

Conflict of interest? Investigating the costs and
benefits of herbivore defense using the model plant
Boechera stricta.

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Abstract

Boechera stricta, a native forb common to the Rocky Mountain range in western North America, is a model species at the forefront of genetics and ecology. Over millennia, in response to herbivory, plants have developed secondary metabolite defensive compounds to deter wide ranges of herbivores. Glucosinolates represent one such type of compound produced by Brassicaceous plants. However glucosinolates may act as cues for oviposition for specialist herbivores and thus may lead to a conflict of interest for their host plant. This study focused on whether contrasting glucosinolate chemical profiles confer costs or benefits to overall fitness in relation to defense against specialist herbivores. I combined field observations from two separate studies to infer whether glucosinolate chemical profile influences specialist oviposition and feeding behavior, and if this behavior may result in selection for plant chemistry, thus influencing phenotypic diversity in defensive strategy. Overall, I found that there was no significant response in specialist oviposition or abundance based on chemical profile. However, in natural populations of *B. stricta*, plants on which *Euchloe ausonides* were present had significantly higher levels of herbivore damage to fruits than plants on which the specialist was absent. These results indicate that plants that attract specialist herbivores suffer a reproductive disadvantage, but that host plant chemical profile does not appear to influence that attraction. Factors influencing specialist herbivore oviposition, presence/absence, and abundance remain unknown.

Introduction

Herbivorous insects often have deleterious effects on plant growth, survival, and reproductive success and thus have the potential to impose natural selection on the defensive traits of plants (Crawley 1983; Marquis 1992). Nearly 90 million years ago, the ancestors of *Brassica spp.* (mustards,

cabbage, etc.) and related plants evolved biologically active secondary metabolite compounds known as glucosinolates that can have multiple ecological effects, including plant defense against herbivores, microbial community structuring, and cueing of oviposition and feeding by some specialist herbivores (Edger et al. 2015; Halkier and Gershenzon 2006; Hopkins et al. 2009). Examples of plant secondary metabolites include cyanogenic glycosides, glucosinolates, alkaloids, and terpenoids (Mithöfer and Boland 2012).

Many studies on the role of plant secondary metabolites and their interactions with herbivores involve glucosinolates, compounds produced by plants in the Brassicaceae family (Louda and Mole 1991). These compounds are generally recognized as plant defenses because they are transformed to harmful products upon plant damage, a characteristic of induced defense. In general, glucosinolates and their hydrolysis products have adverse effects on a wide range of herbivores (Giamoustaris and Mithen 1995; Halkier and Gershenzon 2006). However, similar to other classes of anti-herbivore defenses, glucosinolates are not equally effective against all herbivores; certain specialists have been found to feed on glucosinolate-producing plants without any deleterious consequences. Thus, while glucosinolates poison and deter most generalist herbivores, they may in turn function as feeding or oviposition cues to specialists (Mewis et al. 2002; Miles et al. 2005; Rojas 1999). Because of this potential to attract herbivores rather than deter them, the benefits in producing glucosinolates are not always obvious (Textor and Gershenzon 2009). Coevolution of plants and herbivores provides an opportunity for studying the role of cost-benefit tradeoffs in trait evolution and phenotypic diversity. As herbivory and resistance are both costly to plants, linking genotype to fitness in nature allows for connecting genes to ecologically relevant traits that are either transient variants on their way to being eliminated because they are deleterious, or on their way to fixation because they are beneficial (Mitchell-Olds et al, 2007).

In addition to having a diversity of effects on different herbivore species, glucosinolate chemical composition is also variable. There are around 120 known glucosinolates produced by plants in the family Brassicaceae. These compounds share a similar chemical structure, but differ in the amino acid precursors from which they are derived, and their variable side chains (Benderoth et al. 2006). In nature, plant defense traits are often polygenic and vary quantitatively among species and populations. Some plants in the Brassicaceae, such as *Boechera stricta*, also show high levels of heritable within-species variation in chemical defense strategies (Rushworth et al. 2011, Prasad et al. 2012). Hence, they provide ripe opportunities to investigate how natural selection may act to maintain natural phenotypic and genetic variation. To date, the ecological and evolutionary processes that maintain natural diversity in this and other complex traits have remained unknown or poorly understood (Pierterse and Dicke 2007).

