The influence of variable plant defense expression on the sequestered defense of an aphid against a coccinelid predator

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Abstract

We investigated the impact of cardenolide expression and sequestration on plant fitness in a tritrophic system of neotropical milkweed, *Asclepias curassavica* L., specialist aphid, *Aphis nerii* B. de F., and polyphagous coccinellid predator *Cycloneda munda* Say. Our objective was to determine whether the plant species can resolve the lethal plant defense paradox. Aphid sequestration of cardenolides from the host plant increased at lower aphid densities in the presence of coccinellids. *C. munda* reduced the growth rate of aphid colonies but was negatively impacted by aphid cardenolides such that survivorship was zero at the end of the fourth instar. Aphid growth rates were also negatively impacted by plant cardenolides but the relationship of whole-plant cardenolide response to aphid biomass and colony size was unclear. This is in contrast to earlier work that looked at local plant response and found a density-dependent reduction and induction of leaf cardenolide concentration. Plant fitness correlates of biomass change and flower production did not differ between experimental groups. The evidence thus indicates that cardenolides present a tradeoff for plants in the presence of sequestering herbivores and the third trophic level.

Introduction

While all angiosperm plants examined to date have extensive systems of secondary metabolites that are used defensively against herbivores and pathogens, many plant systems also attract specialist herbivores that can not only detoxify the defensive chemicals but can also sequester the chemicals for use against their natural enemies (Duffey 1980, Nishida 2002). This poses a problem for plants that Malcolm (1995) termed the “lethal plant defense paradox” because the sequestered plant defenses are usually characterized as small, toxic molecules and Price et al. (1980) had considered that large, digestibility reducing chemical defenses posed another paradox for plants. They termed this the “sublethal plant defense paradox” in which they argued that investment in large molecules such as complex polyphenolics can result in reduced plant fitness because herbivores are forced to consume more plant biomass. Price and colleagues considered that the paradox is resolved by natural enemies because compounds such as tannins make herbivores more vulnerable to predation and plants benefit from the third trophic level. In contrast, Malcolm (1995) considered that the problem plants face when sequestering herbivores use their toxic chemical defenses against the third trophic level is more likely to be resolved by the plants rather than by natural enemies.

Attempts have been made to resolve this paradox using different study systems. Malcolm and Zalucki (1996) suggested that milkweed cardenolide induction occurs rapidly in response to feeding damage by first instar larvae of the monarch butterfly when the herbivore is most vulnerable, but then returns to constitutive levels after several days to minimize sequestration in later instars. Fordyce (2001) hypothesized

that a similar mechanism occurs in *Aristolochia californica* but was unable to resolve the paradox because a delayed return to constitutive defense levels did not seem to help the lacewing predator *Chrysoperla* against larvae of the specialist sequestering pipevine swallowtail butterfly (*Battus philenor)*. Most recently, Arab and Trigo (2011) presented data suggesting that *Brugmansia suaveolens* foregoes chemical induction when attacked by specialist larvae of *Placidina euryanassa* and instead invests its resources into compensatory growth.

Milkweeds in the genus *Asclepias* (Apocynaceae) are well known because they are exploited by iconic sequestering herbivores such as the monarch butterfly, *Danaus plexippus*, and the oleander aphid, *Aphis nerii*, and has become a popular study system for chemically-mediated interactions among plants and herbivores since Poulton (1914) called for American chemists to determine the basis of aposematism in monarch butterflies (Roeske et al., 1976; Malcolm 1981, 1986, 1989, 1990, 1992, 1995; Malcolm and Brower, 1989; Malcolm and Zalucki, 1996; Martel and Malcolm, 2004; Bingham and Agrawal, 2010). The genus *Asclepias* has a widespread distribution In North, Central and South America, and the Caribbean (Woodson 1954; Fishbein et al., 2011) and throughout their range they are attacked by both the leaf-chewing larvae of monarch butterflies and all stages of the phloem-sucking aphid, *A. nerii,* which, like the monarch, can sequester toxic cardenolide defenses from milkweed leaves and stems (Rothschild et al., 1970; Malcolm, 1981, 1986, 1989, 1990, 1992). *A. nerii* is distributed widely wherever milkweeds are found (Malcolm, 1991) and thus presents a fitness challenge to its host plants.

In a previous study with the neotropical milkweed, *Asclepias curassavica*, Martel and Malcolm (2004) found a localized response whereby the plant initially reduced its cardenolide concentration in response to low densities of *A. nerii* biomass, followed by induction as aphid density increased. Other studies have also found that a reduction in plant defense can occur during the early stages of herbivore attack (Musser et al., 2002; Prado and Tjallingii, 2007). These results suggest that reduction of chemical defense may offer a resolution to the lethal plant defense paradox by making sequestering specialist herbivores more vulnerable to natural enemies. Blaauw and Malcolm (in prep) tested this possibility and found that a polyphagous coccinellid species shows a domed or type-4 functional response when attacking different densities of *A. nerii* because aphids sequestered lower amounts of cardenolide at low density and higher amounts at high density.

While Martel and Malcolm (2004), Blaauw and Malcolm (in prep) and Couture and Malcolm (in prep) examined localized plant responses on the leaf containing the aphid colony and the opposite leaf, only recently has the entire plant fitness perspective been included in a study on the lethal plant defense paradox (Arab and Trigo, 2011) and no study has considered the tritrophic interaction of milkweed-herbivore-predator from the whole plant perspective. In a recent review, Agrawal (2011) considers the literature on aphid effects on plant performance measures and cardenolide chemistry to be equivocal. Hence our objective was to investigate whether the dynamics of cardenolide reduction and induction can occur systemically in *A. curassavica* in response to changing densities of *A. nerii* and if these dynamics have differential fitness

consequences in the presence or absence of a polyphagous coccinellid predator, *Cycloneda munda*.

Methods and Materials

Plant Culture. 50 neotropical *Asclepias curassavica* L. were pot-reared from seed at the Western Michigan University greenhouse complex. Seeds were collected in 2009 by Steve Malcolm *from A. curassavica* growing along a roadside by Amboro National Park in Huaytu, near Buena Vista, Santa Cruz Department, Bolivia. Plants were grown in MetroMix^{TM} 300 soilless medium for 4 months in a greenhouse and then acclimated outside in nylon mesh cages (August 20th) for 1 month before the start of the experiment (September 20th).

Broad Windsor fava bean seeds, *Vicia faba* L., were purchased from the Victory Seed Company (victoryseeds.com). Plants were pot-reared from seed in MetroMix^{TM} 300 soilless medium in a greenhouse. Once mature, the adult plants were transferred to a growth chamber for use as pea aphid culture. The chamber room was set at 16:8 L:D cycle at 26°C.

Insect Culture. Oleander aphids, *Aphis nerii* B. de F., were maintained on *A. curassavica* in a greenhouse room separated from the hostplant cultures. These cultures were originally established from natural populations on common milkweed (*Asclepias syriaca* L.) in Kalamazoo County, Michigan, USA.

