

Effects of Plant Diversity on Selection for Insect Resistance Traits in *Boechera stricta*

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Abstract

Evolution by natural selection is influenced by an organism's environment. Biodiversity, an aspect of the biotic environment, is rapidly changing due to anthropogenic activity, so its impacts on selection must be better understood. In this study, the effects of diversity on selection for an insect resistance gene and trait were studied in *Boechera stricta*. In naturally-occurring *B. stricta*, abundances of morphospecies in 1x1-meter plots containing the focal species were recorded. Damage and fitness data were also collected. Fitness was shown to decrease significantly across damage, confirming the theoretical basis of the study and allowing damage to proxy fitness, though this was not the case in a model that considered phenological stage as a covariate. Richness was shown to predict damage with marginal significance ($p = 0.0614$), but a Shannon-Weaver Index and Simpson Index analysis yielded no meaningful results. A principal components analysis (PCA) allowed researchers to identify species that correlated most strongly with the third PCA axis, which significantly predicted damage. 3 of those top 5 species abundances significantly correlated positively with damage directly, without the PCA axis as an intermediate. These results show the importance of community composition, which accounts for species identity, in insect damage. It also points to the importance of low-density, low-abundance species, which may not create a strong signal in indices like Simpson, but which affect richness more strongly, and some of which were shown to influence damage through the PCA. Further study is certainly necessary to understand which sorts of species might be most important in influencing damage and the extent of their potential influence.

Introduction

In recent history, anthropogenic activities have accelerated changes in biodiversity by means such as global climate change, land use conversion, and invasive plant introduction. These shifts might have evolutionary consequences; as such, it is becoming increasingly important to understand the effects of diversity on the evolution of organisms within changing ecosystems. This will require greater study of the effects of community heterogeneity on the selective pressures organisms face. An integrative view of evolution and diversity must be maintained and further developed to understand the complexities of the large-scale transitions taking place in Earth's biota.

In plants, species diversity has long been recorded to facilitate interspecific ecological interactions within a community (Isbell et al. 2009). Some of those ecological interactions can have evolutionary consequences. For example, a greater variety of species increases the opportunity for a plant of one species to interface with another heterospecific plant, which can select for traits that allow the focal plant to survive in that interaction. In a more indirect sense, all organisms modify their environments to a certain extent (Matthews et al. 2014), so greater diversity implies more and different ways in which the environment is modified, which can then go on to influence selective pressures. In addition, higher community heterogeneity may favor a plant's interspecific competitive ability over intraspecific competition, which could change the functions in which a plant invests more energy (Utsumi et al. 2013).

Ecological processes like herbivory that necessarily involve interspecific interactions can act as important drivers of evolutionary change in a species. Indeed, plant defense is a model in evolutionary ecology because of its community-level implications beyond the focal species (Agrawal 2011). Because plant defense is an important function in maintaining fitness and survival, defense can face strong selective pressure. Selection can theoretically be affected by increased plant diversity. With greater community diversity, direct interspecific plant-plant interactions including competition and facilitation

increase proportionally. Plant diversity can also indirectly affect herbivore-mediated selection, as the species of plants that are present within a community might affect which associated herbivores are present. Stated succinctly, diversity can affect the ecological process of herbivory, which can then influence selection for plant defense traits.

While plant diversity is an important aspect of the biotic environment that can affect ecological processes, reported diversity effects on herbivory have been mixed. Some propose a positive relationship between plant richness and herbivory due to associational susceptibility. In this framework, diversity increases a focal plant's vulnerability to herbivory based on different plants around it that draw herbivores (Haddad et al 2001). Other studies with similar results have cited increased the attraction of increased resource diversity as a putative explanation for a positive diversity-herbivory relationship (Crutsinger et al. 2006). This mechanism of diversity increasing herbivory is called associational susceptibility. Its converse, associational resistance, has also been recorded, however. Andow has theorized that higher plant diversity would undermine herbivory due to changing resource concentrations; fewer specialist attacks would take place due to decreased density of the species on which it feeds (1991). Unsicker et al. experimentally found that relationship (2006), which they also explained through specialist enemy release. These mixed results indicate that the relationship between plants' environments and natural selection by herbivores is context-dependent due, perhaps, to variation in the specificity of insect-plant herbivory and to the more general complexity of natural habitats. As Ellner puts it, "the devil is in the details" (2013). Thus, the importance of heterospecific plant communities on plant defense evolution remains unclear.

The local diversity-plant defense relationship has received some study in mustards, the system to be used in this proposed study. In an experiment on *Brassica nigra*, Lankau and Strauss studied concentrations of sinigrin, a plant defense chemical, and found that species diversity depends on genetic variation in the extent of production (2007). Without genetic variation, either the highest sinigrin-

producing genotype of focal *B. nigra* or another species would dominate. They also found that genetic variation depends on species diversity because selection would fix plant defense traits at its lowest levels if not for competing species.

Given the significance of plant defense in eco-evolutionary study, *Brassicaceae* provides an ideal study system. The family's members produce secondary metabolic compounds called glucosinolates that deter herbivory (Koenig and Weigel 2015). Glucosinolates have a genetic basis and a clear chemical phenotype, facilitating eco-evolutionary study of insect resistance within the family. Within *Brassicaceae*, I will study the mustard *Boechera stricta*, native to my location in Colorado. The process of glucosinolate production is highly involved, but an advantage of studying *B. stricta* is that the ratios of the type of glucosinolates, which are produced from different amino acids, maps to a single set of quantitative trait loci, Branched Chain Methionine Allocation (*BCMA*) (Koenig and Weigel 2015). This makes studying selection effects on variation in defense genetically tractable, as complex phenotype differences in plant defense chemicals can be associated with their underlying gene.

