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The influence of plant modularity on the defense against specialist insect herbivory

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THE INFLUENCE OF MILKWEED MODULARITY ON DEFENSE AGAINST APHID HERBIVORY

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Abstract

Little work has been done to determine the relationship between modularity and chemical defense induction against herbivory in herbaceous plant species. We investigated this topic using *Asclepias syriaca*, common milkweed, and one of its specialist aphid herbivores, *Aphis nerii*. We studied milkweeds of varying modularities growing in the prairies of Pierce Cedar Creek Institute in Hastings, MI and introduced *A. nerii* to the plants, monitoring growth of plants and aphid populations over time. After *A. syriaca* leaves and *A. nerii* were harvested from study sites, leaf samples from aphid and control treatments were analyzed to measure variation with degree of modularity in cardenolide concentration, leaf hair length, leaf size, and aphid population growth rate. Although we predicted that plant chemical defense expression would be lower in more modular milkweed genets and aphid performance would be higher in these genets, we were unable to reject the null hypotheses that milkweed modularity has no effect on the level of plant chemical defense expression in response to aphid herbivory or on measures of aphid performance such as population growth rates or cardenolide sequestration. We conclude that the high variability in aphid treatments among replicates was too high to detect responses. We also conclude that our aphid "sinks" for leaf phloem resources were too subtle to induce leaf defenses and detect significant responses in both plants and aphids to variation in milkweed modularity.

Introduction

Here, we describe experiments to measure the influence of plant modularity on defense against specialist insect herbivory using a specialist aphid feeding on a highly modular milkweed species in the genus *Asclepias* (Apocynaceae). While we know that modular, clonal trees in Finland can show long term induction of chemical defenses in response to insect herbivores (Honkanen and Haukioja 1998), we know little about the relationship between modularity and chemical defense against herbivores in smaller, more ephemeral and less apparent (Feeny 1976) herbs. Many herbaceous plant species show considerable degrees of modularity with plants growing as a single individual, or genet, comprised of many stems or ramets. Notable examples in the US Midwest include common goldenrod, *Solidago altissima* (Heath *et al.* 2014), and common milkweed, *Asclepias syriaca* (Malcolm *et al.* 1989), that both grow in large and conspicuous, single-species patches.

Although much is known about basic chemical defenses in these plants (Agrawal 2007), nothing is known about how modularity influences chemical defense investment and expression. Given this obvious gap in our knowledge about plant defenses, we focused on the interaction between the common milkweed, *Asclepias syriaca*, and one of its specialist aphid herbivores, *Aphis nerii*, because the common milkweed is the most modular of all the approximately 130 *Asclepias* species in North America (Woodson 1954; Malcolm *et al.* 1989; Wyatt and Broyles 1994; Van Zandt and Agrawal 2004; Agrawal *et al.* 2009). Common milkweeds have multiple ramets (aboveground shoots) that make up the genet, so that a single plant looks like a patch of plants of the same species.

More modular genets have more ramets and in the case of the common milkweed, *A.*

syriaca, the plant has evolved underground stems that bud to produce the aboveground stems or ramets and high levels of modularity so characteristic of this widely distributed species (Woodson 1954; Malcolm *et al.* 1989). These underground stems generate what we call "unconstrained" modularity; if grown undisturbed, the species may produce extremely large genets that may have hundreds to thousands of ramets. Other *Asclepias* species show "constrained" modularity with the number of ramets constrained to considerably smaller genets by the size of the root mass (Woodson 1954).

Milkweeds produce steroidal toxins called cardenolides as a chemical defense that can be induced in response to herbivory (Malcolm, 1991), but it is not known whether these defenses vary with modularity. *Aphis nerii* is a common, phloem-feeding milkweed specialist that is conspicuously colored yellow and black to signal to predators that it sequesters these toxins from milkweeds and uses them for their own chemical defenses against natural enemies (Malcolm 1986, 1989, 1990, 1991, 1992; Agrawal and Malcolm 2002; Agrawal *et al.* 2004; Martel and Malcolm 2004; Mooney *et al.* 2010).

Here we describe our research on the influence of milkweed modularity on aphid herbivory by using genets of the common milkweed as independent replicates across a range of modularities. Common milkweed at the Pierce Cedar Creek Institute (Hastings, MI) grows in patches that vary widely in size, and each of these patches represent a single genet.

We expected variation among genets in both constitutive and inducible chemical defense expression because the probability of a single ramet being attacked by an herbivore should be lower in a large genet with many ramets than in a small genet with few ramets. In addition, we expect aphid population growth to occur at a higher rate in highly modular milkweeds, and at a lower rate in less modular milkweeds. We test the null hypotheses that milkweed modularity has

no effect on the level of plant chemical defense expression in response to aphid herbivory or on measures of aphid performance such as rates of increase or cardenolide sequestration.

Methods

Milkweed surveys. The distribution of most *A. syriaca* genets at Pierce Cedar Creek Institute in Hastings, MI was not known prior to this study. Therefore, starting on June 12, 2017, we began surveying the property for common milkweed genets and gave each individual genet its own unique number, or genet ID. A genet was defined as a visually distinct cluster of ramets. For each genet we recorded GPS coordinates, circumference of the genet, and number of ramets per genet. To gather information on ramet characteristics, we took measurements of ramet height, number of leaves per ramet, distance to nearest neighbor of each ramet, reproductive stage (budding, flowering, producing seed pods, or absence of seed pods), and specialist insect herbivores present on each ramet. For genets with greater than, or equal to, five ramets, we recorded these data for five ramets per genet. For genets with less than five ramets, we recorded these data for all ramets in the genet. This survey took approximately one month.

We then determined the most suitable genets to investigate the effects of modularity on chemical defense induction via introduced aphid herbivory. The most suitable genets fit under the criteria of ± one standard deviation from the average genet in characteristics of ramet height, leaf number, and herbivore density per genet. The most suitable genets also did not show signs of budding or flowering at the time of survey. From a list of suitable genets, 20 experimental genets were randomly selected for aphid introduction. These genets ranged in modularity from 1 ramet per genet to 105 ramets per genet.

We also surveyed the milkweeds for all insect herbivores so that we had measures of background herbivory at PCCI. Michigan has a total of 11 specialist herbivore species that are classified within six different feeding guilds that feed on *A. syriaca.* Among these specialist herbivores is the common phloem-feeding milkweed specialist Winged alate adult *A. nerii* are only produced when necessary for dispersal at high density (Agrawal 2004; Agrawal *et al*. 2004; Zehnder and Hunter 2007; Tao and Hunter 2013), and therefore their presence can be used as a criterion for aphid population density at carrying capacity.