However, understanding how selection operates on variable plant defenses is complicated by interactions with coevolved specialist herbivores. In response to the “arms-race” to circumvent plant defenses (Erich and Raven 1964), insects have developed several methods of bypassing chemical barriers that would otherwise prevent them from feeding, growing, and reproducing on defended host plants (Mello and Silva-Filho 2002). Specifically, for Brassicaceae specialists, otherwise defensive glucosinolates can act as potent oviposition and feeding stimulants for more than 25 insect species in the Coleoptera, Lepidoptera, and Diptera, and new cases of specialization are expected to continue to arise over time (Hopkins et al. 2009). In many species, the bidirectional interactions between host plant defense chemistry and specialist herbivore behavior remains uncharacterized, and the implications of these interactions for natural selection on defense traits is unknown. In this study, I addressed the following questions:

1. Does host plant chemical profile influence specialist oviposition preference and feeding behavior?

2. What are the implications of specialist herbivore behavior on selection for plant chemistry, and how might this influence maintenance of natural phenotypic diversity in defense strategies?

Materials and Methods

Study organisms: *Boechera stricta*

Natural history: *Boechera stricta* (Brassicaceae) is a short-lived North American forb that occurs throughout a broad range of habitats in the Rocky Mountains of the western United States (Anderson et al. 2015). *Boechera stricta* is one of the most morphologically and molecularly well-defined *Boechera* species, making it an excellent model for genetic and genomic studies (Schranz et al. 2007). *Boechera stricta* has a glucosinolate polymorphism; within and among populations, glucosinolates may be synthesized from either branched-chain (BC) amino acids or methionine (Met; Schranz et al. 2009). In *B. stricta*, the major BC-derived glucosinolates are derived from valine and isoleucine, and are uncommon among other members of the Brassicaceae (Windsor et al. 2005).

Experimental genotypes: To better understand how natural selection is operating on glucosinolate variation in nature, the Mitchell-Olds lab group has developed near-isogenic lines (NILs), which have been bred to be nearly genetically identical, differing only in a small chromosomal region (<<1 centiMorgan) containing the gene controlling glucosinolate profile (*BCMA*: branched-chain/methionine allocation; Prasad et al. 2012; Mitchell-Olds et al., *unpub.*). I used these plants to link known variation in chemical profile to observational preferences in herbivore oviposition.

Additionally, ongoing studies in the Mitchell-Olds lab utilize genome wide association studies (GWAS), an approach to linking broad-scale genetic and phenotypic variation (Anderson et al. 2011; Josephs et al. 2017). GWAS explicitly tests for the association between phenotypic traits and allelic

variation at many loci (Anderson et al. 2011), utilizing plant genotypes collected from across a broad geographic region spanning many environmental gradients. I used GWAS accessions planted into a common garden to assess oviposition preference and herbivory in plants that naturally vary in both glucosinolate profile and many other traits. I censused 14 replicates of the 411 genotypes represented in this study for specialist herbivore oviposition and subsequent herbivory and fitness. Each of these genotypes has already had its glucosinolate profile characterized in laboratory studies (Mojica, Mitchell-Olds, et al., unpub.). Specific glucosinolate profiles are referred to as “BB” and “MM”: the NIL genotypes homozygous for the branched-chain GS allele or methionine GS allele, respectively.

Natural Populations: My own datasets were supplemented with an observational study of natural *B. stricta*-*E. ausonides* interactions compiled by other members of the Mitchell-Olds lab in 2016 (Carley et al., unpub.). This study examined five quadrats in each of six natural populations, monitoring relative fitness based on factors such as presence of herbivore (*Euchloe* and “other”), leaf herbivory, fruit herbivory, plant height, and GS profile based on HPLC data.

