, 1970). This similarity allows REEs to replace calcium in a number of physiological processes in plants, including root growth, photosynthesis and flowering (Babula et al., 2008; Liu & Hasenstein, 2005; Pickard, 1970; Yang et al., 2015). Still, little information exists about the levels of contamination in plant tissues at sites with elevated soil contamination levels. Plants, in their role as primary ecosystem producers, represent a direct entryway for REE contaminants into food webs. The impact of REEs, and more specifically of Nd and Ce, on wildlife are lacking in the scientific literature.

Species feeding directly on plant tissues with elevated levels of REEs may likewise accrue these metals in their body. For instance, Cowgill (1973) detected uptake of several REEs, including Nd and Ce, by waterlilies (*Nymphaea odorata*) and subsequently found these metals in the aphids (*Rhopalosiphum nymphaeae* (L.)) feeding on this species. Concentrations in aphids were higher than their aquatic plant host, suggesting biomagnification.

Contact with REE contaminated substrates may also adversely affect invertebrates. Custer et al. (2009) observed occurrence of some REEs, including Ce and Nd, in over 90% of their aquatic insect samples from two locations with contaminated sediment. Decreased soil macrofauna diversity has been linked to areas of REE contamination (Li et al., 2010). Further evidence of accumulation of REEs in insects is lacking in the literature. Consumed by other arthropods, amphibians, fish, reptiles, birds and small mammals, insects represent a substantial portion of the biomass available in wildlife food webs. Accumulation of REEs in insects may represent a direct route of exposure for species feeding on contaminated prey. The effects on health, growth and survival of wildlife, including insects, consuming REE pollution remain unknown.

1.4 *Melanoplus sanguinipes* as a model species

The objective of this study is to assess the impact of REE pollution on terrestrial herbivores using the lesser migratory grasshopper, *Melanoplus sanguinipes* (Fabricius) (Orthoptera: Acrididae). This medium-sized rangeland grasshopper (adult females: 20-29 mm, 0.301±0.008 mg; adult males: 20-26 mm, 0.257±0.006 mg (Johnson, 2002)) is an ideal candidate as it is native to Canada and is the most commonly known grasshopper in North America (Johnson, 2002). *Melanoplus sanguinipes* accepts a wide variety of host plants and has a short development cycle (approximately 35 days under field conditions) of 5 instars, with females often requiring a 6th instar (Schell et al., 2005). Acridids (i.e., grasshoppers) while generally intolerant of metal pollution, often survive contamination (Schmidt, 1986). They form a significant part of the diet of birds, small mammals, reptiles, amphibians and spiders because of their abundance and high individual weight, in some cases forming 20-30% of the arthropod biomass available for consumption (Schmidt, 1986). If exposure to REEs results in accumulation in the body, Acridids may represent an exposure route for these other organisms. The sublethal effects produced in *M. sanguinipes* exposed to contamination may be indicative of effects on other wildlife similarly consuming contaminated plant/invertebrate material.

1.5 Objectives

Through a captive rearing approach, *M. sanguinipes* individuals were exposed to either Ce or Nd contaminated substrate or food. The impact of pollutants can be assessed over the full spectrum of biological organization, from the subcellular to ecosystem level. With this increasing biological organization comes increased realism of the experiments, at odds with the repeatability and interpretation of the data. Using this integrated approach, the effects of REEs were assessed following both acute (chapter 2) and chronic (chapter 3) exposure using classical toxicology endpoints (survival, body burden, size).

Acute toxicity testing, using elevated doses of a contaminant over a very short period, allows the opportunity to determine if toxins are accumulated, what effects are apparent and if further investigation is required. I tested the hypothesis that REEs would accumulate in *M. sanguinipes* and would be toxic to exposed individuals. I predicted this accumulation would result in increasing concentrations in the body with increasing dose resulting in reduced survival and biomass.

Often overlooked in toxicity testing are more subtle, sublethal effects that nonetheless affect an organisms ability to thrive in its environment. I tested the hypothesis that prolonged exposure to persistent pollutants would increase accumulation and sublethal toxic effects. I predicted that accumulation would increase with dose and with age and that toxic effects would result in reduced growth, delayed development to adulthood and reduced survival to reproductive maturity and reduced investment in reproduction. Growth is considered to be a sensitive indicator of toxicity for many species and is a common measure in studies of chronic toxicity (Azevedo-Pereira & Soares, 2010). Effects on development, reproduction and lifespan can have broader implications for populations if there are fewer adults to produce the next generation.

Sublethal effects resulting from exposure to toxins may represent trade-offs between development and detoxification. Detoxification of ingested toxins like Ce are assumed to be energetically costly and this may be reflected in changes in energy reserves (Wang et al., 2020). These changes in energy reserves are biomarkers of the sublethal changes that occur following exposure to toxins (Lagadic et al., 1994). As metal intoxication increases, changes may occur in metabolic capacity to accommodate energy requirements for detoxification. Alternately, in some cases, exposed individuals might decrease metabolic activity to limit accumulation of toxins in the tissues. Citrate synthase is a pace making enzyme and the first step of the tricarboxylic acid (TCA) cycle (Wiegand & Remington, 1986). Quantifying CS activity can provide an indication of these shifts in energy demands. In chapter 4, I tested the hypothesis that chronic ingestion of Ce would be energetically costly to detoxify and predicted that this cost would be evident in changes in energy reserves and energy production. Energy stores (lipids, carbohydrates and proteins) and energy production (TCA cycle) were assessed to look for indication of shifts in energy budgets, or a cost of detoxification.

While metabolism is an endogenous source of reactive oxygen species (ROS), metal intoxication can increase production of ROS (Ercal et al., 2001). Metals can donate an electron to molecular oxygen, forming a superoxide anion. This triggers a cascade of reactions that result in the formation of a toxic hydroxyl radical (Ahmad, 1995). In response to ROS, insects rapidly synthesize antioxidant enzymes like catalase (CAT) and biotransformation enzymes like glutathione-s-transferase (GST) to resist oxidative stress from ROS (Ahmad, 1995). The mobilization of antioxidant and biotransformation responses are indicative of an organism increasing efforts to rid themselves of unwanted contaminants.

If not eliminated through antioxidant and biotransformation processes, ROS can damage macromolecules (lipids, proteins, carbohydrates and nucleic acids) causing oxidative stress (Ahmad, 1995), partially accounting for the toxicity of some metals, particularly heavy metals (Ercal et al., 2001). Toxic effects of REEs at the cellular level, measured by lipid peroxidation and loss of redox balance, provides direct evidence of this cellular damage resulting from encountered contaminants. I tested the hypothesis that increasing accumulation of Ce in the body would cause toxic effects at the cellular level in the form of increased reactive oxygen species. I predicted that rising levels of ROS would be evident from increases in the activity of CAT and

GST to rid themselves of unwanted Ce and to prevent oxidative stress. Lastly, I predicted that excess ROS resulting from chronic exposure to Ce would result loss of redox balance, quantified by the ratio of GSH:GSSG, and an increase in cellular damage identified through lipid peroxidation.

Finally, in chapter 5, I bring together the key findings of each chapter and highlight how the information contributes to our understanding of the risks posed by rising levels of REEs in the environment. Terrestrial systems are poorly studied for these contaminants of emerging concern and directions for future research stemming from this thesis are suggested.

Chapter 2 Acute toxicity of neodymium chloride hexahydrate ($NdCl_3·6H_2O$) and cerium chloride heptahydrate ($CeCl_3·7H_2O$) on the generalist herbivore, *Melanoplus sanguinipes* (Fabricius), feeding on contaminated plant tissues

Note: A portion of the data in this chapter was included in the following published article:

Allison, J.E., Boutin, C., Carpenter, D., Ellis, D.M. and Parsons, J.L., 2015. Cerium chloride heptahydrate ($CeCl_3·7H_2O$) induces muscle paralysis in the generalist herbivore, Melanoplus sanguinipes (Fabricius) (Orthoptera: Acrididae), fed contaminated plant tissues. *Chemosphere*, *120*, pp.674-679.

2.1 Abstract

The economic importance of the rare earth elements (REEs) continues to grow with industrial demand for emerging technologies. Pollution from mining and processing activity is expected to rise with this demand. Plants are known to accumulate REEs, although levels vary with species and soil content. However, the effect on wildlife of ingesting REE contaminated vegetation is not well understood. Here I examined the effect of consuming vegetation with elevated levels of either neodymium (Nd) or cerium (Ce) on the generalist grasshopper, *Melanoplus sanguinipes*. Although a substantial amount of ingested contamination was excreted, accumulation of Nd and Ce in the body occurred at all doses. Reduced growth, increased lethargy and paralysis resulted at the highest dose depending on the metal tested. As dose increased, a greater portion of ingested contamination was incorporated into body, indicating the ingestion exceeded the excretion capability of adults. These results were seen after only 4 days of feeding at elevated concentrations. Metals are persistent pollutants and toxic effects may be underestimated by short-term studies. Exposure to REEs over longer periods of the life cycle warrants further investigation using environmentally relevant concentrations. Given these results, Acridids (i.e., grasshoppers) may have potential as biomonitors of metal pollution as they can accumulate metals without immediate death. They also form an important part of healthy ecosystems forming up to 30% of the arthropod biomass

available for consumption. These results suggest they have the potential to accumulate toxins and pass them to other trophic levels.

2.2 Introduction

The rare earth elements (hereafter REEs) are commonly found in the earth's crust and are termed "rare" because they do not concentrate in pure ore deposits (Hu et al., 2006). REE mining activity has increased with increasing demand for these elements, due primarily to their use in emerging technologies. REEs possess unique properties positioning them as ideal materials for use in emerging technologies, particularly those aimed at reducing fossil fuel reliance (e.g., hybrid cars, wind turbines) (Long et al., 2012). In stark contrast to their use in green technologies, extraction of REEs generally results in large amounts of wastewater and waste material.

Of significant economic importance are neodymium (Nd) and cerium (Ce). Natural crustal levels of vary by location and soil type with average estimates at approximately 40 mg kg^{-1} (Nd) and approximately 66 mg kg^{-1} (Ce) (Greenwood & Earnshaw, 1997; Tyler, 2004). Cerium, the most abundant of the REEs, is comparable to levels of copper (68 mg kg^{-1}) and zinc (76 mg kg^{-1}), two of the most widely studied metals (Greenwood & Earnshaw, 1997; Tyler, 2004). Far less attention has been paid to REEs, possibly because they are not considered to be essential to life nor strongly toxic (Tyler, 2004). As a result, threshold limits and maximum permissible concentrations are poorly established in the literature.

Increased use of REEs in emerging technologies may result in elevated environmental levels, as a result of mining and processing for industrial use. For example, concentrations of Ce in polluted soils from industrial locations in The Netherlands run as high as 900 mg kg^{-1} (Slooff et al., 1993). Elevated Ce levels were found up 6 km from a processing plant in China and were correlated with distance from the source (Li et al., 2010). Environmental levels are expected to rise due to mining activity and disposal of technologies that employ Nd. For example, concentrations in polluted soils from industrial locations in The Netherlands run as high as 400 mg kg^{-1}, about ten times higher than background soil levels (Slooff et al., 1993). Elevated soil concentrations due to mine tailings have been found up to approximately 6 km from a processing plant in China with concentration strongly correlated to distance from the source (Li et al., 2010). Detected Nd levels ranged from 5726 mg kg^{-1} at 0.4 km from the source to 88 mg kg^{-1} at 6 km (Li et al., 2010).

Phosphate fertilizers produced from REE rich monazite rocks, may also be causing substantial increases in REE levels, particularly in agricultural areas (Todorovsky et al., 1997; Turra et al., 2011; Volokh et al., 1990). For example, in 1994 in The Netherlands, phosphate fertilizer application released approximately 85 tonnes of Nd into the environment (Sneller et al., 2000).

Little attention has been paid to REEs, likely because they are considered neither essential to life nor acutely toxic (Tyler, 2004). Plants accumulate REEs from contaminated soil (Carpenter & Boutin, 2013; Ichihashi et al., 1992; Thomas et al., 2014). Accumulation is thought to be linked to the similarity in ionic radii between REEs and calcium (Pickard, 1970). As primary ecosystem producers, plants represent a direct entryway for contaminants into the food chain. Herbivores feeding on plant tissues with elevated levels of REEs may likewise accrue these metals in their bodies. For instance, Cowgill (1973) observed uptake of several REEs, including Nd, by waterlilies (*Nymphaea odorata*) and subsequently found these metals in aphids (*Rhopalosiphum nymphaeae* (L.)) feeding on the lilies. Further evidence of accumulation of REEs in insects is lacking. Consumed by other arthropods, amphibians, reptiles, birds and small mammals, herbivorous insects represent a substantial portion of the biomass available in wildlife food webs.

In this experiment, I tested the hypothesis that REEs, like other metals, would accumulate in insects and would be toxic to exposed individuals. I predicted this accumulation would result in increasing concentrations in the body with increasing dose and that toxic effects would result in reduced survival and biomass. The accumulation and toxicity of Nd and Ce was assessed by exposing *Melanoplus sanguinipes* (Fabricius) (Orthoptera: Acrididae) to elevated concentrations over a 4-day exposure period using contaminated leaf material (*Lactuca sativa*). *Melanoplus sanguinipes* is an ideal candidate for study as it is native to Canada, is found throughout the country and feeds on a variety of plants.

2.3 Materials and methods

2.3.1 Neodymium and cerium doses

Neodymium chloride hexahydrate ($NdCl_3 \cdot 6H_2O$, Sigma-Aldrich, Oakville, ON, CA; CAS: 13477-89-9) and cerium chloride heptahydrate ($CeCl_3 \cdot 7H_2O$ - Sigma-Aldrich, Oakville, ON, CA; CAS: 18618-55-8) were selected due to their high solubility in water. The lowest dose was selected to be representative of the highest accumulation of Nd found in shoot material in previous greenhouse studies by Carpenter and Boutin (2013) (i.e., *Raphanus sativus* shoot concentration of 25.5 mg Nd kg^{-1} dry biomass when grown in soils containing 819 mg Nd kg^{-1} dry soil). Using 30 mg Nd L^{-1} dH_2O as the base dose, two additional doses were selected following a geometric progression of 10 (i.e., 30, 300, 3000 mg Nd L^{-1} dH_2O or nominal concentrations). Neodymium comprises approximately 40.2% of the total mass of $NdCl_3 \cdot 6H_2O$ necessitating the use of 75, 750 and 7500 mg of $NdCl_3 \cdot 6H_2O$.

The highest accumulation of Ce found in shoot material in previous greenhouse studies was 39.4 mg kg^{-1}, i.e., *Solanum lycopersicum* growing in a soil concentration of 978 mg Ce kg^{-1} dry soil, (Thomas et al., 2014). The same progression of doses was selected for Ce (30, 300, 3000 mg Ce L^{-1} dH_2O or nominal concentrations). Cerium comprises approximately 37.6% of the total mass of $CeCl_3 \cdot 7H_2O$ necessitating the use of 80, 800 and 8000 mg of $CeCl_3 \cdot 7H_2O$. All results are based on and reported in terms of mg Nd or Ce kg^{-1} dry biomass of *Lactuca sativa* (contaminated food).

A positive control was used to verify both that the exposure method would successfully deliver Nd and Ce into the diet and to verify if effects were caused by the REEs or chloride. Zinc chloride ($ZnCl_2$, Sigma-Aldrich, Oakville, ON) was selected as a positive control for accumulation as zinc is a necessary element for insect development and is readily incorporated into the body. A dose of 300 mg kg^{-1} was selected to match the midlevel exposure of the dose progression.

2.3.2 Rearing

Twelve female and twelve male replicates were used at each dose, including negative and positive controls, except for the highest Nd dose where only nine healthy females were obtained, for a total of 93 insects. Individuals were selected from a laboratory reared colony maintained on a mixed diet of leaf lettuce, fresh cut wheat treated with a sulfonamide antibiotic (as a prophylaxis against fungal infection) and wheat bran. Full details of the colony rearing can be found in appendix C. Several hours after metamorphosis to the adult stage, healthy individuals (all legs, antennae and wings intact) were sexed based on external genitalia and assigned to a feeding treatment. Due to space limitations, treatments were run in cohorts consisting of one to four female and male adults per dose.

2.3.3 No-choice diet

Individuals were weighed to the nearest 0.01 g before random assignment to either one of the doses, a positive or a negative control. At this time, a sampling error was discovered at the highest Nd dose. Both females and males at this dose had a lower initial weight than other doses (ANOVA and Bonferroni post hoc analysis; Females: $F_{4,47} = 6.84$; $p<0.001$; $F_{4,51} = 6.826$; $p < 0.001$ males). Studies of heavy metal body burdens have shown that the relationship between body burden and size is not linear (van Straalen & van Wensem, 1986). Therefore, accumulation should not depend solely on initial biomass of exposed individuals. Although biomass gain is not a linear function of start weight, we retested the highest dose along with an additional set of controls to verify the findings of the first experiment.

Grasshoppers were placed individually in 500 mL glass jars fitted with aluminum screen lids. A 24-hour starvation period preceded the start of the trials. Based on consumption of healthy individuals determined before these experiments, a feeding ration of 2 g of leaf material per individual per day was used. *Lactuca sativa* L. (var. buttercrunch) was grown from seed (OSC, Waterloo, ON, CA, Lot 14-3170) in soil-less potting mix (Pro-mix MPV, Premier Horticulture Ltée, Rivière-du-loup, QC, CA) for 3-4 weeks to reach adequate size. Ce, Nd and Zn solutions were mixed in 1 L batches and dispensed 100 mL daily into individual glass containers. Control doses consisted of 100 mL of deionized water (dH_2O). Each food ration was weighed to the nearest 0.01 g and soaked overnight in a jar containing 100 mL of solution. One additional lettuce leaf was added to each container for chemical analysis. These leaves were dried and used to estimate water loss of leaves in the food ration and dry weight consumption.

Leaves were air-dried, reweighed, and offered to the grasshopper for a 24-hour feeding period. Each day, remaining food and frass was collected, weighed, placed in bags, and oven-dried to constant weight at 70°C. Individuals were fed in this manner daily for four days, after which grasshoppers were weighed to determine final mass and fasted for a 24-hour period to eliminate gut contents. On the final day, remaining frass was weighed, and dried and individual grasshoppers were frozen. Mass gains were compared on a fresh weight basis between doses as a percentage of the initial weight, i.e., the difference between initial and final weight, divided by the initial weight.

2.3.4 Consumption estimates

Using the extra leaves in each aliquot, the average water loss and percentage dry matter was calculated as 9.7% of the food rations. The percentage dry matter was used to calculate the estimated dry matter available for consumption, following Schmidt and Reese (1986). For each grasshopper, the dry weight of ingested food was calculated as:

$$\text{ingested food (g)} = (\text{ration (g)} * \%\ \text{dry matter}) - \text{dry weight remaining food (g)}$$

for the duration of the 4-day feeding period. This value was converted back to fresh weight consumed by dividing by the percentage dry matter content of *L. sativa* leaves.

2.3.5 Visual assessments (toxicity index)

Visual assessments were made daily on the condition of each grasshopper, their reaction when provoked (lethargic or responsive) and mobility (healthy, slow or paralysed). Assessments of individuals were scored using a semi-quantitative scale for sublethal effects adapted from Isman (1985) to create a toxicity index. Scores ranged from 0 to 4, where:

0 = healthy individuals with normal movements

1 = lethargic reactions; reluctance to move, noticeable reduction in locomotion

2 = noticeable paralysis in one or both hind legs, unable to jump but able to right itself

3 = general paralysis or debilitation of limbs

4 = moribund/dead

The toxicity index was calculated at each dose as the mean toxicity index scores for all females or males within a dose.

2.3.6 Chemical analysis

Frozen grasshoppers were thawed for several minutes and dissected under a dissecting microscope to separate the digestive tract from the rest of the body. Briefly, the dorsal side of the grasshopper was cut open and the gut was separated from the body by cuts posterior to the rectum and anterior to the crop.

Once dried to constant weight at approximately 70°C, samples of soaked leaves and insect frass, body and gut tissues, pooled by gender and dose, were sent to Brooks Rand Labs (Seattle, Washington, USA) for analysis of metal content. Samples were hot block digested with nitric acid (HNO_3) and hydrogen peroxide (H_2O_2), then diluted to volume with deionized (DI) water. Digests are then analyzed by inductively coupled plasma - mass spectrometry (ICP-MS) following the US Environmental Protection Agency (USEPA) Method 1638 (US Environmental Protection Agency, 1996). Due to the high cost of chemical analysis, samples had to be pooled by dose resulting in a single measure for concentration at each dose. Variation between individuals could not be assessed. Quality control data for the chemical analysis can be found in appendix A.

2.3.7 Choice diet

Choice feeding trials, to detect avoidance of dosed food, followed similar methodology to the no-choice trials. The third dose, 300 mg Nd kg^{-1} and 300 mg Ce kg^{-1} dry biomass, was selected. Newly emerged adults (10 female and 10 male) were selected and placed in cages separated by sex (i.e., 10 males per cage and 10 females per cage). A 24-hour starvation period preceded the trial. We mixed Nd and Ce solutions in 1 L batches and used dH_2O as a negative control.

The feeding ration was selected to allow 2 g of leaf material per individual per treatment, or 20 g of dosed and 20 g of control leaf material. All lettuce was grown in the same conditions as in our no-choice trials. Rations were weighed to the nearest 0.01 g and soaked overnight in 900 ml of solution. Following the same methodology used in our no-choice trials, two additional lettuce leaves were added to each container to provide an estimate of water loss of leaves in the food ration.

Once removed from the soaking solution, leaves were air-dried and reweighed. Each cage received one ration of dosed food and one of control food for a 24-hour period. Each day, remaining food was weighed, placed in bags, and then oven-dried to constant weight at 70°C. The process was repeated for four days after which to total amount of each type of food consumed was calculated. Each trial was repeated three times to obtain an average estimate of consumption.

2.3.8 Data analysis

Data were analysed using R language environment (v 4.1.0, R Development Core Team, 2021) and RStudio (v1.4.1717). Normality assumptions were evaluated using the Shapiro Wilks test. Homogeneity of residuals was verified by a Levene's test. Inhibition concentrations, indicating 10, 25 or 50% reduction in percent biomass gains as compared to controls were calculated using non-linear regression models, when model assumptions could be met.

Effects on biomass and consumption were compared between doses using ANOVA with Bonferroni post hoc analysis for parametric data or Kruskal-Wallis nonparametric analysis when data failed to meet model assumptions, and this could not be resolved by data transformation. For Kruskal-Wallis analysis, a Dunn's post hoc comparison and holm correction factor was used. For Nd analysis, the means of the control and high doses were compared between datasets 1 and 2. Datasets were combined only if there was no significant difference in control or high dose means between trials.

For Ce exposure data, mass gains were evaluated using ANCOVA with initial biomass as a covariate. Mass gains were also adjusted for consumption using ANCOVA with initial biomass and fresh weight consumption as covariates to determine if effects on biomass occurred pre- or post-ingestion.

Three nutritional indices were compared across doses: the approximate digestibility (AD), the efficiency of conversion of ingested food to biomass (ECI) and the efficiency of conversion of digested food to biomass (ECD). The method of ANCOVA (Raubenheimer & Simpson, 1992) was used to compare efficiencies by dose.

The AD measures the portion of ingested food that is digested. An ANCOVA between the dry weight of frass and dose, using the estimated dry weight consumption as a covariate provides an approximation.

The ECI, sometimes referred to as the gross growth efficiency, estimates the portion of ingested food converted to biomass (Slansky, 1985). An ANCOVA between final biomass and dose, using both initial biomass and fresh weight consumption as covariates was used.

The ECD, sometimes called the net growth efficiency, measures the portion of digested food converted to biomass (metabolic efficiency taking into account food portions used for respiration) (Slansky, 1985). The ECD was approximated using ANCOVA between final biomass and dose using initial biomass and digested mass as covariates. Digested mass was determined as the difference between fresh weight consumption and frass produced. For all ANCOVA, Bonferroni post hoc analysis was used to determine which doses differed significantly from controls.

For Nd data, the sampling error at the highest dose made analysis by ANCOVA unreliable. Instead, relative growth rate and relative consumption rate were calculated using the Waldbauer approach (Waldbauer, 1968) and compared by ANOVA, where:

$$Relative\ Growth\ Rate = Mass\ Gain/(Average\ Mass * Number\ of\ Days)$$

and

$$Relative\ Consumption\ Rate = Ingested\ Food(Average\ Mass * Number\ of\ Days)$$

For the choice feeding experiments, a linear model was used to identify effects of dose on fresh weight consumption.

2.4 Results

2.4.1 Leaf tissue analysis

Neodymium was detected in all analysed samples (Table 2.1). As a result of soaking leaves in contaminated water, Nd content increased above the nominal doses (0, 30, 300, 3000 mg Nd kg^{-1}) to 1.49, 1550, 3150 and 10300 mg Nd kg^{-1} in the first trial and 4.27 and to 10900 mg Nd kg^{-1} dry biomass for the control and high dose in the second trial (Table 2.2). Cerium concentrations were also much higher than nominal concentration (0, 30, 300, 3000 mg Ce kg^{-1}) at 15.9, 2060, 4480 and 13900 mg kg^{-1} dry biomass (Table 2.2).

2.4.2 Initial weight

Initial weight of females and males was compared across doses to ensure an even distribution of larger and smaller individuals. Due to the sampling error with Nd data, we repeated experiments at the highest dose. In the second set of individuals, initial weight did not differ between control and dosed individuals (ANOVA; Females: $F_{1,22} = 0.452$; p = 0.509; Males: $F_{1,22} = 0.011$; p = 0.916). Combining both data sets together did not completely alleviate the sampling error. Log transformed initial weights for females differed between the high and positive control doses (ANOVA; Females: $F_{4,71}=3.58$; p =0.01), while males at the highest dose had lower initial weights than the low, medium and positive control doses but did not differ from negative controls ($F_{4,75}$ =5.32; $p < 0.001$). Initial weight of individuals across Ce doses did not differ from each other (Female: $F_{3,44}=0.17$, p=0.917; Male: F=2.34, p=0.086).

2.4.3 Biomass inhibition concentrations (IC)

The IC10, IC25 and IC50 values were determined for mass gains as a percentage of initial mass for Nd exposed individuals using a log-logistic, 4-parameter model. Calculated IC values ranged from 4211.834 mg Nd kg^{-1} dry biomass (IC10) to 6697.26 mg Nd kg^{-1} dry biomass (IC50) (Table 2.1). Inhibition concentrations were undetectable from the range of cerium concentrations tested.

Table 2.1 Summary of inhibition concentration (IC) values calculated as the neodymium (Nd) dosage resulting in a 10, 25 or 50% reduction in mass gains (%) of adult *Melanoplus sanguinipes* exposed to contaminated food (*Lactuca sativa*) when compared to controls. Values were determined through log-logistic, 4-parameter regression model.

