Cardenolide Handling by three Specialist Aphid Herbivores of Milkweeds

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In Collaboration With
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Sections

Abstract: It is known that the aposematic aphid, *Aphis nerii* sequesters plant defense toxins called cardenolides from plants of the genus *Asclepias.* Little is known about two other cryptic aphid species, though; *Aphis asclepiadis,* and *Myzocallis asclepiadis.* These aphids employ various modes of defense and this may affect the chemical defense strategy used by the plants on which they feed. These three aphid species do not differ significantly in terms of reproductive rate on *Asclepias syriaca,* but are significantly different in average aphid mass. Additionally, *A. asclepiadis* and *M. asclepiadis* seem to be able to sequester plant cardenolides and further HPLC analysis is underway to confirm this. This could be an example of the evolution of aposematism, wherein organisms first become unpalatable then become brightly colored.

Introduction

Many plants produce compounds to be used as defenses against herbivores (Crawley, 1983). Through their coevolution, herbivores have developed means of dealing with these defensive toxins. In response to herbivory, plants can chemically increase or decrease their defense to subsequent herbivory. This can either deter herbivores from consuming the plant or create niches in which other herbivores can thrive (Karban and Baldwin, 1997). Many herbivores, namely specialists, seem to be unaffected by certain plant toxins. Others have been found to sequester plant defense compounds for their own defense against predators (Berenbaum and Miliczky 1984). Herbivore defensive tactics can be grouped into three general categories; fighting, running and hiding (Malcolm 1992).

Fighting herbivores are ones whose defense includes fighting back against predators trying to harm them. This defense comes in many forms including the presence of spines, claws and chemical defenses. Chemical fighting defense is often coupled with aposematic coloration, which is characterized by structures, colors or other signals that animals which have dangerous attributes use to advertise their danger to predators to avoid being attacked (Edmunds 1974). Aposematic coloration has been found to provide protection to animals from their predators (Edmunds 1974). Thus, animals that are considered to be aposematic no longer avoid predator detection to some degree since even when detected, predators will learn to avoid them (Edmunds 1974). There are limitations to aposematic defense, though, in that for a learning response to develop in predators, some individuals from each generation must be sacrificed (Edmunds 1974).

Fighting defense is very different from a running defense strategy. A running defense includes evading predators instead of acting back against them. Using this defense, organisms must be able to physically avoid a pursuing predator. Thus, wings, fast moving legs, and a general fleeing response to disturbances are typical of a running defense. A hiding defense does not rely on physically harming or evading predators like the fighting and running defenses, but instead relies on its crypticism to avoid

detection by those predators altogether. There are apparent energy trade offs for each of these defenses. Fighting defense provides low vulnerability to predators, but has a high energy cost. Likewise although running herbivores may be vulnerable to predation when caught, they are not often caught due to their speed or agility, and this also comes at a high energy cost. Hiding herbivores use a much different defensive strategy. Their vulnerability to predator attack is high, but they evade detection through their camouflaged nature. This comes at a much lower energy cost (Malcolm 1992). It appears that these defensive strategies may be employed together or change through time.

Each of these three modes of defense of herbivores depends on the ability of these herbivores to take up nutrients from the plants on which they feed. Plants of the genus *Asclepias* (*Apocynaceae*) are the primary producers from which several specialist herbivores feed. Several aphids of the genus *Aphis* and *Asclepiadis* are specialists to milkweeds. Some *Asclepiads* also produce toxins called cardiac glycosides for protection against herbivores. Cardenolides, a subset of these glycosides were the focus of this project. These cardenolides are composed of a basic steroid subunit bound to a five membered lactone ring as well as a sugar side chain (Reichstein et al. 1968). The polar alcohol groups of the sugar are known to attach to cardiac muscle, and the binding sites of these cardenolides are sodium/potassium ATPases. Thus, these toxins disrupt the ionic balance of cells in predators that consume the *Asclepiads* (Malcolm 1991). Cardenolide concentration within herbivore populations can vary based on interspecific and intraspecific differences in host plant cardenolide content and type of plant tissue consumed *(Isman et al. 1976).* Van Zandt and Agrawal showed that plants can respond to environmental stresses such as herbivory by increasing or decreasing physical and chemical defenses as well as nutrients that flow to the affected tissues (2004). The cardenolide content in members of the genus *Asclepias* has been shown to be inducible by varying the type of herbivory on the plant (Van Zandt and Agrawal, 2004). This could have implications for cardenolide levels induced by different aphid species feeding on the *Asclepiads*.

