

EXPLORING GRASSHOPPER FEEDING PREFERENCES  
BASED ON PLANT CARBON TO NITROGEN RATIOS  
ACROSS DIFFERENT SOIL NUTRIENT TREATMENTS

by

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## **An Abstract of the Thesis of**

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Ratios Across Different Soil Nutrient Treatments

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Anthropogenic nutrient enrichment can rapidly change ecosystem functions and diversity, as well as plant tissue composition. This research explores the impacts of soil nutrient enrichment on insect-plant relationships in a rare montane meadow ecosystem in the Western Cascades of Oregon. Montane meadows host a wide variety of diversity. We performed an experimental study at Bunchgrass Ridge, a Long-Term Ecological Research site supporting a long-term nutrient loading experiment. Grasshopper feeding was quantified by observing leaf damage and analyzing leaf tissue chemistry of two plant species, grass *B. carinatus* and legume *L. oregonus*. Leaves for each species were collected and their C:N ratios were analyzed. Our analyses show that soil nutrient enrichment, plant tissue C:N, and plant species do impact grasshoppers' feeding preferences, with plant species having the largest impact. Understanding the ecological drivers that affect threatened montane ecosystems can inform management and policy decisions as we face continuous anthropogenic influences.

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## Table of Contents

Introduction	1
Background	1
Existing Literature	5
Carbon to Nitrogen Ratios	5
Plant Community Responses to Nutrient Enrichment	7
Grasshopper Response to Nutrient Enrichment	8
Insect Feeding Preferences with Altered Soil Nutrients	10
Montane Ecosystems	12
Project Overview and Hypotheses	14
Methods	16
Site and Experimental Setup	16
Data Collection	18
Grasshopper Collection	18
Leaf Damage	19
Carbon to Nitrogen Analysis	19
Instruments Used for Analysis	20
Statistical Analysis	20
Results	23
Grasshopper Feeding Preferences	23
Differences in C:N Ratios Between <i>B. carinatus</i> and <i>L. oregonus</i>	26
Comparing Grasshopper Chewing Across C:N Ratio, Soil Nutrient Treatment, & Plant Species	28
Discussion	32
Grasshopper Responses to Different Soil Nutrient Treatments	32
Carbon to Nitrogen Ratio Analysis	34
Comparison of All Variables and Grasshopper Chewing	37
Study Limitations	38
Future Directions	39
Conclusions	41
Appendix	42
References	46

## List of Figures

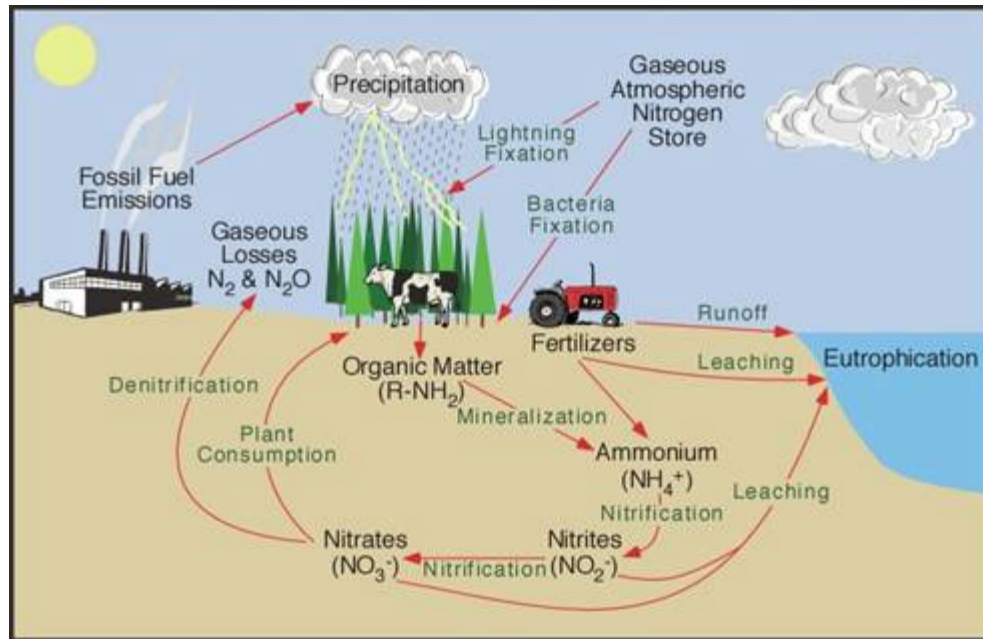
Figure 1. Anthropogenic Nitrogen Cycle	2
Figure 2. Soil Microbial Community Responses to C:N Ratios	3
Figure 3. Conifer Encroachment in Bunchgrass Meadow since 1946	5
Figure 4. Potential Plant-herbivore Stoichiometric Relationships	9
Figure 5. Mountain Brome, <i>Bromus carinatus</i>	12
Figure 6. Legume <i>Lupine oreganus</i>	12
Figure 7. Bunchgrass Ridge Study Site	17
Figure 8. Experimental Design	18
Figure 9. Percent Leaf Area Chewed Across All Soil Nutrient Treatments	24
Figure 10. Percent Leaf Area Chewed Isolated by Nitrogen and Phosphorus Enrichment	25
Figure 11. Carbon to Nitrogen Ratios in Primary Producers Across All Nutrient Treatments	27
Figure 12. C:N Ratios in Plots With and Without Nitrogen Enrichment Across Both Plant Species	28
Figure 13. Mean Percent Leaf Area Chewed versus C:N Ratios Across All Nutrient Treatments	29
Figure 14. MDS Plot Between Both Plant Species	31

# Introduction

## Background

Ecosystem functioning is dependent on the interactions between primary producers and their consumers. Such interactions are the basis for the complex food webs which characterize an ecological community, making their study vital in a constantly changing world. Even though insect herbivores comprise a large portion of ecosystem biomass and have been observed to directly alter plant community structure, their role as consumers has been understudied, especially in montane ecosystems (Hautier et al., 2014; Harpole et al., 2016). As anthropogenic climate change and nutrient enrichment rapidly alter grassland composition and functioning globally, untangling the role of plant-herbivore interactions in these systems is imperative.

Soil nutrient enrichment, especially by nitrogen and phosphorus, is a pressing problem for grassland ecosystems today. Scientists estimate that nitrogen deposition, often in the form of agricultural runoff and fossil fuel emissions, is 7 degrees higher than it was before the Industrial Revolution (Clark & Tilman, 2008). This global change driver disrupts natural cycles such that eutrophication and nutrient leaching, an excess of nitrogen present in the soil, are amplified (Figure 1). While nitrogen deposition can cause an initial increase in plant biomass production, ultimately it decreases plant species diversity (Wei et al., 2013; Stevens et al., 2004). Nutrient enrichment has also been shown to impact carbon, nitrogen, and phosphorus cycles vital to maintaining proper soil nutrient stoichiometry. In fact, it has been shown to weaken soil-microbe interactions, meaning that nitrogen availability at all levels of the food web will be altered (Wei et al., 2013).

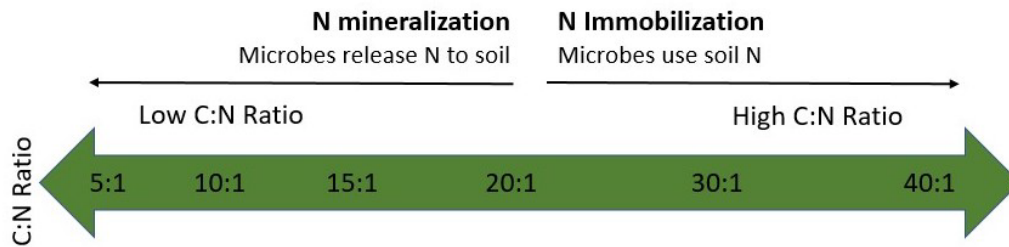


**Figure 1. Anthropogenic Nitrogen Cycle**

**Both natural and the anthropogenic influences of fossil fuel emissions and agricultural fertilizers of nitrogen deposition are represented, as well as the implications for the surrounding environment. Source: The University of British Columbia Okanagan.**

One of the many ways to examine changes within soil nutrient levels is through carbon to nitrogen ratios (C:N ratios). C:N ratios show the mass of carbon to the mass of nitrogen in a particular substance. They are often used in the study of soils, but they can be used with plants, insects, and anything else that researchers find important. They are especially useful for tracking changing flows in food webs. In order to remain healthy, microbes in the soil that fix atmospheric nitrogen ( $N_2$ ) into ammonia ( $NH_3^+$ ) and nitrify it into nitrates ( $NO_3^-$ ) for the plants to use need a C:N content in the soil of roughly 24:1 (USDA, 2011). Eight parts of this are used for physiological functions while the other 16 parts are given off as energy (USDA, 2011).





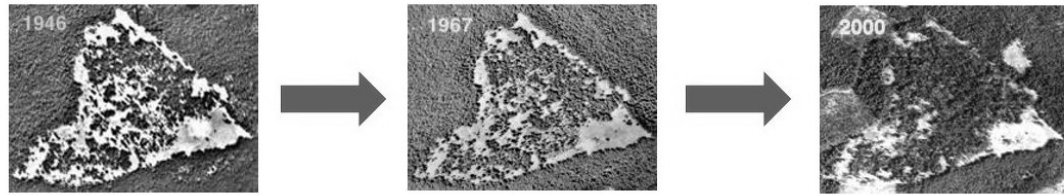
**Figure 2. Soil Microbial Community Responses to C:N Ratios**

C:N ratios vary across different ecosystems, with a ratio of roughly 20:1 being standard. C:N ratios higher than 20:1 induce microbial release of nitrogen into the soil and ratios lower than 20:1 induce microbial use of soil nitrogen. Figure adapted from Schipanski et al., 2014.