***Aphis nerii* treatment -** On July 11, 2017, we introduced one alate adult *A. nerii* to each of our 20 experimental genets, which ranged in modularity from 1 to 105 ramets per genet. *A. nerii* were kept in culture on a single ramet of *A. syriaca* by the main building at PCCI, originating from a culture kept by the authors at Western Michigan University in Kalamazoo, MI. Alates were constrained in a circular clip cage of approximately 7 cm^2 on the underside of one leaf of *A. syriaca*. This leaf was identified as leaf 1 of the genet – the topmost, fully expanded leaf. Clip cages were also attached to the leaf opposite the alate-containing leaf, and on one leaf of a different ramet within the genet. These leaves were identified as leaves 2 and 3 respectively. Leaf 4 was identified as the leaf opposite leaf 3, and did not have a clip cage attached to the leaf. Data were recorded on ramet height (five ramets per genet in genets with five or more ramets, and all ramets per genet in genets with less than five ramets), leaf number of select ramets, stem diameter of select ramets, reproductive characteristics of selected ramets, and herbivore presence on selected ramets. Ramets were selected randomly on the first day of aphid introduction, then surveyed consistently afterward. Photos of leaves 1-4, with a ruler for scale, were taken for leaf area analysis using ImageJ. These data were collected every other day, along

with recording aphid population by identifying the numbers of each of the five *A. nerii* instars and any new alate production in the clip cage on leaf 1 of each genet.

Whole leaves were harvested with clip cages still attached when either (A) the *A. nerii* population in a clip cage became large enough that a new winged alate individual was produced, or (B) after 25 days of introduced aphid presence on the plant. Exceptions were made to option B when necessary, partly because some treatments had to be restarted due to aphid mortality. Leaves were harvested by cutting the base of the petiole using a sharp blade. Latex was allowed to drip out of the petiole until it stopped or significantly slowed. Then leaves were placed in individual plastic bags, labeled with both genet ID and leaf number. In the field, leaves were stored in a cooler filled with ice packs, then transferred to a freezer after completing all fieldwork for the day.

Leaf area analysis. Leaf area was analyzed using ImageJ (https://imagej.nih.gov/ij/) both for leaves in the field and leaves after harvesting. Measurement of leaf area in the field was inconsistent because it was difficult to keep both the plane of a leaf and a camera parallel with an included scale. Thus we analyzed leaf area from digital images taken post-harvest on a lab bench with a camera on a photographic stand and an included scale.

Leaf hair analysis. Leaf hair length was measured by removing a 1 cm^2 disc from a frozen wet leaf with a cork borer. A 1 mm-wide strip was then cut from the leaf disc using two parallel razor blades. The leaf strip was then set on its side under a microscope so the leaf hairs were visible, then a digital image of the enlarged leaf strip was captured and leaf hair length was measured in ImageJ. Leaf discs were stored between glass microscope slides and kept at 0°C between periods of analysis.

Water content measurement. Masses of harvested leaves and aphid bodies were recorded prior to lyophilization to determine their wet weight. After drying (described in detail in the next section) masses of harvested leaves and aphid bodies were again recorded to determine their dry weight. Subtracting the dry mass from the wet mass gives the water content of the material.

Cardenolide extraction. Cardenolides were extracted from both *A. nerii* and *A. syriaca* leaves. Using a paintbrush, *A. nerii* were removed from leaves into the clip cage within which they had been contained. The clip cage was then placed in the lyophilizer and the sample was dried. Once dry, each sample was weighed and ground with 4 ml methanol in an 18 x 150 mm glass tube using a motorized homogenizer. The methanol extract was poured into a labeled centrifuge tube. The homogenizer post was rinsed with 2 ml methanol and added to the centrifuge tube. Samples were vortexed and then sonicated in a 55°C water bath for 10 minutes. Samples were centrifuged at high speed for 10 minutes, and the supernatant was poured into a labeled 13 x 100 mm glass tube. The precipitate was washed with 2 ml methanol, vortexed, then centrifuged again for 10 minutes. This supernatant was poured into the same labeled 13 x 100 mm glass tube. The labeled glass tubes containing 6 ml methanolic extract each were placed in a nitrogen evaporator at 60°C until dry. The dried extract was resuspended in 1 ml acetonitrile, vortexed, and sonicated for one minute. The solution was then filtered through a 0.45 μm Luer-lock syringe filter into a 1 ml autosampler vial ready for HPLC analysis. This procedure was repeated for all aphid samples.

To prepare leaves for cardenolide extraction, leaves in their frozen state were cut in half down the midrib, and each half was "feathered" by making three to four diagonal cuts along the leaf. The leaf was labeled using a piece of tape attached to the petiole of both halves of the leaf.

Leaves were placed in a -80°C freezer for 15 minutes, then moved to a lyophilizer. After two days in the lyophilizer, leaves were moved to a 50°C oven to dry completely. Once a leaf was dry, it was taken from the oven and weighed to determine its dry weight. One half of the leaf was kept intact in a petri dish, while the other half of the leaf was ground using a mortar and pestle to produce a fine powder. Around 0.2g of ground leaf powder was weighed and added to a labeled 15 ml centrifuge tube. This was repeated for each leaf harvested, only varying when a leaf was so small that both halves needed to be ground to reach 0.2g leaf material. To the ground leaf material in the centrifuge tube, the cardenolide extraction process continued for leaf material as outlined for aphid material above. This procedure was repeated for all leaf samples.

Cardenolide analyses of plant and insect extracts in acetonitrile were based on the method of Wiegrebe and Wichtl (1993) on a Waters gradient HPLC system with WISP autosampler, 600E pump, 996 diode array detector and Millennium 2010™ chromatography software. The reverse-phase elution gradient was acetonitrile:water at 1.2 $ml \cdot min^{-1}$ at 40˚C, with 20% acetonitrile at start, 32% after 35 min., 40% after 45 min., 50% after 55 min., then back to 20% at 61 min., and 20% at 65 min., on a 250-4 LiChroCART® RP-18 column packed with LiChrospher® 100, 5µm (E. Merck) with a 10 mm guard column. 20 µl sample injections were separated for 65 minutes with 10 minute equilibration intervals between samples and cardenolides were detected at 218.5 nm and identified by their symmetrical spectra between 205 and 235 nm and a λ_{max} of between 214 and 224 nm. Cardenolide concentration for each peak (µg/0.1g sample DW) was calculated from a calibration curve with the external cardenolide standard digitoxin (Sigma, St Louis, Missouri). Only cardenolide peaks reported by Millennium 2010® software as consistently pure were considered for analysis.

Statistical analyses. Data were analyzed with JMP version 13.0 (SAS Institute 2016) using analyses of variance (ANOVA) and analyses of covariance (ANCOVA) among regressions of plant and aphid variables against milkweed genet size for ramets treated with aphids and 3 controls.