Measure	Log-Logistic
IC10	4211.83 ± 2371.62
IC10 95% CI	-477.58– 8901.25
IC25	5311.10 ± 3024.28
IC25 95% CI	-668.82 – 11291.01
IC50	6697.26 ± 4404.70
IC50 95% CI	-2012.18 – 15406.69

2.4.4 Neodymium relative consumption and relative growth

Relative consumption rate of Nd contaminated food was unaffected by dose for females ($F_{4,71}$=0.15; p=0.964) and males ($F_{4,75}$=3.08; p=0.417). Data transformation did not resolve heteroscedasticity, but the lack of relationship between dose and relative consumption was confirmed by non-parametric Kruskal-Wallis analysis (Females: χ^2=0.72; d.f.=4, p=0.949; Males: χ^2=3.26; d.f.=4, p=0.516).

Relative growth rate was unaffected by dose for females ($F_{4,71}$=1.97; p=0.108; Figure 2.1A), however male growth rate was impacted by Nd dose (χ^2=28.47; d.f.=4; p<0.0001; Figure 2.1B). Post hoc analysis indicated that the high dose had a lower relative growth rate than individuals at the control (p=0.008), low (p<0.001) and medium (p=0.0001) doses.

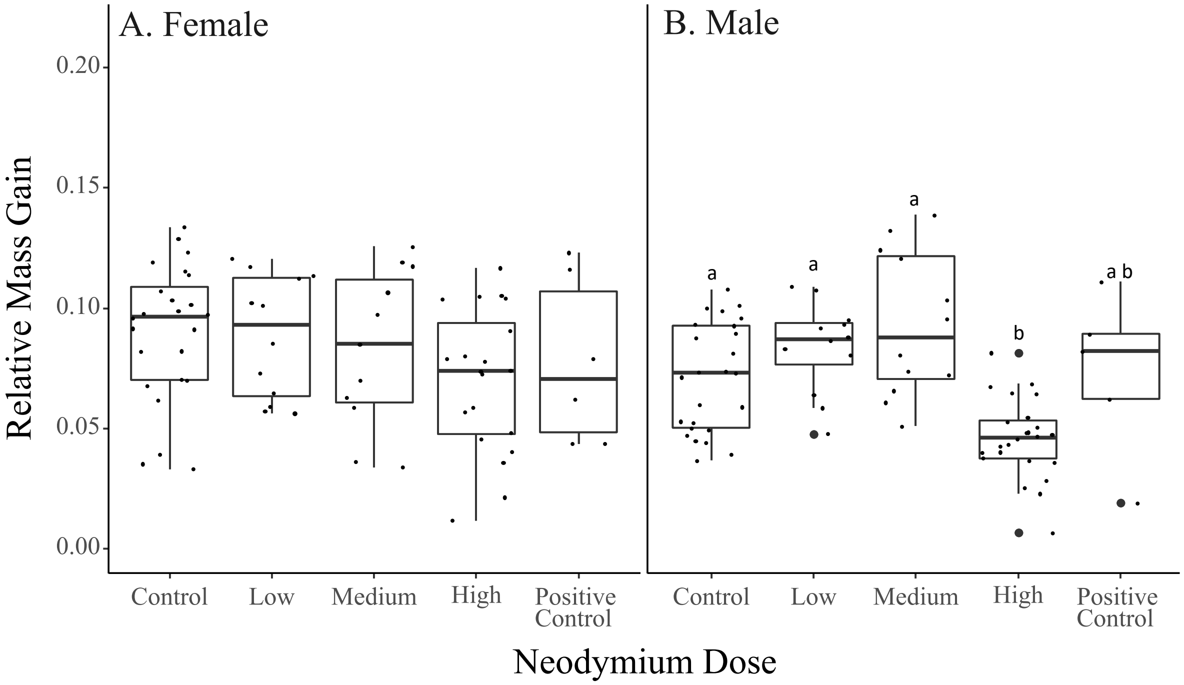

Figure 2.1 Relative mass gain of adult (A) female and (B) male *Melanoplus sanguinipes* exposed to neodymium during a 4-day exposure period. Measured doses in diet (*Lactuca sativa* leaves) (in mg kg^{-1}) were: Control = 1.49, Low = 1550, Medium = 3150, High = 10300, Positive Control (zinc) = 4180. Larger circles represent outliers and letters indicate doses that differ from each other.

2.4.5 Cerium consumption and growth

Consumption was unaffected by Ce dose when adjusted for initial weight (Female: $F_{1,43}$=0.41, p=0.525, Dose: $F_{3,43}$=0.09, p=0.963; Male: $F_{1,43}$=2.06, p=0.159, Dose: $F_{3,43}$=1.18, p= 0.327). Final mass was also unaffected by Ce dose (Female: $F_{3,44}$=0.26, p=0.851; Male: $F_{3,44}$=1.82, p=0.157). When adjusted for initial mass, dose was still not significant, only initial mass was significant (Female: $F_{1,43}$=102.00, p=6.379e^{-13}; Dose: $F_{3,43}$=0.57, p=0.638; Male: $F_{1,43}$=53.02, p=5.043e^{-09}; Dose: $F_{3,43}$=0.50, p=0.683).

Female biomass gains were unaffected by dose ($F_{3,44}$=0.51, p=0.68; $F_{3,44}$=0.53, p=0.667). Dose was still insignificant when female biomass gains were adjusted for initial mass (Initial Mass: $F_{1,43}$=6.71, p=0.013; Dose: $F_{3,43}$=0.57, p=0.638) and adjusted for consumption

(Consumption: $F_{1,43}$=6.87, p=0.012; Dose: $F_{3,43}$=0.482, p=0.696). Initial mass and consumption were the best predictors of mass gain during the experiment.

Likewise, males mass gain was unaffected by dose, when adjusted for initial weight (Initial Mass: $F_{1,43}$=0.006, p=0.940; Dose: $F_{3,43}$=0.50, p=0.683) or adjusted for consumption (Consumption: F=4.54, p=0.039; Dose: $F_{3,43}$=0.42, p=0.742). Interestingly, mass gains were not affected by initial mass for males, only by consumption.

2.4.6 Nutritional indices

2.4.6.1 Approximate digestibility (AD)

Female AD did not change with dose (Consumption: $F_{1,43}$=37.99, p=2.111e^{-07}, Dose: $F_{3,43}$=0.42, p=0.742). On the other hand, dose did influence male AD (Consumption: $F_{1,43}$=91.52, p=3.256e^{-12}; Dose: $F_{3,43}$=2.99, p=0.0414). Post hoc analysis using the estimated marginal means indicates that the high dose had a lower AD compared to the controls (p=0.044).

2.4.6.2 Efficiency of converting ingested food to biomass (ECI)

Female ECI did not differ by dose ($F_{3,42}$=0.55, p=0.651). Both initial weight ($F_{1,42}$=6.19, p=0.017) and consumption ($F_{1,42}$=0.55, p=0.016) were significant. Consistent with earlier results, male ECI was unaffected by dose ($F_{3,42}$=0.42, p=0.741). Initial weight did not predict mass gain ($F_{1,42}$=0.29, p=0.590) while consumption was significant ($F_{1,42}$=4.75, p=0.035).

2.4.6.3 Efficiency of converting digested food to biomass (ECD)

Similarly, ECD did not differ by dose for females ($F_{3,42}$=0.55, p=0.651) or males ($F_{3,42}$=0.43, p=0.735). As anticipated, for females, both the initial weight ($F_{1,42}$=7.16, p=0.011) and the digested mass ($F_{1,42}$=4.22, p=0.046) were significant in explaining mass gain. For males, neither the initial mass ($F_{1,42}$=0.29, p=0.593) nor the digested mass ($F_{1,42}$=3.72, p=0.061) explained mass gain.

2.4.7 Choice feeding

Choice experiments showed no significant effect of sex or dose on consumption of Nd dosed food ($F_{3,8}$=0.87; d.f.=8; p=0.494; Figure 2.2A) nor Ce dosed food ($F_{3,8}$=0.51, d.f.=8, p=0.688; Figure 2.2B).

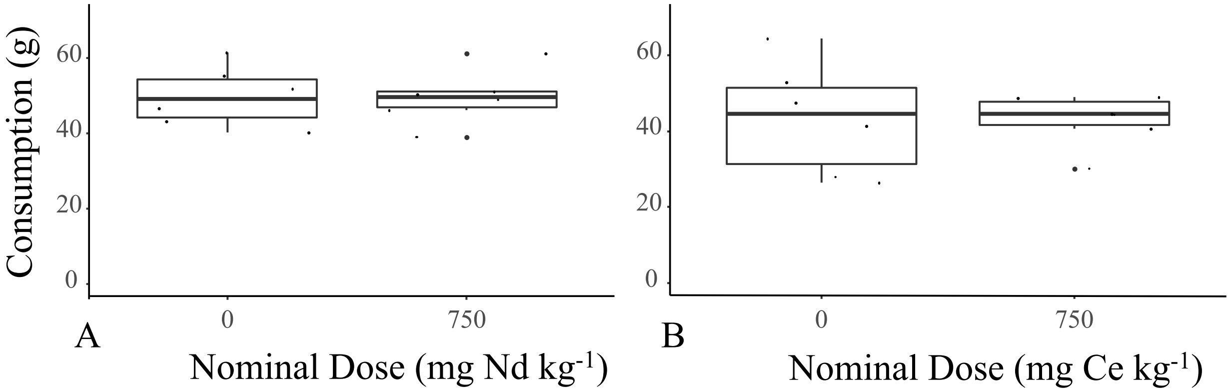

Figure 2.2 Effect of (A) neodymium (Nd) or (B) cerium (Ce) on consumption of adult *Melanoplus sanguinipes* when offered a choice between dosed and control food types**.**

2.4.8 Visual assessments

Visually, adult *M. sanguinipes* exposed to Nd and Zn contamination were more lethargic when provoked than adults fed a control diet. Toxicity index indicates that effects were seen at the medium and high doses for both females and males, with the zinc controls experiencing similar levels of toxicity (Figure 2.3).

In contrast, adults exposed to Ce contamination were not more lethargic than controls. However, calculated toxicity index scores increased with dose starting at the medium dose for males, and the highest dose for females (Figure 2.4). After one day of exposure, one male at the third dose was unable to move his antenna. After three days, two additional males at the highest dose experienced impaired movement in one or both rear legs. The rear legs were held in an extended position behind the body with no bend in the femur/tibia joint. After four days, the rear leg of a female at the highest dose was found in the same extended position. Two additional males at the highest dose were found to have impaired movement of the rear legs and antenna respectively.

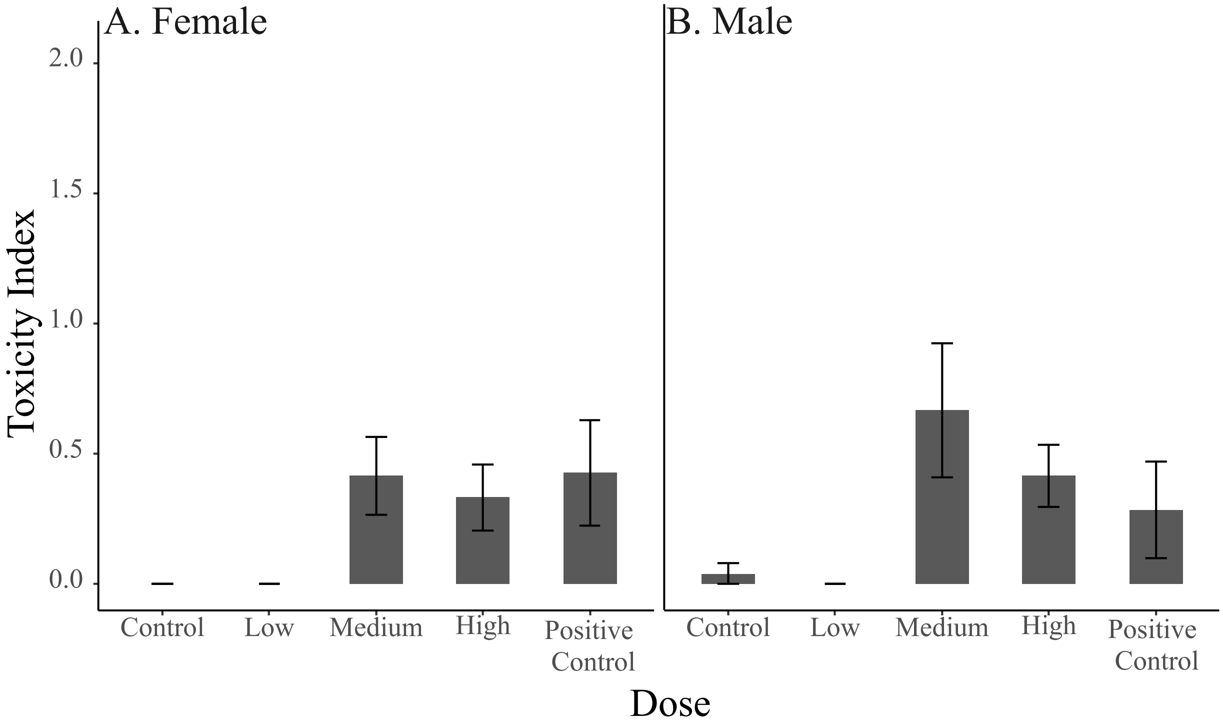

Figure 2.3 Effect of neodymium (Nd) on adult (A) female and (B) male *Melanoplus sanguinipes* after 4 days of exposure to contaminated diet (*Lactuca sativa* leaves). Toxicity scores (see materials and methods for detailed description) represent the mean score (± standard error) at each dose, using a semi-quantitative scale ranging from 0 - 4 where 0 = healthy, 1 = noticeable reduction in locomotion, reluctance to jump, 2 = noticeable paralysis in one or both hind legs, unable to jump but can right itself, 3 = general paralysis or debilitation of limbs, 4 = moribund/dead. Measured doses in diet (in mg kg^{-1}) were: Control = 1.49, Low = 1550, Medium = 3150, High = 10300, Positive Control (zinc) = 4180.

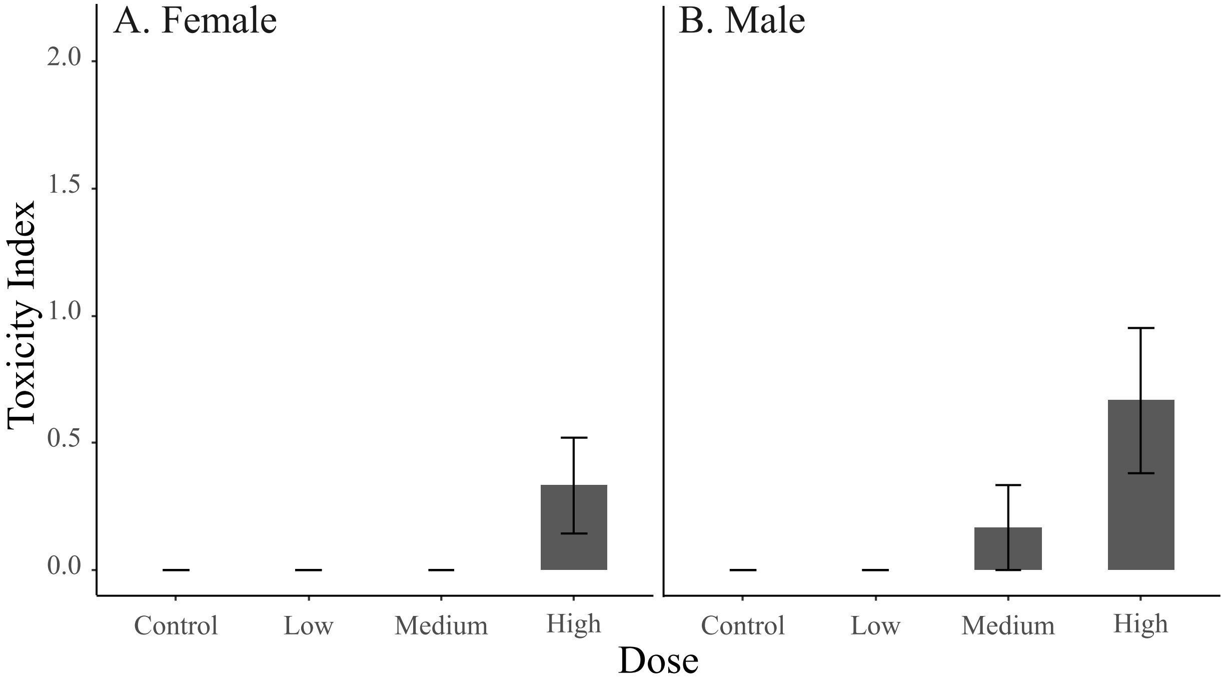

Figure 2.4 Effect of cerium (Ce) on adult (A) female and (B) male *Melanoplus sanguinipes* after 4 days of exposure to contaminated diet (*Lactuca sativa* leaves). Toxicity scores (see materials and methods for detailed description) represent the mean score (± standard error) at each dose, using a semi-quantitative scale ranging from 0 - 4 where 0 = healthy, 1 = noticeable reduction in locomotion, reluctance to jump, 2 = noticeable paralysis in one or both hind legs, unable to jump but can right itself, 3 = general paralysis or debilitation of limbs, 4 = moribund/dead. Measured doses in diet (in mg kg^{-1}) were: Control = 15.9, Low = 2060, Medium = 4480, High = 13900.

2.4.9 Tissue accumulation

Nd accumulated in the body, intestine and frass at all doses tested (Table 2.2). Accumulation was greatest in the frass with concentrations higher than that of the offered food. As expected, zinc accumulation in the body was high compared to the similar Nd dose (3150 mg Nd kg^{-1}), indicating that our dosing process allows for uptake of the metals as intended. At lower doses, the majority of ingested Nd contamination was excreted; however, as dosage increased, the ratio of incorporated versus excreted toxin declines (i.e., frass:body) for both females and males (Table 2.3) indicating that more of the ingested Nd is making its way into the body.

With respect to the ratio of concentration between body tissue and food ration (Table 2.3), the ratio in tissues was highest at the control dose, followed by a sharp decline as dose increased. When considering only the dosed samples, the ratio in intestine as compared to the food ration increased with increasing dose. This indicates that intake and transfer into the gut was proportional to contamination levels, although subsequent transfer to the tissues or elimination from the body varied by dose.

Measured concentrations of Ce in tissues are reported in Table 2.2. As with Nd, accumulation was greatest in the frass, exceeding the food concentration. Although a considerable amount of the ingested contamination was excreted, females and males showed increasing concentration of Ce with dose, indicating accumulation (Table 2.3). When considering only the dosed samples, the ratio of incorporated versus excreted Ce by males (i.e., frass:body) fell as dose increased, indicating that more of the ingested Ce was accumulated in the body, while for females there was some variation by dose.

Table 2.2 Comparison of the nominal and measured concentrations of neodymium (Nd), cerium (Ce) or zinc (Zn) in diet (*Lactuca sativa* leaves. Background levels were measured in control leaves soaked in deionized water (dH_2O). Measured concentrations, including background levels, were used in subsequent analyses of effects.

Analyte	**Dose**	**Nominal Dose (mg L^{-1} dH_2O)**	**Measured Dose (mg kg^{-1} dry biomass)**	**Measured Dose (background adjusted) (mg kg^{-1} dry biomass)**	**MDL***	**MRL****
Nd	Negative Control	0	1.49	0	0.006	0.02
Nd (Exp 2)	Negative Control	0	4.27	0	0.08	0.2
Nd	Low	30	1550	1548.51	0.6	1.59
Nd	Medium	300	3150	3148.51	0.57	1.53
Nd	High	3000	10300	10298.51	1.16	3.1
Nd. (Exp 2)	High	3000	10900	10895.73	1.24	3.3
Ce	Control	0	15.9	0	0.006	0.02
Ce	Low	30	2060	2044.1	0.61	2.04
Ce	Medium	300	4480	4464.1	0.57	1.89
Ce	High	3000	13900	13884.1	2.93	9.77
Zn	Positive Control	300	4180	4178.51	4.13	20.7

*MDL=method detection limit

**MRL= method reporting limit

Table 2.3 Concentration ratios of neodymium (Nd), cerium (Ce) and zinc (Zn) accumulation in the frass and tissues (body and intestine) and ratio of excreted and incorporated metal in the body and intestines of female and male *Melanoplus sanguinipes* adults exposed to contaminated food (*Lactuca sativa* leaves). Numbers represent analyses of pooled tissue samples corresponding to the sex/tissue/dose combination.

Sex	**Dose**	**Analyte**	**Concentration Ratios**					
			Body: Food	**Intestine: Food**	**Frass: Food**	**Frass: Body**	**Frass: Intestine**	**Intestine: Body**
Female	Control	Nd	0.15	0.10	1.00	6.77	9.93	0.68
	Control	Nd (Exp. 2)	0.17	10.42	1.68	9.68	0.16	60.14
	Low	Nd	0.002	0.02	2.15	1110.00	130.08	8.53
	Medium	Nd	0.003	0.03	2.86	835.19	104.16	8.02
	High	Nd	0.005	0.04	2.36	518.10	56.25	9.21
	High	Nd (Exp. 2)	0.006	0.04	3.37	545.32	95.08	5.74
	Positive Control	Zn	0.08	0.19	1.52	19.51	8.22	2.37
Male	Control	Nd	0.28	0.26	251.68	892.86	961.54	0.93
	Control	Nd (Exp. 2)	0.18	0.33	3.35	18.82	10.07	1.87
	Low	Nd	0.002	0.09	2.38	1281.25	251.02	5.10
	Medium	Nd	0.004	0.04	2.94	676.64	82.77	8.18
	High	Nd	0.004	0.07	2.54	567.10	37.97	14.94
	High	Nd (Exp. 2)	0.007	0.08	2.17	316.78	26.61	11.91
	Positive Control	Zn	0.07	0.19	1.56	21.85	8.31	2.63
Female	Control	Ce	0.014	2.044	0.981	70.91	0.48	147.73
	Low	Ce	0.002	0.005	2.583	1418.67	497.20	2.85
	Medium	Ce	0.005	0.007	2.723	516.95	405.32	1.28
	High	Ce	0.003	0.016	2.928	900.44	187.56	4.80
Male	Control	Ce	0.013	0.098	8.742	661.90	89.10	7.43
	Low	Ce	0.001	0.010	2.791	2712.26	269.95	10.05
	Medium	Ce	0.002	0.008	2.857	1643.13	341.33	4.81
	High	Ce	0.004	0.077	2.698	735.29	35.05	20.98

2.5 Discussion

Although REEs are known to accumulate in plants (Carpenter & Boutin, 2013; Thomas et al., 2014; Tyler & Olsson, 2005), little is known about the effect on herbivores feeding on contaminated plant tissues. In this study, the toxicity of Nd and Ce to herbivorous insects when exposed to contaminated vegetation was assessed using the generalist grasshopper *M. sanguinipes* as a model species. Acridids (i.e., grasshoppers) make ideal environmental indicators as they are generally intolerant of pesticide and heavy metal pollution (Schmidt, 1986). They are known to form a substantial portion (i.e., 20-30%) of arthropod biomass in ecosystems with minimal chemical inputs (Schmidt, 1986). Abundance and high individual weight make them ideal prey for birds, small mammals, reptiles and amphibians (Schmidt, 1986).

2.5.1 Inhibition concentrations

High concentrations of Nd were required to reduce insect biomass gains by 50% as indicated by IC50 values. Females were more sensitive than males in terms of subtle toxic effects (IC10 and IC25). IC values could not be calculated for Ce based on the range of concentrations tested. Although more baseline data from contaminated sites is required, it is likely there would be few acute effects arising from short term exposure to either Nd or Ce.

These results are consistent with findings regarding heavy metal pollution. Acute toxicity of metals is generally low. More often, physiological injury caused by toxic metals result in decreased growth or reproduction (Posthuma & Van Straalen, 1993). Along with prolonged nymphal development and shortened adult life span, Schmidt et al. (1992) found that adults of *Aiolopus thalassinus* (Saltatoria: Acrididae) had reduced fresh weight when exposed to mercury and lead. Exposure to cadmium also caused reductions in female biomass. In *Locusta migratoria*, cadmium and mercury poisoning have been linked to injury to fat bodies and reduced energy reserves (Martoja et al., 1983). Fat body injuries were also seen in German cockroaches (*Blattela germanica*) exposed to cadmium, mercury and chromium (Zhang et al., 2001).

2.5.2 Consumption

In this study, both relative consumption rate of Nd food and consumptions of Ce food (adjusted for initial weight) were unaffected by dose. This should be interpreted with care. Estimates of consumption are subject to error, which can be magnified when consumption is less than 80% of available food (Schmidt & Reese, 1986). In this study, insects were provided a substantial ration to feed *ad libitum* and as such may be subject to a higher degree of error.

2.5.3 Growth

Relative growth rate was unaffected by Nd dose for females, however male relative growth rate was reduced at the highest dose when compared to control individuals as well as males at the low and medium doses. By contrast, Ce final mass was unaffected by dose even when adjusting for initial mass. Mass gain during the experiment was unaffected by dose when adjusted for initial mass and consumption. For females, initial mass and consumption were the best predictors of mass gain. For males, mass gain was not influenced by initial mass only by consumption during the experiment.

2.5.4 Choice experiments

Comparisons of mass gains both unadjusted by consumption and adjusted for consumption can highlight differences between avoidance of contaminated food and toxicity following ingestion (Horton & Redak, 1993). We followed up with a choice experiment and found that dosed food was consumed as readily as contaminated food. There was no indication that adults were deterred from consuming either Nd or Ce contaminated food consistent with our earlier results and further suggesting that effects on biomass occurred post-ingestion.

2.5.5 Nutritional indices

For Ce, the efficiencies of converting ingested and digested food to biomass (i.e., ECI and ECD) were compared. Both were unaffected by dose for females and males indicating no pre or post ingestion toxic effects. However, at the highest dose, there was a drop in the approximate digestibility for males when compared to controls, suggesting less of the ingested food was digested.

2.5.6 Visual assessment

Visually, as dose increased, adults exposed to Nd showed increased lethargy and reluctance to move when provoked. Subtle toxic effects on behaviour and locomotion are not often included in short-term studies but might be more sensitive measures of toxicity at lower doses. Studies of aquatic organisms have found locomotion to be a consistently sensitive measure of toxic stress due to environmental contaminants (Little & Finger, 1990). For Ce treated individuals, the calculated toxicity index increased with dose. Visual assessments during the experiment showed paralysis, predominantly in males, at the two highest doses. Reduced locomotion may affect weight in the long-term due to reduced ability to forage. Paralysed individuals may also find themselves more susceptible to predation, passing toxins to other trophic levels. Similar paralysis was only observed in one individual exposed to Nd and none of the Zn treated individuals, suggesting effects are attributable to the ingested metal and not chloride.

The paralysis may be a result of ingested Ce blocking calcium channels, as has been found with other REEs. Lanthanum is a strong calcium channel blocker due to its similar ionic radius and higher valence (3^+) (Craig et al., 1999). Blocking calcium uptake can lead to muscle membrane depolarization, resulting in sustained muscle contraction or tetanic paralysis. This effect has been observed in cockroach (*Periplaneta americana*) leg muscles (Washio & Miyamoto, 1983), frog (*Rana temporaria*) Sartorius muscle fibres (Glavinović et al., 1989) and in snake (*Thamnophis sp.*) muscle fibres (Coniglio et al., 1993) treated with lanthanum. Evidence from the literature suggests the condition is not reversible once exposure ceases (Coniglio et al., 1993). Increasing environmental concentrations of Ce may increase competition for calcium binding sites in exposed organisms.