Adding organic or inorganic nutrients changes soil nutrient composition, and thus C:N ratios. For example, the addition of a substance with a C:N ratio higher than 24:1 results in immobilization, or a nitrogen deficit, because soil microbes then must process excess soil nitrogen to compensate for the added carbon (Figure 2). When something with a C:N ratio lower than 24:1 is added, soil microbes leave excess nitrogen in the soil, resulting in mineralization (USDA, 2011). The above information only applies for when the soil is under balanced conditions. Soil microbial communities are very diverse, and this diversity is directly tied to the amount of carbon and organic biomass in the soil (Bastida et al., 2021). Anthropogenic nutrient enrichment, however, by quickly changing basic C:N ratios in the soil, decreases the diversity of soil microbial communities (Chen et al., 2019; Niu et al., 2016). Despite the importance of balanced C:N ratios for whole ecosystem functions, the effects of anthropogenically altered C:N ratios on food webs is vastly understudied (Wei et al., 2013). Here, I

investigate how changes in soil nutrient composition affects plant-grasshopper interactions in a montane meadow ecotype.

Montane meadows are an ecologically important, rare ecosystem in the Cascade Range, comprising less than 5% of the region's area. Generally found at subalpine elevations, mountain meadows are highly stratified, meaning that vegetation species are layered in a vertical arrangement based on different plant heights (Mayor et al., 2017). These few meadows are local biodiversity hotspots essential for organisms that cannot thrive beneath tree cover. They provide a vast array of ecosystem services such as natural fire breaks and belowground watersheds (University of Washington, 2016; Aparecido et al., 2018). In the Cascades, they are threatened mainly due to conifer encroachment, so it is of high ecological importance to study them now (Figure 3). Conifer encroachment in the montane meadows of the Cascade Range has occurred since the 1800s. Trees like pines and Douglas firs are most common in these areas, and they both have different survival and reproduction patterns from each other. This lessens competition for nutrients and space, creating rapid transformation from meadow to forest. ("Study Area," n.d.).



**Figure 3. Conifer Encroachment in Bunchgrass Meadow since 1946**

**Conifer encroachment has progressively turned an open montane meadow into a forest, disrupting the ecosystem and the organisms living there. Source: The University of Washington**

### **Existing Literature**

#### *Carbon to Nitrogen Ratios*

A common method of understanding how nitrogen deposits affect soil and plant ecosystems, C:N ratios tell researchers the mass of carbon versus the mass of nitrogen in a sample (USDA, 2011). C:N ratios are not exclusively used in terrestrial ecosystems but can be used in both freshwater and marine systems. Aquatic C:N ratios are normally around 4:1-10:1, while terrestrial ones are much higher at 24:1 (Myers, 1994). In terrestrial ecosystems, C:N ratios are measured primarily in fungi, soil, and plants. Plants are the primary focus of my study, but here I include information about different C:N uses.

Regarding fungi, many studies have examined the impacts of fungal growth on introducing additional nitrogen deposits and changing C:N ratios in their ecosystems. One study found that between ammonium sulfate and urea deposits, two nitrogen-rich sources, urea deposits enhance fungal growth better than ammonium sulfate. Additionally, they found that soil C:N ratios between 10:1 and 50:1 allow for increased fungal growth and anything smaller inhibits this (Mantovani et al., 2007). Different

species of fungi have varying C:N ratios present in their ecosystems, ranging anywhere from 15:1 to 117:1, while 20:1 is ideal for most species (MacCanna, 1984; Srivastava and Bano, 1970; Chang & Miles, 1989). C:N ratios are also very important on growth and sporulation of mushrooms. Both the vegetative feeding stage and the sporulation phase of fungi's life cycle require nutrients from the soil, but it is unclear if each stage requires the same C:N ratio within the soil or within fungal tissues (Gao & Liu, 1975). Fungi is an important group of organisms when talking about C:N ratios because fungi are decomposers who control nutrient levels in soils. Understanding C:N impacts on fungi can improve knowledge on how plants absorb nutrients and what ideal soil microbial communities look like for this process.

In most soils, the ideal C:N ratio for microorganisms responsible for converting atmospheric nitrogen into inorganic nitrogen for plants usage is 24:1. Primary producers, however, have a wide range of C:N ratios within their tissues (USDA, 2011). One study found that producers symbiotically associated with fungi in temperate forests have C:N ratios that are more heavily dictated by low levels of nitrogen instead of high levels of carbon, especially when the soil C:N is already high (Zhu et al., 2018). Other studies have examined differences in C:N ratios across different biomes and found that globally the variation is generally narrow (Cleveland & Liptzin, 2007). However, within the same ecosystem, different plant species require very different levels of nitrogen, carbon, and phosphorus; these differences alter localized microbial community composition. Further, already existing soil nutrients influence plant competition (Bell et al., 2014). Because of this, changing nutrient levels play a very important role in plant community composition.

### *Plant Community Responses to Nutrient Enrichment*

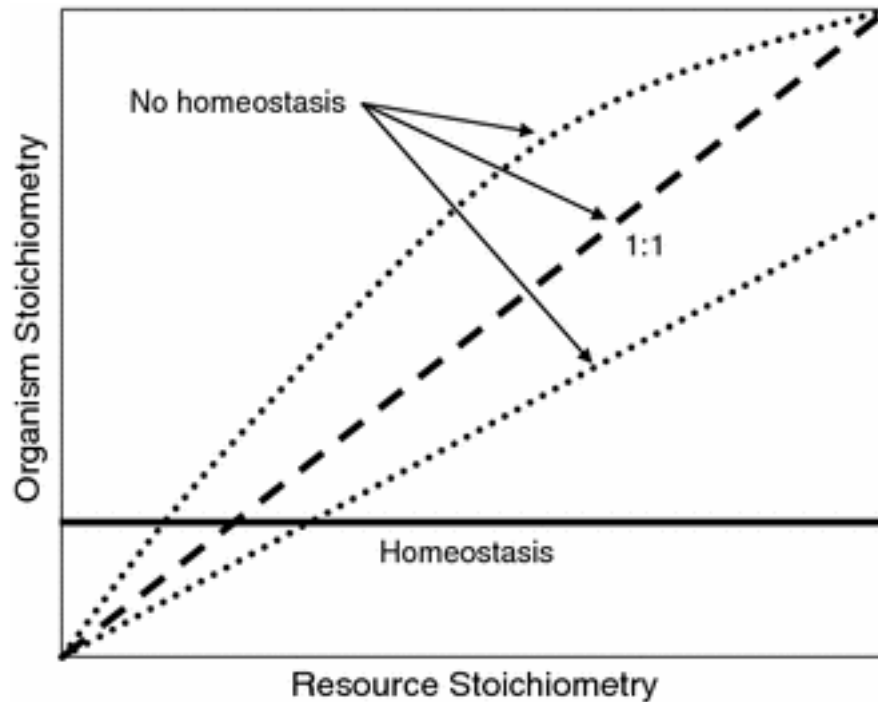
Nitrogen and phosphorus enrichment is primarily the result of water runoff from the agricultural fertilizers, as they leach into surrounding environments through storm drains and roadways. These fertilizers typically contain nitrogen, phosphorus, potassium, and other nutrients that are known to have impacts on plant communities. In this study, nutrient additions of nitrogen, phosphorus, and potassium were experimentally added to the natural meadow system. Changing soil nutrient levels significantly impacts primary producer diversity, tissue chemistry, and community composition (Harpole et al., 2016; Elser et al., 2000). In general, nutrient enrichment increases above ground biomass productivity and reduces biodiversity. It also increases the amount of plant residue present on top of the soil, which further decompose to produce more usable nutrients. These impacts are only made worse over time (Seabloom et al., 2021). Agricultural fertilizers are large producers of nutrient deposits across grasslands, which directly induces plant species diversity loss as well as general destabilization and inability to adapt to changing conditions (Hautier et al., 2020). In most ecosystems, an imbalance of soil nutrients favors non-native species invasion, negatively impacting community composition and diversity (Knauf et al., 2021).

Nitrogen and phosphorus are known to limit plant communities. Co-limitation is more important in plant communities where both phosphorus and nitrogen levels are low, while higher levels of nutrients create an imbalance where one affects the plant system more than the other (Harpole et al., 2016). Nutrient enrichment can also have an indirect impact on plant growth. For example, one study found that nutrient enrichment

in the form of synthetic bovine urine increased plant growth to such a high degree that competition for sunlight eventually limited growth (Renne et al., 2006).

#### *Grasshopper Response to Nutrient Enrichment*

Since insects feed on plants, changes in grassland plant communities directly impact insects (Tao & Hunter, 2012; Throop & Lerdau, 2004). These changes can alter an insect herbivore's ability to maintain internal homeostasis, affecting stoichiometric ratios between plants and herbivores (Figure 4). In one study, researchers found that long term nitrogen loading in grassland communities decreases plant species richness but increases abundance and biomass. This triggers a similar pattern in insects: plant diversity loss decreases insect species richness but increases abundance (Haddad et al., 2000). In other grassland communities, grasshopper densities are positively correlated with nitrogen whereas phosphorus has no effect (Loaiza et al., 2011). Higher trophic levels of mammalian herbivores also influence nutrient availability in soils, oftentimes increasing nitrogen content in both soils and plants. It is unclear, however, how this increase affects overall grasshopper diversity and abundance (Haddad et al., 2001).