Milkweeds (*Asclepias spp.*) get their common name from the fact that they all seem to produce milky latex. They have opposite or whorled leaves and showy flowers (Weiss and Dickerson 1921). The genus *Asclepias* is diverse in the life strategy used by its members and different species are found in different geographical locations (Price and Willson 1979), so several members may have different defensive characteristics. *A. syriaca* has a profound root system that connects individual clones (ramets) and may influence its defense. This study focuses on the common milkweed, *Asclepias syriaca*, which is found throughout North America and is known to produce cardenolides (Woodson 1954, Hoch 1961, Price and Willison 1979)

While milkweeds photosynthesize energy and store nutrients, their herbivore parasites leech some of those nutrients for their own growth and defense. One herbivore, *Aphis nerii* seems to also use the plants' cardenolides for its own defense by sequestering them (Rothschild et al. 1970). *Aphis nerii* is a bright yellow aphid and seems to be aposematic. It was observed to be densely aggregated in the field. Because of this, *A. nerii* seems to be employing a fighting defense against its predators. *A. nerii,* when fed to spiders was found to disrupt the ability of those spiders to build symmetrical webs, impairing their fitness (Malcolm 1989). Aposematic coloration (such as that of *A. nerii*) has been shown by Rothschild and Reichstein (1974) to come about after the presence of defensive compounds. In this way, herbivore populations can be well defended against predators before they alert them of their location. After web disruption of spiders fed *A. nerii*, when another individual was placed in the web, the spider would not attack it or would be delayed in its response (Malcolm 1989). *A. nerii* has also been found to have an association with ants which may tend it variably. Bristow (1991) found that *A. nerii* feeding on floral tips where cardenolide content is relatively low were much more likely to be tended by ants than those feeding on leaf tips which are high in cardenolides. This study focused on the role of cardenolides in phloem feeding *A. nerii*.

While much is known about the cardenolide sequestration of *A. nerii*, much less is known about

that of two other aphids, *A. asclepiadis* and *Myzocallis asclepiadis.* It is known that these two aphids are commonly found on *A. syriaca* in nature (Weiss and Dickerson 1921). *A. asclepiadis* seems to be polymorphic and can range in color from olive green to dark green with tiny white dots. Alate adult *A. asclepiadis* seem to be black in color. Apterous adults also occur but seem to maintain the typical green colors. *A. asclepiadis* was observed to be densely aggregated on plants in the field and does not seem to use an abandoning tactic similar of some aphids. Due to these observations, *A. asclepiadis* seems to be using some type of hiding defense. Ants seem to also play a role in the *A. asclepiadis* system, and have been observed eating the honeydew extract that *A. asclepiadis* secretes. According to Mooney and Agrawal (unpublished data), *A. asclepiadis* has a slower growth rate, differences with ant tending, and ability to sequester defensive cardenolides than *A. nerii*. There may be a mutualism between *A. asclepias* and one or more species of ants in which the ants protect the aphids in return for their honeydew food reward (Mooney and Agrawal 2005). Thus, *A. asclepiadis* may be instead using some type of hybrid hiding/fighting defense strategy where the fighting is masked as recruiting other fighters for defense.

Like *A. asclepiadis*, little is known about *M. asclepiadis.* Aphids of this species also tend to be cryptic, often having a translucent green color with later instars exhibiting a pattern of light red spots late in the season. All adults of *M. asclepiadis* seem to be winged. These aphids were observed to be highly mobile in the field and tended to be widely dispersed. When disturbed with light, *M. asclepiadis* mobilized and early instars typically ran around leaf surfaces while adults tended to fly away. Based on these observations, *M. asclepiadis* seems to use some type of running defense, or possibly a running/hiding hybrid defense. Because these three aphids seem to use such diverse defensive strategies and are all specialists on the *Asclepias* genus, they seemed to be an ideal target for this study.

Other than milkweeds and aphids, there is another trophic level that has an impact on this system. Aphid predators have a positive impact on plants since they are removing dangerous

herbivores. Because of the lethality of some aphids due to sequestered cardenolides, some predators may eat one aphid, die and never impact the aphids or plants in the system. These have been termed excluded predators. Some predators seem to be able to consume aphids and not be affected and thus change the aphid and plant community structure (Included predators). Others are in between these extremes and may eat an aphid and be affected but still be able to consume aphids later and affect the community. These have been termed peripheral predators. Through their defense compound production, plants can change the predators that are considered included or excluded (Malcolm 1992). Milkweeds have been shown to induce and reduce their cardenolide levels based on the presence and density of herbivores (Martel and Malcolm 2004).

This study aims to uncover the relationship between the varying life histories of three aphid species and the chemical response of *A. syriaca.* As a null hypothesis, we hypothesized that the three aphids *A. nerii, A. asclepiadis,* and *M. asclepiadis,* would not vary in their cardenolide content. This would suggest that the apparent aposematism of *A. nerii*, and crypticism of *A. asclepiadis* and *M. asclepiadis* have no effect on the chemical defenses that these aphids exhibit. Since these aphid species seem to be defending themselves from predators in different ways, however, alternative hypotheses were formed. We alternatively hypothesized that (1.) *A. nerii* would sequester cardenolides from *A. syriaca*, (2.) *A. asclepiadis* would not sequester cardenolides from *A. syriaca*, and (3.) *M. asclepiadis* would not sequester cardenolides from *A. syriaca*.

