

Effects of phenological stage and temperature on *Ligusticum porteri*'s volatiles and trophic interactions

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Abstract:

Volatile organic compounds (VOCs) are emitted by plants as signals to other organisms and are interrelated with atmospheric chemistry and climate change. Typically, phenology advances and temperature increases with climate change. How floral volatiles of *Ligusticum porteri* influences its multitrophic interactions and changes with increased temperature is unknown. We investigated how elevated temperature and phenological stage affects both the volatiles released by host plants and the performance of intraguild Miridae predators and their competitor/prey, aphid *Aphis helianthi*. Throughout the study we monitored phenological stage and insects present on *L. porteri*. Open top warming chambers (OTC) increased the temperature surrounding the plants by 0.81°C on average and were removed after 23 days for the advanced phenology treatment group, though no significant phenological advancement occurred ($p=0.682$). The OTC remained on plants for the advanced phenology plus increased temperature treatment group. Silicon tubing (ST) captured floral volatiles, and was analyzed by thermal desorption coupled to gas chromatography mass spectrometry (TD-GC-MS) to quantify volatiles released. Ten aphids from existing colonies were placed onto test of suitable condition. Miridae abundance was correlated to phenological stage and the amount of photosynthetically active radiation (PAR) ($p=2.782 \times 10^{-11}$) and found to drive plant damage ($p=0.02716$). The ability for aphids to colonize host plants was related to the amount of plant damage. Thus, Miridae populations seem to indirectly negatively impacting aphid performance, even though elevated temperatures typically increase aphid abundance. The volatile profile is expected to correlate with phenological stage, which may explain Miridae abundance patterns. Additionally, the defense volatiles may play a role in why aphid ability to colonize host plants. As temperature increases and phenology advances with the changing climate, the biotic interactions associated with phenology may be stronger than direct effects of temperature.

Introduction

Climate change has direct and indirect impacts on the ecosystems through alteration of phenology and volatile organic compounds (VOCs) released by plants. The phenology of many organisms have advanced due to climate change, which can cause phenological mismatches, where phenology of organisms and their food source are no longer synchronous (Both, van Asch, Bijlsma, van den Burg, & Visser, 2009; Schwartzberg et al., 2014). The advancement of flowering time and consequences for pollination mutualism has received considerable attention, though phenological mismatches across multiple trophic levels also has many ecological implications and deserves investigation.

VOCs are released for a variety of reasons, such as a defense mechanism, pollinator attractant, plant-plant communication or stress response (Yuan, Himanen, Holopainen, Chen, & Stewart, 2009). Climate change alters volatiles released by plants directly through increased temperature, CO₂ and O₃ (Guenther, Zimmerman, Harley, & Monson, 1993; Yuan et al., 2009). Rising temperature enhances enzyme activity, raises vapor pressure, and decreases diffusion pathway resistance which increase VOC emissions in the short term (Penuelas & Staudt, 2010). Increased CO₂ levels causes different responses of VOC emission in plants, which can depend upon metabolic and enzymatic responses (Yuan et al.,

2009). VOC emissions vary by plant organ, circadian rhythms, age and individual (Knudsen, Tollsten, & Bergstrom, 1993; Yuan et al., 2009). VOC carbons are key signals in host plants, aphid herbivores and their predators and mutualists. Aphids use antennal olfactory sensilla to detect plant volatiles, which influence their behavior after landing on a potential host plant (Powell, Tosh, & Hardie, 2006). For example, the presence of plant defense volatiles lead aphids to deem plants less suitable as hosts (Powell et al., 2006).

A predator which also competes with prey for limited resources is an intraguild predator (Holt D. & Huxel R., 2007). Members of the Miridae family can be intraguild predators and commonly feed upon Aphididae (Chouinard, Bellerose, Brodeur, & Morin, 2006; Lucas, Fréchette, & Alomar, 2009; Mooney et al., 2016). *Aphid helianthi* and Miridae such as *Lygus* are both commonly found on *Ligusticum porteri* (Apiaceae), where Miridae are a known aphid predator (Mooney et al., 2016). However, the extent to which Miridae impact aphid populations via resource competition or reducing host plant quality has not been explored.