The visual assessments made here were limited to lethargic reactions when provoked and obvious paralysis of limbs. In a more detailed study of impaired locomotion, Bayley et al. (1995) found reductions in distance travelled by adult female *Pterostichus cupreus* (Coleoptera: Carabidae) exposed to elevated levels of copper. Females were active for 51% as much time and moved at 71% the speed of controls (Bayley et al., 1995). Studies of aquatic organisms have found locomotion to be a consistently sensitive measure of toxic stress due to environmental contaminants (Little & Finger, 1990). Detailed studies linking altered locomotion to toxic stress in terrestrial invertebrates are lacking. This link may be important to distinguish actual toxic effects of contaminants, such as metals, from alterations in behaviour (e.g., consumption deterrence).

2.5.7 Tissue accumulation

Adults were able to excrete the majority of ingested Nd and Ce, with frass concentrations increasing with dose for both elements. The elimination process is an essential route for terrestrial invertebrates, including many insects (Dallinger, 1993). Unwanted metals ingested by terrestrial arthropods can be either assimilated and detoxified or eliminated. During the elimination process, toxic metals are stored in vesicles of the digestive cells which are then moved to the lumen of the gut or excretory system and eliminated in solid waste (Dallinger, 1993). For example, in addition to high assimilation rates, carabid beetles can efficiently excrete metal-containing vesicles (Janssen et al., 1991).

Despite the level of excretion of Nd and Ce, intestine and body concentrations increased with dose. The influence of accumulation may not be as important as the influence of metabolically active Nd or Ce in the toxic effects we observed (e.g., reductions in mass gain, paralysis). Regardless of current tissue concentration, toxic effects occur once the rate of uptake exceeds the rate of excretion and/or detoxification long enough for the metabolically available concentration to exceed the critical threshold of the study organism (Rainbow, 2007). The accumulation seen in our results suggests that the uptake rate of both Nd and Ce exceeded that of excretion. As dose increased, the ratio of excreted to incorporated (i.e., frass:body) Nd declined, suggesting incorporation of a higher proportion of ingested Nd into the body. A similar pattern was seen in males exposed to Ce dosed food, while female ratios were variable.

2.6 Conclusion

The doses used in this study were higher than concentrations reported from plants growing at natural sites, however, there is currently little information regarding plant concentrations at contaminated sites near mining/processing activity. Evaluating acute toxicity at high doses can be useful to identify contaminants that merit further investigation. In this study, toxic effects were most evident at the highest dose, but accumulation in the body tissues occurred at all doses.

The current experiment was limited to four days of feeding. Although acute toxicity of metals is generally low, they are persistent and short-term studies might underestimate toxic effects and increased mortality. For example, Laskowski (2001) showed a gradual increase in mortality in pea aphids (*Acyrthosiphon pisum*) from cadmium exposure over a 22-day period that would have gone unnoticed in short-term studies. Although toxic effects were not detectable at all doses during the four-day feeding trial, it is possible that exceeding the excretion rate over longer-term exposure periods would yield the same toxic effects seen at higher doses. Further investigation over the life cycle of exposed herbivores using environmentally relevant concentrations might shed more light on the levels of REEs in consumed food that cause toxic effects.

Chapter 3 Whole organism effects of chronic exposure to cerium or neodymium over the life cycle of a terrestrial invertebrate, *Melanoplus sanguinipes*.

3.1 Abstract

Rare earth elements (REEs) are contaminants of emerging concern. Pollution levels continue to rise worldwide as demand for these valuable elements increases. Plants are known to accumulate REEs, but little is known about the fate of REEs in biota consuming contaminated plants, especially in terrestrial ecosystems where pollution originates. Invertebrate herbivores have a dual role in terrestrial ecosystems as both primary consumers and as prey for consumption by birds, mammals, amphibians, and other invertebrates. In this study, I examined the fate and effect of REEs on terrestrial invertebrates using *Melanoplus sanguinipes* as a model species. In separate experiments, grasshoppers were exposed to cerium (Ce) or neodymium (Nd) from egg hatch through their entire life cycle until death. Measures of accumulation, elimination, survival, mass, and development times were compared between doses and contrasted between REEs. The duration of development to the adult stage, number of developmental instars, sex ratio and adult longevity were followed. For Ce exposed females, the number of emerged offspring from contaminated substrate and the number of eggs developed at adult maturity were counted. Cerium was accumulated in the body at higher levels than Nd. Survival was negatively impacted for Ce exposed individuals. Females and males carried body concentration of Ce and Nd. We also found that males experienced reduced adult mass, longer development times to reach the adult stage and shorter adult lifespans. Contaminated substrate did not affect nymphal incubation period or emergence. Exposed females did not have reduced numbers of eggs in their abdomens, although the viability and accumulation of Ce in the eggs is unknown. Consumption of Ce not only has consequences for the growth and survival of terrestrial invertebrates, the high concentrations of both Ce and Nd in exposed individuals may have implications for other species ingesting *M. sanguinipes* as prey.

3.2 Introduction

Although termed “rare”, REEs are abundant throughout the Earth’s crust although not in the large deposits characteristic of other metals (Hu et al., 2006). While China is the world’s largest producer of REEs, following recent export reductions, Canada is set to increase its mining activity of these valuable metals (Long et al., 2012). In tandem, demand for REEs continues to rise for use in emerging technologies, particularly those aimed at reducing reliance on fossil fuels (e.g., hybrid cars and wind turbines). In stark contrast to their use in green technologies, extraction of REEs produces large amounts of wastewater and material.

Of significant economic importance are neodymium (Nd) (atomic number 60, atomic mass 144.24 g mol^{-1}) and the most abundant of the REEs, cerium (Ce) (atomic number 58, atomic mass 140.12 g mol^{-1}). Nd is used to create neodymium-iron-boron permanent magnets which are important for numerous technologies including MRI machines (Stone, 2009), hybrid-electric vehicles and wind turbines (Chu, 2010). Ce is commonly used in catalytic converters and contributes to the manufacture of magnets and electrodes (Chu, 2010; Sabiha-Javied et al., 2010). Increased mining activity to meet the demand for emerging technologies may result in elevated environmental levels, due in part to mining waste and in part to landfills holding discarded REE containing technologies.

Elevated levels of REEs from direct mining and processing have been discovered adjacent to facilities in China. Soil concentrations in excess of 1000 mg kg^{-1} (for Nd) have been documented up to one kilometer from the source (Li et al., 2010; Liang et al., 2014). This pollution may not be limited to the areas immediately surrounding processing plants. Elevated soil concentrations of REEs due to mine tailings have been found up to approximately 6 km from a processing plant in China, with concentration strongly correlated to distance from the source (Li et al., 2010). Detected Nd levels ranged from 5726 mg kg^{-1} at 0.4 km from the source to 88 mg kg^{-1} at 6 km. Cerium levels were even higher at 12170 mg kg^{-1} at 0.4 km from the source and only dropped to 205 mg kg^{-1} at 6 km (Li et al., 2010). Concentrations in polluted soils from industrial locations in The Netherlands run as high as 400 mg kg^{-1} for Nd and 900 mg kg^{-1} for Ce, about ten times higher than the background soil levels of 40 and 80 mg kg^{-1} respectively (Slooff et al., 1993). Oil-refining activity in the U.S. also results in the release of REEs to the environment, reported at 60-80 tonnes per day (Slooff et al., 1993). As REE containing technologies are discarded, levels of contamination are expected to rise around landfill sites. The full impact of this source of pollution for local environments remains unknown.

In addition to direct mining and industrial pollution, phosphate fertilizer production and application is increasing levels of all REEs in the environment. These fertilizers are commonly applied on the Canadian prairies, with 1990 levels at approximately 775 000 tons (IPNI, 1999). Release of REEs, including Nd and Ce, following fertilizer application has been reported. In 1994 in the Netherlands, roughly 85 tonnes of Nd was released into the environment from phosphate fertilizer production (Sneller et al., 2000). The application of phosphate fertilizers may already be causing substantial increases in REEs, including Nd and Ce, especially in agricultural areas (Todorovsky et al., 1997; Turra et al., 2011; Volokh et al., 1990).

Plants are known to accumulate REEs, including Nd and Ce, in their root and shoot tissue through contaminated soils (Carpenter & Boutin, 2013; Thomas et al., 2014). Species feeding directly on plant tissues with elevated levels of REEs may likewise accrue these metals in their body. In previous acute studies with Nd and Ce, accumulation in the body of *Melanoplus sanguinipes* was correlated with increasing dose (Chapter 2).

Studies to assess the long-term consequences of REE ingestion on terrestrial species are rare. While acute toxicity may be low, metals are persistent pollutants and mortality and sublethal toxic effects may be underestimated by sort-term studies. For example, a gradual increase in pea aphid (*Acyrthosiphon pisum*) mortality was seen after 22 days of exposure to cadmium, a result that would have gone unnoticed in short-term studies (Laskowski, 2001). Often, exposure to toxic metals causes decreased growth or reproduction (Posthuma & Van Straalen, 1993).

In this study, I seek to understand the effect of chronic exposure to Ce and Nd at the whole organism level using *Melanoplus sanguinipes* (Fabricius) (Orthoptera: Acrididae) as a model species. This rangeland grasshopper is the most commonly known species in North America (Johnson, 2002). Acridids (i.e., grasshoppers) while generally intolerant of metal pollution, often survive contamination (Schmidt, 1986). They form a significant part of the diet of birds, small mammals, reptiles, amphibians and spiders because of their abundance and high individual weight, in some cases forming 20-30% of the arthropod biomass available (Schmidt, 1986).

In this experiment, I tested the hypothesis that prolonged exposure to persistent pollutants increases accumulation and sublethal toxic effects. I predicted that accumulation would increase with dose and with age and that toxic effects would result in reduced growth, delayed development to adulthood and reduced survival to reproductive maturity. I further predicted that active detoxification of ingested REEs would leave less energy for investment in reproduction resulting in fewer eggs produced by reproductively mature females.

Using a captive rearing approach, freshly emerged *M. sanguinipes* nymphs were exposed to Ce or Nd throughout their life cycle, both in their diet and via sand where egg pods were laid by adults and through the 5-6 instars and adult stage until mortality. Measures of accumulation of REEs, survival, growth and development times were compared between doses and contrasted for the two REEs. For Ce, the impact of contaminated oviposition substrate on nymph emergence and incubation period was compared between doses, and the number of eggs in the abdomen of exposed females was counted at reproductive maturity. Along with our acute exposure results, this study will add to our understanding of the risk rising environmental levels of REEs might pose to the growth, survival, and reproduction of terrestrial invertebrates.

3.3 Materials and methods

3.3.1 Selected rare earth elements and doses

Individual *M. sanguinipes* were harvested from a colony maintained on a 16:8 day:night cycle with an average temperature of 30.8±0.26°C. Insects are reared on a mixed diet of leaf lettuce, fresh cut wheat and a mixture oats (80%), bran (15%) and skim milk powder (5%). Full rearing protocol can be found in appendix C.

Ce and Nd were selected for the chronic exposure study. The chloride forms of both elements, cerium chloride heptahydrate ($CeCl_3 \cdot 7H_2O$ – Sigma-Aldrich, Oakville, ON, CA; CAS: 18618-55-8) and neodymium chloride hexahydrate ($NdCl_3 \cdot 6H_2O$, Sigma-Aldrich, Oakville, ON, CA; CAS: 13477-89-9) were used due to their high solubility in water.

The same dose progression was used for oviposition substrate and diet. Doses were selected to be representative of accumulation found in studies with plants growing in contaminated soil, 39.4 mg kg^{-1} in *Solanum lycopersicum* for Ce (Thomas et al., 2014) and 25.5 mg Nd kg^{-1} in *Raphanus sativus* (Carpenter & Boutin, 2013). Resulting dose progressions were 40, 160, 640 mg Ce kg^{-1} and 30, 150, 750 mg Nd kg^{-1}.

3.3.2 Oviposition substrate

Newly emerged, healthy adults (all limbs, antennae, and wings intact) were paired within 48-hours of emergence and assigned to either Ce contaminated, Nd contaminated or control substrate. Each pair was provided with control diet and cotton soaked in dH_2O for hydration. Each week for 4 weeks, adults were provided with a specimen cup filled with 100 mL of commercial playground sand for oviposition substrate.

Substrate was contaminated with either Ce or Nd 24-hours prior to exposure to allow the contaminant to settle in the substrate. The nominal concentrations in the sand were 40, 160, 640 mg Ce kg^{-1} and 30, 150, 750 mg Nd kg^{-1}. Every 2-3 days, the substrate was moistened with up to 5 ml dH_2O to prevent any developing eggs from dehydrating. Each week, the specimen cup was removed and replaced with a new cup containing either control or contaminated substrate. Once removed from the rearing arena, cups were covered with a specimen cup lid and incubated for up to 21 days at 30°C until egg hatch.

Cups were checked every 2-3 days and hydrated with 5-10 ml of dH_2O to prevent dehydration. Once the first nymph emergence was observed, the number of emerging nymphs was counted daily until no further emergence was recorded for 3 days. Cups were incubated for a period of 28 days before it was considered to have produced no offspring. A sample of sand from each cup was taken just after dosing and a sample at the end of the 28 days to account for any settling of the REEs that may have occurred during the exposure period. Sample of sand from each time period were pooled by dose and sent for chemical analysis. The average concentration of the sand from the pre- and post-exposure period is reported.

3.3.3 Oral dose exposure

Hatchlings were collected and fresh weight was recorded to the nearest 0.001 g within 24-hours of emergence. Nymphs were reared individually in 100 ml specimen cups fitted with a lid vented using galvanized aluminum insect screening to allow air flow. Each container was fitted with a 2.0 ml microcentrifuge tube filled with water and capped with a shortened piece of dental cotton, a short stick to act as a perch for moulting and a ration of either contaminated or control diet.

Nymphs were reared from egg hatch until death to monitor growth, development and survival at each REE dose. Three times per week, individuals were checked for overall health and development and containers were replenished with fresh food and water. The diet was made from commercial guinea pig food pellets (Hagen Living World Extrusion Diet, Rolf C. Hagen Inc., Montreal, Quebec) consisting of 15.0% crude protein, 3.0% crude fat and 14% crude fiber. This commercial formulation was selected based on its similarity to synthetic diets used for rearing of *M. sanguinipes* in previous research by Ward et al. (1983). The guinea pig diet is sold in pellet form which is too hard for the mandibles of *M. sanguinipes*, a leaf chewing insect. Palatability was increased by grinding pellets to a fine grind using an electric coffee grinder and mixing the ground pellets with an agar solution to a ratio of 3% (w/v) based on the formulation used by Ward et al. (1983). For dosed diets, the desired weight of REE was dissolved in the agar just prior to mixing with guinea pig food. Final dose progressions in the sand were 40, 160, 640 mg Ce kg^{-1} and 30, 150, 750 mg Nd kg^{-1}. Full details of the diet preparation can be found in appendix B.

Shed exoskeletons indicating a moult had occurred, were collected, and pooled by dose for chemical analysis of REE content. The date was recorded to assess development times, frass was collected, and the individual was weighed to determine fresh weight at the beginning of the life stage. Collected frass was dried to constant weight and sent for chemical analysis of REE content pooled by life stage. Once an individual had reached the adult stage, sex was recorded, and they were fed three times weekly and frass was collected for chemical analysis. When an individual died, the life stage and date of death were recorded.

A subset of adults (12 females per dose) exposed to cerium were dissected at reproductive maturity (14 days post emergence to the adult stage). The number of eggs in the abdominal cavity was counted an indicator of reproductive potential.

3.3.4 Chemical analysis

Analysis was conducted by Brooks Applied Labs (Bothell, WA, USA). Samples were hot block digested with nitric acid (HNO_3) and hydrogen peroxide (H_2O_2), then diluted to volume with DI water. Digests were then analyzed by inductively coupled plasma mass spectrometry (ICP-MS) following the US Environmental Protection Agency (USEPA) Method 1638 (US Environmental Protection Agency, 1996). Due to the high cost of chemical analysis, samples had to be pooled by dose resulting in a single measure for concentration at each dose. Variation between individuals could not be assessed. Full chemical analysis results and quality control data for the chemical analysis can be found in appendices D and E.

3.3.5 Data analysis

Data were analysed using R language environment (v 4.1.0, R Development Core Team, 2021) and RStudio (v1.4.1717). Normality assumptions were evaluated using the Shapiro Wilks test and homogeneity of residuals assumptions were verified by the Levene test.

The effects of Nd or Ce dose on the mass, development time, adult longevity, and reproductive endpoints (number of emerged offspring, incubation period, number of eggs) were evaluated using ANOVA with Dunnett post hoc to determine which doses differed from controls when model assumptions could be met. The development time from egg hatch to adult was inverse transformed to resolve normality of residuals for both females and males exposed to Ce. The incubation period of eggs in Ce contaminated sand was also inverse transformed to resolve normality of residuals. A Kruskal-Wallis non-parametric analysis with Dunn post hoc test was used where violations of normality or homogeneity of residuals could not be resolved with data transformation. For Nd, female development time, lifespan and the number of developmental instars were analysed using Kruskal-Wallis test. For Ce, male lifespan and the number of eggs developed by exposed females were evaluated with a Kruskal-Wallis test. Binomial logistic regression was used to evaluate the number of developmental instars used to reach adulthood (5 or 6) and the sex ratio of emerged adults. Overall survival was evaluated with a non-parametric Kaplan-Meier estimate of the survival probability over time.

3.4 Results

3.4.1 Accumulation and elimination

3.4.1.1 Oviposition substrate and diet

Ce and Nd were recovered from all oviposition substrate samples. Background levels in control substrate was 48.5.30 mg Ce kg^{-1} and 17.15 mg Nd kg^{-1}. Concentration of Ce and Nd increased corresponding to increased dose and were generally higher than the expected dose. The final dose progressions were Ce: 48.5, 114.6, 321.0, 865.0 mg kg^{-1} and Nd: 17.2, 79.0, 260.5, 1084.5 mg kg^{-1}.

Likewise, Ce and Nd were recovered from all diet samples. Background concentrations in control diet were low at 0.41 mg Ce kg^{-1} dry biomass and 1.17 mg Nd kg^{-1} dry biomass. The final dose progressions in the diet were Ce: 0.41, 37.3, 202, 894 mg kg^{-1} and Nd: 1.17, 45.6, 239, 720 mg kg^{-1}. Full chemical analysis results can be found in Appendix D and E.

3.4.1.2 Grasshopper accumulation

Melanoplus sanguinipes had Ce in their bodies at all life stages tested and concentration increased with dose at each stage when compared to controls (Figure 3.1). Adult females showed the highest concentration of Ce, over 600 times that of the control females. Late juvenile instars

(instars 4-6) by contrast show lower concentrations of cerium than earlier life stages (instars 1-3) (Figure 3.1A). For Nd, chemical analysis was limited to adult stages, which showed accumulation of Nd at all doses (Figure 3.1B). Adult males showed the highest concentration of Nd. The concentration in males at the highest dose was over 600 times that of control males (Figure 3.1B). At all stages, body concentrations were lower than the dosed food for both Ce and Nd (Figure 3.1 A&B).

Shed exoskeletons contained increasing concentrations of Ce and Nd with increasing dose. The measured concentration in exoskeletons were 4.54, 7.5, 14.7 and 23.2 mg kg^{-1} for Ce at the control, low, medium and high doses respectively. There was only enough tissue to measure Nd concentrations at the control, medium and high doses which were 1.8, 16.3 and 42.9 mg kg^{-1} respectively. This represents a 5-fold increase in Ce concentration at the highest dose over the controls and a 23-fold increase for Nd. Full chemical analysis results can be found in Appendix D and E.

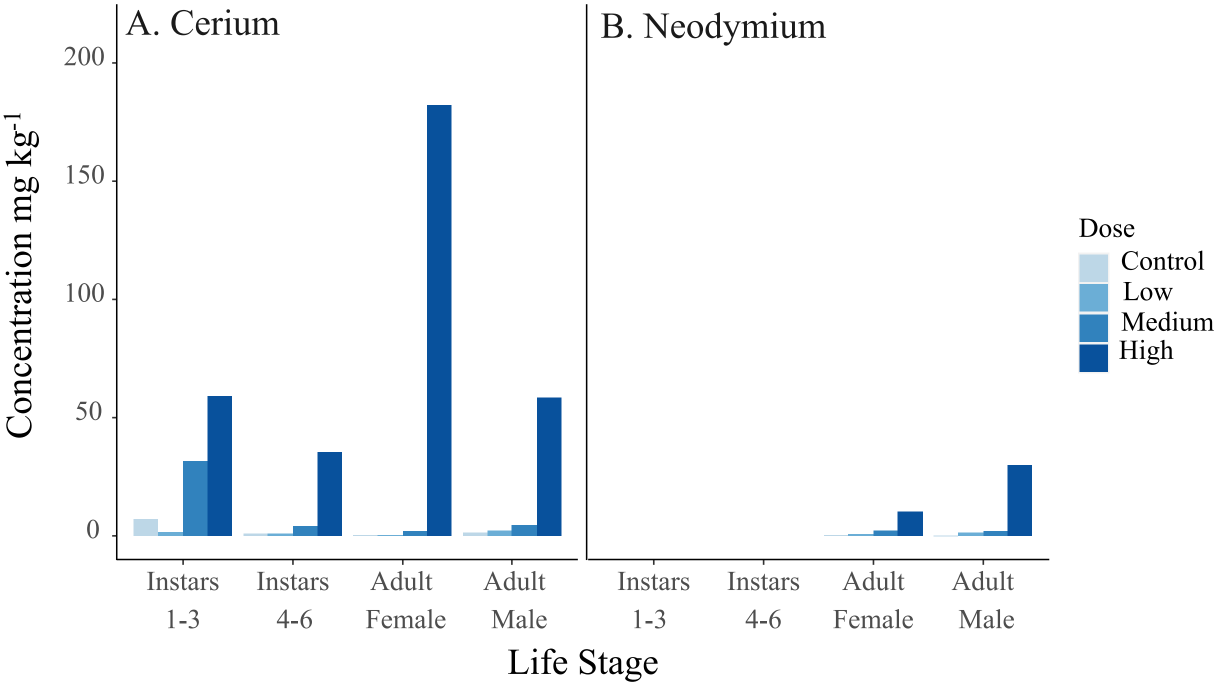

Figure 3.1 Measured concentration of (A) cerium (Ce) and (B) neodymium (Nd) accumulated in whole bodies of *Melanoplus sanguinipes* exposed to contaminated food from egg hatch until death. Numbers represent analysis of pooled tissue samples for early juvenile (instars 1-3), late juvenile (instars 4-6) and adult the life stages at each tested concentration. Measured Ce doses (mg kg^{-1}): Control = 0.41, Low = 37.3, Medium = 202.0, High = 894.0; Measured Nd doses (mg kg^{-1}): Control = 1.17, Low = 45.6, Medium = 239.0, High = 720.0.

3.4.1.3 Grasshopper elimination

Ce and Nd concentration in the frass was greater than that in the body or shed exoskeletons. Concentrations increased with increasing dose for both REEs at all life stages tested (Figure 3.2 A & B).

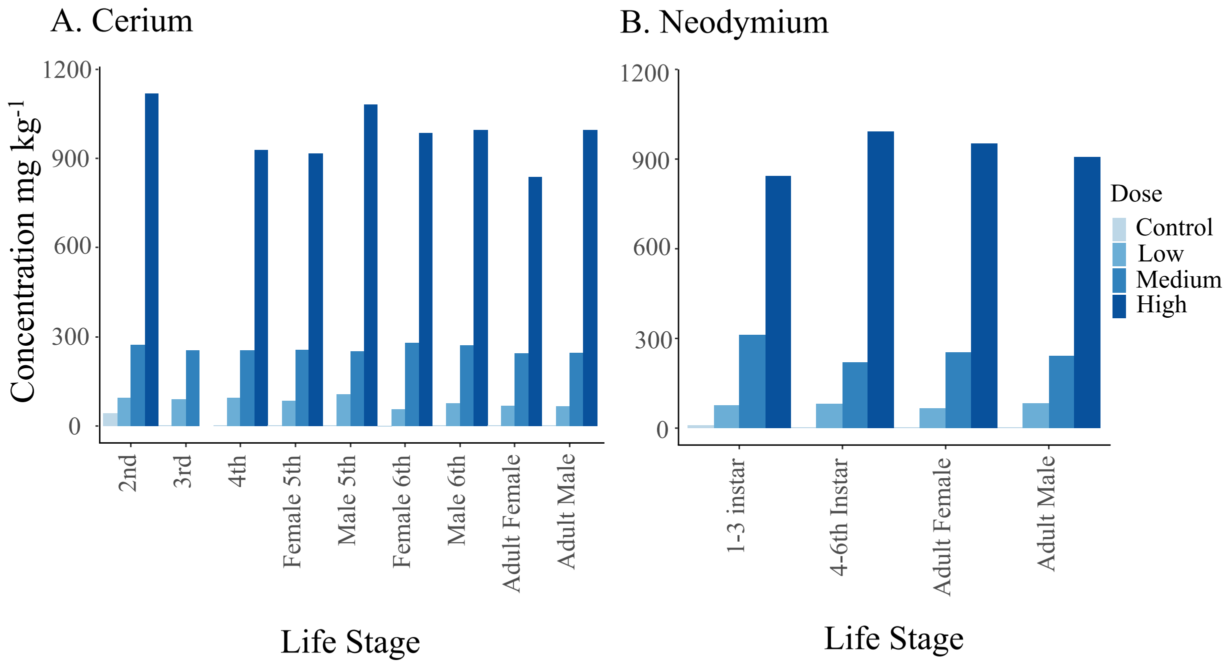

Figure 3.2 Measured **c**oncentration of (A) cerium (Ce) or (B) neodymium (Nd) eliminated in the frass of *Melanoplus sanguinipes* exposed to contaminated food from egg hatch until death. Numbers represent analysis of pooled tissue samples for the life stage-dose combinations. Measured Ce doses (mg kg^{-1}): Control = 0.41, Low = 37.3, Medium = 202.0, High = 894.0; Measured Nd doses (mg kg^{-1}): Control = 1.17, Low = 45.6, Medium = 239.0, High = 720.0.

3.4.2 Growth and development

3.4.2.1 Mass

Adult female mass was unaffected by Ce (Female: $F_{3,79}$=1.09; p=0.357; Figure 3.3A), while male mass trended downward with increasing Ce dose (Male: $F_{3, 101}$=2.48; p=0.066; Figure 3.3B). Dunnett post hoc analysis indicates males at the highest dose had lower mass compared to controls (p=0.03). Neither female ($F_{3, 46}$=2.44; p=0.076; Figure 3.3A) nor male ($F_{3, 30}$=1.17; p=0.336; Figure 3.3B) mass was impacted by exposure to Nd.