Materials and Methods

Insect Culture. Aphid cultures of *A. nerii* and *A. asclepiadis* were established from natural populations feeding on *A. syriaca* in Kalamazoo County, MI. Cultures were created from the same original populations at Pierce Cedar Creek Institute in Hastings, MI and Western Michigan University in Kalamazoo, MI. These cultures were enclosed in mesh bags to protect the colonies from predators

and herbivores, which could affect the constitutive levels of cardenolides available to the aphid cultures. These bags were placed on native *A. syriaca* plants for 7 days prior to experimental trials to allow for acclimatization and aphid reproduction. Due to the dispersed nature and mobility of *M. asclepiadis*, these aphids were not isolated in a culture prior to experimental trials but instead were removed from naturally occurring *A. syriaca* prior to each trial.

Plant Selection. A. syriaca plants were used from sites on the Western Michigan University campus and at Pierce Cedar Creek Institute. One trial of each aphid species and time interval was used in each genet selected, with each replicate being located at a different genet. There were thus 10 genets used in the study. Plants were selected from each genet and were at least 1 meter in height and without significant initial herbivore destruction. Trial plants were selected at random from each genet and plants were not reused after trials.

Aphid density sampling was done for the three aphid types in one of two ways. For the widely dispersed *M. asclepiadis*, nearest neighbor data were taken at sites at WMU by measuring the distance from one aphid to the nearest neighboring aphid, distance to the nearest infected plant to the plant of those aphids, and the distance to the nearest uninfected plant. Due to the aggregated distribution of *A. nerii* and *A. asclepiadis*, these density measurements were taken in the laboratory using a dissecting microscope connected to a computer with MetaMorph digital imaging software. Again, the nearest neighboring aphid technique was used with a Nikon binocular microscope with a Javelin digital camera to measure distances from the head capsule of an aphid to the head capsule of its nearest neighbor.

Clip cages were created to provide a consistent environment for aphid growth. The Clip cages used in this experiment were fabricated in our laboratory and were composed of two PVC (polyvinyl chloride) rings held together so that leaves could be placed in the center. Each cage had an interior diameter of 2.9 cm and interior felt rings for leaf protection. The exterior of the PVC rings on each cage was fashioned from mesh netting (0.1 mm weave) to protect and enclose the trials. Metal spring

loaded clips were used to secure the halves of cages together and provide access before and after trials. Prior to use, cages were washed in a hot soap and water bath for 20 minutes and rinsed in deionized water for 5 minutes to remove any volatiles produced during fabrication.

To begin each experimental trial, two fifth instar aphids from each species were placed into a clip cage which was clipped onto the first fully developed leaf from the apical meristem of each experimental *A. syriaca* plant. For *A. nerii* and *A. asclepiadis*, two apterous aphids were used in each trial, but for *M. asclepiadis* which produces only alate adults, two alate fifth instars were used. The aphids were placed in the half of the cage on the underside of the leaf as they are found in nature. As a control, clip cages were also placed on separate plants in each genet that contained no aphids. The trial duration varied and consisted of 2, 4, 8, and 16 day intervals. Leaf size measurements were conducted before and after the trials in terms of length and width of the experimental and control leaves and the total number of leaf pairs on each plant was recorded. Evidence of other herbivory was noted upon completion of each trial.

Once trials were completed, leaves with clip cages attached were frozen at -80 °C until all other trials were completed. Once finished, all trails with leaves and aphids were placed into a Labconco Freeze Dryer 8™ freeze-drying apparatus and dried until brittle. Aphids were emptied of clip cages and sorted by instar. The total number of aphids of each instar was recorded. Total aphid mass and leaf mass were taken for all samples. Leaves were then homogenized using a mortar and pestle into a fine powder. Leaf stems and other parts that failed to homogenize were discarded from the sample. The recollected mass was again taken and about 0.2 grams of the homogenized leaf sample was placed into a centrifuge tube. Leaf samples were then suspended in 4 ml methanol, vortexed, sonicated for 10 minutes in a Branson 1200® sonicator and hot water bath, and centrifuged for 10 minutes in a Dynac™ centrifuge at high speed. The supernatant was collected and the pellets were resuspended in an additional 2 ml methanol, vortexed, sonicated for 10 minutes in a hot water bath, centrifuged for 10

minutes and the 2 ml supernatant was added to the original supernatant.

After being weighed, aphid trials were placed in test tubes, homogenized using a Brinkman™ homogenizer for 1 minute in 2 ml methanol, vortexed, and decanted into centrifuge tubes. The test tubes used for homogenization were washed with an additional 2 mL methanol, which was vortexed and added to the centrifuge tubes. These samples were vortexed, sonicated for 10 minutes in a hot water bath, centrifuged for 10 minutes at high speed, and decanted into glass culture tubes. Again, 2 mL methanol was added to resuspend the sample and the process was repeated and the supernatant was decanted into the original tubes.