In this study, we investigated how elevated temperature, phenological stage and plant damage may be correlated with insect populations and floral volatile production of *L. porteri*. As snow melts earlier with the changing climate, *L. porteri*'s phenology has advanced (Iler et al., 2013). Miridae abundance also positively relates with earlier snowmelt (Mooney, unpublished data, Figure 1). Whereas, aphid abundance is related to direction of phenological mismatch with host plant, where year with later host plant phenology correlates with lower aphid populations (Mooney, unpublished data). Additionally, though aphid growth rates increase due to direct effects of elevated temperature, we were interested in how aphid- Miridae interactions respond to increased temperature and thus may impact aphid populations (Barton & Ives, 2014; Mooney et al., 2016). We hypothesized that greater abundance of Miridae with advancing phenology increases predation of aphids and reduces host plant quality, and thus indirectly decreased aphid populations. Additionally, the role of chemical cues in Miridae-aphid interactions of *L. porteri* is unknown. In general, benzenoids, isoprenoids and fatty acid derivatives are the primary groups of floral volatiles (Knudsen et al., 1993), where monoterpene hydrocarbons typically constitute over 90% of floral volatiles of the Apiaceae family (Valterovat & Xdn, 1994). Temperature strongly correlates to an increase in terpenoid emissions (Yuan et al., 2009), thus we suspected that increasing *L. porteri* temperature would increase overall quantity of floral volatile emissions. Floral terpene emission rates reach a maximum during the temperature of their peak flowering month (Farre-Armengol, Filella, Llusia, Niinemets, & Peñuelas, 2015). We suspect a greater quantity of floral volatiles present during full anthesis, though were curious how volatiles differ throughout *L. porteri* development. Additionally, since light has been found to drive volatile emissions, such as monoterpene (Kuhn et al., 2004), we also investigated how light influences floral volatile emissions. We suspected volatile quantities and composition would relate to Miridae abundance and ability of aphids to colonize host plants.

Methods

Four populations of *Ligusticum porteri* were sampled; two in shady aspen understory and two in the sun to accurately represent the regions which it naturally grows. Figure 2 displays site locations in relation to the Rocky Mountain Biological Laboratory in Gothic, Co. Each population had three treatment

categories of five randomly selected *L. porteri* with flowering stalks: ambient (C), accelerated phenology (AP) and accelerated phenology with increased temperature (APT). To increase temperature surrounding plants and ideally accelerate phenology, an open top warming chamber (OTC) created of metal tomato cages covered with 4 mm clear polyethylene (Film-Gard, Evansville, IN) surrounded plants, following the methods of Baron and Ives (2014). The warming chambers remained on *L. porteri* throughout the experiment for APT treatment and were removed after 23 days for the AP treatment. The C treatment consisted of unaltered plants surrounded by tomato cages. Phenological stage was observed every two-three days using a scoring system adopted from Lindsay (1982) (Table 1). The terminal, primary, secondary and tertiary compound umbels were scored; if there was more than one compound umbel in a position the average was recorded. Weighted averages were then calculated, with terminal umbel scores multiplied by 4, primary by 3, secondary by 2 and tertiary by 1.

To determine how much the OTC increased the temperature surrounding plants, HOBO data loggers (OnSet Computers, Bourne, MA) were placed on a randomly selected C and OTC *L. porteri* per plot; HOBO data was collected five times throughout the study, and replaced on a randomly selected C and OTC plant. HOBOs measured temperature every five minutes throughout the experiment. We measured photosynthetically active radiation (PAR) over each plant on a cloudless day using a light meter (LI-250A Light Meter, LiCor, Lincoln, NE) and quantified the average amount and variance of light at each study site.

Volatile sampling of each plant in decent condition plus one sample of ambient air per plot occurred two days after OTC removal, following an adopted method from (Kallenbach et al., 2014). Plants were sampled if rays and stems were green and upright and thus potentially capable of hosting aphids. Terminal umbel headspace was sampled using polyethylene terephthalate (PET) chambers with three holes in the lid and three lateral holes to allow airflow (Kallenbach et al., 2014). Three 10mm sections of silicon tubing (ST) were placed in the chamber interior and removed after 48 hours of exposure. Which compound umbels were enclosed in the PET chambers, their phenological stage and any insects present were recorded at the time of ST removal. ST were placed into 2 mL glass vials and sent to a commercial laboratory for analysis (Altitude Lab Solutions, Highlands Park Ranch, CO). The lab performed thermal desorption and gas chromatography mass spectrometry (TD-GC-MS) analysis to quantify amounts of total volatiles against known standards. Analysis of variation volatile emissions will include only plant-specific volatiles and not include those identified from ambient-air sampling.

To account for plant damage, the number of rays and number of rays with damage were counted on each compound umbel and used to calculate the proportion of ray damage. Visual estimations of the percent of flower/seed damage were also made for each compound umbel. Damage was defined as a plant part which was brown and/or no longer upright. An overall damage index was created for each plant by averaging the proportion of ray damage and decimal conversion of percent flower/seed damage. These same damage quantifications were also made for only the umbels enclosed in the PET chambers to investigate how volatile emission may be related to plant damage.

