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UNIVERSITY OF CALIFORNIA  
RIVERSIDE

Tritrophic Mutualisms in a Changing Climate

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

Doctor of Philosophy  
in  
Evolution, Ecology, and Organismal Biology

by

Andrea M. Keeler

December 2022

Dissertation Committee:  
Dr. Nicole Rafferty, Chairperson  
Dr. Erin Wilson Rankin  
Dr. Marko Spasojevic

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This Dissertation of Andrea M. Keeler is approved:

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Committee Chairperson

University of California, Riverside

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The text of Chapter 2 of this dissertation is a re-print of the material as it appears in: “Legume germination is delayed in dry soils and in sterile soils devoid of microbial mutualists: species-specific implications for upward range expansions”, *Ecology & Evolution* 2022. The co-author listed in this publication, Nicole E. Rafferty, directed and supervised the research which forms the basis for this dissertation.

## **Dedication**

This dissertation is dedicated to my sister, Amanda; my students, from whom I've learned the most; and my beloved pets, Neko and Mist.



## ABSTRACT OF THE DISSERTATION

### Tritrophic Mutualisms in a Changing Climate

by

Andrea M. Keeler

Doctor of Philosophy, Graduate Program in Evolution, Ecology, and Organismal Biology  
University of California, Riverside, December 2022  
Dr. Nicole E. Rafferty, Chairperson

Mutualistic species interactions are ubiquitous. Every species on Earth is involved, directly or indirectly, in a net beneficial partnership. Through shifts in partner phenology and distribution, climate change can disrupt mutualistic interactions, resulting in altered interactions and modified communities. However, research has focused on pairwise mutualisms, neglecting the fact that species interact with multiple mutualists simultaneously. In this dissertation, I explore the effects of climate change on nitrogen-fixing soil microbes, legume plants, and their pollinators. Climate change is predicted to affect the activity and ranges of belowground mutualists which will lead to changes in host plant germination timing and success, water use efficiency, floral traits, and, consequently, bee pollinator behavior. In the following chapters, I use a multi-year snowmelt timing manipulation, historical legume leaf tissue samples, and common garden techniques to assess the effects of belowground mutualism loss via climate stress on legume functional traits and legume-solitary bee interactions. I find evidence that climate warming, advanced snowmelt, and drought can lead to a short-term loss of interactions between soil microbes and leguminous plants, consequently leading to shifts in germination phenology, the quality floral rewards, plant nitrogen content, and consequently, pollinator floral preferences. Such costs could translate into reduced fitness and novel selection pressures for bees and flowering plants in the short term. My dissertation highlights the importance of studying multiple

mutualisms in a climate change context and serves to identify the effects of soil microbial mutualism loss on legumes and on higher-order mutualists, such as bees.

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## Introduction

Mutualisms, or interspecific interactions in which each partner incurs a net fitness increase, are ubiquitous in nature (Boucher et al. 1982, Bronstein 1994). Every species on Earth is involved in at least one mutualistic partnership, directly or indirectly (Bronstein 2015).

Mutualisms have shaped the atmosphere, hydrosphere, and lithosphere throughout Earth's history (Fath 2007, Bronstein 2015). The importance of mutualisms is seen in every system: mutualisms provide essential ecosystem services (e.g., seed dispersal, pollination), are vital to the survival, growth, and reproduction of multitudes of organisms (e.g., gut endosymbionts), play a large role in global nutrient cycles (e.g., N<sub>2</sub>-fixing bacteria), and have shaped Earth's biodiversity (Stebbins 1970, Clay and Holah 1999, Little et al. 2004, Van Der Heijden et al. 2008, Fenice 2021).

Evolutionary transitions enabling the diversification of life, such as the evolution of the eukaryotic cell and the movement of plants to land, were made possible by mutualistic interactions (Margulis 1970, Pirozynski and Malloch 1975). Despite their notable importance, mutualisms were not seriously studied until the late twentieth century (Boucher et al. 1982), but theoretical advancements are growing.

Anthropogenic global climate change is rapidly altering biotic and abiotic features of the planet and is altering the demography, evolution, phenology, physiology, and distributions of species (Hughes et al. 2003, Parmesan 2006, Miller-Rushing et al. 2010, Scaven and Rafferty 2013, Burkle et al. 2013, Kudo and Cooper 2019, Richman et al. 2020). These changes could lead to shifts in the strength and persistence of mutualisms (Tylianakis et al. 2008, Kiers et al. 2010). Many historical studies focus on how a single species or population will respond to anthropogenic global climate change, without considering how the mutualistic interactions in which they are involved will be affected (Wolfe et al. 2005, Franks et al. 2014). Mutualisms form only when receptive partners can locate one another in time and space. Thus, the effects of climate change

on mutualisms are complex as partners may respond differently to climatic cues (Visser and Both 2005, Chakraborty et al. 2014), where differential responses among partners could lead to the loss of mutualisms within a growing season and mutualism breakdown at an evolutionary timescale (Kiers et al. 2010, Werner et al. 2018). Thus, there is concern that climate change will disrupt interactions via reduced interaction strength and mutualism loss.

Mutualism breakdown, or the permanent, evolutionary loss of a mutualism, can occur in three non-mutually exclusive ways: coextinction of the partners, mutualism abandonment, and novel partner interactions (Sachs and Simms 2006, Sachs et al. 2011, Bronstein 2015). Mutualism loss, on the other hand, is typically a stress-induced loss of a facultative mutualistic interaction that takes place during all or part of a growing season. Rafferty et al. (2015) predict that certain traits of a mutualism may predispose a partnership to loss (Rafferty et al. 2015). For example, a non-symbiotic partnership which lacks co-dispersal is more likely to become lost than a symbiosis that is vertically transmitted as co-dispersal ensures the persistence of a mutualism in space; importantly though, joint dispersal may not ensure an intact interaction (Wornik and Grube 2010). Short-term, seasonal mutualisms may be more susceptible to mismatch than aseasonal interactions, and mutualists which confer multiple benefits are less susceptible to breakdown as selection to maintain these highly beneficial interactions is strong (Palmer et al. 2010). It is important to note that there is substantial evidence that some mutualisms exhibit traits that buffer them against the short- or long-term absence of a partner (Bronstein et al. 2004).

The effects of global climate change on the persistence of mutualisms have profound implications for community- and ecosystem-level phenomena (Kardol et al. 2010, Brosi and Briggs 2013, Rafferty et al. 2015). For example, climate change has non-randomly decreased the abundances and diversity of pollinator species via phenological and spatial mismatches (Burkle et al. 2013). The advanced phenology of spring ephemerals but not the associated pollinators in

Japan due to warmer temperatures has led to significantly decreased seed set, potentially reducing future population sizes of the affected plants and altering pollinator behavior and fitness (Kudo and Hirao 2005, Kudo and Cooper 2019). Declines in population sizes following mutualism loss have been seen in animal-pollinated plants and in ant-tended acacia trees (Cox and Elmqvist 2000, Palmer et al. 2008), and these declines can cascade to affect other mutualists, such as seed dispersers, though some animal-pollinated plants have evolved abiotic means of pollination in the long-term absence of pollinators (Whitehead 1969, Ruan et al. 2009, Panique and Caruso 2020). The loss of mycorrhizal mutualists could have negative implications for terrestrial carbon sequestration in tree biomass, though in the absence of mutualistic mycorrhizae, some plants have evolved traits which negate the need for these mutualists, such as cluster roots (Neumann and Martinoia 2002). The fitness consequences of a mutualism loss or breakdown are generally partner-specific and are thus difficult to predict (Kiers et al. 2010). Mutualisms are rarely one-to-one interactions, and each species generally interacts with multiple mutualists; the loss of one mutualism will have consequences for the other interactions a former partner was involved in (Bronstein et al. 2004).

In this dissertation, I identify responses of soil microbe-legume-pollinator mutualisms to changes in snowmelt date, temperature, and precipitation. I showcase a suite of common garden experiments which manipulate soil moisture and test differences between home (current elevation) vs. away (beyond the current elevation), and sterilized vs. unsterilized soils. These experiments, in combination with field manipulations which advance snowmelt timing, help us to address the effects of climate change on soil microbe-legume mutualisms. Simultaneously, I quantified long-term isotopic trends in historical herbarium samples to understand trends in rhizobia-legume interactions over time (1929-2019). Finally, I used various lab techniques to identify changes in *Osmia lignaria* bee feeding behavior that could follow a loss of the mutualism

between legumes and rhizobia, and the subsequent changes in nectar amino acids generated by such a loss. Specifically, this dissertation addresses the following three topics corresponding to three dissertation chapters: 1) changing climatic conditions alter legume functional traits and interactions with belowground mutualists; 2) legume germination is delayed in dry soils and in sterile soils devoid of microbial mutualists; and 3) female *Osmia lignaria* prefer synthetic nectar enriched with amino acids.

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## Chapter 1

### Changing climatic conditions alter legume functional traits and interactions with belowground mutualists

#### Abstract

Warmer than average temperatures and decreased annual precipitation caused by climate change are affecting the growth and reproduction of many species, including flowering plants. Climatic shifts may affect angiosperm functional traits, such as water use efficiency and nectar sugar concentration, which can in turn alter the strength and incidence of interactions between plants and their mutualists. Climatic stress can separate mutualists, leading to additional alterations in partner traits which may have consequences for plant fitness. Here, we explore how warming temperatures, early snowmelt, and below-average annual precipitation (snow and summer monsoonal rains) can affect plant functional traits such as water use efficiency and lead to a loss of interaction with symbiotic rhizobia, thereby altering legume nitrogen content and leaf carbon and nitrogen isotopic signatures. In a snowmelt manipulative experiment, we found that early snowmelt increased nectar sugar concentrations, reduced plant total nitrogen, shifted the activity and phenology of plant-important rhizobia, and decreased legume water use efficiency relative to ambient snowmelt conditions. Decreased soil nitrogen inputs from senescing legume plants may alter competitive outcomes and growth rates of heterospecific plants in the local area. Additionally, an analysis of historical herbarium specimens revealed that warmer, drier conditions over time are associated with a loss of interactions between legumes and rhizobia and decreased plant water use efficiency. These two independent lines of evidence suggest that changing climate may destabilize mutualistic interactions and shift plant functional traits, ultimately affecting nitrogen inputs and plant community dynamics.

## Introduction

The effects of climate change-induced shifts in temperature and precipitation on plant species are profound (Suttle, Thomsen and Power, 2007; Jones and Berkelmans, 2010; Kudo and Cooper, 2019; Körner, 2021; Vorkauf *et al.*, 2021). Climate change impacts can be especially severe in high elevation systems where the growing season is short and plant phenology is closely linked to snowmelt timing (Inouye, 2008; Lambert, Miller-Rushing and Inouye, 2010; Pardee *et al.*, 2019). In alpine and subalpine areas, climate change is predicted to reduce snowpack and advance the timing of snowmelt (Keller, Goyette and Beniston, 2005), decreasing soil moisture earlier in the season, thereby exacerbating summer droughts (Sloat *et al.*, 2015). Because growing season lengths are ephemeral, an especially stressful year in the alpine or subalpine can be severely detrimental to plant fitness; early snowmelt and dry summer conditions can cause wildflowers to produce smaller and fewer flowers resulting in reduced seed set (Gezon, Inouye and Irwin, 2016; Pardee *et al.*, 2019). Indeed, as water is commonly the most limiting environmental factor for plant growth, low moisture plus elevated temperatures can combine to negatively affect plant water use efficiency (WUE; as measured by mean values of leaf  $\delta^{13}$  carbon (C)), increase plant stress, and decrease plant productivity (Cregg, 1993; Cordell *et al.*, 1998; Suttle, Thomsen and Power, 2007). Furthermore, mutualistic soil microbes often confer strong positive effects on alpine plant survival, growth, and phenology, and on landscape-scale patterns of floral and faunal diversity (Reynolds *et al.*, 2003; Wardle *et al.*, 2004; Berg *et al.*, 2010; Lau and Lennon, 2011; Wagner *et al.*, 2014; Eldridge *et al.*, 2021), but little is known regarding the impact of drought stress on plant-soil mutualist relationships. To date, the effects of climate warming, early snowmelt, and soil drying have seldom been described for mutualistic interactions between plants and soil microorganisms (Blankinship, Niklaus and Hungate, 2011; Classen *et al.*, 2015; Keeler, Rose-Person and Rafferty, 2021).

While climate change is expected to increase stress on individual plants, it may also disrupt their mutualistic relationships with other species (Skogen, Holsinger and Cardon, 2011; Pringle *et al.*, 2013; Remke *et al.*, 2021) where mutualists can become decoupled due to stress-induced partner dormancy or non-receptivity (Keeler, Rose-Person and Rafferty, 2021); this is especially detrimental in cases where partners are specialized or when there is limited alternate partner availability. Such loss of temporal or spatial overlap with belowground microbial mutualists may lead to decreased plant fitness and diminished distributions (Simonsen *et al.*, 2017; Harrower and Gilbert, 2018). Ultimately, mutualism loss could result in rapid population declines and reduced partner stress tolerance, potentially leading to the local extinction of interacting species (Burkle, Marlin and Knight, 2013).

Mutualistic soil microbes, including facultative plant growth-promoting soil microbes, can ameliorate stressors for plants and help them withstand the harsh conditions associated with global climate change (Ngumbi and Kloepper, 2016; Fitzpatrick, Mustafa and Viliunas, 2019). However, too much stress can strain the interaction, with some facultative mutualisms potentially being lost within a single growing season (Stanton-Geddes and Anderson, 2011; Rafferty, CaraDonna and Bronstein, 2015; Heide *et al.*, 2021; Keeler & Rafferty, 2022). For example, nitrogen (N)-fixing bacteria provide fixed N to legume plants in exchange for photosynthate and protection within a root nodule. Drought and temperature stress lead to the rapid inhibition of facultative, symbiotic N<sub>2</sub>-fixation and denodulation in legumes (Serraj, Sinclair and Purcell, 1999; Zahran, 1999; Hungria and Vargas, 2000; Valentine, Benedito and Kang, 2010; Aldasoro, Larrainzar and Arrese-Igor, 2019). The legume can expel current and reject new mutualists when rhizobial reward quality and/or quantity decreases, a common occurrence under stressful abiotic conditions (Douglas, 2008). Additionally, many bacteria, including some rhizobia, can undergo metabolic stasis when conditions become unfavorable, leading to mutualism loss (Blagodatskaya

and Kuzyakov, 2013; de Vries *et al.*, 2018; Schimel, 2018). Once in stasis, the microbe can no longer interact with the legume or contribute to ecophysiological processes such as N<sub>2</sub>-fixation (Zahran, 1999; Vriezen, de Bruijn and Nüsslein, 2006). If legumes are not receptive to hosting rhizobia or if the bacteria are dormant in stressful conditions, the mutualism can be lost in the short-term.

If the legume-rhizobia mutualism is temporarily lost due to climate change, legume plants will have significantly less N in their tissues and likely a lower photosynthetic capacity, leading to reductions in fitness (Stitt and Schulze, 1994; Onoda *et al.*, 2004). One key indicator of such mutualism loss is altered N isotope values. The discrimination between <sup>15</sup>N and <sup>14</sup>N in biological N<sub>2</sub> fixation is very high, where N<sub>2</sub>-fixing plants typically have a  $\delta^{15}\text{N}$  signature at or less than 0 since there is high discrimination for <sup>14</sup>N by the nitrogenase enzyme (Handley and Raven, 1992). Thus, mutualism loss will result in plants that exhibit a positive, relatively high  $\delta^{15}\text{N}$ , indicating a shift from use of atmospheric ( $\delta^{15}\text{N}$  values of zero) to soil-based N reserves ( $\delta^{15}\text{N}$  values greater than zero). In addition, total N in the leaves of N<sub>2</sub>-fixing plants is, in most cases, higher than that of non-fixing plants grown on the same sites (McLauchlan *et al.*, 2010). A high tissue N could benefit plants by enabling relatively higher rates of photosynthesis, water acquisition, and growth during the short subalpine growing season (Wright, Reich and Westoby, 2003). Low plant tissue N is often associated with a loss of foliar C (Wright *et al.*, 2004; Osnas *et al.*, 2013); as N often limits the rate of photosynthesis in plants, decreases in usable N translate to less fixed C. Thus, leaf  $\delta^{13}\text{C}$  may also change with warmer, drier conditions. Due to differences in the mass of <sup>13</sup>C and <sup>12</sup>C, plants will preferentially take up lighter <sup>12</sup>C. During times of low water availability, some plants are able to close their stomata and discriminate less against <sup>13</sup>C, thereby increasing the ratio of <sup>13</sup>C to <sup>12</sup>C (less negative  $\delta^{13}\text{C}$  value relative to Vienna Pee Dee Belemnite (V-PDB)), which has been associated with higher WUE (Farquhar and Richards, 1984;

Farquhar, Ehleringer and Hubick, 1989). For example, in high elevation conditions, observations of relatively low  $\delta^{13}\text{C}$  are common (Wang *et al.*, 2017), and values decrease with soil drying (Treydte *et al.*, 2016). Decreases in plant WUE are associated with decreased plant productivity (Hatfield and Dold, 2019), which will affect interactions between legumes and their mutualists, such as rhizobia, which consume photosynthate.

Warmer and drier than average conditions may negatively affect legumes and their interactions with  $\text{N}_2$ -fixing bacteria. Specifically, dry, warm soils may not support these facultative mutualistic interactions which could negatively affect legume phenology, fitness, and demography (Lau and Lennon, 2011; Wagner *et al.*, 2014), which could in turn affect interactions between legumes and other mutualists (e.g., pollinators). We explore the effects of warming temperatures, decreased snowpack, early snowmelt, and reduced summer monsoonal rainfall on high elevation (2853-3110 m) legumes and their interactions with  $\text{N}_2$ -fixing bacteria. Because warmer temperatures and low precipitation can lead to a longer, more intense period of drought before mid-summer monsoon rains, and because drought stress can inhibit  $\text{N}_2$ -fixation in mutualistic rhizobia, shallow-rooted legume species may be at high risk of mutualism loss and subsequent N limitation (Harte and Shaw, 1995; Serraj, Sinclair and Purcell, 1999; Valentine, Benedito and Kang, 2010; Slominski, German and Burkle, 2018). Here, we applied in situ field experiments manipulating the date of snowmelt to test our hypothesis that, with increasing climate stress, legumes will lose their interactions with non-obligate mutualistic  $\text{N}_2$ -fixing bacteria, which will affect legume functional traits such as nectar sugar concentration and plant isotopic values. In addition, we used a novel functional trait analysis of herbarium specimens to address our second hypothesis that legumes may have decreased interaction strength with rhizobia and altered water use efficiency due to early snowmelt and drought stress.