To remove methanol from vials, nitrogen gas was blown onto the test tubes while they were held in a hot water bath. After methanol removal, 1 ml HPLC grade acetonitrile was added to the test tubes and the tubes were vortexed and sonicated for 30 seconds. The solvated mixtures were decanted into a 5 mL syringe equipped with a Millipore™ filter. The solvents were pushed through the filter into 1ml HPLC vials for HPLC analysis. Between samples 1 mL acetonitrile was passed through the syringe and filter twice to remove any remaining cardenolides. The syringe and filter were blotted with a Kimwipe™ to remove remaining acetonitrile. Each Millipore™ filter was used 10 times or until physical resistance developed.

Sample analyses were performed using the method of Wiegrebe and Wichtl (1993) on a Waters gradient HPLC system with WISP autosampler, 600E pump, 2996 diode array detector and Millennium32™ chromatography software. The reverse-phase elution gradient 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 and were separated by 10 minute equilibration at 20% acetonitrile.

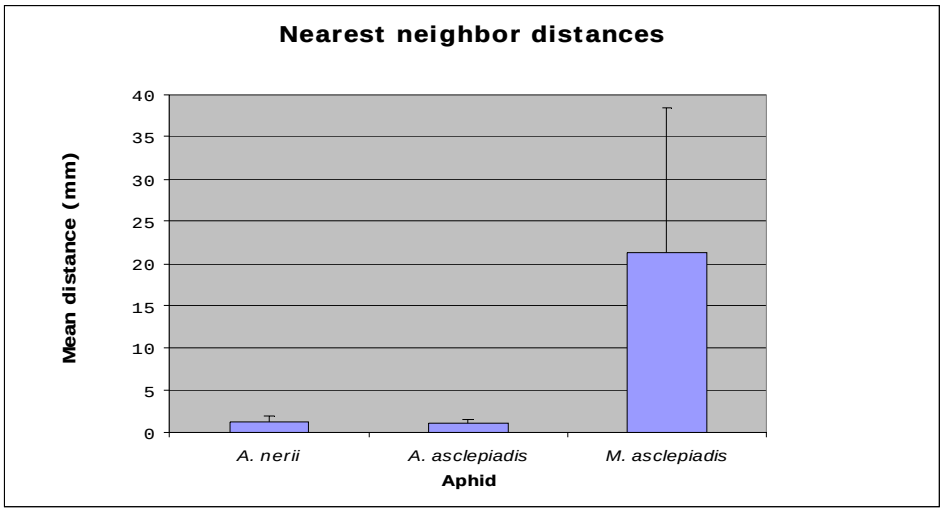
Cardenolides were detected at 218.5 nm and identified by their symmetrical spectra between

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 cardenolide standard digitoxin (Sigma, St Louis, Missouri). Only cardenolide peaks reported by Millennium software as consistently pure were considered for analysis.

Results

There was a significant difference among nearest neighbor distances for tall three aphid species (Figure 1, ANOVA $F_{2, 224} = 68.7$, $P<0.0001$). *A. nerii* and *A. asclepiadis* were not significantly different from each other with nearest neighbor means of 1.28 and 1.03 mm, respectively ($t<1.97$, $P>0.05$), but these two aphids were both significantly more aggregated than *M. asclepiadis* ($P<0.05$) with a mean nearest neighbor distance of 21.29 mm (Figure 1).

Figure 1. Nearest neighboring aphid distances for each aphid species and standard error bars showing the comparatively broad distribution of *M. asclepiadis* to *A. nerii and A. asclepiadis*.



The average leaf size change for 2 day trials of A. nerii was found to be 0.6655 cm^2, with a standard deviation of 1.1540 cm^2 (n=10). For A. asclepiadis that underwent the 2 day treatment, the average leaf size change was found to be 1.0725 cm^2, with standard deviation of 1.6870 cm^2 (n=10). The 2 day treatment of M. asclepiadis showed an average leaf size change of 0.5860 cm^2, with a

standard deviation of 0.6187 cm^2 (n=10). The control leaves showed a size change of 0.9795 cm^2 over the 2 day period, with a standard deviation of 1.0441 cm^2 (n=10). For the 4 day interval of A. nerii, the average leaf size change was found to be 0.3310 cm^2, and the standard deviation was calculated to be 0.5257 cm^2 (n=10). The replicates of A. asclepiadis showed an average growth of 0.7480 cm^2 and a standard deviation of 0.7249 cm^2 over the 4 day interval (n=10). The average leaf growth of the M. asclepiadis trials was found to be 1.3385 cm^2, with a standard deviation of 1.4175 cm^2 (n=10). The control trials for the 4 day interval showed an average leaf size change of 0.8005 cm^2, and a standard deviation of 1.1241 cm^2 (n=10). The 8 day trials of A. nerii showed an average leaf size change of 0.6800 cm^2, with a standard deviation of 0.6613 cm^2 (n=9). The A. asclepiadis trials of the same time interval showed an average growth of 2.2339 cm^2 and a standard deviation of 1.5810 cm^2 (n=9). The M. asclepiadis trials showed an average growth of 0.6650 cm^2, with a standard deviation of 0.9837 cm^2 (n=10). The control trials over the 8 day interval showed an average leaf growth of 1.4022 cm^2, with a standard deviation of 2.1931 cm^2 (n=9). For the 16 day trials, those infected with A. nerii showed an average leaf growth of 1.6150 cm^2 and a standard deviation of 1.6000 cm^2 (n=9). The trials infected with A. asclepiadis for 16 days showed an average growth of 2.2706 cm^2, with a standard deviation of 2.9746 cm^2 (n=9). The M. asclepiadis trials showed an average growth of 2.6665 cm^2 and a standard deviation of 0.9680 cm^2 (n=10). The 16 day controls showed an average growth of 2.2120 cm^2, and a standard deviation of 2.3375 cm^2 (n=10). The leaf area change results are summarized in Figure 2. An analysis of variance (ANOVA) showed a significant effect of time treatment on leaf growth ($F_{(3, 154)}$ = 7.5195, $P<0.0001$), indicating that longer time intervals produced larger leaf area change (Figure 3). Aphid treatment and control did not show a significant ANOVA result on plant growth ($F_{(3, 154)}$ = 1.5392, P= 0.2067).