Pea aphids, *Acyrthosiphon pisum* Harris, were obtained from Dr. Jenn Brisson (University of Nebraska). *A. pisum* were maintained on fava bean plants in a sealed greenhouse chamber.

The spotless ladybug, *Cycloneda munda* Say, was obtained from *A. syriaca* patches infested with *A. nerii* in Berrien County, Michigan, USA. Ladybugs were kept in Petri dishes with pea aphid prey in a growth chamber at the Western Michigan University greenhouse complex. The growth chamber was set to a 16:8 L:D cycle at 26°C. Petri dishes were checked daily for coccinellid eggs. Eggs were isolated into separate Petri dishes to prevent cannibalism. *C. munda* were fed pea aphids *ad libitum* and were given fresh moist cotton balls daily.

Experimental Procedure. 50 potted, 5-month-old *Asclepias curassavica* were placed in individual nylon mesh cages distributed 2 meters apart among 10 rows and 6 columns as shown in Table 1. The plants were randomly assigned to three treatments of intact controls (10 “C” replicates), *A. curassavica* + *A. nerii* (20 “N” replicates), and *A. curassavica* + *A. nerii* + *C. munda* (20 “S” replicates). The experiment was conducted in a mowed outdoor area enclosed on all sides by brick building (Table 1). The cages were tied to metal stakes for stability and plants were placed in watering trays to avoid drought stress.

Before the start of the experiment plants were acclimated to the conditions and checked daily for pests that were removed. One week was allowed between the start of the experiment and the last pest removed to minimize possible induction of plant defenses.

Five alate, 5th instar *Aphis nerii* were taken from the colonies maintained on *A. curassavica* and placed on each N and S replicate. The aphids were allowed to reproduce and feed freely from the internal phloem of *A. curassavica* (Botha *et al.,*

1977). Numbers of each aphid instar and plant response variables were recorded 3 times every week. When the aphid density reached between 20-60 aphids on an S replicate, 20 *Cycloneda munda* eggs were placed close to the aphid colony on the top of the closest leaf or on the flowers. If an aphid colony had dropped to 10 or fewer aphids then 5 more 5^{th} instar alates were placed on the replicate. If the 20 coccinellid eggs all experienced mortality then 20 new eggs were placed on the same replicate.

The harvesting of replicates was started one month after the experiment started and was done by harvesting the highest aphid density replicates first (those that produced winged alate aphids) and with a mixed balance of C, N and S group replicates (October 18^{th}-October 31^{st}). Generally, 20 replicates were harvested every week and the typical ratio was 1:2:2 for C:N:S replicates harvested. When harvested, the plant stem was severed at the bottom of the stem and immediately bagged in a 50 L black plastic bag and placed in a -80°C freezer. The roots were rinsed off and as much soil as possible was removed before storage in a 3.8 L Ziploc bag at -80°C.

Once frozen, the above-ground plant material was removed from the bag and the aphids were collected with a camel-hair brush and placed into a Petri dish to be counted and weighed. The stems, and leaves were trimmed and weighed separately to obtain the wet mass of the sample. The aphids were also weighed separately. To quantify the systemic cardenolide response of the plant, a random sample of stems and leaves were lyophilized for each replicate. All aphids from a sample were also lyophilized.

Phytochemical Analysis. Procedures followed those of Malcolm and Zalucki (1996) based on Wiegrebe and Wichtl (1993). Plant samples were lyophilized, weighed

and ground into a fine powder using a mortar and pestle. Approximately 50 mg of each sample was placed into a 15 ml centrifuge tube and mixed with 4 ml 100% methanol (aqueous), vortexed and then sonicated in a water bath for 10 minutes at 55˚C. The samples were then centrifuged at high speed for 10 minutes and each supernatant was decanted into a 13 x 100 ml glass tube. The remaining precipitate was washed with 2 ml methanol, vortexed and centrifuged and the supernatants pooled. The methanolic extracts were dried using a nitrogen evaporator in a 60°C water bath. Dried samples were then resuspended in 1 ml acetonitrile, vortexed, sonicated for a few seconds, and filtered through a 0.45 µm luer-lock syringe filter into a 1 ml autosampler vial ready for analysis by high performance liquid chromatography (HPLC).

Insect samples were lyophilized, weighed, and dried samples were then placed in a 15 ml glass tube with 2 ml methanol, homogenized with a motorized homogenizer, vortexed, and the extract was sonicated in a water bath for 10 minutes at 55˚C. The samples were then centrifuged at high speed for 10 minutes and each supernatant was decanted into a 13 x 100 mm glass tube. The remaining precipitates were washed with 2 ml methanol, vortexed and centrifuged and the supernatants were pooled to give a total insect extract volume of 4 ml. Samples were dried with a nitrogen evaporator in a 60°C water bath and then the dry extract was resuspended in 1 ml of acetonitrile. The resuspended samples were vortexed and filtered through a 0.45 µm luer-lock syringe filter on a 3 ml plastic syringe into labeled 1 ml autosampler vials ready for HPLC analysis.

HPLC analyses of cardenolides in the prepared samples were performed using 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 over 65 min. was acetonitrile:water at 1.2 ml·min^{-1} at 40˚C, with 20% acetonitrile at start, to 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). Sample injections were 20 µl. Samples were separated by 10-minute equilibration at 20% acetonitrile.

Cardenolides were detected at 218.5 nm and identified by four characteristics: a symmetrical absorbance spectrum between 205 and 235 nm, a λ_{max} of between 213 and 224 nm, a purity 1 angle below 90.00, and a percent area greater than 0.25. Cardenolide concentration for each peak (µg/0.1g sample DW) was calculated from a calibration curve with the cardenolide standard digitoxin (Sigma, St Louis, Missouri). Concentration values (µg/0.1g) were then adjusted for error by multiplying the cardenolide value by the difference of the measured digitoxin concentration and the actual concentration of digitoxin used. Only cardenolide peaks reported by Millennium 2010® software as consistently pure were considered for analysis.

Data Analysis. Collected data were analyzed with R statistical software (v 2.14). Data were analyzed for normality (Shapiro-Wilk Q-Q plot), homogeneity of variances (Bartlett's test) and the presence of outliers. Data that violated any of these statistical assumptions were analyzed using nonparametric tests. Indication of where nonparametric tests are used is given in the Results section.

In order to compare the influence of predation and plant cardenolide chemistry on aphid population dynamics, the average aphid intrinsic rates of increase were calculated for the N (-coccinellids) and S (+coccinellids) treatment groups by fitting the data to the exponential model in the R program ($N_t = N_0 \times e_{rt}$; R function = nls). The data range in all cases was after any alates were added up until the time of harvest of the replicate. The average aphid intrinsic rates of increase were compared between the two groups with a T test.