Next, aphid host plant preference was studied by placing ten aphids from existing colonies onto test plants in suitable condition, following the same criteria use for volatile sampling. To encourage

colonization, aphids were surrounded by mesh bags around *L. porteri* for two days. Aphid population size and other insects present were observed every two-three days for eleven days.

Data Analysis

Insects and phenology

ANOVA analysis was performed on the phenological advancement of OTC versus C plants for the first 23 days of OTC presence, the time of OTC presence for AP group, to determine if AP phenology significantly differed from the control plants at that time. To determine what factors impacted the advancement of phenological stage, multiple linear mixed models were run using the nlme package in R. Models were run with plant ID treated as a random effect, and data points collected when phenology was unable to be scored (NA) excluded. Anova was performed on models to determine their significance using the car package in R. If significant, a Tukey HSD post hoc test was performed.

Linear mixed models were also created, following the same criteria, to determine factors influencing Miridae abundance and plant condition. General linear models were created for what affected the initial establishment of aphid colonies, from the time of adding the ten aphids to plants and removing protective mesh bags. Factors influencing aphid colony survival, after bag removal, were determined from linear mixed models of plants excluding plants without aphid colony establishment. To determine if temperature surrounding OTC versus C plants differed, an ANOVA analysis was performed. All of the above analysis were performed in R version 3.2.3 (2015-12-10),

Floral Volatile Organic Carbons

To identify which compounds from the different treatment groups varied, non-metric multidimensional scaling (NMDS) was performed using the 'vegan' package in R (CITE). The 'envfit' function in vegan determined what volatiles vary with phenological stage or temperature treatment. Once compounds that vary with treatments were identified, we used general linear models to determine if the emission of a specific volatile varies among treatments (control plants, advanced phenology and advance phenology plus elevated temperature). If a treatment effect was detected ($P < 0.05$), we used post-hoc tests to identify pairwise differences. Any pairwise differences observed between plants with advanced phenology versus those with advanced phenology plus elevated temperature indicated an effect of temperature independent of phenology alone.

Results

After 23 days of the OTC enclosure, there was no difference in rate of phenological advancement of the OTC versus control plants ($\chi^2 = .1678$, $Df = 1$, $p = .682$). Though, temperature surrounding plants did significantly differ between control and treatment groups, with an overall average of .087°C increase ($F = 129474$, $p = 2e-16$) (Table 2). Analysis including treatment as a factor thus only used two treatment groups, of increased temperature and ambient temperature. Phenological stage depended upon the Census, or sampling, number ($\chi^2 = 6103.1$, $Df = 1$, $p = 2.2e-16$).

The amount of light at each plot significantly varied ($X^2=345.13$, $Df=3$, $p<2.2e-16$, Table 3). Figure 3 displays how the PAR of shade plots did not differ from each other, but were less than PAR at Sun 1, which has less PAR than Sun 2.

Miridae abundance was positively correlated with the interactive effect of phenology and PAR ($X^2=44.3246$, $Df=1$, $p=2.782 e-11$); as phenology advances by 3.225 and as PAR increases by $.00699 \mu\text{mol s}^{-1} \text{ m}^{-2}$, the probability of Miridae abundance increases (Figures 4 and 5).

Damage

It was noted during observations that plants with damage had less, if any, insects on them. By July 29th, when plant damage data was recorded, many plants had been damaged for several censuses. To determine if the number of Miridae previously present on plants impacted plant health, the average number of Miridae on plants until census 6, the midway point when plant damage had been noticed, was calculated. The total amount of damage was positively correlated with the interactive effect of warming or control surrounding temperature plus the average number of Miridae until census 6 ($X^2=4.8807$, $Df=1$, $p=0.02716$, Figure 6)

Aphid colonization

12 plants were visually determined as unsuitable aphid host plants and two plants had aphids naturally colonize at the time of aphid addition. Therefore we added aphids to 9 APT, 11 AP and 13 C plants. Since OTC chambers were already removed from AP plants when aphids were added, 9 aphid additions on plants were in a warming chamber and 24 were exposed to unaltered environmental conditions.

Initial aphid colony establishment depended upon the total amount of plant damage ($X^2=28.273$, $Df=1$, $p=1.053 e-7$), whereas the proportion of plant damage decreased by 1.4, the probability of aphid colony establishment increased by 1. Though, both average number of Miridae ($X^2=4.5925$, $Df=1$, $p=.03211$) and the interactive effect of plant damage and average number of Miridae per plant was significantly correlated to the initial survival of aphid colonies ($X^2=4.6885$, $Df=1$, $p=.03037$). Neither light ($X^2=1.97152$, $Df=1$, $p=.1603$), treatment ($X^2=0$, $Df=2$, $p=1$), any of their interactive or additive effects influenced aphid initial survival.