Figure 2. Average leaf growth chart summarizing the four time treatments and aphid treatments used. Error bars represent standard deviation.

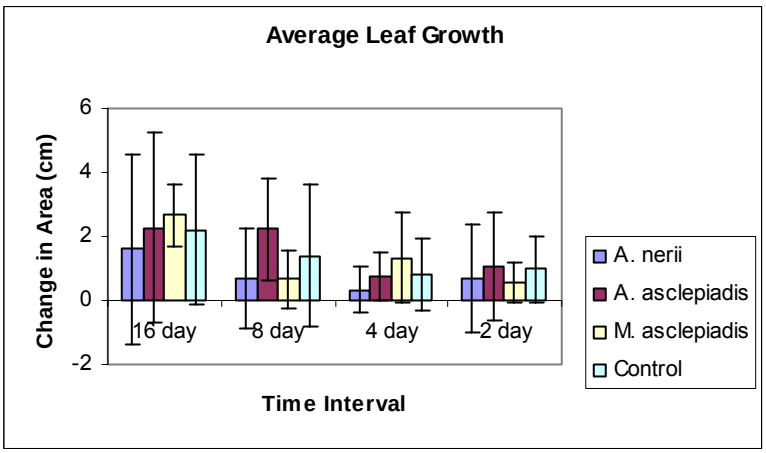
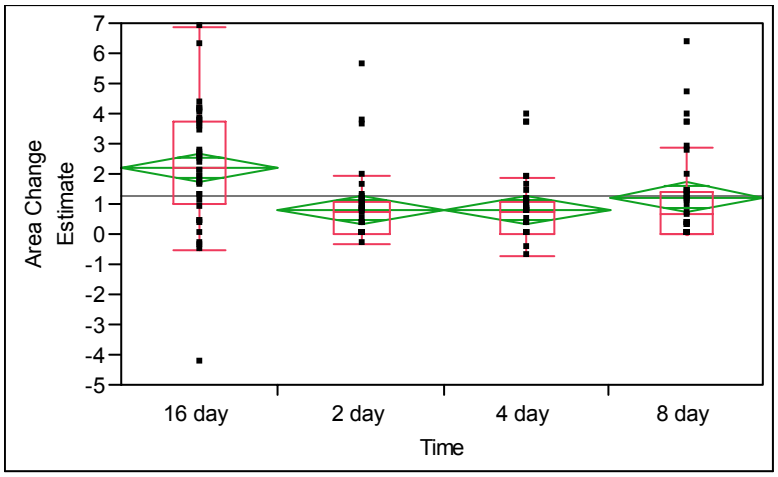


Figure 3. One way Analysis of Leaf Area Change Estimate by Time. Boxes and whiskers represent quantiles.



The aphid reproduction per day was calculated by dividing the final number of aphids in each trial by the number of days for which the trial was conducted. For the 2 day trial of A. nerii, the average reproduction per day was found to be 7.20 aphids per day, with a standard deviation of 4.45 aphids per day (n=10). The A. asclepiadis trials were found to have 6.95 aphids per day over the 2 day interval with a standard deviation of 3.74 aphids per day (n=10). The 2 day trials of M. asclepiadis showed an average of 6.85 aphids per day with a standard deviation of 2.24 aphids per day (n=10). The 4 day A. nerii trials showed an average reproduction of 5.10 aphids per day and a standard deviation of 1.94 aphids per day (n=10). The A. asclepiadis trials showed 5.13 aphids per day over the 4 days, with a