## Methods

### *Study system*

This study was conducted using contemporary and historical field samples collected from The Rocky Mountain Biological Laboratory (RMBL; N 38° 52.2928', W 106° 58.671') located in the Maroon Bells-Snowmass Wilderness area near Gothic, Colorado, USA. The RMBL area, comprised of vast, open subalpine meadows dominated by perennial wildflowers and patches of aspen-fir forests, experiences a very low N deposition rate (~2 kg N/ha/year), which provides a unique opportunity to explore ecologically important legume-rhizobia interactions in this very N-limited region (Vitousek and Howarth, 1991; Bobbink *et al.*, 2010; Slominski, German and Burkle, 2018). Additionally, despite it being a subalpine ecosystem, water is the most limiting resource for plant growth at RMBL (Burkle & Irwin 2010; Campbell *et al.* 2010), making it an ideal site for addressing the impacts of drought stress on plant-soil mutualist interactions.

Subalpine plant communities, like those at RMBL, are especially sensitive to changes in climate due to short growing seasons (Parmesan, 2006; Hülber, Winkler and Grabherr, 2010). At RMBL, mean summer temperatures have increased by 2.0°C since 1973 and are expected to continue to increase over the next century (Miller-Rushing and Inouye, 2009; Overpeck and Udall, 2010; Pederson *et al.*, 2011). Precipitation is minimal in the early growing season (June), and moisture comes from snowmelt and July monsoon rains. Based on a 116-year gauge record (1906–2021) of the Gunnison River which runs through the RMBL area, the current 21-year drought (2000–2021) is the worst drought in the southwestern United States in over 1,000 years (Gangopadhyay *et al.*, 2022). Along with increased desert dust deposition in the alpine (Steltzer *et al.*, 2009), higher temperatures and drought are associated with earlier spring snowmelt (3.5 days earlier per decade from 1974-2012) and increased year-to-year variation in snowmelt date

(20% more variable per year from 1974-2008; Lambert, Miller-Rushing and Inouye, 2010; Overpeck and Udall, 2010; CaraDonna, Iler and Inouye, 2014). With earlier snowmelt and reduced precipitation, early summer soil moisture has decreased (Blankinship *et al.*, 2014). These climate change-induced shifts result in earlier, longer dry seasons prior to North American July monsoon rains (Clow, 2010; Kittel *et al.*, 2015; Sloat *et al.*, 2015). Soil moisture can alter the ability of plants to produce and maintain flowers; based on resource allocation theory, when a plant flowers, water and nutrients are diverted away from other functions such as vegetative growth (Gleeson and Tilman, 1992; Bazzaz and Grace, 1997). Such conditions will increase water stress on plants and soil microbes and have important implications for plant fitness (Inouye, 2008; Berdanier and Klein, 2011; Waser and Price, 2016). When drought-stressed, plants often produce fewer, smaller flowers as they are an energetically costly organ in terms of formation and water loss through inflorescences (Caruso, 2006; Zhang and Brodribb, 2017; Phillips *et al.*, 2018).

### *Selection of plant species*

We studied the two native, nectar-producing legume plant species present in the Maroon Bells-Snowmass Wilderness area, *Lathyrus lanszwertii* var. *leucanthus* (hereafter *Lathyrus leucanthus*) and *Vicia americana* (Fabaceae). We verified that both of these species form root nodules and host N<sub>2</sub>-fixing bacteria in the field at several points across the valley. Both species have shallow roots extending ~10 cm down before spreading laterally; as shallow soils are expected to be affected by increases in temperature and drought more strongly than deeper soils, these legume species may be susceptible to environmental stress (Blankinship *et al.*, 2014). Both are perennial vines that are common in the RMBL area and produce nutrient-rich rewards that attract native pollinators; these species are not capable of autonomous self-pollination and rely on insect pollinators for reproduction (Xingwen, 2021). Historical *L. leucanthus* and *V. americana*



leaf collections are housed within the RMBL herbarium, with specimens dating back to the 1920s (Image 1.1).

### *Experimental design and setup*

In 2019, snowmelt timing was manipulated across two adjacent valleys at RMBL (Image 1.2). Snowpack in 2019 was within average based on long-term RMBL data (Xingwen, 2021). The early snowmelt manipulation design began with black plastic 50% shade cloth, which was laid atop the snow of early snowmelt plots in late May of each year (2017-2019), decreasing albedo and accelerating the date of early-summer snowmelt by up to 17 days (Steltzer *et al.*, 2009; Xingwen, 2021); this simulates the effects of climate change and increases the time between snowmelt and July monsoon rains (Wipf and Rixen, 2010). One of the two plots within each site was randomly assigned to receive the accelerated snowmelt treatment, while the other was unmanipulated in terms of snowmelt and served as a control. This was done at each of our five sites (10 total plots), all located at least 800 m from one another (2853-3110 m); each site had a manipulated and a control plot measuring 10 x 14 m with a 1 m buffer strip around the perimeter. Paired plots were at least 5 m apart from one another and were similar to each other in terms of aspect, slope, canopy cover, plant community composition, and soil texture. Snowmelt was monitored weekly at each site and the black shade cloth was removed when >80% of the ground was snow-free. Our advanced snowmelt plots melted out more quickly than control plots by  $8.29 \pm 2.01$  days (mean  $\pm$  SE; Xingwen, 2021).

### *Field Data Collection*

In 2019, we collected soil and *L. leucanthus* leaf samples from control and advanced snowmelt plots at RMBL. Later-emerging *Vicia americana* samples were not collected from field plots due to extremely late green-up in summer 2019. At least three individual *L. leucanthus*

plants were marked per plot at the start of the growing season. Many subalpine species, including *L. leucanthus*, are highly clonal. To ensure collection of leaves from separate individual plants, we marked *L. leucanthus* that were at least 2 m from each other. Once fully emerged and mature (> 5 true leaves), samples from new, open leaves were collected weekly from each marked focal plant in each plot; we collected a minimum of 6 mg dry mass per sample (Reisinauer 1879, Peterson and Fry 1987). Newer leaves were collected to avoid internal N translocation from senescing leaves and to capture the N reserves that influenced the growth of that leaf. At the same time, in-field, non-destructive nodule counts, an estimate of the presence and strength of legume-rhizobia interactions (Magnoli and Lau 2020, Gano-Cohen et al. 2020), were made by revealing and visually inspecting the roots of marked focal plants in each plot. A 10 cm-deep, 5 cm-radius hole was dug near a marked plant; the root of *L. leucanthus* typically abruptly ends at 10 cm and then spreads laterally to other clones. The downward root plus all lateral roots in a 5-cm radius were visually inspected for nodules. From non-focal individuals, nodules were collected to see if they contained rhizobia (pink coloration within the nodule), and they all did (n = 15; 3 per plot). In addition to legume leaf tissue samples and nodule counts, we measured soil moisture twice weekly using 3-cm in-soil probes (VWC %; Spectrum Field Scout TDR 100 Soil Moisture Probe; Spectrum Technologies, Aurora, Illinois, USA).

Open legume flowers were counted weekly in each plot as the metric of floral abundance. Legume nectar sugar concentration (10 randomly selected, open flowers of different plants per plot) were measured with microcapillary tubes, a ruler, and a handheld refractometer (Bellingham + Stanley, Eclipse 0-50 BRIX Nectar, Xylem Inc., Washington DC, USA). The nectary in *L. leucanthus* is at the bottom of the flower. To collect a nectar sample, we gently squeezed the keel of the flower to open it, then inserted a microcapillary glass tube into the base of the flower. Capillary action pulled the nectar into the tube, and we recorded the length (mm) of nectar within

the tube. We then expressed the nectar onto the refractometer and recorded the concentration of nectar on the BRIX scale (percentage sucrose equivalent; Corbet, 2003). For each sampling date, we calculated a plot-average volume ( $\mu\text{l}$ ) of nectar by comparing the length of the column of nectar with the total length and volume of the microcapillary tube. Finally, three soil samples were collected at least once per week from each plot and site for eight weeks. 10-cm soil cores were collected from around the roots on non-focal *L. leucanthus* plants. Samples were homogenized, sifted for rocks and roots, stored in sterile bags, then dried until analysis. In total, we collected 40 soil samples and 76 legume leaf samples.

#### *Herbarium Samples*

We collected legume leaf samples from stored historical specimens from the RMBL area. Herbaria collections represent unique archives of plant tissue chemistry, including isotopic signals, which provide insight into metabolic processes at the time of collection. The RMBL Herbarium houses *L. leucanthus* and *V. americana* samples, all collected from the area between the Maroon Bells-Snowmass Wilderness area and the city of Crested Butte, CO. Twenty-two total specimens from the RMBL Herbarium were sampled from plants living between 1929-2014 and were chosen based on location, average snowpack and temperature of the collection year, and based on the number of leaves pictured in the digital collection ( $> 6$  mg dry mass per sample (Peterson and Fry, 1987)). Leaf samples were collected from 10-11 specimens per species (Table 1.1). To our knowledge, no N-containing compounds have been used in the RMBL Herbarium for sample preparation or tissue preservation.

#### *Isotope Analyses*

Current *L. leucanthus* and historical *L. leucanthus* and *V. americana* leaf samples were secured in paper envelopes and dried at  $70^{\circ}\text{C}$  for at least three days or until the material could

snap easily. Leaves from non-N<sub>2</sub>-fixing plants, *Aquilegia coerulea* (Ranunculaceae) and *Geranium richardsonii* (Geraniaceae), were also collected from the RMBL area (though outside of the manipulation treatment plots) to set a non-fixer baseline for plant  $\delta^{15}\text{N}$ , total N,  $\delta^{13}\text{C}$ , and total C. These plants produce nectar and emerge at a similar time as, and co-flower with our focal legumes. Leaf and soil samples were sent to the University of California, Davis Analytical Laboratory where they were ground and homogenized to the point where matter was the consistency of a fine dust. Samples were enclosed in combustible stable isotope tin (Sn) capsules for analysis at the University of California, Davis Stable Isotope Facility. All tins were organized into 96-well plates, combusted, and analyzed with continuous flow isotope ratio mass spectrometer (IRMS configured using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK)). The final delta values are expressed relative to international standards VPDB and air for carbon and nitrogen, respectively (Sharp, 2017). We considered  $\delta^{15}\text{N}$  values lighter than 0 per mille as an intact interaction between rhizobia and legumes;  $\delta^{13}\text{C}$  values heavier than -20 per mille to represent C4 photosynthesis and lighter values to represent C3 photosynthesis (Delwiche *et al.*, 1979). Lower, negative  $\delta^{13}\text{C}$  values also indicate a lower water use efficiency (Farquhar, Ehleringer and Hubick, 1989); carbon isotope fractionation is highly correlated with the ratio of photosynthetic carbon assimilation to transpiration and, therefore, with plant WUE (Farquhar and Lloyd, 1993).

#### *Data analysis*

To assess how soil moisture was affected by day of year, we used linear mixed-effects models (LMM) where soil moisture was the response variable, sampling day of year (where day 1 = January 1), snowmelt treatment, and the interaction between these factors were predictors, and

site was held as a random effect to account for the non-independence of multiple samples from the same site. Similarly, we used LMMs to understand how nectar sugar concentration varied in response to snowmelt treatment, day of year, and the interaction between those two factors. We used generalized linear mixed-effects models (GLMM) with a Poisson error distribution to examine variation in maximum floral abundance (count) in response to snowmelt treatment, date of collection, and the interaction between those two variables.

To investigate variation in soil and *L. leucanthus* leaf total N,  $\delta^{15}\text{N}$ , total C, and  $\delta^{13}\text{C}$  and in nodule count between control and early snowmelt treatments, we used LMMs. In separate models, nodule count, total N,  $\delta^{15}\text{N}$ , total C, or  $\delta^{13}\text{C}$  served as the response, with snowmelt treatment and day of year as fixed effects. Site was included as a random effect in all models. For historical herbarium specimens, we used LMs to examine the variation in both *L. leucanthus* and *V. americana* leaf total N,  $\delta^{15}\text{N}$ , total C, and  $\delta^{13}\text{C}$  over time (years) and in different abiotic scenarios (1929-2019 average annual temperature, 1929-2019 summer monsoonal precipitation, 1929-2019 snowpack data, and the interactions between these factors). With the uneven sampling effort in the 2019 field season vs. what was stored in the herbarium, we ran these models with and without the 2019 data. All results trended in the same direction with and without 2019, though total C analyses were sensitive to whether 2019 was included. Species identity was not a significant predictor in our models, so all plant samples were pooled.

We used likelihood ratio tests to compare the fit of full models which include all variables and interactions to that of sequentially reduced models. Here, we report the best fitting models and only the significant results (estimate  $\pm$  SE). For the historical data, many years were not represented in the herbarium collections; a non-parametric bootstrap method was applied to these data to assess the accuracy of our statistical estimates ( $R^2$ ) and to make inferences about our long-term abiotic parameters (Efron and Tibshirani, 1994). This bootstrapping process consisted

of 10,000 simulations of our LMMs which established the associated 95% confidence intervals of the bootstrapped data. All analyses were conducted in R version 4.0.2 (R Core Team 2019).

## **Results**

### ***Snowmelt Timing Manipulation***

In 2019, soil moisture in each treatment decreased significantly during the season (LMM:  $0.18 \pm 0.06$ ,  $t_{67} = 3.004$ ,  $p < 0.001$ ). Based on a post-hoc assessment, control plots had significantly higher soil moisture than early snowmelt plots for the first 21-28 days after snowmelt (LMM:  $2.18 \pm 0.92$ ,  $t_{33} = 2.37$ ,  $p < 0.01$ ). At 28 days post-snowmelt, control and early snowmelt treatments ceased to differ in soil moisture (LMM:  $-0.58 \pm 1.36$ ,  $t_{33} = -0.43$ ,  $p < 0.66$ ). Cumulative growing season floral abundance, or the maximum flower count during the 2019 growing season, was not affected by snowmelt manipulation (GLMM:  $3.58 \pm 0.25$ ,  $z_{49} = 13.84$ ,  $p < 0.001$ ). Nectar sugar concentration was higher in the early snowmelt treatment at  $21 \pm 4\%$  versus  $12 \pm 3\%$  in the control treatment (LMM:  $0.22 \pm 0.04$ ,  $t_{26} = 5.29$ ,  $p < 0.0001$ ). Nodule number per plant was not significantly different between snowmelt treatments (LMM:  $0.01 \pm 0.05$ ,  $t_{50} = 0.19$ ,  $p < 0.42$ ).

### ***Total Nitrogen***

Total N of leaves ranged from  $141.73 \pm 33.77 \mu\text{g}$  (average  $\pm$  SD) in early snowmelt plots to  $146.87 \pm 45.49 \mu\text{g}$  in control plots (Figure 1.1A). Leaves lost N as the growing season progressed (LMM:  $0.70 \pm 0.04$ ,  $t_{75} = 14.75$ ,  $p < 0.0001$ ). In control settings, non-N<sub>2</sub>-fixer leaf tissue N was  $132.97 \pm 4.47 \mu\text{g}$  in the early season (leaves collected on July 11th) and lower ( $109.27 \pm 3.37 \mu\text{g}$ ) in the late season (August 22nd). We identified interactive effects of day of year and snowmelt treatment on total N of legume leaves (LMM:  $0.71 \pm 0.05$ ,  $t_{67} = 13.59$ ,  $p < 0.0001$ ); total N decreased more in control than in early snowmelt leaves over the course of the

2019 growing season. In soils, total N increased with time while snowmelt treatment was not a significant predictor of soil N (LMM:  $0.25 \pm 0.03$ ,  $t_{39} = 6.62$ ,  $p < 0.0001$ ; Figure 1.1A). Season average total N of soils was  $56.78 \pm 22.04 \mu\text{g}$  in control plots and  $56.4 \pm 20.2 \mu\text{g}$  in early snowmelt plots.

#### *Interactions between legumes and $N_2$ -fixing bacteria: $\delta^{15}\text{N}$*

Over the entire growing season, there was no significant difference in legume leaf  $\delta^{15}\text{N}$  in control vs. early snowmelt plots (LMM:  $-0.62 \pm 1.22$ ,  $t_{67} = 0.472$ ,  $p < 0.69$ ; Figure 1.1B). In control plots, leaf  $\delta^{15}\text{N}$  averaged  $-0.027 \pm 0.688$  while in early snowmelt plots, leaf  $\delta^{15}\text{N}$  was  $0.031 \pm 0.559$ . Leaf  $\delta^{15}\text{N}$  of two non-fixer species (*A. coerulea* and *G. richardsonii*) was always above zero ( $6.13 \pm 1.04$ ). The  $\delta^{15}\text{N}$  of soils was always above zero and differed between treatments (LMM:  $7.03 \pm 1.20$ ,  $t_{37} = 5.81$ ,  $p < 0.0001$ ; Figure 1.1B). We did not detect any effects of day of year or the interaction between day of year and snowmelt treatment on soil  $\delta^{15}\text{N}$ .

#### *Total Carbon*

There was no effect of snowmelt treatment on leaf total C (LMM:  $1329.13 \pm 12.9$ ,  $t_{75} = 103.0$ ,  $p < 0.0001$ ; Figure 1.1C). Leaf total C was relatively higher, at  $1314.97 \pm 70.31 \mu\text{g}$  in control plots vs.  $1327.56 \pm 74.74 \mu\text{g}$  in early snowmelt plots while relatively lower soil total C values ranged from  $603.37 \pm 236.28 \mu\text{g}$  in control plots to  $606.11 \pm 222.62 \mu\text{g}$  in early snowmelt plots. There was a significant increase in soil C over the season where we found an interactive effect of day of year and snowmelt treatment on soil C (LMM:  $2.94 \pm 0.40$ ,  $t_{38} = 7.28$ ,  $p < 0.0001$ ; Figure 1.1C); total C increased more in control soils than in early snowmelt soils over the course of the 2019 growing season.

### *Plant water use efficiency: $\delta^{13}\text{C}$*

There was an interactive effect of day of year and snowmelt treatment on leaf  $\delta^{13}\text{C}$ , where values decreased over the course of the season (LMM:  $0.02 \pm 0.01$ ,  $t_{75} = 1.75$ ,  $p < 0.04$ ; Figure 1.1D). Leaves in the control treatment were relatively enriched compared to those in the early snowmelt treatment, but both steadily decreased over the course of the summer. Leaf  $\delta^{13}\text{C}$  of non-fixers dropped from  $-27.11 \pm 0.09$  on June 11th to  $-28.15 \pm 0.10$  on August 22nd. All values, legume and not, were consistent with C3 photosynthesis. Soil  $\delta^{13}\text{C}$  was not different between treatments, where values ranged from  $-25.44 \pm 0.45$  in control plots to  $-25.59 \pm 0.56$  in early snowmelt plots.