Plant quality also dictated which of the colonies were able to survive for at least five days ($X^2=4.6091$, $Df=1$, $p=.0318$). Of plants which aphids were added to, only four plants still had aphids present after five days. Though plants were monitored for a longer period of time, no factors determining aphid success were detectable.

Discussion

The phenology of OTC plants did not advance significantly after 23 days and to move along the experiment we removed the chambers for the AP group only. There may have been no noticeable phenological effect because the chambers were not placed around plants early enough in the season. OTC have been found to advance bud burst (Gillespie, Jonsdottir, Hodkinson, & Cooper, 2013), and OTC were added when flowers were on the verge of bud burst with an average phenological score of 1.246627 ± 0.6629762 . Since the average OTC temperature increase of by 0.80744°C in this experiment was within a typical range of effective OTCs, of $0.4\text{-}1.8^\circ\text{C}$, duration and time of OTC installment is the

most plausible explanation for treatment groups lack of phenological advancement (Bokhorst et al., 2013; Cornelius, Heinichen, Drosler, & Menzel, 2014; Gillespie et al., 2013; Marion et al., 1997). Treatment groups were therefore indicative of different surrounding temperatures only.

As expected, census number impacted phenological stage, since plants advance in development through time. This indicates that the phenological scoring system accurately captured plant development. Since light significantly differed by plot location, using light as a factor in linear mixed models captures some environmental variation that is due to site location.

The correlation of Miridae abundance and phenology indicates that plant cues associated with flowering stages, such as volatile emission, may attract Miridae. The positive relationship of Miridae abundance and PAR is consistent with prior findings of increased insect abundance with greater light exposure (Mooney et al., 2016).

Damage

Throughout the experiment plant condition began to deteriorate due to Miridae feeding and lack of rain. We quantified damage to consider how the condition of the plant may be confounding insect observations and impact other results. The midway point of the first set of census data was used as the cutoff point for calculating average Miridae abundance on plants because at that point plants had been noted as damaged and any plant somewhat damaged was then allowed to continue deterioration until the time of damage quantification.

Aphids

Aphid colony establishment and survival five days after addition depended upon plant condition, and not on any abiotic factors. Thus, though aphids may be positively impacted by direct impacts of increased temperature, plant host quality had a greater impact on aphid abundance (Barton & Ives, 2014). Since Miridae abundance was positively correlated with plant damage, this suggests Miridae indirectly negatively affect aphid populations more so than a 0.807 °C temperature increase.

Volatiles

The results of volatiles were not back from the lab by the time of the end of the undergraduate research program. We expect the total quantity of volatile production to be greater during peak flowering, around phenology stage four to five. This would correlate with peak Miridae abundances and suggest that the volatiles attract Miridae to plants and be consistent with previous findings (Raguso & Weiss, 2015). It is unknown how the composition of volatiles will differ by phenological stage. Additionally, if particular compounds differ between plants which aphids were able to colonize versus not colonize, defensive compound released may interacting with plant damage to impact aphids.

Biotic factors, such as the abundance of an intraguild predator, Miridae, seem to impact ability of aphids to colonize host plants more so than the expected positive effects of increased temperature. Thus, as the climate warms and phenology advances, indirect biotic effects may be stronger than direct abiotic impacts on insect communities.

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Tables and Figures:

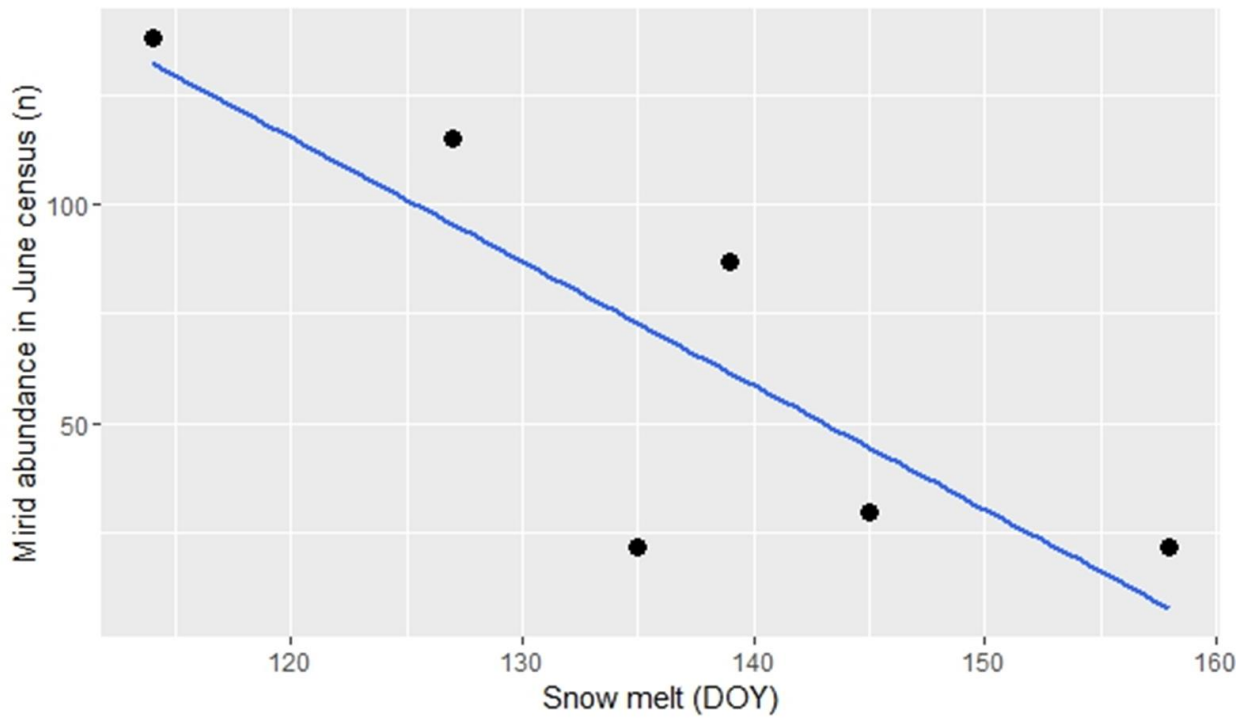


Figure 1: Miridae Abundance in June versus Snowmelt date. Each dot corresponds to a one year, where snow melt date data collected from Inouye, unpublished data.