The effects of a species' plant defense phenotype on the local diversity have been studied in mustards (Lankau and Strauss 2007), but the converse – the consequence of surrounding plant communities on the success of a plant's defense – has received little investigation. In a formal meta-analysis, for example, Cardinale et al. could not find sufficient data on the relationship between herbivory and primary producer richness to support a hypothesis (2011). They found just 10 terrestrial experiments that attempted to quantify the way plant diversity modifies herbivore impacts on producer biomass based on producer fitness. This suggests the need for more research on the relationship between an individual's surrounding diversity and its experienced herbivory.

In this study, I confirmed the complexity of community-level interactions like plant diversity, insect herbivory, and selection by the environment. I was able to begin to parse out important determinants of the impact aspects of diversity have on selection through studying insect resistance

traits. I surveyed sites with both manipulative and observational plots to ask about the effects of surrounding plant communities and their diversity on selection of plant defense. By studying endogenous populations of *B. stricta* across several sites, I was able to ask the following questions:

1A. Do naturally-occurring *B. stricta* plants experience different selective pressures on insect resistance depending on neighboring plant diversity?

1B. Is species diversity *per se* or species composition a stronger influence on the selective pressures plants face on insect resistance?

I combined my observational work of naturally occurring *B. stricta* with a manipulative study of experimental arrays that vary in allele frequencies for the BCMA gene. Specifically, I was interested in the following questions that informed my data collection:

2A. Does neighboring plant diversity influence herbivory?

2B. Does the BCMA allele favored by natural selection change depending on the context of the surrounding plant community? Or does the extent to which one allele is favored depend on that context?

Methods

Study Species

Boechera is at the forefront of natural experimentation because wild populations can be studied, and it is related to the heavily researched *Arabidopsis thaliana* (Koenig/Weigel 2015). Its native range includes the Rocky Mountains of Colorado. Its small genome, self-fertilization, and short lifespan make it ideal as a model species, and there are thus many resources available regarding its genetics.

[IMAGE 1]

All mustards produce glucosinolates – amino acid-derived secondary metabolites that are meant to deter herbivory. The polymorphic *BCMA* gene in *B. stricta* is either derived from methionine or

derived from the branched-chain amino acids valine or isoleucine (Koenig and Weigel 2015). Genotypic variation in the glucosinolate produced has been shown in mustards to determine insect herbivore consumption of plants (Bidart-Bouzat and Kliebenstein 2008), which speaks to the value of studying the *BCMA* gene and its associated phenotype in the context of herbivory.

Experimental plants were put in the ground as part of another concurrent experiment in the Mitchell-Olds Lab. These known genotypes on near-isogenic lines, bred to be nearly genetically identical except for the gene of interest. This allows testing for selection effects on a single gene and its accompanying trait (Rushworth et al. 2011). They were planted by direct seeding into a mix of 75% Fafard 4P Mix and 25% Sunshine MetroMix 350 in the Duke University greenhouse (Durham, NC). All plants were put into 98-cell cone-tainer racks. Racks were misted under plastic domes for the first week of life and subsequently bottom-watered daily. When shipped to Colorado, they were about twelve weeks old. The plants are arranged in a randomized complete block design with different genotypes spatially randomized across each rack. In the field, whole racks were transplanted directly into the ground by digging a 1'x2'x6" hole. The rack was placed into it, level with the ground, to allow it access by local insect communities. Plants were bottom-watered several times weekly and supplemented with top-watering in times of drought stress. The plants were not vernalized and thus fitness data from fruiting bodies could not be collected.

Experimental Design

The experiment took place across six sites throughout the Gothic Valley, termed "Upper Avery," "Lower Avery", "Ross's Meadow," "Maxfield Meadow", "Kid's Camp", and "401 Trail" (See "MAPS").

Within each site, ten experimental plots of 98 plants each were planted as full racks, for a total of 5,880 plants across all sites. They were protected from mammalian herbivory by wire cages. For diversity assessments, I identified a one meter-squared quadrat in which the rack of experimental plants

is centered. I assessed abundances of plant morphospecies at the end of the experiment immediately prior to retrieving the racks to the lab. I scored each identified species for percentage vegetation cover, as it can often be difficult to distinguish true individuals from aboveground stalks sharing a root system. Percent cover was measured using ocular estimation in five-percent increments. For any plants that occupied less than five percent of a quadrat, they were denoted as “minor species” and a total percent coverage value was assigned to the minor species as a whole. That coverage value was divided by the number of minor species, and each minor species was finally assigned the resulting percent coverage. For example, if two minor species were identified and minor species occupied five percent of the ground in a quadrat, each species would be assigned 2.5 percent ground coverage. This was seen as the least biased method of evaluating ground coverage due to a lack of confidence in estimating percentages under five percent. It was also seen as the most quantitative solution. In the lab, racks were assessed for height, number of leaves, number of damaged leaves, and average percent damage of damaged leaves only by ocular estimation in 5% increments. In between disinterring and censusing, racks were placed in a screenhouse to prevent further herbivory.

In a recent review on eco-evolutionary feedbacks, Sharon Strauss recommended multiple approaches to studying communities, including field experiments that manipulate both community and genotypic diversity and observational data to complement experimental approaches (2014). Considering this, five to ten 1x1-meter natural quadrats were also laid out where abundant *B. stricta* – usually five plants minimum – had been observed. Across the six sites, I observed a total of 37 quadrats and 251 naturally-occurring *B. stricta*. I surveyed each quadrat once weekly over the course of three weeks. At the quadrat level, I first visually identified every vascular plant morphospecies and collected voucher specimens for the plants I identified with less than 100% certainty. I estimated percent ground cover using the same method described above for diversity data collection surrounding the experimental plots. At the plant level, I scored the *B. stricta* for herbivory using an ocular estimation of percent leaf

area removed in five-percent increments. Data on potential covariates such as plant size and developmental stage was collected. Survival data was also taken for the *B. stricta* in all plots. For fitness measures, I surveyed each plant for the number of fruits, average fruit length, and visible fruit damage of the *B. stricta*.