### ***Herbarium Specimens***

#### *Total Nitrogen*

Snowpack and summer monsoonal rains did not significantly affect leaf N (Figure 1.2AII; Figure 1.2AIII) but leaf total N did increase over time (LM:  $0.05 \pm 0.002$ ,  $F_{1,78} = 415.1$ ,  $p < 0.0001$ ; Figure 1.3A). Rising annual temperatures significantly affected leaf total N (LM:  $1.96 \pm 0.48$ ,  $F_{3,76} = 135.4$ ,  $p < 0.0001$ ; Figure 1.2AI). Using a  $R^2$  bootstrap method to determine the fit of our data over time, we created a confidence interval related to the  $R^2$  of our historical data where the bootstrap  $R^2$  value was 0.97. The bias, or the difference between the bootstrap  $R^2$  and the original data  $R^2$ , was 0.001. The 95% bias-corrected confidence interval was between 0.95 and 0.98.

#### *Interactions between legumes and $N_2$ -fixing bacteria: $\delta^{15}\text{N}$*

Above average annual temperatures led to leaf  $\delta^{15}\text{N}$  of  $1.016 \pm 1.81$ , while below average annual temperatures yielded significantly lower legume leaf  $\delta^{15}\text{N}$  values of  $-0.138 \pm 0.405$  (LM:



$0.02 \pm 0.01$ ,  $F_{3,76} = 7.64$ ,  $p < 0.0001$ ; Figure 1.2BI). Leaf  $\delta^{15}\text{N}$  increased, becoming positive from 1929 - 2019 (LM:  $<0.0001 \pm 0.0001$ ,  $F_{1,78} = 20.54$ ,  $p < 0.0001$ ; Figure 1.3B). Using a  $R^2$  bootstrap method on leaf  $\delta^{15}\text{N}$  over time, the bootstrap  $R^2$  value was 0.21. The bias, or the difference between the bootstrap  $R^2$  and the original data  $R^2$ , was 0.0006. The 95% bias-corrected confidence interval was between 0.10 and 0.32.

### *Total Carbon*

Average annual temperature had a significant positive effect on leaf total C (LM:  $17.06 \pm 3.22$ ,  $F_{3,76} = 295.5$ ,  $p < 0.0001$ ; Figure 1.2CI). Total C in leaves increased in leaves over time (LM:  $0.65 \pm 0.008$ ,  $F_{1,78} = 73.73$ ,  $p < 0.0001$ ; Figure 1.3C). Based on bootstrapping, we used a  $R^2$  value was 0.996 and our bias was  $< 0.0002$ . The 95% bias-corrected confidence interval was between 0.991 and 0.998.

### *Plant water use efficiency: $\delta^{13}\text{C}$*

We detected an interactive effect of average annual temperature, summer monsoonal rain totals, and snowpack on leaf  $\delta^{13}\text{C}$  (LM:  $0.0009 \pm 0.0005$ ,  $F_{4,75} = 8615$ ,  $p < 0.0001$ ). Leaf  $\delta^{13}\text{C}$  decreased over time, leading to leaves with a lighter, very negative delta signature (LM:  $<0.0001 \pm 0.0001$ ,  $F_{1,78} = 20.54$ ,  $p < 0.0001$ ; Figure 1.3). Using a  $R^2$  bootstrap method to assess the change in leaf  $\delta^{13}\text{C}$  over time, the  $R^2$  value was 0.998 and the bias was  $< 0.0001$ . The 95% bias-corrected confidence interval was between 0.997 and 0.998.

## **Discussion**

One of the major aspects of climate change is a predicted increase in the duration, frequency, and intensity of drought, caused by warming temperatures and decreasing annual precipitation (Sloat *et al.*, 2015). In North American high elevation systems, water is often the

most limiting resource for plants (Burkle & Irwin 2010; Campbell et al. 2010). Limited soil moisture, whether from early snowmelt or low snowpack and monsoonal rainfall, is known to affect plant functional traits. In this study, we observed negative effects of low soil moisture via early snowmelt on plant water use efficiency, where legume plants expressed traits of worsened WUE with increasing temperatures and drought. Lack of plasticity in WUE may not allow these plants to adjust to the warming, drying climate, both over the course of a single growing season and over a century of climate change (Ehleringer 1989; Farquhar *et al.*, 1989; Goud *et al.*, 2021). Additionally, mutualisms between legumes and N<sub>2</sub>-fixing bacteria ensure plant access to fixed nitrogen, another limiting resource for plants in many environments, including the subalpine (Powers and Tiffin, 2010; Vitousek *et al.*, 2013), but the facultative nature of the symbiosis means that it is easily affected by soil moisture and temperature stress (Serraj, Sinclair and Purcell, 1999; Valentine, Benedito and Kang, 2010). Using stable isotope analysis of herbarium samples and leaves collected from plots where snowmelt date was experimentally advanced, we found that this mutualism may be retained within a season but may be lost over recurrent stressful years.

### ***Snowmelt Manipulation***

In our manipulative field study, early snowmelt led to reduced soil moisture early in the season, but treatments were equally dry 21-28 days after snowmelt as all soils became drier during the growing season. As water inputs from snowmelt were the only source of water early in the season, the advanced snowmelt treatment served to prolong dry conditions. With no July monsoon rains in 2019, plots remained dry until snowfall in early September. Advanced snowmelt did not affect the number of flowers present, but it did increase the nectar concentration of those flowers. A thicker nectar consistency in drier conditions is common; warmer, drier

conditions tend to lead to more evaporation of the water in nectar, leaving a thicker, more concentrated sugar slurry behind (Pacini, Nepi and Vesprini, 2003; Mu et al., 2015).

Legume leaf N was significantly greater than foliar N in non-legume plants, as expected (Vergutz *et al.*, 2012). Leaf N content for both legume and non-fixer species was higher after snowmelt than it was later in the season which is common in high elevation ecosystems (Sorensen *et al.*, 2020). Soil microbial species begin mobilizing N early in the season, often before snowmelt, freeing N for plant use. Total N in leaves decreased over the summer, as plants reallocated N from leaves to flower and fruit production (Martínez-Alcántara *et al.*, 2011). Total N in soils increased over time as plants senesced and added N to the soils (Figure 1.1A). As leaf N was relatively low at the start of the season in early snowmelt conditions, there may be effects of snowmelt timing on photosynthesis and growth. A central tenet of plant physiological ecology is that leaf C will increase with leaf N, as the large enzyme Rubisco is a limiting factor for photosynthetic output (Wright *et al.*, 2004; Osnas *et al.*, 2013). If leaf N was significantly lower in early snowmelt plots than in control plots early in the season, Rubisco synthesis may have been affected, altering photosynthetic rates. Also, short-statured, shallow-rooted species like *L. leucanthus* often struggle to compete with other plants for light, another factor mediating photosynthetic output (Tilman, 1987; Suding *et al.*, 2005; Skogen, Holsinger and Cardon, 2011). It has been shown in this system that, in dry conditions, soil N addition promotes photosynthetic capacity in *L. leucanthus* but not in deeper-rooted legume species like *Lupinus argenteus* (Fatichi, Pappas and Ivanov, 2016). Early snowmelt, low light, and N limitation, possibly due to a loss of the mutualism with N<sub>2</sub>-fixing bacteria in dry conditions, are all predicted to negatively affect photosynthetic productivity, thus legume fitness.

Early snowmelt had no effect on legume-rhizobia interactions; plant  $\delta^{15}\text{N}$  remained near zero (and significantly lower than soil  $\delta^{15}\text{N}$  values) throughout the season, indicating intact

interactions with N<sub>2</sub>-fixing bacteria (Figure 1.1B). Similarly, nodule counts did not differ between control and early snowmelt conditions, reinforcing the isotopic data. Soil  $\delta^{15}\text{N}$  values were relatively more enriched in early snowmelt plots, likely because early snowmelt shifted soil microbial activity and subsequent  $^{14}\text{N}$  use, leaving behind heavier  $^{15}\text{N}$  in the soil (Schimel, 2018).

Total C in soils increased over the growing season, likely due to plant senescence and mobilization of previously sequestered C (Figure 1.1C). Total C in leaves did not shift over the summer, a surprising finding given that leaf N decreased in early snowmelt conditions and over the season; as N and C are closely linked, we expected a decrease in leaf C with leaf N (Wright *et al.*, 2004; Osnas *et al.*, 2013). This may indicate the presence of structural, non-mobile carbon in the leaves later in the season. There was similarly no difference in soil or leaf C between snowmelt and control plots, indicating that early snowmelt may not affect C sequestration in leaves or in soils.

Water use efficiency, as measured by leaf  $\delta^{13}\text{C}$  values, decreased, becoming increasingly negative, in both treatments. By the end of the season, plants in control and early snowmelt plots had indistinguishable  $\delta^{13}\text{C}$  values, and thus water use strategies. Often, in drier conditions, plants invest in strategies to alleviate drought stress, such as closing stomata, leading to increased WUE (Kao, Chiu and Chen, 2000; Van de Water, Leavitt and Betancourt, 2002). Plants in drier conditions in our study had larger negative values of  $\delta^{13}\text{C}$ , indicating a preference for  $^{12}\text{C}$ , thus open stomata and a low WUE. These low  $\delta^{13}\text{C}$  values could similarly indicate a nutrient limitation related to stomatal closure (Kirschbaum, 2004). In either of these situations, the stomata of the plants in our treatments were open and plants were losing water to their environments. Decreased plant WUE is associated with decreased plant productivity (Hatfield and Dold, 2019), which will affect interactions between legumes and their mutualists. Plant

growth-promoting soil microbes, such as rhizobia, and pollinators alike use photosynthate resources, and a decrease in plant productivity could lessen the reward quality and quantity associated with the mutualism, which could lead to mutualism loss (Keeler, Rose-Person and Rafferty, 2021). Alternatively, larger negative leaf  $\delta^{13}\text{C}$  values may indicate that plants were obtaining lighter C from respiring soil rather than more enriched bulk atmospheric C, which is possible for low-lying plants like our focal legumes (Ehleringer *et al.*, 1986). Soil  $\delta^{13}\text{C}$  was consistently enriched over the season, and treatments did not differ in terms of  $\delta^{13}\text{C}$  values. As soil  $\delta^{13}\text{C}$  is usually shaped by the senescing plants that inhabit that soil and microbial activity following plant senescence (Gleixner, Bol and Balesdent, 1999), the consistently low, C3 photosynthesis-like  $\delta^{13}\text{C}$  values of soil in this area are not surprising given the abundance of C3 angiosperms.

### ***Herbarium Specimens***

Very few studies have assessed change in plant traits over historical time, even though there can be strong interseasonal variation within individual perennials over time (Mason *et al.*, 2020). One such study assessed the effects of  $\text{CO}_2$  on two mangrove species, noting that rapidly rising atmospheric  $\text{CO}_2$  levels over the past two centuries have led to changes in plant carbon sequestration, among other traits (Reef *et al.*, 2014). As surprisingly few datasets have examined the long-term response of long-lived plants to climate change, our results are both unique and novel. Considering herbarium samples collected from the RMBL area between 1929 to 2019, there was a positive trend in leaf N over time, possibly due to increased N deposition, though the rate of deposition is extremely low in the RMBL area (Vitousek and Howarth, 1991; Bobbink *et al.*, 2010; Slominski, German and Burkle, 2018).

Leaf  $\delta^{15}\text{N}$  increased over time, where historical legumes were more often negative and lighter (more  $^{14}\text{N}$  than  $^{15}\text{N}$ ) than current legumes (Figure 1.3B). This trend indicates a gradual interaction weakening between legumes and  $^{14}\text{N}$ -assimilating rhizobia, a novel finding in a natural system. In contemporary conditions, legumes were more often relying on soil N, which is relatively heavy. Leaves of plants in warmer years were more enriched than plants in cooler years, possibly because rhizobia are sensitive to temperature and will become dormant at high temperatures (Hungria and Vargas, 2000). This trend was the same in below vs. above average snowpack conditions, where the interaction between legumes and rhizobia was stronger in higher snowpack years, likely because soil moisture strongly affects the formation and strength of this interaction (Zahran, 1999; Valentine, Benedito and Kang, 2010; Aldasoro, Larrainzar and Arrese-Igor, 2019).

Mean annual temperature had a significant effect on leaf C, where high temperatures led to high carbon sequestration in leaves (Figure 1.2CI). As temperature influences the rate of photosynthesis, higher-than-average temperatures would increase photosynthetic rate, thereby increasing leaf C. Similarly, and as seen in other long-term studies, increases in atmospheric  $\text{CO}_2$  levels has led to a rise in sequestered C over time (e.g., Reef *et al.*, 2014; Figure 1.3).

Leaf  $\delta^{13}\text{C}$  of legume plants decreased over the 90-year-period, possibly reflecting decreasing atmospheric  $\delta^{13}\text{C}$  values, a direct result of fossil fuel emissions (Treydte *et al.*, 2001). Decreasing leaf  $\delta^{13}\text{C}$  corresponds to a lack of plasticity in and a decreased overall WUE (Assouline and Or, 2013), which will negatively affect plant fitness. As in the snowmelt manipulation, this could be caused by a nutrient limitation related to improper stomatal closure, or possibly a lack of plasticity in stomatal counts. To our knowledge, this is a novel finding

## ***Conclusions***

In this study, we found that an early snowmelt field treatment reduced plant total nitrogen, shifted the interactions between plant-important rhizobia early in the season (leaf  $\delta^{15}\text{N}$ ), and decreased legume water use efficiency (leaf  $\delta^{13}\text{C}$ ). An analysis of historical herbarium specimens revealed that warmer, drier climatic conditions led losses of interactions between legumes and rhizobia (increasing leaf  $\delta^{15}\text{N}$ ) and decreased plant water use efficiency (leaf  $\delta^{13}\text{C}$ ). Both of these findings suggest that a changing climate may destabilize mutualistic interactions and shift plant functional traits. A disruption of the mutualism between legumes and rhizobia could affect the fitness and phenology of the legume host, the floral resources of the plant through altered C and N quantities and qualities, and the behavior and fitness of pollinators. Similarly, a disruption in this mutualism may affect legume-mycorrhizal interactions; increases in plant N from rhizobia benefit not only the plant but also other root symbionts (Larimer *et al.*, 2014). Losses of plant N could lead to shifts in the mutualisms between legumes and mycorrhizal fungi, further disrupting water acquisition in these species. At plant senescence, the amount of biologically usable N and C in the local system will also be altered, in turn influencing local community composition. For example, legumes enhance the growth of other plant species by increasing soil N (Spehn *et al.*, 2002); changes in biologically available soil N could have a large effect on ecosystem function and competitive outcomes of other plants, and thus species diversity (McInnes and Haq, 2007; Keller and Lau, 2018). In addition to community and population responses, rhizobia may, through natural selection, dispose of the gene segment responsible for the initiation and maintenance of their mutualism with legumes (Sullivan *et al.*, 1996; Sullivan and Ronson, 1998; Denison and Kiers, 2004; Sachs, Skophammer and Regus, 2011). The loss of this segment of DNA ultimately causes a complete breakdown of the mutualism. All in all,

warming, drying climatic conditions may negatively affect the ecologically important interaction between legumes and rhizobia, plant fitness, and interactions between legumes and other species.



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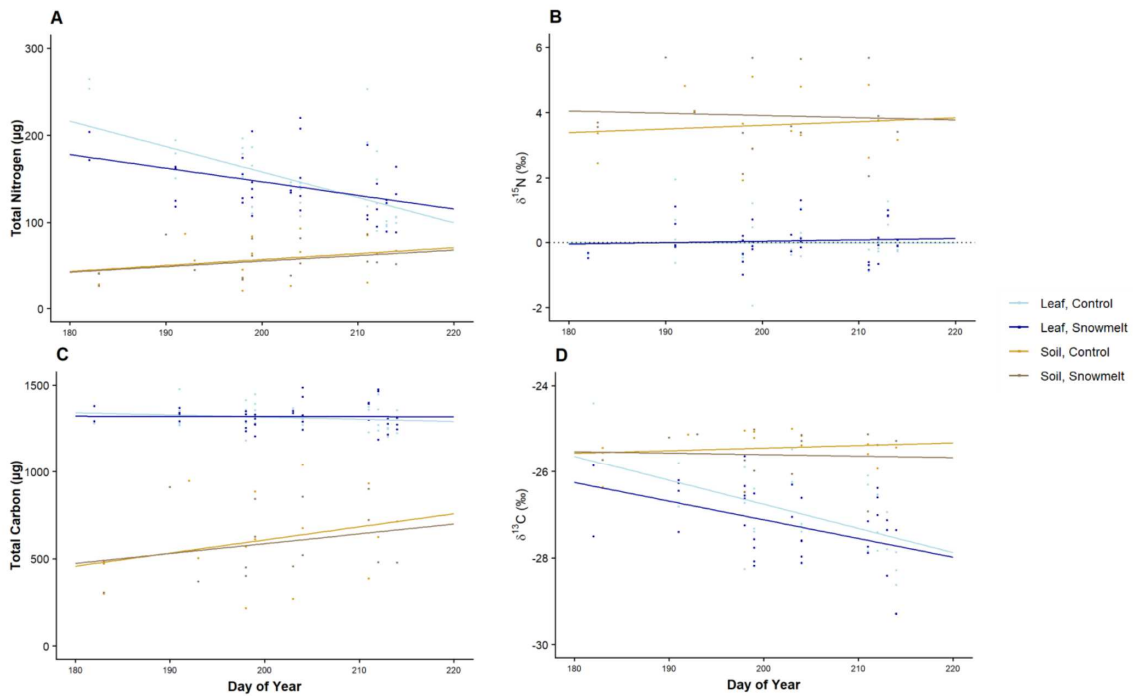
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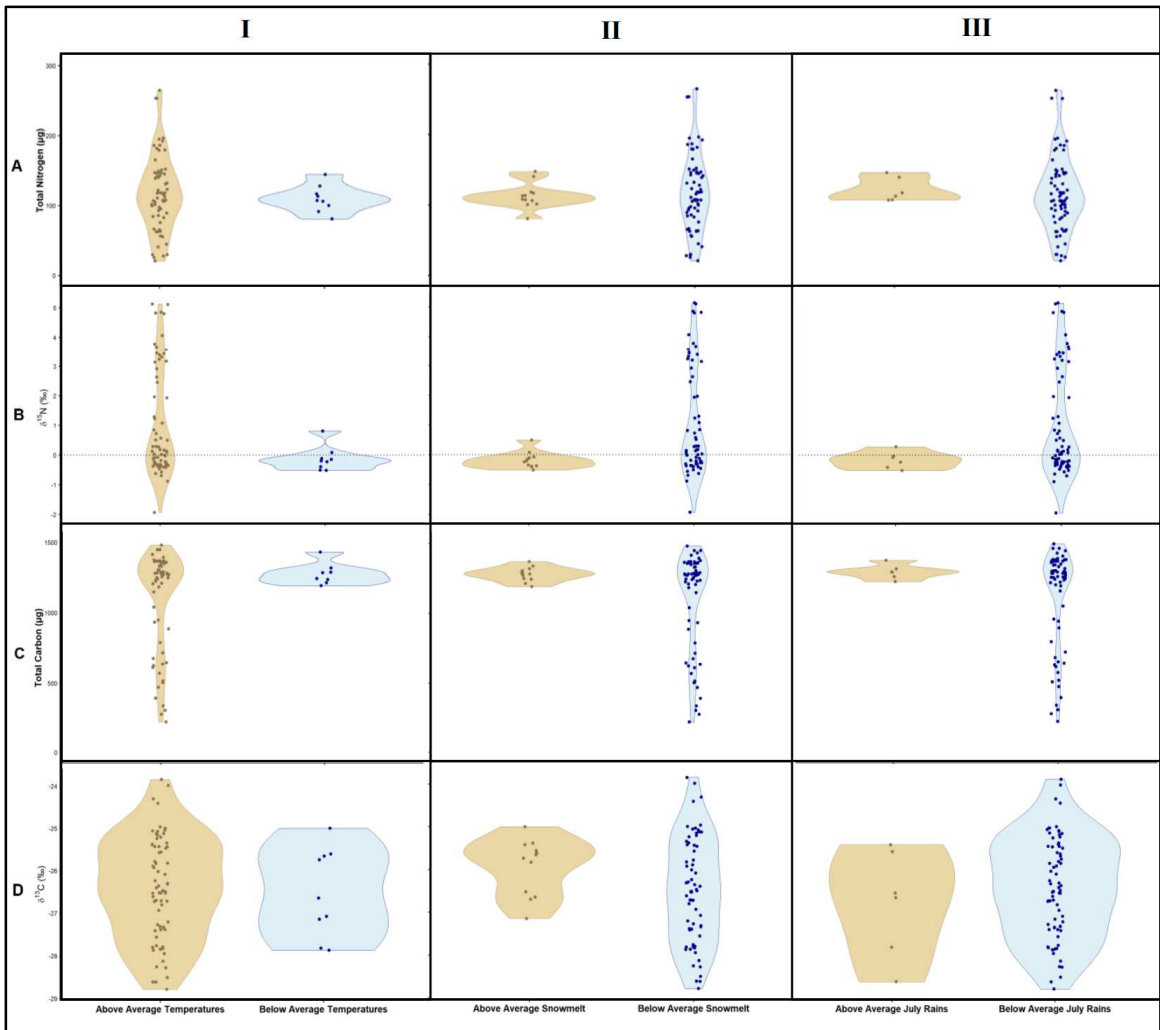
## Tables, Figures, & Images