Study organisms: specialist herbivores

As specialist herbivores, I focused primarily on *Euchloe ausonides* (Pieridae), a known glucosinolate specialist that is commonly observed on *B. stricta* and some other wild mustard species near Gothic, CO (L. Carley, pers. comm.). Little is known about *E. ausonides* as it has been extirpated from most of its natural range. Its current range is believed to span from the California coast to the Great Lakes region, USA (Opler 1966). The most widespread known mechanism by which specialist Lepidopterans, including *Euchloe* spp., deal with plant defenses in the Brassicaceae is deflection or bypass of glucosinolate hydrolysis (Kliebenstein et al. 2005). This counter-defense utilizes a nitrile-specifier protein expressed in the caterpillar midgut that redirects the hydrolysis of glucosinolates to less

harmful nitriles (Edger et al. 2015). Nitrile-specifier protein appears to be a key biochemical counter-adaptation that allows insects to feed on plants containing glucosinolates. I caught 28 female butterflies from areas around RMBL in Gothic, CO for use in oviposition experiments with glucosinolate NILs, and monitored all other plants (GWAS and natural populations) for ambient levels of oviposition by this species.

Study Site

My study was conducted at the Rocky Mountain Biological Laboratory in Gothic, CO (38.9585° N, 106.9878° W). For my experiments, I utilized two common gardens used by the Mitchell-Olds lab group: one ~0.5 miles southwest of the Gothic town-site (“Gothic Garden”; Figure S1), and one ~10 miles north of Gothic in Schofield Park (“Schofield Garden”). GWAS and NIL plants at both common gardens have been previously planted in a randomized complete block design in the fall, allowed to overwinter under natural conditions, and are primed to bolt, flower, and reproduce the following summer (*sensu* Anderson et al. 2011; Prasad et al. 2012). I used existing plants in these gardens to perform observational and manipulative research *in situ*.

Data Collection

Over nine weeks from June 2 to August 4, 2017, I assessed whether variation in the defensive chemistry of *B. stricta* influenced the reproductive and feeding behavior of the specialist Lepidopteran herbivore *Euchloe ausonides* (*Pieridae*). Furthermore, I assessed what the consequences of *E. ausonides* oviposition choice would have on herbivory and overall plant fitness. I performed two studies to

investigate the role of specialist insect behavior in natural selection on plant chemistry: paired-choice tests and field observations.

Paired-choice tests: In order to determine whether plant chemical profile influences specialist oviposition behavior, I executed controlled, paired-choice tests with gravid female *E. ausonides* butterflies at Gothic Garden. I utilized the existing layout of experimental NIL plants and selected pairs of plants in close physical proximity that had contrasting glucosinolate profiles. For each trial, I enclosed each pair of plants in a 1' x 1' x 1' flight cage and released one butterfly into the flight cage at 10:00 am. I returned and checked each flight cage 24 hours after the initiation of each trial to assess the selection of oviposition sites based on the number of eggs laid and the chemical profile of the host plant chosen. Because I anticipated having more available pairs of NIL plants than gravid female butterflies, and because butterflies can oviposit multiple times onto different plants (Renwick and Chew 1994; R. Steward, *pers. comm.*), I utilized each individual butterfly in up to three paired-choice tests.

Field observations and natural variation in plant chemistry: In late summer, I conducted a second, non-manipulative study at Schofield Garden. Here, I assessed natural oviposition preferences of ambient *Euchloe ausonides* populations in response to known, quantitative glucosinolate profile variation in the Mitchell-Olds GWAS plants. I also utilized additional field data collected by other members of the Mitchell-Olds lab, from natural populations of *B. stricta* that previously had their chemical phenotypes characterized via high-performance liquid chromatography (Carley et al., *unpub.*). The goal of these two studies was to determine whether *Euchloe* preference based on glucosinolate profile is still detectable among plant genotypes that exhibit broad-scale genetic and phenotypic diversity in addition to variation in the *BCMA* gene.