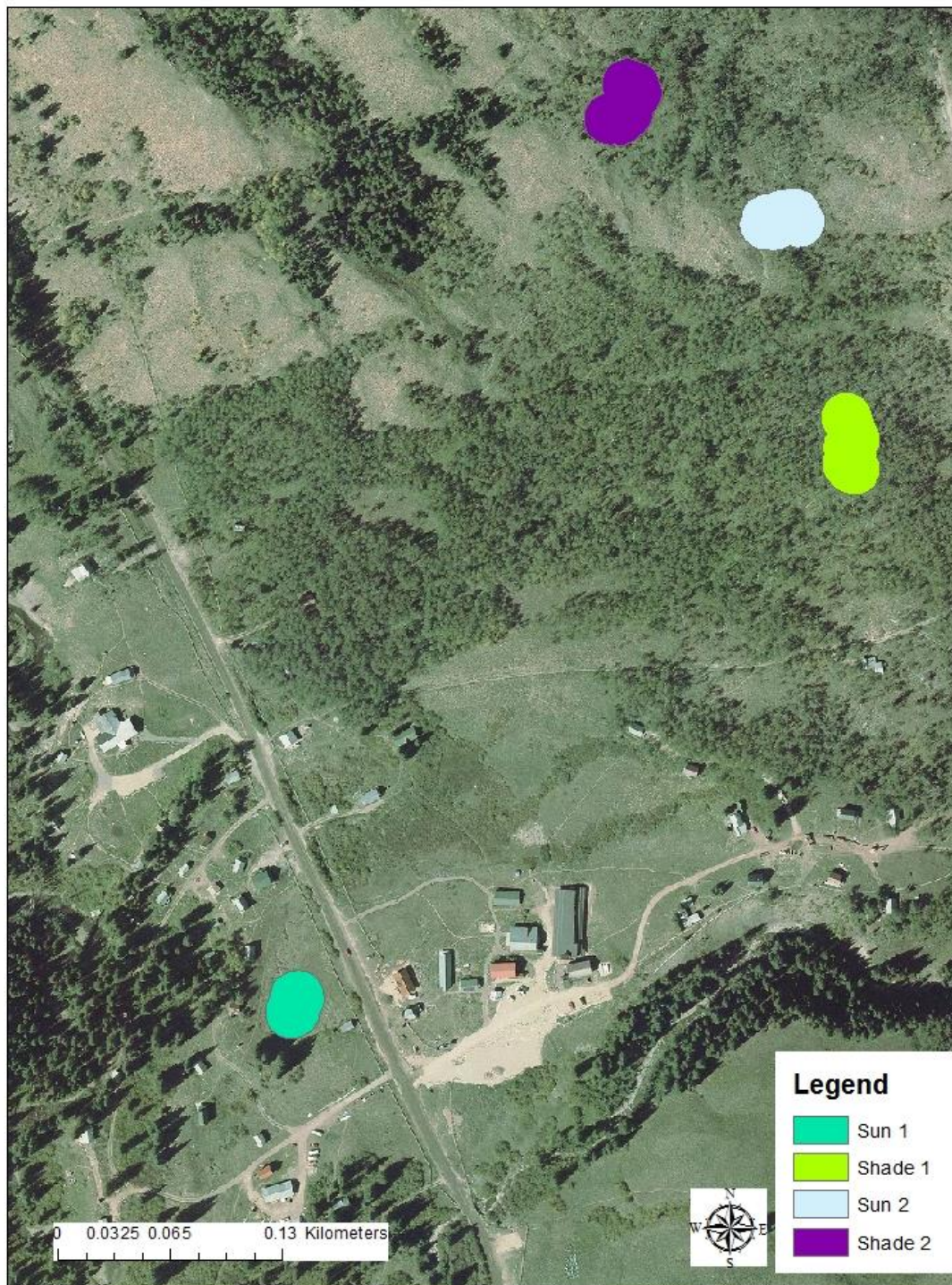


Figure 2: Site locations in relation to the Rocky Mountain Biological Laboratory in Gothic, Co.

Table 1: Phenological Scoring Guide

Phenology Score	Description
0	Compound umbel partially enclosed by bract. No separation of individual umbels. Tight buds.
1	Compound umbel separated from bract. Umbels are very close together and unable to be distinguished.
2	Umbels are separated. Some floral cracking evident.
3	Exterior flowers in full anthesis on the exterior of umbels. Flowers in the center of umbel are buds.
4	All flowers in full anthesis.
5	Petals fallen on exterior of umbel.
6	All petals shed.
7	Fruit developing
8	Fruit have elongated and widened
NA	Damaged flower/fruit

Table 2: Average temperature $\pm$ standard deviations of data recorded every five minutes using HOBO data loggers (OnSet Computers, Bourne, MA). Loggers were placed on randomly selected control and open top warming chamber (OTC) plants per plot.

Plot	Control (°C)	OTC (°C)	Difference (°C)
All	15.32919 $\pm$ 10.189	16.13662974 $\pm$ 11.44975828	0.80744
Sun 1&2	15.88122 $\pm$ 11.84861	17.08293 $\pm$ 13.69582	1.201718
Shade1&2	14.77683 $\pm$ 8.165311	15.16007 $\pm$ 8.427026	0.383239
Sun 1	15.08975 $\pm$ 12.23157	16.08231 $\pm$ 13.58904	0.992562
Sun 2	16.6224 $\pm$ 11.43602	18.01999 $\pm$ 13.73705	0.399421
Shade 1	15.21095 $\pm$ 8.408626	15.610367 $\pm$ 8.5073131	1.397587

Shade 2	14.370801 ± 7.9144085	14.7125813 ± 8.32768312	0.3417801
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Table 3: The average and standard deviation of photosynthetically active radiation (PAR) per study plot. PAR was measured over each plant with a light meter (LI-250A Light Meter, LiCor, Lincoln, NE).

Plot	Average Photosynthetically Active Radiation (PAR)
Sun 1	1321.467 ± 10.3863 $\mu\text{mol s}^{-1} \text{m}^{-2}$
Sun 2	1607.933 ± 46.0529 $\mu\text{mol s}^{-1} \text{m}^{-2}$
Shade 1	311.9683 ± 242.4549 $\mu\text{mol s}^{-1} \text{m}^{-2}$
Shade 2	268 ± 347.6283 $\mu\text{mol s}^{-1} \text{m}^{-2}$