Catalog Number	Species	Year Collected
RMBL0001799	<i>Lathyrus lanszwertii</i> var. <i>leucanthus</i>	1958
RMBL0001801	<i>Lathyrus lanszwertii</i> var. <i>leucanthus</i>	1958
RMBL0001797	<i>Lathyrus lanszwertii</i> var. <i>leucanthus</i>	1963
RMBL0001796	<i>Lathyrus lanszwertii</i> var. <i>leucanthus</i>	1965
RMBL0009503	<i>Lathyrus lanszwertii</i> var. <i>leucanthus</i>	2008
RMBL0011668	<i>Lathyrus lanszwertii</i> var. <i>leucanthus</i>	2009
RMBL0009797	<i>Lathyrus lanszwertii</i> var. <i>leucanthus</i>	2011
RMBL0011032	<i>Lathyrus lanszwertii</i> var. <i>leucanthus</i>	2013
RMBL0011034	<i>Lathyrus lanszwertii</i> var. <i>leucanthus</i>	2013
RMBL0010501	<i>Lathyrus lanszwertii</i> var. <i>leucanthus</i>	2014
RMBL0001975	<i>Vicia americana</i>	1929
RMBL0001977	<i>Vicia americana</i>	1958
RMBL0001979	<i>Vicia americana</i>	1958
RMBL0001973	<i>Vicia americana</i>	1963
RMBL0001976	<i>Vicia americana</i>	1963
RMBL0001978	<i>Vicia americana</i>	1963
RMBL0001983	<i>Vicia americana</i>	1965
RMBL0001982	<i>Vicia americana</i>	1995
RMBL0011614	<i>Vicia americana</i>	2009
RMBL0011688	<i>Vicia americana</i>	2009
RMBL0009802	<i>Vicia americana</i>	2011

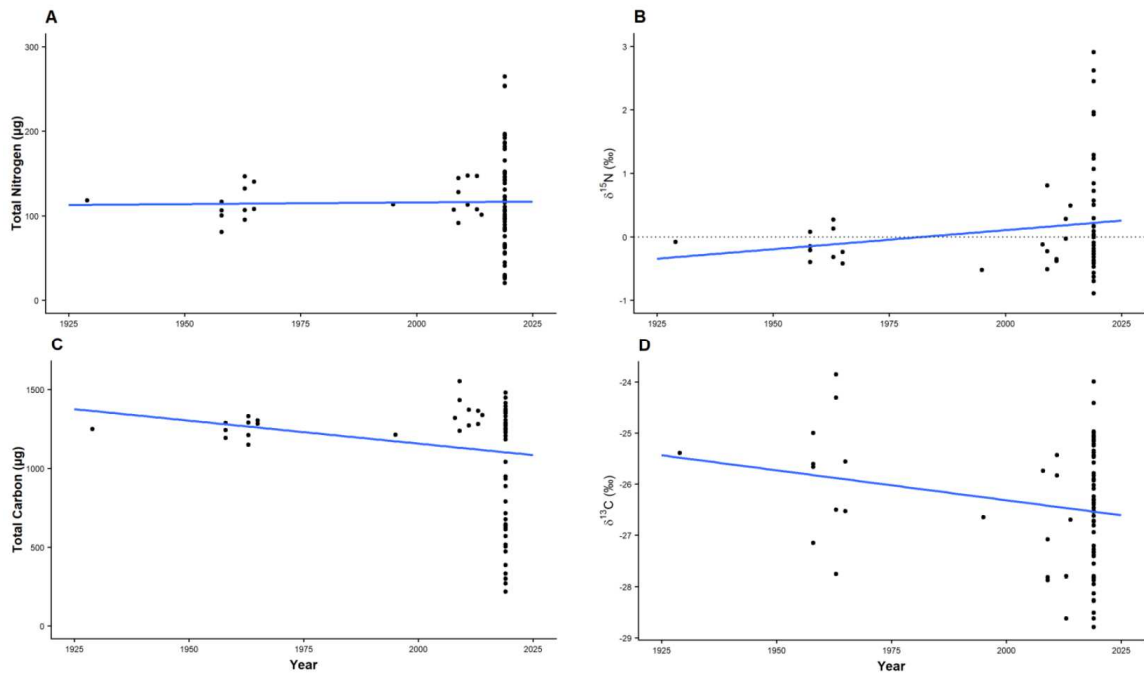
**Table 1.1.** Herbarium samples collected from the Rocky Mountain Biological Laboratory Herbarium.



**Figure 1.1.** *Lathyrus leucanthus* leaf (blue) and soil (brown) total N (A),  $\delta^{15}\text{N}$  (B), total C (C), and  $\delta^{13}\text{C}$  (D). Darker colors indicate raw data and linear fits for the snowmelt treatment while lighter colors represent control conditions. Total N (A),  $\delta^{15}\text{N}$  (B), total C (C), and  $\delta^{13}\text{C}$  (D) of *L. leucanthus* depended on the day of the year within the growing season.



**Figure 1.2.** Herbarium leaf total N (A),  $\delta^{15}\text{N}$  (B), total C (C), and  $\delta^{13}\text{C}$  (D) in three above (brown) or below (blue) average climatic conditions. Violin plots showcase the raw data, where points are jittered for clarity, and the density of the data within the interquartile range.



**Figure 1.3.** Herbarium-kept legume leaf total N (A),  $\delta^{15}\text{N}$  (B), total C (C), and  $\delta^{13}\text{C}$  (D) over time from 1928 to 2019. Raw data and linear fits are shown.



**Image 1.1.** Left to right: a *Vicia americana* plant, a *Lathyrus leucanthus* plant, and a *L. leucanthus* herbarium specimen collected in 1958 and used for analysis in this study.



**Image 1.2.** Five sites located in two valleys at RMBL in Gunnison County, CO. The blue circle indicate the location of the RMBL field station and the white circles indicate the approximate site locations where collections occurred. Prominent peaks in the area are labeled for reference. An example photograph of the experimental snowmelt treatment is shown to the right for the site at elevation 2853 m where the manipulated plot is located on the left (black shade cloth) and the paired control plot is located on the right.

## Chapter 2

### **Legume germination is delayed in dry soils and in sterile soils devoid of microbial mutualists: species-specific implications for upward range expansions**

#### **Abstract**

Climate change is affecting species and their mutualists and can lead to the weakening or loss of important interspecific interactions. Through independent shifts in partner phenology and distribution, climatic stress can separate mutualists temporally or spatially, leading to alterations in partner functional traits and fitness. Here, we explored the effects of the loss of microbial mutualists on legume germination success and phenology. In particular, we assessed the effects of mutualism loss via soil sterilization, increased drought, and introduction to novel soils found beyond the current distributions of two focal legume species in subalpine environments. Through common garden experiments in controlled environments, we found evidence that soil sterilization (and consequent microbial absence) and dry soils caused species-specific phenological delays of 2-5 weeks in germination, likely as a result of interaction loss between legumes and specialized germination-promoting soil microbes, such as mutualistic rhizobia. Delays in germination caused by a mismatch between legumes and beneficial microbes could negatively affect legume fitness through increased plant-plant competition later in the season. Additionally, we found evidence of the presence of beneficial microbes beyond the current elevational range of one of our focal legumes which may allow for expansion of the leading edge, though harsh abiotic factors in the alpine may hinder this. Alterations in the strength of soil microbe-legume mutualisms may lead to reduced fitness and altered demography for both soil microbes and legumes.



## Introduction

Mutualisms are essential and beneficial species interactions which profoundly influence the structure, productivity, and stability of communities (Bruno *et al.*, 2003; Wardle *et al.*, 2004; Leff *et al.*, 2018). Mutualistic interactions provide ecosystem services such as nutrient cycling (Wall and Moore, 1999) and increase partner stress tolerance (Lau and Lennon, 2012; de Zelicourt, Al-Yousif and Hirt, 2013; David *et al.*, 2020). Mutualisms between soil microbes and plants, such as the rhizobia-legume and the nearly-ubiquitous mycorrhizal fungi-plant symbioses, are especially ecologically important for plant growth and fitness, as well as community composition and dynamics (Klironomos, 2002; Reynolds *et al.*, 2003; Van Der Heijden and Bardgett, 2008; Mangan *et al.*, 2010). These mutualisms may become increasingly important as the environment becomes more stressful as a result of anthropogenically-induced global climate change (Allan and Soden, 2008; Dai, 2012; Lau and Lennon, 2012; Gehring *et al.*, 2017; Porter *et al.*, 2020). Environmental context can determine the level of investment made by mutualists, such that the net benefits of mutualisms are often greater in more stressful environments (Pringle *et al.*, 2013; Remke *et al.*, 2021). However, some soil microbes enter a state of dormancy in stressful environments, meaning they do not interact with the plant host under increased stress, which can affect host plant distributions and functional traits (Stanton-Geddes and Anderson, 2011; Simonsen *et al.*, 2017; Werner *et al.*, 2018). Indeed, declines in active partner abundances can destabilize mutualisms (Tylianakis *et al.*, 2008; Kiers *et al.*, 2010), altering plant functional traits and overall fitness (Worchel, Giauque and Kivlin, 2013; Fitzpatrick, Mustafa and Viliunas, 2019).

Mutualistic interactions may become decoupled if one partner is dormant or non-receptive for part of the year while the other is not (Rafferty, CaraDonna and Bronstein, 2015).

Bacteria in particular, including the naturally and agriculturally important nitrogen (N)-fixing rhizobial bacteria (Harris, Pacovsky and Paul, 1985) and other plant growth-promoting rhizobacteria (PGPR), are susceptible to desiccation and death in dry conditions (Schimel, Balsler and Wallenstein, 2007; de Vries and Shade, 2013; Ngumbi and Kloepper, 2016; de Vries *et al.*, 2018; Xu and Coleman-Derr, 2019). To avoid losing water to their environment, many bacterial cells can enter a state of dormancy (Lennon and Jones, 2011; Schimel, 2018). In N-fixing rhizobia, soil drying has been shown to induce dormancy in free-living cells and inhibit N-fixation in symbiotic bacteroid cells, leading to denodulation (Zahran, 1999; Hungria and Vargas, 2000; Vriezen, de Bruijn and Nüsslein, 2006; Aldasoro, Larrainzar and Arrese-Igor, 2019) and short-term mutualism loss. Similarly, active, free-living bacterial cells in dry soils may have difficulty forming interactions with plant roots because low soil moisture negatively affects the signaling abilities of soil bacteria and plants (Schimel, 2018; Williams and de Vries, 2020). As a result, the mutualism between legumes and soil microbial species, such as PGPR, can weaken in the short term due to climate change-induced soil drying.

When active, soil microbial mutualists often ameliorate environmental stress and help host plants overcome limitations, allowing plants to persist in conditions otherwise intolerable (Defosse *et al.*, 2011; Redman *et al.*, 2011; Lau and Lennon, 2012; Rodríguez-Echeverría, Lozano and Bardgett, 2016; Petipas *et al.*, 2017; David, Thapa-Magar and Afkhami, 2018; Bennett and Meek, 2020; David *et al.*, 2020). For this reason, soil microbe-plant mutualistic partners are often able to inhabit a broad range of habitats (Stachowicz, 2001; Bruno, Stachowicz and Bertness, 2003; Rodriguez-Cabal, Noelia Barrios-Garcia and Nunez, 2012; Afkhami, McIntyre and Strauss, 2014; Harrison *et al.*, 2018). For example, by increasing plant access to N, phosphorus (P), and water, N-fixing bacteria and arbuscular mycorrhizal fungi (AMF) allow plants to expand their ranges into otherwise unsuitable, nutrient-poor habitats (Halvorson, Smith

and Franz, 1991; Stachowicz, 2001; Afkhami, McIntyre and Strauss, 2014; Hayward *et al.*, 2015; Harrison *et al.*, 2018). Additionally, agricultural studies have demonstrated that some soil microbial species are able to promote host seed germination by excreting phytohormones, thereby increasing germination success in newly colonized habitats (Atzorn *et al.*, 1988; Noel *et al.*, 1996; Bastian *et al.*, 1998; Tsavkelova *et al.*, 2007; Miransari and Smith, 2009; Kumar, Dubey and Maheshwari, 2011; Namvar and Sharifi, 2011; Meena *et al.*, 2012; Ngumbi and Kloepper, 2016; Wu *et al.*, 2016). Conversely, the absence of mutualists can negatively affect population persistence and limit species distributions (Pellmyr, 2003; Nuñez, Horton and Simberloff, 2009; Mueller *et al.*, 2011; Harrower and Gilbert, 2018; Benning and Moeller, 2021b). Indeed, this has been documented in some soil microbe-plant mutualisms (Stanton-Geddes and Anderson, 2011; Simonsen *et al.*, 2017). Thus, the ability of a plant to successfully establish in a new habitat depends on not only dispersal and the physical conditions in the novel range but also biotic factors, including the presence of mutualists (van der Putten *et al.*, 2010; HilleRisLambers *et al.*, 2012; Brown and Vellend, 2014).

The role of soil microbe-plant mutualisms in shaping the geographic distributions of plant species is still little-understood (Classen *et al.*, 2015; Bueno de Mesquita *et al.*, 2016; Benning and Moeller, 2021a). As plants, including legumes, continue to expand their leading range edges up altitudinally and poleward latitudinally in response to climate change (Chen *et al.*, 2011; Harrison *et al.*, 2018), they may encounter harsh environments, made potentially more stressful by the absence of mutualistic partners. Legumes often require exposure to soils that have been pre-inoculated with compatible soil microbes to establish and persist in dry N- and P-poor soils such as those found in the alpine and subalpine (Parker, 2001; Simonsen *et al.*, 2017; Darcy *et al.*, 2018; Bueno de Mesquita *et al.*, 2020). The absence or reduced abundance of microbial mutualists beyond the current range of a population could impair plant fitness and hinder leading

range expansion (Miransari, 2010; Peay, Garbelotto and Bruns, 2010; Sedlacek *et al.*, 2014; Lankau and Keymer, 2016; Wu and Ying-Ning, 2017; Hu *et al.*, 2022). Non-co-dispersed, horizontally transmitted symbionts, including legumes, rhizobia, and some other PGPR, may be at high risk of becoming spatially mismatched as they may track climate differently (Keeler, Rose-Person and Rafferty, 2021). Legumes that interact with specialized mutualists may be less likely to find a compatible partner in novel habitats and thus may fail to establish (Simonsen *et al.*, 2017), while legumes that have been successful in expanding into novel ranges without a historical partner may have benefitted from their ability to relax their partner choice mechanisms and establish interactions with generalist mutualists (Harrison *et al.*, 2017; Younginger and Friesen, 2019). However, compared to the historical interactions, new interactions in a novel habitat may not confer equivalent benefits to the host plant (Bueno de Mesquita *et al.*, 2018; Werner *et al.*, 2018).

A spatial or dormancy-induced loss of a mutualism, even for part of a season, could lead to reduced germination stimulation by soil microbes which could decrease plant germination success (David *et al.*, 2020; Eldridge *et al.*, 2021), delay host plant germination phenology, and alter downstream phenophases, such as flowering onset (Namvar and Sharifi, 2011), which will alter phenological overlap and interaction strengths among host plants and pollinators (Rafferty and Ives, 2012; Rafferty, Bertelsen and Bronstein, 2016). A shift in flowering phenology without a corresponding shift in pollinator phenology could decrease the fitness of both mutualists (Rafferty and Ives, 2011; Schenk, Krauss and Holzschuh, 2018; Kudo and Cooper, 2019). Germination phenology is especially important, as germinants are particularly vulnerable to stress relative to other stages of the plant life cycle. Seedlings that emerge in stressful environments, including in environments lacking certain facultative mutualists, have lower survival (Donohue *et al.*, 2010). Germination timing also shapes competitive outcomes and reproductive success

(Fowler, 1984; Leverett, 2017). Studies evaluating the interactions between seeds and soil microbes during the germination process in natural systems are limited (but see: Shade *et al.*, 2017; Tobias *et al.*, 2017; Nelson *et al.*, 2018), though the importance of these interactions could become greater in a changing climate; seed germination stimulation by microbes may strengthen (David *et al.*, 2020), or weaken via stress-induced microbial dormancy (Schimel, 2018).