**Figure 4. Potential Plant-herbivore Stoichiometric Relationships**

**The solid line represents an organism in homeostasis and the dotted lines represent examples of an organism not in stoichiometric homeostasis with its producers. Source: Cleveland & Liptzin, 2007**

Insect herbivory can mitigate nutrient-induced plant diversity loss by reducing light competition amongst plants (Borer et al., 2014; Crawford et al., 2021). Because nutrient enrichment increases plant biomass, plants experience more intense competition for access to light. Insect herbivores can ameliorate this by reducing plant biomass through consumption and so increasing light availability. There is a limit to how exhaustive exclusively insect herbivory reversal can be. One study found that grasslands with only insect herbivory could not offset increased biomass nearly as well as grasslands with both insect herbivory and mammalian herbivory (Borer et al., 2020).

### *Insect Feeding Preferences with Altered Soil Nutrients*

Insect herbivores have an interdependent relationship with plant communities. There is not, however, a universal pattern that every plant-herbivore relationship follows. There are bottom-up relationships where lower trophic levels like plants have a stronger influence over insects through reducing their consumption resources (Li et al., 2020). There are also top-down relationships where consumers control ecosystems through predation (Li et al., 2020). It is hard to predict which trophic structure a specific ecosystem will adhere to, making it difficult to understand the ecological drivers that influence these relationships.

Insects are primary consumers in grassland ecosystems, as they directly influence the growth of the plants they eat. Nitrogen has long been considered the primary limiting nutrient affecting insect feeding behaviors because of its tripled abundance in insect tissue as opposed to plants, but phosphorus is also considered a possible limiting factor for similar reasons (Rode et al., 2017; Ritchie, 2000; Huberty & Denno, 2006). One study found, however, that neither nitrogen nor phosphorus are the drivers for grasshopper growth (Rode et al., 2017). Furthermore, while nitrogen is present in higher quantities in insects than phosphorus, nitrogen deposition does not cause phosphorus to be limiting, nor does it cause insects to seek out phosphorus rich food sources (Tao & Hunter, 2012; Rode et al., 2017). Conversely, nitrogen and phosphorus may be equally limiting to herbivorous insects (Lemoine et al., 2014). While feeding preferences based on nutrient alterations have not been extensively studied beyond nitrogen and phosphorus, abiotic factors like water availability may have larger roles in insect herbivory than nutrient limitation does. Additionally, nitrogen



enrichment may affect stoichiometric nitrogen concentrations in plants more than it does in insects (Sagers & Goggin, 2007; Ritchie, 2000; La Pierre & Smith, 2016; Jamieson & Bowers, 2012).

In this project, *B. carinatus* (Figure 5), a grass species, and *L. oreganus* (Figure 6), a legume species, were studied. Legume tissues are incredibly nitrogen-rich because they fix nitrogen themselves, something that grass species cannot do (Haddad et al., 2001; Mattson, 1980). As a result, legumes have a much higher tissue nutrient content than grasses do. As stated earlier, insects are specialized to specific resources based on their own needs, so there may be food preferences based on that alone. Legumes and grasses have different soil microbial communities associated with their roots, with legumes' being more diverse (Zhou et al., 2017). This plant-soil feedback interaction can affect insect herbivory across different plant functional groups, but there is not a general pattern for all insects across all ecotypes (Kos et al., 2015). These variations, along with increased nutrient enrichment, must be explored to fully understand how anthropogenic nutrient levels will affect grassland ecosystems.



**Figure 5. Mountain Brome, *Bromus carinatus***

**Forbs of *B. carinatus* with evidence of flowering. Source: Kramer, 2010, via the California Native Plant Society**



**Figure 6. Legume *Lupine oreganus***

**Leaves and flowers of *L. oreganus* at Bunchgrass Meadow. Source: Altmire, 2021**

### *Montane Ecosystems*

Montane ecosystems are found around the world in temperate, Mediterranean, and tropical areas. Typically, they contain high levels of carbon within the soil, and

have historically acted as carbon sinks. In the Sierra Nevadas in California, montane ecosystems represent only 2% of the geographical area, but hold 12-31% of the carbon found in the mountain range (Norton et al., 2011).

Montane soils are rich in minerals and support shallow water tables throughout the growing season, limiting larger plant species growth. The smaller species that grow here create thick root mats under the soil, which help stabilize soils against erosion. They are adept at carbon sequestration due to the minerals' ability to stabilize large C inputs and anoxic soil conditions (Newcomb et al., 2017; Whiting & Chanton, 2001). Destabilizing factors like urbanization, grazing, and recreational activities, however, have increased erosion and created prolonged aerobic soil periods. This has changed some montane ecosystems from carbon sinks into atmospheric carbon sources (Reed et al., 2020).

Montane soil is alluvial, meaning that it has been deposited by surface water, and soil compositions in terms of depth and materials change depending on whether the herbaceous ecosystem is upslope or downslope (Vankat, 2013). Upslope and downslope montane ecosystems have different soil nitrogen concentrations, with downslope sites having more nitrogen availability, and upslope sites having lower microbial biomass (Weintraub, Brooks, & Bowen, 2017). One study found that long-term warming decreases soil microbial growth and reduces carbon content in montane ecosystems (Purcell et al., 2022). Decreasing soil microbe content can decrease the amount of usable nitrogen in an ecosystem, altering the amount present for plants to use in their tissues, which could set off a cascade of nitrogen deficiency in consumers as well.

## Project Overview and Hypotheses

Here, I investigate how one kind of plant-insect interaction, grasshopper herbivory, changes as a montane meadow undergoes long-term soil nutrient enrichment. Understanding these interactions in response to an anthropogenic influence will be important for informing grassland management and ecological conservation decisions.

Within this study, I have three main research questions:

1. Do grasshoppers prefer *B. carinatus* or *L. oregonus* as a food source? Does food differ by treatment?
2. How do C:N ratios differ in *B. carinatus*, a grass, and *L. oregonus*, a legume, across experimental treatments?
3. How do C:N ratios, soil nutrient enrichment, and plant species influence grasshopper herbivory?

In order to answer these research questions, I came up with these three hypotheses:

1. *L. oregonus* will be preferred by grasshoppers because it is more nitrogen rich. This makes it a more sought-after food source.
2. C:N ratios will be lower in *L. oregonus* because it is a legume. Legumes have nitrogen fixing bacteria present within their root nodules that directly impact the nitrogen cycle. Since this species does not have to rely entirely on soil microbes to fix nitrogen, nitrogen is more readily available regardless of what the soil ecosystem looks like. In plots where multiple nutrients are present, the plots containing nitrogen will lower C:N ratios in general, but *L. oregonus* will be lower than *B. carinatus*.

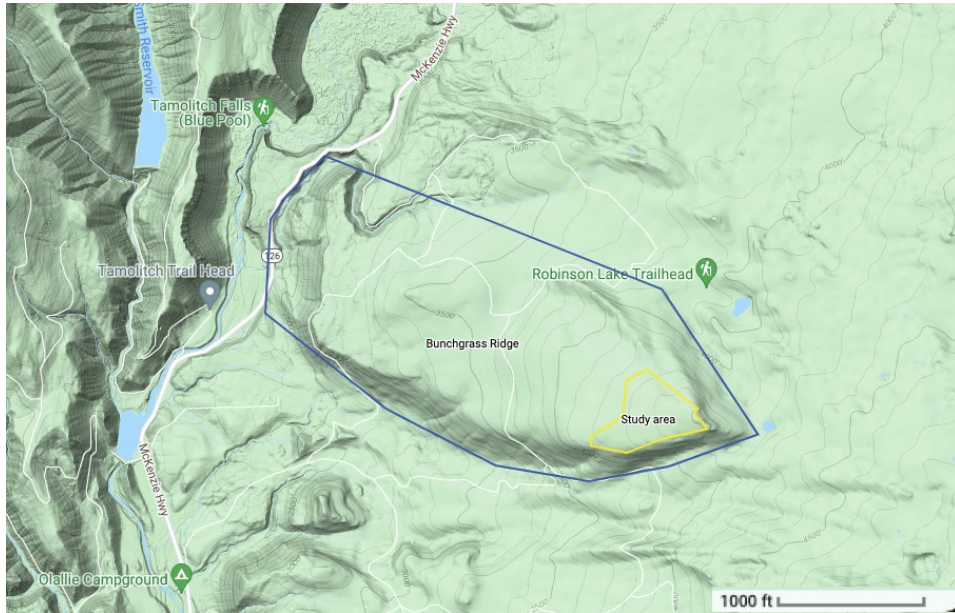
3. Nitrogen has long been thought of as the limiting nutrient for insect herbivores, and some studies also show that phosphorus may be limiting as well. Because of this, plants in nitrogen-enriched plots with lower C:N ratios will have more leaf damage because of their higher nitrogen content. This will be particularly expressed in *L. oreganus*.