Several different measures of diversity that indicate slightly different types of species composition were used to avoid *a priori* exclusions of certain aspects of community heterogeneity. Richness, a total count of species considering each species the same; Shannon-Weaver Index, an index considering both richness and evenness; and Simpson Index, a similarity index weighting dominant species, were all used. In addition, the percentage of each quadrat that was measured as bare ground was used as the converse of vegetation cover with the hypothesis that more bare ground would lower herbivory on *B. stricta*.

[FIGURE 1]

Data Analysis

From raw data at the quadrats of naturally-occurring *B. stricta*, leaf area removed (LAR), as a measure of herbivory damage, was calculated as the fraction of leaves that were damaged multiplied by the percent damage.

$$LAR = \left(\frac{n_{damaged}}{n_{total}} \right) * \%_{damaged}$$

Fitness was calculated as the number of fruits (f) times the average fruit length, and damage was included in that fitness calculation by subtracting fruit area damaged. This was done through multiplying the number of damaged fruits times the percent damaged fruits and subtracting this from the total number of fruits before multiplying by average fruit length.

$$\omega = f * length_{fruits} - f_{damaged} * length_{fruits} * \%_{damaged}$$

Further analyses used the log-transformed versions of those values. Several diversity indices including richness, Shannon-Weaver Index, and Simpson Index were calculated using R and Excel 2013. Whittaker's Beta Diversity will also be assessed going forward. As fitness and damage were calculated at the plant level and diversity indices were calculated at the quadrat level, mean LAR and fitness were calculated per quadrat through R version 3.3.1.

All statistical analyses were done in JMP 11 or 12 using mixed-effects regression models unless otherwise stated. A principal components analysis was also calculated through JMP 11. I found an axis of variation in my data through a principal components analysis that significantly predicted data. After finding species whose abundances correlated most strongly with that axis, I was able to test the effect of those individual species' abundances on damage, as well. In general, I tested for relationships between various measures of diversity as predictors of damage. I calculated selection gradients, the way in which fitness changes across a phenotype – in this case, damage. However, I was not able to show any significant quadrat-level coefficients, as I was limited by the sparse *B. stricta* plants per quadrat. For this reason, I used damage as my proxy for fitness. For the sake of this summer, I measured natural selection on the composite trait of insect damage, which is influenced by plant chemistry along with other plant properties. Using this inferred phenotype, I assessed diversity effects on plant defense traits. Later on, once the true chemical phenotype of the *BCMA* gene is known in the natural *B. stricta*, I will also be able to calculate the effect of diversity on the selection gradient for BC ratio.

I plan to do similar analyses for the quadrats containing experimental *B. stricta*. I have not yet run these analyses, as the lab finished censusing damage data as of 5 August 2016. Within the genotypes used in this experiment, the lab has observed a consistent negative correlation between damage and fitness. For experimental plots, I will determine selection coefficients between the two *BCMA3* alleles by assessing variation in damage across alleles. Selection coefficients show the way in which fitness changes across genotypes, analogous to selection gradients for phenotypic traits. The

change in fitness, ability to survive and reproduce, across genetic variation in the population graphically represents this difference. In this case, the selection differential is the difference in fitness, measured through insect damage, between *BCMA* alleles. Since those plants did not flower this summer, fitness is determined through survival and insect resistance. As the genetic information is known for these plants, the effect of surrounding plant diversity on the selection differential for the plants by genotype can be calculated. I plan to assess whether my measures of diversity in the experimental plots – including, again, richness *per se*, Shannon-Weaver Index, Simpson Index, and Whittaker's Beta Diversity – can predict the selection coefficients. In addition, through another principal components analysis, I want to try and relate individual species abundances directly to damage.

Results

All results currently presented are for quadrats with naturally-occurring *B. stricta* only. Results from experimental plots are currently being analyzed. Results shown here are from the second of three rounds of surveying. The earliest survey was determined to have not captured peak damage and by the third survey, many plants had zero live, green leaves so there were fewer data points.

Fitness and damage were significantly correlated when accounting for site as a random effect ($p = 0.003^*$, $R^2 = 0.113$). Models were also run including elevation, but site and elevation captured too much of the same variance for the model to run effectively. When accounting for phenological stage – rosette, bolting only, flowering only, early fruit production with flowers, late fruit production with flowers, or fruits only – this significance disappeared due to the overwhelming importance of phenological stage in fitness. **[FIGURE 2]**

Richness provided a nearly statistically significant explanation of variation in damage **[FIGURE 3]**, but no other considered measure of diversity had a clear relationship with damage by herbivory **[FIGURE 4]**.

A Principal Components Analysis (PCA) was run to capture the variation between species abundance data points across all sites in a few axes of variation. The five most explanatory axes were run as predictors of damage, and the third axis (PCA3) was found to be a significant predictor. After this, the species abundances that correlated most with that axis were run as predictors of damage directly, and three of the top five species indeed significantly predicted it. **[FIGURE 5]**

Discussion

While these analyses provide preliminary evidence of some relationship between diversity, interspecific interactions, and herbivory, the bottom line seems to be that complex processes like interspecific interactions are hard to capture without heavy data collection. For example, the variation in abundances of 66 species in the 37 wild quadrats required 36 axes to explain; in other words, the quadrats examined varied extensively when considering species identity and abundance. The most explanatory axis, PCA1, explained less than 9 percent of the variation in the data. **[FIGURE 7]**

I was able to show that fitness and damage were indeed correlated across all sites. This supports previous results on the direct negative relationship between damage and fitness (Mitchell-Olds unpublished). This finding is important firstly because the theory underlying the project – that plant defense faces selective pressure – is accurate. Second, it allows me to relate fitness to damage in further analyses and focus on the predictive ability of various diversity analyses on damage. That said, accounting for phenological stage as an ordinal covariate explained the fitness variation so well that it overwhelmed any signal from damage. This makes sense, as fitness is measured as average fruit length times the number of fruits. Thus, plants that differ in phenological stage – for example, those that have just begun producing fruits versus those that have matured – will differ in fitness strongly regardless of damage. Taking fitness data at single time points was thus a limitation of the measurement. This “loss of

signal” also supports the idea that complex interactions such as damage from higher trophic levels is hard to capture without extensive data collection.