Two plant fitness correlates were used: flower numbers and proportional change in above-ground biomass. In order to calculate the proportional change in plant biomass, the initial plant biomass needed to be estimated based on the number of leaves and plant height at the start of the experiment (above-ground mass could not be weighed without sacrificing the plant). Thus, a linear model was fitted to the final plant biomass predicted from stem height and leaf number: Plant biomass (g) = 0.415 + 0.0524 x Height (mm) + 0.159 x Leaf number {r^2 = 0.687; F = 43.82; SE = 6.14; d.f. = 40; p (Height) < 0.0001; p (Leaf number) = 0.015}. This linear model was then used to predict initial plant biomasses at the start of the experiment. The statistical assumptions of this linear model are acceptable to an alpha level of 0.2 or greater.

Results

Aphid population dynamics. Out of the 40 replicates with aphids (both N & S treatments), 37 showed a pattern of growth with time that best fit the positive exponential model (Table 2). Aphid intrinsic rates of increase were not correlated with the initial population size over the data range (r^2 = 0.00040; F = 0.014; SE = 0.0019; d.f.

= 39; p = 0.906). Coccinellids in S treatments significantly reduced aphid intrinsic rates of increase measured as *r* individual/individual per hour when compared with aphids without coccinelids (N) (N mean = 0.00715±0.00045, S mean = 0.00583±0.00037; t = 2.333; d.f. = 37; p = 0.026).

Aphid population numbers were linearly and positively correlated with the final weighed aphid biomass (r^2 = 0.785; F = 139; SE = 78.62; d.f. = 38; p < 0.001). Thus, aphid population numbers are used for the rest of the analysis.

Cardenolide concentrations. There were no significant differences in stem cardenolide concentrations among the groups (Kruskal-Wallis test; chi squared = 0.81; d.f. = 2; p = 0.67; Table 3). However, there were significant differences among the groups in leaf cardenolide concentrations that reflected induction of leaf cardenolides by aphids (Kruskal-Wallis test; chi squared = 6.49; d.f. = 2; p = 0.039; Table 3).

Aphid cardenolide concentrations were negatively correlated with the final aphid colony size according to a power relationship (r^2 = 0.479; t = 5.23; SE = 196.9; p < 0.001; Figure 1). There was also a significant interaction effect of the final aphid colony size with the presence and absence of coccinellids on the aphid cardenolide concentration (F = 3.83; p = 0.031; Figure 1). Systemic plant cardenolide response did not influence aphid cardenolide concentrations because neither stem cardenolide concentrations (r^2 = 0.021; F = 0.72; SE = 218.6; d.f. = 33; p = 0.402; Figure 2) nor leaf cardenolide concentrations (r^2 = 0.044; F = 1.51; SE = 252.8; d.f. = 33; p = 0.229; Figure 3) significantly predicted aphid cardenolide concentrations. However, there was a significant interaction effect of experimental group (± coccinellids) and leaf

cardenolide concentration on aphid cardenolide concentration (F = 3.97; d.f. = 1; p = 0.0288) whereas neither of these predictors were significant by themselves in an ANCOVA test (p (group) = 0.106; p (leaf cardenolide) = 0.217; Figure 3). There was no similar interaction effect of stem cardenolides and experimental group on the aphid cardenolide concentrations (F = 0.50; d.f. = 2; p = 0.61).

Leaf cardenolide concentrations had a negative linear relationship with aphid colony size (r^2 = 0.162; t = 2.53; SE = 5.96; d.f. = 33; p = 0.0165; Figure 4). However this linear relationship does violate the normal distribution assumption and it is unclear what nonlinear function it could fit. There is a significant interaction effect between aphid colony size and experimental group (±coccinellids) on leaf cardenolide concentration (F = 3.50; d.f. =2; p = 0.042). The interaction effect might be a cause of the skewed distribution. In contrast, stem cardenolide concentrations had no relationship with aphid colony size (r^2 = 0.002; t = 0.23; SE = 6.10; d.f. = 33; p = 0.82; Figure 5). In this case there was also no influence of experimental group or interaction effect of experimental group and aphid colony size on stem cardenolide concentration (F = 0.88; d.f. = 1; p (experimental group) = 0.35; F = 0.81; d.f. = 2; p (aphid colony size : experimental group) = 0.45).

Coccinellid survivorship. *Cycloneda munda* was unable to complete its development with *A. nerii* as its sole food source and none survived past third instar (Figure 6). The coccinellid larvae did consume some aphids and impacted the rate of increase of the aphids (Table 2). Coccinellid survivorship to second instar was negatively impacted by higher aphid cardenolide concentrations because replicates where *C. munda* did not survive to second instar had significantly higher aphid cardenolide concentration than

replicates where *C. munda* did survive to second instar (mean = 323.4 µg/0.1g and 135.9 µg/0.1g, respectively; SEM = 110.4 µg/0.1g and 95.6 µg/0.1g; p = 0.047).

Plant response and fitness. Two correlates for plant fitness were used: the estimated proportional change in plant biomass and the production of flowers. The proportional change in plant biomass did not differ among the groups (F = 1.167; d.f. = 2; p = 0.321; Table 4). The proportional changes in plant biomass were unrelated to final aphid colony size among the experimental groups (+aphids, ±coccinellids) (r^2 = 0.02; t = 0.86; SE = 0.16; d.f. = 34; p = 0.40; Figure 7). There was no significant interaction term among aphid colony size and experimental group (F = 0.50; d.f. = 2; p = 0.61). Flower production was not different among the three groups (Kruskal-Wallis test; chi-squared = 0.90; d.f. = 2; p = 0.61; Table 5). Between the experimental groups (+aphids, ±coccinellids), the production of flowers was also unrelated to final aphid colony size (r^2 < 0.001; t = 0.05; SE = 5.49; d.f. = 38; p = 0.96) and also unrelated to aphid colony size with presence of coccinellids as a covariate (F = 0.329; d.f. = 1; p = 0.570).

Discussion

In contrast to coccinellid-free N treatments, aphids at low density that were exposed to coccinellid larvae in S treatments had strikingly high cardenolide concentrations (Figure 1), but at high aphid densities these concentrations reduced to those of aphids without coccinellids. This suggests that the initial presence of coccinellid larvae triggers enhanced sequestration by *A. nerii* perhaps because initial aphid colonies are comprised of first and second instar aphids and sequestration may be a scale-dependent phenomenon.

In the absence of coccinellid predators we found no clear relationship between plant cardenolide concentration and aphid density (Figures 4 and 5) although a negative correlation between leaf cardenolide concentration and aphid colony size is suggested by the data. This was in contrast to previous work in which *A. curassavica* reduced its constitutive leaf cardenolide concentration at low densities of *A. nerii* and then induced cardenolides as aphid density increased to levels that were significantly higher than constitutive concentrations (Martel and Malcolm, 2004). In this earlier work the leaf response occurred in both the leaf to which aphids were restricted and in the nearest leaf opposite the infested leaf. Our results differed in that we examined a random sample of all leaves and aphids were free to feed from phloem from any part of a mature plant. This plant level response appears to eliminate the leaf-specific signal found by Martel and Malcolm (2004) and Blaauw and Malcolm (in prep.). Another species of milkweed, *A. viridis*, can also reduce its cardenolide concentration in response to *A. nerii* attack (Zehnder and Hunter, 2007). However, the direction of causation in the present study is unclear. Perhaps higher cardenolide concentrations negatively impacted aphid growth rates with consequent reduced colony sizes. Although *A. nerii* is an adapted specialist, this interpretation is consistent with previous results indicating that cardenolides do depress the growth rate of *A. nerii* (Agrawal, 2004).