## **Methods**

### **Site and Experimental Setup**

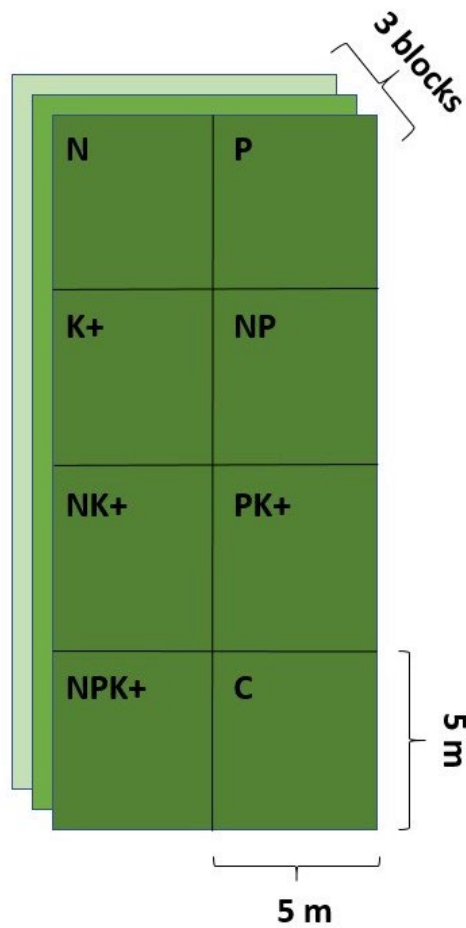
This experiment took place at Bunchgrass Meadow, a montane meadow found within the 100 ha space of Bunchgrass Ridge in the H.J. Andrews Experimental Forest. The ridge is located on unceded Confederated Tribes of Grand Ronde and Confederated Tribes of Siletz Indians land. Bunchgrass ridge is part of the H.J. Currently, it is managed by Oregon State University and the United States Forest Service where many research institutions conduct studies. Bunchgrass Meadow (Bunchgrass) is located in the southeastern region of Bunchgrass Ridge (Figure 7); here, there is a more than decade long experiment by the Nutrient Network (NutNet), a global research collective that monitors ecosystem response to soil nutrient enrichment in grasslands around the world. In order to develop management strategies, University of Washington also conducts a variety of experiments in this and other montane meadows in the Pacific Northwest.

At Bunchgrass, the experimental design was implemented and is maintained by NutNet. Here, there are three randomized blocks with eight plots measuring 5m x 5m with different nutrient treatments in each. Each block contains a control and the following treatments: nitrogen, phosphorus, potassium, nitrogen and potassium, phosphorus and potassium, nitrogen and phosphorus, and all three nutrients (NPK). Treatments are replicated three times, with one replicate in each block. Treatments are maintained by adding 10g m<sup>-2</sup> of each nitrogen, phosphorus, and potassium annually such that these nutrients gradually accrue in the soil (Figure 8).



**Figure 7. Bunchgrass Ridge Study Site**

**The blue area represents the entirety of Bunchgrass Ridge while the yellow area represents Bunchgrass Meadow, where this study took place, within the Western Cascade Range in Oregon. The site proximity to Highway OR 126 and other landmarks is shown with a scale included in the bottom righthand corner. Source: Google Maps.**



**Figure 8. Experimental Design**

Each square represents one of eight nutrient treatments with a replicate in each block. Figure adapted from Altmire, 2020.

## Data Collection

### *Grasshopper Collection*

Grasshoppers are the most influential chewing insect herbivores at this site; this made them the focus of the study. However, it is important to note that Bunchgrass's insect community is diverse such that grasshoppers are not the only insect herbivores active throughout a field season. Since the chewing activity of other insect herbivores is marginal when compared to grasshoppers, data collected centered only around



grasshoppers. To measure abundance, the Onsanger ring count method was used. In each plot, there were metal rings that measured between 8-25 centimeters. Weekly, the grasshoppers per ring were quantified; the density was calculated and extrapolated outwards to represent the entire plot.

### *Leaf Damage*

In each plot, leaf damage was estimated for six focal plant species: *Carex abrupta*, *Bromus carinatus*, *Lupine oregonus*, *Festuca idahoensis*, *Penstemon diffusa*, and *Lathyrus latifolius*. In this study, however, only *Bromus carinatus* and *Lupine oregonus* were analyzed due to their overall abundance across all plots. 30 leaves of each species were collected from each plot at peak biomass (July 30, 2021). In the laboratory, leaf damage per leaf was quantified categorically: 0%-25%, 26%-50%, 51%-75%, 76%-100%. Chewing damage was identified by physical holes or chunks of leaf that had been removed in the shape of a bite mark or the size of a grasshopper's mouth. We also monitored grasshopper sucking, which is characterized by discolorations on leaves in similar sizes to chewing marks.

### *Carbon to Nitrogen Analysis*

Carbon to nitrogen ratios for *B. carinatus*, a grass, and *L. oregonus*, a legume, were analyzed. 30 leaves were collected for each species from each plot and homogenized to create one sample. Only the leaves were removed from each plant. In total, there were 55 plant samples. Samples were frozen until they were prepared for C:N analysis. Preparation entailed drying the samples at 60 degrees Celsius for 48 hours and grinding them into fine powder using a mortar and pestle. Three milligrams of each

ground up sample was measured on a microbalance to an accuracy of 0.001mg and placed into a tinfoil cup to be put in an elemental analyzer. After preparation, samples were transported to Oregon State University's Soil Health Lab in Corvallis, Oregon to be analyzed in an Elementar vario macro cube, a machine that can determine the ratios of carbon, nitrogen, and other stable isotopes in plant tissues and soil.

### **Instruments Used for Analysis**

The Elementar Vario Macro Cube was operated by the Soil Health Lab at Oregon State University. The Soil Lab analyzed elemental concentrations in samples based on the Dumas Method. The Dumas Method, a form of dry combustion, is a process often used in chemistry to obtain concentrations of elements in a particular substance. The samples used in this study were combusted at 1,185 degrees Celsius in the presence of oxygen; this releases carbon dioxide, water, and nitrogen. Samples were then passed over columns containing a solution that can absorb the carbon dioxide and water off the sample. Finally, the machine uses thermal conductivity to remove excess water or carbon dioxide, leaving only the sample behind (McClements, n.d.). The ratio is observed through infrared spectroscopy, and numerical values are generated based on the waves emitted. Kristen McAdow and Adam Fund, two researchers at the Soil Health Lab, personally ran the samples for this project.

### **Statistical Analysis**

Statistical analysis on the data was performed using the R software package, specifically the libraries tidyverse, dplyr, ggplot2, lme4, car, permute, vegan, and MASS, to examine whether the independent variables of soil nutrient treatments, plant

species, and C:N ratios had a significant impact on grasshopper feeding preferences (Wickham et al., 2019; Wickham et al., 2021; Wickham, 2016; Bates et al., 2015; Fox & Weisberg, 2019; Simpson, 2022; Oksanen et al., 2020; Venables & Ripley, 2002). A 2-Way ANOVA was first used to determine whether grasshopper feeding preferences in the form of leaf damage were affected by soil nutrient treatment and plant species ( $\alpha=0.05$ ). An additional interaction test was used to determine the interrelatedness of soil nutrient treatment and plant species in terms of how they impact grasshopper feeding. Due to emerging trends in the data, 2-Way ANOVAs were performed to ascertain the significance of the presence of nitrogen and the presence of phosphorus in the soil on grasshopper feeding ( $\alpha=0.05$ ).

More 2-Way ANOVAs were run with the C:N ratio data, one to determine if C:N ratio variance was caused by soil nutrient treatments and plant species, one to isolate the effects nitrogen enrichment has on C:N ratios, and one to examine C:N ratios, plant species, and the co-variance between the two and how they affect chewing behaviors ( $\alpha=0.05$ ). Additionally, an OLS regression generated lines of best fit to predict grasshopper chewing damage based on grass species, soil nutrient treatment, and C:N ratios. Finally, an MDS plot was created with a Bray-Curtis similarity test as the distance metric to examine grasshopper chewing patterns across various nutrient treatments. An ANOSIM was used to confirm statistical separation between the nutrient treatments.

The Shapiro Test of Normality and the Levene Test for Equality of Variances were performed for each ANOVA to see if the data met the assumptions of normal distribution and equal variances. The assumptions were not met for most ANOVAs, but

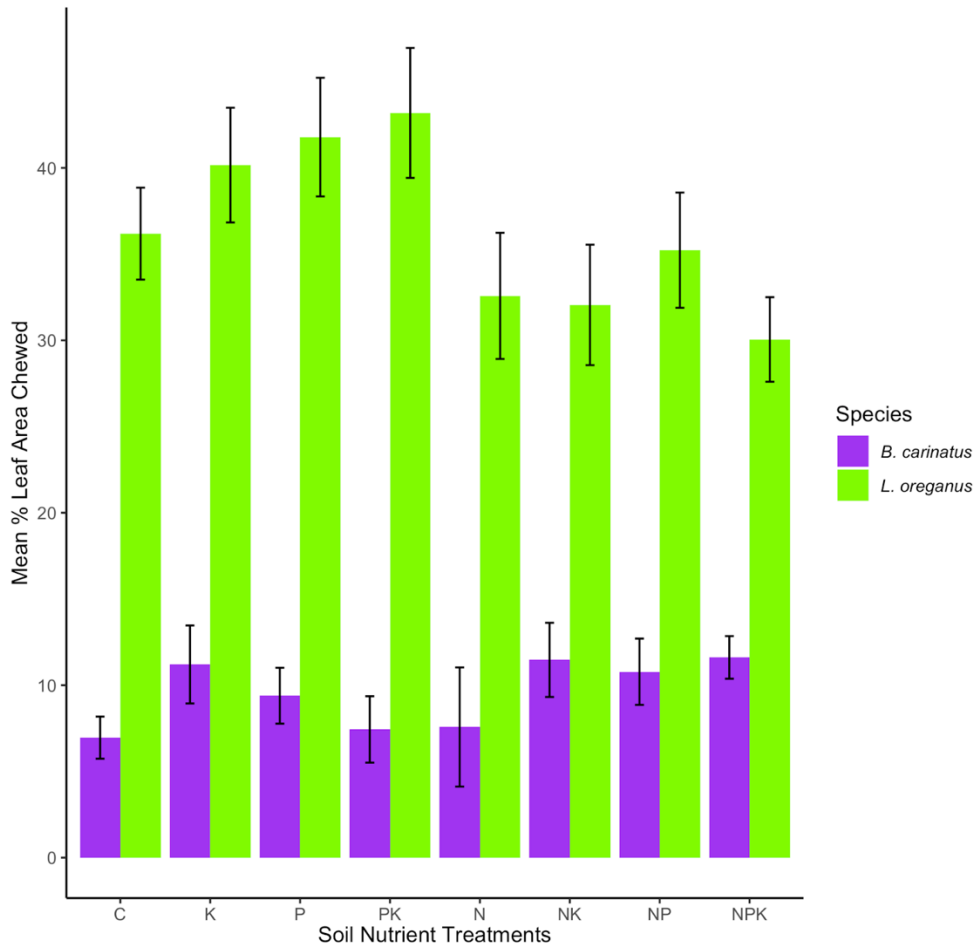
the ANOVAs were performed anyway due to the large sample size. I got approval from the Masters student I worked with to proceed with statistical analysis.