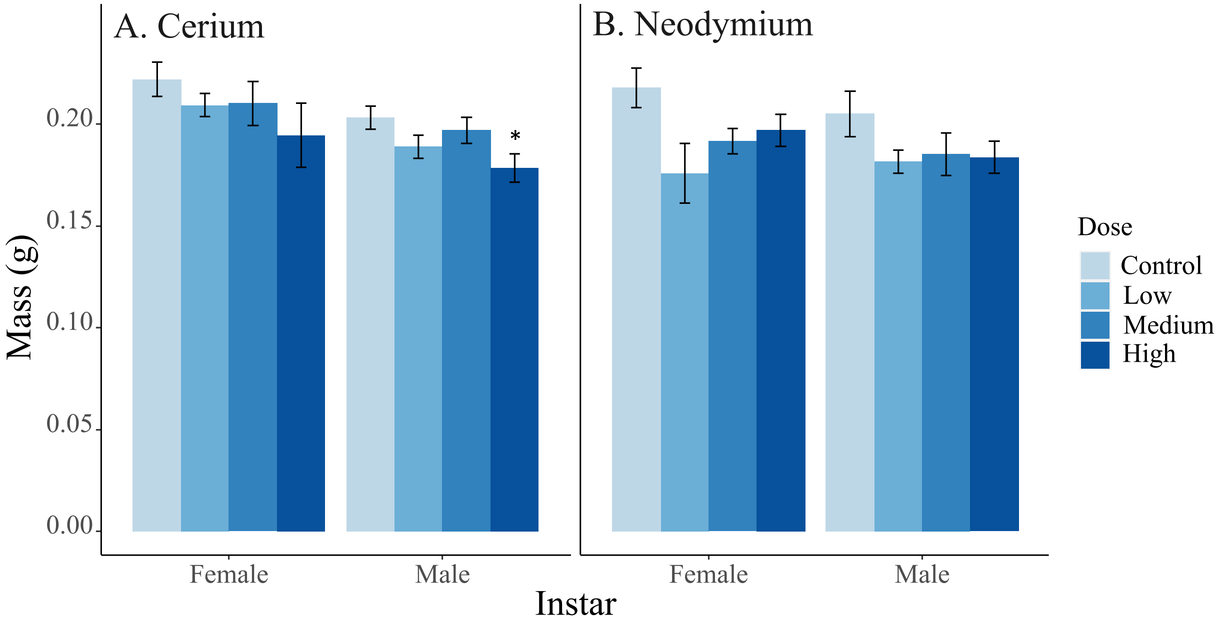

Figure 3.3 Mean mass (± standard error) of newly emerged *Melanoplus sanguinipes* adult females and males exposed to (A) cerium (Ce) or (B) neodymium (Nd) from egg hatch through development to the adult stage. Measured Ce doses (mg kg^{-1}): Control = 0.41, Low = 37.3, Medium = 202.0, High = 894.0; Measured Nd doses (mg kg^{-1}): Control = 1.17, Low = 45.6, Medium = 239.0, High = 720.0.

3.4.2.2 Duration of development

Exposure to an increased dose of Ce did not affect the duration of development from egg hatch to adult emergence ($F_{3, 82}$=0.86; p=0.467; Figure 3.4A) while males showed an increasing trend of development time ($F_{3, 107}$=2.37; p=0.075; Figure 3.4A). Dunnett post hoc analysis indicates a prolonged development time at the highest dose when compared to controls (p=0.028).

Exposure to Nd did not affect female development (χ^2=1.15; d.f.=3; p=0.7645; Figure 3.4B), but did affect males ($F_{3, 31}$=3.14; p=0.039; Figure 3.4B). Males exposed to the lowest dose had a shorter development period than did controls (p=0.022, Figure 3.4), which may be due to the smaller sample size obtained in the low dose group (n=4).

Nymphs develop through 5 or 6 instars to reach the adult stage. The proportion of emerged adults using 6 instars to reach adulthood shows a decreases trend for females exposed to Ce (χ^2=3.74, 3 d.f., p<0.291) and increases for males (χ^2=8.42, 3 d.f., p<0.038). Females exposed to Nd use a 6th developmental instar less often (χ^2=16.41, 3 d.f., p<0.001) at both the medium (p=0.0036) and high (p=0.0258), while males show an increasing trend of using an extra developmental stage (χ^2=2.003, 3 d.f., p<0.572).

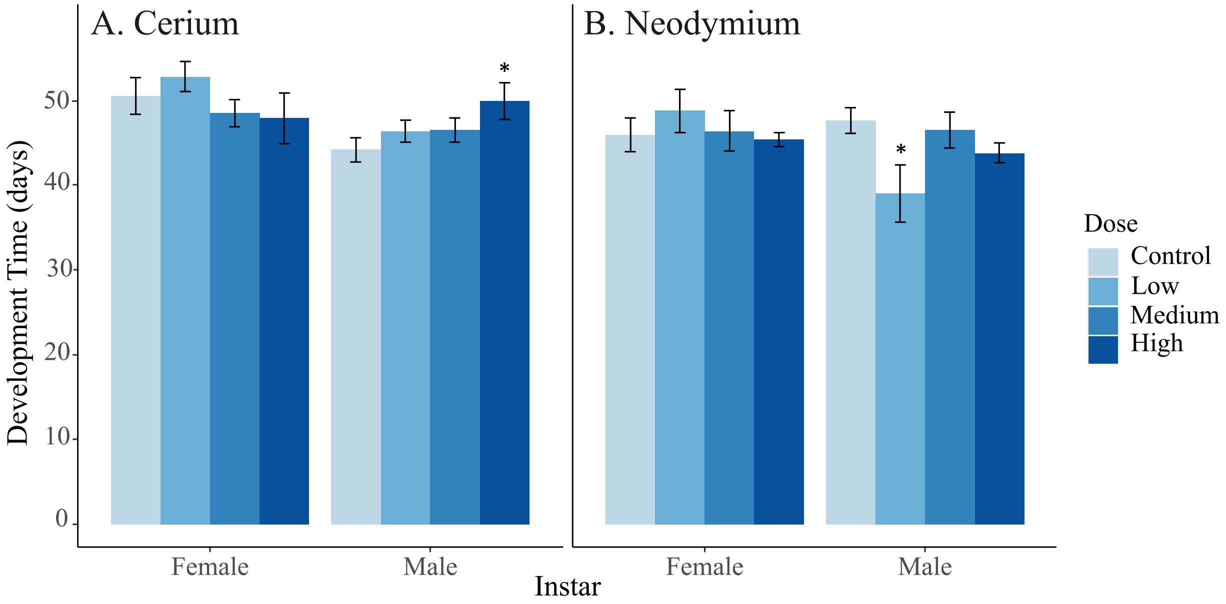

Figure 3.4 Mean duration of development (± standard error) for *Melanoplus sanguinipes* females and males exposed to (A) cerium (Ce) or (B) neodymium (Nd) from egg hatch to adult emergence. Measured Ce doses: Control = 0.41, Low = 37.3, Medium = 202.0, High = 894.0; Measured Nd doses: Control = 1.17, Low = 45.6, Medium = 239.0, High = 720.0.

3.4.2.3 Survival

Cerium negatively affected survival of exposed individuals (χ^2=35.6, 3 d.f., p<0.001, Figure 3.6). The individuals exposed to the control dose had better survival that those exposed to the medium (p=0.013) and high (p<0.001) doses. Survival of those exposed to the low dose did not differ from controls but was better than those exposed to the medium (p=0.013) and high (p<0.001) doses. Individuals at the medium dose also had better survival than those exposed to the high dose (p=0.023). Neodymium exposure did not impact survival (χ^2=3.3, 3 d.f., p=0.4, Figure 3.6). The sex ratio of individuals reaching the adult stage did not differ for Ce (χ^2=4.05, 3 d.f., p=0.256) or Nd (χ^2=0.68, 3 d.f., p=0.878).

Female adult lifespan showed a decreasing trend with Ce exposure ($F_{3,\,69}$=2.63, p=0.057). Male lifespan was negatively affected by Ce dose (χ^2=10.71, 3 d.f., p=0.013) at the highest dose (p=0.015). Exposure to Nd did not negatively affect adult female (χ^2=3.42, 3 d.f., p=0.331) or adult male lifespan ($F_{3,31}$=2.01, 3 d.f., p=0.133).

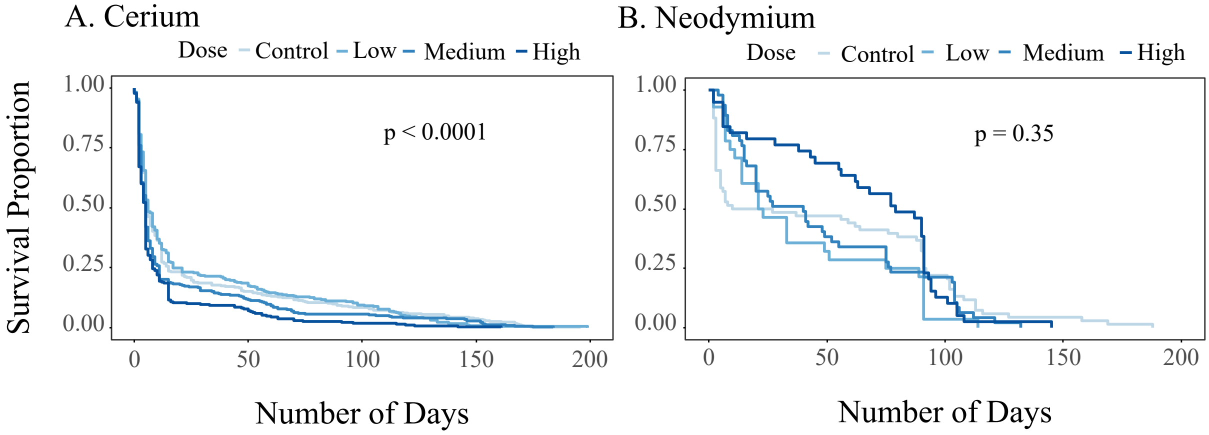

Figure 3.5 Survival of *Melanoplus sanguinipes* exposed to (A) cerium (Ce) or (B) neodymium (Nd) from egg hatch to adult emergence. Measured Ce doses (mg kg^{-1}): Control = 0.41, Low = 37.3, Medium = 202.0, High = 894.0; Measured Nd doses (mg kg^{-1}): Control = 1.17, Low = 45.6, Medium = 239.0, High = 720.0.

3.4.2.4 Reproduction

While the number of deposited eggs were not counted, the presence of Ce in the oviposition substrate did not affect the total number of emerged nymphs during the first 4-weeks of female oviposition ($F_{3,43}$=0.50; p=0.685) nor was the incubation period prolonged by exposure to Ce

($F_{3,40}$=0.84; p=0.483). Females exposed to Ce contaminated food did not develop fewer eggs in the first clutch of eggs (χ^2=0.34, 3 d.f., p=0.953).

3.5 Discussion

Despite widespread use of REEs for emerging technologies, agricultural applications, and growing landfill waste, the long-term consequences of exposure for terrestrial species have not been widely studied. Plants are known to accumulate REEs and in their role as primary producers, may be a source of REE contamination for herbivores feeding directly on tissues with elevated levels of REEs. Insects consuming REE contaminated plants may in turn become a source of REEs for other animals consuming them as prey. Here the lesser migratory grasshopper, *Melanoplus sanguinipes*, was used as model species to understand the effect of consuming REEs for insects feeding directly on plants in contaminated ecosystems.

Following our dosing process, we recovered Ce and Nd from all diet and oviposition substrate samples. Background levels in control substrates were higher than expected for both Ce and Nd. Concentrations recovered from both sand and diet increased corresponding to increased dose.

3.5.1 Accumulation and elimination

Accumulation of both Ce and Nd occurred at all doses and increased with dose within a given life stage. Adult female body concentration at the highest dose tested was 3 times higher than males and 600 times higher than control females. The concentration in males exposed to the highest dose was about 40 times higher than controls. Accumulation of Nd was much lower for adult females, with those at the highest dose only 21 times higher than controls, while the males accumulated over 600 times the level of controls. Males exposed to Nd have a body concentration almost 3 times as high as the females.

Late juvenile stages (instars 4-6) had lower concentration of Ce in the body than at earlier or later life stages. At all life stages, body concentrations were lower than the dosed food for both Ce and Nd suggesting the risk of biomagnification is low.

Shed exoskeletons also contained detectable amounts of Ce and Nd that increased with increasing dose. While levels of Ce in the body were generally higher than Nd, Nd appears to concentrate in the exoskeleton at a higher level than Ce. The measured concentration in exoskeletons at the highest dose is 5 times that of controls for Ce but 23 times the control levels for Nd.

Most of the ingested contamination was eliminated from the body. Ce and Nd concentration in the frass was greater than that in the body or shed exoskeletons and often greater than the concentration in the ingested food. Concentration increased with increasing dose with adult males excreted a higher concentration of Ce than females at the highest dose. In contrast, females excrete a higher concentration of ingested Nd than do males.

Despite excreting the majority of the ingested REEs, both Ce and Nd accumulated in the body. It is unclear from this study where the ingested REEs ended up in the body and there is little information in the literature about the fate of ingested REEs for terrestrial invertebrates. Accumulation in species like *M. sanguinipes* can have consequences for other trophic levels consuming them as prey. REEs such as Ce and Nd may enter the food web via insects and find their way into predator species that might not otherwise be at risk of exposure (Custer et al., 2009). For example, insectivorous birds from mining impacted areas had concentrations of REEs in their livers that were similar to levels in insects (Custer et al., 2009). MacMillan et al. (2017) found patterns of accumulation of REEs in arctic ecosystems that showed high levels of accumulation at the base of an arctic food web including lichens/moss, marine invertebrates and freshwater invertebrates, with lower levels in the muscle tissue of vertebrate consumers. However, the fate of consumed REEs in the body is unknown, as are the long-term consequences of this exposure for most terrestrial species. Future studies should focus on understanding what happens to REEs like Ce and Nd that do cross the gut barrier, where they are stored, and what systems might be affected.

3.5.2 Growth and development

Unwanted metals ingested by terrestrial arthropods can be either assimilated and detoxified or eliminated. The elimination process is an essential route for terrestrial invertebrates, including many insects (Dallinger, 1993). For example, in addition to high assimilation rates, carabid beetles possess efficient excretion of metal-containing vesicles or cells (Janssen et al., 1991). In this experiment, the concentration of Ce and Nd eliminated in solid waste was often higher than the offered food. However, this detoxification of metals is considered to occur through energetically costly processes (Lagisz et al., 2005), which may come at the expense of growth and development.

3.5.2.1 Mass

Growth is considered to be a sensitive indicator of toxicity for many species and is a common measure in studies of chronic toxicity (Azevedo-Pereira & Soares, 2010). For example, mercury has been shown to reduce growth in *Chironomus riparius* larvae at sublethal concentrations (Azevedo-Pereira & Soares, 2010). In our experiment, only males exposed to the highest Ce dose had a reduction in adult mass. Cerium exposure did not negatively impact the mass of females reaching the adult stage, nor did Nd exposure affect adult mass of exposed individual, female or male.

Studies with heavy metals have shown similar reductions in mass of other Acridid species. Adult body mass of the Acridid, *Aiolopus thalassinus* was reduced for females and males exposed to mercury, while only female mass was decreased by exposure to cadmium (Schmidt et al., 1991a; Schmidt et al., 1992). Adults exposed to high concentrations of lead also had reduced body mass, although in this case the males were more strongly affected (Schmidt et al., 1992). Other species of acridid are similarly affected. Malakar et al. (2009) found body mass was reduced in adult *Oxya fuscovittata* exposed to mercury ($HgCl_2$) during development. The authors observed that treated food was consumed less than control food which may have accounted for the loss in growth (Malakar et al., 2009).

Avoidant behavior has also been demonstrated in grasshoppers, with species from 11 different genera of Orthoptera showing avoidance of leaves high in selenium (Freeman et al., 2007). While we don't have information about consumption in this study, our previous studies on acute exposure suggest that consumption was unaffected by Ce and Nd dose (Chapter 2).

While it is widely thought that deterrent effects on herbivores are reflective of avoidance of a toxic effect, many deterrent responses seem to be unrelated to post-ingestion toxicity (Bernays, 1991). This has been documented in congeneric species such as *Melanoplus bivittatus* (Harley & Thorsteinson, 1967). Even without direct toxic effects, a lack of energy reserves from inadequate food can have negative consequences for exposed individuals from less effective excretion due to water shortages to less effective detoxification resulting from a lack of protein (Bernays, 1991). Given that males had lower body concentrations of Ce than females, it is possible that male growth was curtailed when energy was used for detoxification of ingested cerium. Further experiments are needed to understand the energy reserves of exposed females and males.

3.5.2.2 Duration of development

The duration of development through juvenile instars to the adult stage was prolonged for males exposed to the highest dose of cerium. In contrast, males exposed to Nd had a shorter development period to the adult stage at the lowest dose, although the small sample size at the lowest does (4 individuals) makes it difficult to understand if this is a real trend or a result of the small sample size. Female development through juvenile instars was unaffected by either Ce or Nd.

This prolonged development for males at the highest dose may be attributable to a 6th juvenile development stage. The 6th instar is generally used by females (Pfadt, 1994), however in our experiments, the proportion of males that used 6 juvenile instars to reach adulthood increased at the medium and high Ce doses but was unaffected by Nd exposure. Males that undergo 6 developmental instars may be able to utilize this extra developmental stage to gain enough energy and mass to transition to the adult stage.

In contrast, fewer females that reached adulthood used 6 juvenile instars for development for both Ce and Nd, although this trend is only significant for Nd. Females exposed to the medium and high Nd dose undergo a 6th instar less often than do controls. Females that are large enough to transition directly to the adult stage at the 5th juvenile instar more often survive to the adult stage.

Delays in development have been demonstrated in other acridids. Exposure to mercury and cadmium delayed development of *Aiolopus thalassinus* nymphs in both the exposed individuals and in the F2 generation (Schmidt et al., 1991a; Schmidt et al., 1992). *Oxya fuscovittata* showed increases in total rearing time to the adult stage when exposed to mercury ($HgCl_2$) and the delays were even more severe for the F2 generation (Malakar et al., 2009). In this study, we only followed the F1 generation and found such delays in male development times. It remains unclear what effect this might have on reproduction, and the development of offspring of exposed adults.

3.5.2.3 Survival

Exposure to cerium negatively impacted survival of individuals exposed to the medium and high doses. There was a trend of fewer females surviving to the adult stage, but it was not significant, suggesting female and male survival was equally impacted by exposure to Ce. Exposure to Nd did not affect survival.

This impact on survival seems to occur at the adult stage. Ce negatively affected adult female lifespan at the medium and high doses, although this was only significant for the medium dose. Males at the highest Ce dose also had a shortened adult lifespan. Consistent with overall survival, Nd had no effect on either female or male adult lifespan. Similar results were seen in *Drosophila melanogaster* exposed to cerium sulfate. Both mean life span and maximum lifespan was shortened for females and males (Huang et al., 2010). Curtailed lifespan has been demonstrated in the grasshopper, *Aiolopus thalassinus* exposed to mercury, cadmium and lead and the effect carried over into the F2 generation (Schmidt et al., 1991a; Schmidt et al., 1992). The shortened adult lifespan for *M. sanguinipes* can have negative consequences for population growth if fewer individuals are available to produce the next generation of offspring. In turn, vertebrate populations may suffer without healthy grasshopper populations as a source of animal protein (Malakar et al., 2009).

3.5.2.4 Reproduction

The number of emerged nymphs over the first 4 weeks of oviposition did not differ between females exposed to control and cerium contaminated substrate, nor did the incubation period of the deposited eggs change. This contrasts with the effects of cerium on fertility that have been documented for *Drosophila melanogaster*. Reduced numbers of emerged female and male neonates were seen at doses between 16 – 1024 mg L^{-1} Ce (Huang et al., 2010). Nymphal hatching is reduced by exposure to mercury in another species of grasshopper, *Aiolopus thalassinus*, but did not prolong development time (Devkota & Schmidt, 1999). In contrast, hatching of eggs of the grasshopper *Eyprepocnemis plorans* was delayed following mercury exposure, but hatch rates were higher than controls (Devkota & Schmidt, 1999). It's thought that protection provided by the large egg volume and thicker foam surrounding *E. plorans* eggs results in a higher tolerance to toxins. In this study, we did not count the total number of eggs deposited by females. More comprehensive studies of reproduction could shed light on the number of deposited eggs in relation to emerged neonates.

While the concentration of eggs incubated in Ce contaminated substrate in this study is unknown, the lack of effect on number of developed nymphs and incubation period suggest *M. sanguinipes* neonates are similarly protected by the egg pod that surrounds a clutch of eggs deposited by the female. In experiments with the heavy metals mercury, cadmium and lead, there was no reduction in the number of eggs laid by females, but analysis of the eggs produced by exposed females showed metal content in the eggs that was correlated with the initial metal concentrations (Schmidt et al., 1991a). The authors suggest the eggs may serve as a mode of elimination of excess heavy metals for females. In this study, females carried a higher concentration of Ce than did males and similarly had no reduction in egg production. It is possible that females are ridding themselves of excess Ce through this mechanism. Further study is needed to determine the Ce content of the eggs laid in treated substrate and in the eggs produced by exposed females.

Freshly emerged females generally mature their first clutch of eggs in the first 10-14 days after adult emergence. Following exposure to Ce contaminated food during development, the number of eggs in the female abdomen at reproductive maturity did not differ between doses. In contrast, heavy metals such as mercury and cadmium have been shown to decrease the number of eggs produced by *Aiolopus thalassinus* (Schmidt et al., 1992). Hatch rate of the eggs produced by exposed females was reduced and the viability of eggs produced by the untreated F2 generation were also reduced (Schmidt et al., 1992). *Locusta migratoria* females injected with cadmium lacked mature eggs in the abdomen at 2 weeks post emergence to the adult stage, possibly due to injury to the fat body (Martoja et al., 1983). In this study, we have no information about the viability of the eggs following maternal exposure to cerium. Negative impacts on fertilization success have been documented in sea urchins (*Paracentrotus lividus*) following sperm exposure to 10^{-5} M Ce (IV) (Oral et al., 2010). Further experimentation is needed to understand the viability and health of offspring produced by adults exposed to REE contamination, and the implications for the F2 generation.

3.6 Conclusion

The long-term consequences of REE exposure on terrestrial invertebrates is not well understood. This study demonstrates that chronic exposure to REEs has negative consequences for *M. sanguinipes* and the effects differed for Ce and Nd and between females and males.

While *M. sanguinipes* was able to excrete a substantial portion of ingested REEs, accumulation in the body occurred at all doses, with Ce accumulating in the body at higher levels than Nd. Females had higher concentrations of Ce than males, while the opposite was true for Nd with males showing higher concentrations than females. Body concentrations remained below that of the contaminated food, suggesting the risk of biomagnification is low. Despite this, both Ce and Nd accumulated in *M. sanguinipes* and may in turn be passed on to predators consuming them as prey with unknown consequences.

Ingestion did have negative consequences for exposed individuals with Ce showing more toxic effects than Nd. Cerium delayed development of males and emerged adult males had lower body mass. Cerium negatively impacted survival through a reduced adult lifespan for both females and males. While the sex ratio was not significantly affected, there was a trend towards decreased females at the adult stage. Females that are large enough to transform to the adult stage at the 5th instar survive to adulthood. However, they carry a higher concentration of cerium in their bodies. Males may be expending more energy on detoxification resulting in delayed development and reduced mass but also the lower accumulation when compared to females. Further testing is needed to look at the energy reserves, energy production and detoxification enzymes in exposed individuals to understand how energy budgets are shifting in response to ingestion of different REEs.

While effects on reproduction were not found in this experiment, there is no information about the viability of eggs produced by exposed females and the REE content of offspring. Future experiments should examine the full impact on reproduction following exposure to REEs. Nonetheless, the effects on development and lifespan found here can have broader implications for the population if there are fewer adults to produce the next generation. Insects are not only primary consumers, but also are also a food source for higher trophic levels. Grasshoppers like *M. sanguinipes* form a substantial portion of the biomass available for consumption. Factors that affect grasshopper population can produce negative impact on the ecosystem if vertebrate populations lose this valuable source of animal protein.

Chapter 4 Effect of cerium on the body composition, energy metabolism and redox balance of *Melanoplus sanguinipes*.

4.1 Abstract

Rare earth elements (REEs) are contaminants of emerging concern with pollution levels rising worldwide as demand for these valuable elements increases. Plants are known to accumulate REEs, as are terrestrial herbivores feeding on contaminated tissues. The fate of REEs in biota consuming contaminated plants, particularly in terrestrial ecosystems where pollution originates, is poorly understood. With increasing intoxication from metal ingestion, effects at the sub organismal level may become apparent. Increased maintenance costs in individuals that must actively detoxify ingested metals may cause reductions in energy stores. Increased demand for energy for detoxification may be reflected in increased metabolic activity. Metal ingestion can lead to increases in reactive oxygen species (ROS) which can damage macromolecules (lipids, proteins, carbohydrates and nucleic acids) causing oxidative stress. Antioxidant and biotransformation defenses scavenge ROS to prevent damage. If not eliminated, ROS can react with lipids in cell membranes causing lipid peroxidation and a loss of redox balance (the ratio between reduced (GSH) and oxidized glutathione (GSSG)) as glutathione is used to oxidize ROS. Here energy reserves (lipid, glycogen, free carbohydrates and protein stores) were quantified in reproductively mature female *Melanoplus sanguinipes* following chronic exposure to Ce during their development. Changes in mitochondrial content associated with increased energy demand were characterized using the activity of the mitochondrial enzyme citrate synthase (CS). Mobilization of antioxidant (catalase (CAT)) and biotransformation (glutathione-s-transferase (GST)) defenses were quantified along with lipid peroxidation and redox balance to identify cellular damage. The results indicate that aerobic capacity is elevated at the lowest dose. There was no increase in either CAT or GST activity and lipid peroxidation did not indicate cellular damage. Redox balance was

similarly unaffected suggesting that level of ROS in *M. sanguinipes* may not be increased by Ce ingestion.

4.2 Introduction

The rare earth elements (REEs) are contaminants of emerging concern. Growing demand for REEs for use in emerging technologies has led to increased mining activity worldwide (Long et al., 2012). Along with mining waste, landfill waste from discarded technologies and application of phosphate fertilizers rich in REEs in agricultural systems is causing an increase in levels of REEs in the environment. Cerium compounds are widely used to increase yield and quality of crops in China (Huang et al., 2010). Many REEs are considered environmentally safe. For example, in addition to their use in agriculture as a component of phosphate fertilizers, lanthanum carbonate is prescribed in cases of kidney failure (Mehrotra et al., 2008). Cerium, neodymium, yttrium, and ytterbium are added to cardiovascular stents to decrease degradation rates (Drynda et al., 2009).

Despite their uses and the continued rise in environmental REE contamination, little is known about the long-term consequences of exposure for terrestrial species. Plants are known to accumulate REEs and in their role as primary producers, they represent a route of exposure for herbivorous species. Despite efficient excretion capabilities, the lesser migratory grasshopper, *Melanoplus sanguinipes* (Fabricius) (Orthoptera: Acrididae) accumulates REEs from their diet with consequences ranging from delays in development to the adult stage, reductions in adult mass and reduced survival (Chapter 3).