Linking herbivore preference and behavior to natural selection: In all studies, I aimed to monitor plants after *Euchloe* oviposition was complete and through future life stages of the butterfly.

However, in Gothic Garden, no eggs matured to hatch, and therefore no larvae emerged to feed on the host plants; in Schofield garden, damage and fitness censuses on GWAS plants are ongoing. Because of these limitations, I focused on previous data from natural *B. stricta* populations (Carley et al., *unpub.*) to assess the effects of *Euchloe* oviposition on subsequent plant herbivory and fitness.

Covariates and additional data collection: In all studies, other plant and environmental traits may have influenced butterfly behavior and larval herbivory aside from host plant chemistry. As such, I recorded plant height, phenological stage, number of branches and flowers per plant, as well as the weather, temperature, and time of day that each paired-choice trial was initiated.

Data Analysis

I conducted all statistical analysis in JMP 13.0, utilizing matched-pairs tests and linear mixed-effects models. In general, I tested for the effect of glucosinolate variation on oviposition preference by modeling the number of eggs laid on plants in response to their known glucosinolate genotypes and/or phenotypes. For my paired-choice tests, I analyzed oviposition preference (# eggs laid) in response to NIL plant genotype using a matched-pairs test. For the observational studies, I analyzed oviposition preference (# eggs laid) in response to the mean glucosinolate profile for each of the 411 genotypes represented in the GWAS study or the measured *in situ* glucosinolate profile for wild populations. For these observational analyses, I fit linear mixed-effects models including plant height and number of branches as covariates as well as random effects of site for natural populations or position (“block”) within Schofield Garden for the GWAS plants.

Results

Paired-choice tests on glucosinolate NILs: There was no significant difference in the number of *Euchloe* eggs laid on *B. stricta* NILs depending on *BCMA* genotype (matched-pairs test, $N = 8$, $df = 7$, $P = 1$; Figure 1).

As defensive chemical profile alone may not be the sole determinant in oviposition preference, I tested whether covariates such as plant height and flower number differed between the *BCMA* NILs used in my paired choice tests. There was a marginally significant difference in height within each trial (matched-pairs test, $N = 8$, $df = 7$, $P = 0.0560$; Figure 2A), and a significant difference in number of flowers (matched-pairs test, $N=8$, $df = 7$, $P = 0.033$; Figure 2B), with plants of the MM glucosinolate genotype having more flowers at the time of the oviposition trials.

Observations in natural populations: Using previously collected data from 6 natural populations near RMBL, I utilized multivariate models to test whether (1) glucosinolate phenotype (BC-ratio) predicts *Euchloe* presence or absence, and (2) whether *Euchloe* presence or absence predicts herbivore damage to fruits or leaves. While *Euchloe* were slightly commoner on plants with high BC-ratio, I found no significant difference in *Euchloe* presence between GS phenotype ($n=111$, $p=0.42581$; Figure 3). With regard to herbivory, I found that *Euchloe* presence predicted herbivore damage to fruits ($n=213$, $p=0.0001$; Figure 4A), but not to leaves ($n=213$, $p=0.5761$; Figure 4B).

Discussion

Glucosinolate variation and specialist herbivore visitation: The intention of this study was to test whether variation in glucosinolate profile may result in differing levels of oviposition and subsequent herbivore damage on *Boechera stricta* by the specialist lepidopteran herbivore *Euchloe ausonides*. Data from two separate studies showed that there was no significant relationship between glucosinolate genotype (Figure 1) or phenotype (Figure 3) and the presence of *Euchloe ausonides*. Thus, glucosinolate chemical profile alone does not seem to be a strong indicator of specialist herbivory and oviposition.

Linking specialist abundance to herbivory and fitness: In response to larval feeding influencing herbivory on *Boechera*, the 2016 data from natural populations shows that glucosinolate profile alone does not influence the presence or absence of *Euchloe* specialists in observed trials. However, the presence of *Euchloe* did influence *Boechera* fruit damage (Figure 4A, although no trend was seen with leaf damage (Figure 4B). Thus, presence or absence of *Euchloe* does have overall fitness consequences, but is not solely mediated by variation in GS profile.