The milkweed clade contains several other adapted specialist herbivores and some of them have been shown to be negatively impacted by cardenolides. Several studies have shown that early instars of the monarch butterfly, *D. plexippus*, are negatively impacted by cardenolides (Zalucki et al., 1990; Zalucki and Brower, 1992; Malcolm and Zalucki, 1996; Zalucki et al., 2001; Agrawal 2005). Similarly, it has been

found that the red milkweed beetle, *T. tetrophthalmus*, has reduced survivorship on higher cardenolide milkweed species (Rasmann and Agrawal, 2011).

Nonetheless, qualitative chemical defenses still come at a cost to the plant. In the present study, it was found that *C. munda* had a reduced survivorship at higher aphid cardenolide concentrations. Previous work has also shown that sequestration of toxic cardenolides presents a dilemma for the plant because Fordyce (2001) showed that lacewing mortality increased when *A. californica* induced its aristolochic acid concentration following pipevine swallowtail feeding.

In the present study, the evidence indicates that cardenolides presented a tradeoff for the plant. There is some evidence that cardenolides depressed the growth rate of *A. nerii*, but decreased the survivorship of *C. munda*. The critical test of this tradeoff is the measurement of plant fitness correlates. The proportional change in plant biomass and the production of flowers are two correlates that do not appear to indicate the plant was impacted either negatively or positively by its cardenolides. In particular, neither final aphid colony size nor the presence of coccinellids predicted plant performance.

Previous work has shown that aphid herbivory has an unclear effect on plant performance traits. In a comparison of the effects of *A. nerii* feeding on four different plant species, Zehnder and Hunter (2007) show that *A. viridis* and *A. incarnata* biomass are negatively impacted by *A. nerii* but *A. syriaca* and *A. tuberosa* are not. Increasing tolerance to herbivory by adapted specialists might be a common strategy among *Asclepias* spp. and might be influenced by plant modularity and ability to compensate

for metabolite losses to aphid herbivory. Agrawal and Fishbein (2008) conducted a phylogenetic analysis of 38 *Asclepias* species and found evidence for an increase in regrowth ability but a general decline in resistance traits such as cardenolides.

Thus, the balance of evidence from the present study is that the lethal defense paradox is indeed present in the *A. curassavica* system but is balanced by the negative impact of cardenolides on *A. nerii* and the relative tolerance of the plant to aphid herbivory.

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Tables

Table 1. Replicate position in the experiment (C = plants only (10), N = plants + aphids (20), S = plants + aphids + coccinellids (20))*

N	S	C	N	S	S
N	S	N	N	S	C
S	C	N	S	S	
S	N	S	C	N	S
N	C	N	N	S	
C	N	N	S		
N	S	C	N		
C	S	N	S		
S	N	S	C	N	
S	N	N	S	C	

*Rows and columns varied according to space limitations

Table 2. Nonlinear least squares regression of raw aphid count per replicate fitted to the exponential model ($N_t = N_0 * e^{r*Time}$). The N and S replicates are without and with ladybugs, respectively.

Replicate	N0	r	p value (r)	SE	t	df
N1	9	0.0069	<0.0001	28.52	61.6	7
N2	15	0.0096	<0.0001	48.85	116.5	6
N3	2	0.0089	<0.0001	9.54	148.6	7
N4	3	0.0048	<0.0001	4.272	31.6	7
N5	4	0.0095	<0.0001	32.03	135.4	7
N6	4	0.0073	<0.0001	20.51	73.6	7
N7	23	0.0081	<0.0001	117.1	82.8	7
N8	4	0.001	<0.0001	238.2	44.1	7
N9	6	0.0073	<0.0001	17.37	122.6	7
N10	3	0.0075	<0.0001	17.77	76.1	7
N11	2	0.0081	<0.0001	17.21	79.4	7
N12	5	0.009	<0.0001	60.74	100.7	7
N13	6	0.0073	<0.0001	32.88	68.12	7
N14	2	0.0094	<0.0001	6.14	552.5	7
N15	2	0.0077	<0.0001	5.62	180.1	7
N16	9	0.0056	<0.0001	24.2	42.4	7
N17	9	0.0057	<0.0001	24.2	42.4	7
N18	7	0.0058	<0.0001	25.2	35.4	7
N19	8	0.0059	<0.0001	20.79	52.2	7
N20	5	0.0081	<0.0001	95.1	35.8	7
S1	4	0.0049	<0.0001	56.63	19.0	7
S2	2	0.0068	<0.0001	102.7	20.9	7
S3	4	0.0066	<0.0001	127.7	28.0	7
S4	5	0.0073	<0.0001	475.9	16.5	7
S5	1	NA	NS	NA	NA	NA
S6	4	0.0062	<0.0001	122.2	22.9	7
S7	5	0.0073	<0.0001	388.7	19.8	5
S8	3	0.0052	<0.0001	49.77	19.9	7
S9	2	0.0069	<0.0001	139.3	16.0	7
S10	9	0.0058	<0.0001	354.8	13.2	6
S11	4	0.0051	<0.0001	87.69	14.2	7
S12	5	NA	NS	NA	NA	NA
S13	6	0.0081	<0.0001	718.5	22.9	5
S14	1	0.0069	<0.0001	84.1	13.5	7
S15	12	0.0052	<0.0001	320.8	12.2	6
S16	6	NA	NS	NS	NS	NS
S17	21	0.006	<0.0001	692.5	17.5	5
S18	6	0.0016	<0.01	16.62	5.5	7

S19	4	0.0044	<0.0001	65.84	10.7	7
S20	7	0.0045	<0.0001	91.57	15.3	6

Mean r_N = 0.0072, SEM = 0.00045; Mean r_S = 0.0058, SEM = 0.00037; p = 0.026

Table 3. Group Differences in Cardenolide concentrations.

	Plants (C)	SEM	Plant + Aphid (N)	SEM	Plant + Aphid + Coccinellids (S)	SEM
Mean Stem card (µg/0.1g)	8.36	3.20	5.31	0.95	7.28	2.02
Mean Leaf Card (µg/0.1g)	2.21*	0.61	6.93*	1.39	4.75	1.70
Mean Aphid Card (µg/0.1g)	NA	NA	98.07	28.16	220.31	73.6

* = differences significant to an alpha level of 0.05.

Table 4. Group mean proportional changes in plant biomass. No group differences are significant ($F = 1.17$; d.f. = 2; $p = 0.32$).