standard deviation of 1.86 (n=10). The average reproduction per day of M. asclepiadis over the 4 day interval was found to be 5.95 aphids per day, with a standard deviation of 2.01 aphids per day (n=10). The 8 day trials of A. nerii showed an average reproduction rate of 5.49 aphids per day and a standard deviation of 1.51 aphids per day (n=9). The A. asclepiadis 8 day trials showed an average reproduction rate of 3.88 and a standard deviation of 1.28 (n=9). The 8 day M. asclepiadis trials showed an average reproduction rate of 3.26 aphids per day and a standard deviation of 1.92 aphids per day (n=10). The 16 day trials of A. nerii showed an average reproduction of 7.08 aphids per day and a standard deviation of 1.95 aphids per day (n=9). The A. asclepiadis trial of the same time interval showed an average reproduction of 4.92 aphids per day and a standard deviation of 5.44 aphids per day (n=9). The 16 day M. asclepiadis trials had an average reproduction of 5.49 aphids per day, and a standard deviation of 2.61 aphids per day (n=10) (Figure 4). ANOVA was performed on the reproduction rate of the various aphids by aphid species and did not show statistical significance ($F_{(2,\ 119)} = 0.7720$, P=0.4644). The reproduction rate was examined against time treatment and showed a significant trend (Figure 5, $F_{(3,\ 119)} = 4.9501$, P=0.0029). Presence of other herbivores at the end of trials was recorded and an analysis of variance was conducted against aphid reproduction rate without significance ($F_{(2,\ 119)} = 1.0199$, P=0.3146).

Figure 4. Graphical representation of aphid reproduction in aphids per day plotted against time treatment. Bars represent aphid species. Standard deviation is represented by error bars.

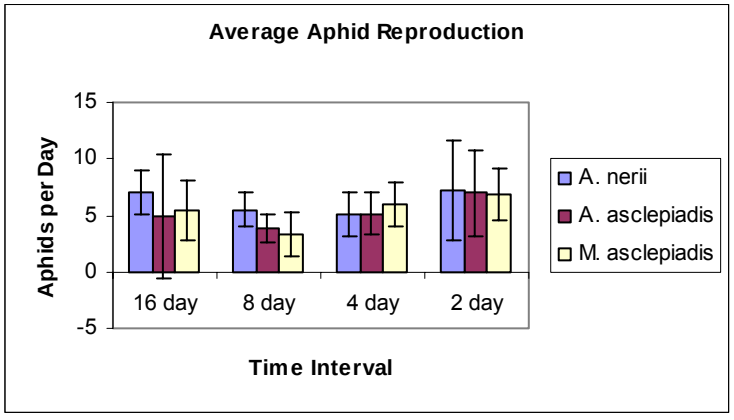
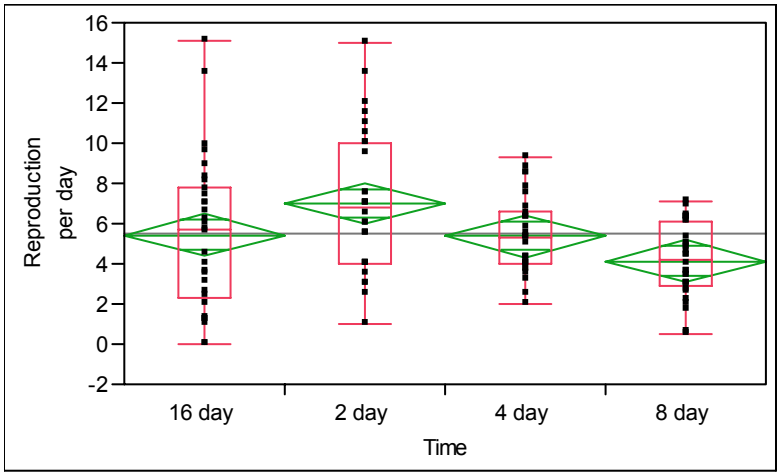


Figure 5. One way analysis of aphid reproduction per day by time. Boxes and whiskers represent quantiles.



The total aphid mass was standardized by dividing by the number of days of each trial. For 2 day A. nerii trials, the average aphid mass per day was found to be 0.335 mg, with a standard deviation of 0.261 mg (n=10). The A. asclepiadis trials for the 2 day interval showed an average aphid mass per day of 0.291 mg and a standard deviation of 0.166 mg (n=9). The trials of M. asclepiadis over the 2 day interval showed an average aphid mass per day of 0.099 mg and a standard deviation of 0.025 mg (n=10). For the 4 day trials, A. nerii had an average aphid mass per day of 0.251 mg and a standard deviation of 0.086 mg (n=10). A. asclepiadis showed an average aphid mass per day of 0.169 mg and a standard deviation of 0.085 mg (n=10). For M. asclepiadis over the same interval, the average was 0.110 mg and the standard deviation was 0.043 mg (n=9). For the 8 day interval, A. nerii showed an average aphid mass per day of 0.576 mg and a standard deviation of 0.337 mg (n=8). A. asclepiadis showed an average aphid mass per day of 0.151 mg and a standard deviation of 0.072 mg (n=9). The average aphid mass per day of M. asclepiadis over the 8 day interval was found to be 0.069 mg, and the standard deviation was found to be 0.043 mg (n=10). The A. nerii trials showed an average aphid mass per day of 0.917 mg for the 16 day trials, with a standard deviation of 0.652 mg (n=9). The A. asclepiadis trials showed an average of 0.164 mg and a standard deviation of 0.156 mg (n=9). For the 16 day interval, M. asclepiadis showed an average aphid mass per day of 0.180 mg and a standard deviation of 0.126 mg (n=10) (Figure 6). One way ANOVA showed a significant difference in aphid

mass per day compared against aphid species, but not against time treatment (Figure 7, $F_{(2,\ 119)}$ = 19.1055, P<0.0001) and ($F_{(3,\ 119)}$ = 2.5411, P=0.0598) respectively. The mass of an average aphid was calculated by dividing the total aphid mass by the number of aphids present at the end of each experimental trial. These differences were examined using one way ANOVA and showed statistical significance (Figure 8, $F_{(2,\ 117)}$ = 29.2409, P<0.0001).