Limitations and future directions: In Gothic Garden, due to high over-winter mortality, I was only able to find five pairs of plants with contrasting GS profiles that had bolted and flowered in close enough proximity to perform paired-choice tests. With only five paired plants for experimental choice tests, I was limited in the number of tests I could run daily. Additionally, butterfly mortality was high, and I only successfully utilized four butterflies could be during paired choice trials. This limited the statistical power of my experimental work and made it difficult to draw robust conclusions from the paired choice oviposition trials. In follow-up studies, I recommend using pre-potted plants to improve experimental conditions. Also, as both tests were performed in the same geographic range, performing multiple trials in separate habitats and elevations would provide detailed information as to whether the results presented here are consistent across multiple environments.

Greater understanding of the life-history of the specialist herbivore *Euchloe ausonides* would also help future studies, as the peak time and geographic range for flight, mating, and oviposition of this non-model species are still poorly characterized. In future studies, the time frame of butterfly emergence and oviposition should be well timed with the performance of experimental oviposition trials in order to maximize butterfly yield and successful oviposition.

Conclusions: Plant defenses have coevolved with their insect herbivore counterparts over millennia. Understanding what forces maintain the balance of natural phenotypic variation in defensive

profile is a fundamental goal in evolutionary ecology. Specialist preference for one phenotype over another would likely lead to directional selection and reduced phenotypic variation over time. However, non-preference (as observed) among other factors may allow for maintenance of natural phenotypic variation, as is observed in *B. stricta* glucosinolate phenotype.

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Figures:

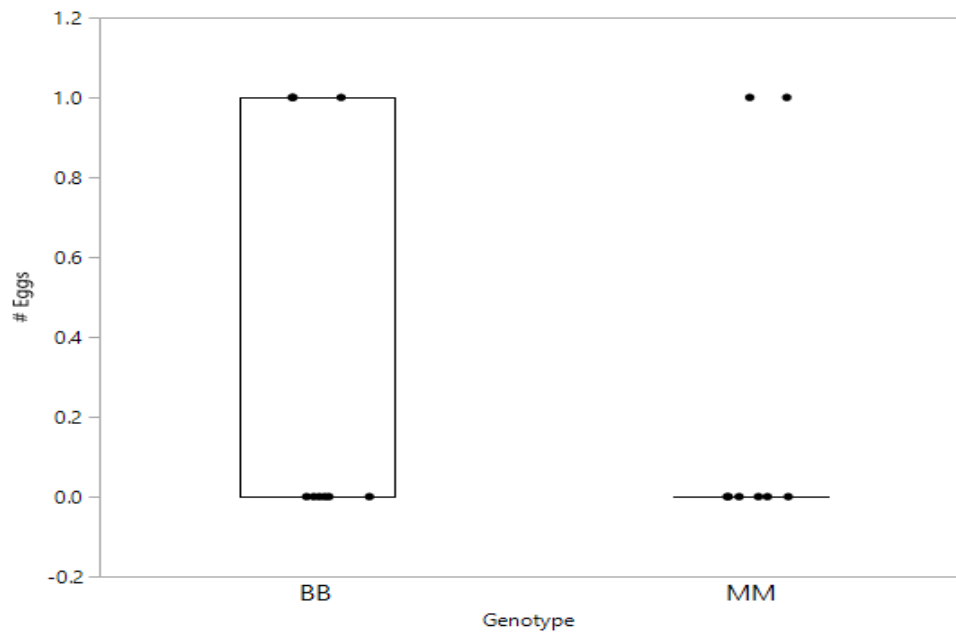


Figure 1. Number of eggs oviposited on *Boecheria stricta* in paired-choice trials in response to glucosinolate chemical composition: BB (branched-chain) vs. MM (methionine).