## Results

### Grasshopper Feeding Preferences

Grasshopper feeding preferences across all nutrient treatments were quantified for both *B. carinatus* and *L. oregonus*. Across all soil nutrient treatments, grasshoppers ate more *L. oregonus* than *B. carinatus* (Figure 9).

For *L. oregonus*, feeding levels were lower in all plots where nitrogen was present. Plots with the highest chewing levels were ones that contained phosphorus (P, PK). This difference was not apparent in plots with a combination of nitrogen and phosphorus, but it is important to note that NP plots had the highest levels of chewing across all nitrogen containing plots. This same pattern did not appear for *B. carinatus*, and there appears to be little variance in the percentage of chewed leaf area across all soil nutrient treatments, although the chewing levels were slightly higher when nitrogen was present in the plots. A 2-Way ANOVA ( $\alpha=0.05$ ) showed that there was significance between leaf damage and the nutrient treatment, species, and the interaction between the soil treatments and the species (Table 1).

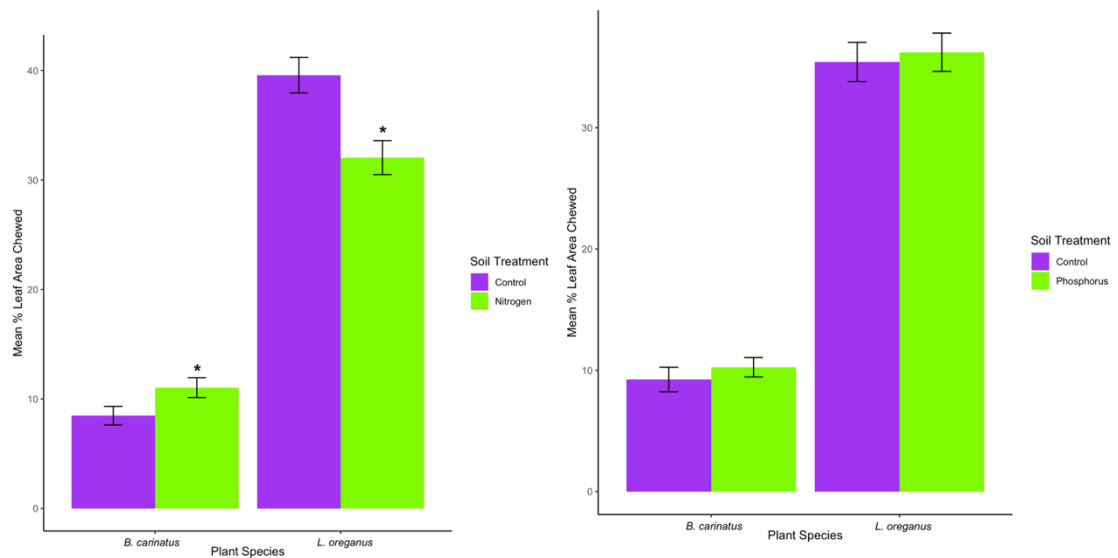


**Figure 9. Percent Leaf Area Chewed Across All Soil Nutrient Treatments**

Chewing percentages for each treatment were calculated across all three blocks ( $25.299 \pm 0.782$ ,  $SD=32.149$ ). Treatments are ordered by the presence of nitrogen enrichment in the soil.

To understand the effects of nitrogen, I isolated nitrogen-containing plots (N, NK, NP, NPK) and compared them with control plots (Figure 10, left). For *L. oreganus*, mean chewing levels were lower in plots containing nitrogen, while for *B. carinatus*, mean chewing levels were very similar, but slightly higher in plots containing nitrogen. A 2-Way ANOVA ( $\alpha=0.05$ ) showed there is a significant interaction between grasshopper feeding and nitrogen presence in the plots (Table 2). For phosphorus-containing plots, however, there was very little difference in the percent of leaf area

chewed by grasshoppers for both species across treatments (Figure 10, right). A 2-Way ANOVA ( $\alpha=0.05$ ) showed that unlike nitrogen additions, phosphorus additions had no statistically significant effect on grasshopper feeding (Table 3). This is interesting to note because phosphorus appeared to be the preferred nutrient in *L. oreganus* based on highest chewing values (Figure 9).



**Figure 10. Percent Leaf Area Chewed Isolated by Nitrogen and Phosphorus Enrichment**

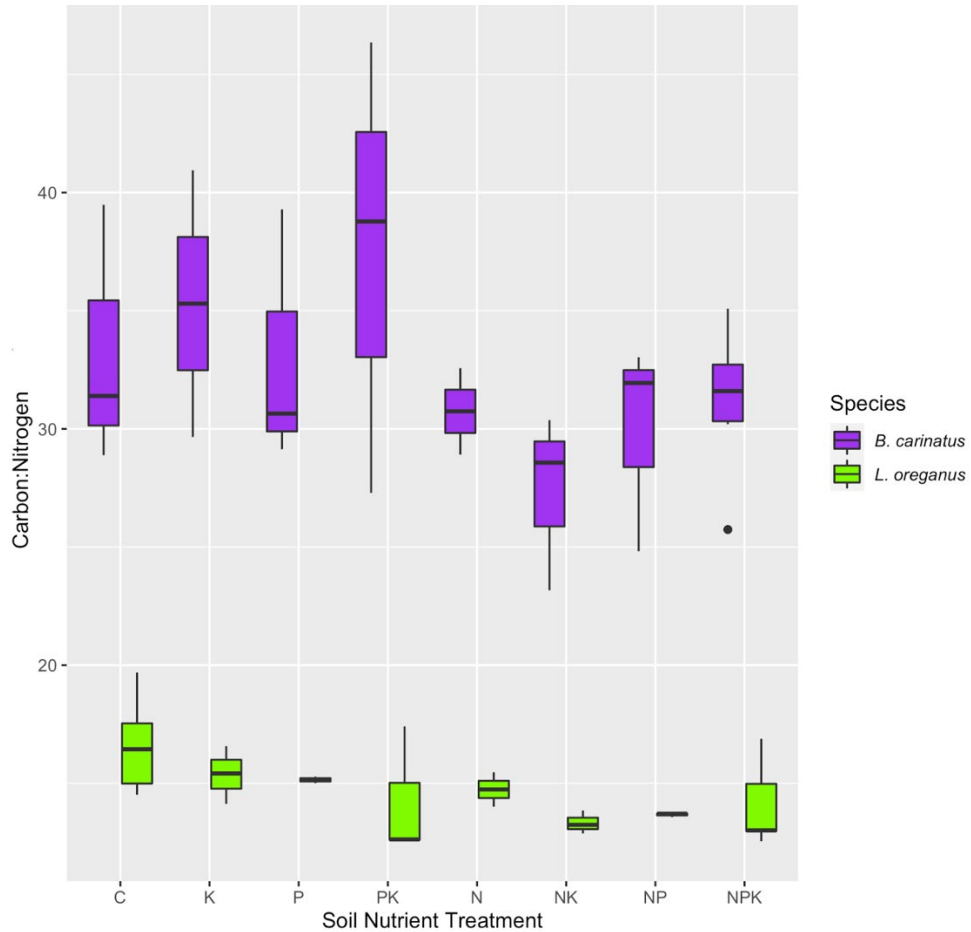
The graph on the left isolates nitrogen from the rest of the treatments ( $22.979 \pm 1.029$ ,  $SD=30.450$ ) and graph on the right isolates phosphorus ( $24.405 \pm 1.024$ ,  $SD=31.694$ ) across all three blocks. Control plots in each graph are comprised of plots that do not contain the nutrients being specifically tested for. An asterisk indicates significance at a 95% confidence interval.

I tested the interaction between the independent variables of plant species and nutrient treatment ( $\alpha=0.05$ ) and it yielded a significant interaction. For *L. oreganus*, this interaction was significant. For *B. carinatus*, however, it was not significant (Table 4). This shows that treatment did have a significant effect on grasshopper feeding in *L. oreganus* but not in *B. carinatus*, which is consistent with the trends seen in Figure 9.