Here, we explore how germination traits of two legumes are affected by the absence of their mutualisms with soil microbial species. Environmental stress, namely drought, and the possible absence of suitable soil microbes in the expected future ranges of our focal legumes may affect legume germination success and timing. Because germination success can be stimulated by microbes, we hypothesize that legumes in sterilized soils devoid of microbes will have lower germination success and delayed germination phenology. Conversely, if partners co-occur, we predict that interaction strength will increase with increasing stress (David *et al.*, 2020), such that germination success and phenology are unaltered despite high-stress (novel range or drought) conditions. To investigate the potential consequences of short-term loss of the interactions among legumes and soil microbes, we ask if the success and timing of germination are affected by i) soil sterilization, ii) foreign soils collected from elevations higher than the current distributions of these legumes, or iii) limited soil moisture. Overall, we aim to address how disruptions in plant-soil microbe mutualisms may prevent range expansion and affect plant germination traits.

## **Methods**

### *Study system*

This study was conducted using seeds and soils collected from The Rocky Mountain Biological Laboratory (RMBL; N 38° 52.2928', W 106° 58.671') located in the Maroon Bells-Snowmass Wilderness area near Gothic, Colorado, USA. The RMBL area is characterized by

vast, open subalpine meadows dominated by perennial wildflowers and patches of aspen-fir forests. Subalpine plant communities, like those at RMBL, are especially sensitive to changes in climate due to short growing seasons and upward range limitations (Parmesan, 2006; Hülber, Winkler and Grabherr, 2010). These subalpine plant communities therefore offer an excellent system to address questions on climate change effects on plant-soil microbe interactions. In the last several decades at RMBL, snowpack has decreased, the date of spring snowmelt has shifted earlier (3.5 days earlier per decade from 1974-2012), and year-to-year variation in snowmelt date increased by 20% from 1974-2008 (Lambert, Miller-Rushing and Inouye, 2010; CaraDonna, Iler and Inouye, 2014). Additionally, June precipitation has decreased significantly since the 1980s and July monsoon rains are delayed or non-existent (data accessible at <https://www.gothicwx.org/>). Temperatures at RMBL have risen to date and are expected to continue to increase over the next century and total precipitation is expected to decrease (Overpeck and Udall, 2010). Decreased snowpack, earlier snowmelt, and decreased June precipitation are predicted to result in earlier, longer dry seasons prior to July monsoon rains (Clow, 2010; Kittel *et al.*, 2015), which is likely to affect the species and their interactions in this system.

### *Selection of plant species*

We studied the two native, nectar-producing legume plant species present in the Maroon Bells-Snowmass Wilderness area, *Lathyrus lanszwertii* var. *leucanthus* and *Vicia americana* (Fabaceae). Both are perennial vines that are common in the RMBL area and produce nutrient-rich rewards that attract native pollinators (unpublished data, A. M. Keeler). We verified that both of these species form root nodules and host N-fixing bacteria in the field and in controlled common garden settings (Image 2.1), and that they host arbuscular mycorrhizal fungi (AMF) and

dark septate endophytes (DSE) in the field. These species are known to host AMF across their range and facultative DSE at higher elevations near RMBL (unpublished data, RMBL). However, after using previously established methods for alpine plants (Schmidt *et al.*, 2008), staining and microscopy revealed neither fungal group on or in the roots of these legumes in our controlled common garden setting, likely because fungal spores can quickly decay in cool, wet soils if stored there for a month or more (Gottlieb, 1950; Varga *et al.*, 2015), as our soils were. We note that, using the same staining methods, we have verified the presence of AMF in roots of other species grown from seeds collected at some of the same sites near RMBL, increasing our confidence that AMF were absent in our soils for this study. Along with AMF, DSE, and rhizobia, it is likely that various phylotypes of Acidobacteria, nitrifying taxa (e.g., *Nitrospira* spp. and *Thaumarchaeota* spp.), *Thelephora* (Agaricomycetes), *Hebeloma* (Agaricomycetes), *Archaeorhizomyces* (Archaeorhizomycetes), *Tetracladium* (Leotiomycetes), and other endophytes, such as fine root endophytes (FRE), were present in our soils, as these taxa are common in the soils around RMBL after snowmelt (Orchard *et al.*, 2017; Sorensen *et al.*, 2020).

*Vicia americana* is widely distributed across North America while *L. leucanthus* is located solely in mountainous regions of western North America. The ranges of *L. leucanthus* and *V. americana* in the RMBL region extend from ~2700 to 3500 m in elevation. Observational data suggest that the elevational ranges of both species (and their bumblebee pollinators) have expanded upward in the last 40 years, and leading edges are expected to continue to expand (Pyke, 1982; Pyke, Inouye and Thomson, 2012; Pyke *et al.*, 2016). These observations come from systematic surveys conducted in the 1970s (Pyke, 1982), wherein the presence/absence of *L. leucanthus* and *V. americana* was noted along transects that span elevational gradients in the RMBL area, and the systematic resurveying in 2015-2018 of some of those same transects and others that span similar elevations (described herein). For example, whereas neither species was

recorded as present at the highest-elevation survey point (3394-3442 m) along the Washington Gulch transect in the 1970s (Pyke, 1982), both species were found there in 2015-2018. In the 1970s, the highest elevation survey point at which the focal legumes were documented was 3333-3393 m (Pyke, 1982).

#### *Collection of soils and seeds*

We collected soils and seeds from multiple populations across gradients that span the current and expected future elevational ranges of our focal plant species at RMBL in summer 2017 and summer 2018 to understand how the loss of microbial partners, soil origin, and drought may affect subalpine legume germination traits. Soils and seeds were collected within 10 m of transects that traverse the Washington Gulch (403), Gothic Mountain, and Baldy Mountain trails (3200-3500 m in elevation; Image 2.2). Within the current range of our focal legume species, soils were collected from within a 10 cm radius of the nearest legume to a depth of 15 cm, just past the rooting depth of *L. leucanthus* and *V. americana*, and where beneficial soil microbial species are likely to be at higher densities in the soil (Komatsu and Simms, 2020). A soil corer was centered over a focal legume and soils were exhumed from that core. To collect soils from elevations beyond the upper range limits of *L. leucanthus* and *V. americana* populations (>3500 m, just beyond treeline), we sampled at least 3 sites per transect after verifying that neither species occurred at those elevations, which ranged from 3500-3800 m. Similar to lower elevations, soils were collected from a 10 cm radius, 15 cm deep core. As no focal legumes were present at higher elevations, collection sites were chosen haphazardly; collections were made near plants such as *Lupinus argenteus*, *Castilleja* species, and various grasses and rushes. All soils were put on ice and transported back to the RMBL field station where they were stored at 4° C. Soils were kept on ice for a day during transportation to UC Riverside, then stored at 4° C. Soils



from each elevational zone were homogenized each year to standardize nutritional content and soil texture. Seeds were haphazardly collected from mature fruits (i.e., pods) within 10 m of these same transects; seeds were collected from plants if pods were beginning to dehisce. One to six pods were collected per maternal plant and each pod contained one to three seeds. Only unparasitized seeds were used in experiments. In total, 347 *L. leucanthus* and 1059 *V. americana* seeds were collected and used for these experiments. Seeds were cold stratified at 4° C for 45 days, the recommended time for these species (personal communication, Mike Bone, Denver Botanic Gardens). All seeds were weighed to the nearest 0.1 mg before sowing, except for the *V. americana* seeds sown in the drought experiment. The average seed mass was  $15.7 \pm 7.4$  mg (mean  $\pm$  SD) for *L. leucanthus* and  $10.9 \pm 8.7$  mg for *V. americana*.

#### *Experimental design and setup*

To assess the effects of drought and soil origin, we designed two separate common garden studies (Table 2.1). We grew our focal legume species in sterile background soils inoculated with field-collected soils (Collins, 2019; David *et al.*, 2020). To control for abiotic differences across soil collection points, background soils were small-batch sterilized (double autoclaved within 12 hours at 121° C for 90 minutes, containers 0.5-1.5 L) and then were added to sterile pots (66 mL with drainage holes; Ray Leach Cone-tainer, Stuewe & Sons, Tangent, Oregon, USA); background soils consisted of 57% sand, 43% peat moss, and various minerals. This relatively high ratio of inoculum to background soils was used in the likely case of low microbial biomass in soil samples. Though the majority of the soil in each pot was the same across treatments, there may have been slight differences in the abiotic properties of inoculants. We then added field-collected soil inoculum to these sterile background soils; 85% of the total soil volume was made up of sterile background soil, while the other 15% of total soil volume was

made up of field-collected soil inoculum. Background and inoculum soils were thoroughly mixed. In addition to twice sterilizing pots and background soils, half of all field-collected soils were twice autoclaved prior to use; in sterile soils there can be no interactions between legumes and soil microbes, mimicking a complete loss of the possible mutualisms. These soils are referred to as sterile herein, but autoclaving does not ensure sterility; these soils are nearly sterile and contain very little, if any, microbial biomass.

Wild-collected *L. leucanthus* and *V. americana* seeds were surface sterilized for 20 minutes in a 10% bleach solution, briefly soaked in four subsequent sterile water baths, then allowed to dry (Oyebanji *et al.*, 2009; Collins, 2019); seeds were sterilized to isolate the effects of the soil type and moisture treatments. Individual sterile, dry seeds were weighed, sown directly into separate soil-filled pots at the same depth, then covered with ~10 g of treatment soil (one seed per pot). Each treatment soil was housed on a separate tray to reduce movement of microbes from pot to pot via watering or air circulation (Image 2.3). Legumes were placed in a growth chamber (Conviron MTR30) at a temperature and photoperiod regime reflecting that of the early growing season (germination period of the focal species) at RMBL (day: 21° C, 50% relative humidity; night: 4.4° C, 20% relative humidity; 12:12 h light:dark cycle).

*Legume germination traits in sterilized soils and soils from beyond the current elevational range: implications for upward range expansion*

We grew individuals of both legume species in separate factorial experiments that manipulated the presence of soil microbes (unsterilized vs. sterilized soil conditions) and elevational origin of the soil (within the current ranges vs. beyond the current ranges of the focal legumes) in soils collected from RMBL. Soil treatments comprised: unsterilized, current range soils (unmanipulated, microbially active, current range of the focal legumes; *L. leucanthus*: n =

95 seeds; *V. americana*: n = 76 seeds); unsterilized, beyond the current range soils (unmanipulated, microbially active, beyond the current range of the focal legumes; *L. leucanthus*: n = 78 seeds; *V. americana*: n = 59 seeds); sterilized, current range soils (double autoclaved, microbially sterile, current range of the focal legumes; *L. leucanthus*: n = 96 seeds; *V. americana*: n = 71 seeds); and sterilized, beyond the current range soils (double autoclaved, microbially sterile, beyond the current range of the focal legumes; *L. leucanthus*: n = 78 seeds; *V. americana*: n = 53 seeds; Table 2.1).

#### *Legume germination traits in dry soil conditions: implications for climate change-induced soil drying*

Separately, we grew *V. americana* in factorial experiments that manipulated the presence of microbes (unsterilized vs. sterilized conditions) and the soil moisture level (dry vs. well-watered), where all soils were from within the current range and began at the same soil moisture VWC%. Half of all pots were placed into the well-watered treatment while the other half were placed in the dry treatment. The well-watered treatment was watered with sterile, twice autoclaved water every other day for 10 weeks while the dry treatment was watered every other day for 2 weeks then once every week for 8 weeks; approximately 3 mL of water was added to each pot at every watering. Soil treatments comprised: unsterilized, well-watered soils (n = 200 seeds); sterilized, well-watered soils (n = 200 seeds); unsterilized, dry soils (n = 200 seeds); and sterilized, dry soils (n = 200 seeds; Table 2.1).

#### *Data collection*

Seedling germination phenology, or the date of germinant emergence from the soil, was monitored every other day for 10 weeks. In total, we documented the timing of germination for

225 seeds (16% of the 1406 seeds sown germinated). Many alpine species, including our focal legumes, are highly clonal and are therefore expected to have a low rate of germination (Angevine, 1983; Eriksson, 1989; Callaghan *et al.*, 1992). Indeed, germination success in both species tends to be low (4-11%) in natural conditions around RMBL and in laboratory settings, even when methods to crack the seed coats (e.g., sulfuric acid treatment) are used (personal communication, Mike Bone, Denver Botanic Gardens; unpublished data, N. E. Rafferty). Seeds that did not successfully germinate within 10 weeks were removed from pots and replanted in unsterilized soils with adequate water and monitored for 10 additional weeks; none of these seeds germinated after replanting.

#### *Data analysis*

To examine variation in germination success in soils that differed in soil sterility and elevational origin, we constructed generalized linear models (GLM) with binomial error. To investigate variation in germination latency in soils that differed in soil sterility and elevational origin, we used linear models (LM). To these models (with either germination success or latency as the response), we introduced species, seed mass, soil elevational origin, and soil sterility as predictors. The importance of seed mass for germination success or latency may depend on soil type, such that seed mass may be positively related to germination in sterilized soils but less important for seeds in unsterilized soils; we therefore also included the three-way interaction between seed mass, soil origin, and soil sterility as a predictor. Because species was a significant predictor in all models, we fitted separate models for *L. leucanthus* and *V. americana*. We used the same approach to examine variation in germination success and latency in response to soil moisture, except we omitted species and seed mass as predictors because only *V. americana* was used in that experiment and the seeds were not weighed. We used likelihood ratio tests to

compare the fit of nested models, starting with the full model and comparing the fit of reduced models, and we report the best fitting models. If a three-way interaction was significant, then we retained all two-way interactions and main effects in the model. Post-hoc Tukey tests were used to test for significant pairwise differences between categorical soil treatments. All analyses were conducted in R version 4.0.2 (R Core Team, 2019).

## Results

### *Interactive effects of soil sterility, soil elevational origin or soil moisture treatment, and seed mass*

We start by describing the best-fitting models for each species, first for germination success and then for germination latency, before turning to specific contrasts of interest. For *L. leucanthus* germination success in the context of soil elevational origin, we detected a significant 3-way interaction between soil sterility, soil elevational origin, and seed mass, indicating that the effects of seed mass on germination success depended on soil type (GLM:  $-0.16 \pm 0.07$ ,  $z_{346} = -2.17$ ,  $p < 0.03$ ; Figure 2.1). For *V. americana* germination success relative to soil elevational origin, the best-fitting model included only soil sterility, soil origin, and the interaction between the two as predictors. Thus, for this species, seed mass did not significantly affect germination success. For *V. americana* germination success in the context of soil moisture, the best-fitting model included only the main effect of water treatment and no interactions.

For *L. leucanthus* germination latency relative to soil elevational origin, the best-fitting model included a significant 3-way interaction between soil sterility, soil origin, and seed mass (LM:  $-5.33 \pm 1.46$ ,  $t_{15} = -3.65$ ,  $p < 0.002$ ; Figure 2.1). For *V. americana* germination latency pertaining to soil elevational origin, only soil sterility and seed mass were retained as predictors

in the best-fitting model. For *V. americana* germination latency in the context of soil moisture, no significant interactions were detected, and the best-fitting model included only soil sterility and soil moisture treatment.

#### *Legume germination traits in sterilized soils*

There was no effect of soil sterilization on *L. leucanthus* or *V. americana* germination success. Germination success of *L. leucanthus* did not differ in sterilized vs. unsterilized soils from the current range (Tukey test:  $p < 0.16$ ; Figure 2.2). However, in unsterilized, current range soils, heavier *L. leucanthus* seeds were more likely to germinate (Figure 2.1). Similarly, for *V. americana*, germination success did not differ in sterilized vs. unsterilized current range soils (Tukey test:  $p < 0.13$ ; Figure 2.2).

For *L. leucanthus* in current range soils, the onset of germination in sterilized soils was delayed by about two weeks compared to germination in unsterilized soils (Tukey test:  $p < 0.03$ ; Figure 2.2). In unsterilized, current range soils, *L. leucanthus* seeds germinated  $28.0 \pm 14.8$  (mean  $\pm$  SE) days after sowing, while in sterilized soils, seeds germinated in  $69.0 \pm 26.0$  days. In unsterilized, current range soils, heavier seeds germinated more quickly (Figure 2.1). For *V. americana*, the delay in germination in sterilized vs. unsterilized current range soils approached significance (Tukey test:  $p < 0.06$ ; Figure 2.2); seeds in unsterilized current range soils germinated in  $30.2 \pm 5.3$  days vs.  $40.0 \pm 6.9$  days in sterilized soils. Nodules were found on some of the plant roots of both species in the unsterilized but not in the sterilized treatments.

#### *Legume germination traits in soils from beyond the current elevational range: implications for upward range expansion*

Germination success of *L. leucanthus* did not differ in unsterilized soils from within vs. beyond the current elevational range (Tukey test:  $p < 0.71$ ; Figure 2.2). There was no difference in germination success in sterilized vs. unsterilized soils from beyond the range (Tukey test:  $p < 0.98$ ). However, in sterilized soils from beyond the range, heavier *L. leucanthus* seeds had a higher probability of germination (Figure 2.1). For *V. americana*, germination success was significantly lower in unsterilized soils from beyond the current elevational range compared to that within the range (Tukey test:  $p < 0.01$ ; Figure 2.2). There was no significant difference in germination success between sterilized vs. unsterilized soils collected from beyond the current elevational range (Tukey test:  $p < 0.71$ ).

Seeds of *L. leucanthus* and *V. americana* sown in unsterilized soils from within vs. beyond their current elevational range showed no significant difference in germination timing (*L. leucanthus* Tukey test:  $p < 0.73$ ; *V. americana* Tukey test:  $p < 0.95$ ; Figure 2.2). In unsterilized, beyond the current range soils, heavier *L. leucanthus* seeds germinated more quickly (Figure 2.1). Notably, *L. leucanthus* seeds germinated more quickly in sterilized beyond the current elevational range soils than in sterilized current range soils (Tukey test:  $p < 0.01$ ), and this was not the case for *V. americana* (Tukey test:  $p < 0.94$ ). Regardless of soil origin, heavier *V. americana* seeds germinated more quickly than lighter seeds (LM:  $-1.83 \pm 0.32$ ,  $t_{15} = -5.75$ ,  $p < 0.00001$ ).

#### *Legume germination traits in dry soil conditions: implications for climate change-induced soil drying*

*Vicia americana* seeds in unsterilized, dry soils had lower germination success than those in unsterilized, well-watered soils (Tukey test:  $p < 0.03$ ; Figure 2.3). Germination success was  $26 \pm 2\%$  in unsterilized, well-watered soils, higher than the  $15 \pm 2\%$  seen in unsterilized, dry soils. Similarly, there was greater germination success in sterilized, well-watered conditions vs.

sterilized, drought conditions (Tukey test:  $p < 0.01$ ). Seeds in unsterilized, dry conditions also experienced delayed germination compared to those in unsterilized, well-watered soils (Tukey test:  $p < 0.0001$ ; Figure 2.3). In unsterilized soils under dry conditions, seeds germinated in  $50.2 \pm 1.8$  days, while in unsterilized soils under well-watered conditions, seeds germinated in  $35.2 \pm 1.9$  days. Lastly, *V. americana* seeds planted in unsterilized, well-watered soils germinated more quickly and readily than those in sterile, dry conditions (germination latency Tukey test:  $p < 0.01$ ; germination success Tukey test:  $p < 0.001$ ; Figure 2.3).