Figure 6. Graphical summary of aphid mass change per day, grouped by aphid species. Mass is represented in mg and time is represented in days. Error bars represent standard deviation.

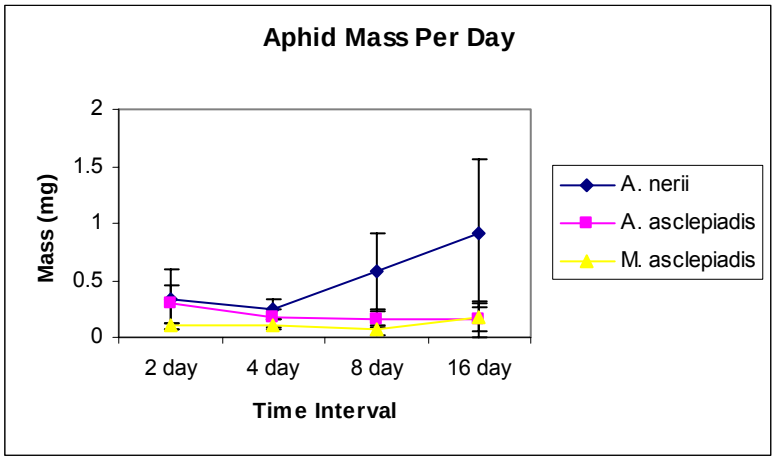


Figure 7. One way ANOVA graphical representation of aphid mass per day plotted by aphid species. Mass is expressed in grams.

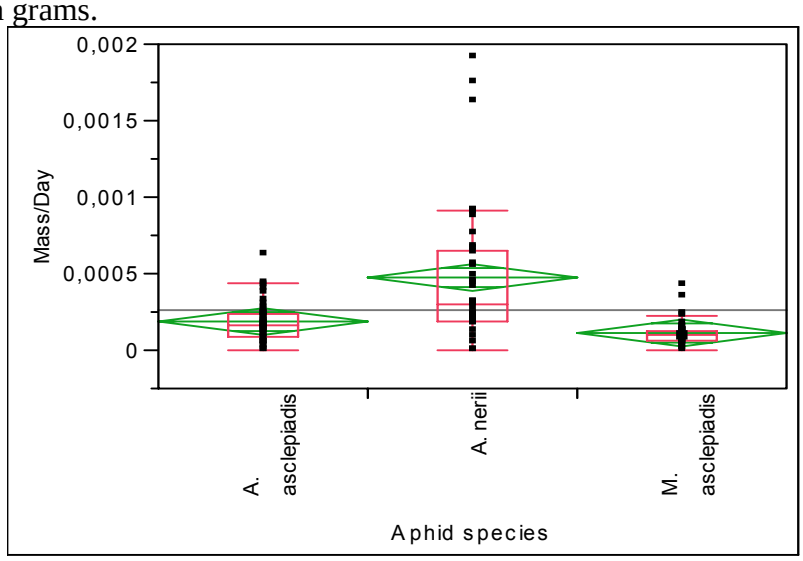
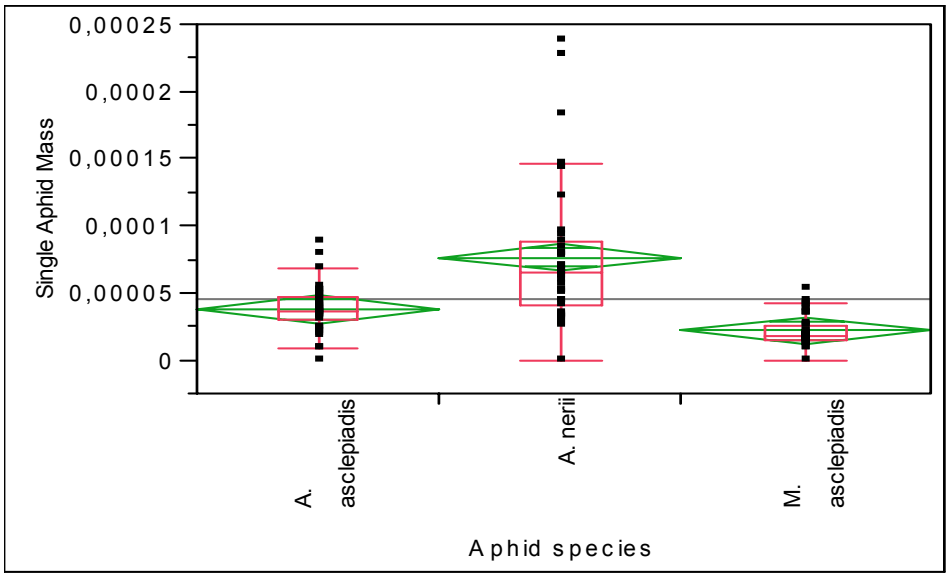


Figure 8. One way ANOVA graphical summary of average single aphid mass plotted against aphid species.