Results

We found 208 genets of *A. syriaca* at PCCI and these were located almost exclusively in open grassland or restored prairie habitats as shown in Figure 1. The frequency distribution of ramets per genet showed the expected negative binomial distribution with almost half of the genets being comprised of 1 to 5 ramets per genet (Figure 2A). The largest 3 genets had 250, 278 and 300 ramets and the mean ramet density among genets was 4.17 ramets/m^2 (±0.36SE with a range from 0.18 to 25.13 ramets/m^2).

Among the 208 genets we found 327 individual insect herbivores distributed among 8 species in 4 feeding guilds (Figure 2B). Leaf chewers included larvae of the monarch butterfly *Danaus plexippus* (Lepidoptera, Danaidae), adults of the longhorn beetle *Tetraopes tetrophthalmus* (Coleoptera, Cerambycidae), and adults of the weevil *Rhyssomatis lineaticollis* (Coleoptera, Curculionidae); phloem suckers included the aphids *Myzocallis asclepiadis* and *Aphis asclepiadis* (Homoptera, Aphididae); larvae of the fly *Lyriomyza asclepiadis* (Diptera, Agromyzidae) are leaf miners; larvae of the weevil *R. lineaticollis* are stem borers; and the lygaeid bugs *Lygaeus kalmii* and *Oncopeltus fasciatus* (Hemiptera, Lygaeidae) are seed predators. We did not find *Aphis nerii* feeding naturally on *A. syriaca* genets at PCCI.

The randomly selected experimental genets ranged in size from 1 to 105 ramets with a mean ramet number of 17.94 (±6.16SE) and a mode of 2.0 that reflects the binomial distribution

of all genet sizes. Within these experimental genets leaves with aphids were larger than the leaf directly opposite on the same ramet (wet weights of leaves with aphids compared with 3 controls, ANOVA $F_{3.67} = 3.54$, P = 0.02 (Figure 3A), and areas of leaves with aphids compared with 3 controls, ANOVA $F_{3.67} = 4.88$, P = 0.004 (Figure 3B)).

The lower or abaxial leaf surface of *A. syriaca* is obviously hairy, with a dense mat of trichomes and although there was a trend towards longer leaf hairs on leaves with aphids, the differences in leaf hair lengths among the aphid and control treatments were not significant (Figure 4, ANOVA $F_{3,67} = 0.55$, P = 0.65).

Similarly, when we measured the cardenolide concentrations of aphid and control leaves there were no significant differences among the four treatments (Figure 5, ANOVA $F_{3,67} = 1.09$, P = 0.36), although there was a trend towards some induction of cardenolide in leaves on which aphids were caged (Figure 5). The cardenolide concentrations of leaves from aphid and control treatments were regressed against ramet number and comparisons among the regressions by ANCOVA also failed to detect any significant differences among the treatment slopes (Figure 6, whole model ANCOVA $F_{7,67} = 0.84$, P = 0.56). Neither treatment (F=1.05, P = 0.38) nor ramet number (F = 2.41, P = 0.13) had a significant influence on leaf cardenolide. While the regression for the aphid treatment does have the highest intercept on the cardenolide axis, we think the lack of significance among the treatments is mostly driven by the large variation in rates of increase of the aphids in the clip cage treatment. The final harvested aphid dry weights show considerable variation (Figure 7) despite similar lengths of time on ramets in the different genet sizes. These when the final harvested aphid dry weights are plotted against ramet number (Figure 7) the large variation precludes any significant linear relationship (Aphid dry weight (g) = 0.0048 - 2.98e-5*Ramet number, ANOVA $F_{1,16} = 0.86$ NS).

In order to compare aphid growth rates among genets of different sizes, we calculated a simple measure of population increase as r_{max} based on the natural logarithm of maximum aphid density in the 7 cm^2 clip cage divided by the time in days to reach that number from the single alate aphid that initiated the experiment (r_{max} = LN(maxN/days)). Aphid population growth rates (r_{max}) showed no significant relationship with milkweed ramet number (Figure 8A, $F_{1,17} = 0.47$, P = 0.50). The aphid r_{max} also showed no relationship with aphid cardenolide concentration (µg/0.1g) (Figure 8B, $F_{1,17} = 0.78$, P = 0.39) and so there seemed to be no measurable cost to the aphid of sequestering cardenolides from the phloem of *A. syriaca.* We also found no relationship between aphid cardenolide concentration (µg/0.1g) and host plant ramet number (Figure 8C, $F_{1,17} = 0.52$, P = 0.48), although the aphids with the highest cardenolide concentrations were found on the smallest genets.

Discussion

In this experiment, we investigated whether *A. syriaca* chemical defense induction varied with regard to genet size, using *A. nerii* as experimental herbivores. While it is well known that *A. syriaca* chemically defends itself with toxic cardenolides, it is not known if larger genets of *A. syriaca* were more or less chemically defended than smaller genets. By introducing experimental populations of *A. nerii* to a range of representative *A. syriaca* individuals, monitoring aphid and plant growth over time, and collecting material for chemical analysis, we hoped to fill this gap in our knowledge.

Our results showed a significant difference between the size of aphid treatment leaves compared to control leaves (Figure 3). We suspect that the aphids, as phloem feeders, may induce the leaves to photosynthesize at a more rapid rate as the plant detects lower levels of

photosynthetic nutrients in the leaf. However, it is difficult to say definitively whether there is a biological explanation behind this difference, as we selected aphid treatment leaves with partiality to large size in order to support the clip cage. For future studies, we suggest selecting unbiased, random leaves on each ramet for aphid introduction in order to further test this relationship.

Other areas of our research did not generate significant differences, but did yield data that suggested trends toward supporting our alternative hypothesis that constitutive and inducible chemical defense expression of the milkweeds would vary with modularity. Although cardenolide levels were not significantly different between the four leaf treatments, aphid treatment leaves did have noticeably higher mean cardenolide levels than control leaves (Figure 5). Moreover, mean abaxial hair length of both aphid and intra-ramet control leaves is noticeably longer than the inter-ramet and opposite control leaves (Figure 4). In addition, with ANCOVA analysis (Figure 6), it can be seen that the aphid treatment regression is higher than the cluster of control regressions with lower intercepts on the y-axis. The aphid treatment regression does have a statistically significantly higher y-intercept than the controls, again suggesting support for our alternative hypothesis. Overall, however, our data are not strong enough to reject the null hypothesis. We think that the driving factor behind our statistically insignificant results is that the size of the aphid populations in comparison to leaf size were too small for the plants to produce a significant response. Thus the source:sink ratio between *A. syriaca* leaves and *A. nerii* was too high. Many of the aphid replicates also failed to reproduce as can be seen in Figure 7 of the harvested aphid weights showing very wide variance, despite having started each replicate with a single alate and allowing each replicate to reproduce for the same amount of time.