	Plants (C)	Plant + Aphid (N)	Plant + Aphid + Coccinellids (S)
Mean	0.24	0.15	0.15
SEM	0.06	0.04	0.04

Table 5. Group mean flower production. No group differences are significant (Kruskal-Wallis test; chi-squared = 0.90; d.f. = 2; p = 0.61).

	Plants (C)	Aphid + Coccinellids (N)	Plant + Aphid + Coccinellids (S)
Mean	5	2.1	3.1
SEM	2.3	0.9	1.5

Figures

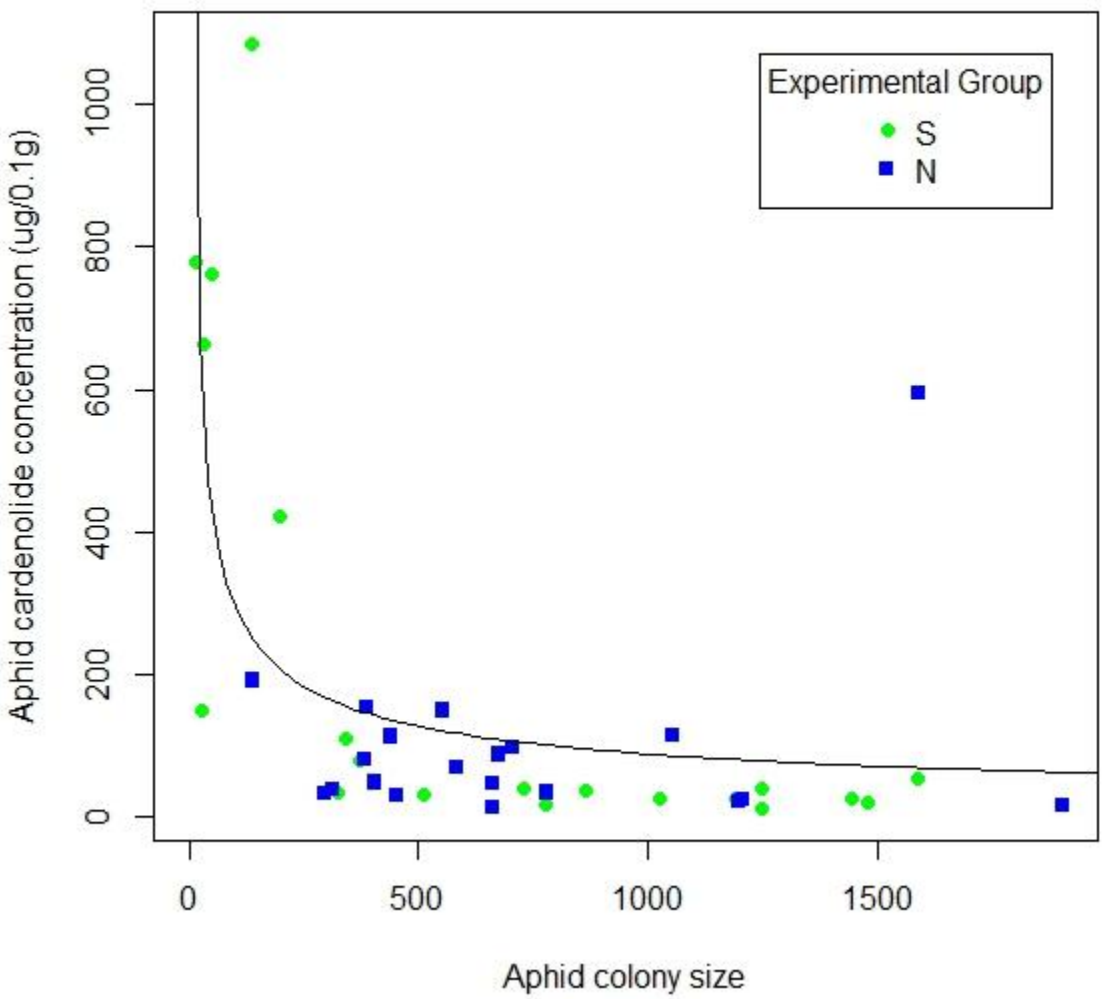

Figure 1. [S = +coccinellids; N = -coccinellids] Nonlinear least squares regression of aphid cardenolide concentration as a function of aphid count. Aphid cardenolide concentration = 3345.25 * Aphidcount$^{-.526}$ (t = 5.23; SE = 196.9; d.f. = 38; p (exponential term) < 0.001). The relationship is different among the S and N groups (F = 3.83; d.f. = 2; p = 0.031)