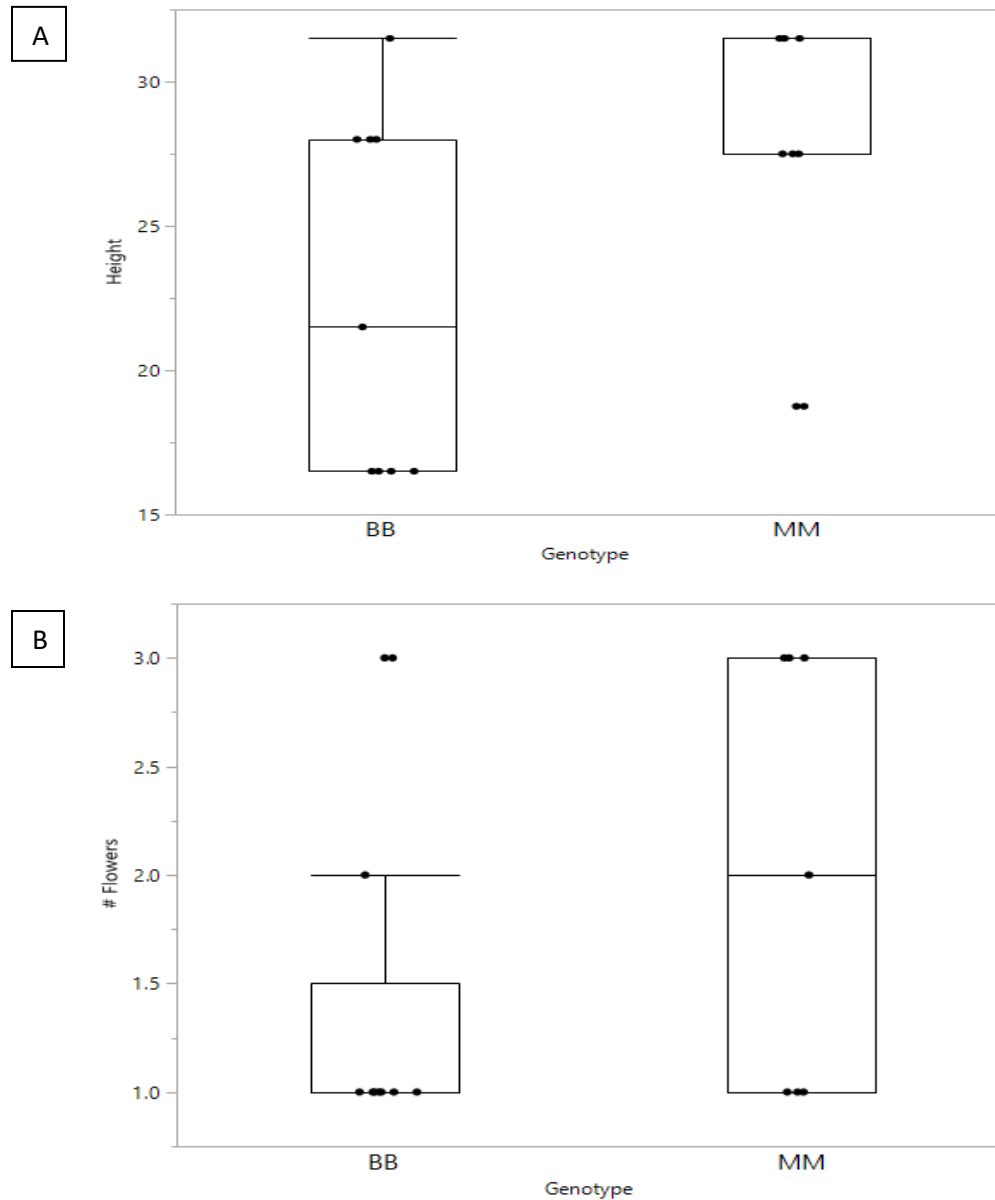


Figure 2. Variation in non-GS traits among BCMA genotypes in matched-pairs trials. A: MM plants were marginally taller than BB plants. B: MM plants had more flowers at the time of oviposition trials than BB plants.