## Discussion

Short-term weakening or loss of beneficial partnerships is becoming increasingly common as our climate rapidly changes, and repeated instances of loss can lead to a mutualism breakdown (Kiers *et al.*, 2010; Werner *et al.*, 2018). The loss of a mutualism can alter the distribution, functional traits, and survival of both partners. In this study, we found that a forced loss of interaction between legumes and root mutualists can have consequences for plant traits, where legume germination timing, but not success, was negatively affected by sterilized, microbe-free soils. Specifically, our results demonstrate that the onset of germination of *L. leucanthus* in sterilized soils depleted of microbes was delayed by about two weeks (Figure 2.1). However, germination probability was low for *L. leucanthus*, with only a few seeds germinating in sterile conditions, suggesting further study is warranted. Similarly, delays in germination in *V. americana* approached significance. These results suggest that some legume seeds and seedlings may benefit from interacting with plant growth-promoting soil microbes shortly after germinating and possibly even before germination via germination stimulation. Germination timing is a particularly important trait, as it affects a plant's competitive interactions and hardiness to frost and drought, and individuals that survive this fragile life stage are more likely to reach



reproductive stages. The observed stimulation of germination by soil microbial mutualists is well known in systems such as the orchid-fungal mutualism (Arditti, 1967; Dressler, 1981), in certain legume-rhizobia mutualisms (Miransari and Smith, 2009), and in plant interactions with *Pseudomonas fluorescens* (Moeinzadeh *et al.*, 2010), but this is the first known evidence of this phenomenon occurring in the legumes of this subalpine system. Further research on the timing of this stimulation, by isolating initial shoot growth from seed splitting at germination, will allow us to better understand the effects of these seed-microbe interactions (Walsh *et al.*; 2021). This work contributes to the growing number of findings that the community of soil microorganisms around a seed influences germination timing, which may affect plant fitness (Mordecai, 2012; Lamichhane *et al.*, 2018; Das *et al.*, 2019; Eldridge *et al.*, 2021).

As climatic patterns shape the natural distributions of species, changing climate conditions strongly influence species' ranges (Chen *et al.*, 2011; Becker-Scarpitta, Vissault and Vellend, 2019), typically promoting range expansion toward higher latitudes and elevations and range contractions away from lower latitudes and elevations (Davis and Shaw, 2001; Parmesan, 2006; Lenoir and Svenning, 2015). Because the presence of mutualists can serve to expand the range of a partner by ameliorating abiotic stressors in novel environments (Halvorson, Smith and Franz, 1991; Stachowicz, 2001; Afkhami, McIntyre and Strauss, 2014; Hayward *et al.*, 2015; Harrison *et al.*, 2018) and the absence of a mutualist can constrict the range of a partner (Nuñez, Horton and Simberloff, 2009; Simonsen *et al.*, 2017; Harrower and Gilbert, 2018), these mutualist-hosting legumes may not be able to expand their elevational ranges upward if compatible soil microbes are not present beyond the current range. This study demonstrates that the leading range edges of *L. leucanthus* may not be restricted to 3500 m as active, compatible microbial species may be present at higher elevations; germination timing and success of this species in higher-elevation soils mirrored that in current range soils. Abiotic soil properties at

higher elevations may also be conducive to earlier germination of *L. leucanthus*, as seed germinated more quickly in sterilized soils from beyond the range than in sterilized soils from within the range, though very few seeds of this species germinated in sterile conditions. In contrast, *V. americana* germination success was over five times lower in novel, beyond the range soils, indicating that beneficial microbe strains specific to *V. americana* plants may be absent or at low abundances at higher elevations, leading to reduced germination success. Although sequencing and quantification of both within- and beyond the range soils are needed to confirm the presence and abundance of active microbes, the comparable germination timing and success in *L. leucanthus* in novel vs. current elevational range soils suggests that compatible *L. leucanthus*-specific soil microbes may facilitate the leading range expansion of this legume.

Germination-triggering soil wetting events are becoming less frequent and less intense in many areas due to climate change (Saatkamp *et al.*, 2019). One of the predictions of the stress gradient hypothesis is that mutualistic interactions increase in strength with increasing stress (Callaway *et al.*, 2002; David *et al.*, 2020). Legumes grown in stressful conditions may not exhibit reduced germination success and initial survival relative to those in less stressful conditions as long as microbial mutualists can buffer the abiotic stress by stimulating germination, provisioning N, P, and water, and reducing root parasitism, thereby increasing plant performance (Figueiredo *et al.*, 2008; Pawar *et al.*, 2014; Jemo *et al.*, 2017; Marinković *et al.*, 2019; Tankari *et al.*, 2019). Conversely, stressful, dry soils promote microbial dormancy, preventing beneficial microbes from stimulating germination or interacting with the seed or plant (de Vries *et al.*, 2018). In this controlled common garden study, legume seeds subjected to dry conditions were less likely to germinate and had significantly delayed germination compared to those in well-watered soils, likely due to stressful abiotic conditions for both the seed and the microbes. This reduced germination stimulation may reflect a drought-induced loss of the

mutualism. If a plant germinates later than the optimal time, plant-plant competition will be greater and the ability to acquire limited resources such as water, light, P, and N will be reduced (Lloret, Casanovas and Peñuelas, 1999; Leverett, 2017). Downstream phenological patterns, such as flowering time, could also be delayed, affecting pollinator visitation rates and reproductive output (Rafferty and Ives, 2012).

Though microbes compatible with *L. leucanthus* may be present at high elevations, abiotic conditions at these elevations are relatively harsh; low soil moisture, high UV exposure, and high winds, among other factors common above the treeline, could limit focal legume establishment beyond 3500 m (Normand *et al.*, 2009). In addition to this, mammalian seed and seedling herbivores may limit seedling establishment in novel areas (Bueno de Mesquita *et al.*, 2020; Lynn, Miller and Rudgers, 2021). In this study, stressful, dry conditions led to decreased percent germination and delayed germination; drier alpine conditions may not allow this mutualism to establish or persist and may hinder a continued upward range expansion. Interestingly, *Lupinus argenteus*, a co-occurring rhizobia- and AMF-hosting legume, occupies higher elevations than *L. leucanthus* or *V. americana*. The PGPR which interact with *L. argenteus* may stimulate the germination of other legume species (Hirsch, Lum and Allan Downie, 2001), though sequencing of both within- and beyond the range soils is needed to confirm the observational evidence of soil microbes occurrence in both ranges. Another avenue to be explored is that of the seed microbiome (Nelson, 2018), specifically the epiphytic microbial community for horizontally transmitted mutualists like rhizobia and other PGPR bacteria. The seed coats of both focal legume species are not smooth; if seeds fall to the ground and accumulate mutualists before dispersal, an upward range expansion may be more likely to occur because the partners would co-occur spatially, though joint dispersal of legumes and rhizobia and other microbes has seldom been studied (Porter, Faber-Hammond and Friesen, 2018; Wendlandt *et al.*, 2021), and joint

dispersal does not always imply interaction (Wornik and Grube, 2010). Sequencing of soils and dispersed seeds would be useful to test this possibility.

In addition to soil microbe-mediated germination stimulation, seed traits, such as seed mass, are important components of germination success and timing (Lord, Westoby and Leishman, 1995; Venable *et al.*, 1998; Thompson, 2008). It is thought that heavier seeds are an adaptation for overcoming stressful conditions, like drought, during seedling establishment (Wulff, 1986), as larger seeds increase seedling persistence via greater internal resource provisioning (Leishman and Westoby, 1994; Lebrija-Trejos *et al.*, 2016; Harrison and LaForgia, 2019). The transition from seed to seedling can be a defining period in the life history of a plant (Muscarella *et al.*, 2013; Larson *et al.*, 2015), and here, we found that heavier *L. leucanthus* seeds germinated more quickly and tended to have greater germination success than lighter seeds. This trend was especially pronounced when seeds were planted in the most stressful conditions, sterile soils collected from beyond the current range of *L. leucanthus* (Figure 2.1). For *V. americana*, germination success was not affected by seed mass, but heavier seeds germinated earlier regardless of soil origin. In the absence of epiphytic microbes, other factors such as seed mass and abiotic components of the seed environment (e.g., soil moisture, available nutrients) become more important (Lamichhane *et al.*, 2018). In these scenarios, heavier seeds are predicted to be more vigorous, and thus germinate at a higher and faster rate.

A drawback to using a sterile soil treatment is that sterilization removes not only plant growth-promoting soil mutualists but also all other potential soil microbes. Based on field observations near the study area, *L. leucanthus* and *V. americana* interact not only with rhizobia but also with AMF and DSE (unpublished data, RMBL). Through staining and microscopy, neither of these fungal symbionts were found on or in any of the plant roots in this study, but

nodules were found on some of the plants in unsterilized, current elevation soils. As simultaneous infection by multiple belowground mutualists can additively benefit the plant (Afkhami and Stinchcombe, 2016), future work that assesses the effects of native rhizobial, AMF, DSE, FRE, and other PGPR bacterial infection on legume functional traits would be valuable.

Symbiotic rhizobia in extreme environments have lost the genes responsible for the initiation and maintenance of their mutualism with legumes due to natural selection; the maintenance of this non-essential portion of the genome is costly to the bacteria in harsh environments (Sullivan *et al.*, 1996; Sullivan and Ronson, 1998; Denison and Kiers, 2004; Sachs, Skophammer and Regus, 2011). The loss of this segment of DNA ultimately causes a complete breakdown of the mutualism, only likely after numerous short-term losses of the mutualism. A climate change-induced breakdown in the mutualism between legumes and rhizobia will have significant effects on legume germination, phenology, and N-acquisition, which could affect higher-order mutualists, such as pollinators (Keeler, Rose-Person and Rafferty, 2021), and plant community structure (Suttle, Thomsen and Power, 2007). Just as floral traits such as nectar quality can be directly related to soil nutrient availability (Mevi-Schutz and Erhardt, 2005; Burkle and Irwin, 2009), short- or long-term loss of the interaction between plants and soil microbial species due to mutualism loss or breakdown will indirectly affect floral traits by altering host plant nutrient acquisition (Gwata *et al.*, 2003; Megueni *et al.*, 2006; Namvar and Sharifi, 2011; Ballhorn, Kautz and Schädler, 2013), which could cascade to affect pollinator behavior and legume reproductive success (Keeler, Rose-Person and Rafferty, 2021). The long-term fitness consequences of this particular mutualism loss are generally unknown (Berg *et al.*, 2010; Kiers *et al.*, 2010), though slower growth and lower quality floral rewards in these pollinator-dependent, pollen-limited plants (Xingwen, 2021) may further decrease reproductive success and thus recruitment in a warming, drying climate.

In this controlled common garden study, we found evidence that active plant germination-promoting microbial species enabled legume germination and advanced germination timing. Soil sterilization (and consequent microbial absence) and dry soils caused germination to be delayed by 2-5 weeks as a result of interaction loss between legumes and germination-promoting soil microbes. Additionally, we documented the presence of beneficial soil microbes beyond the current elevational range of one of our focal legume species which may allow for expansion of the leading range edge of *L. leucanthus* but not *V. americana*, suggesting *V. americana* may require more specialized interactions with soil microbial species. As soils dry and changing climatic conditions reshape legume upper range edges, beneficial soil microbial species may become inactive or absent, altering legume germination timing and success and ultimately affecting legume demographics and interactions with other mutualists.

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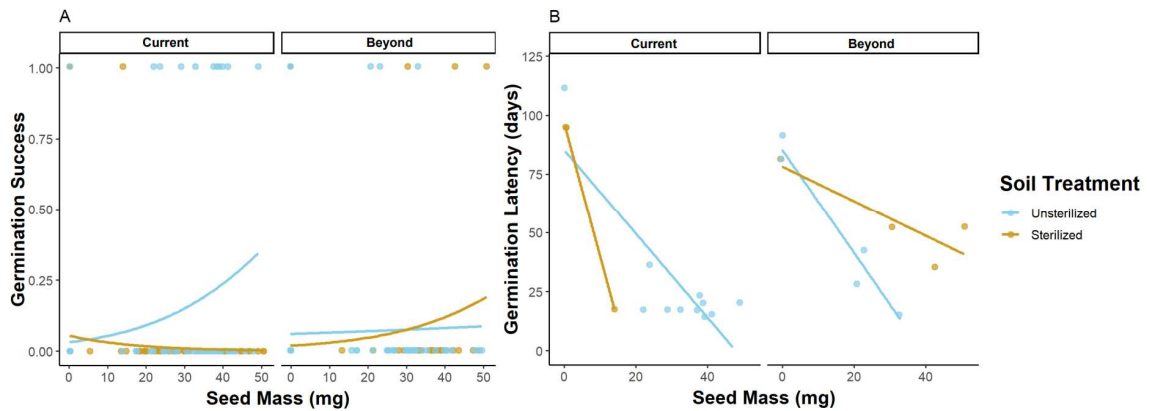
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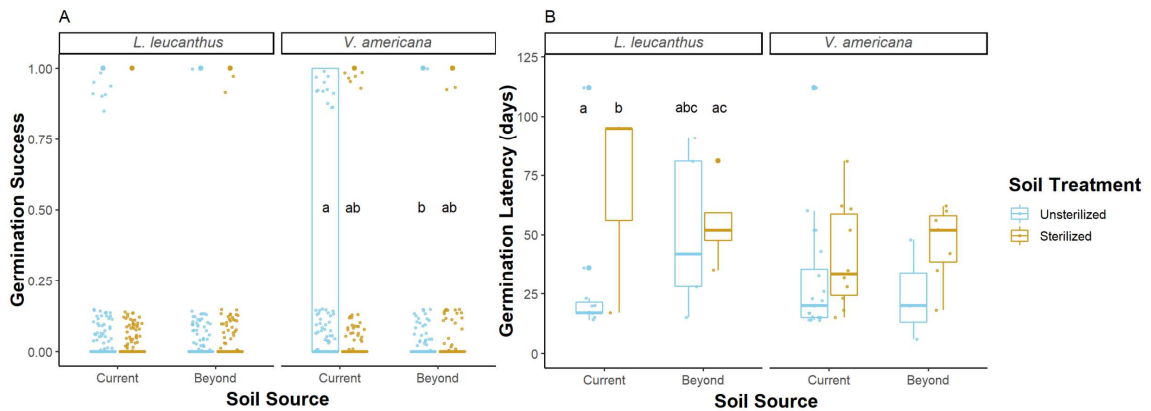
## Tables, Images, & Figures

Soil Type	Seed Count: <i>L. leucanthus</i>	Seed Count: <i>V. americana</i>
Unsterilized, Current Range	95	76
Unsterilized, Beyond the Current Range	78	59
Sterilized, Current Range	96	71
Sterilized, Beyond the Current Range	78	53
Unsterilized, Well-Watered	n/a	200
Unsterilized, Drought	n/a	200
Sterilized, Well-Watered	n/a	200
Sterilized, Drought	n/a	200

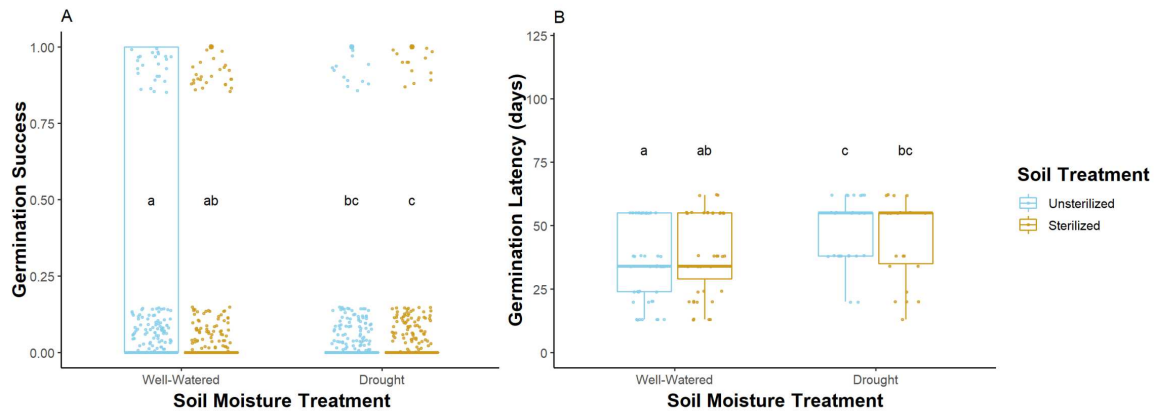
**Table 2.1.** Experimental design showing the number of individually potted seeds planted in each soil type.



**Figure 2.1.** Germination success (A) and germination latency (B) of *L. leucanthus* depended on the interaction between seed mass, soil treatment (unsterilized vs. sterilized), and soil origin (current = within the elevational range; beyond = beyond the current elevational range). In current range soils, heavier seeds were more likely to germinate in unsterilized soils than in sterilized soils (A, current). Beyond the current elevational range, greater seed mass increased germination success in sterilized soils (A, beyond). Greater seed mass advanced germination to varying degrees, depending on soil type (B). Lines represent binomial (for germination success) or linear (for germination latency) fits.



**Figure 2.2.** Germination success (A) and germination latency (B) for *L. leucanthus* and *V. americana* seeds by soil treatment (unsterilized vs. sterilized) and soil origin (current = within the elevational range; beyond = beyond the current elevational range). Microbial absence (sterilized soil) did not significantly alter *L. leucanthus* or *V. americana* germination success (A), but significantly delayed *L. leucanthus* germination within the current range (B; compare unsterilized, current vs. sterilized, current); this trend approached significance for *V. americana*. *Vicia americana* seeds planted in soils from beyond their elevational range had lower germination success than seeds planted in soils from within their current range (A; compare unsterilized, beyond vs. unsterilized, current). Points are jittered for clarity. Each bolded bar represents the mean germination value within the interquartile range.