Metal accumulation can occur because excretion capacity must be balanced with water loss, restricting the elimination capacity for terrestrial species (Dallinger, 1993). These excess metals will be accumulated through the gut epithelia (Dallinger, 1993). Many insects have detoxifying mechanisms, transporters, immunity and peritrophic membranes to resist toxic substances (Wang et al., 2020). While detoxifying processes may limit the toxicity of ingested metals, they are considered to be energetically costly (Lagisz et al., 2005). Based on the assumption that the energy budget of an organism is limited, an increase in energy expended to detoxify ingested metals may leave less energy available for growth and development.

Lipids, stored as an energy reserve in the insect fat body, can account for up to 50% of total dry weight (Arrese & Soulages, 2010). Acting as the main metabolic center, insect fat bodies are analogous to vertebrate adipose and liver tissue (Arrese & Soulages, 2010). Reduced energy stores linked to exposure to cadmium and mercury have been shown in *Locusta migratoria* (Martoja et al., 1983). Histochemical testing revealed that exposure to cadmium caused reduced glycogen stores and mercury resulted in reduced lipid stores in the fat bodies (Martoja et al., 1983). Changes in energy stores may be reflective of increased maintenance metabolism costs associated with detoxification.

Exposure to toxins can lead to alterations in energy production and enzyme regulation. For example, up-regulation of enzymes involved in energy production has been found in bees following exposure to nicotine (Rand et al., 2015). Similarly, neodymium increased metabolic capacity of *Mytilus galloprovincialis* (Freitas et al., 2020). This increased energy is thought to be used for detoxification. It is unclear from the literature if REE exposure causes similar increases in the metabolic machinery of insects.

At some level of exposure, the detoxification and storage capacity of unwanted metals may be exceeded and additional metals that enter the body are metabolically available (Rainbow, 2007). These may bind with amino acids and interfere with the metabolic role of the protein (Rainbow, 2002). REEs that enter the body may be particularly problematic as they have the same atomic radius and share similar chemical properties with ions like calcium, magnesium, iron and manganese (Evans, 1983). The resulting affinity for binding sites of similar elements may allow them to substitute themselves for other metallic ions, a result already established in studies with plants (Babula et al., 2008; Liu & Hasenstein, 2005; Pickard, 1970; Yang et al., 2015).

The similarity between some REEs and calcium has been investigated as the primary mechanism of toxicity, mainly in aquatic invertebrates. Gadolinium has been established as a calcium channel blocker in four species of sea urchin (*Paracentrotus lividus*, *Arbacia lixula*, *Heliocidaris tuberculata* and *Centrostephanus rodgersii*) resulting in inhibition or alteration of growth (Martino et al., 2017). Lanthanum is also a strong calcium channel blocker due to its similar ionic radius (Craig et al., 1999). In insects, blocking of calcium uptake can lead to depolarization of muscle membrane potential, resulting in sustained muscle contraction or tetanic paralysis. This effect has been observed in cockroach (*Periplaneta americana*) leg muscles treated with lanthanum (Washio & Miyamoto, 1983) and *M. sanguinipes* legs and antennae following exposure to Ce (Allison et al., 2015). Evidence from the literature suggests that the condition is not reversible once exposure to lanthanum ceases (Coniglio et al., 1993).

Ingested metals often result in increases in reactive oxygen species (ROS) (superoxide anions, hydroxyl radicals and hydrogen peroxide). ROS are produced when an electron is donated from the metal to the molecular oxygen used for oxidative metabolism to generate ATP (Ahmad, 1995). The superoxide anion formed in this process triggers a series of reactions that result in a toxic hydroxyl radical (Ahmad, 1995). Antioxidant defenses like catalase (CAT) scavenge ROS to prevent damage caused by oxidative stress (Ahmad, 1995).

Glutathione, a key antioxidant used to protect organisms from ROS, converts ingested toxins to less harmful substances that can be excreted. It is conjugated to xenobiotics by glutathione-S-transferase (GST) (Lukkari et al., 2004). An increase in GST activity is often used as an indirect measure of metal exposure (Freitas et al., 2020). Mobilization of antioxidant (CAT) and biotransformation (GST) responses are indicative of an organism's efforts to rid themselves of unwanted contaminants like Ce.

Under conditions of extreme stress or prolonged stress from chronic exposure, protein damage may occur. This can lead to a decrease in the activity of enzymes, limiting individual's capacity to avoid damage due to ROS (Freitas et al., 2020). Reductions in activity of superoxide dismutase (SOD) has been linked to an overproduction of ROS in *Mytilus edulis* from polluted areas (Manduzio et al., 2004) and in *Tapes philippinarum* exposed to copper (Matozzo et al., 2001).

Under conditions of oxidative stress, when the capacity to eliminate ROS through antioxidant and biotransformation has been overwhelmed, excess ROS in the body can damage lipids, proteins, carbohydrates, and nucleic acids (Ahmad, 1995) accounting for the toxicity of some metals, particularly heavy metals (Ercal et al., 2001). ROS that react with lipids contained in cell membranes cause lipid peroxidation, or damage to the cell membrane (Ahmad, 1995). Following damage, cell membranes are unable to control the efflux or influx of substances crucial to normal cell physiology (Ahmad, 1995). Measuring levels of lipid peroxidation can provide an indication of cellular damage resulting directly from ingestion of metals.

Damage from ROS may play a role in REE toxicity. Increases in ROS following exposure to REEs have been linked to cell apoptosis in mouse mitochondria (Liu et al., 2003), rat cortical neurons (Xia et al., 2011) and human embryo liver (Ye et al., 2011). Following exposure to low levels of lanthanum, gadolinium and ytterbium, mouse mitochondria showed decreased membrane potential and elevated cellular ROS (Liu et al., 2003). Similarly, mouse liver hepatocyte apoptosis resulted from exposure to cerium chloride (Zhao et al., 2012). Among invertebrates. the freshwater mussels, *Dreissena polymorpha*, did not show an increase in ROS following exposure to gadolinium (Hanana et al., 2017). The nature and extent of toxic effects of metabolically active REEs is poorly understood for terrestrial invertebrates.

Redox balance (the ratio between reduced (GSH) and oxidized glutathione (GSSG)) can be used to identify and estimate levels of oxidative stress. The reduced form of glutathione (GSH) scavenges ROS (Couto et al., 2016) and when mitigating lipid peroxidation, GSH is oxidized to GSSG (Ahmad, 1995). A decrease in the ratio of GSH/GSSG can indicate increased demand for GSH to neutralize ROS through oxidation to the GSSG form.

There are few experiments demonstrating the role of these mechanisms for invertebrates exposed to REEs. *Mytilus galloprovincialis* exposed to neodymium show higher metabolic capacity and increased CAT activity but appear unable to mobilize biotransformation defenses (GST) (Freitas et al., 2020). It remains unclear what, if any role, these mechanisms play in the detoxification of rare earth elements for terrestrial invertebrates.

In this experiment, I tested the hypothesis that chronic ingestion of Ce would be energetically costly to detoxify. I predicted this cost of detoxification would lead to detectable depletion of energy stores as energy is diverted and increased metabolic capacity to meet energy demands. Energy reserves (lipid, glycogen, free carbohydrates and protein) and aerobic capacity (citrate synthase activity (CS)) were quantified in reproductively mature female *M. sanguinipes* following chronic exposure to Ce during their development to test this hypothesis. I further tested the hypothesis that increasing accumulation of Ce in the body would cause toxic effects at the cellular level. I predicted that levels of ROS would increase resulting in mobilization of antioxidant and biotransformation defences, characterized through increased activity of CAT and GST to rid themselves of unwanted Ce and to prevent oxidative stress. Lastly, I predicted that excess ROS resulting from chronic exposure to Ce would result in loss of redox balance, quantified by the ratio of GSH:GSSG, and an increase in cellular damage identified through lipid peroxidation.

4.3 Materials and methods

4.3.1 Selected rare earth elements and doses

Individual *M. sanguinipes* were exposed to Ce from egg hatch throughout their development following our previous methodology (Chapter 3). Briefly, individuals were exposed to cerium chloride heptahydrate ($CeCl_3 \cdot 7H_2O$ - Sigma-Aldrich, Oakville, ON, CA; CAS: 18618-55-8) using a dose progression of 40, 160, 640 mg Ce kg^{-1}. The lowest dose in the progression was representative of accumulation found in studies with plants growing in contaminated soils (i.e., *Solanum lycopersicum* growing in a soil concentration of 978 mg Ce kg^{-1} dry soil) (Thomas et al., 2014). All results are based on and reported in terms of mg Ce in dry mass of diet based on chemical analysis performed by Brooks Applied Labs (Bothell, WA, USA). Briefly, samples were hot block digested with nitric acid (HNO_3) and hydrogen peroxide (H_2O_2), then diluted to volume with deionized (DI) water. Digests are then analyzed by inductively coupled plasma - mass spectrometry (ICP-MS) following the US Environmental Protection Agency (USEPA) Method 1638 (US Environmental Protection Agency, 1996). Results of the chemical analysis and quality control data can be found in appendix D & E.

Hatchlings were reared individually from egg hatch in 100 ml specimen cups fitted with a lid vented using galvanized aluminum insect screening to allow air flow. Each container was fitted with a 2.0 ml microcentrifuge tube filled with water and capped with a shortened piece of dental cotton, a short stick to act as a perch for moulting and a ration of either contaminated or control diet. Individuals were fed ad libitum and three times per week, were checked for overall health and development and containers were replenished with fresh food and water. The diet was made from commercial guinea pig food pellets (Hagen Living World Extrusion Diet, Rolf C. Hagen Inc., Montreal, Quebec) consisting of 15.0% crude protein, 3.0% crude fat and 14% crude fiber following. Diet preparation details can be found in Appendix B.

Females were dissected for extraction of energy stores between 12- and 16-days following emergence to the adult stage. This period corresponds with reproductive maturity which occurs in the first 2 weeks of reaching the adult stage. Adults were in a post absorptive state and were weighed and then the head, wings and legs were removed. The body cavity was opened from the pronotum to the tip of the abdomen along the dorsal midline to reveal the reproductive structures. The digestive tract was removed from the body cavity and the body was bisected at the first abdominal section.

4.3.2 Energy stores extraction

The abdomen and reproductive tissue were weighed to the nearest 0.01 mg and frozen at -80°C in 2 ml micro tubes (Axygen Scientific MCT-200-C) until energy stores could be extracted. All chemicals for extraction of energy stores were obtained from Sigma Aldrich (Oakville, ON, CA). Extraction of energy stores (glycogen, free carbohydrates, lipids, and abdominal protein) was done following Lorenz (2003) with some modifications for grasshoppers. Briefly, 200 µl of sodium sulfate (Na_2SO_4) and 300 µl of 75% Methanol (MeOH) diluted in distilled water (DI) was added to the abdominal tissue. All manipulations were carried out on ice. The mixture was first minced with scissors and then homogenized with three 10 s bursts at 10,000 rpm with 30 s cooling intervals using a Polytron homogenizer (Polytron PT1300D, Kinematica AG, Malters, Switzerland) fitted with a 7mm rotor stator tip until no identifiable parts remained. The homogenizer tip was rinsed with 600 µl of chloroform methanol ($CHCl_3$: MeOH) (1:1, v:v) to remove any remaining homogenate. The 600 µl of $CHCl_3$:MeOH was then combined with the abdominal homogenate. The homogenate was mixed using a vortex mixer and centrifuged for 10 minutes at 21,000 g and 4°C (Sorvall Legend Micro 21r, Thermo scientific, Osterode, Germany). All further centrifugation was similarly conducted at 21,000 g and 4°C for 10 minutes.

The supernatant was removed and held in reserve for extraction of lipids and carbohydrates. The pellet was suspended in 300 µl of $CHCl_3$:MeOH (1:1, v:v), mixed by vortex and further broken down using sonic vibration (VC750 Ultrasonic Processor, Sonics Materials, Inc., CT, USA) for 3 minutes (40 kHz for 5 s per cycle with 30 s rest periods). All further sonication was done using the same conditions (40 kHz for 5 s per cycle with 30 s rest periods). The homogenate was then mixed by vortex and centrifuged. The supernatant was combined with the supernatant from the previous step for lipid and free carbohydrate extraction. The pellet was further processed for protein and glycogen content.

4.3.2.1 Lipid and free carbohydrate extraction

The combined supernatants were mixed with 500 µl of $CHCl_3$ and 300 µl of 1M sodium chloride (NaCl) and mixed by vortex. The solution was then centrifuged resulting in two phases. The aqueous epiphase was removed quantitatively and dried using a speed-vac at 35°C. The resulting precipitate was dissolved in 400 µl of H_2O, mixed by vortex and aliquots were used to assess the quantity of free carbohydrates in each sample.

The organic hypophase was dried at room temperature overnight and the resulting precipitate was dissolved in 1000 µl of hexane (C_6H_{14}) and 500 µl of 1M NaCl. The sample was mixed by vortex and aliquots used for characterization of lipid levels.

4.3.2.2 Protein and glycogen extraction

The remaining pellet was dried completely in a thermo block (Eppendorf Thermostat plus MTP, Hamburg, Germany) at 70°C and then suspended in 500 µl of Na_2SO_4 saturated 66% ethanol (EtOH). This was mixed by vortex and the pellet was then broken down using a glass stir rod initially followed by sonic vibration to completely dissolve the pellet. The mixture was then mixed by vortex and centrifuged.

The supernatant was quantitatively discarded, and the pellet was dried completely in a thermo block at 70°C. Once dry, 200 µl of 10% potassium hydroxide (KOH) was added to the pellet which was then mixed by vortex, further broken down using a sonicator, mixed again by vortex and finally macerated for 30 minutes at 99ºC in a thermo block. The sample was mixed by vortex every 10 minutes during the 30-minute period.

Following the 30 minutes of maceration, a 50 µl aliquot was removed and mixed with 150 µl H_20 for protein characterization. To the remaining mixture, 150 µl of 95% EtOH was added and allowed to stand for 10 minutes before centrifugation. The supernatant was quantitatively discarded, and the pellet was dried completely at 70°C in a thermo block. The resulting precipitate was dissolved in 600 µl of DI heated at 99°C on a hot plate. The sample remained at 99°C on the thermo block and aliquots were taken for determination of glycogen content.

4.3.3 Energy stores quantification

All assays were performed in triplicate at room temperature (24°C) and the average measurement was used to calculate activity or content. Measurements were made in 96 well plates (Corning Costar 21) using a plate spectrophotometer (Synergy 2 Multi-Detection Microplate Reader, Biotek Instruments, Winooski, VT, USA). For all assays, substrate concentration was verified to ensure saturating conditions and the sensitivity of the assay to increases in activity was verified by doubling the volume of homogenate. Assays were performed over multiple days with doses distributed across assay dates. Standard curves were run alongside samples with each analysis.

4.3.3.1 Lipids

Lipid measurements were made using the phosphovanillin method (Kauffman and Brown, 2014) with the modification of dissolving hexane in soybean oil instead of chloroform as in Thomson et al. (2014). The phosphovanillin reagent was made by dissolving 600 mg of vanillin ($C_8H_8O_3$) (Sigma Aldrich CAS: 121-33-5) in 100 ml of hot DI and then adding 400 ml of 85% phosphoric acid (H_3PO_4) (Sigma Aldrich, CAS:7664-38-2). Standard curves were prepared by dissolving soybean oil (Sigma Aldrich, CAS: 8001-22-7) in C_6H_{14} (1 mg ml^{-1}) and diluting with DI to final concentrations of 0, 0.01, 0.02, 0.04 and 0.08 mg g^{-1} lipid. Extractions were diluted 25, 50, or 100 x in vanillin phosphoric acid reagent (final volume 5.0 ml). Extracts and standards were heated at 90°C in a water bath (Isotemp 210, Fisher Scientific, MA, USA) to evaporate any solvent. Sulfuric acid (H_2SO_4) (200 µl) (Fisher Brand, CAS: 7664-93-9) was added to the tube and it was heated for a further 10 minutes. Vanillin phosphoric acid reagent was then added and allowed to cool for 5 minutes to allow the red color to develop. Individual standard curves were used for each plate and absorbance was read at 560 nm.

4.3.3.2 Glycogen and free carbohydrate

Glycogen and free carbohydrate content were measured separately using the anthrone method described by Kauffman and Brown (2014) with an anhydrous glucose standard. The anthrone reagent was made mixing 385 mL 98% H_2SO_4 with 150 mL DI. To this solution, 750 mg of anthrone ($C_{14}H_{100}$) (CAS:90-44-8) was added, mixed well and stored at 4°C. Standard curves were prepared by mixing glucose solution (1 mg ml^{-1}) with DI to final concentrations of

0, 0.005, 0.01, 0.02, 0.03 and 0.04 mg g^{-1} glucose. Extractions were diluted 25, 50, or 100 x in anthrone reagent to a final volume of 600 µl to obtain a measure within the range of the standard curve. In triplicate, 200 µl of each standard and glycogen or free carbohydrate extract was added to a well on the 96 well plate. Standards and extracts were then heated at 99°C for 17 minutes in a thermo block and allowed to cool for 5 minutes. Individual standard curves were used for each plate and absorbance was read at 625 nm.

4.3.3.3 Abdominal protein

Abdominal protein content was determined using the bicinchoninic acid assay (BCA) Protein Assay kit from Sigma Aldrich (Sigma-Aldrich, B9643-IL-KC). This assay relies on the reduction of Cu^{3+} to Cu^{2+} and then Cu^{1+} under conditions of 50 mM potassium phosphate (KPO_4) buffer, 1 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.0), BCA working reagent (50 parts bicinchoninic acid, sodium carbonate, sodium tartrate, sodium bicarbonate in 0.1 M NaOH, pH 11.25 and 1-part Copper (II) sulfate pentahydrate, 4%). Samples were diluted 50 times and standard curves were prepared by mixing bovine serum albumin (BSA) protein standard with buffer (50 mM potassium phosphate buffer, 1 mM EDTA (pH 7.0)) to final concentrations of 0, 10, 20, 50, 100, 200 µg g^{-1}. Samples were read at 562 nm following a 10-minute incubation period.

4.3.4 Tissue extraction

Thorax tissue was stored at -80°C until assays could be performed. Frozen thoraces were bisected down the midline to produce two equal halves. Each half was weighed to the nearest 0.01 mg. One half of the thorax was used for characterization of thorax protein, metabolic capacity (citrate synthase activity (CS)), antioxidant defenses (catalase (CAT)) and biotransformation defenses (glutathione-s-transferase (GST)). The second half was used for measurement of redox balance (ratio of reduced to oxidized glutathione) (GSH:GSSG) and lipid peroxidation (LPO).

4.3.4.1 Tissue preparation for thorax protein, citrate synthase activity, antioxidant and biotransformation defenses

Frozen half thoraces were homogenized in 19 volumes of ice-cold homogenisation buffer (50 mM KPO_4, pH 7.0 at 24°C, 1mM EDTA, 5 mM dithiothreitol (DTT) and 0.5% Triton X-100). All manipulations were carried out on ice. The tissue was minced with scissors, then homogenized with three 10 s bursts at 10,000 rpm with a 30 s rest interval in between using a Polytron homogenizer fitted with a 7 mm rotor stator tip until no identifiable parts remained. Homogenate was then sonicated for 3 minutes (40 kHz for 5 s per cycle with 30 s rest periods). All further sonication was done using the same conditions (40 kHz for 5 s per cycle with 30 s rest periods). Samples were then centrifuged at 5000 g for 5 mins at 4°C. The resulting supernatant was used for all assays.

4.3.4.2 Tissue preparation for reduced and oxidized glutathione and lipid peroxidation

Homogenization of the second half of the thorax was performed following Rahman et al. (2006). Briefly, frozen half thoraces were homogenized in 19 volumes of ice-cold homogenisation buffer (0.1 M KPO_4, pH 7.5 at 24°C, 5 mM EDTA, 0.6% sulfosalicylic acid ($C_7H_6O_6S$) and 0.5% Triton X-100). All manipulations were carried out on ice. The tissue was minced first with scissors then homogenized with three 10 s bursts at 10,000 rpm with 30 s cooling intervals using a Polytron homogenizer fitted with a 7 mm rotor stator tip until no identifiable parts remained. Homogenate was then sonicated for 3 minutes. The homogenate was then divided, with 100 μl transferred to another microcentrifuge tube and treated with 2 volumes of methanol chloroform (2MeOH: $1ChCl_3$) for characterization of LPO. Both homogenates were centrifuged at 5000 g for 5 mins at 4°C. The resulting LPO samples were then topped up by adding 1/3 the volume of DI and centrifuged again at 3000 g for 5 mins at 4°C. The top phase was discarded, and the bottom phase was frozen at -80°C until LPO could be measured.

4.3.5 Enzyme assays

All assays were performed in duplicate, and the average measurement was used to calculate activity or content. Measurements were made in 96-well plates using a plate spectrophotometer adjusted to maintain 25°C. For all assays, substrate concentration was verified to ensure saturating conditions and the sensitivity of the assay to increases in activity was verified by doubling the volume of homogenate. All enzyme activities are reported in U g^{-1} of thorax tissue where 1 U = 1 μmol substrate min^{-1}. Assays were performed over multiple days with doses distributed across assay dates.

4.3.5.1 Thoracic protein

Thoracic protein content was measured using the same methodology as abdominal protein. Samples were diluted 20 x and standard curves were prepared by mixing bovine serum albumin (BSA) protein standard with buffer to final concentrations of 0, 25, 125, 250, 500, 1000 μg g^{-1}. Samples were read at 562 nm following a 10-minute incubation period.

4.3.5.2 Citrate synthase

Citrate synthase activity was quantified using the method described in Darveau et al. (2005). Briefly, the assay follows the appearance of 5-thio-2-nitrobenzoic acid (TNB) from 5,5-dithiobis-2-nitrobenzoic acid (DTNB), under conditions of 50 mmol Tris HCL, pH 7.4 at ambient temperature (24°C), 0.3 mmol acetyl CoA, 0.1 mmol DTNB, and 0.5 mmol oxaloacetate. Sample homogenate was diluted 50 times and maximum activity (V_{max}) was measured at 412 nm for 5 min at 25°C using the extinction coefficient ε=13.6. Background activity was measured without oxaloacetate and subtracted when observed.

4.3.5.3 Antioxidant defenses

CAT activity was determined using the Catalase Kit from Sigma-Aldrich (CAT100, MilliporeSigma Canada Ltd, Oakville, ON). This assay follows the formation of formaldehyde when catalase react with methanol in the presence of peroxide (H_2O_2). Conditions of the assay were 100 mM KPO_4 buffer pH 7.0 at ambient temperature (24°C), 90% methanol, 8.82 M H_2O_2, 10 M KOH, Purpald (4-amino-3-hydrazino-5-mercapto-1,2,4-triazole) in 0.5 M hydrochloric acid (HCl), potassium periodate in 0.5M KOH. Samples were diluted 50 times using buffer (25 mM KPO4 buffer pH 7.5 at ambient temperature (24°C), 1 mM EDTA, 0.1% BSA). Standard curves were prepared with 4.25 M formaldehyde to final concentrations of 0, 5, 15, 30, 45, 60, 75 µM formaldehyde and catalase activity was measured at 540 nm.

4.3.5.4 Biotransformation defenses

Glutathione-s-transferase activity was quantified using the method described in Carregosa et al. (2014) which is adapted from Habig et al. (1974). The assay follows the appearance of GS-DNB conjugate formed when GST catalyzes the reaction between reduced glutathione (GSH) and 1-chloro-2,4,-dinitrobenzene (CDNB):

$$GSH + CDNB \xrightarrow{GST} GS - DNB\ Conjugate + HCL$$

under conditions of 0.1M KPO_4 buffer (pH 6.5) at ambient temperature (24°C), 60 mM CDNB, 10 mM GSH. Sample homogenate was diluted 50 times and maximum activity (V_{max}) was measured at 340 nm for 5 minutes. Enzyme activity was calculated from a time interval during which activity was linear using the extinction coefficient, ε=9.6 $mM^{-1}cm^{-1}$.

4.3.5.5 Cellular damage

Lipid peroxidation was determined using the PeroxiDetect kit from Sigma-Aldrich (PD1, MilliporeSigma Canada Ltd, Oakville, ON). This assay follows the formation of iron (Fe^{3+}) that occurs when peroxides oxidise to Fe^{2+} then Fe^{3+} using working colour reagent (25 mM ferrous ammonium sulfate in 2.5 M sulfuric acid, 4mM BHT, 125 μM xylenol orange (3,3"bis[N,N-bis(carboxymethyl)aminomethyl]-o-cresolsulfonephthalein, sodium salt) in 90% methanol. Samples were diluted 5 times and standard curves were prepared with tert-Butyl Hydroperoxide (t-BuOOH) to final concentrations of 0, 0.01, 0.02, 0.04, 0.08, 0.12, and 0.16 nM t-BuOOH. Samples were read at 560 nm to determine the nmol peroxide g^{-1}.

4.3.5.6 Redox balance

Determination of GSH and GSSG was done following the method described by Rahman et al. (2006). The assay follows the appearance of 5-thio-2-nitrobenzoic acid (TNB) from 5,5-dithiobis-2-nitrobenzoic acid (DTNB), under conditions of GSH: 0.1M KPO_4 buffer pH 7.5 at ambient temperature (24°C), 5mM EDTA, 0.1% Triton X-100, 0.6% sulfosalicylic acid ($C_7H_6O_6S$), 250U ml^{-1} glutathione reductase (GR), 0.67 mg ml^{-1} DTNB, 0.67 mg ml^{-1} NADPH; GSSG: 0.1M KPO_4 buffer pH 7.5 at ambient temperature (24°C), 5mM EDTA, 0.1% Triton X-100, 0.6% $C_7H_6O_6S$, 250U ml^{-1} glutathione reductase (GR), 0.67 mg ml-1 DTNB, 0.67 mg ml^{-1} NADPH, 200 μl ml^{-1} triethanolamine, 111.1 μl ml^{-1} 2-vinylpyridine. The standard curve for GSH was prepared by adding GSH to 0.1M KPO4 buffer pH 7.5 at 24°C, 5 mM EDTA, hereafter KPE buffer (1 mg ml^{-1}) to final concentrations of 0, 0.41, 0.81, 1.63, 3.25, 6.5, 13 and 26 nmol ml^{-1} GSH. Standard curves for GSSG were prepared by adding GSSG to KPE buffer (2 mg ml^{-1} to final concentrations of 0, 0.21, 0.41, 0.81, 1.63, 3.25, 6.5 and 13 nmol ml^{-1} GSSG. Sample homogenate was not further diluted for GSSG assays but was diluted 5 times for GSH activity, and V_{max} was measured spectrophotometrically at 412 nm for 5 minutes for both GSH and GSSG. Activity was expressed as nmol g^{-1} thorax tissue using the calibration curve.

4.3.6 Data analysis

Data were analysed using R language environment (v 4.1.0, R Development Core Team, 2021) and RStudio (v1.4.1717). Normality assumptions were evaluated using the Shapiro Wilks test and homogeneity of residuals assumptions were verified by the Levene test.