We have several suggestions to improve this experiment. Aphid population growth was unexpectedly slow in this experiment, and few populations reached carrying capacity within the clip cage as indicated by the production of new alates as a density dependent signal of carrying capacity in the clip cage. Had this been anticipated, we may have selected a greater number of replicate genets for aphid treatments, or introduced more alates per genet within one clip cage, then chose to study in more detail the genets whose aphid populations grew at a rapid rate. We also may have chosen to study more genets overall, increasing the sample size and potentially lowering standard error. We also believe it would have been beneficial to study more genets with 30 to 100 ramets in order to analyze a more balanced dataset, although genets of that size were rarer at PCCI than smaller genets. Additionally, we think that constraining aphids to a whole leaf on one ramet using a leaf-sized mesh bag, rather than a smaller clip cage, may allow for better aphid performance and increased plant response from a smaller source:sink ratio.

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Figures

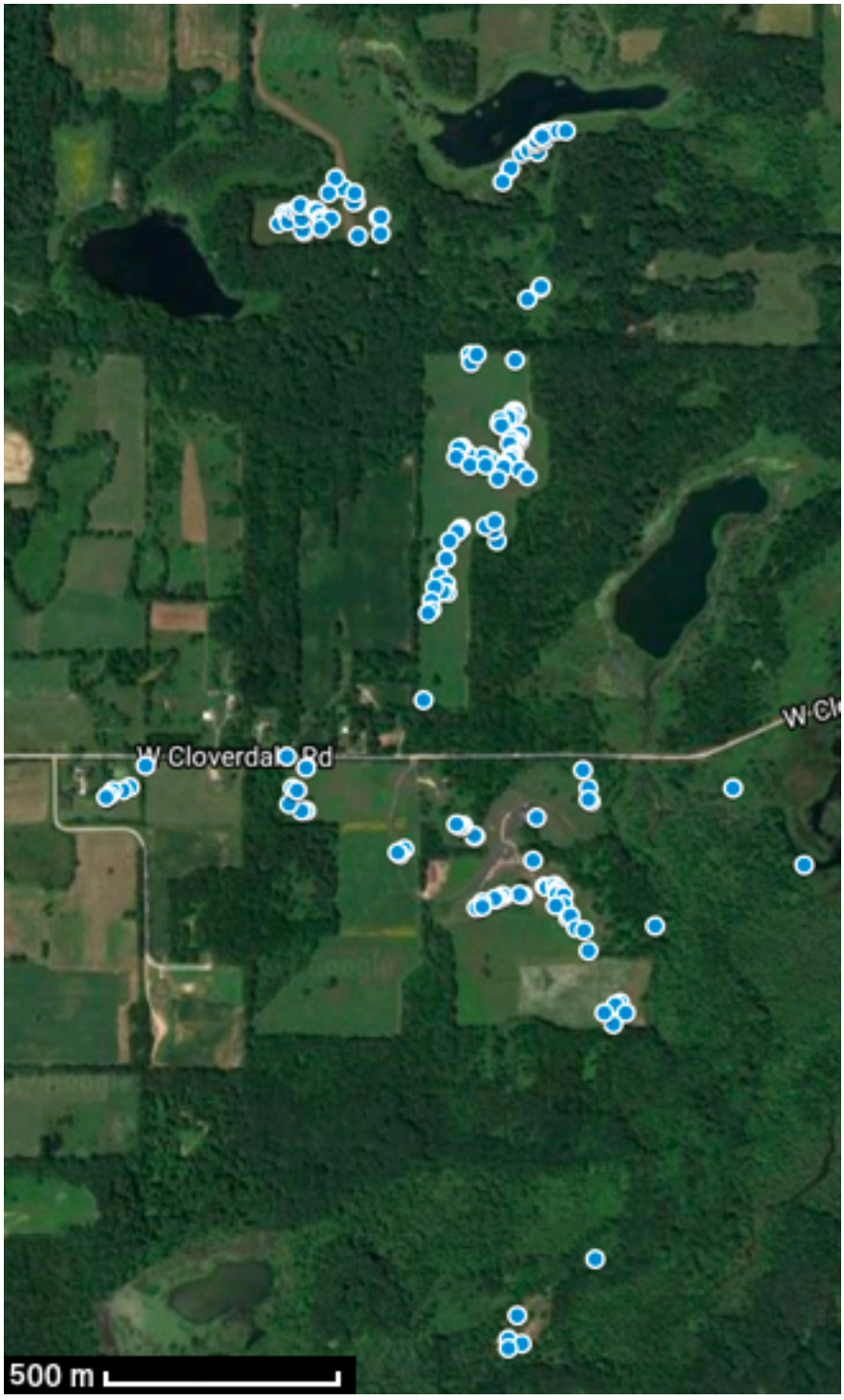


Figure 1. Distribution of 208 genets of the common milkweed, *Asclepias syriaca,* at Pierce Cedar Creek Institute, June 2017.