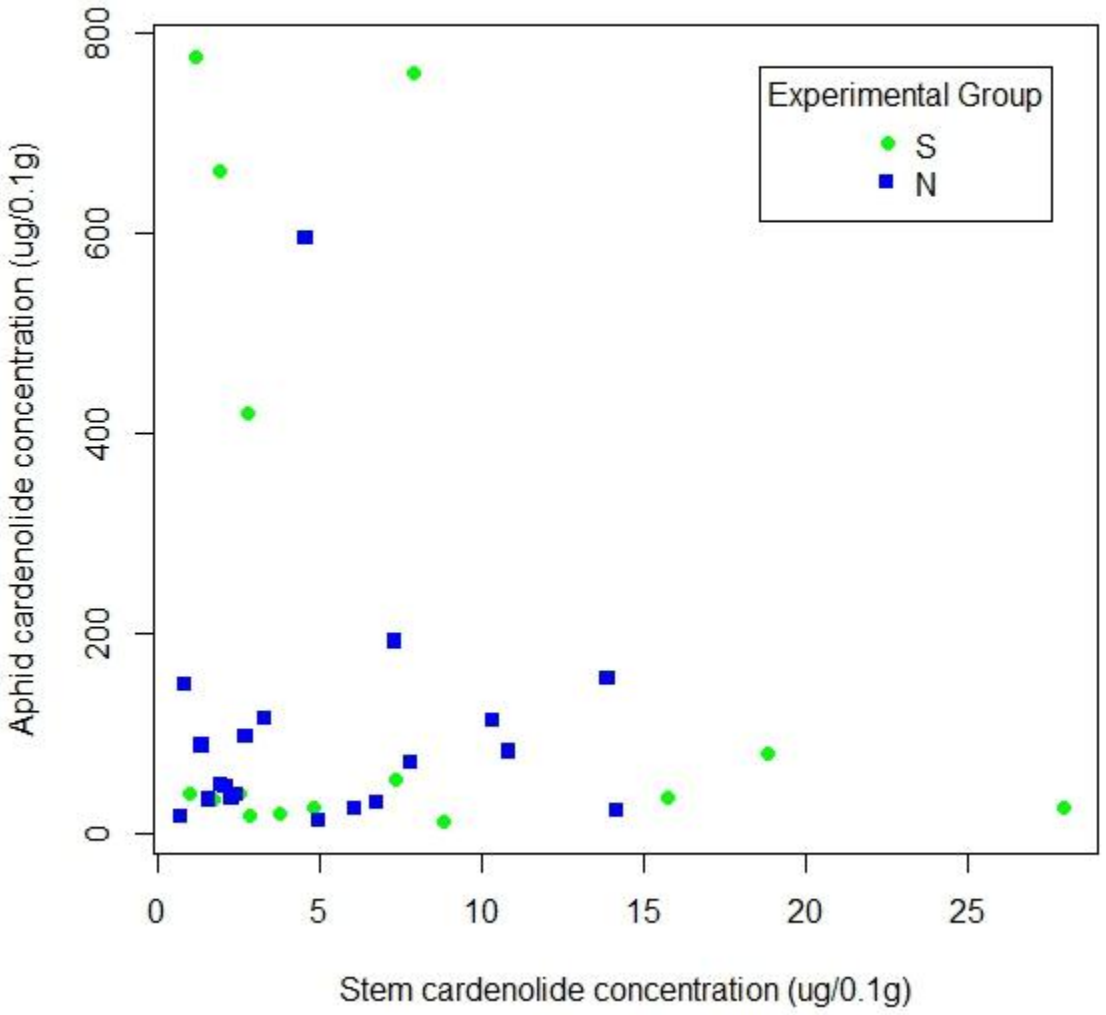

Figure 2. [S = +coccinellids; N = -coccinellids] Aphid cardenolide concentration has no clear relationship with stem cardenolide concentration.

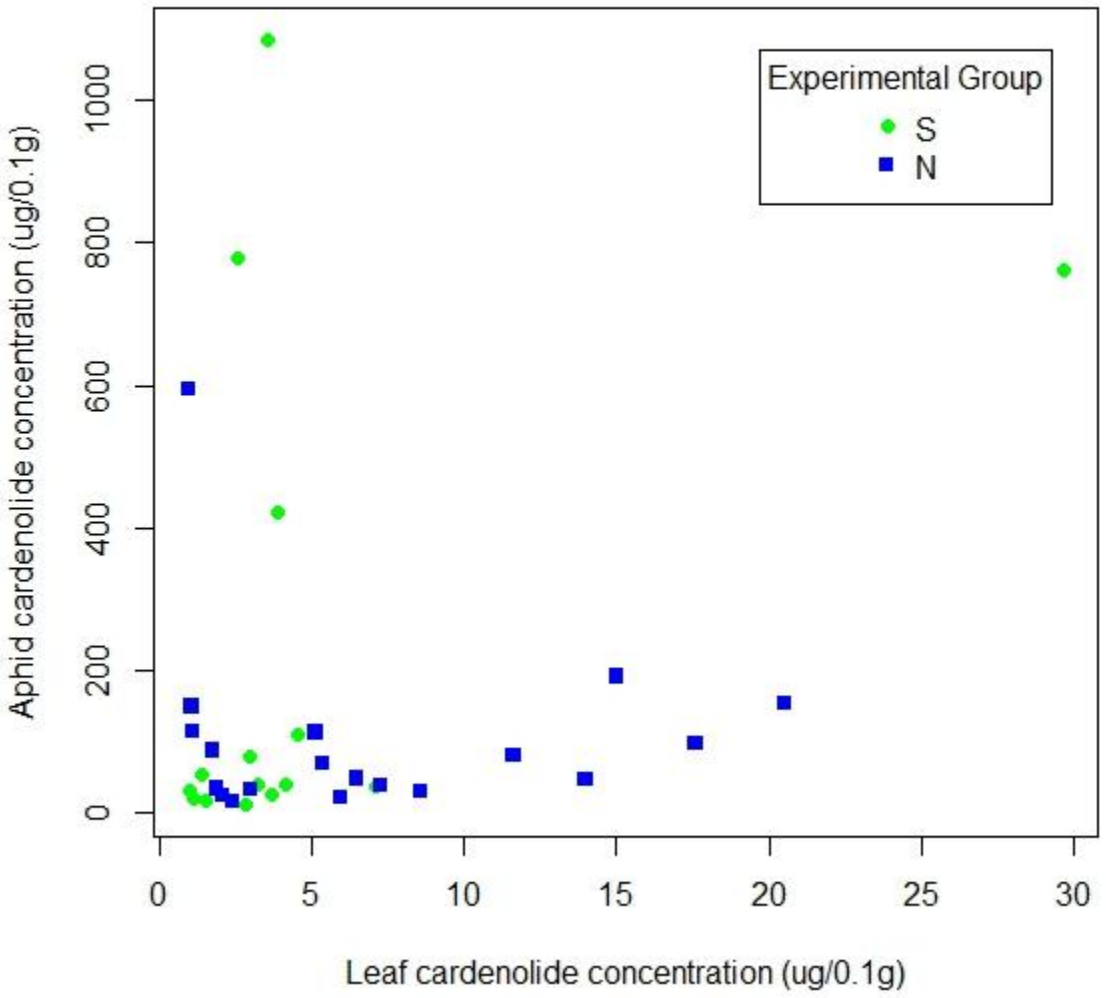

Figure 3. [S = +coccinellids; N = -coccinellids] The overall relationship between aphid cardenolide concentration and leaf cardenolide concentration is unclear but the relationship is different among the S and N groups ($F = 3.97$; d.f. = 2; $p = 0.029$).