### **Differences in C:N Ratios Between *B. carinatus* and *L. oreganus***

Because grasshoppers are typically nitrogen-limited, plants with lower C:N ratios are more nutritious than those with higher ratios, making them more desirable for grasshoppers. CHN elemental analysis showed that C:N ratios in *B. carinatus* were higher than ones in *L. oreganus* across all nutrient treatments, meaning that there was more carbon compared to nitrogen in *B. carinatus* than in *L. oreganus*. *B. carinatus* also displayed a larger variation of C:N ratios in each soil enrichment treatment (Figure 11). A 2-Way ANOVA exploring the effects of both soil nutrient treatment and plant species on C:N ratios further confirmed that differences in C:N ratios between plant species were statistically significant. However, the data showed that treatment did not have a significant effect on C:N ratios, and the covariance between treatment and species did not affect C:N ratios (Table 5).

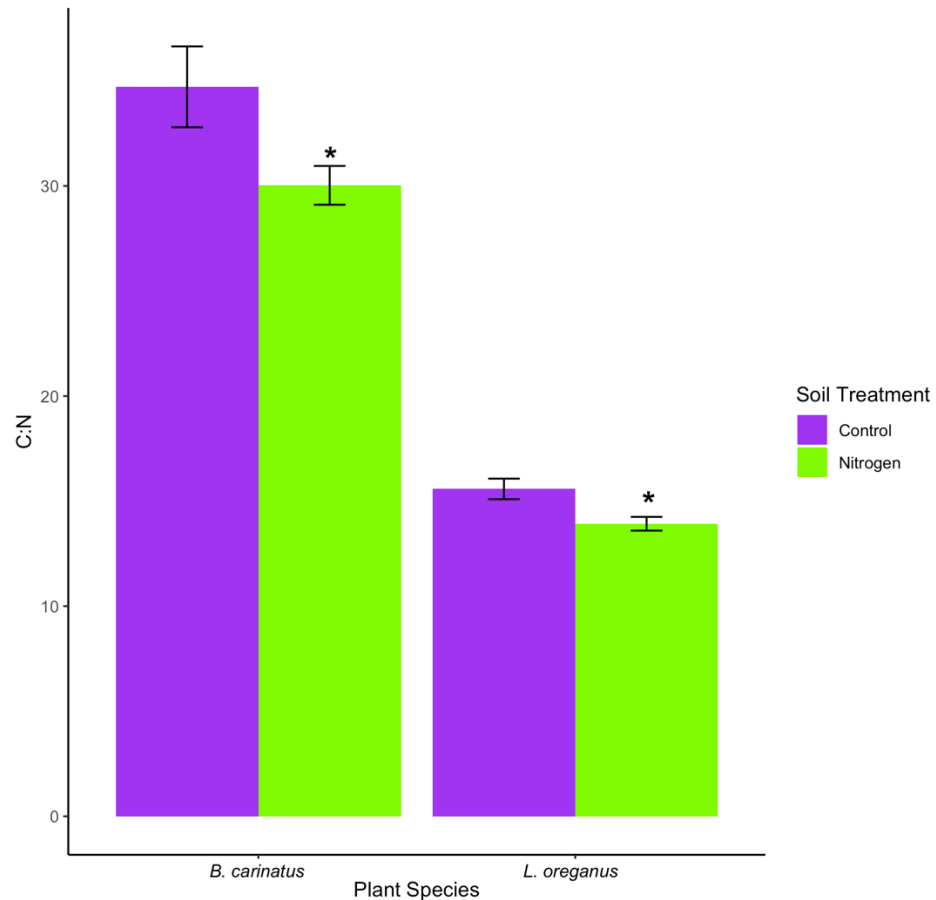




**Figure 11. Carbon to Nitrogen Ratios in Primary Producers Across All Nutrient Treatments**

Each boxplot represents the spread of the data from the minimum to maximum values found across all three experimental blocks ( $22.633 \pm 1.283$ ,  $SD=9.517$ ).

Again, I isolated nitrogen-enriched plots and compared them to control plots, this time for C:N ratios. In both *B. carinatus* and *L. oreganus*, C:N ratios were lower in plots containing nitrogen enrichment. The difference in nitrogen and control ratios were higher in *B. carinatus*, which corresponds with the trends demonstrated in Figure 11 (Figure 12). A 2-Way ANOVA comparing C:N ratios across nitrogen-enriched plots and plant species confirmed that nitrogen did have a significant effect on C:N ratios in both species (Table 6).



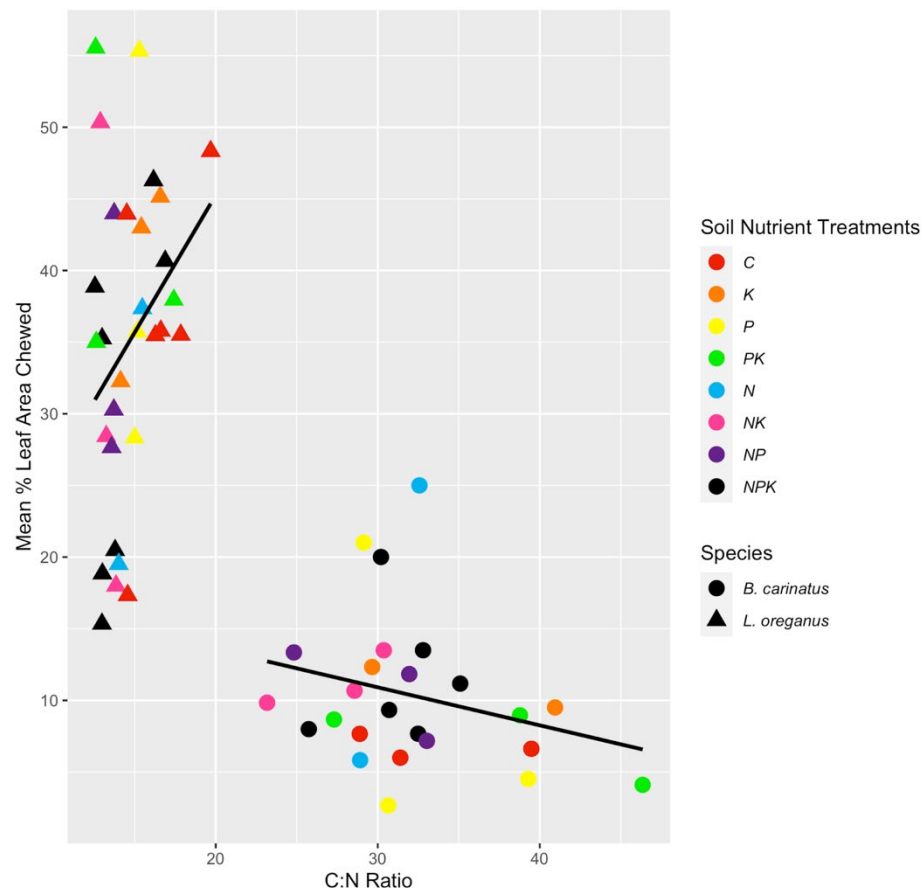
**Figure 12. C:N Ratios in Plots With and Without Nitrogen Enrichment Across Both Plant Species**

The results from all three experimental blocks are shown ( $21.698 \pm 1.592$ ,  $SD=8.569$ ). An asterisk indicates significance at a 95% confidence interval.

### **Comparing Grasshopper Chewing Across C:N Ratio, Soil Nutrient Treatment, & Plant Species**

Across all soil nutrient treatments, *B. carinatus* samples had higher C:N ratios and lower chewing values than *L. oreganus*. In *B. carinatus*, plots containing any form of nitrogen enrichment (N, NP, NK, NPK) had higher average chewing damage than those without nitrogen. *L. oreganus* samples, on the other hand, had low C:N values which correspond with more nutritious plants, and higher chewing percentages. Plots

without nitrogen (C, K, P, PK) typically had higher chewing damage than those without in *L. oreganus* (Figure 13). While nutrient treatment did not have a significant impact on C:N ratios, C:N and plant species did significantly impact grasshopper chewing (Table 7).



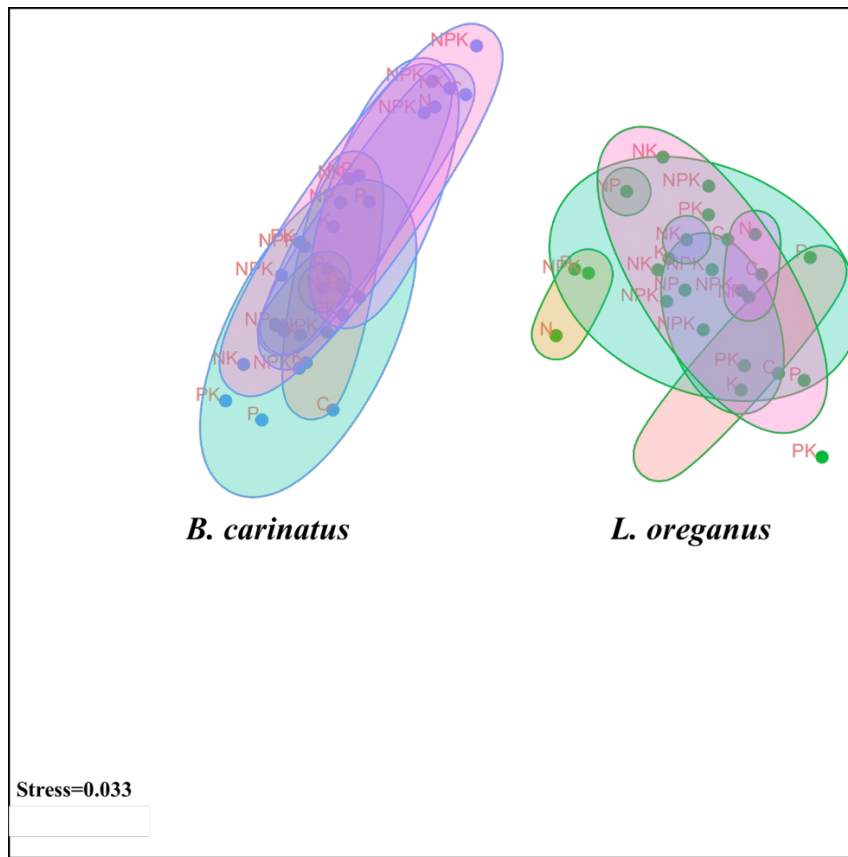
**Figure 13. Mean Percent Leaf Area Chewed versus C:N Ratios Across All Nutrient Treatments**

The average percent leaf area chewed ( $25.299 \pm 0.782$ ,  $SD=32.149$ ) is tested for against all three independent variables. Colors represent different soil nutrient treatments and shape represents plant species. Lines of best fit are shown for each species.