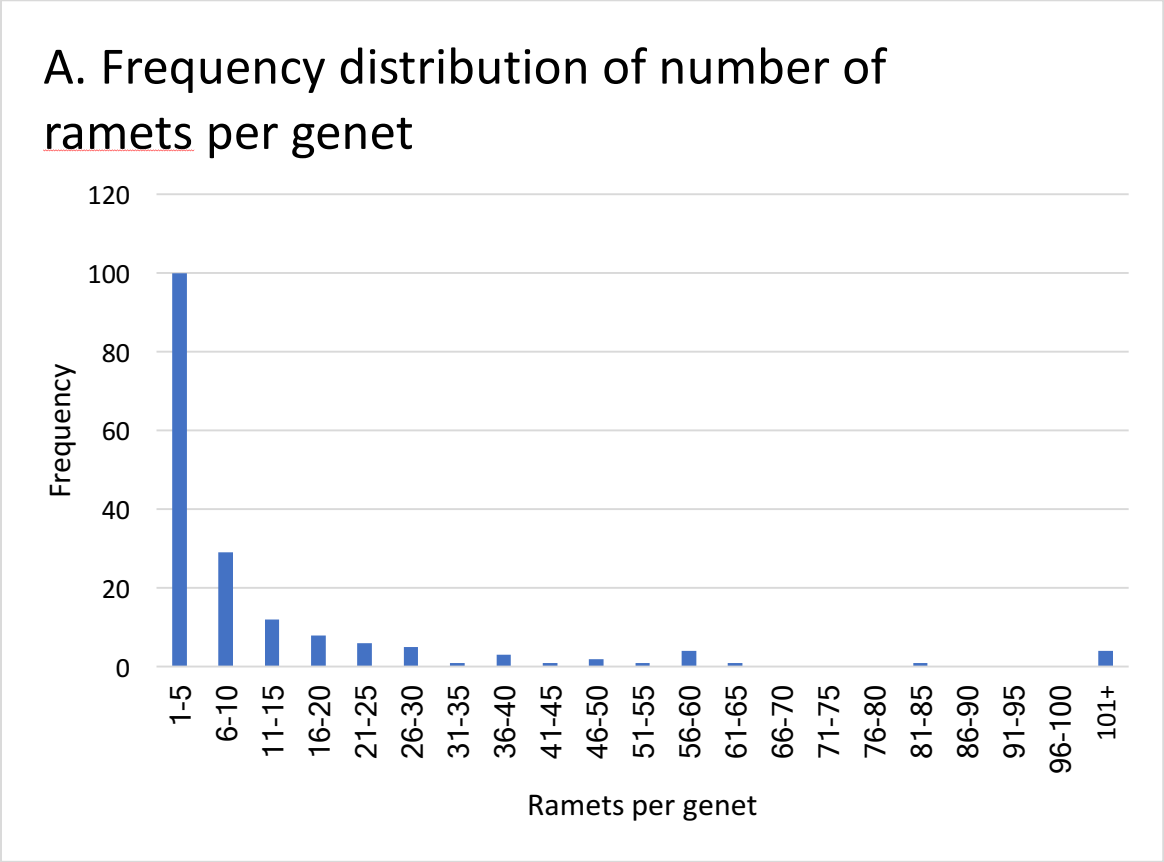


Figure 2. (A) Frequency distribution of 2924 ramets distributed among 208 genets at PCCI.

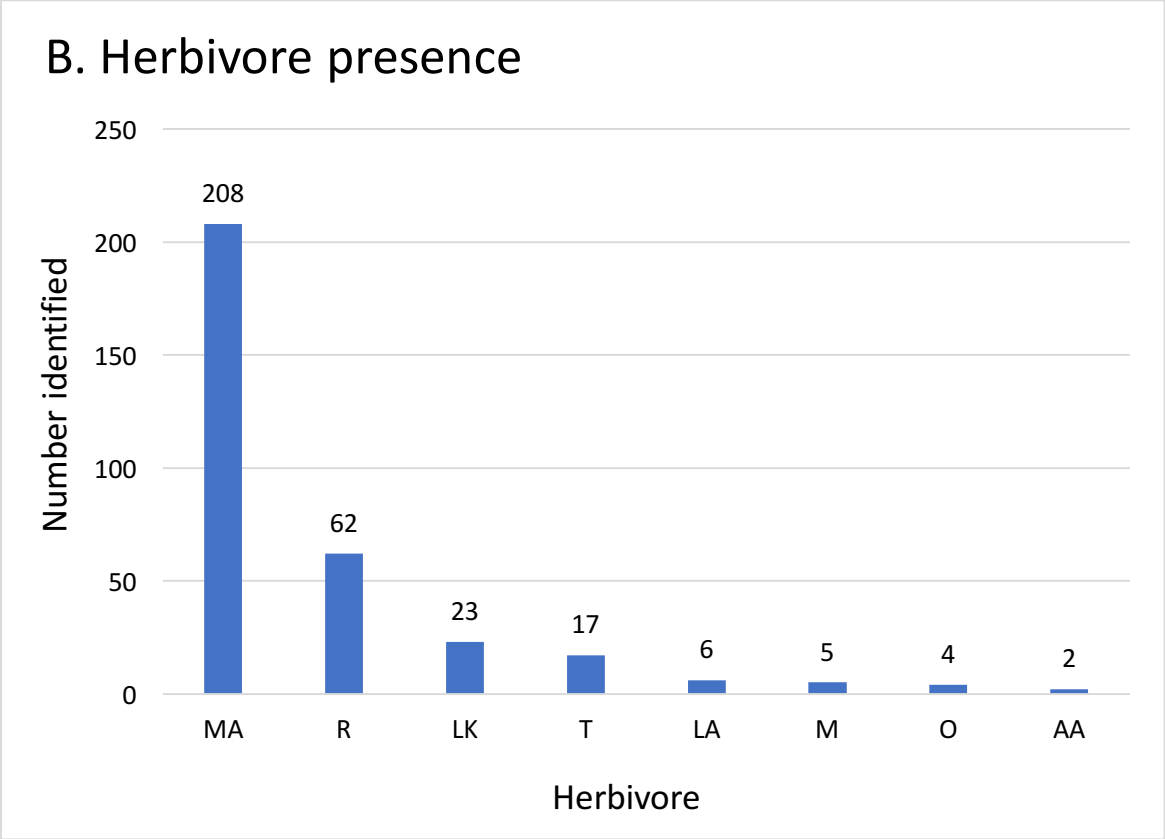
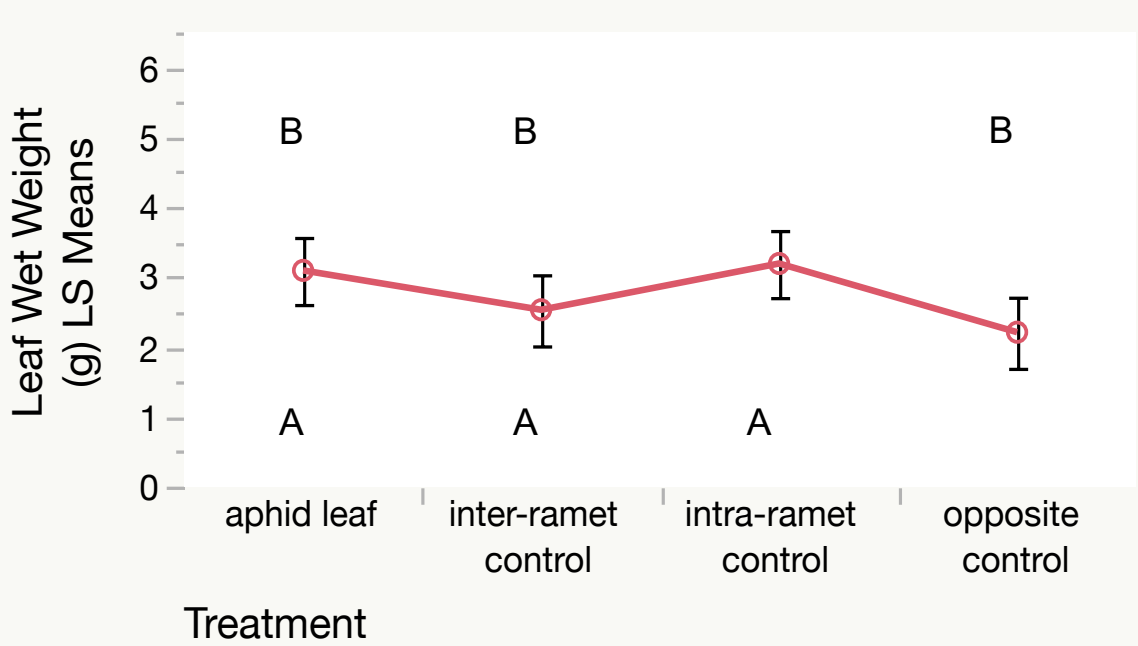