Richness showed a marginally significant result but no other measure of diversity did. The PCA informed the idea that community composition – in other words, the identities of the species present – matters for the relationship between diversity and damage. These results are not intuitive to interpret, but it is possible that richness accounts for minor species far more than the Shannon-Weaver or Simpson Index was. One of the species that was shown to significantly predict damage, *Viola praemorsa*, was a low-density and low-abundance species. This supports the idea that the presence of some species, even if they are not present at great quantities, might be especially important.

Overall, these preliminary results point to the importance of understanding the mechanisms by which various species surrounding the focal species might influence insect-mediated selection. With a sample size of 37 quadrats, diversity indices alone that did not account for composition could not sufficiently explain variation in insect damage. That said, analyses such as the PCA that assessed variation in species abundances across quadrats, took species identity into account. The PCA, along with some individual species abundances, significantly correlated with damage, which has been shown to explain differences in fitness. The interesting and obvious next question is about what causes some species to especially affect insect feeding on *B. stricta*. In other words, what mechanisms drive interspecific interactions that promote or deter herbivory? While these results are certainly preliminary, they are exciting because they can direct future research towards understanding community dynamics by including species identity and with the goal of understanding mechanisms of species interactions.

With the current data collected, I have the capacity to test several more potential predictors of damage – and, indirectly, fitness. Given the significant predictive ability of three of five species abundances noted in quadrats, I want to go ahead and test the effects of all 66 species’ abundances. I

also plan to quantify species turnover within sites through Whittaker's Beta Diversity, and test its effect on damage. As species identities are important for this index, as they were for the PCA, the PCA results provide encouragement for its potential predictive ability. In addition, three of the five species best explained by the third PCA axis were grasses. Two of those grasses' abundances significantly negatively correlated with damage (see Figure 6). This preliminary result seems to support the previously mentioned model of associational susceptibility, in which greater resource diversity attracts more insects to feed. In such a model, functional group is important because different species of the same functional group may provide similar resources. For this reason, I want to test the effect of percent ground cover that is grass on damage. I anecdotally noticed throughout the field season that grassier meadows seemed to have more insects, but I have not quantified that nor have I quantified the relationship between grass coverage and plant damage.

While the results presented in this paper are preliminary, they provide a direction for further research that both considers the identity of species when examining the effects of community heterogeneity on selection dynamics and takes a mechanistic approach to understand what about those species causes herbivory to increase depending on their abundances. The "reality" of community ecology, as explained by Agrawal in a review of insect herbivore-plant coevolution, is that its dynamics cannot be explained through simple pairwise interactions (2011). Such complex types of "diffuse coevolution," with reciprocal selection effected by plants on insect herbivores and vice versa (Fox 1981), require much more data and much more analysis than a summer project can capture. That said, the data and accompanying analyses presented here inform future analyses that I can do to explain variation in plant herbivory and future data collection by other scientists interested in community heterogeneity and its effects on selection.

Figures

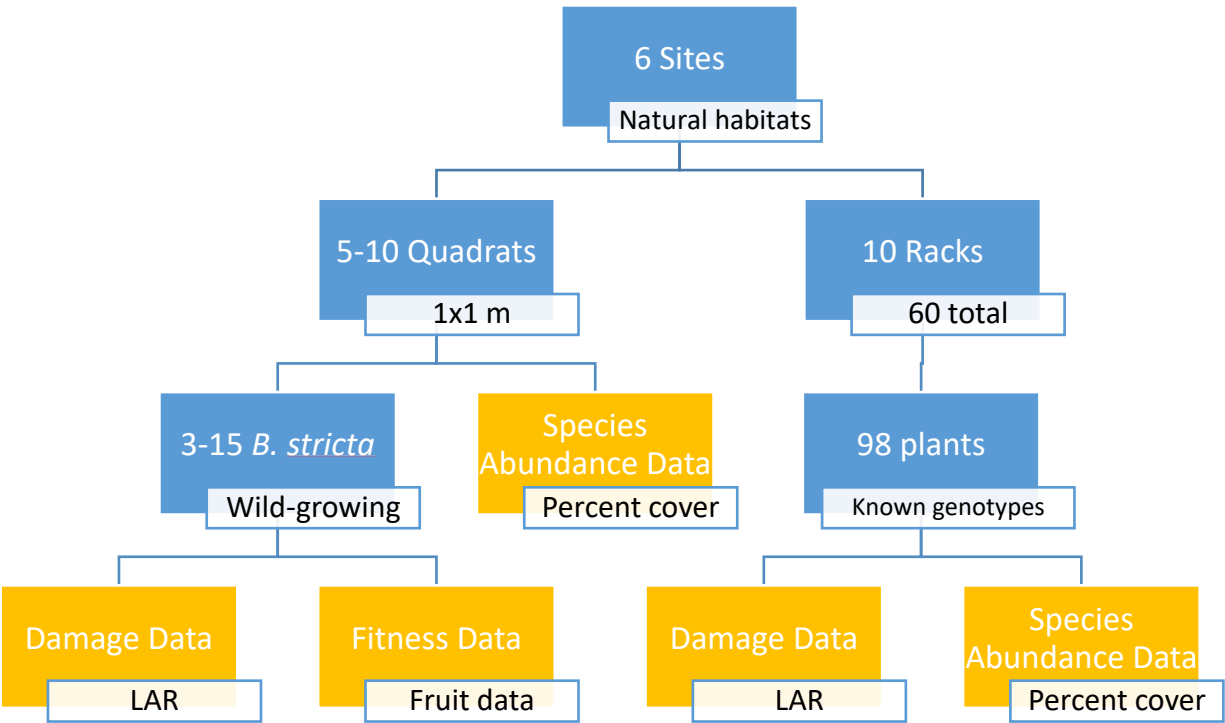


Fig. 1: Graphical explanation of experimental design. Data taken in orange.