The effects of Ce dose on energy stores (lipid glycogen, free carbohydrate and protein) and biochemical markers (CS, CAT, GST, LPO, GSH, GSSG) were evaluated using ANOVA with Dunnett post hoc to determine which doses differed from controls when model assumptions could be met. Lipid, thoracic protein, CAT, GST, GSH and GSSG data were log transformed to resolve normality of residuals. A Kruskal-Wallis non-parametric analysis with Dunn post hoc test was used for glycogen stores as issues of normality of residuals could not be resolved with data transformation.

4.4 Results

4.4.1 Energy reserves

Lipid content was not impacted by Ce dose ($F_{3, 36}$= 0.77, p=0.518, Figure 4.1). Glycogen content was variable but did not change with increasing dose (χ^2=0.23; 3 d.f., p=0.973, Figure 4.1) nor did free carbohydrates ($F_{3, 30}$= 0.20, p=0.892, Figure 4.1). Neither abdominal protein ($F_{3, 36}$= 1.43, p=0.248, Figure 4.1) nor thoracic protein ($F_{3,34}$=0.64, p=0.593, Figure 4.2) were affected by exposure to Ce.

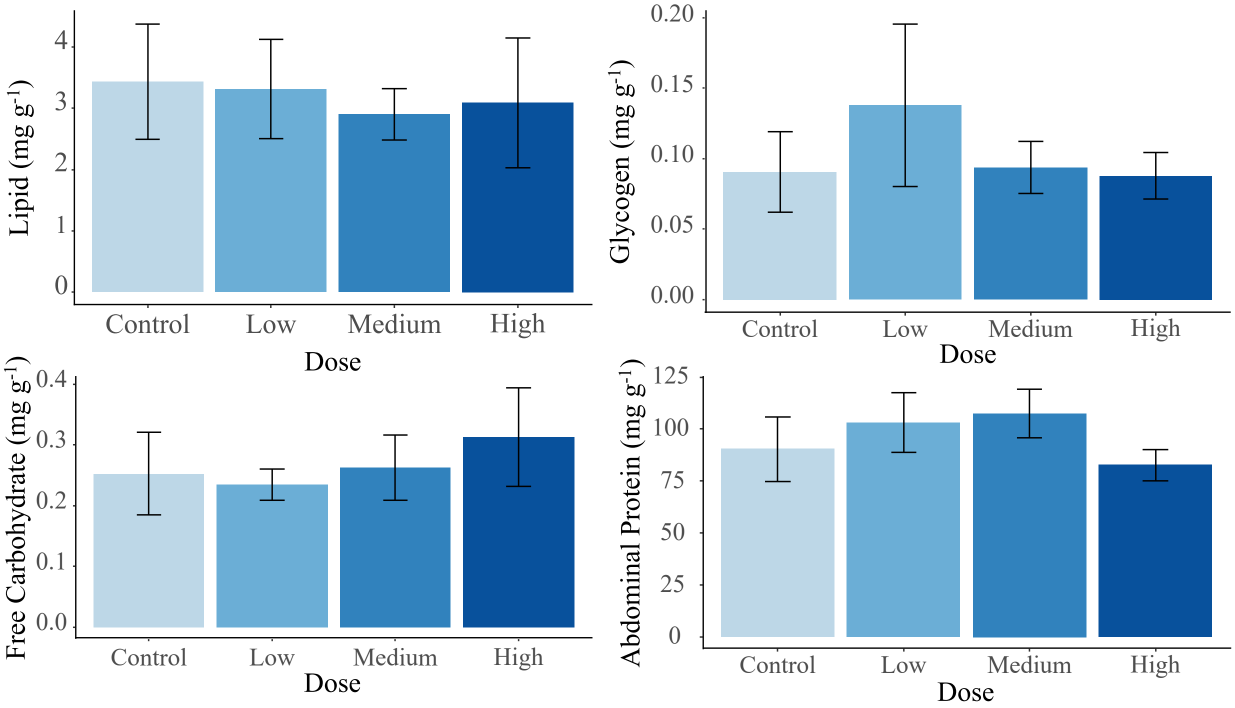

Figure 4.1 Mean energy stores (± standard error) (lipid, glycogen, free carbohydrate and protein) in the abdomen of adult female *Melanoplus sanguinipes* exposed to cerium (Ce) in their diet from egg hatch until reproductive maturity. Measured doses in diet (in mg kg^{-1}) were: Control = 0.41, Low = 37.3, Medium = 202.0, High = 894.0.

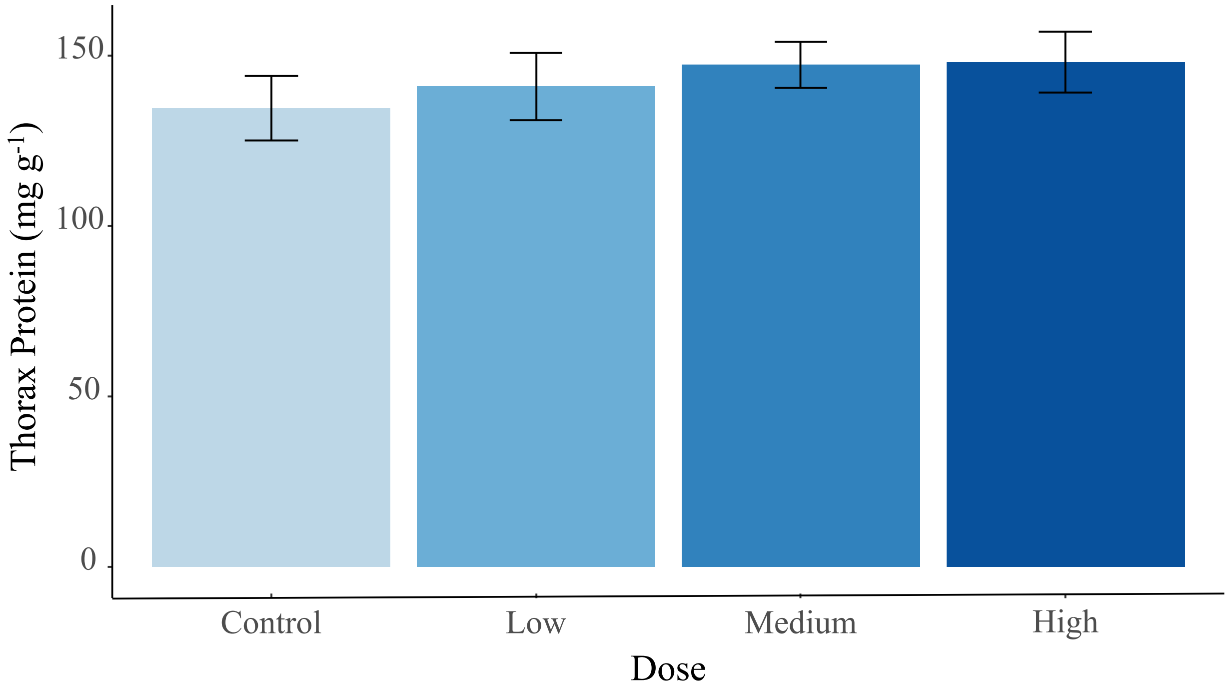

Figure 4.2 Mean protein content (± standard error) in the thorax of adult female *Melanoplus sanguinipes* exposed to cerium (Ce) in their diet from egg hatch until reproductive maturity. Measured doses in diet (in mg kg^{-1}) were: Control = 0.41, Low = 37.3, Medium = 202.0, High = 894.0.

4.4.2 Metabolic activity and biochemical markers

Citrate synthase activity shows a trend of increased activity at low doses of Ce ($F_{3,34}$=2.63, p=0.066, Figure 4.3). Post hoc analysis showed that activity at the lowest dose was significantly higher than that of the controls (p=0.0257). Neither CAT ($F_{3,34}$=1.07, p=0.373, Figure 4.3) or GST ($F_{3,34}$=0.64, p=0.595, Figure 4.3) activity was elevated in response to Ce ingestion.

Redox balance was not affected by exposure to Ce ($F_{3,36}$=0.65, p=0.589, Figure 4.4) and levels of lipid peroxidation did not differ between doses and the controls ($F_{3,24}$=0.61, p=0.613, Figure 4.4).

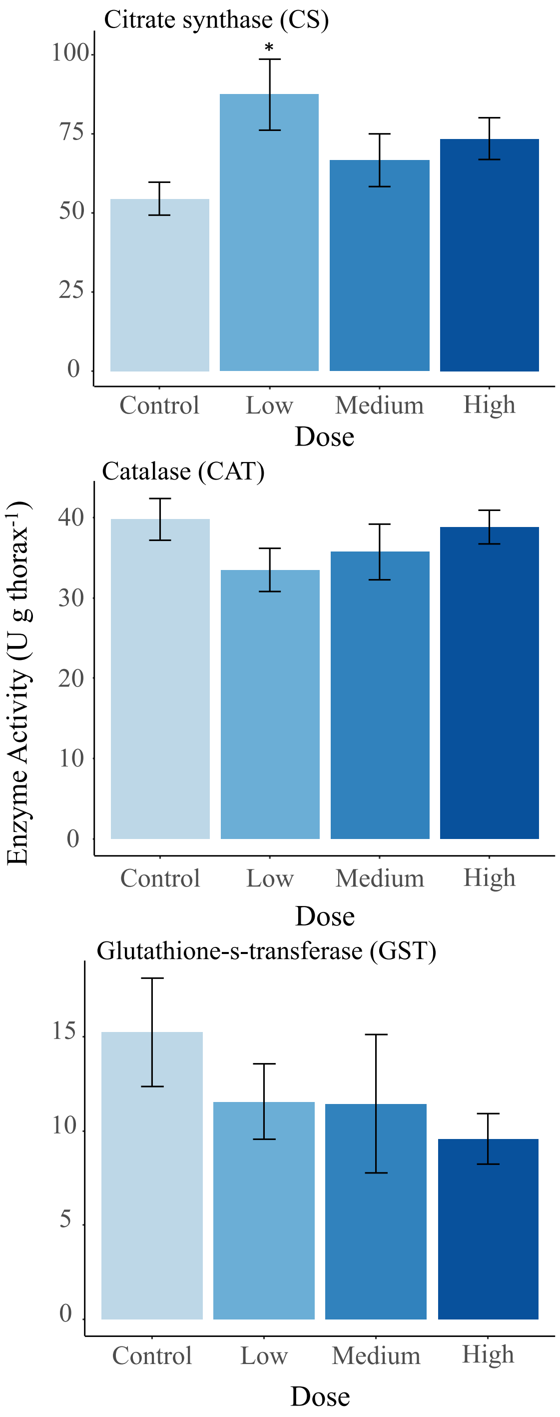

Figure 4.3 Mean (± standard error) metabolic capacity (citrate synthase, CS), antioxidant defenses (catalase, CAT) and biotransformation defenses (glutathione-s-transferase, GST) defences of adult female *Melanoplus sanguinipes* exposed to cerium (Ce) in their diet from egg hatch until reproductive maturity. Activity is reported in U g^{-1} of thorax tissue where 1 U = 1 µmol substrate min^{-1}. (*) represents significantly different dose ($p=0.0257$). Measured doses in diet (in mg kg^{-1}) were: Control = 0.41, Low = 37.3, Medium = 202.0, High = 894.0.

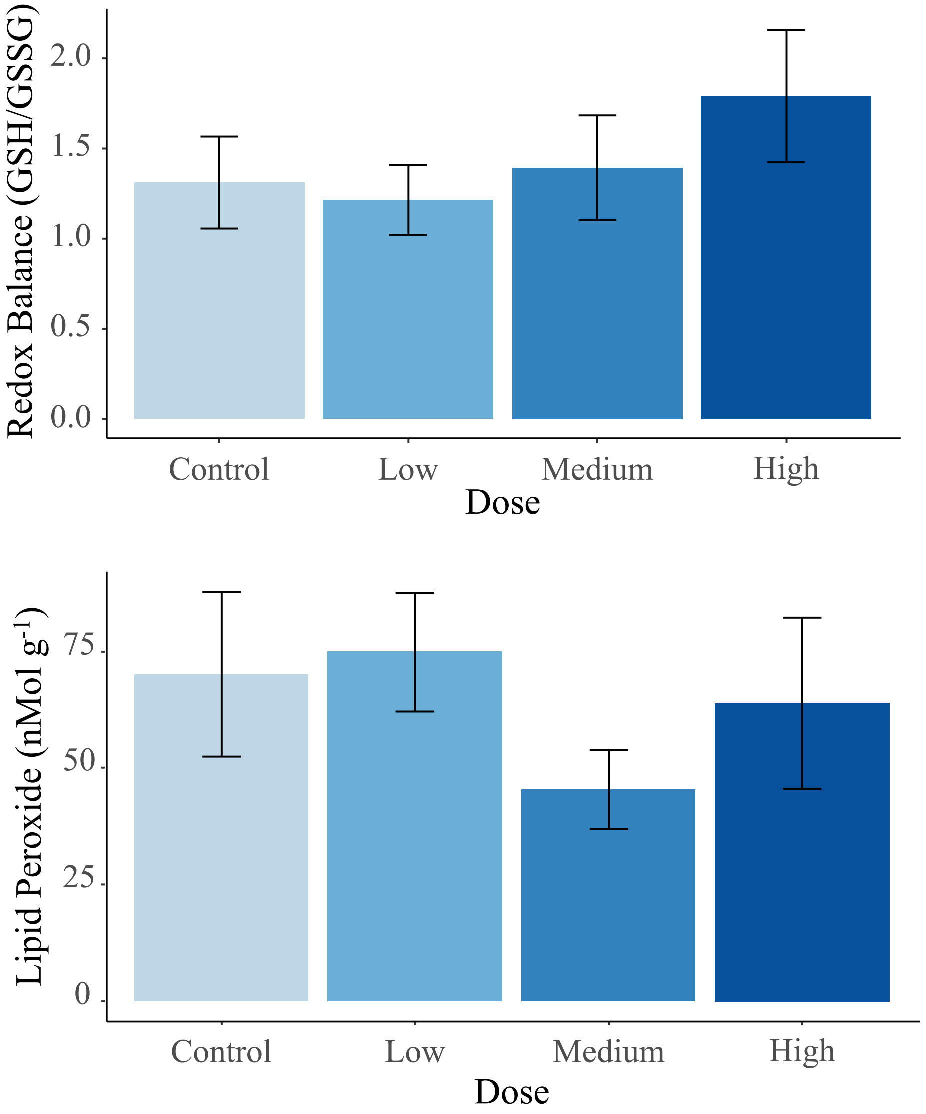

Figure 4.4 Mean (± standard error) redox balance (GSH:GSSG) and lipid peroxidation (LPO) of adult female *Melanoplus sanguinipes* exposed to cerium (Ce) in their diet from egg hatch until reproductive maturity. Measured doses in diet (in mg kg^{-1}) were: Control = 0.41, Low = 37.3, Medium = 202.0, High = 894.0.

4.5 Discussion

Increased energy expenditure to detoxify ingested metals may be reflected in decreases in energy reserves (Lagadic et al., 1994; Wang et al., 2020). The insect fat body is an organ responsible for the release of the two main energy reserves, glycogen and fat, used to fuel many biological processes, including growth and reproduction (Arrese & Soulages, 2010). Glycogen, a stored form of glucose, is readily mobilized and reserves can be depleted in response to increased energy demands associated with stress (Lagadic et al., 1994). For example, exposure to cadmium resulted in fat body injury and reduced glycogen stores in *Locusta migratoria* (Martoja et al., 1983). Protein damage can occur under extreme or prolonged stress from chronic exposure to toxins (Freitas et al., 2020). Free carbohydrates (glucose) concentration can also vary in response to stress, although the transitory nature of free glucose concentrations limits its usefulness as a stress indicator (Lagadic et al., 1994). Increases in metabolic rates have also been documented for organisms living in metal polluted environments and are thought to be linked to increased energy demands for detoxification (Lagisz et al., 2005). Citrate synthase is a pace making enzyme and the first step of the tricarboxylic acid (TCA) cycle (Wiegand & Remington, 1986). Increases in CS activity can indicate that aerobic capacity has increased and can be used to meet increased energy demands.

Although they possess efficient excretion capabilities, *M. sanguinipes* accumulate REEs from their diet that must be actively detoxified (Chapter 3). I hypothesized that chronic exposure to Ce would be energetically costly to detoxify resulting in decreased energy reserves and increased energy production to meet demand for detoxification.

Contrary to expectation, energy stores in reproductively mature females (lipid, glycogen, free carbohydrate and protein) were unaffected by exposure to Ce. Aerobic capacity (citrate synthase activity) was increased at the lowest dose tested but did not stay elevated as dose increased. This suggests that detoxification of ingested Ce did not exert an energetic burden on exposed females.

These results are in direct contrast with research with freshwater invertebrates. Mussels (*Mytilus galloprovincialis*) exposed to neodymium show lowered glycogen content compared to controls and increased metabolic capacity at low doses (Freitas et al., 2020). While female *M. sanguinipes* in this study had increased CS activity compared to controls, activity levels did not stay elevated as dose increased. While females had the capacity for increased energy production, further experiments are needed to determine if energy production is increased following Ce exposure. Mussels exposed to REES would be in constant contact with contaminated water, perhaps increasing the urgency of detoxification. *Melanoplus sanguinipes* females by contrast excretes most of the ingested contamination and may be able to avoid increased energy expenditure on detoxification. However, females also accumulated Ce at a higher level than males exposed to the same dose. Characterization of lipid and glycogen stores in adult males would indicate if males deplete energy reserves in favour of detoxification of ingested Ce.

An increase in metal accumulation can also lead to the production of reactive oxygen species (ROS). Many insects have developed mechanisms to resist toxic substances, and these enzyme activity levels are important for detoxification (Wang et al., 2020). Detoxification of metals can be accomplished through biotransformation, a process by which ingested metals are converted to less harmful substances that can be excreted. GST is one such enzyme that conjugates glutathione to a number of toxins, including metals, as well as products of oxidative stress induced by excess ROS (Abdelfattah et al., 2021). GST activity is often increased in the presence of pollutants to protect cells from damage (Lagadic et al., 1994). Antioxidant enzymes like catalase (CAT) resist oxidative stress from ROS (Ahmad, 1995). I tested the hypothesis that increasing accumulation of Ce would cause increased reactive oxygen species that would result in elevated levels of antioxidant and biotransformation defenses. However, female *M. sanguinipes* did not mobilize antioxidant and biotransformation defenses in response to Ce exposure. This result is surprising given that these defenses have been mobilized in other species exposed to REEs. For example, CAT levels were elevated in *Mytilus galloprovincialis* in response to neodymium (Freitas et al., 2020) and in *Drosophila melanogaster* exposed to low doses of Ce, with declines in activity at higher doses (Huang et al., 2010). It is possible that CAT and GST do not play a role in detoxification of ingested Ce for this species. For example, CAT and GST levels in the heteropteran, *Oncopeltus fasciatus* were unaffected by exposure to cadmium (Cervera et al., 2003). However, glutathione reductase activity was induced, suggesting that glutathione was involved in protection against oxidative damage caused by cadmium (Cervera et al., 2003). Ahmad and Pardini (1990) suggest that superoxide dismutase (SOD) activity is the main response to prooxidant exposure in insects. Further experiments with other antioxidant and biotransformation enzymes might provide a better understanding of the mechanisms used by *M. sanguinipes* in response to ingested Ce.

Similarly, females did not show a loss of redox balance, nor was there evidence of lipid peroxidation in response to Ce ingestion. Like CAT, the reduced form of glutathione (GSH) also scavenges ROS (Couto et al., 2016) resulting in the oxidation of GSH to GSSG (Ahmad, 1995). Redox balance (the ratio between reduced (GSH) and oxidized glutathione (GSSG)) can be used to identify and estimate levels of oxidative stress (Freitas et al., 2020). If not eliminated, excess ROS that react with lipids contained in cell membranes cause lipid peroxidation (Ahmad, 1995). This damage leaves cells unable to control the efflux or influx of substances crucial to normal cell physiology (Ahmad, 1995). Lipid peroxidation can be particularly damaging to insects not only because lipids are essential to maintain cell membrane integrity, but also because it can impair a number of physiological functions that depend on lipids (e.g., synthesis of cuticular hydrocarbons to prevent desiccation, ecdysone synthesis) resulting in impaired development and poor survival (Ahmad, 1995).

My results are in direct contrast to findings with mussels (*Mytilus galloprovincialis*) exposed to neodymium where declines in redox balance were seen (Freitas et al., 2020). Similarly, increases in LPO have been found in and *Drosophila melanogaster* exposed to Ce (Huang et al., 2010) and in *Mytilus galloprovincialis* exposed to neodymium (Freitas et al., 2020). Glutathione reductase (GR) is the enzyme responsible for converting oxidised glutathione back to its reduced form. Assessing levels of GR in *M. sanguinipes* exposed to Ce might clarify if activity levels are increased to maintain redox balance.

It is worth noting that in both the aforementioned experiments, the tissue tested for LPO was in direct contact with the ingested REE. The tissues of mussels submerged in contaminated water are in constant contact with Nd. Similarly, *Drosophila melanogaster* digestive tissue, which would have been in direct contact with ingested Ce, were included in characterization of LPO levels. In this experiment, thorax muscle tissue was used to determine levels of antioxidant and biotransformation defenses, lipid peroxidation and redox balance. Future experiments should examine enzyme activity, levels of LPO and redox balance in tissues in direct contact with ingested Ce, e.g., the digestive system.

4.6 Conclusion

With rising environmental levels of REEs, there is a need to better understand the impact this may have on exposed wildlife. REEs accumulated in soils of heavily polluted areas are taken up by plants and passed on to organisms feeding on contaminated tissues. Terrestrial invertebrates, particularly insects, may be at increased risk of exposure and are known to accumulate ingested REEs. The fate of accumulated REEs and the role of detoxification system has not been well established for terrestrial invertebrates.

In this experiment, female *M. sanguinipes* energy reserves were not impacted by chronic exposure to Ce. At the lowest dose, metabolic capacity was increased, although further experiments are needed to determine if energy production was increased. Antioxidant and biotransformation defenses were not mobilized in response to Ce exposure and redox balance was unaffected suggesting ROS were not increased. Testing with a wider range of antioxidant and biotransformation defenses would provide a better understanding of the mechanisms used by this species to detoxify ingested Ce and maintain redox balance. Similarly, there was no indication of lipid peroxidation in the tissue tested. Future experiments with tissues in direct contact with ingested Ce might provide a better indication of the damage, if any, following exposure to Ce.

Chapter 5 Synthesis and conclusions

The rare earth elements (REEs) are contaminants of emerging concern. Demand for REEs continues to grow for use in emerging technologies and has led to increased mining activity worldwide (Long et al., 2012). Pollution from mining and processing these elements is expected to rise. Along with mining waste, landfill waste from discarded technologies is causing an increase in levels of REEs in the environment. In terrestrial environments, phosphate fertilizers rich in REEs continue to be applied in agricultural systems. Cerium compounds are widely used to increase yield and quality of crops in China (Huang et al., 2010). Many rare earth elements are considered to be environmentally safe and are used in agricultural and medical applications. They are key components of phosphate fertilizers, lanthanum carbonate is prescribed in cases of kidney failure (Mehrotra et al., 2008) and cerium, neodymium, yttrium, and ytterbium are added to cardiovascular stents to decrease degradation rates (Drynda et al., 2009). Nonetheless, little is known about the long-term consequences of exposure to REEs, especially in terrestrial systems where pollution originates.

Of significant economic importance are neodymium (Nd) and the most abundant of the REEs, cerium (Ce). Neodymium-iron-boron permanent magnets are among the strongest magnets and are used in MRI machines and electronics (Stone, 2009), hybrid-electric vehicles (approximately 1 kg per 5 vehicles) and wind turbines (approximately several hundred kg per turbine) (Chu, 2010). Cerium (III) oxide is also commonly used in the manufacture of catalytic converters and as a fuel additive to reduce carbon monoxide emissions (Chu, 2010; Sabiha-Javied et al., 2010).

Plants accumulate REEs, including Ce and Nd from contaminated soil (Carpenter & Boutin, 2013; Thomas et al., 2014). As primary ecosystem producers, plants represent a direct entryway for contaminants into the food chain. Invertebrate species feeding on plant tissues with elevated levels of REEs may likewise accrue these metals in their bodies. Insects have a dual role not only as primary consumers, but also as a food source for higher trophic levels. Accumulation of REEs in insects may represent a direct route of exposure for species feeding on contaminated prey with unknown consequences.

The impact of REEs, and more specifically of Ce and Nd, on terrestrial insects are lacking in the scientific literature. Shortened lifespan and oxidative stress has been demonstrated in *Drosophila melanogaster* exposed to cerium (Huang et al., 2010). Accumulation of REEs was detected in aphids (*Rhopalosiphum nymphaeae*) consuming contaminated water lilies (*Nymphaea odorata*) (Cowgill, 1973) and decreased soil macrofauna diversity has been linked to areas of REE contamination (Li et al., 2010). More recently, MacMillan et al. (2017) reported high concentrations in terrestrial invertebrates sampled in arctic ecosystem. Further evidence of accumulation, and more importantly the consequences of exposure to REEs for insect growth, reproduction, health, and survival is lacking in the literature.

In this thesis, the terrestrial grasshopper *Melanoplus sanguinipes* (Fabricius) (Orthoptera: Acrididae) was used to fill in some of these knowledge gaps. Acridids have been well studied in toxicity literature, as the focus of pesticide, heavy metal, and trace metal research. Given that grasshoppers form 20-30% of the biomass available for consumption in healthy ecosystems (Schmidt, 1986), factors that affect grasshopper population can produce negative impacts on the ecosystem if vertebrate populations lose this valuable source of animal protein.

Evidence from this work shows *M. sanguinipes* not only consumes REEs indiscriminately, but also accumulate ingested REEs in their bodies with consequences for survival and health. With environmental levels set to rise well beyond historic levels, these findings suggest several important consequences not only for species health and survival but also ecosystem health.

5.1 Acute exposure to high levels of cerium and neodymium

While environmental levels of REEs continue to rise, it is unclear from the literature if this has an impact on terrestrial species. It has been well established in the literature that plants accumulate REEs from the soil (Carpenter & Boutin, 2013; Ichihashi et al., 1992; Thomas et al., 2014) among others. In many places, phosphate fertilizers rich in REEs are used to increase plant yield. It is unclear if insects will consume REEs and what consequences might follow ingestions. Research with heavy metals suggests that acute toxicity is generally low (Posthuma & Van Straalen, 1993), and that excretion is an essential route for terrestrial invertebrates to rid themselves of ingested metals (Dallinger, 1993).

In chapter 2, I established that *M. sanguinipes* consumes both Ce and Nd indiscriminately when presented with a contaminated diet and further that metal accumulation in the body increases with dose. Consistent with heavy metal research, the concentrations in insect frass were much higher than in the body and acute effects were few. However, two important findings resulted from this research. First, that even extremely high levels of Ce or Nd in the diet did not affect survival over a short (96-hour) exposure period. While the body concentration continued to rise with dose, grasshoppers survived the exposure. This has implications for species consuming grasshoppers as prey items. Species like *M. sanguinipes* may represent a source of REE pollution for birds and small mammals that might otherwise not be at risk of exposure.