Figure 2. (B) 327 insect herbivores, from 8 different species were identified on ramets of *A. syriaca* at PCCI. MA = *Myzocallis asclepiadis* (Homoptera, Aphididae), R = *Rhyssomatis lineaticollis* (Coleoptera, Curculionidae), LK = *Lygaeus kalmii* (Hemiptera, Lygaeidae), T = *Tetraopes tetrophthalmus* (Coleoptera, Cerambycidae), LA = *Lyriomyza asclepiadis* (Diptera, Agromyzidae), M = *Danaus plexippus* (Lepidoptera, Danaidae), O = *Oncopeltus fasciatus* (Hemiptera, Lygaeidae), AA = *Aphis asclepiadis* (Homoptera, Aphididae).

A. Leaf wet weight



B. Leaf area

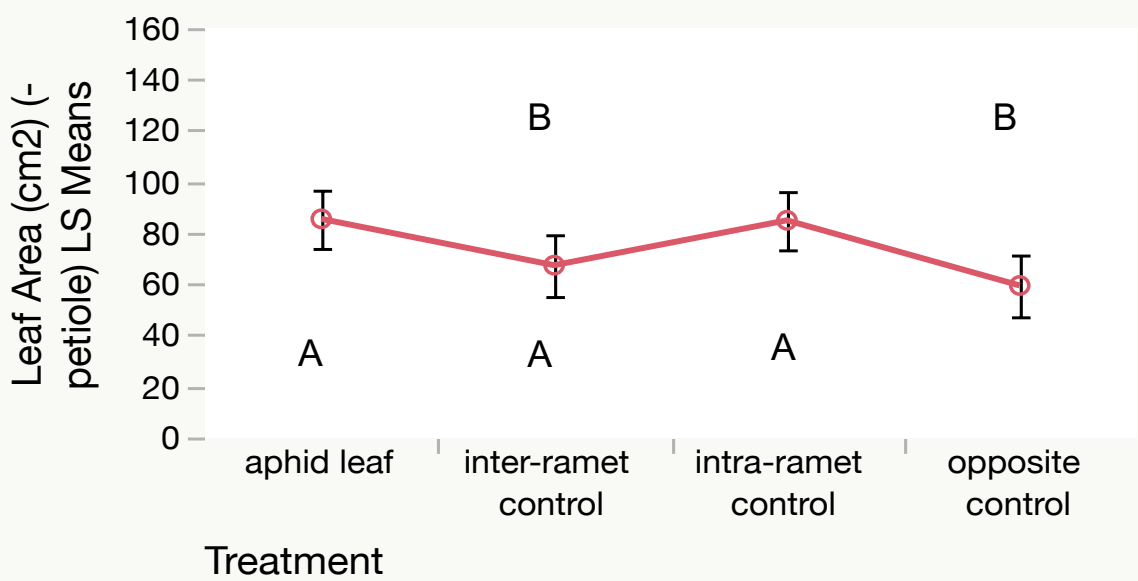


Figure 3. Comparisons of least square mean (±SE) leaf wet weights (A) and leaf areas (B) among aphid and 3 control treatments. Letters represent significant differences from Tukey comparisons among means.

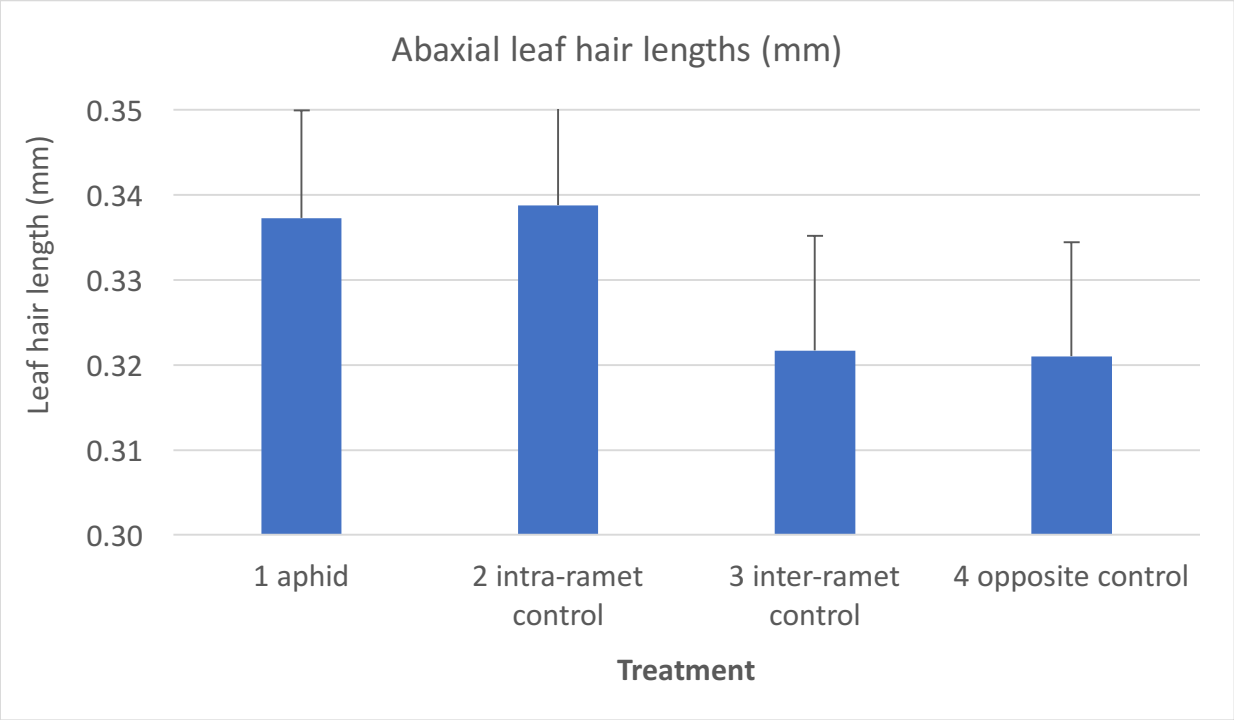


Figure 4. Least square means (±SE) of abaxial leaf hair lengths (mm) in aphid and control treatments.