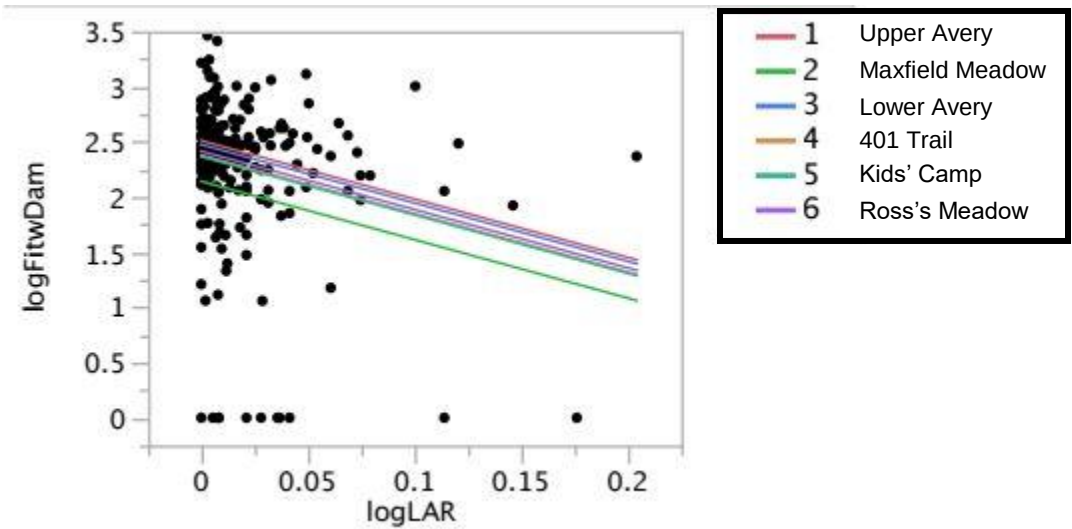


Fig. 2 – Random effects model of fitness predicted by leaf area removed, accounting for site. Each point is an individual plant, color-coded by site ($p=0.0003^*$, $R^2 = 0.1096$)

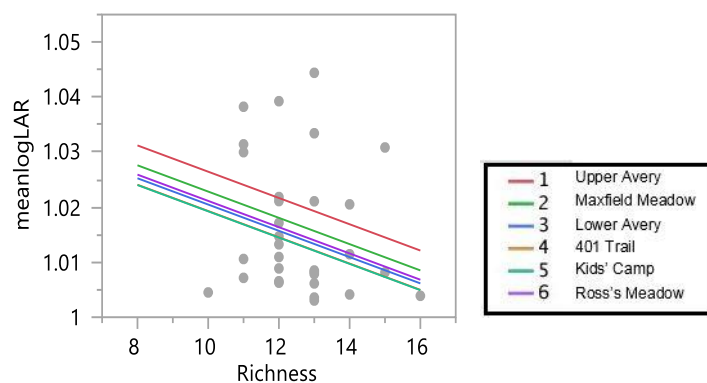


Fig. 3 – LAR as a function of richness at the quadrat level with site included as a random effect ($p = 0.0614$, $R^2 = 0.113$).

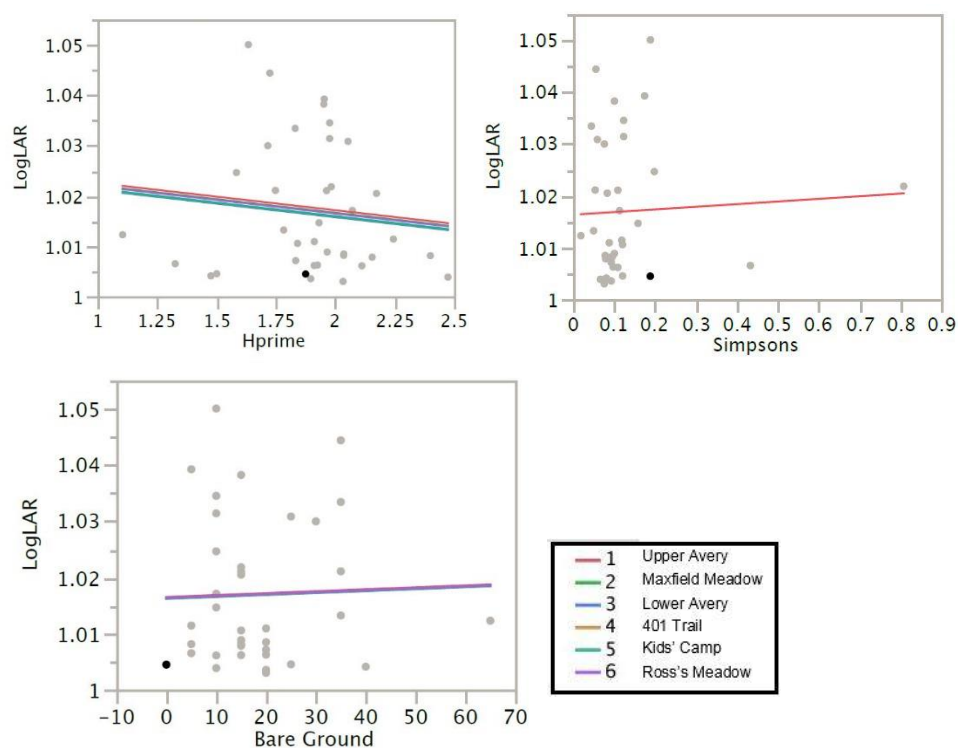


Fig. 4 – No clear relationships shown between Shannon-Weaver Diversity ($p = 0.5349$, $R^2 = 0.01573$), Simpson Diversity ($p = 0.7607$, $R^2 = 0.002686$), or percent coverage by bare ground ($p = 0.8547$, $R^2 = 0.00484$).