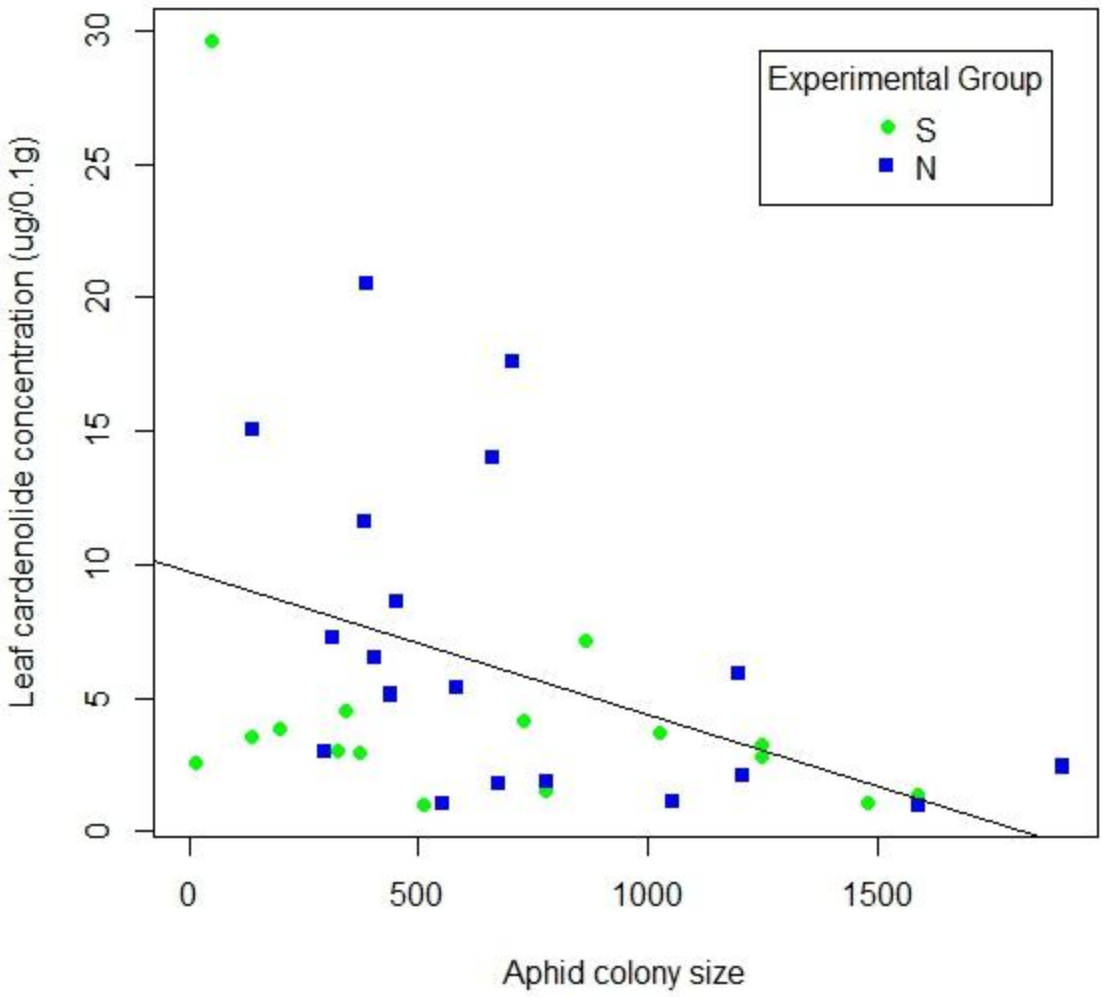

Figure 4. [S = +coccinellids; N = -coccinellids] Leaf cardenolide concentration has a negative relationship with aphid colony size (r^2 = 0.162; t = 2.53; SE = 5.96; d.f. = 33; p = 0.017). The skewedness of the data does violate the normality assumption. There is a significantly different relationship between the two groups (F = 3.50; d.f. = 2; p = 0.042).

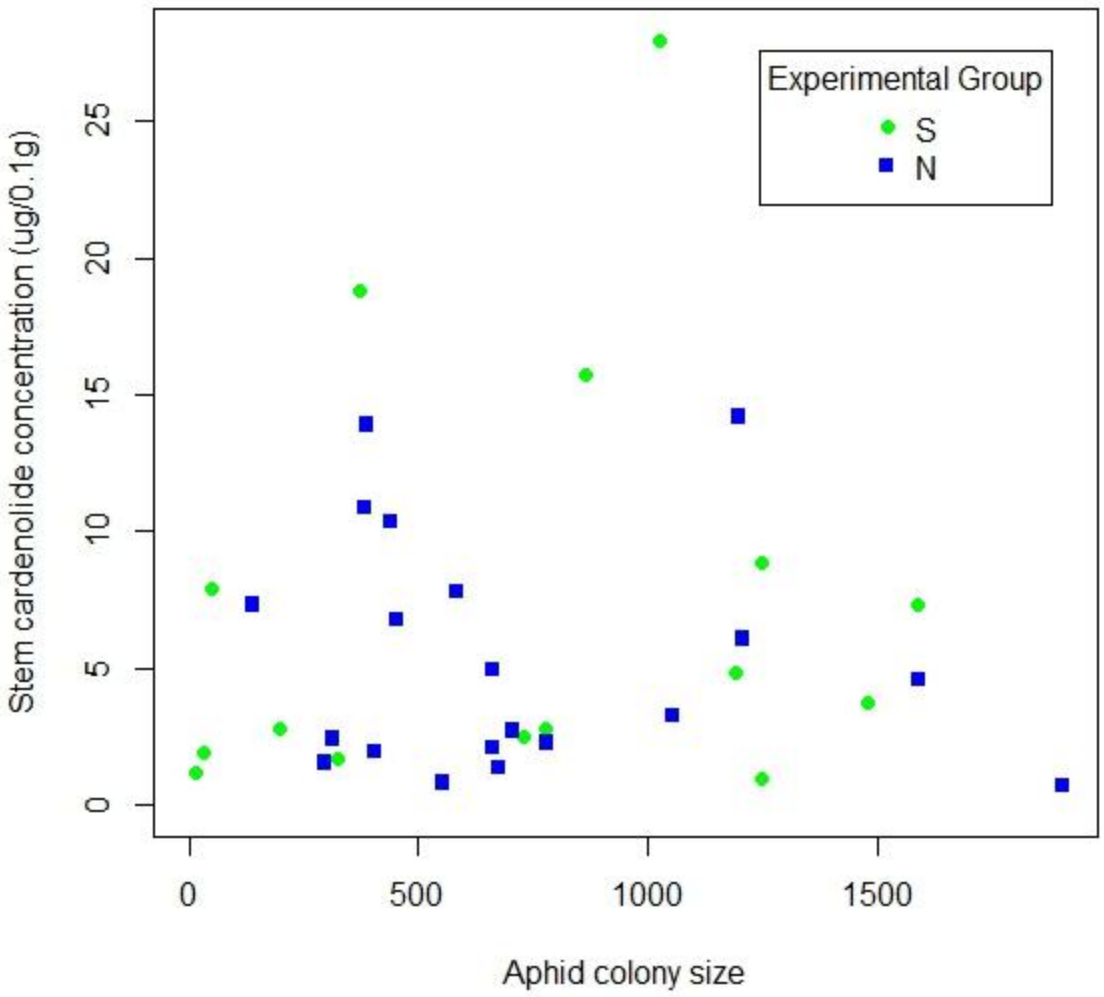

Figure 5. [S = +coccinellids; N = -coccinellids] There is no clear relationship between stem cardenolide concentration and aphid colony size. There are no significant group or interaction effects {F = 0.88; d.f. = 1; p (experimental group) = 0.35; F = 0.81; d.f. = 2; p (aphid colony size : experimental group) = 0.45}.