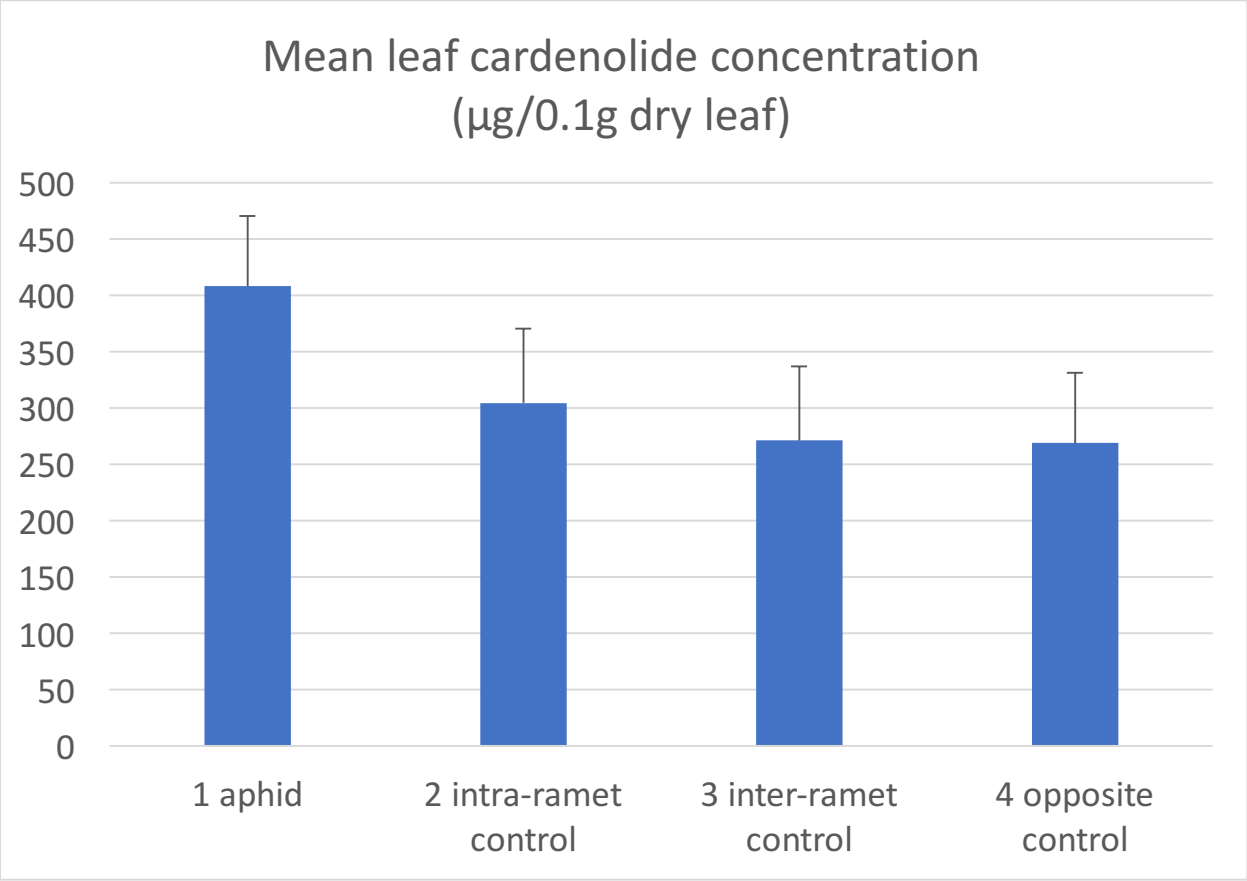


Figure 5. Mean leaf cardenolide concentration (µg/0.1g dry leaf ±SE) in ramets from aphid and 3 control treatments. There were no significant differences among the data distributions ($F_{3,67} = 1.09$, $P = 0.36$), despite the trend for some induction of cardenolides in leaves with aphids.

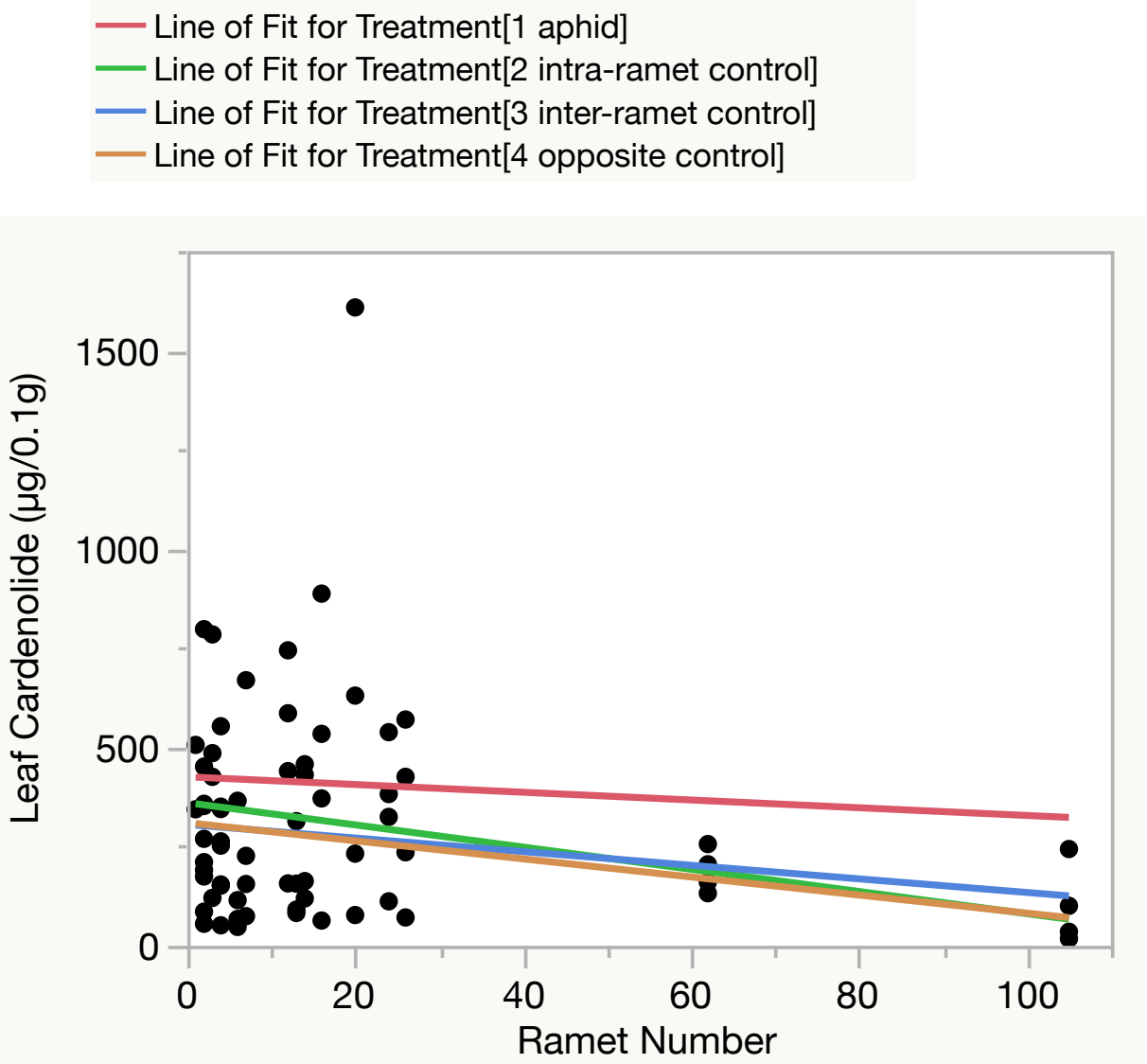


Figure 6. Linear regressions of leaf cardenolide (µg/0.1g dry leaf) against ramet number for aphid and 3 control treatments. There was no significant effect of ramet number on leaf cardenolide among the aphid and 3 control treatments (see Results)