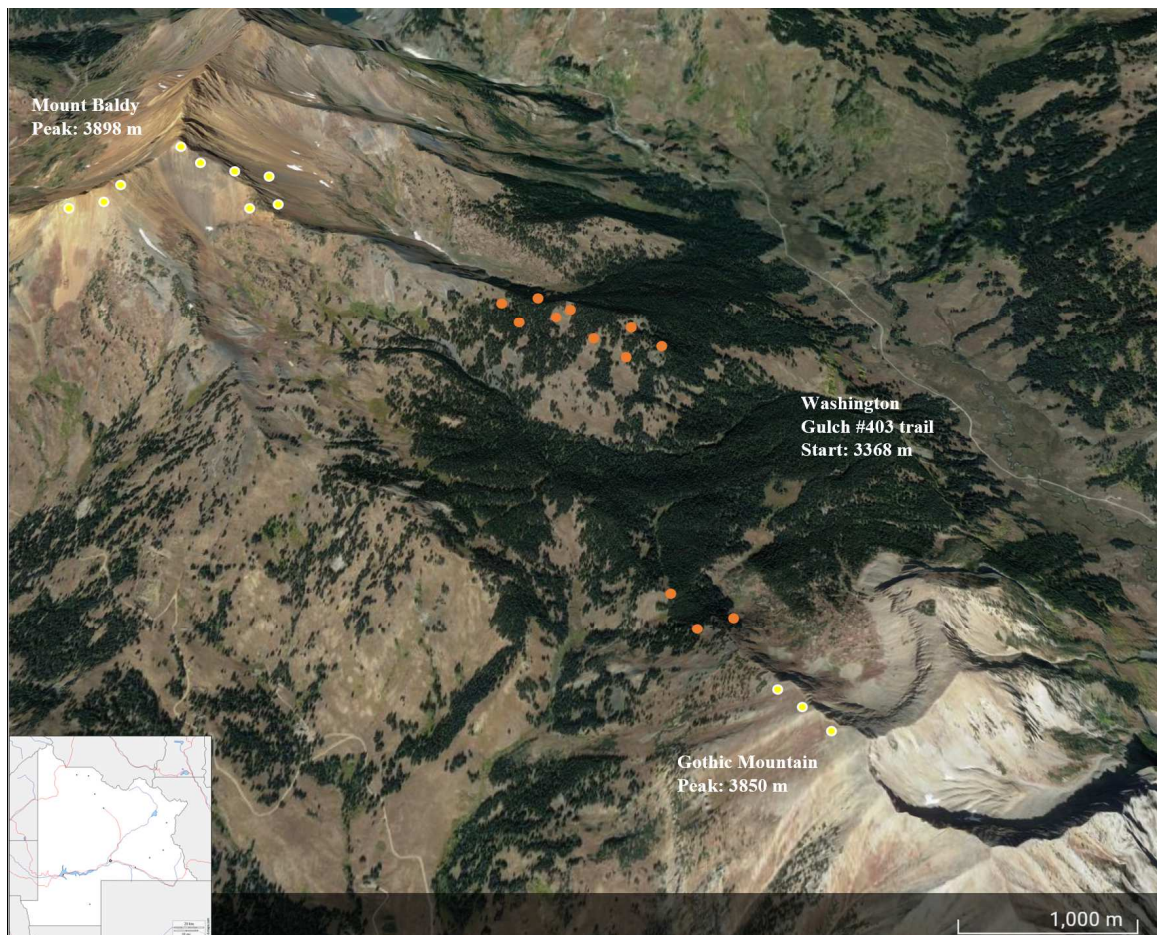


**Figure 2.3.** Germination success (A) and germination latency (B) for *V. americana* seeds by soil treatment (unsterilized vs. sterilized) and soil moisture treatment (well-watered vs. drought). Germination success was lower in unsterilized, dry soils than in unsterilized, well-watered soils (A). In dry soils, unsterilized or sterilized, seeds germinated later than those in unsterilized, well-watered soils (B). Germination success and timing did not differ significantly between seeds planted in dry conditions (unsterilized, drought vs. sterilized, drought) or well-watered conditions (unsterilized, well-watered vs. sterilized, well-watered). Points are jittered for clarity. Each bolded bar represents the mean germination value within the interquartile range.

**Image 2.1.** Root nodules found on plants grown in unsterilized, current range soils.

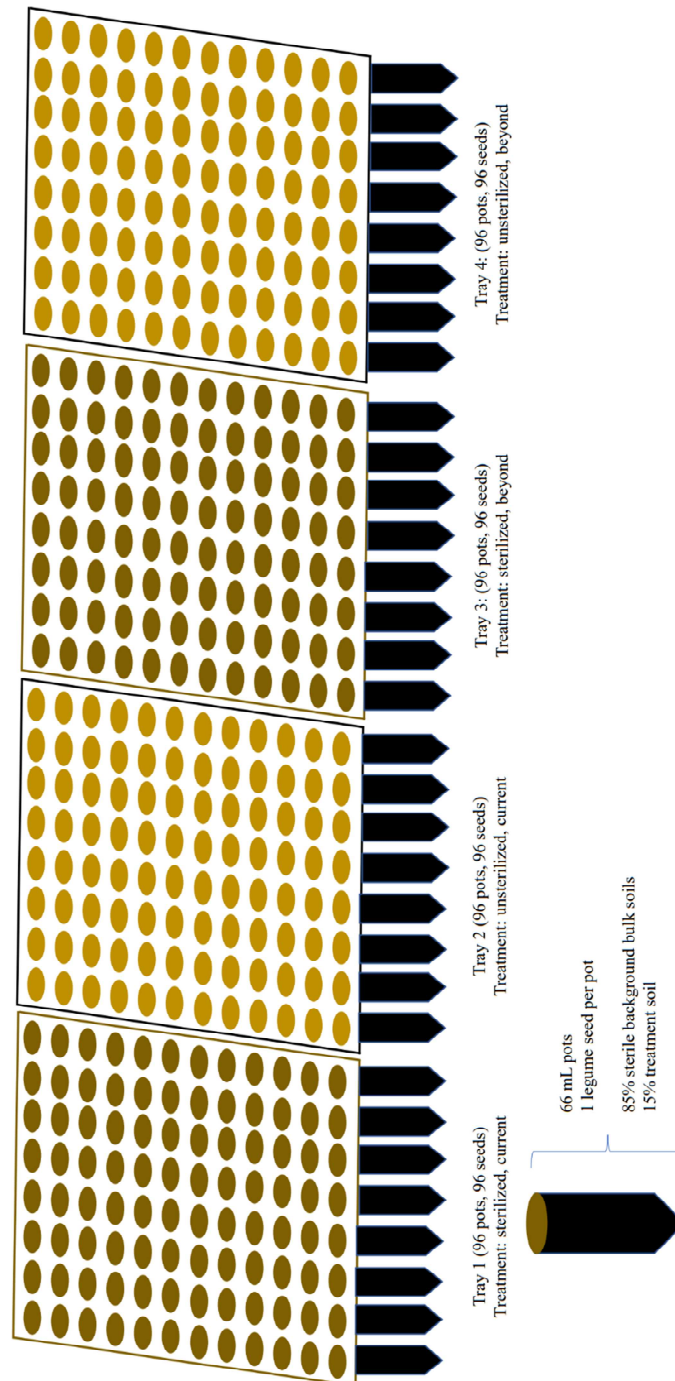


**Image 2.2.** Soil collection sites at RMBL in Gunnison County, CO. Yellow dots are approximate areas where soil was collected beyond the current range of the legumes on Mt. Baldy (upper left peak; 38.9926°N, 107.0462°W) and Gothic Mountain (lower right peak; 38.9564°N, 107.0106°W). Orange dots are approximate locations where soil was collected near *L. leucanthus* and *V. americana* patches.





**Image 2.3.** Common garden experimental design. Each tray contained a single treatment type to reduce movement of water and/or microbes between pots. There were 96 pots and 96 seeds per tray (one seed per pot). We show 4 example trays, but there were 15 total trays.



## Chapter 3

### Female *Osmia lignaria* prefer synthetic nectar enriched with amino acids

#### Abstract

Amino acids are critical nutritional components of the nectar of flowering plants and can influence pollinator foraging behavior. Many insect pollinators prefer nectar solutions containing specific amino acids, but the strength and sometimes the direction of preferences differ by sex and are often life history-dependent. Nectar amino acid composition and quantity can change over a flowering season, both within individual plants and within a flower. Plant stress caused by climate change can serve to alter nectar amino acids and consequently the feeding behavior of insect pollinators. Here, we used choice tests to examine the preferences of the solitary bee *Osmia lignaria* in relation to legume nectar amino acid presence or absence. Female *Osmia lignaria* preferred nectar solutions containing amino acids over those without, whereas no preference was detected in males. In addition, females consumed more nectar than males. These results demonstrate that female *O. lignaria* seek out and preferentially consume nectar containing amino acids, behaviors likely to affect fitness and offspring provision quality. Climate change-induced shifts in nectar amino acid composition and availability may lead to altered pollinator and plant fitness.

## Introduction

Solitary bees are important pollinators of both wild and agricultural plants (Batra, 1995; Bosch, Bosch and Kemp, 2002). The economic and ecological roles of solitary bees in pollination have received much interest in recent decades, due in part to the worldwide pollinator decline (Kearns, Inouye and Waser, 1998; Bosch, Bosch and Kemp, 2002; Bosch, Kemp and Trostle, 2006; Winfree *et al.*, 2007; Garibaldi *et al.*, 2013; Kline and Joshi, 2020). There are over 17,000 described bee species globally, many of which visit crops (Nabhan and Buchmann, no date; Michener, 2000; Klein *et al.*, 2007; Garibaldi *et al.*, 2013). As agriculturally-important honey bee populations decline due to factors such as parasitic mites and Colony Collapse Disorder (Stokstad, 2007), solitary bees may become increasingly important in croplands. Utilizing the ecosystem services of solitary bees in natural and agricultural lands will not only increase pollinator biodiversity but can ensure ecosystem functioning by buffering the negative effects of honey bee declines (Winfree *et al.*, 2007; Bartomeus *et al.*, 2013). Still, with global climate change, floral rewards can be altered, which may affect solitary bee feeding preferences, flower visitation rates, and plant and pollinator fitness (Takkis *et al.*, 2015; Brunet and Van Etten 2019; Powers *et al.* 2022). Thus, our understanding of the feeding preferences of model pollinator species forms an important resource for solitary bee conservation in our changing climate.

Insect pollinators, including solitary bees, make numerous foraging decisions, discriminating between flowering patches of variable reward quality (Watt, Hoch and Mills, 1974; Heinrich, 1976; Howell and Alarcón, 2007), and these foraging decisions can directly influence pollinator fitness (Behmer, 2009). Many studies have focused on the effects of varying nectar sugar concentrations and pollen quality on pollinator behavior, and a growing number of studies have determined the effects of nectar amino acids on pollinator behavior (Broadhead and Raguso, 2021; Dmitruk *et al.*, 2022; McCombs *et al.*, 2022).

Behavioral preferences for nectar amino acids have been documented in foraging animals across a variety of taxa including flies, ants, bees, and butterflies (Alm *et al.*, 1990; Rathman, Lanza and Wilson, 1990). Nitrogen is the core component of all amino acids, and insect reproductive success depends on the nitrogen content of larval provisions, among other things. For example, the oocytes of *Osmia californica* remained small when given nitrogen-poor rewards (Cane, 2016). In contrast, the oocytes of females with access to high nitrogen rewards reached full size following emergence, enabling them to lay eggs. Honey bees (*Apis mellifera*) and heath butterflies (*Coenonympha pamphilus*) consumed more high amino acid nectar than sugar-only nectar, indicating that amino acids contributed to pollinator attraction and feeding (Alm *et al.*, 1990; Cahenzli and Erhardt, 2013); in heath butterflies, consumption of high protein nectar led to increased reproductive success. In some cases, nectar amino acids interfered with the ability of bat pollinators to detect sugar concentrations (Rodríguez-Peña *et al.* 2013). Thus, the response to amino acids in floral nectars varies by pollinator taxon (Tiedge and Lohaus 2017; Roguz *et al.* 2019). While these studies have drawn links between nectar amino acids and pollinator feeding preference and fitness, in other studies, amino acids did not affect nectar preference (Broadhead and Raguso, 2021).

The composition and concentration of nitrogenous (N) compounds such as amino acids in nectar can vary within plant species, even changing within individuals in a growing season (Baker and Baker, 1973; Lüttge, Stichler and Ziegler, 1985; Gardener and Gillman, 2002; Petanidou *et al.*, 2006). Altered nectar amino acid composition can have immediate effects on flower visitation and can have consequences for pollinator fitness (Nepi *et al.* 2012; Scaven and Rafferty, 2013; Parachnowitsch, Manson and Sletvold, 2019). Variation in nectar quality can influence the activity, abundance, and diversity of pollinators (Potts *et al.*, 2004, 2006; Willmer, 2011; Junker *et al.*, 2013; Parachnowitsch and Manson, 2015; Hicks *et al.*, 2016; Egan *et al.*,

2018). Thus, changes in nectar N can cascade through trophic levels, potentially restructuring ecological communities. As nectar is an important resource for the daily energetics of foraging bees, and acquired N is a key determinant of reproduction in insects (Behmer and Joern, 1993), plant access to N may strongly mediate bee foraging behavior through changes in nectar amino acids. The influence of non-sugar nectar nutrients, such as amino acids, on insect pollinator foraging behavior has received relatively little attention, but this is a rapidly growing area of interest.

In this study, naive, unmated *Osmia lignaria* of both sexes were used to determine whether this solitary bee exhibits any preference for nectars containing concentrations of amino acids that reflect field-realistic concentrations found in the nectar of a leguminous wildflower that hosts nitrogen-fixing bacteria. With soil drying and warming, there may be a loss of the interaction between legumes and nitrogen-fixing bacteria, causing a decrease in nectar amino acid quantities, thus affecting pollinator preference. We hypothesized that bees would preferentially consume nectar containing amino acids due to innate preferences for higher-quality rewards. Similarly, we hypothesized that females, which are typically larger and provision nests with N-rich resources (Bosch and Vicens, 2006; Cane, 2016), would show a stronger preference for high quality, amino acid-rich rewards relative to males. Overall, we aim to address how nectar amino acids may alter solitary bee feeding preferences.

## **Methods**

### *Study species*

Blue orchard bees (*Osmia lignaria*; Hymenoptera: Megachilidae) were obtained from Mountain West Mason Bees (Riverton, UT, USA) for this study. *Osmia lignaria* are native to North America and are polylectic. In addition to being important pollinators for orchard crops, they also visit the flowers of wild *Cercis*, *Lathyrus*, and *Vicia* (Fabaceae). Indeed, Fabaceae floral

rewards are important for the maximal growth of larvae and adults as these rewards are extremely nutritious and nitrogen-rich (Levin and Haydak, 1957). Similar to other wild solitary bees, female *O. lignaria* collect large amounts of pollen and nectar for the provisioning of their offspring. Males do not participate in nest construction and provisioning; they visit flowers to collect rewards only for their own consumption. *Osmia* species are able to identify the presence of floral nectars in the wild, though the specific cues are unknown (Howell and Alarcón, 2007).

*Osmia lignaria* used in behavioral trials were emerged from cocoons. Cocoons were sorted by sex and weighed to the nearest 0.001 g (Figure S3.3). Cocoons were stored at 4°C and 70% relative humidity prior to emergence, then incubated in individual plastic cups at room temperature (~25°C) in April 2022. Bees typically emerged within 24 h of being incubated. Bees of both sexes remained unmated for the duration of the experiment.

#### *Artificial nectar solutions*

Nectar was synthetically developed based on the amino acid composition and sugar concentrations of the legume *Vicia americana* (Chalcoff, Aizen and Galetto, 2006; McAulay, 2018; McAulay, Killingsworth and Forrest, 2021). Artificial nectar solutions were created using pure standards of equal parts arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, and valine, with non-essential amino acids alanine, cystine, glycine, proline, serine, and tyrosine (Sigma-Aldrich). For bees, the ideal composition of essential amino acids in rewards is roughly equal parts arginine, isoleucine, leucine, lysine, phenylalanine, threonine, and valine, though histidine and methionine are also important (de Groot, 1953). The standardized sugar content of our nectar was 20% sucrose (m/m); though *V. americana* nectar contains sucrose, fructose, and glucose, only sucrose content was measured in the field. The 20% sucrose value is based on the average sugar concentration of *V. americana* nectar in the Gothic, CO area. The amino acid + sugar nectar solution was made from 2.5  $\mu$ moles of amino acid

solution per mL of 50  $\mu$ L sucrose solution and contained all essential amino acids (de Groot, 1953; McAulay, 2018). The sugar-only nectar solution was made of 50  $\mu$ L sucrose and water. Sugar concentrations were held constant across treatments. The nectar solutions were dyed two different colors (blue or red) with a non-toxic food dye and placed into microcentrifuge tubes. The dye color to which each type of nectar solution was assigned was switched weekly to control for any effect of color on feeding preference, though a pilot choice test demonstrated no color preference by bees.

#### *Experimental design and setup*

A dual-choice feeding test was used to determine the preference of bees for artificial nectar containing amino acids + sugar or sugar only (Image 3.1). Inverted, clear plastic 60 ml cups with the two nectar solutions were presented (Wilson Rankin *et al.*, 2020). A small (2 mm) feeding hole was made at the bottom of each microcentrifuge tube. Tubes were weighed before and after the addition of solutions to the nearest 0.001 g. Two microcentrifuge tubes were installed through opposite sides of each plastic cup so that both the amino acid + sugar and the sugar-only solutions were equally accessible. The addition of amino acids does not affect nectar viscosity significantly (Heyneman, 1983). Still, we measured the evaporation constants of our nectar solutions by measuring evaporation for 30 dual-choice test cups that were not exposed to bees. There was no difference in the rate of evaporation between nectar solution types (amino acid + sugar vs. sugar-only;  $t_{59} = 0.05$ ,  $p < 0.47$ ).

Naive, newly emerged adults were used in choice tests (1 bee per cup). In total, we weighed 163 female cocoons and 125 male cocoons. After the 24-hour emergence period, 42.3% of females emerged while 20.0% of males emerged (Figure S3.3); unemerged bees were determined to be deceased. The dual-choice cups were adhered to a 7 or 10 cm filter paper (Whatman), then were placed on white paper and exposed to natural photoperiod. After being

placed into individual dual-choice cups, bees were allowed to forage for 24 h at 20°C. After 24 h elapsed, filter papers were removed and scanned, the nectar tubes were weighed to the nearest 0.001 g (empty tubes and tubes filled with nectar were pre-weighed), and the total area of dyed fecal exudates on the filter paper was quantified to the nearest 0.001 cm<sup>2</sup> using ImageJ (Rasband 2018; Image 3.2).

### *Data analysis*

To test whether bees exhibited preferences for either nectar solution type (amino acid + sugar vs. sugar-only) we constructed a linear model. We calculated the proportion of the dyed fecal area that corresponded to the color for the amino acid + sugar nectar solution by dividing the area of each dyed fecal area by the total dyed fecal area for both nectar solutions. For the purposes of calculating these proportions, we excluded any dyed fecal area that corresponded to a mixture of the two colors as we could not differentiate between feces originating from red vs. blue dye when mixed together. We also excluded any bees that did not feed on nectar during the 24-hour trial period. We applied a log + 1 transformation to the proportion data to achieve a normal distribution and included sex and cocoon mass as predictors.