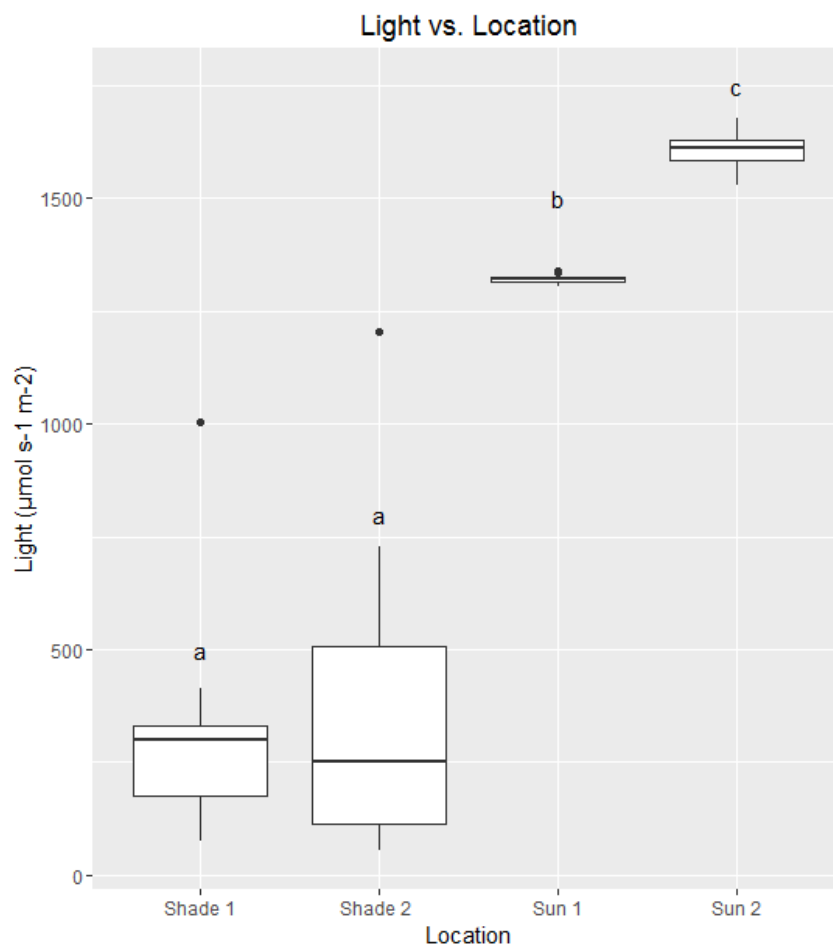


Figure 3: Photosynthetically Active Radiation (PAR) versus Plot Location.

The letters above the boxplots represent if plot locations differ from each other based on Tukey HSD post hoc analysis.

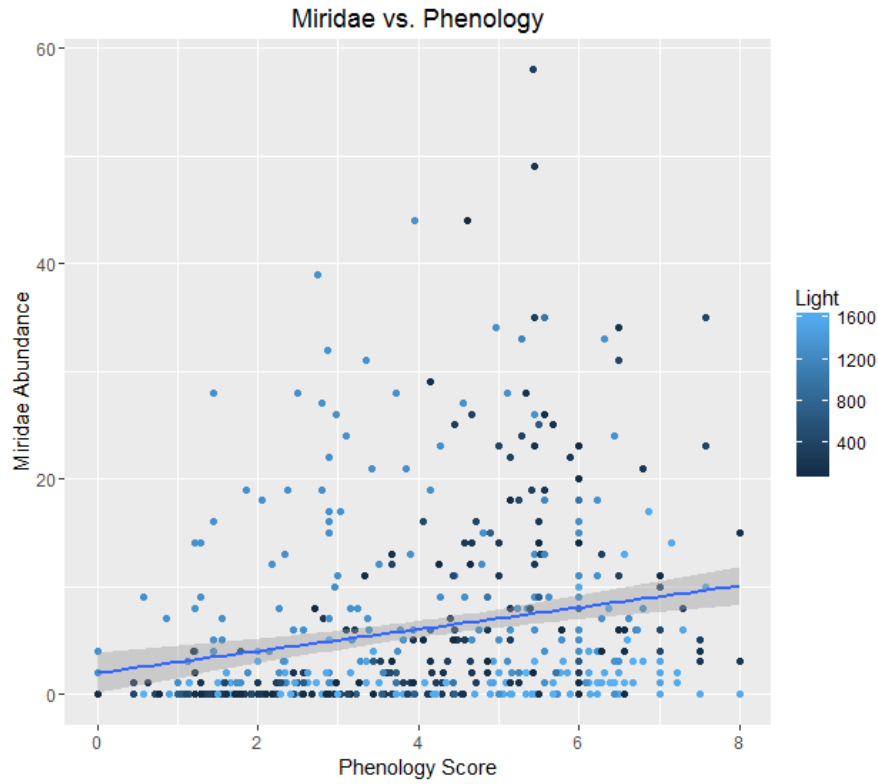


Figure 4: Miridae Abundance vs. Phenology Score. A linear regression line is displayed with the 95% confidence interval in grey.