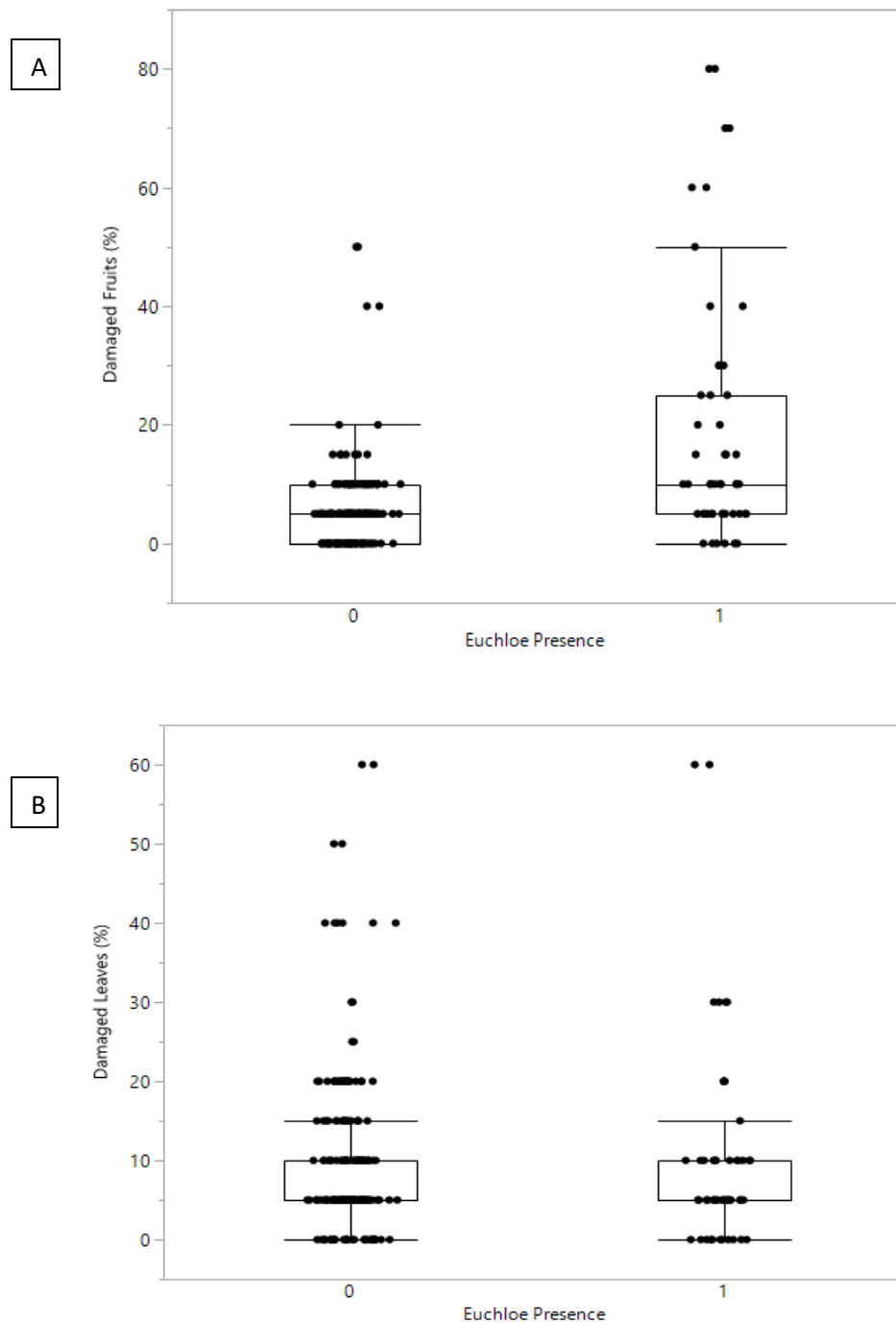


Figure 4. Impact of *Euchloe* presence on herbivory in endogenous *B. stricta*. A: Percent fruit area removed is significantly impacted by *Euchloe ausonides* presence. B, Percent damage to leaves did not vary depending on the presence or absence of *Euchloe*.