The second important finding from this experiment was that exposure to high levels of Ce resulted in paralysed limbs (legs and antennae). REEs, including Ce, have the same atomic radius and share similar chemical properties with ions like calcium, magnesium, iron and manganese (Evans, 1983) and can substitute themselves for other metallic ions, a result already established in studies with plants (Babula et al., 2008; Liu & Hasenstein, 2005; Pickard, 1970; Yang et al., 2015). Blocking calcium uptake can lead to muscle membrane depolarization, resulting in sustained muscle contraction or tetanic paralysis. Among animals this has been observed in frog (*Rana temporaria*) sartorius muscle fibres (Glavinović et al., 1989) and in snake (*Thamnophis sp*.) muscle fibres (Coniglio et al., 1993) treated with lanthanum. Among insects, this effect has been observed in cockroach (*Periplaneta americana*) leg muscles treated with lanthanum (Washio & Miyamoto, 1983). My work in Chapter 2 has established this effect in vivo with *M. sanguinipes* exposed to high concentrations of Ce. Reduced ability to move can affect an individual's mass over the long-term due to reduced ability to forage. Paralysed individuals may also find themselves more susceptible to predation, increasing the risk of exposing other trophic levels. It remains unclear if Ce or Nd are substituting themselves for other metallic ions in insects. Further work needs to be done to determine which physiological processes are affected and the range of concentrations and exposure periods that produce effects.

5.2 Whole organism effects of REE exposure

REEs like Ce and Nd are persistent pollutants and effects on organisms may be underestimated by short term exposure studies. Research with heavy metals suggests that the consequences of living in a metal polluted environment can vary depending on the metal and species tested. Like heavy metals, acute toxicity of Ce and Nd to *Melanoplus sanguinipes* was generally low, although longer term exposure can have more subtle impacts on growth, development, reproduction, and survival.

Accumulation in the body occurred at all doses. Concentrations were lower in the body than in the contaminated food, suggesting the risk of biomagnification is low. However, in Chapter 3, survival was negatively impacted for Ce exposed individuals. Females carried a higher body concentration of Ce, while males carried a higher body concentration of Nd. Males and females responded differently to REE pollution. While fewer females were produced, their mass and development times were normal. Males in contrast experienced developmental delays and reduced adult mass. Adult lifespan was also reduced by exposure to Ce. These findings can have broader implications for populations if fewer individuals are available to contribute to the next generation. The number of eggs produced by exposed females was not impacted by exposure to Ce, but further research is needed to understand the viability of eggs produced by exposed females, and the impact on the health of emerged offspring.

Importantly, my work in Chapter 3 suggests that classical toxicity testing, focusing on 50% reductions in growth and survival over a portion of the life cycle of exposed species would miss these more subtle effects. Differences in patterns of accumulation and effects between females and males, and between REEs, as well as the possibility of effects on subsequent generations highlights the need for more thorough investigation of persistent pollutants like REEs, individually and in mixture, over longer developmental periods as they are encountered in the environment.

5.3 Subcellular effects

The impact of exposure to pollutants can be assessed over the full spectrum of biological organization, from the subcellular to ecosystem level. With this increasing biological organization comes increased realism of the experiments, at odds with the repeatability and interpretation of the data (Persoone and Janssen, 1994). Many toxicological studies focus on whole organism effects investigating survival and growth. Subcellular experimentation has the advantage of short exposure periods but must be linked with higher those levels of organization to be ecologically relevant (Coen & Janssen, 1997). For example, changes in energy reserves can be used biomarkers of the sublethal changes that occur following exposure to toxins (Lagadic et al., 1994).

In chapter 4, an increase in aerobic capacity following chronic exposure to Ce was identified in females. Activity of key biotransformation enzymes was unaffected and there was no evidence of a loss of redox balance or cellular damage. The results suggest that detoxification of ingested Ce did not impose an energetic burden on exposed females. Given that females and males responded differently to Ce ingestion, further studies are needed to determine if and how males respond at the subcellular level. Experiments with Nd would help to understand if the differences in organismal level effects seen in Chapter 3 translate to differences in subcellular effects.

5.4 Future research directions

One of the challenges with assessing the risk of REEs for terrestrial invertebrates is the lack of available data. Little attention has been paid to REEs, likely because they are considered neither essential to life nor acutely toxic (Tyler, 2004). As a result, threshold limits and maximum permissible concentrations are poorly established in the literature. In the available studies, there are few acute effects in response to exposure lending itself to a no-effect perception surrounding the accumulation of REEs in terrestrial environments. The lack of data concerning background levels of REEs, levels in polluted ecosystems and the levels of contamination in plant tissues at contaminated sites further complicates any assessment of the environmental risk of REEs for terrestrial species. While my thesis has provided some insight into the accumulation and effects of exposure for terrestrial invertebrates, this has led to more questions that warrant further study.

5.4.1 Assessment of terrestrial species

In this thesis, there is evidence that herbivorous insects accumulate REEs from their diet (Chapter 2 and 3). Research with heavy metals suggests acute toxicity of metals is low (Posthuma & Van Straalen, 1993). Consistent with this, I found that female growth was inhibited by high doses of Nd, but there were few other whole organism level effects following acute exposure to Ce and Nd (Chapter 2). Nonetheless, accumulation occurred at all doses tested for acute (Chapter 2) and chronic (Chapter 3) exposure periods. Through all experiments, as dose increased, a greater portion of ingested contamination was incorporated into body, indicating the ingestion rate exceeded the excretion capability of adults.

While this work has contributed to our understanding of the risk of rising environmental levels of REEs, there are few other studies available for terrestrial invertebrates exposed to REEs. In my future research, I will focus on testing other species to understand if effects are consistent across species, sexes and REEs. A focus on species that may be more sensitive to metal pollution, those with alternate life histories (e.g., insects with a holometabolous development cycle (egg, larva, pupa, adult)) and those that may be at higher risk of exposure such a soil dwelling species (e.g., crickets) will be provide a better understanding of the risk of REE exposure for terrestrial invertebrates.

5.4.2 Population level effects

In my third chapter, an initial assessment of reproductive potential was made to look for links between exposure to REEs and reduced fertility. Contaminated substrate did not alter the number of emerged nymphs, but it’s been hypothesized that larger egg volume and the foam surrounding the eggs of grasshoppers can offer protection from contaminants in substrate (Devkota & Schmidt, 1999). In chapter 3, females showed a much higher concentration of Ce than did males. The possibility that females use their eggs as a means of elimination of metals has been raised (Schmidt et al., 1991b). In this study we have no information about the viability and Ce concentration of eggs produced by exposed females, but their higher body concertation raises the possibility that their eggs may be contaminated.

In my future work, the viability of eggs in contaminated soil will be thoroughly investigated for species with different approaches to oviposition, e.g., species that lay eggs directly in substrate with no protective foam casing. Similarly, I would like to continue to work with the F2 generation to understand the impact of reproductive potential following exposure to REEs. I believe that understanding the viability of eggs laid by exposed females and the concentration in eggs and in emerged offspring will provide critical information about the long-term consequences of REEs for populations of terrestrial invertebrates. These effects in turn have consequences for species consuming terrestrial invertebrate as prey and may suffer from a loss of this valuable source of protein.

5.4.3 Ecosystem level effects

Terrestrial invertebrates play dual role in ecosystems, acting as both primary consumers and as a food source for higher trophic levels. In chapter 3, the results indicate the risk of biomagnification of REEs may be low. Concentrations in exposed individuals were consistently lower than the offered food (Chapter 3). Nonetheless, accumulation occurred at all doses, and increased with increasing dose. This opens the possibility that terrestrial species like *M. sanguinipes* may become a source of pollution for species consuming them as prey. MacMillan et al. (2017) identified low levels of REEs in muscle tissue of wild caught species of vertebrates with higher concentrations in the liver (eiders, ringed seal, caribou, ptarmigan and snowshoe hare). Continuing this line of inquiry, I would like to understand how REEs might cycle through ecosystems using a mesocosm approach to look at the risk of transfer between trophic levels.

5.4.4 Fate of ingested REEs

Due to their close association with the environment, invertebrates are uniquely at risk from pollutants (McClellan-Green & Romano, 2007). Chapter 2 and 3 identified that *Melanoplus sanguinipes* consumes REEs indiscriminately and can accumulate high concentrations in their body, however the fate of REEs in the body remains unclear. Interestingly, ingestion of high levels of Ce over a short duration induces muscle paralysis in the legs and antennae of this species (Chapter 2). This is thought to be a result of REEs blocking calcium channels resulting in tetanic paralysis. This suggests that at least some of the ingested Ce is in the muscle tissue but does not provide a complete picture of the tissues affected.

Many insects have detoxifying mechanisms, transporters, immunity and peritrophic membranes to resist toxic substances (Wang et al., 2020). While detoxifying processes may limit the toxicity of ingested metals, they are considered to be energetically costly. Resulting changes in energy reserves are biomarkers of the sublethal changes that occur following exposure to toxins (Lagadic et al., 1994). In chapter 4, slight reductions in lipid and protein reserves may be indicative of shifting energy budgets to detoxify ingested Ce.

The role of detoxification systems has not been well established for REEs. This thesis adds to our understanding of this process. However, I was limited to testing only females and limited in the range of antioxidant and biotransformation defenses that could be tested. Catalase (CAT) and glutathione-s-transferase (GST) were selected for assays. While CAT was unaffected by Ce dose, GST levels showed a slight decline (Chapter 4). It is possible that these enzymes don’t play a role in the detoxification of Ce. Alternately, it is possible that Ce ingestion damages proteins and interferes with the function of detoxification enzymes. Given that males experienced stronger effects from ingesting Ce at the whole organism level (Chapter 3), a more complete evaluation of both females and males is needed. In my future research, I will further explore this question of the fate of REEs in the body, the mechanism of toxicity and which, if any, defenses are mobilized in response. Given that effects differed between females and males at the whole organism level, these studies would provide a better understanding of the link between subcellular effects and the consequences at the whole organism level.

5.5 Conclusions

With environmental levels of REEs set to rise, there is a need to better understand the impact this may have on exposed wildlife, particularly in understudied terrestrial systems. Terrestrial invertebrates, such as insects, may be at increased risk of exposure. While research has been done to assess the impact of REEs on wildlife, my thesis work suggests more research is needed to understand the fate and effect of REEs in terrestrial species. There is a need not only for testing REEs individually but also in mixture with other REEs, as they are encountered in agricultural systems and polluted areas. Sublethal endpoints such as locomotion, reproduction, energy stores, redox balance should be incorporated into toxicity testing to understand the full impact of persistent pollutants on individuals and populations. Further, the cycling of REEs in terrestrial systems, from plants to invertebrates and higher trophic levels could help identify species at risk and the unexpected consequences of exposure. By continuing to add to our understanding of the fate and effect of ingested REEs, including sublethal effects, we can evaluate the risk of exposure for terrestrial species.

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Appendices

Appendix A Quality control for chemical analysis of *Melanoplus sanguinipes* bodies, intestines, frass and diet (*Lactuca sativa* leaves) following acute exposure to cerium, neodymium or zinc.

Table A.1 Accuracy and precision of the chemical analysis by inductively coupled plasma mass spectrometry (ICP-MS) of cerium (Ce), neodymium (Nd) or zinc (Zn) content in *Melanoplus sanguinipes* bodies, intestines, frass and diet (*Lactuca sativa* leaves). Analysis was performed by Brooks Rand Labs (now Brooks Applied Labs, Seattle, WA, USA) following the US Environmental Protection Agency (USEPA) Method 1638 (US Environmental Protection Agency, 1996).

Matrix	Analyte	Description	Native Sample (mg kg^{-1})	Spike (mg kg^{-1})	Results (mg kg^{-1})	Recovery %	Limits %	RPD	Limits %	Batch - Sample
Biota	Ce	Laboratory Fortified Blank 1402005		30.09	28.26	94%	75-125			B140017-BS1
Biota	Ce	Certified Reference Material (0822035, NIST 1547 - peach leaves)		10	9.02	90%	N/A			B140017-SRM1
Body (Female)	Ce	Duplicate 1351036 - 11	0.22		0.22			1%	30	B140017-DUP4
Body (Female)	Ce	Matrix Spike 1351036-11	0.22	80.2	82.21	102%	70-130			B140017-MS4
Body (Female)	Ce	Matrix Spike Duplicate 1351036-11	0.22	123.2	125.1	101%	70-130	41%	30	B140017-MSD4
Body (Female)	Ce	Duplicate 1351036-12	3.75		3.43			9%	30	B140017-DUP5
Body (Female)	Ce	Matrix Spike 1351036-12	3.75	124	131	103%	70-130			B140017-MS5
Body (Female)	Ce	Matrix Spike Duplicate 1351036-12	3.75	113.9	117.5	100%	70-130	11%	30	B140017-MSD5
Body (Female)	Ce	Duplicate 1351036-13	23.63		11.71			68% *	30	B140017-DUP6
Body (Female)	Ce	Matrix Spike 1351036-13	23.63	130.6	151.1	98%	70-130			B140017-MS6

Matrix	Analyte	Description	Native Sample (mg kg^{-1})	Spike (mg kg-1)	Results (mg kg^{-1})	Recovery %	Limits %	RPD	Limits %	Batch - Sample
Body (Female)	Ce	Matrix Spike Duplicate 1351036-13	23.63	127.1	142.8	94%	70-130	6%	30	B140017-MSD6
Biota	Zn	Laboratory Fortified Blank (1330042)		150	137.8	92%	75-125			B131919-BS1
Biota	Nd	Laboratory Fortified Blank (1330042)		500	467.3	93%	75-125			B131919-BS2
Biota	Zn	Certified Reference Material 1245076, DORM-4)		52.2	49.33	95%	75-125			B131919-SRM1
Biota	Zn	Certified Reference Material 1201032, DOLT-4 Dogfish Liver Certified for TM)		116	111.3	96%	75-125			B131919-SRM2
Biota	Nd	Laboratory Fortified Blank (13344061)		150	144	96%	75-125			B131977-BS1
Biota	Zn	Laboratory Fortified Blank (13344061)		150	160.1	107%	75-125			B131977-BS1
Biota	Zn	Certified Reference Material 1245076, DORM-4)		52.2	50.87	97%	75-125			B131977-SRM1
Biota	Zn	Certified Reference Material 08222035, NIST 1547 - peach leaves)		17.9	19.7	110%	75-125			B131977-SRM2
Diet (*Lactuca sativa*)	Nd	Duplicate	0.19		0.21			8%	30	B131977-DUP1
Diet (*Lactuca sativa*)	Nd	Matrix Spike	0.19	307.4	307.4	100%	70-130			B131977-MS1
Diet (*Lactuca sativa*)	Nd	Matrix Spike Duplicate	0.19	284.1	259.4	91%	70-130	17%	30	B131977-MSD1
Diet (*Lactuca sativa*)	Nd	Duplicate	1549		2002			25%	30	B131977-DUP2
Diet (*Lactuca sativa*)	Nd	Matrix Spike	1549	316.5	1628	NR	70-130			B131977-MS2
Frass	Nd	Duplicate	1.49		8.01			137 %	30	B131977-DUP3

Matrix	Analyte	Description	Native Sample (mg kg^{-1})	Spike (mg kg-1)	Results (mg kg^{-1})	Recovery %	Limits %	RPD	Limits %	Batch - Sample
Frass	Nd	Matrix Spike	1.49	298.8	298.1	99%	70-130			B131977-MS3
Frass	Nd	Matrix Spike Duplicate	1.49	285.2	293.7	102%	70-130	1%	30	B131977-MSD3
Body (Male)	Nd	Duplicate	ND		0.25			N/C	30	B131977-DUP4
Body (Male)	Zn	Duplicate	298.1		295			1%	30	B131977-DUP4
Body (Male)	Nd	Matrix Spike	ND	700.9	622	89%	70-130			B131977-MS4
Body (Male)	Zn	Matrix Spike	298.1	700.9	942.3	92%	70-130			B131977-MS4
	Nd	Laboratory Fortified Blank (1310038)		1000	981.5	98%	75-125			B140274-BS1
Female Body	Nd	Duplicate	0.74		1.71			80%	30	B140274-DUP5
Female Body	Nd	Matrix Spike	0.74	2295	2301	100%	70-130			B140274-MS5
Female Frass	Nd	Matrix Spike Duplicate	0.74	2314	2182	94%	70-130	5%	30	B140274-MSD5
Female Frass	Nd	Duplicate	7.16		8.61			18%	30	B140274-DUP6
Female Frass	Nd	Matrix Spike	7.16	2276	2214	97%	70-130			B140274-MS6
Female Frass	Nd	Matrix Spike Duplicate	7.16	2357	2271	96%	70-130	3%	30	B140274-MSD6

ND: Native sample results and/or associated duplicate result were below the method detection limit, relative percent difference was not calculated **(N/C)**

NR: Matrix was spiked at <= 25% of the native sample concentration, therefore the recovery was not reported (NR)

RPD: Relative Percent Difference

***** Duplicate precision (RPD) was not within acceptance criteria, results are estimated

Table A.2 Method blanks and reporting limits for chemical analysis by inductively coupled plasma mass spectrometry (ICP-MS) of cerium (Ce), neodymium (Nd) or zinc (Zn) content of *Melanoplus sanguinipes* bodies, intestines, frass and diet (*Lactuca sativa*). Analysis was performed by Brooks Rand Labs (now Brooks Applied Labs, Seattle, WA, USA) following the US Environmental Protection Agency (USEPA) Method 1638 (US Environmental Protection Agency, 1996). **MDL:** Method detection limit; **MRL:** Method reporting limit.

Batch-Sample	Matrix	Analyte	Results (mg kg^{-1})	Average	Limit	Standard Deviation	Limit	MDL	MRL
B140017-BLK1	Biota	Ce	-0.0002						
B140017-BLK2	Biota	Ce	-0.0001						
B140017-BLK3	Biota	Ce	-0.0002						
B140017-BLK4	Biota	Ce	-0.0002	0	0.01	0	0	0.003	0.01
B131919-BLK1	Biota	Nd 142	0.002						
B131919-BLK2	Biota	Nd 142	0.002						
B131919-BLK3	Biota	Nd 142	0.001						
B131919-BLK4	Biota	Nd 142	0.005	0	0.01	0	0	0.003	0.008
B131919-BLK1	Biota	Zn 66	0.19						
B131919-BLK2	Biota	Zn 66	0.06						
B131919-BLK3	Biota	Zn 66	0.08						
B131919-BLK4	Biota	Zn 66	0.03	0.09	1	0.07	0.2	0.2	1
B131977-BLK1	Biota	Nd 142	0.0004						
B131977-BLK2	Biota	Nd 142	0.0004						
B131977-BLK3	Biota	Nd 142	0.0005						
B131977-BLK4	Biota	Nd 142	0.0005	0	0.01	0	0	0.003	0.008
B131977-BLK1	Biota	Zn 66	0.08						
B131977-BLK2	Biota	Zn 66	0.18						
B131977-BLK3	Biota	Zn 66	0.07						
B131977-BLK4	Biota	Zn 66	0.09	0.11	1	0.05	0.2	0.2	1
B140274-BLK1	Biota	Nd 143	-0.003						
B140274-BLK2	Biota	Nd 143	-0.003						
B140274-BLK3	Biota	Nd 143	-0.003						
B140274-BLK4	Biota	Nd 143	-0.002	0	0.08	0	0.03	0.03	0.08

Appendix B Diet preparation for chronic exposure to either cerium or neodymium

Ingredients

100 ml dH_2O
300 mg Agar
100 g commercial Guinea pig food*
$CeCl_3 \cdot 7H_2O$ or $NdCl_3*6H_2O$ (hereafter Ce or Nd)

Equipment

10 labelled 60 x 15 mm Petri plates
100 ml graduated cylinder
250 ml flask or beaker
500 ml beaker (or larger)
Squeeze bottle of dH_2O
Hot Plate
Coffee grinder
Flexible forceps
Spoons for dispensing and stirring
3 Weigh dishes of 2 sizes (one large, two small)

Instructions

1. In the larger dish, weigh out ~100 g of guinea pig food pellets. Slightly more than 100 g is needed since there will be transfer loss from the grinder.
2. Grind the guinea pig food pellets in the coffee grinder. This may take several short bursts to reach a fine consistency. Remove any food that is well ground to another container and put the remaining chunks of food back in the grinder for another pass. Repeat as necessary until all the food is of the same, fine consistency.
3. Once the guinea pig food is ground to a fine consistency, add it to the 500 ml beaker.
4. Measure 100 ml of dH_2O using a graduated cylinder – add the water to a 250 ml flask or beaker.
5. Weigh out 300 mg of agar.
6. Add the agar to the water.
7. Mark the water height on the beaker.
8. Heat on the hot plate (maximum setting) until the agar starts to boil. Allow to boil for 1 minute. Watch carefully and turn off the heat and remove the flask from the hot plate once the mixture starts to rise – do not allow it to overflow the flask.
9. Allow the agar mixture to cool for 5 minutes.
10. For control diet, move on to step 14.

11. For dosed diet, weight out the necessary amount of Ce or Nd chloride for the dose you are preparing. Set aside.
12. Add the measured amount of Ce or Nd chloride to the flask. A small amount of dH_2O can be used from the squeeze bottle to dissolve the metal in the weigh dish so it is easily poured into the flask. Use a small amount of dH_2O from the squeeze bottle to rinse any remaining Ce or Nd in the weigh dish into the flask.
13. Swirl the flask around to mix the contents, ensuring all the Ce or Nd dissolves.
14. Refill the flask with dH_2O from the squeeze bottle until the water level is back to the original mark.
15. Pour the agar mixture into the beaker with the ground guinea pig food pellets. Pour slowly and mix as you go so the ground food at the bottom of the dish doesn't remain dry.
16. Add 5 g of the mixed diet to each petri dish and allow to cool completely.
17. Wrap each plate with Parafilm and put in the freezer until needed.

* Guinea Pig Food used in these experiments:

Living World Extrusion Diet for Guinea Pigs, Rolf C. Hagan Inc., Montreal, QC

Ingredients:

Dehydrated alfalfa meal, wheat middlings, soybean hulls, ground corn, soybean meal, vitamins (vitamin E supplement, vitamin B12 supplement, calcium L-ascorbyl-2-monophosphate, folic acid, niacin, d-calcium pantothenate, vitamin A supplement, riboflavin, pyridoxine hydrochloride, thiamine mononitrate, biotin, menadione sodium bisulfite complex (source of vitamin K activity), vitamin D3 supplement), minerals (cobalt sulfate, ferrous sulfate, magnesium sulfate, zinc oxide, sodium selenite, copper sulfate, manganous oxide, calcium iodate), lecithin, salt, calcium carbonate, dicalcium phosphate, yeast culture, ground flaxseed, DL-methionine, choline chloride, yeast extract, alfalfa nutrient concentrate, yucca schidigera extract.

With nutrient ratios of:

Crude Protein 15.0% (min), Crude Fat 3.0% (min), Crude Fibre 14.0% (max), Moisture 12.0% (max), Calcium 0.7% (min), Calcium 1.1% (max), Phosphorus 0.4% (min), Salt 0.6% (min), Salt 0.9% (max), Vitamin A 2,500 IU/lb (min), Ascorbic Acid (Vitamin C) 100 mg/lb (min), Vitamin D3 200 IU/lb (min), Vitamin E 10 IU/lb (min) (http://ca-en.hagen.com/Small-Animal/Nutrition/Extruded/60463, Last Accessed January 12th 2023).

Appendix C Rearing methods for *Melanoplus sanguinipes* colony

Acknowledgment

This *Melanoplus sanguinipes* colony was established in 2013 by donation of eggs from a colony at Agriculture and Agri-Food Canada's (AAFC) Saskatoon Laboratory. The AAFC colony was started and is maintained by Dr. Martin Erlandson.

Safety Precautions

Appropriate clothing and personal protective equipment must be worn when working with the grasshopper colony (see list of requirements).

Rearing Procedures

General Rearing Introduction

Grasshoppers are housed in wooden cages, with Plexiglas windows at the front and insect screening on the floor to allow frass to fall through. The screened floor sits above a wooden bottom that serves to contain the frass, food and sand wastes that fall through the screened floor. Each cage is equipped with an incandescent light bulb that allows the grasshoppers to bask and raise their internal body temperature. The cages are plugged into a timer providing 16:8 day:night cycle. The grasshopper colony should be maintained at or around 30°C. The colony consists of grasshoppers at all stages of development to ensure a healthy population. New hatchling cages are set up bi-weekly and cages are shut down once all juveniles have matured to the adult stage.

Figure C.1 *Melanoplus sanguinipes* colony rearing cages. Each wooden cage is fitted with a Plexiglass front, insect screening floor, a 40-watt incandescent lightbulb for warmth and perches for grasshoppers.

Feeding and Cleaning

Grasshopper cages are cleaned, and food is replenished on Monday, Wednesday and Friday. Wheat plants can be used when they are about 15 cm tall, approximately 7 days after planting. Each cage should get cuttings from 1-2 pots of wheat, depending on the age and size of the grasshoppers in the cage (e.g., a cage of newly emerged 1st and 2nd instar grasshoppers will eat less than a cage of 4th and 5th instars). Wash the cut wheat thoroughly to remove any traces of soil and allow it to dry. While the wheat is drying, it should be sprayed with the sulfa solution as a prophylaxis against fungal infection.

Grasshopper are fed green or red leaf head lettuce leaves. The amount of lettuce needed in each cage will vary based on the age and size of the grasshoppers (e.g., a cage of newly emerged 1st and 2nd instar grasshoppers will eat less than a cage of 4th and 5th instars). Lettuce should be thoroughly washed and dried before reaching the grasshopper cages. The biggest threats to our grasshopper colony are pesticide residues and nematode parasites from soil contamination on the purchased lettuce. Thoroughly washing the lettuce should help to reduce/eliminate these risks. Remove the outer lettuce leaves that are most at risk of contamination, and any leaves that are mouldy or otherwise unsuitable. Remove the thick midrib at the bottom third of the leaves as the grasshoppers do not generally eat this part and tear the remaining leaves into strips to be washed and dried.

While the lettuce and wheat are drying, put on a lab coat, gloves and a mask remove the old food from the insect cages. Starting with the youngest juvenile cage and work up in age until the adult cages. For each cage, open the lid carefully as there may be a number of grasshoppers at the top edges and on the inside of the lid. Collect any uneaten lettuce and wheat leaves. Remove the dish containing any remaining dry food mixture. If the hatchling cage has an egg pot(s), moisten the sand with deionized water. Check for first instar emergence in the hatchling cages. If first instars are seen, remove the covering on the egg cup to allow the young hoppers to escape. Mark the date of emergence on the cage.

Egg pots in the hatchling cage should be removed once enough hatchlings have emerged (usually 3-4 days after hatching begins). Remove the egg pot from the cage, ensuring that there are no juvenile hoppers either inside or on the outside of the pot. Once all egg cups have been removed, 1-2 clean sticks can be added to the cage for the hoppers to climb. Discard sand and wash the egg cups to be reused.