An OLS regression showed that interactions between C:N ratios, plant species, and soil nutrient treatments combine to account for a line of best fit that can explain

roughly 70% of the variation within grasshopper feeding data ( $R^2=0.70$ ). *B. carinatus* was the reference group, meaning that the values obtained through the analysis represent *L. oreganus* in comparison to *B. carinatus*. Interactions between plant species and treatments created the largest differences in chewing leaf damage across all other factors, the highest being a 1,092.97 unit increase in chewing in *L. oreganus*. While many interactions had multi digit increases or decreases in chewing percentages compared to *B. carinatus*, most were not significant ( $\alpha=0.05$ ). Despite the lack of significance for most individual interactions between chewing and the different variations of the independent variables themselves, the overall model suggested a significant interaction ( $p=8.62e-5$ ).

Finally, an MDS plot, which fit the data well (stress=0.033), showed clear separation between the treatments used for *B. carinatus* and *L. oreganus* (Figure 14). However, each species showed an overlap of the ellipses, meaning that there was not a clear difference in the treatment effect on the chewing percentages within each species. There was less overlap within *L. oreganus* between each treatment, which is consistent with the results provided in Figure 7. These results were confirmed by an ANOSIM analysis (Global  $R=0.96$ ,  $p=0.001$ ).



**Figure 14. MDS Plot Between Both Plant Species**

**The circles represent groups of closely related chewing percentages by nutrient treatment with 70% similarity to each other. Overlapping circles indicate similarity between different groups.**

## Discussion

### Grasshopper Responses to Different Soil Nutrient Treatments

Based on the results of this project, it is clear that grasshoppers favor *L. oregonus* as a food source over *B. carinatus*, which supports my first hypothesis. However, feeding preferences for *L. oregonus* were reduced with nitrogen addition. Nitrogen is widely considered an important nutrient for grasshopper feeding and growth, meaning that nitrogen levels in primary producers help dictate the size and growth of grasshopper populations (Rode et al., 2017). Nitrogen nutrient enrichments had lower chewing values in *L. oregonus*. Generally, phosphorus-enriched plots had higher chewing values, meaning that phosphorus may be more limiting than nitrogen, but only in *L. oregonus*.

While phosphorus enrichment appeared to increase grasshopper chewing in *L. oregonus*, there was no statistical significance when the plots were isolated. Nitrogen enrichment, however, did cause significance. This may be explained by the nature of *B. carinatus* as a grass species. Grass species are low in nutrients compared to legumes, so nitrogen enrichment may make them much more attractive to grasshoppers. Phosphorus enrichment, however, may have only had marginal effects compared to nitrogen, potentially explaining their differences in significance. This does not negate the explanations of phosphorus's importance explained in this section, but the severity may differ across different grassland ecosystems.

One possible explanation for phosphorus importance is that ribosomal RNA (rRNA) is phosphorus rich. rRNA takes genetic code from messenger RNA and is able to actively use it for protein synthesis, which is highly important for somatic cell growth

in grasshoppers (Rode et al., 2017). Grasshoppers and other insect herbivores are often phosphorus rich and therefore need to consume plants with high phosphorus levels in order to maintain their internal stoichiometric balance (Ibanez et al., 2017). This is explained through the growth rate hypothesis, which states that both plants and insect herbivores need phosphorus rich nutrient sources to make more rRNA which in turn stimulates faster protein synthesis (Matzek & Vitousek, 2009).

Another possible explanation for grasshoppers' preference for phosphorus may be related to plant community composition. Long term nitrogen and phosphorus additions can change ecosystem functions and decrease plant community diversity. However, their impacts on community composition drives aboveground biomass production, which in turn affects insect herbivores and their food availability (Avolio et al., 2014). Nitrogen enrichment may also negatively affect plants' ability to reabsorb both nitrogen and phosphorus, and this overall interaction with plant community composition changes insect herbivore preferences (Lü et al., 2020).

This alone does not explain why grasshoppers preferred phosphorus rich legumes, as leaf stoichiometry is not always the only defining factor for feeding preferences, but it is a good hypothesis to explain general trends for the scope of this project. I did not measure the stoichiometric ratios of phosphorus along with carbon and nitrogen because I was not aware of how important phosphorus would be to this experiment and due to time constraints. Future work would benefit from comparing plant stoichiometric contents of both nitrogen and phosphorus in terms of grasshopper feeding.

## Carbon to Nitrogen Ratio Analysis

My second research question explored C:N ratios in *B. carinatus* and *L. oreganus* and how these ratios influenced grasshopper feeding preferences. My hypothesis was mostly supported, as there was statistical significance between the C:N ratios of the two species. *B. carinatus* did have higher C:N ratios across all nutrient treatments as well as a larger spread of ratios at each treatment. Additionally, ratios in *B. carinatus* were lower in nitrogen-enriched plots. For *L. oreganus*, ratios across all treatments had a much smaller spread and were fairly similar.

Across both plant species and all soil treatments, plant C:N was roughly 22.633:1. According to Figure 2, this should cause microbial immobilization, meaning that microbes absorb nitrogen in the soil. This in turn can affect the amount of usable nitrogen present in the soil for plant use. In control plots, plant C:N ratios were around 22.141:1. In nitrogen-enriched plots, however, plant C:N ratios were very slightly lower at 21.698:1, meaning that enrichment may not have caused soil microbes to release or use more nitrogen than they normally would have.

The main explanation for the lower C:N ratios in *L. oreganus* is due to its status as a legume. Legumes have nitrogen fixing bacteria within their roots, allowing for more nitrogen absorption and retention in legume based ecosystems, making them much more nutrient rich (Suter et al., 2015). Grasses like *B. carinatus* cannot fix nitrogen, making their nitrogen contents lower and their C:N ratios higher, therefore less nutritious for insect herbivores.

As nutrients were added to the soil in this experiment, the legume C:N ratio consistently decreased, with the control plots being the highest and the NPK+ plot being



the lowest. This shows that these legumes were able to retain the added nutrients effectively. Legumes also can reduce nitrogen loss and runoff from pastures and agricultural sites when they are part of the primary producer cover, which may be a way to mitigate nutrient pollution into surrounding areas (MacAdam et al., 2022; Suter et al., 2015). Legume nitrogen fixation is one way to stabilize soil ecosystems through producing an optimal amount of usable nitrogen. However, as climate change begins to affect salinity, temperature, and water availability in soils, this will also affect legumes' abilities to fix nitrogen and support ecosystem functions through this fixation (Sulieman & Tran, 2015).

My second research question grouped both the C:N ratios and the soil nutrient treatments together and asked how they both affected leaf damage via grasshopper herbivory. This hypothesis was also supported because *L. oregonus* samples across all nutrient treatments had statistically significant lower C:N ratios and higher leaf damage values than *B. carinatus* did. Plants with lower C:N ratios contain a higher amount of nitrogen in proportion to the carbon in the rest of the plant, which makes them more nutritious and more sought after as a food source. Grasses have less nutrients than legumes, making them less optimal for grasshoppers, which was shown through the lower leaf damage levels. This was further explained by the strong significance C:N ratios caused on chewing behaviors but relatively low significance that plant and C:N co-variation had (Table 7). C:N ratios can cause significance on their own but adding plant species as another factor explaining variation in the data reduces that significance.

Due to anthropogenic climate change and fossil fuel emissions, atmospheric carbon dioxide levels are rapidly increasing. Under these elevated conditions, legumes

are able to increase their nitrogen fixation levels, creating more usable nitrogen in the soil (Lam et al., 2012). This provides both positive and negative impacts to the surrounding ecosystem, as it creates more resources for plants to utilize, which often increases above ground plant biomass, but it also drives a shift towards nonnative grasses in many ecosystems (Alpert & Maron, 2000; Huenneke et al., 1990). Additionally, increased atmospheric carbon dioxide increases legume C:N ratios, which may make legumes less desirable as a food source (Lam et al., 2012). It is unclear whether this same trend will occur at Bunchgrass Meadow due to its isolation from most anthropogenic carbon dioxide sources, but there is existing research from the H.J. Andrews Experimental Forest conducted on carbon dioxide effects on these ecosystems (Pierson et al., 2021; Harmon, 2021).