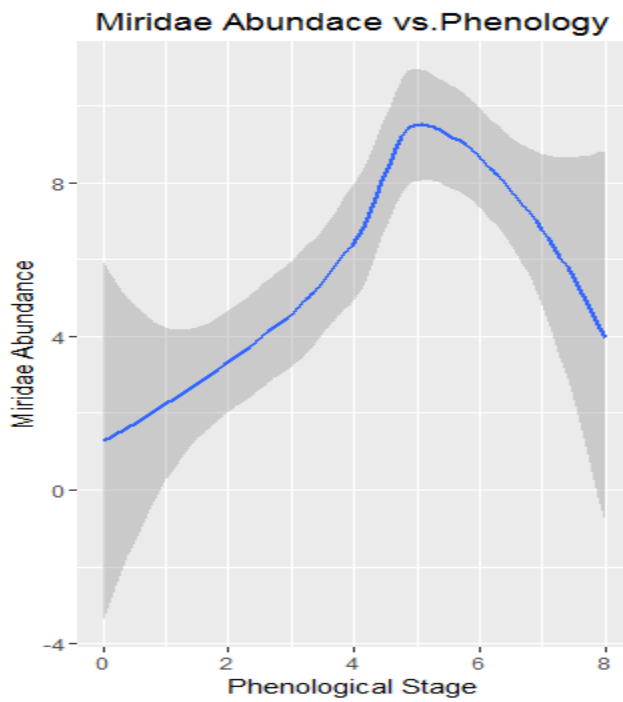


Figure 5: Miridae Abundance vs. Phenological Stage. A logically weighted polynomial curve is shown, with 95% confidence intervals in grey.

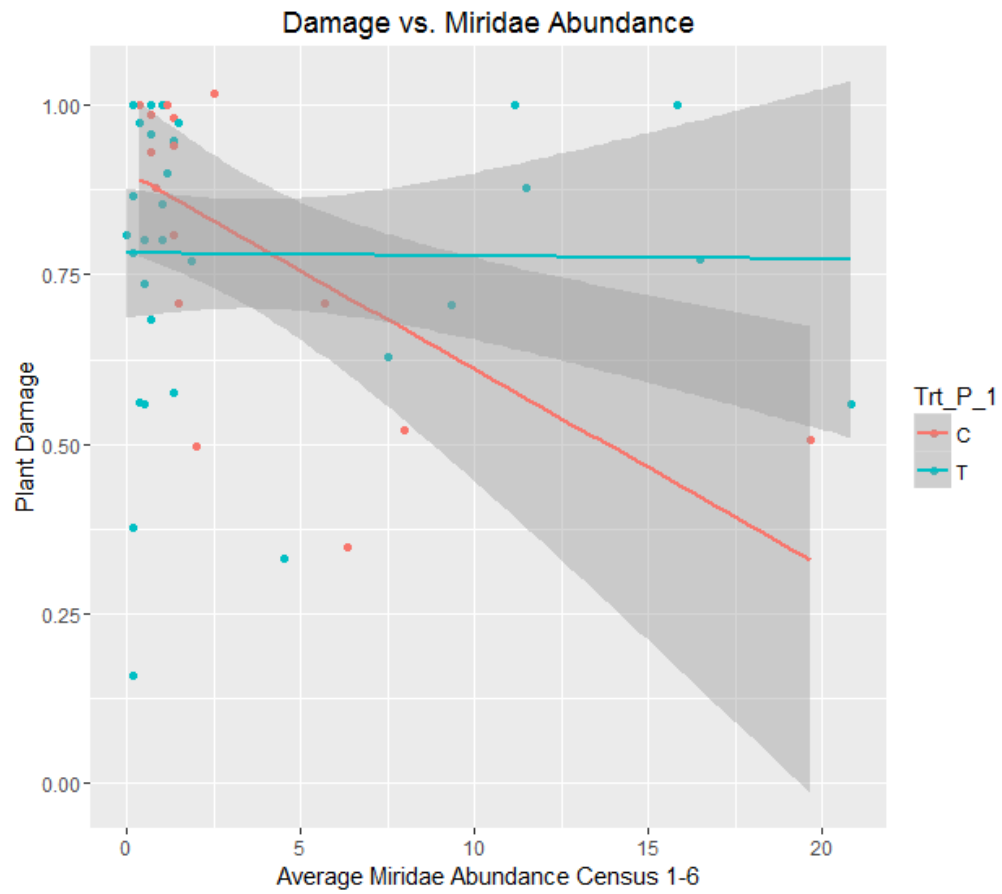


Figure 6: Damage vs. Miridae abundance with linear regression lines displayed for control and treatment plants. Grey regions represent 95% confidence intervals.