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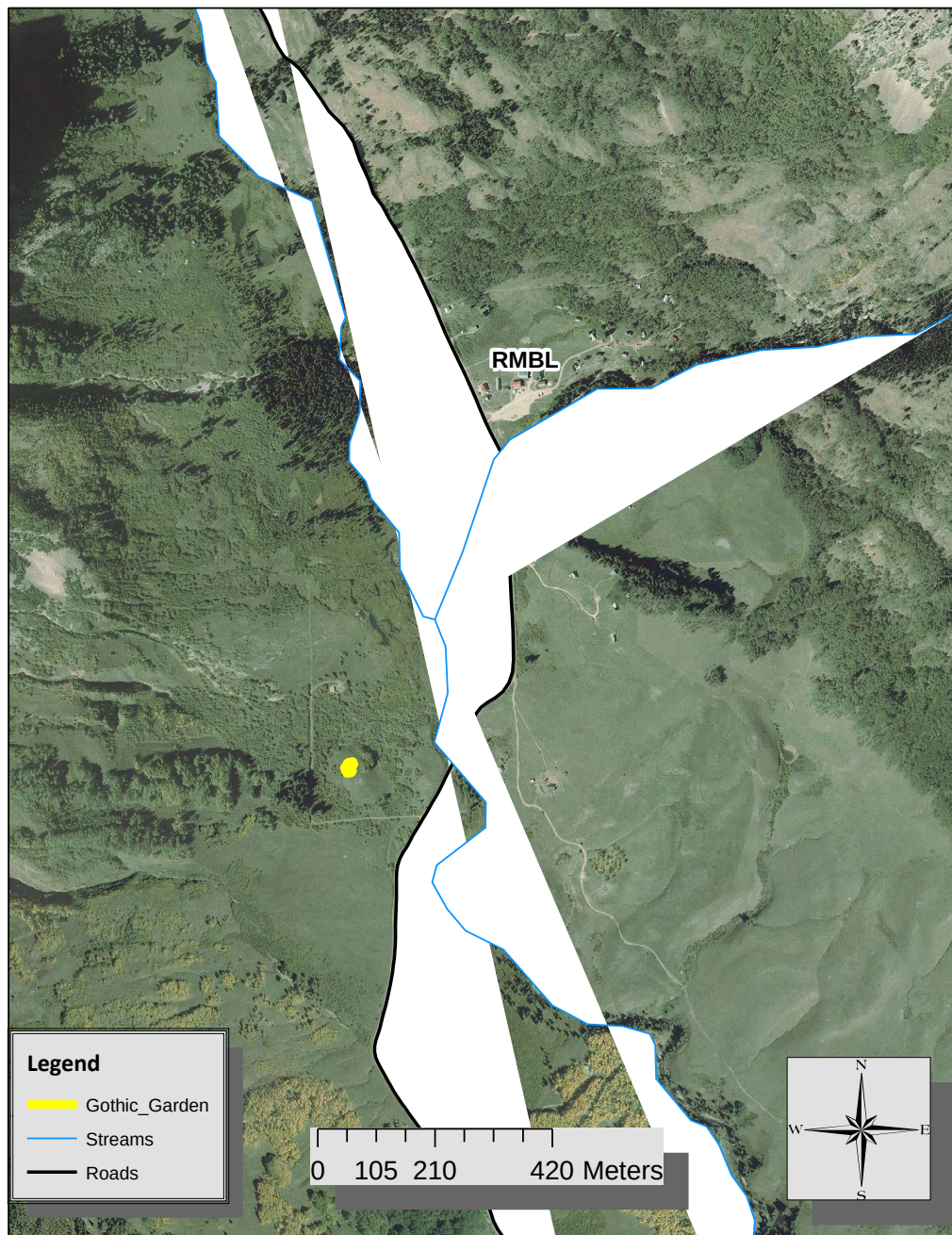


Figure S1. Location of Gothic Garden (yellow), the site of experimental oviposition trials, relative to RMBL.