To test whether there was a relationship between total amount defecated and bee sex, we constructed a linear model with the total area of filter paper covered by dyed feces (of any color) as the response; we included only data for bees that defecated during the trial period. To this model, we added sex and cocoon mass as predictors.

To understand whether there was a relationship between total amount consumed and bee sex, we used a linear model with the solution consumed (of any color) as the response variable and bee sex as a factor; we included data for all bees. To this model, we added sex and cocoon mass as predictors. All analyses were conducted in R version 4.0.2 and the package *lme4* was used to run linear models (Bates *et al.* 2015; R Core Team 2019).



## Results

Based on amounts defecated, female bees preferentially consumed the amino acid + sugar nectar solution (LM:  $0.74 \pm 0.19$ ,  $t_{40} = 3.98$ ,  $p < 0.003$ ; Figure 3.1), whereas males did not prefer one nectar type over the other, although they tended to also prefer the amino acid + sugar solution (LM:  $-0.23 \pm 0.12$ ,  $t_{40} = -1.92$ ,  $p < 0.062$ ; Figure 3.1). After accounting for sex differences, there was no significant effect of cocoon mass on preference (LM:  $-4.19 \pm 2.61$ ,  $t_{42} = -1.60$ ,  $p < 0.11$ ).

As indicated by total area covered by dyed feces, females consumed more of the nectar solutions than males (LM:  $1.18 \pm 0.14$ ,  $t_{42} = 8.29$ ,  $p < 0.0001$ ; Figure 3.2A). On average, feces from females covered  $0.56 \pm 0.09$  cm<sup>2</sup> of the filter papers while feces from males covered  $0.18 \pm 0.09$  cm<sup>2</sup> of the filter papers. As in the preference model, after accounting for sex differences (Figure S3.3), there was no significant effect of cocoon mass on total amount defecated.

Overall, females consumed more of the nectar solutions than males based on the amount of liquid displaced over the 24-hour feeding period (LM:  $0.01 \pm 0.004$ ,  $t_{284} = 4.50$ ,  $p < 0.00001$  Figure 3.2B). Females consumed an average of  $0.041 \pm 0.004$  g of nectar solution while males ingested  $0.001 \pm 0.003$  g of solution. There was no significant effect of cocoon mass on total nectar solution consumed.

## Discussion

Here, we explored the feeding preferences of naive solitary bees in the context of decreased floral reward quality. In this study, we found that female *O. lignaria* preferred the nectar solutions containing both sugar and amino acids over a sugar-only solution. Females tended to consume both nectar types, then gravitate towards consuming the amino acid-containing nectar (Figure 3.1). Interestingly, males of the same species did not prefer one nectar type over the other, though they generally leaned towards consuming the solution containing

amino acids. Males typically consumed one nectar type or the other, and rarely consumed both types within a feeding trial. These results indicate that females are choosier than males regarding the amino acids in nectar.

Not only did female *O. lignaria* prefer nectar solutions containing amino acids, female solitary bees consumed more of both types than males did, likely because nectar consumption is positively related to their provision size and quality, and thus their reproductive success (Goodell, 2003; Splitt, Schulz and Skórka, 2022). This was true both in terms of nectar solution displaced from the dual-choice arenas and the area of fecal matter measured. As female *Osmia* provision their brood cells with nectar, pollen, salivary exudates, and other materials, female foraging decisions directly affect the survival and development of their offspring. Additionally, there is isotopic evidence for essential amino acid transfer from floral rewards to pollinator eggs, meaning that female feeding preferences directly affect the quality of the next generation (O'Brien, Boggs and Fogel, 2003). Beyond provisions, nectar amino acid consumption during the adult phase has implications for basic energy requirements for maintenance and flight (Willers, Schneider and Ramaswamy, 1987; Levin *et al.*, 2017). Thus, early and regular feeding upon protein-rich rewards can increase some pollinators' lifetime reproductive output and overall fitness.

In this study, we used realistic, field-based quantities of amino acids to create our nectar solutions. As climate change can alter the scent and taste of nectar (Howell and Alarcón, 2007; Gallagher and Campbell, 2017; Phillips *et al.*, 2018; Russell and McFrederick, 2021; McCombs *et al.*, 2022), resource quality shifts may affect bee pollinator foraging decisions. Selective visitation by pollinators can affect the fitness of both the pollinators and the insect-pollinated plants involved in these interactions (Thomson, 1988; Kudo and Cooper, 2019). There is evidence that some pollinators respond to single amino acid solutions or to random blends of amino acids, but responses vary significantly (Blüthgen and Fiedler, 2004; Broadhead and Raguso, 2021).

Though it would be beneficial to test if bees prefer nectar produced by plants affected by varying levels of global climate change in a natural system, our preference test demonstrates that *Osmia lignaria* can discriminate between synthetic nectar solutions on the basis of their amino acid content.

More broadly, climate change is altering interactions between legumes and nitrogen-fixing bacteria, which will alter amino acid quantities in nectar (Southwick, 1984; Pyke, 1991; Rusterholz and Erhardt, 1998). Despite the importance of the mutualism between legumes and N-fixing bacteria, there are few studies on the effects of N-fixing bacteria on plant-pollinator interactions (Keeler, Rose-Person and Rafferty, 2021). As many pollinators feed on these floral rewards, a decrease in nectar amino acid reward quality could negatively affect feeding behavior and bee fitness. A decrease in floral reward quality or availability with climate change could alter *Osmia* reproductive output by increasing time and energy allocated to foraging; if flowers provide lower quality rewards, more time and energy will be required to provision a brood cell (Gauthier, 2019). Population decreases due to smaller, lower-quality provisions could reduce pollination success of crops and natural plant communities. As plant and bee reproductive success is directly affected by their interaction, decreased feeding preference on wild or agricultural legumes due to low reward quality could reduce legume and pollinator fitness.

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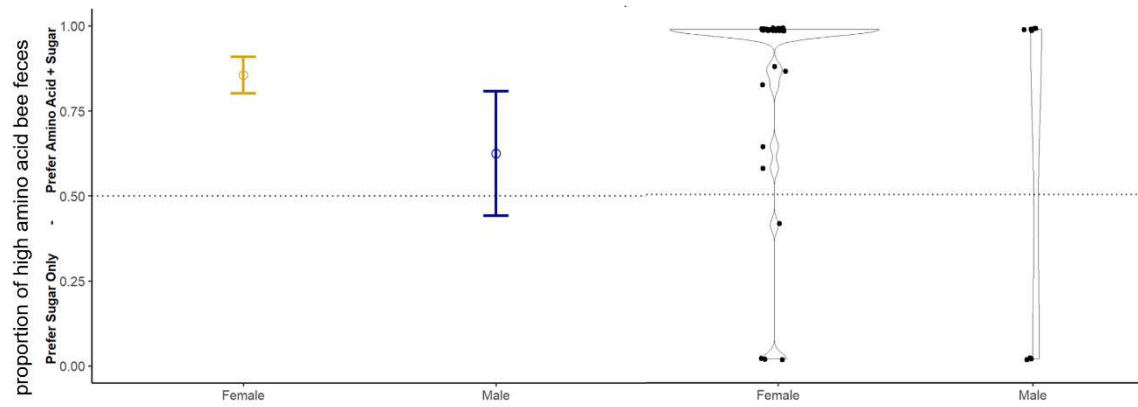
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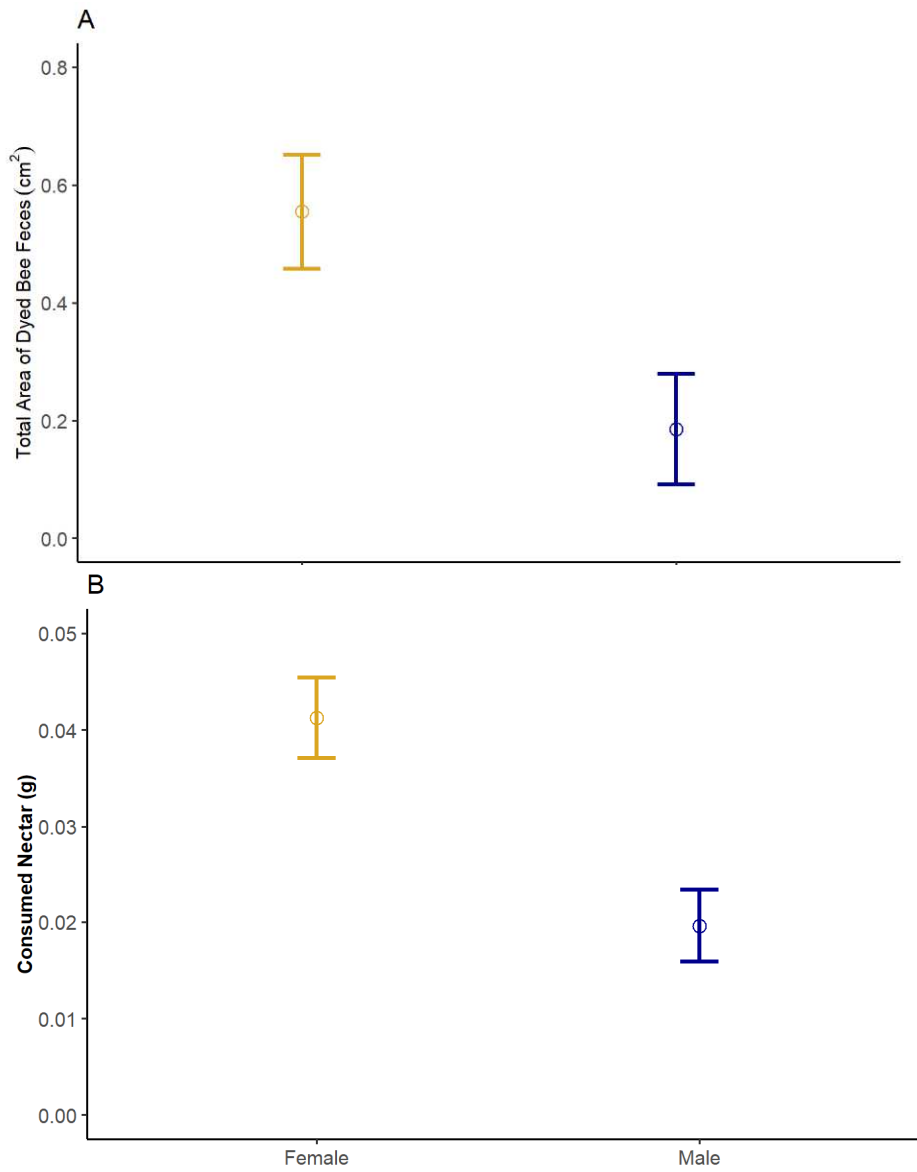
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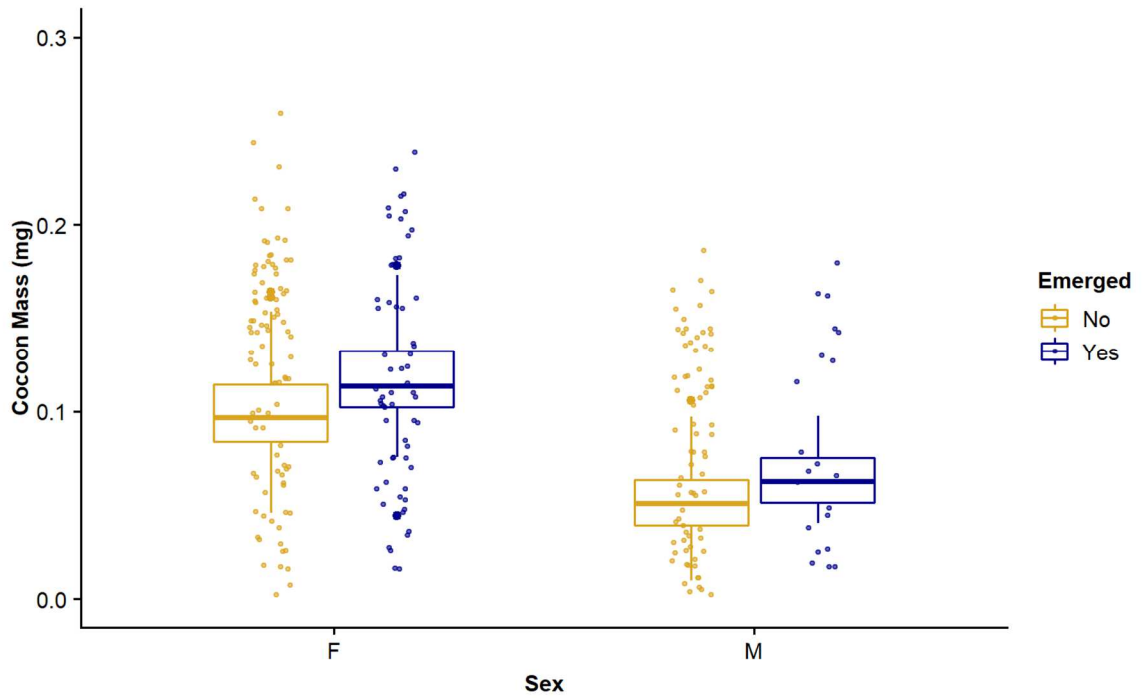
## Figures & Images



**Figure 3.1.** Nectar preferences of female and male *O. lignaria*. Data above the dotted line show a preference for high quality nectar while data below the dotted line indicate preference for nectar free from amino acids. Females preferred higher quality nectar containing amino acids while males showed slight, but insignificant preference for nectar solutions containing amino acids. Circles represent the mean  $\pm$  SE (left) and raw data are shown (right).



**Figure 3.2.** Total fecal matter area (A) and consumed nectar (B) of female and male *O. lignaria*. Males are represented by blue lines while females are shown in gold. Female bees defecated (A) and consumed (B) more dyed nectar than males. Circles represent the mean +/- SE.

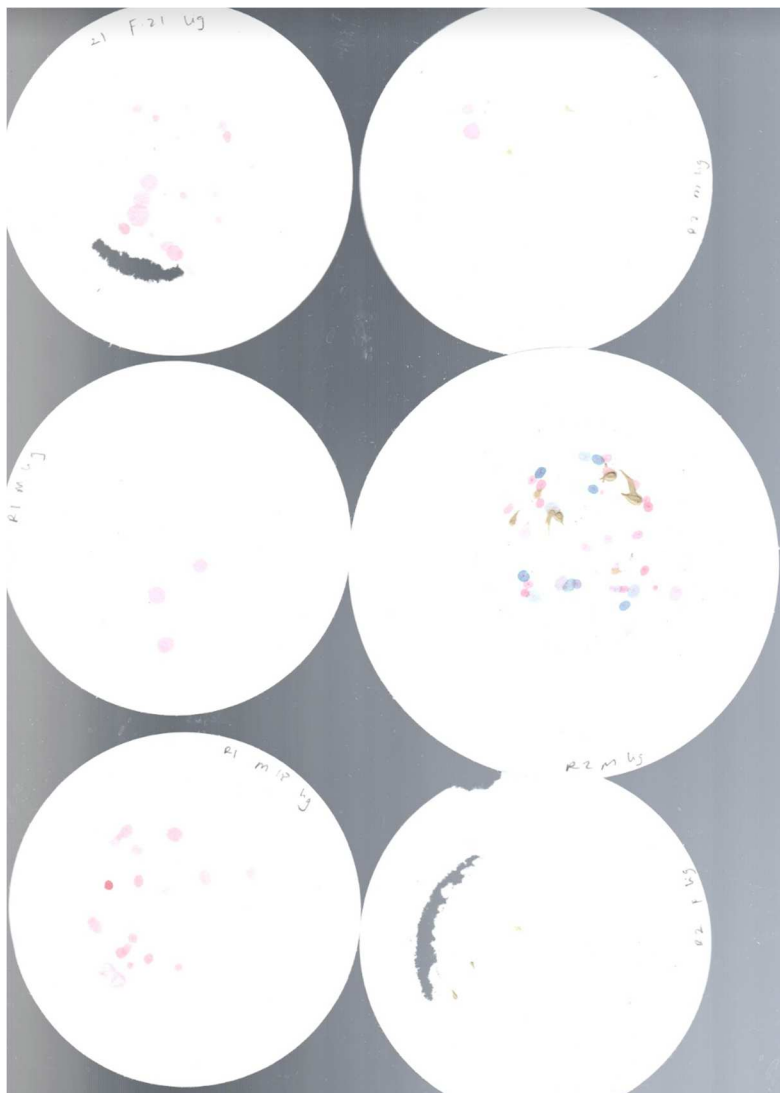


**Figure S3.3.** Emergence success of female and male *O. lignaria*. Golden bars indicate the mass of cocoons that did not show emergence while blue bars show the mass of successfully emerged adult bees. Females were marginally more likely to emerge than males (LM:  $-0.21 \pm 0.12$ ,  $t_{286} = -1.68$ ,  $p < 0.09$ ), and heavier cocoons were more likely to contain living adult bees than lighter cocoons. Points are jittered for clarity. Each bolded bar represents the mean mass within the interquartile range.

**Image 3.1.** *Osmia lignaria* male probing for nectar in a dual choice test. April 2022.



**Image 3.2.** *Osmia lignaria* dyed fecal matter scanned and uploaded to ImageJ for quantification.



## Synthesis

Mutualism loss among formerly interacting partners, even temporarily, can have direct negative effects on the species involved and indirect effects on other mutualists. Using multi-year in situ research plots at the Rocky Mountain Biological Lab (CO, USA) which experimentally advance snowmelt timing and historical legume leaf samples, we were able to assess how increased drought duration caused by increasing temperatures and early snowmelt affect the legume-rhizobia mutualism. We found increased legume nectar sugar concentration, instability in the legume-rhizobia mutualism shortly after snowmelt, and decreased legume water use efficiency within a summer, plus decreased water use efficiency and fewer interactions between legumes and rhizobia over the last 90 years (Chapter 1). Early snowmelt and warmer temperatures may contribute to seasonal losses in the legume-rhizobia mutualism. Based on these findings, we assessed the effects of mutualism loss on legume germination traits using a controlled common garden approach. We found evidence that soil sterilization and dry soils caused species-specific phenological delays in germination (Chapter 2). Additionally, we found evidence of the presence of beneficial microbes beyond the current elevational range of one of our focal legumes. Changes in germination timing could have severe consequences for the fitness and future range expansions of these legumes and for the biodiversity of the local community. Finally, floral functional traits, such as nectar sugar concentration, changed with a mutualism loss between legumes and rhizobia (Chapter 1). These changes can go on to affect pollinator feeding behavior and both pollinator and plant fitness. We observed that female solitary *Osmia lignaria* bees preferred high-quality nectar containing amino acids, while males showed no preference for nectar type (Chapter 3). Mutualism loss is likely to become more common with anthropogenic global climate change, and the research outlined in this dissertation advances our understanding of the effects of interaction loss, particularly in subalpine systems.