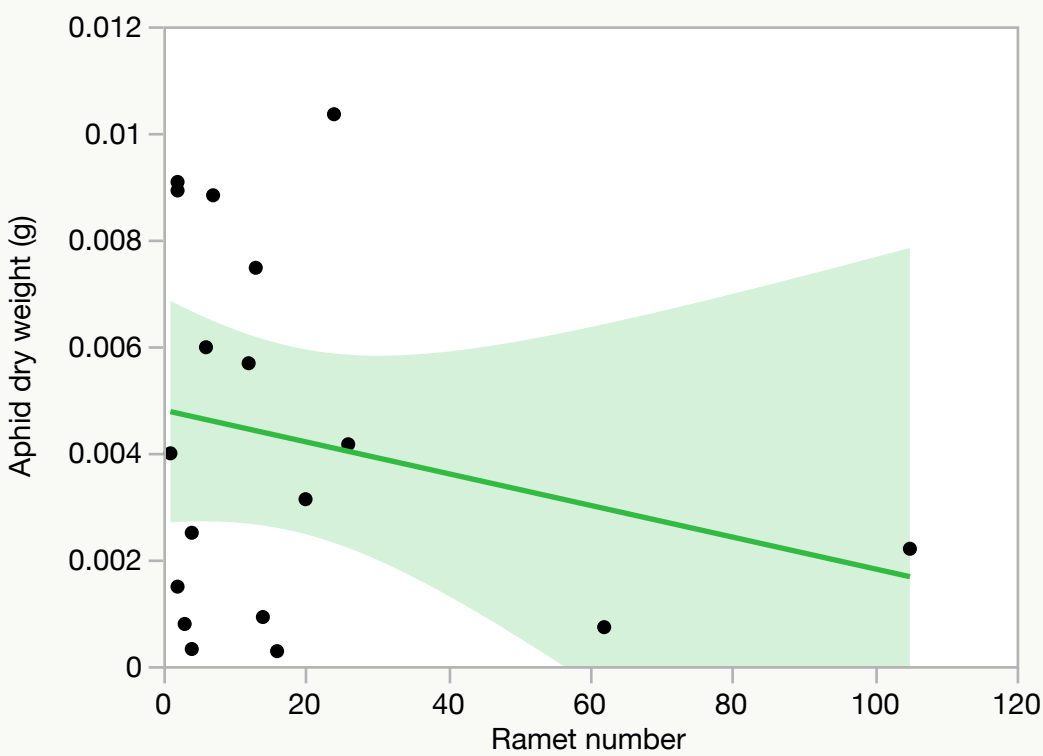
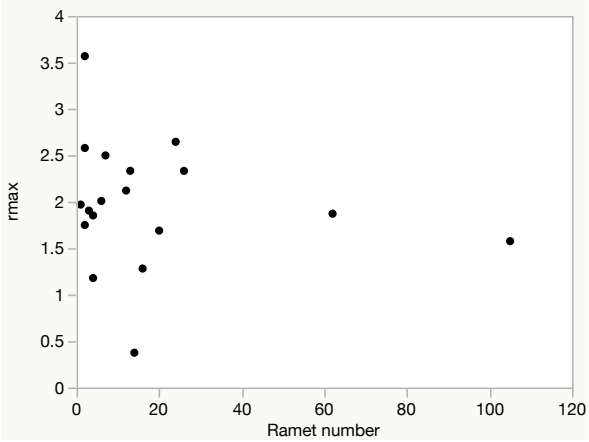
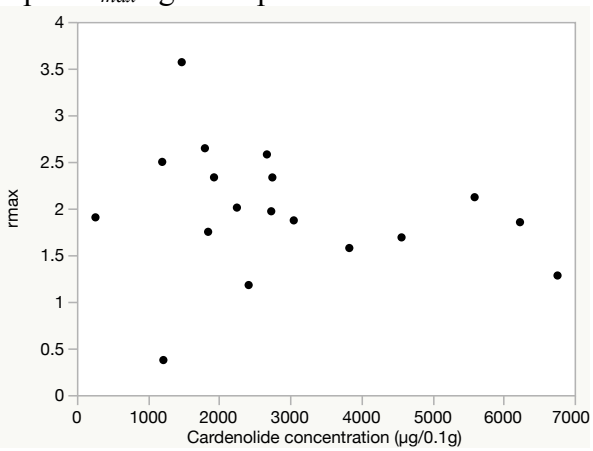


Figure 7. Relationship between dry aphid biomass (g) and number of ramets per genet.

A. Aphid r_{max} against host plant ramet number



B. Aphid r_{max} against aphid cardenolide concentration (µg/0.1g)



C. Aphid cardenolide concentration (µg/0.1g) against host plant ramet number

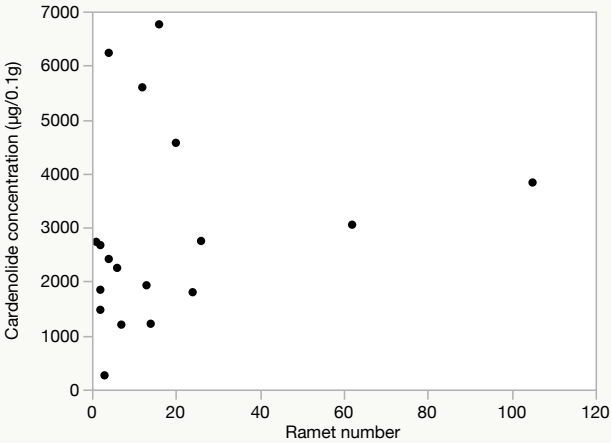


Figure 8. (A) Aphid r_{max} against ramet number, (B) Aphid r_{max} against aphid cardenolide concentration (µg/0.1g), (C) aphid cardenolide concentration (µg/0.1g) against ramet number.