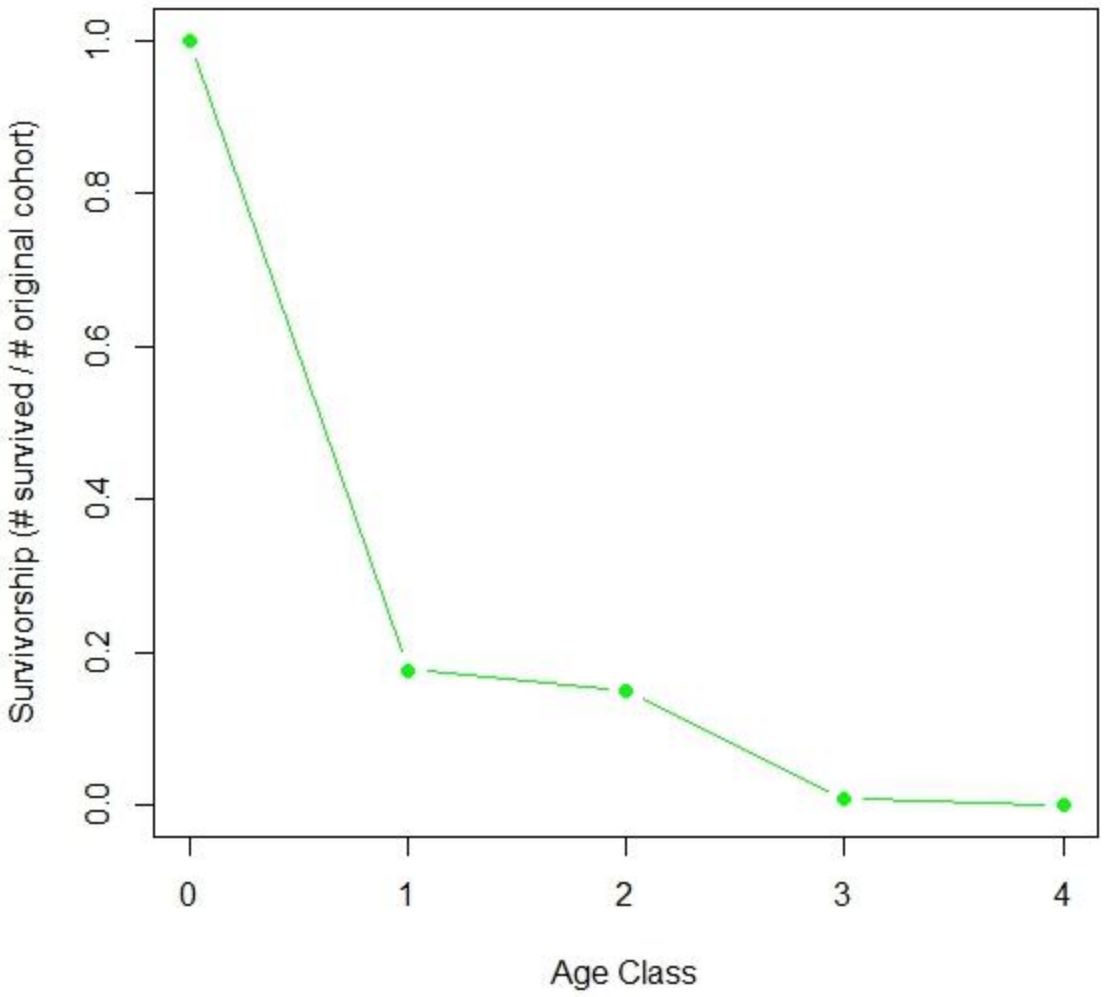

Figure 6. *Cycloneda munda* survivorship (0 = egg; 1-4 = larval instars).

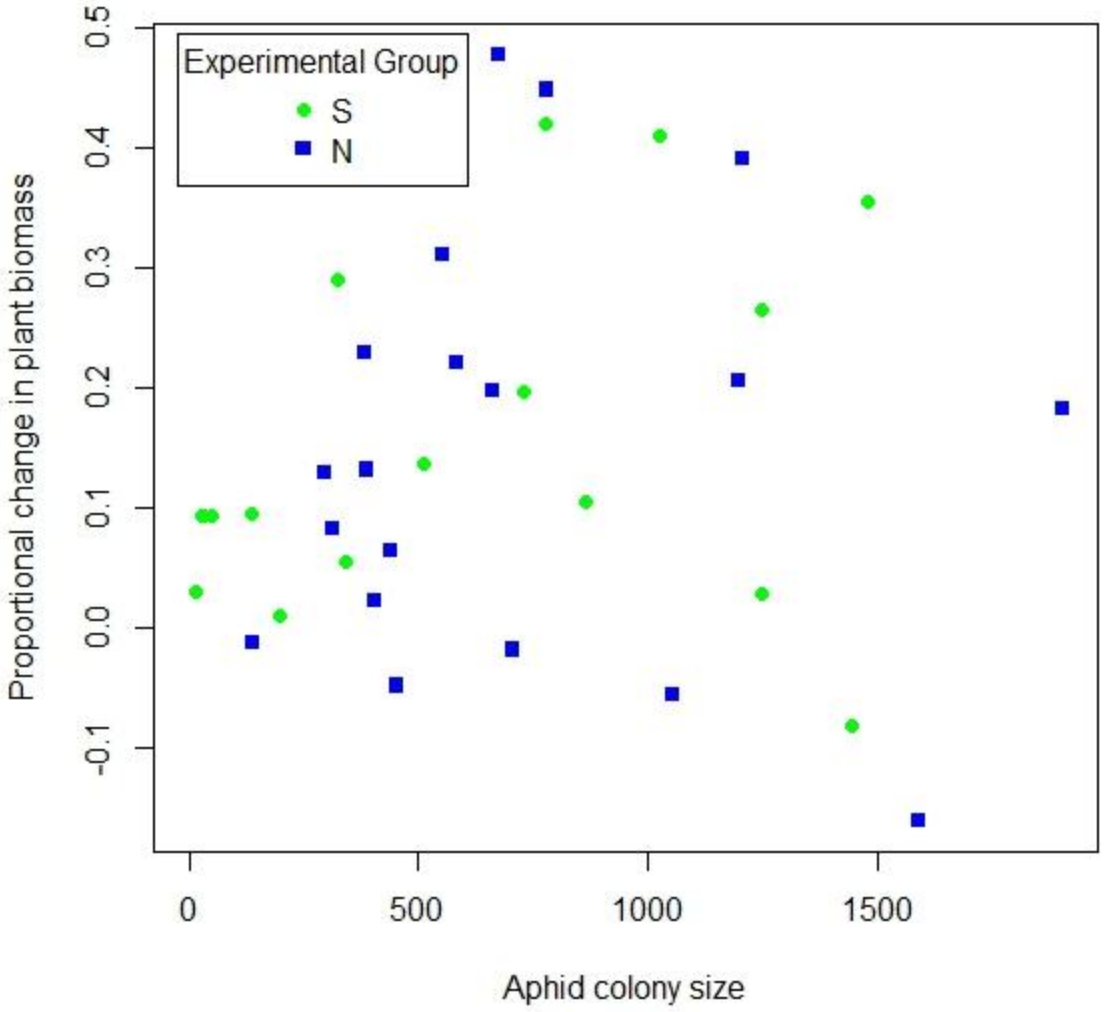

Figure 7. Proportional change in plant biomass was not influenced by aphid colony size (r^2 = 0.02; t = 0.86; SE = 0.16; d.f. = 34; p = 0.40). There was no significant interaction term among aphid colony size and experimental group (F = 0.50; d.f. = 2; p = 0.61).