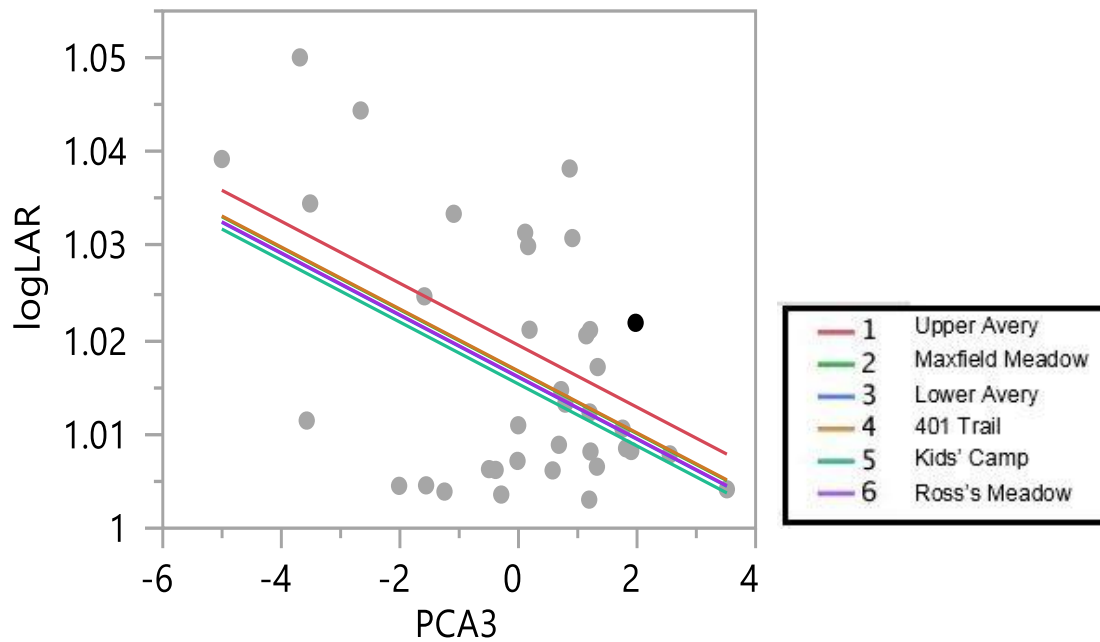


Fig. 5 - Third most highly correlated axis of variation in a Principal Components Analysis shown to significantly correlate with damage by herbivory ($p = 0.0030^*$, $R^2 = 0.1617$).

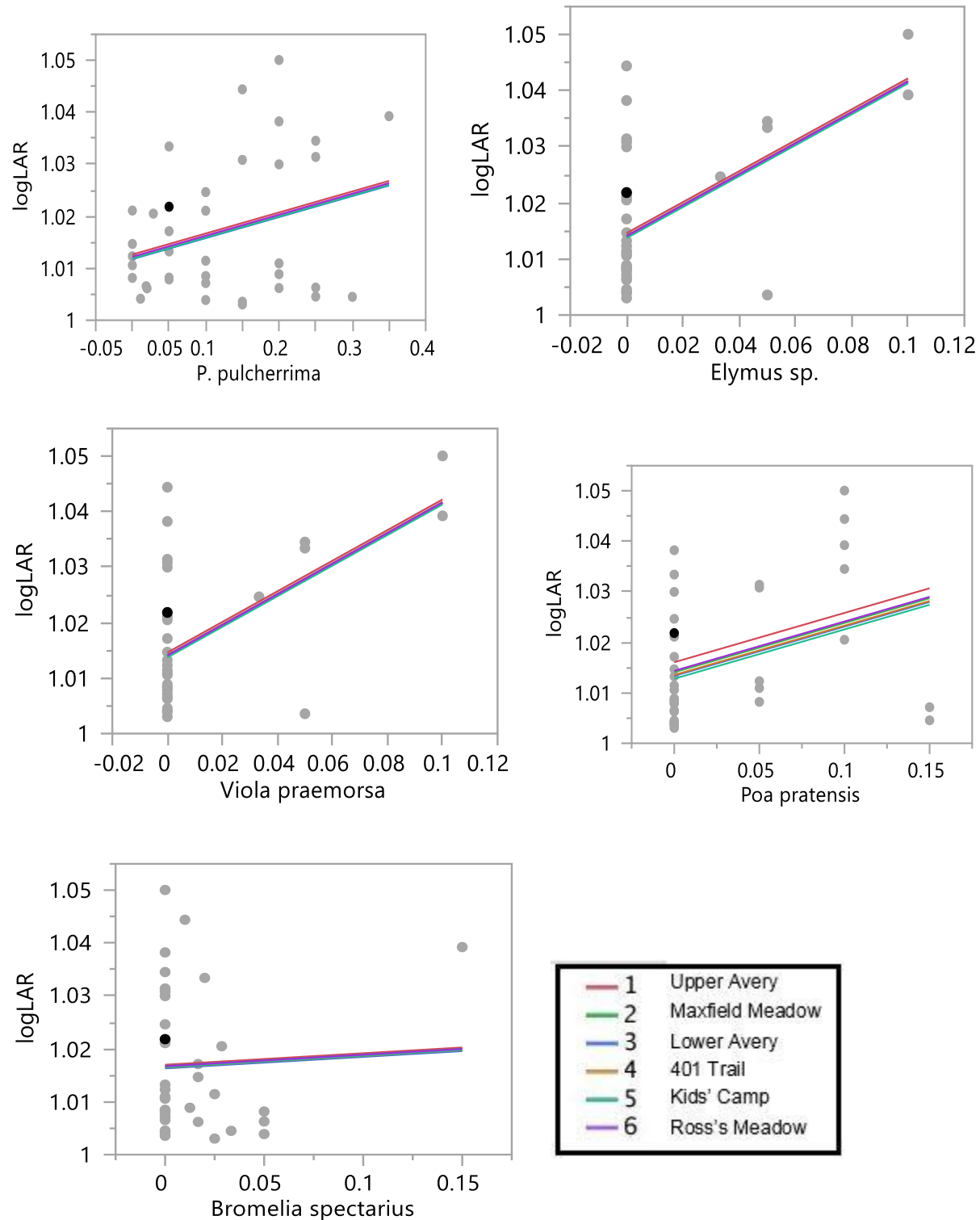


Fig. 6 – Species whose abundances were most explained by PCA3 were modeled directly with damage by herbivory. Significant relationships were found for three of the five most closely correlated species: *Viola praemorsa* ($p = 0.0006^*$, $R^2 = 0.2930$), *Poa pratensis* ($p = 0.0398^*$, $R^2 = 0.06193$), *Elymus* sp.