Check for adult emergence in the cage with the oldest nymph and move any freshly emerged adults to a breeding cage. If this is the first adult emergence in the cage, mark the date of first emergence on the cage label and set up a new breeding cage to house the adults. Freshly moulted adults should be allowed to dry their wings and harden before being moved to an adult cage – this can take several hours. Remove any dead adults from the adult cage and any dead nymphs, blackened or mouldy exuviae from the juvenile cages. If necessary, grasshoppers can be transferred to a new, clean cage. Record the date of transfer on the cage label and ensure that the label is moved to the new cage.

Once cages are cleaned, fill a clean dish with enough dry food mixture (80% oats, 15% bran, 5% skim milk powder) for a couple of days and place one in each cage. The amount of dry food will depend on the number and age of the grasshoppers in the cage. On Fridays, ensure there is enough for the weekend. Add the now dry wheat and lettuce to each cage. Take care not to crush any small or newly moulted individuals. Ensure that the food does not cover the egg pots in either the adult or hatchling cages.

Figure C.2 *Melanoplus sanguinipes* colony breeding cage with freshly added wheat, head lettuce and dry food mixture (80% oats, 15% bran, 5% skim milk powder) and oviposition substrate (sand).

Egg Laying

On Mondays, Wednesdays, and Fridays, new egg cups are prepared for the adult grasshopper cage(s). Egg cups consist of a 500 ml clear plastic cup filled about 1/3 of the way with the rocks from sand sifting, and then topped off with sifted sand and moistened with 80-100 ml of deionized water. There should be a 1-2 cm gap between the top of the sand and the top of the cup to allow room for emerging nymphs. Label the cup with the date using a waterproof marker. It is important to change the egg pots regularly as the females will not oviposit in a cup that they find to be overly full. Instead, egg pods will be deposited in other parts of the cage, and offspring will be lost. When an egg cup is removed from a cage, carefully pour off the frass from the top of the removed egg cup and cover the cup with plastic wrap. Poke several small holes in the plastic wrap to allow air circulation and secure with an elastic band. The egg cup should be dated and moved to the refrigerator (4°C) for up to 3 months before incubation or incubated right away.

Figure C.3 Cup filled with sifted sand and *Melanoplus sanguinipes* eggs laid by colony females.

Weekly Tasks

Wheat Planting

New trays of wheat are planted weekly, the number will depend how many cages are currently in use in the colony. In general, one tray of wheat will feed about 5-6 cages for 2 days. We use organic whole wheat seed, planted in 1-2 inches of pro-mix soil in 2 x 6 rectangular seedling pots. Fill each pot about halfway with lightly packed soil, pressing gently until the surface of the soil is level. For each pot, distribute wheat seeds evenly and densely over the surface of the soil. Place a small scoop of the remaining soil over the seeds. Spread the soil evenly to cover, but do not compact. There should be 1 – 2cm of space at the top of the pot to ensure the seeds are not washed out during watering.

New Hatchling Cage (new cage every other week)

Use a clean cage with a lid that closes securely. Check the fine screen mesh on the cage floor and replace if there are any holes. Add a 40-watt light bulb to the cage and plug in to a timer. Add 2-4 stored egg pots to the new hatchling cage and label with the date.

Adult Breeding Cage

Once a juvenile cage starts to produce adults we try to start a new adult cage to house them (depending on available space and available cages). Use a clean cage with a lid that closes securely and check screening for any holes. Add sticks for perches and a 40-watt incandescent light bulb to the cage (plug in to a timer). Make a new label for the cage with the date.

Sift Sand

Sand used for female oviposition should be sifted to remove any large rocks or debris that may damage the eggs. Ordinary playground sand can be sifted using fine mesh screening. The rocks can be kept and used to fill the bottom half of the oviposition cups and the sifted sand to top up the cups. Sift enough to fill three oviposition cups for the following week.

Wash Cages

It is important to wash the insect cages as soon as possible after removing all grasshoppers to minimize the dust, dirt and disease that can spread between colony cages. Remove the light bulb and everything from the cage. Wash in the sink in a couple of inches of warm water with a small amount of soap. Scrub walls and bottom of the cage to remove any dirt. Rinse very well and set to dry.

Discarding Unused Plant Material

Wheat plants that mature beyond what is usable for the colony can be safely disposed of in the compost. Wash empty pots with soap and water and rinse well. If mould becomes a problem on the wheat plants (seen around the top of the soil), the seedling pots and the trays can be rinsed in 10% bleach prior to washing. Leave the trays to soak for 10 minutes and then rinse well to remove any bleach residue before washing with soap and water.

Room Cleanup

Wash the countertop area where the cage sat with bleach diluted to 10% in water and rinse well. Empty any garbage and wash and dry dishes.

Disease and Infection Prevention and Control

Disease and infection can be minimized and/or prevented in the grasshopper colony by providing good quality food and maintaining a clean rearing room. Grasshopper food should be thoroughly washed before feeding and should originate from a known source (no plants collected from outdoors). Feeding should be done as frequently as possible (at least 3 times per week) to reduce the likelihood of cannibalism. Dead individuals should be removed frequently.

Cages and instruments that come into contact with the colony (including cage floors, sticks for climbing, Plexiglas among others) should be cleaned frequently and sterilized with bleach. During infections outbreaks, grasshoppers should be moved to clean cages more frequently (as frequently as once per week). Cages should be sterilized with 10% bleach during outbreaks (cage availability permitting) and washed with warm water and soap as usual. Through several washing and sterilization cycles, the number of disease spores will be reduced. Cages and any bleached parts/equipment should be thoroughly rinsed before moving any grasshoppers into the cage. It is important not to overcrowd cages as this can also endanger the health of the colony.

Chemical Supplies

Sodium sulfamethazine 25%

Dominion Veterinary Laboratories
4L bottle
http://domvet.com/sodiums.html

Mixing Sulfa Solution

Sulfa solution should be diluted to half strength (12.5%) using deionized water. Diluted solution can be kept in a spray bottle and used as necessary to prevent the spread of moulds and fungus on plant material to the grasshopper colony

Appendix D Chemical analysis of oviposition substrate, diet and *Melanoplus sanguinipes*.

Table D.1 Comparison of the nominal (expected) and measured concentrations of cerium (Ce) or neodymium (Nd) based on the average amount measured in oviposition substrate (sand) samples before oviposition and following the egg incubation period. Background levels were measured in control sand treated with deionized water (dH$_2$O). Average measured concentrations, including background levels, were used in subsequent analyses of effects.

REE	Nominal Dose (mg kg^{-1})	Measured Dose (mg kg^{-1}) (Pre-Exposure)	MDL*	MRL**	Measured Dose (mg kg^{-1}) (Post Exposure)	MDL*	MRL**	Average Measured Dose (mg kg^{-1})
Ce	0	49.3	0.02	0.06	47.6	0.02	0.06	48.5
Ce	40	148	0.02	0.06	81.1	0.02	0.06	114.6
Ce	160	358	0.02	0.06	284	0.02	0.06	321.0
Ce	640	986	0.06	0.18	744	0.06	0.17	865.0
Nd	0	14.2	0.019	0.193	20.1	0.02	0.197	17.2
Nd	30	71.7	0.018	0.185	86.3	0.019	0.191	79.0
Nd	150	347	0.019	0.186	174	0.019	0.188	260.5
Nd	750	1410	4.81	48.1	759	4.88	48.8	1084.5

***MDL:** method detection limit

****MRL:** Method reporting limit

Table D.2 Comparison of the nominal (expected) and measured concentrations of cerium (Ce) or neodymium (Nd) based on the average amount measured in diet. Background levels were measured in control diet treated with deionized water (dH_2O). Measured concentrations, including background levels, were used in subsequent analyses of effects.

REE	Nominal Dose (mg kg^{-1})	Measured Dose (mg kg^{-1})	MDL*	MRL**
Ce	0	0.41	0.009	0.03
Ce	40	37.3	0.008	0.02
Ce	160	202	0.02	0.05
Ce	40	894	0.09	0.26
Nd	0	1.17	0.004	0.039
Nd	30	45.6	0.004	0.04
Nd	150	239	4.63	46.3
Nd	750	720	4.87	48.7

***MDL:** Method detection limit

****MRL:** Method reporting limit

Table D.3 Measured concentration of cerium (Ce) or neodymium (Nd) in the shed exoskeletons of *Melanoplus sanguinipes* exposed to contaminated food from egg hatch through development until death. Numbers represent analysis of pooled tissue samples for all juvenile life stages (instar 2-6) at each dose.

REE	Nominal Dose (mg kg^{-1})	Measured Dose (mg kg^{-1})	Tissue Concentration (mg kg^{-1})	BAF* (Body:Diet)	MDL**	MRL***
Ce	0	0.41	4.54	11.1	0.04	0.13
Ce	40	37.3	7.5	0.2	0.04	0.11
Ce	160	202	14.7	0.1	0.06	0.17
Ce	640	894	23.2	0.0	0.28	0.8
Nd	0	1.17	1.8	1.5	0.019	0.192
Nd	150	239	16.3	0.1	0.025	0.25
Nd	750	720	42.9	0.1	0.016	0.158

***BAF:** bioaccumulation factor

****MDL:** method detection limit

*****MRL:** Method reporting limit

Table D.4 Measured concentration of cerium (Ce) or neodymium (Nd) in whole bodies of *Melanoplus sanguinipes* exposed to contaminated food from egg hatch through development. Numbers represent analysis of pooled tissue samples for the life stage-dose combinations.

REE	Life Stage	Diet Nominal Dose (mg kg^{-1})	Diet Measured Dose (mg kg^{-1})	Body Concentration (mg kg^{-1})	BAF* (Body:Diet)	MDL**	MRL***
Ce	1 to 3	0	0.41	7.21	17.6	0.07	0.21
Ce	1 to 3	40	37.3	1.59	0.0	0.07	0.2
Ce	1 to 3	160	202	31.6	0.2	0.1	0.28
Ce	1 to 3	640	894	59	0.1	0.15	0.44
Ce	4 to 6	0	0.41	1.19	2.9	0.08	0.24
Ce	4 to 6	40	37.3	1.15	0.0	0.05	0.14
Ce	4 to 6	160	202	4.41	0.0	0.12	0.36
Ce	4 to 6	640	894	35.6	0.0	0.18	0.52
Ce	Adult Female	0	0.41	0.3ª	0.7	0.22	0.65
Ce	Adult Female	40	37.3	0.44	0.0	0.02	0.06
Ce	Adult Female	160	202	1.92	0.0	0.02	0.06
Ce	Adult Female	640	894	182	0.2	0.1	0.28
Ce	Adult Male	0	0.41	1.33	3.2	0.02	0.06
Ce	Adult Male	40	37.3	2.39	0.1	0.02	0.06
Ce	Adult Male	160	202	4.58	0.0	0.02	0.06
Ce	Adult Male	640	894	58.3	0.1	0.03	0.09
Nd	Adult Female	0	1.17	0.502	0.4	0.004	0.036
Nd	Adult Female	30	45.6	0.875	0.0	0.015	0.149
Nd	Adult Female	150	239	2.35	0.0	0.004	0.038
Nd	Adult Female	750	720	10.5	0.0	0.006	0.062
Nd	Adult Male	0	1.17	0.047ª	0.0	0.006	0.062
Nd	Adult Male	30	45.6	1.44	0.0	0.038	0.378
Nd	Adult Male	150	239	2.17	0.0	0.008	0.08
Nd	Adult Male	750	720	30.1	0.0	0.012	0.119

ªMeasure falls between MDL and MRL and is considered an estimate; *BAF bioaccumulation factor; **MDL: method detection limit; ***MRL: Method reporting limit

Table D.5 Measured concentration of cerium (Ce) or neodymium (Nd) in frass of *Melanoplus sanguinipes* exposed to contaminated food from egg hatch through development. Numbers represent analysis of pooled tissue samples for the life stage-dose combinations.

REE	Life Stage	Diet Nominal Dose (mg kg^{-1})	Diet Measured Dose (mg kg^{-1})	Frass Concentration (mg kg^{-1})	MDL*	MRL**
Ce	1 to 2	0	0.41	42.5	0.03	0.1
Ce	1 to 2	40	37.3	95.5	0.02	0.06
Ce	1 to 2	160	202	274	0.39	1.13
Ce	1 to 2	640	894	1120	1.02	2.96
Ce	3	0	0.41	2.94	0.03	0.08
Ce	3	40	37.3	89.1	0.02	0.05
Ce	3	160	202	254	0.21	0.6
Ce	3	640	894	1160	4.23	12.3
Ce	4	0	0.41	1.59	0.02	0.06
Ce	4	40	37.3	93.8	0.02	0.06
Ce	4	160	202	255	0.21	0.61
Ce	4	640	894	928	0.33	0.96
Ce	5th Female	0	0.41	1.19	0.02	0.06
Ce	5th Female	40	37.3	86	0.02	0.06
Ce	5th Female	160	202	257	0.26	0.75
Ce	5th Female	640	894	917	0.93	2.71
Ce	5th Male	0	0.41	1.17	0.02	0.06
Ce	5th Male	40	37.3	106	0.02	0.06
Ce	5th Male	160	202	251	0.21	0.61
Ce	5th Male	640	894	1080	0.35	1.02
Ce	6th Female	0	0.41	0.75	0.02	0.06
Ce	6th Female	40	37.3	56.8	0.22	0.63
Ce	6th Female	160	202	279	0.22	0.65
Ce	6th Female	640	894	985	0.38	1.12
Ce	6th Male	0	0.41	0.97	0.02	0.06
Ce	6th Male	40	37.3	75.3	0.09	0.27
Ce	6th Male	160	202	271	0.21	0.62
Ce	6th Male	640	894	995	0.3	0.87
Ce	Adult Female	0	0.41	1.51	0.02	0.06
Ce	Adult Female	40	37.3	67.5	0.22	0.63
Ce	Adult Female	160	202	245	0.22	0.65
Ce	Adult Female	640	894	839	0.21	0.61
Ce	Adult Male	0	0.41	1.72	0.02	0.06
Ce	Adult Male	40	37.3	67	0.22	0.64
Ce	Adult Male	160	202	246	0.22	0.64
Ce	Adult Male	640	894	995	0.21	0.61
Nd	1 to 3	0	0.41	7.78	0.008	0.077
Nd	1 to 3	30	45.6	74.9	0.014	0.137
Nd	1 to 3	150	239	311	11.7	117
Nd	1 to 3	750	720	843	6.68	66.8
Nd	4 to 6	0	1.17	0.311	0.006	0.059
Nd	4 to 6	30	45.6	78.3	0.004	0.04
Nd	4 to 6	150	239	218	4.67	46.7
Nd	4 to 6	750	720	993	4.3	43
Nd	Adult Female	0	1.17	0.344	0.004	0.038
Nd	Adult Female	30	45.6	66	0.004	0.043
Nd	Adult Female	150	239	252	4.99	49.9

Nd	Adult Female	750	720	952	4.89	48.9
Nd	Adult Male	0	1.17	0.449	0.004	0.04
Nd	Adult Male	30	45.6	81	0.006	0.063
Nd	Adult Male	150	239	239	4.79	47.9
Nd	Adult Male	750	720	907	4.39	43.9

*****MDL: Method detection limit

****** MRL: Method reporting limit

Appendix E Quality control for chemical analysis of *Melanoplus sanguinipes* bodies, intestines, frass and diet (commercial guinea pig food) following chronic exposure to cerium or neodymium.

Table E.1 Accuracy and precision of the chemical analysis by inductively coupled plasma mass spectrometry (ICP-MS) of cerium (Ce) or neodymium (Nd) content in *Melanoplus sanguinipes* bodies, frass, diet (commercial Guinea pig food) or oviposition substrate (sand). Analysis was performed by Brooks Applied Labs (Seattle, WA, USA) following the US Environmental Protection Agency (USEPA) Method 1638 (US Environmental Protection Agency, 1996).

Matrix	Analyte	Description	Native Sample (mg kg^{-1})	Spike (mg kg^{-1})	Results (mg kg^{-1})	Recovery %	Limits %	RPD	Limits %	Batch/Sample
	Ce	Laboratory Fortified Blank (1510061)		25.00	27.48	110	75-125			B150232-BS1
	Ce	Cert. Ref Material (0919050, NIST 2709a)		42.00	43.44	103	N/A			B150232-SRM1
	Ce	Cert. Ref Material (0919053, NIST 2710a)		60.00	46.2	77	N/A			B150232-SRM2
Sand	Ce	Duplicate	23.77		27.21			13	30	B150232-DUP2
Sand	Ce	Matrix Spike	23.77	24.95	49.89	105	70-130			B150232-MS2
Sand	Ce	Matrix Spike Duplicate	23.77	24.30	29.32	105	70-130	1	30	B150232-MSD2
Sand	Ce	Duplicate	49.33		55.06			11	30	B150232-DUP1
Sand	Ce	Matrix Spike	49.33	26.53	66.01	63	70-130			B150232-MS1
Sand	Ce	Matrix Spike Duplicate	49.33	25.61	67.07	69	70-130	2	30	B150232-MSD1
	Ce	Laboratory Fortified Blank 1442029		10.00	9.73	97	75-125			B150346-BS1
Diet	Ce	Duplicate	201.80		221.5			9	30	B150346-DUP1
Diet	Ce	Matrix Spike	894	66.58	635	NR	70-130			B150346-MS1
	Ce	Lab Fortified Blank 1508048		25.00	23.64	95	75-125			B150358-BS1

Matrix	Analyte	Description	Native Sample (mg kg^{-1})	Spike (mg kg^{-1})	Results (mg kg^{-1})	Recovery %	Limits %	RPD	Limits %	Batch/Sample
	Ce	Cert Reference Material 0919050, NIST 2709a		42.00	38.26	91	75-125			B150358-SRM1
Sand	Ce	Duplicate	2597		2852			9	30	B150358-DUP4
Sand	Ce	Matrix Spike	2597	27.19	2888	NR	70-130			B150358-MS4
Sand	Ce	Matrix Spike Duplicate	2597	27.09	2632	NR	70-130	9	30	B150358-MSD4
5th Instar Male Frass	Ce	Duplicate	1.17		1.01	14%	30			B151490-DUP2
5th Instar Male Frass	Ce	Matrix Spike	1.17	19.76	20.03	95%	70-130			B151490-MS2
5th Instar Male Frass	Ce	Matrix Spike Duplicate	1.17	19.92	20.5	97%	70-130	2%	30	B151490-MSD2
Adult Male Frass	Ce	Duplicate	1.72		1.63			5%	30	B151490-DUP3
Adult Male Frass	Ce	Matrix Spike	1.72	19.38	19.53	92%	70-130			B151490-MS3
Adult Male Frass	Ce	Matrix Spike Duplicate	1.72	19.46	21.04	99%	70-130	8%	30	B151490-MSD3
Adult Female Frass	Ce	Duplicate	1.51		0.82			59%	30	B151599-DUP1
Adult Female Frass	Ce	Matrix Spike	1.51	18.66	18.61	92%	70-130			B151599-MS1
Adult Female Frass	Ce	Matrix Spike Duplicate	1.51	18.73	18.57	91%	70-130	0.60%	30	B151599-MSD1
6th Instar Male Frass	Ce	Duplicate	270.9		257.4			5%	30	B151713-DUP1

Matrix	Analyte	Description	Native Sample (mg kg^{-1})	Spike (mg kg^{-1})	Results (mg kg^{-1})	Recovery %	Limits %	RPD	Limits %	Batch/Sample
6th Instar Male Frass	Ce	Matrix Spike	270.9	22.73	288.5	NR	70-130			B151713-MS1
6th Instar Male Frass	Ce	Matrix Spike Duplicate	270.9	20.83	259.4	NR	70-130	11%	30	B151713-MSD1
Adult Male Frass	Ce	Duplicate	995		934.9	6%	30			B151713-DUP2
Adult Male Frass	Ce	Matrix Spike	995	20.92	7.78	NR	70-130			B151713-MS2
Adult Female Body	Ce	Matrix Spike	0.44	20.08	19.42	95%	70-130			B151490-MS6
Adult Female Body	Ce	Matrix Spike Duplicate	0.44	19.61	18.58	93%	70-130	2%	30	B151490-MSD6
	Ce	Laboratory Fortified Blank - 1542007		10	9.43	94%	75-125			B151599-BS1
Biota	Nd	Laboratory Fortified Blank 1612042		5.000	4.773	95%	75-125			B160490-BS1
Biota	Nd	Laboratory Fortified Blank 1612042		5.000	4.891	98%	75-125			B160490-BS2
Adult Female Body	Nd	Matrix Spike 16100004-08	10.55	3.750	12.72	58%	75-125			B160490-MS1
4th, 5th, 6th Instar Frass	Nd	Duplicate 16100004-13	0.311		0.413			28%	25	B160490-DUP2
4th, 5th, 6th Instar Frass	Nd	Matrix Spike 16100004-13	0.311	7.781	7.677	95%	75-125			B160490-MS2

Matrix	Analyte	Description	Native Sample (mg kg^{-1})	Spike (mg kg^{-1})	Results (mg kg^{-1})	Recovery %	Limits %	RPD	Limits %	Batch/Sample
4th, 5th, 6th Instar Frass	Nd	Matrix Spike Duplicate 1610004-13	0.311	6.412	6.28	93%	75-125	2%	25	B160490-MSD2
Adult Female Frass	Nd	Duplicate 1610004-15	0.344		0.375			9%	25	B160490-DUP1
	Nd	Laboratory Fortified Blank 1612042		5.000	4.398	88%	75-125			B160683-BS1
	Nd	Laboratory Fortified Blank 1612042		5.000	6.206	124%	75-125			B160683-BS2
Adult Female Frass	Nd	Duplicate 16100004-27	952.1		923.9			3%	25	B160683-DUP1
Adult Female Frass	Nd	Matrix Spike 16100004-27	952.1	4.907	945.8	NR	75-125			B160683-MS1
Adult Female Frass	Nd	Matrix Spike Duplicate 16100004-27	952.1	4.866	994.5	NR	75-125	N/C	25	B160683-MSDA
Diet	Nd	Duplicate 16100004-30	238.7		242			1%	25	B160683-DUP2
Diet	Nd	Matrix Spike 16100004-30	238.7	4.806	169.7	NR	75-125			B160683-MS2
Diet	Nd	Matrix Spike Duplicate 16100004-30	238.7	4.804	194.6	NR	75-125	-44%	25	B160683-MSD2
	Nd	Laboratory Fortified Blank 1612042		5.000	4.788	96%	75-125			B160491-BS1
Sand	Nd	Duplicate 16100004-34	346.6		354.1			2%	25	B160491-DUP1
Sand	Nd	Matrix Spike 16100004-34	346.6	4.755	358.7	NR	75-125			B160491-MS1

Matrix	Analyte	Description	Native Sample (mg kg^{-1})	Spike (mg kg^{-1})	Results (mg kg^{-1})	Recovery %	Limits %	RPD	Limits %	Batch/Sample
Sand	Nd	Matrix Spike Duplicate 16100004-34	346.6	4.586	470.8	NR	75-125	N/C	25	B160491-MSD1
	Nd	Laboratory Fortified Blank 1612042		5.000	4.6	92%	75-125			B160684-BS1
Sand	Nd	Duplicate 16100004-35	1406		1242			12%	25	B160684-DUP1
Sand	Nd	Post Spike 1610004-35	1406	480.9	1766	75%	75-125			B1606840PS1
Sand	Nd	Post Spike 1610004-35	1406	480.9	1900	103%	75-125			B160684-PS2

ND: Native sample results and/or associated duplicate result were below the method detection limit, relative percent difference was not calculated **(N/C)**

NR: Matrix was spiked at <= 25% of the native sample concentration, therefore the recovery was not reported (NR)

N/A: Not Applicable
RPD: Relative Percent Difference

Table E.2 Method blanks and reporting limits of the chemical analysis by inductively coupled plasma mass spectrometry (ICP-MS) of cerium (Ce) or neodymium (Nd) content of *Melanoplus sanguinipes* bodies, frass, diet (commercial Guinea pig food) or oviposition substrate (sand). Analysis was performed by Brooks Applied Labs (Seattle, WA, USA) following the US Environmental Protection Agency (USEPA) Method 1638 (US Environmental Protection Agency, 1996).

Batch-Sample	Matrix	Analyte	Result (mg kg^{-1})	Average	Limit	Standard Deviation	Limit	MDL	MRL
B150232-BLK1	Sand	Ce	0.008						
B150232-BLK2	Sand	Ce	0.01						
B150232-BLK3	Sand	Ce	0.008						
B150232-BLK4	Sand	Ce	0.003	0.01	0.06	0	0.02	0.02	0.06
B150346-BLK1	Biota	Ce	0.001						
B150346-BLK2	Biota	Ce	0.001						
B150346-BLK3	Biota	Ce	0.001						
B150346-BLK4	Biota	Ce	0.001	0.00	0.01	0.00	0.00	0.003	0.008
B150358-BLK1	Sand	Ce	0.08						
B150358-BLK2	Sand	Ce	0.09						
B150358-BLK3	Sand	Ce	0.08						
B150358-BLK4	Sand	Ce	0.09	0.09	0.09	0.01	0.03	0.03	0.09
B151490-BLK1	Biota	Ce	-0.0002						
B151490-BLK2	Biota	Ce	-0.0002						
B151490-BLK3	Biota	Ce	-0.000004						
B151490-BLK4	Biota	Ce	-0.00004	0.00	0.03	0.00	0.01	0.01	0.03
B151599-BLK1	Biota	Ce	0.0007						
B151599-BLK2	Biota	Ce	0.0002						
B151599-BLK3	Biota	Ce	-0.00008						
B151599-BLK4	Biota	Ce	0.0007	0.00	0.03	0.00	0.01	0.01	0.03
B160490-BLK1	Biota	Nd	0.00						
B160490-BLK2	Biota	Nd	0.00						
B160490-BLK3	Biota	Nd	0.00						
B160490-BLK4	Biota	Nd	0.00	0.00	0.04	0.00	0.003	0.004	0.04
B160491-BLK1	Sand	Nd	0.006						
B160491-BLK2	Sand	Nd	0.000						

Batch-Sample	Matrix	Analyte	Result (mg kg^{-1})	Average	Limit	Standard Deviation	Limit	MDL	MRL
B160491-BLK3	Sand	Nd	0.007						
B160491-BLK4	Sand	Nd	0.007	0.005	0.2	0.003	0.013	0.02	0.2
B160683-BLK1	Biota	Nd	0.00						
B160683-BLK2	Biota	Nd	0.00						
B160683-BLK3	Biota	Nd	0.00						
B160683-BLK4	Biota	Nd	0.00	0.00	1.00	0.00	0.067	0.10	1.00
B160684-BLK1	Sand	Nd	0.0006						
B160684-BLK2	Sand	Nd	0.0000						
B160684-BLK3	Sand	Nd	0.0000						
B160684-BLK4	Sand	Nd	0.0020	0.001	1.00	0.001	0.067	0.10	1.00

MDL: Method detection limit

MRL: Method reporting limit