Discussion

The significant difference in nearest neighbor distances across aphid species further confirms the suspected life history differences of these aphid species. *M. asclepiadis* has a much larger and more variable nearest neighbor distance than *A. nerii* or *A. asclepiadis*, which confirms the observation that *M. asclepiadis* has a dispersed nature. This follows with *M. asclepiadis* being labeled as having a hiding defense. It seems that individuals that are widely dispersed would have a lower chance of being encountered by a predator than a large group of the same individuals. This method of defense could use a sacrifice strategy, where predators may be more likely to encounter a single individual due to a dispersed nature, but encountering one individual does not sacrifice the safety of other individuals.

The insignificant difference between *A. nerii* and *A. asclepiadis* and their relatively close proximity to nearest neighbors suggests other defense strategies. These may represent the same strategy, but due to the aposematic nature of *A. nerii* and the cryptic nature of *A. asclepiadis*, they probably have an aggregated nature for different reasons. For *A. nerii*, it is most likely part of a fighting

defense where the aggregated nature helps alert predators of their location, and when predators encounter the aggregation they will consume an aphid and become unable to consume others. Due to its cryptic nature, *A. asclepiadis* is not likely attempting to attract predators like *A. nerii*. Instead, a large aggregate of aphids may have differences in ant tending from a dispersed group of the same size. *A. asclepiadis* may be better able to recruit ants by being densely aggregated.

The presence of different aphid species does not seem to affect plant growth against the control, as shown by the insignificant ANOVA result. Although this result suggests that the aphid sink is not affecting the plant health in terms of leaf area, this may be due to the relatively small aphid population size for such a large plant with a large root network to other ramets. As one would expect, the trial length significantly affected leaf growth, with 16 day trials growing much more on average than any of the other treatments. Although the aphid presence does not seem to have an effect on the plant growth, time universally does.

The three aphid species also did not show a significant ANOVA result in terms of aphid reproduction. This suggests that although the aphids employ different defensive strategies in terms of density, they still produce offspring at rates that are not significantly different. Not surprisingly, the time treatment that had the largest mean reproduction rate was the two day treatment. This can be explained by the presence of two adult aphids in an empty clip cage, when the density is furthest from the cage capacity. The four and eight day treatments showed decreasing mean reproductive rates, which may indicate a resting and growth phase for the aphids already in the cage. The sixteen day trial showed a surprising result, in which the mean reproduction rate rose to nearly that of the 2 day trial. This could represent the time after the short growth phase when growing aphids are able to reproduce and give an explosion of offspring. It should be noted that some of trial cages were observed to be very densely packed with aphids after sixteen day trials, and clip cage size could become a limiting factor for additional reproduction.

The effect of time treatment did not yield significant differences on aphid mass, but the p value of 0.0598 suggests that time may have some impact to a degree that is not statistically significant. Both the aphid mass per day and the average single aphid mass showed significant results, however. This helps confirm that these aphid species are employing different survival strategies. The aphid mass per day results show the difference between the three aphid species in terms of energy use. *A. nerii* accumulates the most mass per day by far, which indicates that it is the largest nutrient sink on *Asclepias syriaca* of the three aphids studied. It thus requires a proportionately larger amount of nutrients to maintain itself compared to the other aphids. This carries implications for the other aphid species, which showed similar reproductive rates to *A. nerii*, but are much smaller and require much less energy to maintain. It could be that in an aposematic aphid such as *A. nerii*, it is advantageous to take up more nutrients and cardenolides in turn, in order to be as potent as possible to predators. Conversely, the cryptic nature of *A. asclepiadis* and *M. asclepiadis* may cause these aphids to be better served by staying small and thus less detectable by predators.

The initial cardenolide data show a general trend that indicates that all three aphid species carry varying amounts of cardenolides. It is still unclear which species sequesters more by mass, but at this time it appears that A. nerii carries the highest amount, followed by *A. asclepiadis* and *M. asclepiadis*. The remaining majority of samples is currently being analyzed by HPLC and will be added to this manuscript as they are received.

Conclusions

The null hypothesis, that each of the three aphid species would sequester the same amounts of cardenolides appears to be false based on initial cardenolide data. Alternative hypothesis 1, that *A. nerii* sequesters cardenolides from *Asclepias syriaca* appears to be true since initial cardenolide data support this. Alternative hypothesis 2, that *A. asclepiadis* does not sequester cardenolides appears to be false

based on initial data. Finally, alternative hypothesis 3, that *M. asclepiadis* does not sequester cardenolides also seems to be false. Further analysis must be conducted in order to confirm or deny these trends.

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