It is important to note that there was no significance detected between C:N ratios in plant tissues compared to the various soil nutrient treatments or between C:N ratios and covariance between plant species and soil treatments. This could have been caused by the addition of non-nitrogen treatments into the statistical analysis. While there is some evidence of co-limitation between nitrogen and phosphorus, there is very little information about potassium and nitrogen co limitation for temperate grassland ecosystems. Potassium has proven to be a limiting factor for tropical soils as well as a co limiting factor with phosphorus, but its role in soil ecosystems in temperate communities is much less clear (Wright et al., 2011; Santiago et al., 2012). In temperate ecosystems, potassium has been seen to increase biomass when paired with nitrogen enrichment (Kering et al., 2012). Other studies have found that potassium is important for the photosynthetic processes of plants and maintaining stoichiometric homeostasis,

which is tied through above ground biomass, but that there is much stronger co variance between nitrogen and phosphorus (Wang & Moore, 2014; Hou et al., 2018).

### **Comparison of All Variables and Grasshopper Chewing**

Soil nutrient treatment, plant tissues C:N, and plant species each had a different effect on grasshopper feeding preferences, partially supporting my third research question. Nutrient treatment alone had the least severe impact on grasshopper feeding, while plant species had the largest. The nutrient treatment variable groups all eight treatments together and compares them to chewing damage. It does not take into account, however, different plant species and their relative importance to grasshoppers. A possible explanation for this is that regardless of what element(s) was added to the soil, grasshoppers were able to obtain the nutrients they needed. Element stoichiometric ratios are highly important to insect life-history traits, fitness, and physiology, making the ability to find nutrient rich foods essential (Filipiak & Weiner, 2017). I did not analyze the stoichiometric ratios of the grasshoppers themselves, but it appears as if the nutrient additions in all soils contained enough essential nutrients.

The OLS regression model run on all three independent variables proved to be statistically significant, but it only accounted for roughly 70% of variation within the site. The other 30% of the variations may be explained by several other factors like grass species percent cover and composition, competition for particular resources, water availability, sunlight, and predation to name a few (Murray et al., 2013; Branson, Joern, & Sword, 2006). Nutrient availability is clearly very important, but it is not the only defining factor of grasshopper feeding preferences.

## **Study Limitations**

Naturally, there are some limitations to the findings of this study. The elemental analyzer tests only found stoichiometric contents of carbon and nitrogen, but after looking at the results, it would have been beneficial to have the phosphorus and potassium percentages as well. Grasshoppers are known to excrete phosphorus when eating plants that are high in phosphorus, but they do not do this with nitrogen (Zhang et al., 2014). Measuring grasshopper phosphorus excretion is also another way to have general data about the phosphorus contents in plant tissues without having to do elemental analysis, so doing something like this would have also been beneficial to this study.

Bunchgrass is home to ecological studies involving long-term soil nutrient enrichment. Researchers from schools throughout the Pacific Northwest use this area as a study site, so the grasshoppers and other insect herbivores may have adapted to these conditions through natural selection, digestive mechanisms, and developing unique feeding niches, meaning that the data collected may be influenced by this (Behmer, 2009). Long-term ecological studies will be essential, however, for understanding the relationships between changing soil conditions and insect responses.

While we did record percent cover data using the application ImageJ, I did not use the data in my research because of how complex the project already was with three independent variables influencing feeding. After looking at my findings as well as reading previous literature, grass cover composition plays a significant role in insect herbivory, and it would have brought another interesting perspective to my projects. If I

had more time to work on this project, this is something that I would have explored further.

### **Future Directions**

There are many other insect herbivores, in addition to grasshoppers, that are active at Bunchgrass Ridge. Observing their feeding preferences and comparing them to grasshopper preferences under the same experimental conditions may show how different insect herbivores respond to the same change.

While nitrogen, phosphorus, and potassium often leach into ecosystems through agricultural nutrient pollution, carbon dioxide emissions through fossil fuel burning are another prominent anthropogenic aspect of climate change. Increased atmospheric carbon dioxide influences plants' ability to absorb soil nutrients, changing their composition and overall value to insect herbivores. Incorporating elevated atmospheric carbon dioxide into the study site through controlled release may be an interesting way to see how the ecosystem changes under multiple forms of anthropogenic climate change. Bunchgrass Ridge is not a location that currently experiences nutrient pollution or extreme carbon dioxide emissions, but other montane ecosystems around the country and world may, so having a baseline knowledge of the trends will be very helpful to long term ecological studies. The Long-Term Ecological Research (LTER) Program, which the H.J. Andrews Experimental Forest has been a part of since 1980, is a comprehensive way to begin and monitor ecological changes across many different ecosystems in order to gain information on how they are changing (Willig & Walker, 2016; LTER, 2021). The ecological monitoring in HJ Andrews mainly focuses on forest

biology, so it will be important in the future to incorporate montane ecosystems into that as well.

Finally, increased nutrient levels in soil make ecosystems more vulnerable to nonnative invasions and a shift in plant dominance from forbs and legumes to grasses. Observing changes in plant community composition, especially in terms of native versus nonnative status, is very important when trying to understand future threats to unique ecosystems like montane meadows.

## Conclusions

Ecosystems around the world are changing due to various global change drivers, including anthropogenic climate change and soil nutrient enrichment. Understanding how soil microbial communities and primary producers at the base of food webs change in response to this is essential in understanding how consumers at higher levels of trophic systems will respond. This experiment looks at only two species, *B. carinatus* and *L. oreganus*, and their response to various forms of change. How other grasses respond in montane meadows is less clear to me, but that knowledge can only help broaden ecological understanding of these ecosystems. To prepare for these changes, experiments like these must be conducted more frequently and over longer periods of time to best understand the implications of nutrient enrichment for soil and plant communities.

## Appendix

Asterisks in the probability value column indicate a significant interaction at a 95% confidence interval

**Table 1. 2-Way ANOVA Comparing Grasshopper Chewing with Soil Nutrient Treatment, Plant Species, and the Co-Variance Between the Two**

	Degrees of Freedom (df)	Sum of Squares (ss)	Mean Sum of Squares (ms)	Variance Ratio (F)	Probability Value (P)
Leaf damage vs. Nutrient Treatment	7	15,084	1676	1.9366	0.043147*
Leaf damage vs. Plant Species	1	268,457	268,457	310.2030	0.000000*
Leaf Damage vs. co-variance between Nutrient Treatment and Plant Species	7	16,693	1,855	2.1433	0.023395*

**Table 2. 2-Way ANOVA Comparing Grasshopper Chewing with Nitrogen Presence, Plant Species, and the Co-Variance Between the Two**

	Degrees of Freedom (df)	Sum of Squares (ss)	Mean Sum of Squares (ms)	Variance Ratio (F)	Probability Value (P)
Leaf damage vs. Presence of Nitrogen	1	9,791	9,791	11.341	0.00077515*
Leaf Damage vs. Plant Species	1	269,767	269,767	312.464	0.000000*
Leaf Damage vs. Co-Variance between Presence of Nitrogen and Plant Species	1	10,311	10,311	11.944	0.00056199*



**Table 3. 2-Way ANOVA Comparing Grasshopper Chewing with Presence of Phosphorus, Plant Species, and the Co-Variance Between the Two**

	Degrees of Freedom (df)	Sum of Squares (ss)	Mean Sum of Squares (ms)	Variance Ratio (F)	Probability Value (P)
Leaf damage vs. Presence of Phosphorus	1	1769	1796	2.0281	0.1546
Leaf Damage vs. Plant Species	1	273,070	273,070	313.0552	<2e-16*
Leaf Damage vs. Co-Variance between Presence of Phosphorus and Plant Species	1	4	4	0.005	0.9435

**Table 4. Interaction Test Between Plant Species and Nutrient Treatment**

	F Value	P Value
<i>B. carinatus</i>	0.3351	0.09072
<i>L. oreganus</i>	2.7507	0.0116*

**Table 5. 2-Way ANOVA Comparing C:N Ratios with Nutrient Treatment, Plant Species, and the Co-Variance Between the Two**

	Degrees of Freedom (df)	Sum of Squares (ss)	Mean Sum of Squares (ms)	Variance Ratio (F)	Probability Value (P)
C:N Ratios vs. Soil Nutrient Treatment	7	120.7	17.2	1.2186	0.31598
C:N Ratios vs. Plant Species	1	4137.1	4137.1	292.3294	0.00000*
C:N Ratios vs. Co-Variance between Soil Nutrient and Plant Species	7	81.4	11.6	0.8213	0.57556

**Table 6. 2-Way ANOVA Comparing C:N Ratios with Presence of Nitrogen Enrichment, Plant Species, and the Co-Variance Between the Two**

	Degrees of Freedom (df)	Sum of Squares (ss)	Mean Sum of Squares (ms)	Variance Ratio (F)	Probability Value (P)
C:N Ratio vs. Presence of N Enrichment	1	53.6	53.6	4.300	0.04318*
C:N Ratio vs. Plant Species	1	4171.0	4171.0	334.702	<2e-16*
C:N Ratios vs. Co-Variance between Presence of N Enrichment and Plant Species	1	31.0	31.0	2.489	0.12083

**Table 7. 2-Way ANOVA Comparing Grasshopper Chewing with C:N Ratios, Plant Species, and the Co-Variance Between the Two**

	Degrees of Freedom (df)	Sum of Squares (ss)	Mean Sum of Squares (ms)	Variance Ratio (F)	Probability Value (P)
Leaf Damage vs. C:N Ratio	1	7070.2	7070.2	91.9162	5.32e-13*
Leaf Damage vs. Plant Species	1	1348.6	1348.6	17.5327	1.12e-4*
Leaf Damage vs. Co-Variance between C:N Ratios and Plant Species	1	393.1	393.1	5.1106	0.0280777*

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