($p=0.0006^*$, $R^2 = 0.2930$), but not *Potentilla pulcherrima* ($p=0.0830$, $R^2 = 0.07498$) or *Bromelia spectarius* ($p=0.7888$, $R^2 = -0.0099$).

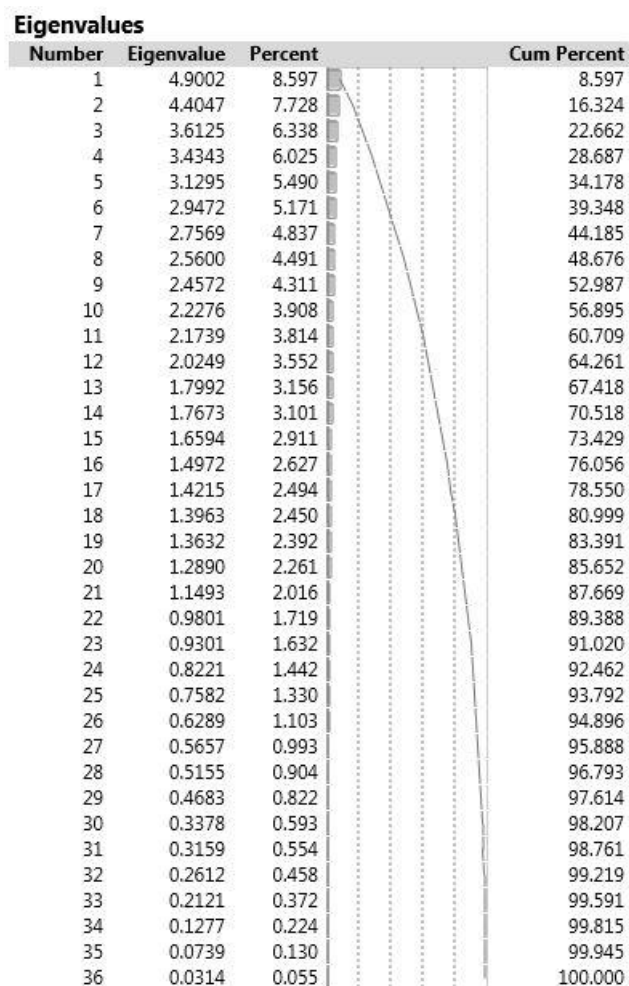


Fig. 7 – A table of each axis of variation from Principal Components Analysis and its associated eigenvalue and percent variation explained.

Images



Image 1 – *B. stricta* experiences herbivory by a variety of insects, such as the one shown.

Literature Cited

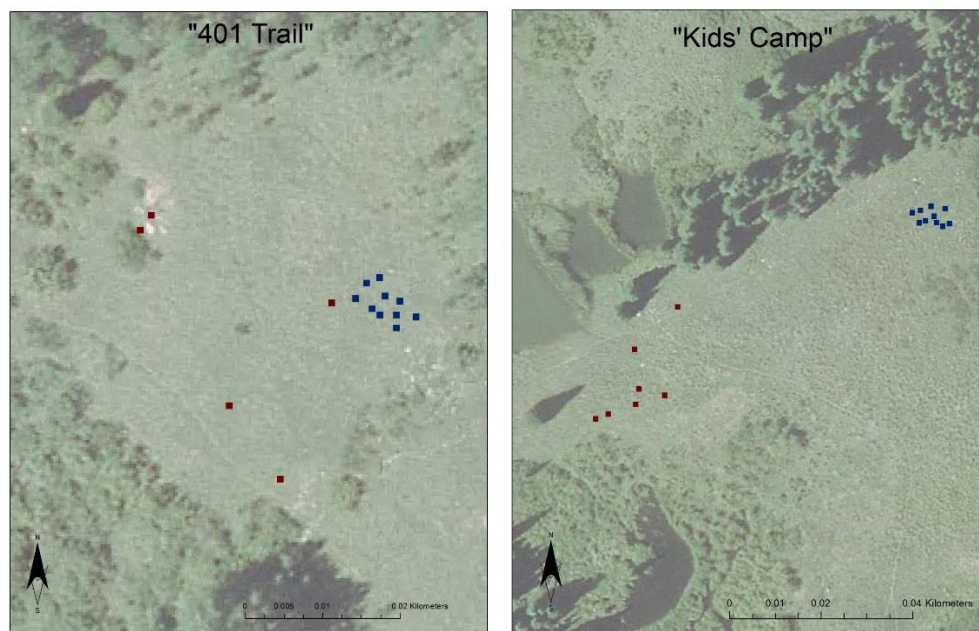
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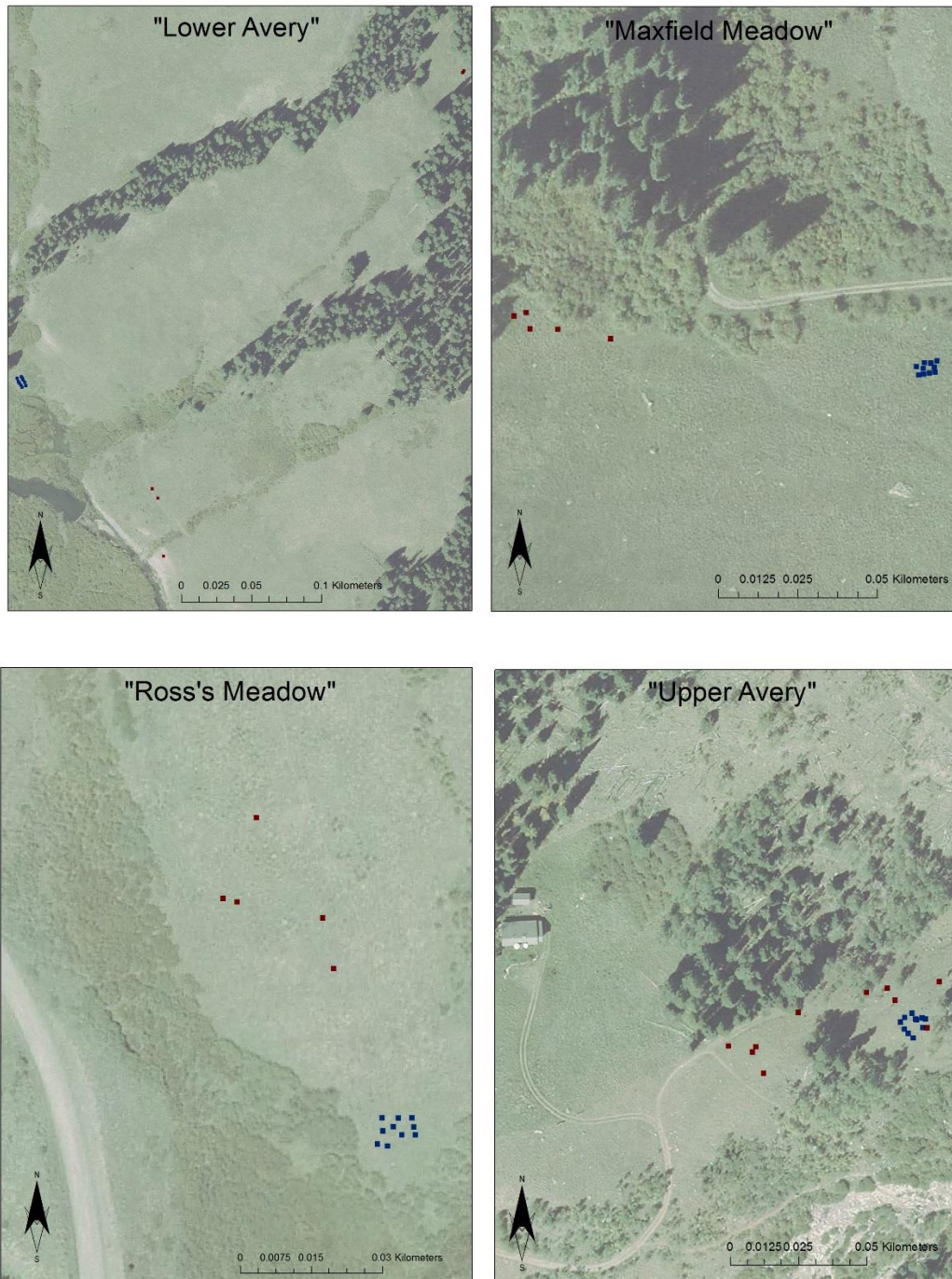
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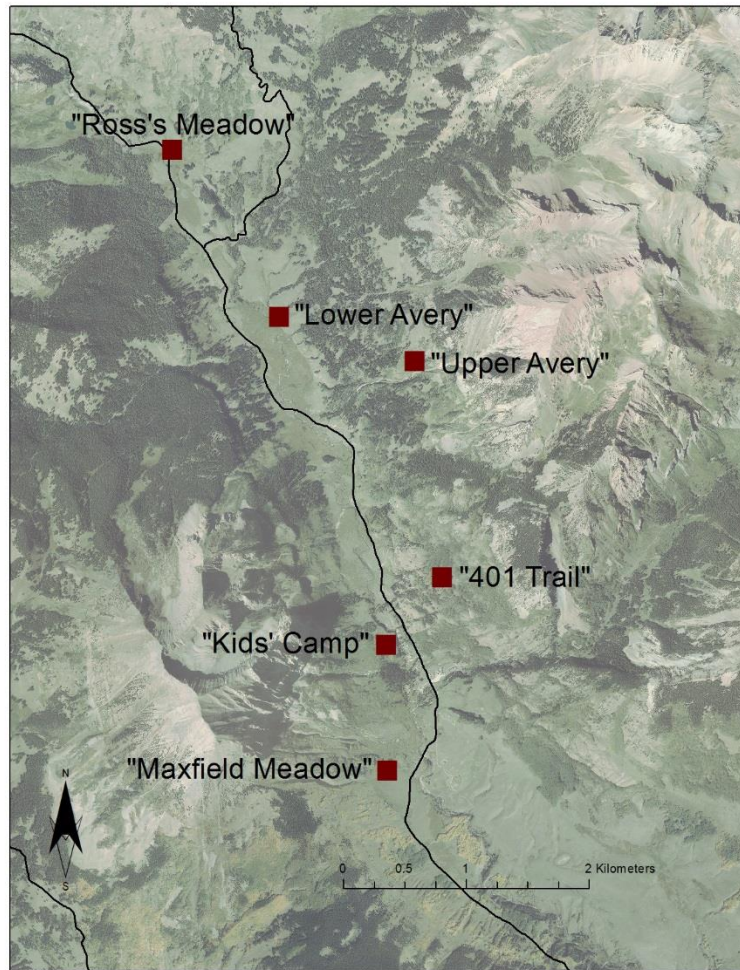
Maps





Maps 1-6 – These maps show the orientation of experimental plots and natural quadrats at each site.

The 10 manipulative racks of plants are shown in dark blue, and the observational racks of plants – ranging in number from 5 to 10 per site – are shown in maroon. North always points toward the top of the page.



Map 7 – This overall map shows the locations of the six sites in Gothic Valley in maroon, with County Road 317 shown in black for reference.